Tomas Bata University in Zlín
Faculty of Technology

Doctoral Thesis

Dispersion systems as carriers of active substances

Disperzní systémy jako nosiče aktivních látek

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ABSTRAKT

Předložená doktorská práce je zaměřena na formulaci, přípravu a charakterizaci vhodných disperzních systémů, které mají schopnost účinně enkapsulovat, uchovávat a uvolňovat účinné látky využívané v kosmetickém, farmaceutickém či potravinářském průmyslu.

Dizertační práce se skládá ze dvou částí. Teoretická část práce je věnována stručnému popisu procesu enkapsulace a nejčastěji využívaných enkapsulačních systémů, s důrazem na problematiku systémů emulzních. Další kapitola se zabývá enkapsulací lipofilních účinných látek. Hlavní část práce tvoří problematika emulzí stabilizovaných pomocí částic. Pozornost je věnována zejména nanočásticím celulózy. Součástí je také krátké srovnání vlastností emulzních systémů připravených v rámci experimentální práce. Závěrečná část přináší přehled experimentálních metod použitých k charakterizaci zmíněných systémů.

Druhá část práce předkládá výsledky získané během doktorského studia formou krátkého shrnutí jednotlivých publikovaných článků. Výzkumné práce v plném znění jsou k dispozici v samém závěru dizertační práce.

ABSTRACT

The doctoral thesis is focused on the formulation, preparation and characterization of the dispersion systems with the ability to carry, effectively encapsulate and release active substances in the cosmetics, pharmaceutical or food industry.

The thesis is divided into two main sections. In the theoretical part, a brief description of encapsulation process and most common encapsulation systems is provided, with emphasis on the emulsion-based systems. The next chapter deals with challenges associated with encapsulation of lipophilic active ingredients. The center theme of the thesis is focused on particle-stabilized emulsions, primarily on those stabilized by nanocellulose particles. Following this, a comparison of properties of prepared systems is presented. Finally, the last section provides an overview of laboratory techniques used for characterization of discussed systems.

The second part of the thesis presents results conducted during the doctoral study in form of short summaries on four research papers. The full-length versions of the papers are available at the end of the publication.

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CONTENT

THEORETICAL BACKGROUND

1. INTRODUCTION TO ENCAPSULATION

Many bioactive compounds (such as flavours, drugs, enzymes, cells, or other materials) cannot be simply incorporated into products in their regular form, and must first be encapsulated within some kind of suitable system before they can be introduced into the final product. Encapsulation is a common practice in the fields of pharmacy, cosmetics and food industry to improve the properties of products. By encapsulation their quality can be enhanced in terms of time and local controlled release, storage stability, protection from chemical, physical, or biological degradation [1-3].

More specifically, encapsulation may be defined as a process in which active ingredients (solids, liquids or gases) are entrapped within a secondary material, which completely embeds them or where they are dispersed in and delivered in small capsules [3, 4]. Capsules may range from nanometre to several millimetres in size and have a multitude of different shapes, depending on the materials and methods used for their preparation. In particular, capsules having diameter in the nanometre range are referred to as nanocapsules, while those with diameter between 3 and 8 μm are called microparticles, microspheres or microcapsules. Capsules larger than 1000 μm are then called macroparticles [3].

The schematic diagram of a capsule is shown in Figure 1a. Generally, capsules consist of a core material, which is also referred to as the active ingredient, internal phase, payload or fill, and a coating also known as the wall, shell, carrier or membrane. The morphology of the internal structure of capsule depends largely on the selected materials and the encapsulation methods that are employed. Capsules can be grouped into two main types: reservoir (core & shell), matrix or their combination (Figure 1b) [3, 5, 6]. For example, micelles, microemulsions, emulsions nanoemulsions can be all classified as matrix type encapsulation systems. Reservoir encapsulation systems include for instance multilayer emulsions, multiple emulsions, liposomes, and polymeric capsules [4, 7].

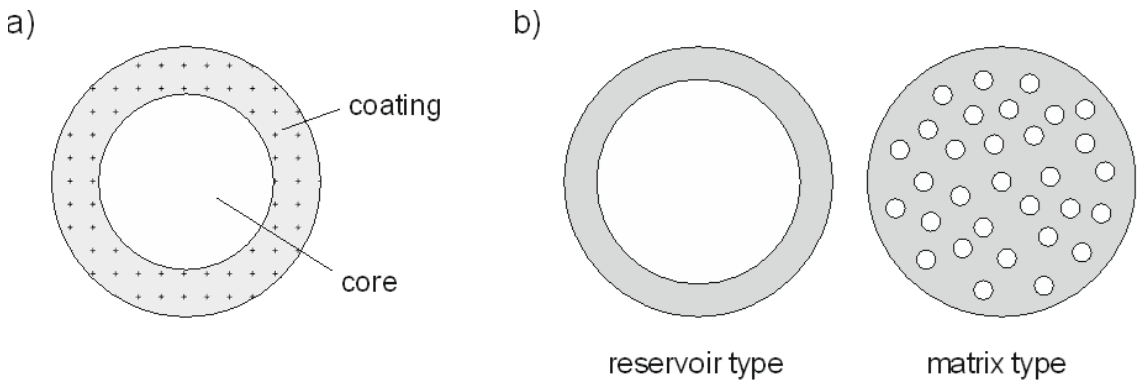

Figure 1(a) Scheme of a capsule. (b) Types of capsules [6].

In encapsulation, the role of coating material is crucial, as it determines the stability of capsule, the efficiency of encapsulation process, and the degree of protection for the core [3, 5, 6, 8]. Importantly, it must be also inert toward active ingredients and preferably nontoxic and biodegradable [3]. Other factors to be considered depend mainly on intended application of the final capsules and include the physicochemical properties of the coating material (solubility, molecular weight, and diffusibility), its film-forming and emulsifying properties, and cost. In practise, when the encapsulated active ingredient is intended for incorporation into foods, the wall materials need to be food grade [9]. In the cosmetic formulations for topical applications, the skin safety and compatibility of encapsulating materials play an important role [10]. The most-used coating materials gaining popularity across a variety of industries are polymers of both synthetic and natural origin. Synthetic polymers typically include poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), acrylic polymers, while commonly used naturally occurring polymers are polysaccharides (gums, starches, celluloses, alginates, cyclodextrins, chitosan), and proteins (gelatine, casein, soy proteins, zein, rice protein) [9, 11-14].

1.1 Encapsulation systems

Due to the number of methods and techniques used for encapsulation, it is difficult to establish a uniform classification of encapsulation systems. In the literature, some authors classify them according to the physicochemical processes involved in encapsulation (i.e. physical, chemical, mechanical) [6], while other classifications are, for example, based on the techniques involved (i.e. atomization, spray coating, coextrusion, and emulsification) [15], or on the morphology of capsules (i.e. matrix type, reservoir type, their combination) [14]. Alternatively, they can also be classified according to the major components used in their fabrication as surfactant-based, emulsion-based, biopolymer-based, or hybrid [1]. Brief summary of encapsulation systems that use water as the dispersing medium is given below (Table 1).

Table 1 Selected examples of colloidal encapsulation systems [1].

Type of Delivery System
Surfactant-based systems
– Swollen micelles
– Microemulsions
Emulsion-based systems
– Liposomes
– Conventional emulsions
– Nanoemulsions
– Multiple emulsions
– Particle-stabilized emulsions
– Multilayer emulsions
– Solid lipid nanoparticles
Biopolymer-based systems
– Microclusters
– Hydrogel particles
– Coated hydrogel particles
– Biopolymer nanoparticles/microparticles
Hybrid systems
– Filled hydrogel particles
– Filled liposomes
– Multilayer liposomes
– Colloidosomes

1.1.1 **Spontaneously-assembled surfactant-based systems**

The main building components for surfactant-based encapsulation systems are surfactants and phospholipids. These surface-active molecules consist of a polar head group and a nonpolar tail group. Different surfactants vary according to the nature of their head and tail groups. The head group may differ in its size, polarity, charge (positive, neutral, negative), and chemical reactivity. The tail group may vary in the number, length, unsaturation, and branching of the nonpolar chains. The functionality of surface-active molecules depends on the nature of the head and tail groups, as well as surrounding environment. Therefore, it is possible to prepare surfactant-based delivery systems with a wide range of structures and functional properties by using different kinds of surface-active molecules. The main driving force for the formation of these system is usually self-assembly of the surfactants based on the hydrophobic effect, and so they are primarily formed spontaneously by simply mixing surfactant, oil, and water together. The main types of surfactant-based systems are micelles, microemulsions, and liposomes (Figure 2) [1, 16].

1.1.2 Emulsion-based systems

The key building components for emulsion-based systems are oil droplets, which may differ in their size, composition, physical state, interfacial characteristics, and structure [16]. Differently from microemulsions and other spontaneously-produced systems, they are required energy input to be formed. Emulsion-based systems are usually fabricated by mixing or blending emulsifier, oil, and water phase together [17]. The principal emulsion-based delivery systems are emulsions, nanoemulsions, particle-stabilized emulsions (Pickering emulsions) and solid lipid nanoparticles (SLNs), but these systems can be used as building materials to construct more complex structures, such as multilayer emulsions, colloidosomes, microclusters, and filled hydrogel particles [1, 18, 19].

1.1.3 Biopolymer-based systems

The key building materials for biopolymer-based delivery systems are biopolymers, such as proteins and polysaccharides. These systems can be fabricated using a variety of different preparation methods depending on the biopolymers involved, and the desired functionality. The most common biopolymer-based delivery systems are molecular complexes and hydrogel particles. Similarly, to emulsion based systems, they can also be used for development of more complex structures, such as filled hydrogel particles [1, 20].

1.1.4 Hybrid systems

The fundamental building components used in surfactant-, emulsion-, and biopolymer-based delivery systems can themselves be used to create structured encapsulation systems with novel or improved functional properties. In general, three main approaches could be used for preparation of more complex structured encapsulation systems: coating, embedding, and clustering. For example, by employment of electrostatic deposition additional biopolymer coatings could be form around charged particles in liposomes, emulsions, SLNs, multiple emulsions, or hydrogel particles. The embedding strategy can be used to trap microemulsions, emulsions, nanoemulsions, SLNs, or multiple emulsions within hydrogel particles. The clustering approach can be used to develop the colloidosomes or microclusters from nanoemulsions, emulsions, multiple emulsions, SLNs, or other types of particles [1, 4, 21].

2. ENCAPSULATION OF LIPOPHILIC ACTIVE INGREDIENTS

Lipophilic active ingredients (LAI) usually refers to diverse class of biological compounds with a wide range of activities, including antimicrobial, antioxidant, anti-inflammatory, health-improving and skin-conditioning properties of which the common and defining feature is their poor solubility in water [22-25]. This broad definition covers a number of different compound groups with various functionalities in the product formulations, such as triglycerides of animal, fish, or plant origin, fatty acids, phospholipids, carotenoids, phytosterols, oil-soluble vitamins, and essential oils with their individual constituents (Table 2) [26, 27]. Selection of the active ingredient therefore depends on the final application of the formulation/product. For example, the food industry has been interested in lipophilic components with health benefits, which can be used in the nutraceuticals and functional food [28, 29]. In the cosmetic industry, the trend continues to shift lipophilic substances from being used in common, daily-care products towards being part of newly-developed active ingredients providing stronger benefits to their targets whether it is the skin, hair, nail, mucous membrane, or teeth [30]. Today, besides the common application in cosmetics such as vehicles (bases), lipophilic ingredients have been used for special purposes in specific products for skin care and treatment or hair care. For example, some products are marketed as having anti-wrinkle and skin enhancement effects [31].

Table 2 Major classes of the most frequent lipophilic active ingredients used in cosmetics, pharmaceutical and food products and their selected examples [1, 10, 31-35].

Lipophilic Active Ingredient	Example
Triacylglycerols (particularly oils and fats rich in essential fatty acids and other bioactive compounds)	Plant oils: almond, apricot, avocado, macadamia, olive, soybean, sesame Fish oils: cod liver, salmon, tuna Plant fats: cocoa, coconut, palm, shea butter
Essential oils and their components	Oils: lemon, cinnamon, clove, thyme, tea tree, rosemary, pine, orange Components: limonene, carvacrol, citral, eugenol, thymol, cinnamaldehyde, linalool, linalyl acetate, eucalyptol
Fatty Acids	oleic (C18:1), conjugated linoleic acid (C18:2), α-linolenic acid (C18:3), eicosapentaenoicacid (C20:5), docosahexaenoic acid (C22:6)
Oil-soluble vitamins	A, D, E
Tocopherols	α-tocopherol
Carotenoids	lycopene, β-carotene, lutein
Phytosterols	stigmasterol, β-sitosterol

2.1 Challenges in incorporating lipophilic actives into products

Despite of the great interest across the different industries to efficiently exploit the full potential of lipophilic ingredients, there is a number of challenges associated with their direct application into products. Examples of the most common formulation issues are summarized in the following sections.

2.1.1 **Poor solubility**

Many lipophilic ingredients of interest have a very low solubility in water (e.g., essential oils, phytosterols, and carotenoids) or they are completely insoluble in aqueous environment (vegetable oils), and therefore they are not readily dispersible in hydrophilic products. This limitation can be overcome by using a suitable encapsulation system. In these systems, lipophilic ingredients are typically used as the hydrophobic core or may be solubilized in the core before the system preparation. Some active ingredients have low solubility in both oil

and water phases (e.g., phytosterols), so they need to be delivered in a crystalline form [21, 36-38].

2.1.2 High melting point

Certain lipophilic bioactives (e. g., phytosterols and carotenoids) are crystalline at storage temperature in their pure form, which leads to sedimentation or give an undesirable appearance of the product. These ingredients should be dissolved within a suitable organic phase, or to be used at temperatures above their melting point. Alternatively, it may be possible to incorporate them as small crystals that remain stable in the product and do not adversely affect the long-term stability or organoleptic properties of the final product [21].

2.1.3 Chemical instability

A number of important lipophilic bioactives, such as fish oils or essential oils are highly susceptible to chemical degradation during processing, storage or utilization, resulting in changes in their biological activity. These changes have a negative effect on the shelf-life, sensory properties, and overall acceptability of the developed products [8, 21]. The rate, extent, and type of chemical degradation depends on the nature of the bioactive agent, encapsulating material, and environmental conditions, such as pH, presence of light, oxygen, prooxidants, and elevated temperatures. For these bioactives, it is necessary to develop encapsulation systems enable to protect the encapsulated component from chemical degradation [21].

2.1.4 Ingredient interactions

Some lipophilic components may physically interact with other components present in in the formulation, which can lead to instability of the system. In this case, it may also be necessary to use a suitable encapsulation system to isolate one component from another in order to avoid their mutual interaction [21].

2.2 Encapsulation systems for lipophilic ingredients

A wide range of systems have been developed to encapsulate lipophilic ingredients (Figure 2). Each of them have their own specific advantages and disadvantages in terms of protection of active substance, delivery, cost, ease of use, biodegradability and biocompatibility [39]. Development of suitable systems can lead to improvement of the stability, water dispersibility, and efficacy of lipophilic ingredients in products, by maintaining/preservation of their biological properties [39, 40].

Among these strategies, emulsion-based systems are considered to be the most-common forms used for encapsulation of lipophilic actives. The thesis is focused on some of them, namely conventional emulsions (macroemulsions), nanoemulsions and mainly on emulsions stabilized with solid particles.

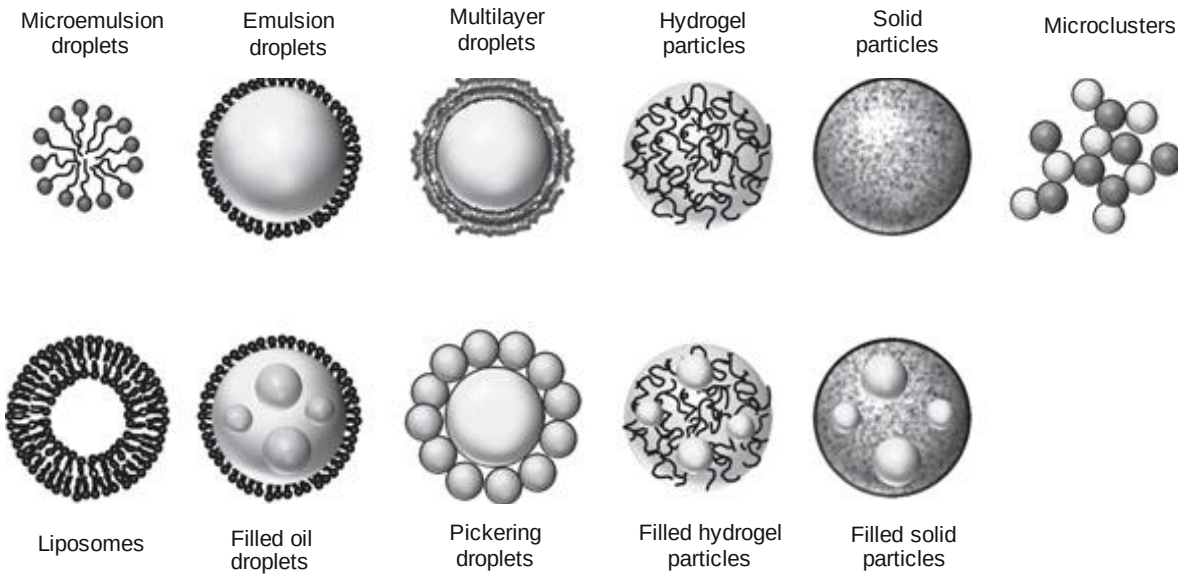

Figure 2 Examples of colloidal systems that can be used to encapsulate lipophilic bioactive agents [1].

3. PARTICLE-STABILIZED EMULSIONS

Emulsions find applications in a numerous industrial processes and commercial products where immiscible liquid phases coexist. Conventionally, emulsions are stabilized by the surfactants or amphiphilic polymers, which adsorb at the oil-water interface and prevent the droplet coalescence due to electrostatic and steric repulsive forces (Figure 3. Left). However, it has been showed that in many cases, they are not biologically compatible and are environmentally harmful. For example, some surfactants cause tissue irritation and even cell damage. These negative effects limit the use of surfactant-based products, especially in cosmetic, pharmaceutical, and biomedical applications [41]. Recently, there has been trend towards surfactant-free emulsions, driven by both legal and consumer requirements. On this basis, emulsions stabilized solely by fine solid particles instead of surfactants, have gained more interest of research and product development throughout various industries [42-44]. These emulsions are known as Pickering emulsions (Figure 3. Right), named after Spencer Umfreville Pickering [45], who reported first scientific study on particle stabilized emulsions. This classic work demonstrated clearly that fine solid particles are capable to remain at an oil–water interface and stabilize emulsion droplets. The presence of particles (usually nano- or microscale) at the interface affects not only the way of preparation, but also the properties of Pickering emulsions. Compared to classical emulsions stabilized by surfactants, particle-stabilized emulsions exhibit superior stability, low toxicity, and in particular cases also stimuli-responsiveness [46]. A wide variety of solid particles can be used in Pickering emulsions, including organic particles (such as cellulose and its derivatives, starch, and zein) or inorganic particles (e.g., silica, titanium dioxide, clay). Recently, thanks to the rapid development of material science, numerous alternative particles can be produced, which further enriches and expands the application of Pickering emulsions into different fields [46, 47]. Pickering stabilization is applied, for example, in emulsions and foams in food, pharmaceutical and cosmetic products.

On the other side, Pickering-type stabilization plays an adverse role in biological waste water purification or oil recovery, in which undesired foams and emulsions are stabilized by bacteria or minerals present in the system [48, 49].

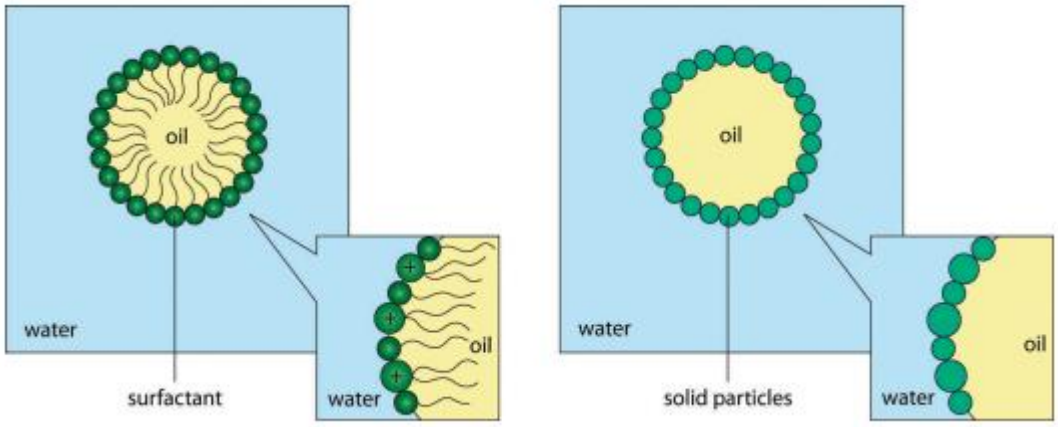

Figure 3 Surfactant-based emulsion (left) and a Pickering emulsion (right) [49].

3.1 Pickering emulsion theory

The Pickering stabilization mechanism is different from that of observed on conventional emulsifiers (surfactants and biopolymers). In principal, the stabilization of emulsions by particles is attributed to their partial dual wettability, resulting in the spontaneous accumulation of dispersed particles at the oil-water interface [50, 51]. This layer of closely packed particles surrounding the droplet provides so-called steric hindrance, which acts as a mechanical barrier and protects the emulsion droplets against coalescence. The assembly of particles at the oil-water interface is allowed by large reduction of total free energy, ΔG, occurring when a particle adsorb at the interface. This can be expressed by equation (1)

$$\Delta G = \pi \gamma_{ow} r_p^2 (1 - cos\theta_{ow})^2 \qquad (1)$$

where r_p is the particle radius, γ_{ow} the interfacial tension at oil-water interfaces and θ the three-phase contact angle measured into the water phase [52]. This energy of attachment of particles to interfaces reaches a maximum at contact angle of 90° and for example, a particle with radius of 0.5 μm at a hydrocarbon/water interface ($\gamma_{ow} \approx 50$ mN m^{-1}) will have $\Delta G \approx 10^6$ $k_B T$ (where k_B is Boltzmann's constant and T is the absolute temperature). Under these conditions, once the particles are attached to the oil/water interface, it is practically impossible to replace them from the interface. Therefore, the particles are considered to be irreversibly adsorbed, which provides an efficient barrier to droplet coalescence [53, 54]. In comparison, the surfactant molecules (≈ 0.5 nm in radius) adsorb and desorb on a relatively faster timescale, and they could be easily detached ($\Delta G \approx 5$ $k_B T$) and not be effective as stabilisers [52, 55]. Also it should be noted that the adsorption of particles does not lead to reduction in the interfacial tension [56].

As it has been mentioned previously, droplet stability in Pickering emulsions is mainly attributed to the steric hindrance provided by a surrounding particulate layer (Figure 4a). However, the presence of other configurations of stabilizing

particles, such as the sharing of adsorbed particles by two droplets, so-called bridging (Figure 4b), or a formation of a three-dimensional particle network between emulsion droplets, may contribute to the stabilization of droplets as well (Figure 4c) [50, 57]. It has been also shown that particles of non-spherical shape, e.g. polymer-based fibres, amorphous clumps or rod-like crystals, can act as stabilizers of Pickering emulsions (Figure 4d) [58].

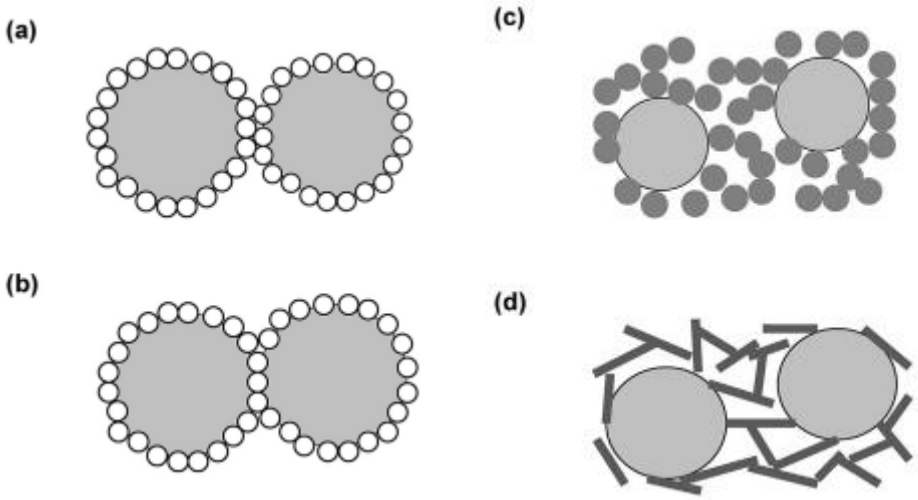

Figure 4 Possible configurations of particles in Pickering emulsions: a) particle bilayer; b) bridging layer; c) 3-D network of interconnected particles; d) stabilization by non-spherical (rod-like) polydisperse particles [58].

3.2 Factors influencing the stability of Pickering emulsions

From Eq. 1 it is evident that the stability of Pickering emulsions is influenced by the properties of the particles adsorbed at the interface. To prepare stable Pickering emulsions, it is necessary to use solid particle of an appropriate size, wettability, and concentration [53, 57]. Other factors involved in stabilization of the emulsions include the oil/water ratio, particle interactions, the ratio of hydrophilic to hydrophobic particles in mixtures, the presence of any other emulsifiers, and pH or ionic strength of the aqueous phase [59]. The mutual particle interactions plays also an important role, as it was already mentioned above [53].

3.2.1 Particle wettability (contact angle)

In order to adsorb at the interface, particles are required to be wetted by both liquid phases of emulsion. This dual particle wettability is the key feature of Pickering stabilization [52]. According to the empirical Finkle's rule [60], the type of emulsion formed, i.e. oil-in-water (o/w) or water-in-oil (w/o) depends on the preferential wettability of the particles in both liquids. In general, the less-wetting liquid becomes the dispersed phase of the emulsion. Similarly to the hydrophilic-lipophilic balance (HLB) number used to describe the preferential wettability of surfactants, the parameter determining particle wettability and a location of the

particle at an interface between oil and water is the contact angle [47, 51, 52, 61]. The contact angle also controls the type of emulsion formed, as can be seen in Figure 5.

By theory, the contact angle measured at the water phase, θ_w, is given by the Young's equation (2)

$$cos\theta_w = \frac{\gamma_{so} - \gamma_{sw}}{\gamma_{ow}} \quad (2)$$

where γ_{so}, γ_{sw}, and γ_{ow} are the solid–oil, solid–water, and oil–water interfacial energies, respectively [62]. In practice, it has been shown that for hydrophilic particles with contact angles $\theta < 90°$ oil-in-water emulsions are formed, whereas hydrophobic particles with contact angle $\theta > 90°$ lead to water-in-oil emulsions [61, 63]. However, the particles very hydrophilic ($0° \leq \theta \leq 20°$) or very hydrophobic ($160° \leq \theta \leq 180°$) would become completely wetted by either the water or oil phase, which leads to the instability at the interface. In this case, the particles become completely dispersed in a single phase, and no stable emulsions can be produced [61-63]. In contrast, the contact angle equal to 90° corresponds to the point of phase inversion, where particles are equally wetted by both phases and the type of emulsion will be governed by the properties of particles as well as properties of liquids forming the emulsion phases [51, 64]. Examples of predominantly hydrophilic particles favouring o/w emulsions cover both particles of inorganic origin, such as metal oxides (unmodified silica, TiO_2, and CuO) [65- 68], and organic-based materials, including polysaccharides (cellulose, chitin nanocrystals, and starch nanocrystals) [69-71]. In case of w/o emulsions, more hydrophobic particles such as polystyrene [72], Fe_3O_4 [68] or crystalline monoacylglycerols [73] have been employed. In addition, the hydrophobicity of some particles, such as silica or cellulose nanocrystals, can be tuned by varying the particle coating, surface modification, or the degree of substitution by functional groups [49, 74-76].

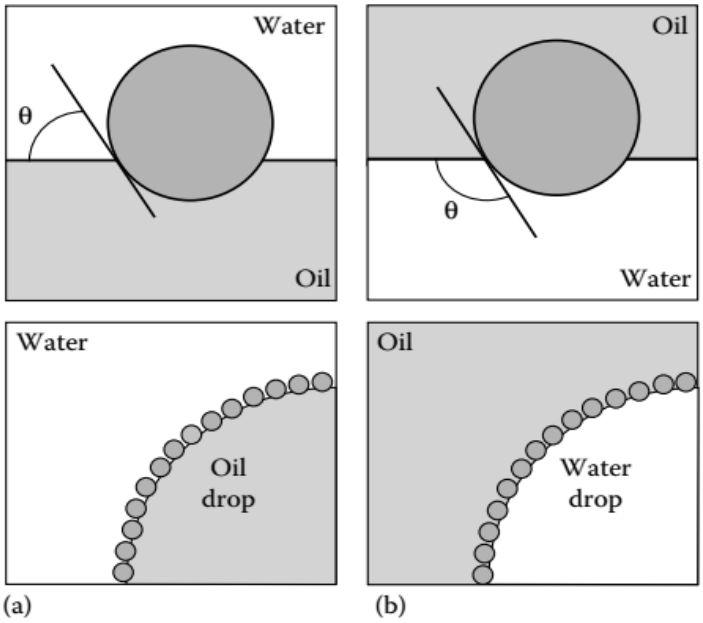

Figure 5 Changes in wettability of solid particle at the oil–water interface and the type of emulsion formed at different contact angles: (a) θ < 90° favouring o/w emulsions, whereas (b) θ > 90° favouring w/o [77].

3.2.2 **Particle size**

Stabilization energy of the particle assembly at the interface increases in proportion to the square of particle size *r* (Eq. 1). This indicates that the larger particles will provide better emulsion stability. For particles above a certain size (≈ 10 nm), the decrease in total free energy under absorption ΔG is much larger than the thermal energy, which leads to an irreversible adsorption of large particles at the interface. On the other hand, very small particles (< 1 nm) are attached to the interface by a ΔG comparable to the thermal energy. As a result, they can easily be replaced from the interface, and they may not be efficient as stabilizers [49, 51, 78].

3.2.3 **Particle concentration**

Particle concentration is also an important factor influencing emulsion stability. In general, the increase in particle concentration (at fixed content of disperse phase) leads to reduction of droplet size, and also improves surface coverage, which imparts to the emulsion additional stability [46, 51]. This trend has however a limit, at which one fraction of particles acts as stabilizer, whereas another fraction would remain non-adsorbed in one of the phases. At this point further decrease in droplet size can only be achieved by improving the emulsification conditions [51, 57, 79]. It has been showed that high particle concentrations do not necessarily lead to a full coverage of the droplet by particles, as in some emulsions stable droplets were observed even without this dense coverage [57, 58].

3.3 Formation of Pickering emulsions

The preparation of a Pickering emulsion involves the dispersion of particles into the continuous phase of an emulsion, following by distribution of the particles, based on their partial wettability, in each of the two immiscible phases [41]. Similarly to classical surfactant-stabilized emulsions, Pickering emulsions are formed by mixing a water and oil phase together in presence of emulsifier, in this case solid particles. As mentioned earlier, the high energy required to remove adsorbed particles from the o/w interface results in the superior stability of Pickering emulsions compared with conventional emulsions. On the other hand, in most cases, in order to prepare a stable Pickering emulsion, the high-energy methods, such as homogenization or sonication, need to be applied to overcome this energy barrier [46]. Various types of Pickering emulsions across different industries have been well-documented in literature, including multiply Pickering emulsions [47, 51].

4. NANOCELLULOSE-STABILIZED EMULSIONS

4.1 Cellulose

Cellulose is the most abundant naturally occurring biopolymer, with about 10^{10}–10^{11} tons produced per year globally [80]. As a renewable, biodegradable, and also non-toxic material, which simultaneously combine low price and excellent mechanical properties, cellulose and its derivatives have played an important role in the development of novel environmentally friendly and biocompatible products [81, 82].

4.1.1 Source materials

Cellulose serves as the main building block of the cell wall structure of higher (vascular) plants, and is often combined with other biopolymers, such as lignin and hemicelluloses [83]. Cellulose can be isolated from various sources. The main sources of cellulose are plants, including wood, bast fibres (flax, hemp), seed fibres (cotton), grasses (bagasse, bamboo) and their agricultural residues. Alternative cellulose feedstocks are marine animals (tunicate), algae, fungi, invertebrates, and bacteria [84-86]. Among the sources, cotton and bast fibres are the purest materials, containing approximately 90 and 70–80% w/w cellulose, respectively. In comparison, the major industrial source of cellulose, wood consists of only 40–50% w/w cellulose [87]. In case of nonconventional sources, the cellulose content in aquatic species is reported to be in the range of 10−40% w/w, and the yield produced by biotechnological process employing bacteria may reach at maximum 0.6 g/g of glucose per day [81].

4.1.2 Chemical composition and structure

Regardless of its source, the cellulose can be characterized as a linear homo-polysaccharide composed of anhydrous *D*-glucose molecules (so called AGU units) linked together by β-1,4-glycosidic bonds. The smallest repeating unit in the polymer, cellobiose, consists of two AGU [83, 87, 88]. The average chain length of cellulose, indicated by the number of AGU unites, is usually expressed as degree of polymerization (DP). The DP of cellulose varies depending on the source material and is of about 10 000 in native wood ($M \approx 2\,000\,000$ g mol^{-1}), as well as on the isolation method. In processes connected with isolation of cellulose from wood the DP further reduces to about 300−3000 ($M \approx 50\,000$−$500\,000$ g mol^{-1}) [81, 83, 87]. Each AGU has six carbon atoms with three hydroxyl groups (at C2, C3, and C6 atoms), which gives cellulose molecule a high degree of functionality. Due to the molecular structure, cellulose possesses advantageous properties, such as hydrophilicity, insolubility in water and most organic solvents, degradability and chirality [87, 89, 90].

Native cellulose does not occur as an isolated molecule but is found in the form of fibrils (Figure 6). Cellulose is synthesized in the cells in the form of individual molecules that crosslink with each other and produce fibrillary structures during

biosynthesis [91]. The basic structural units, known as elementary fibrils or nanofibers, are made of 36 individual cellulose molecules with diameter of about 5 nm. These entities aggregate into microfibrils, which are up to 20 nm in diameter and a few micrometres in length, where the cellulose chains are arranged in crystalline (highly ordered) and amorphous (disordered) regions. These microfibrils further aggregate into larger entities called macrofibrils and cellulose fibres, which are visible under a light microscope [83, 92-95].

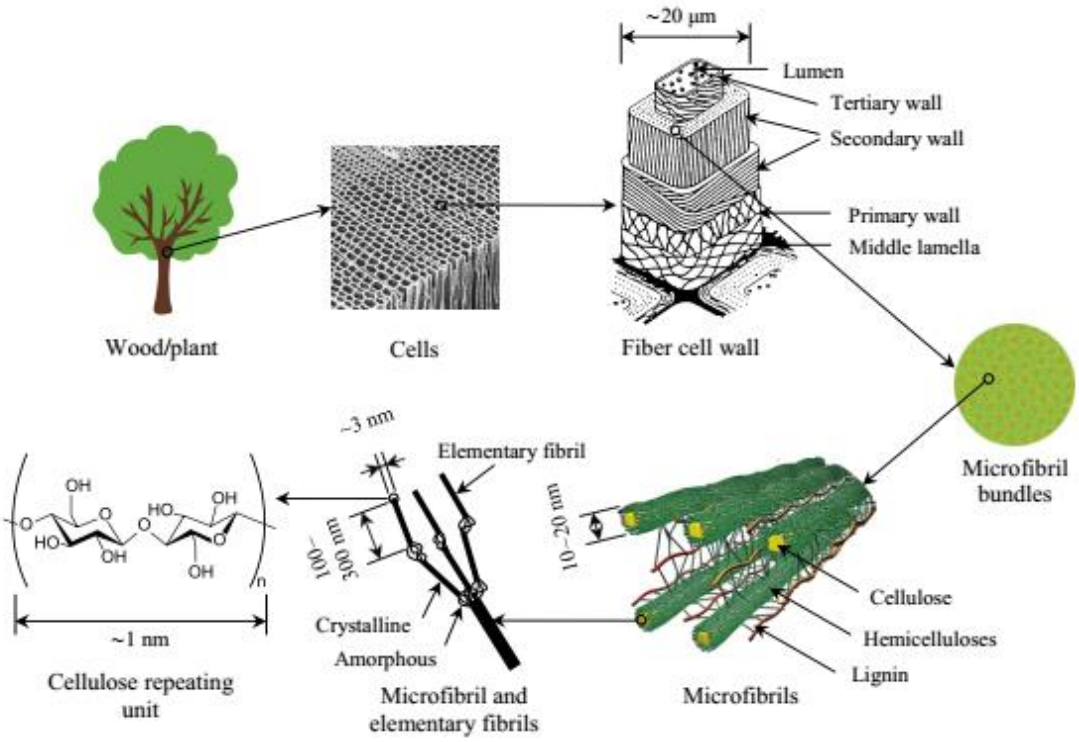

Figure 6 Schematic diagram of the structure and composition of the wood/plant [89].

4.2 Nanocellulose

Cellulose can also be used as a source for nanoscale cellulose materials. The individual cellulose elements with a diameter in nanometre range (< 100 nm) are generally referred to as nanocellulose. Based on the treatment conditions, which affect the dimensions, composition, and properties, the nanocellulose may be classified in three main categories: (i) cellulose nanocrystals (CNCs) also called whiskers; (ii) nanofibrillated cellulose (NFC) alternatively named microfibrillated cellulose (MFC), or cellulose nano-/microfibrils and (iii) bacterial nanocellulose (BNC) or microbial cellulose. However, it should be noted that standard terminology and nomenclature for different types of cellulose nanomaterials has not yet been adopted [89, 96, 97]. Summary of nanocellulose types with average dimensions is presented in Table 3.

In general, CNCs and MFC are produced by top-down methods involving enzymatic/chemical/physical disintegration of cellulose fibers to the nanosized

material. In contrast, BNC is fabricated *via* bottom-up approach from low molecular weight sugars by aerobic bacteria. Irrespective of the particles type, nanocellulose exhibits hydrophilic character, relatively large specific surface area, broad potential of surface chemical modification, and excellent mechanical strength [89, 97]. These properties make the nanocellulose promising source for the various applications such as novel composites, bioactive filters and membranes, food packaging, drug delivery, stimuli-responsive materials, biomedical applications and many others [82, 84, 97].

Table 3 Dimensions of nanocellulose particles from published studies [88].

Cellulose Type	**Diameter (nm)**	**Length (nm)**	**Aspect Ratio**
Microfibrillated cellulose	10–40	> 10 000	> 1 000
Cellulose nanocrystals	2–20	100–600	10–100
Bacterial cellulose	5–6	1 000–9 000	160–1 800

4.2.1 **Microfibrillated cellulose**

Microfibrillated cellulose (MFC) is produced from purified wood-based fibres using a variety of mechanical processes including high-pressure homogenization, high-intensity ultrasonic treatments, and microfluidization [91]. These treatments generate high shearing forces resulting in a strongly entangled and disordered networks of nanofibrils, having both crystalline and amorphous domains [81, 87, 91]. Usually, before performing the final mechanical separation to MFC, the raw materials are pre-treated mechanically, chemically, and/or enzymatically in order reduce energy consumption in the process [98].

Newly produced MFC forms an aqueous gel (2–7% w/w) possessing pseudoplastic and thixotropic properties [99]. Depending on the processing conditions, cellulose fibers can be further disintegrated to nanofibrils with the lateral dimensions from ~ 3 nm, which represents elementary fibrils, to tens of nanometres, corresponding to microfibrils [89, 100]. Typically, MFC has a diameter of 10–40 nm and a length of few micrometres with an aspect ratio (length to diameter ratio, L:D) of nearly 1 000 [88].

4.2.2 **Cellulose nanocrystals**

Cellulose nanocrystals (CNCs) are highly crystalline, rod-shaped particles with nanoscale dimensions. CNCs are commonly obtained through hydrolysis of cellulose-containing materials, which selectively dissolves the amorphous cellulose regions, while leaving the less accessible crystalline parts unaffected [101, 102]. The first report on colloidal cellulose nanocrystals was published by Rånby [103] who treated wood and cotton with concentrated sulfuric acid. Acid hydrolysis by using concentrated mineral acids such as sulfuric and hydrochloric acids is the most common preparation method of CNCs [102]. More recently, CNCs have been obtained by more energy-efficient production, whereby cellulose

materials were oxidized with ammonium persulfate [104] or 2,2,6,6-Tetramethylpiperidin-1-yl)oxyl radical (TEMPO) [105]. The size of the crystallites depends on the hydrolysis conditions and the cellulose source. In general, a milder hydrolysis and/or a more crystalline source material result in larger particles [101]. Selection of hydrolysis/oxidation treatment also determines the surface charge of resulting CNCs. While hydrolysis of cellulose with hydrochloric acid gives uncharged nanocrystals, CNCs produced by sulfuric acid and ammonium persulfate routes are charged negatively, with charge originating from surface bearing sulphate half-esters ($-OSO_3^-$) and carboxyl ($-COO^-$) groups, respectively. Suspensions of uncharged CNCs tend to flocculate or gel at low concentrations (< 0.5% w/v) due to strong interactions between particles. On the contrary, charged CNCs are readily dispersed in water and form stable colloidal dispersions (2–10% w/w) as a result of electrostatic repulsion between particles [87, 97, 101, 106]. However, increasing ionic strength of medium leads to their aggregation caused by shielding of the charges [107, 108].

Morphology and dimensions of CNCs depend mainly on the source of cellulose. Typically, nanocrystals with diameters of 2–20 nm and lengths of 100−600 nm are produced [88]. Compare to MFC, CNCs exhibits lower aspect ratio ranging between 10 and 100.

4.2.3 Bacterial cellulose

Bacterial cellulose (BNC) is produced by *Gluconacetobacter* bacterial strains, as a pure component of their biofilms in aqueous culture media containing source of sugar. The duration time of the process ranges from days up to two weeks [97]. In contrast to CNCs and MFC materials, BNC is obtained directly as a polymer by sequential assembling of low-molecular-weight carbon sources, such as *D*-glucose. BNC is also free from the other unwanted, ballast polymers. The resulting BNC hydrogel has unique properties, such as an extremely fine and pure fibre network structure (80–90% w/w), high degree of polymerization (DP up to 8 000), good mechanical properties in terms of high mechanical strength, biocompatibility, and water holding capability. It is composed of nanofibers with a width of 5–6 nm and a length of few micrometre [87, 88, 92].

4.3 Application of nanocellulose for emulsion stabilization

Various grades of nanocellulose particles, including MFC [109], CNCs [110, 111] and BNC [69, 112], have been successfully used as particulate stabilizer offering sustainable and potentially low-cost replacement of standard surfactants used for preparation of emulsions [113]. In comparison with other types of particulate stabilizers, nanocellulose have shown superior properties when it comes to biocompatibility, biodegradability, low density, thermo-mechanical behaviour and costs [93, 114].

Due to nanocellulose hydrophilic nature, o/w emulsions are preferentially formed [69, 109]. In order to produce w/o emulsions, suitable surface

modifications need to be introduced. These modifications involve for example silylation [76], polymer grafting, [115] or esterification [116]. In this context, the contact angle between a particle and a given liquid–liquid interface is also of great importance and can successfully be controlled by the degree and type of particle functionality. By this way, the emulsions with tailor-made properties for specific applications can be prepared. For example, using multifunctional proteins leads to a self-assembly properties of NFC [117] and grafting of CNCs with poly(*N*-isopropylacrylamide) or poly[2-(dimethylamino)ethyl methacrylate] affords temperature-sensitive or pH-responsive properties to emulsions [41]. Recently, preparation of multiple emulsions, particularly, oil-in-water-in-oil have also been reported by using both native and hydrophobized NFC and CNCs [74]. In addition to ability of cellulose particles to stabilize different types of emulsions, they are also reported to afford systems with reasonably good stability during storage, including those highly resistant to coalescence [118].

Among all three reported cellulose types, the behaviour of CNC particles as emulsion stabilisers is better described in literature and this cellulose type is also more frequently used for preparation of Pickering emulsions. Previous works, for example, clearly documented that unmodified CNCs can efficiently stabilize emulsions and perform better when having a low surface charge density, or when the surface charge is screened by the presence of salts in dispersion medium [119]. Furthermore, lower concentrations of CNCs are needed for stabilization of emulsion droplets when nanocrystals with higher aspect ratios are used. On the contrary, MFC-based emulsions have received less attention and it was reported that, due to its very high aspect ratio, MFC forms in emulsions strongly entangled and disordered networks. This stabilizing network is more evident at higher MFC concentrations and it contributes to overall stability of the emulsions [109, 120]. Regarding bacterial cellulose, its stabilizing properties are not yet fully explored [112], however BNC obtained by hydrochloric acid hydrolysis of bacterial cellulose were successfully used for stabilization of hexadecane/water interface [69].

Until now, the research on the Pickering emulsions have primarily been focused on model systems containing well-defined oils, such as hydrocarbons, as dispersed phase. Most of the studies worked with emulsions having a low content of oil phase, being in the range of 10−30% w/w. Trials to incorporate larger amounts of oil, high internal phase emulsions (HIPEs) with oil fractions higher than 90% w/w stabilized by less than 0.1% w/w CNCs have been recently reported [119]. Recently, several works have also been devoted to emulsion formulations containing oils derived from plant sources. For example, in study by Mikulcová et al. (2016) [110], CNC and MFC particles were used to stabilize emulsions loaded with up to 40% w/w limonene, cinnamaldehyde, or eugenol with more than 95% encapsulation efficiency. In another work, Wen et al. (2014) [111] reported on carboxylated CNCs prepared from corn-cob cellulose by oxidation with ammonium persulfate, which were then used to prepare d-

limonene emulsions. An observed reduction in stability with respect to creaming at low pH or high salt concentration was attributed to an electrostatic screening effect. Currently, CNCs obtained by sulfuric acid hydrolysis treatment of asparagus stems showed a satisfying stabilization for food grade palm oil emulsions [121]. In regard to stabilization properties of MFC, the particles isolated from mangosteen rind were used to prepare stable soybean oil-in-water emulsions at neutral pH [109].

In principle, previous studies have shown that various cellulosic particles derived from different sources, are capable of acting as stabilizing agents of emulsion systems. It was established that these particles adsorb at the oil-water interface, thus providing a steric barrier against droplet coalescence *via* the Pickering mechanism. Moreover, some works reported on contribution to the stabilization from an interconnected network of fibrils present is dispersion phase of emulsion [109, 120]. Despite the significant research progress on Pickering emulsions stabilized by cellulosic particles, more investigations are needed to be done for a better understanding of the surface properties, adsorption and aggregation of particles at the oil-water interface.

5. CHARACTERIZATION OF DISPERSION SYSTEMS

The complexity of the systems studied in this work is reflected by diversity and a broad range of characterization techniques employed, which are introduced in more details in the following sections. Emulsions were primary characterized by their particle sizes measured by photon correlation spectroscopy (PCS) and laser diffraction (LD) techniques. Physicochemical behaviour of the formulations was assessed by zeta potential analysis (ZP), particle charge density titration (PCD), and quartz crystal microbalance with dissipation monitoring (QCM-D). Phase stability of emulsions was studied using both visual observation and optical turbidity measurements. On a microstructural level, the systems were observed by optical microscopy (OM) and atomic force microscopy (AFM).

5.1 Particle size determination

The size and distribution of the particles within a colloidal systems is one of the most important aspects influencing the physicochemical characteristics and functional performance of formulation, including optical properties, rheology, release kinetics and long-term stability [1].

5.1.1 Photon correlation spectroscopy

Photon correlation spectroscopy (PCS), also referred to as dynamic light scattering (DLS), is widely used technique to determine the particle size distribution of colloidal systems with sizes from a few nanometres to several microns. PCS is a rapid, non-invasive and non-destructive technique, suitable for analysis of both dilute and concentrated dispersions (0.001 to 10% w/w), and requires only very small amount of sample (μg to mg quantities) without extensive sample preparation [1, 4, 122].

In the PCS technique, fluctuations in the intensity of scattered light while the particles undergo Brownian motion are analysed at a given angle as a function of time [123]. The frequency of these fluctuations depends on the speed at which the particles move, which again depends on their sizes. In general, small particles cause high intensity fluctuations as they move fast. On the contrary, large particles, with relatively slower motion, undergo lower fluctuations [1, 122]. The observed fluctuations are then analysed to obtain the correlation function and then to convert the characteristic decay of this correlation function into a diffusion coefficient distribution and a particle size distribution. The particle size is calculated by using the Stokes-Einstein equation, which allows to convert the diffusion coefficient to the particle hydrodynamic radius (R_H) as follows,

$$R_H = \frac{kT}{6\pi\eta D_T} \qquad (3)$$

In equation (3) k is Boltzmann's constant, T is the absolute temperature, D_T is the translational diffusion coefficient of the particles and η is the viscosity of the continuous phase of dispersion. It should be noted that for this conversion, the particles are assumed to be spherical and without mutual interactions [124-126]. If the particles are non-spherical, the equivalent or apparent radius of a sphere with the same R_H is calculated. The typical setup for a PCS instrument (Figure 7) consists of an optical unit (laser), a cuvette with sample, a digital correlator, and a detector [123, 126].

PCS allows accurate, reliable, and repeatable analysis of particle sizes in often less than one minute. However, PCS is mostly suitable for monodisperse samples. In case of systems containing particles of different sizes the technique is less reliable because of problems associated with interpreting the more complex autocorrelation decay functions [127, 128]. Additionally, for accurate measurements, transparent, and in general highly diluted, samples are required. At increased concentrations, only relative values of apparent size can be obtained as particle–particle interactions, multiple scattering and a level of turbidity become frequent and can influence the measurements. Common errors during measurements can also result from introduction of air bubbles, dust or use of an unclean cuvette, which can disturb the scattering pattern, and so can lead to artefacts in the analysis [122, 123, 127, 129].

PCS technique has become popular characterization tool across variety of fields, including biology, chemistry, and physics. It is useful for analysis of particles suspended in solvents such as polymers, biomolecules (proteins, nucleic acid), colloidal systems (emulsions, micelles), pigments etc. [124, 126, 130].

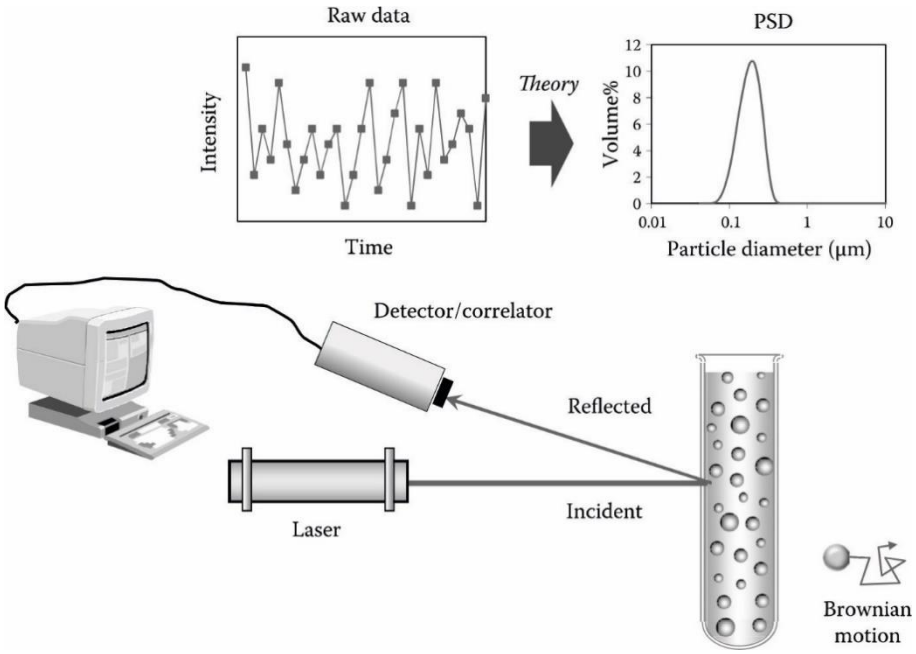

Figure 7 Setup of a PCS instrument [1].

5.1.2 **Laser diffraction**

Laser diffraction (LD) is the most frequently used method for determining the droplet size of emulsions. The LD technique is based on measurement of angular variation in intensity of light scattered as a laser beam passes through a dispersed sample. Principle of the method relies on the fact that large particles scatter light intensely at small angles relative to the laser beam whereas small particles scatter light weakly at large angles. The diffraction angle is, hence, inversely proportional to particle size, and recorded data from the angular dependence of scattering intensity are then used to calculate the size of the particles, with the help of the Mie theory. As a result, the particle size is obtained as a volume equivalent sphere diameter and expressed as (D[4,3]) [131-133].

The commercial LD instruments comprise the main optical unit with laser and detectors, one or more dispersion units (wet or dry), and a measurement cell (Figure 8) [132]. Before analysing a sample, the instrument is usually blanked by measuring the scattering profile from the continuous phase in the absence of sample. This scattering pattern is then subtracted from that of the sample to eliminate extraneous scattering from background sources other than the droplets, such as dust or optical imperfections [127]. The data is then interpreted by the application software to obtain information on particle size. The measurement range of the instruments usually covers particle sizes from 100 nm to 3 mm, in special set-ups up to about 10 mm or down to 30 nm. Main advantage of this technique is its large flexibility to different sample types, allowing to measure the particle size of dispersed dry powders, sprays, gas bubbles, suspensions, and emulsions. Dry powders and sprays can be measured directly without using any liquid dispersing medium. Liquid suspensions and emulsions are measured usually in a recirculating cell, which leads to high reproducibility with the possibility of adding dispersing agents and surfactants to ascertain the primary particle size. The size of the sample needed for analysis ranges from mg to gram quantities depending on particle size, with larger quantities required for dispersed dry powders [123, 132].

Although the LD is a simple, rapid and highly repeatable technique, its use has also certain limitations. Similarly to other scattering techniques, it is restricted mainly to the analysis of highly dilute samples 0.001–1% v/v, depending on their particle sizes. This technique is also less sensitive in case of analyses of non-spherical and highly polydisperse samples [122, 123, 131].

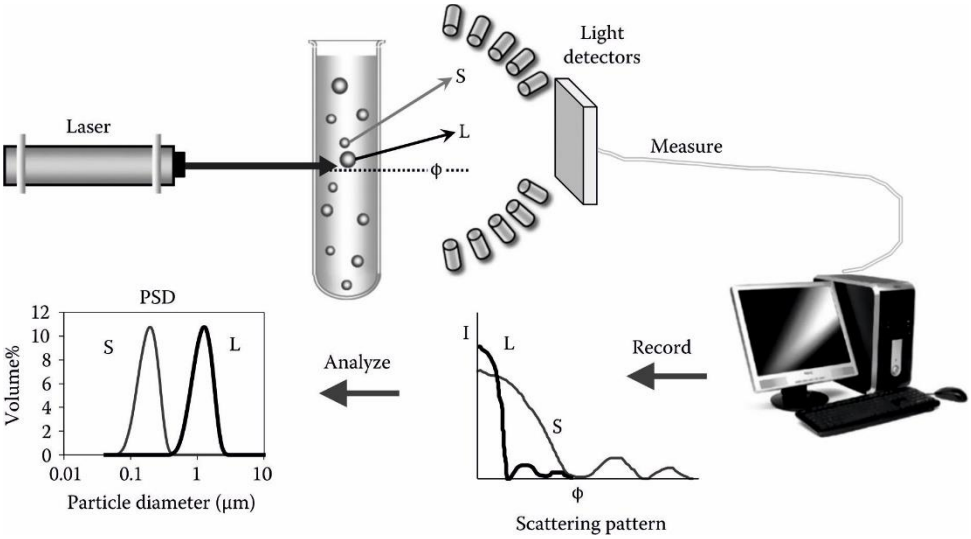

Figure 8 Setup of a LD instrument [1].

5.2 Characterization of electrical properties of emulsions

The particles in most colloidal systems usually possess an electrical charge induced primarily by the presence of ionized substances at their surfaces, such as ionic surfactants, phospholipids, free fatty acids, proteins, ionic polysaccharides, and mineral ions. As a result, electrostatic interactions are responsible for the overall stability and physicochemical properties of the system. The electrical properties of colloidal particles depend mainly on the type, concentration, and presence of ionized moieties at their surfaces, as well as on the ionic strength, pH and physical properties of the surrounding medium. A variety of analytical techniques are available for characterizing the electrical properties of colloids, including the surface charge density (σ) and the zeta (ζ)-potential [1, 127, 134].

5.2.1 Zeta potential analysis

The zeta potential (ZP) corresponds to the electrical potential at the so-called shear plane, which is defined as the distance away from the particle surface below which any counter-ions remain strongly attached to the particle while moving in an electrical field. The ZP can be determined by using a combination of particle micro-electrophoresis with dynamic light scattering measurements [1, 127]. In this technique, the charged particles move by applying an electrical field. The light scattered due to moving particles causes a frequency shift which is proportional to electrophoretic mobility [122]. The zeta potential of the spherical particles is then derived from the electrophoretic mobility (μ) by application of the Henry equation (4),

$$\mu = \frac{2\varepsilon\xi f(\kappa_a)}{3\eta} \qquad (4)$$

where ε is the permittivity, ξ is the zeta potential of the particles, η is the viscosity of the dispersion medium and $f(\kappa_a)$ is the Henry's function [135].

Simple scheme of ZP determination is showed in Figure 9. The sample is placed into a folded capillary cell, equipped with electrodes, and the movement of particles (electrophoretic mobility) towards the electrode of opposite charge is monitored by a laser beam [136].

In general, the ZP is a useful tool for predicting the stability and bulk physicochemical properties of colloids. The influence of pH, ionic strength, or composition of continuous phase on the ζ-potential often provides valuable insights into the major factors contributing to the stability of the dispersion systems. In practice, systems with high ZP values, either positive or negative (+30 mV or -30 mV), are normally considered to be stable with ability to prevent aggregation of the particles or droplets [127, 137]. The minimum concentration of the sample required for analysis is usually between 0.1 and 1 % w/v [136].

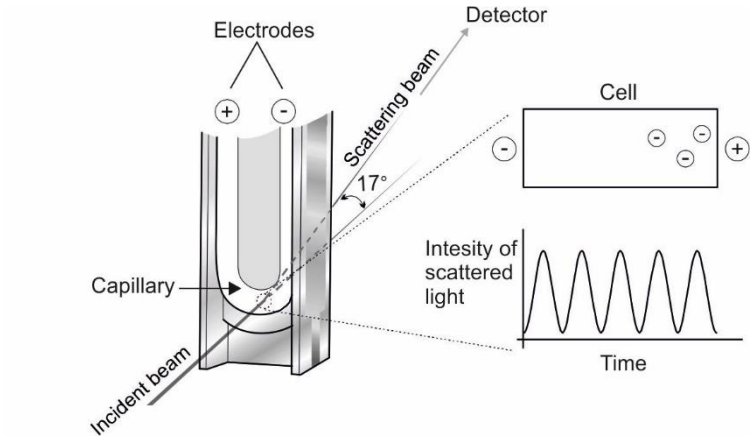

Figure 9 Scheme of zeta potential measurement [136].

5.2.2 **Particle charge density titration**

The particle charge density (PCD) titration is suitable for systems with fixed ionogenic groups on the surface of the particles or droplets [138]. During the analysis, the electrical charge at an interface is determined by titrating polyanionic liquids into the system and calculating the concentration of the ions that adsorb to the interface. For determination of the absolute interfacial charge using this technique, it is necessary to define a reference point of known charge, which is then used as the point of zero charge (0 mV) [1, 127]. The measurement is carried out by the piston-type streaming current device, where a streaming current is used for indication of the end-point of titration, at which all existing charges in the sample are neutralized. In practice, a measurement cycle takes of about 8–10 min [139]. Scheme of the PCD device, consisting typically of a plastic measuring cell with a fitted displacement piston, is given in Figure 10 [140]. Obtained surface charge density values represent the amount of electrical charge per unit surface area, and depend on the type and concentration of stabilizer present at the surfaces, as well as on the properties of the surrounding medium, such as pH, ionic strength, relative permittivity, and temperature [127, 134].

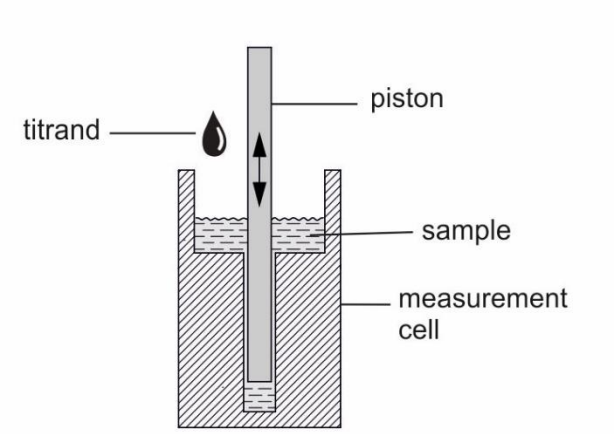

Figure 10 Scheme of the PCD device [140].

5.3 Quartz crystal microbalance with dissipation monitoring

Quartz crystal microbalance with dissipation monitoring (QCM-D) is an advanced technique for surface analysis, which provides a real-time measurement of adsorption of molecules and their interactions with rigid or soft surfaces in vacuum, gas or a liquid environment. The current QCM-D technique enables a precise measurement of adsorbed mass by collecting of changes in both frequency (related to mass/thickness) and dissipation (related to viscoelastic properties) of the quartz crystal. This allows to monitor and characterize thin films on a surface in terms of adsorption, desorption, molecular interactions and structural properties [141, 142].

The main component of the QCM-D device is the piezoelectric quartz crystal or sensor (Figure 11), which can be coated with different materials, for example, metals, metal oxides, polymers, or reactive surfaces. This offers wide range of opportunities for the studying of interactions or affinity between a sample and a certain surface. For high precision measurement, cleanness of the surfaces is critical. Commonly, a contaminated surface alters the affinity of the sample molecules to sensor, which can lead to measurement errors. Different coating layers require different cleaning methods, however a combination of UV/ozone and piranha solution treatment is mostly used [141].

The QCM-D method has a broad range of applications. In particular, it has been used to study *in situ* interactions involving surfactants, proteins, lipids, polymers, cells, bacteria and/or soft matter, by real time monitoring of changes in interfacial dynamics, surface roughness, viscoelasticity, density and mass [141, 143, 144]. Despite some limitations, including harsh working environmental conditions and complicated data analysis, the major advantage, compared with other commonly used surface study methods, is the high sensitivity of measurement (1 ng/cm^2) [141].

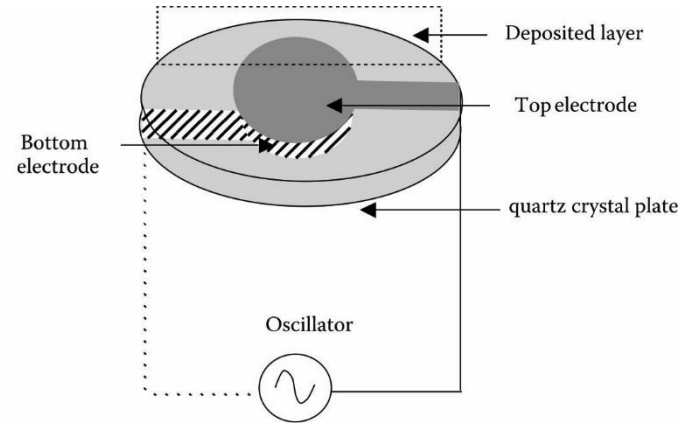

Figure 11 Schematic diagram of QCM-D[145] .

5.4 Phase stability studies

Emulsion are thermodynamically unstable systems with a significant tendency to undergo phase separation. One of the most common instability mechanisms, which occur in food and cosmetics, is the gravitational separation of emulsions [146]. Two common methods used in phase stability studies are simple visual observation by naked eye and instrumental method, laser dispersant tester.

5.4.1 Visual observation

The visual observation is the classical approach to monitor stability of emulsion systems. It is a method with an old tradition, which is still commonly used in research and development of emulsion formulations. By using this procedure, emulsion samples are placed in test tubes, which are stored at different temperatures/environments. For o/w emulsions, destabilization is then indicated predominantly by macroscopic separation of water phase and dispersed phase consisting of emulsion droplets, or by a complete breaking of emulsion into two or more phases. The phases are optically distinct to such degree that their height or volume can be estimated [129]. The destabilization of emulsions is often expressed in terms of the emulsion index (EI) (Figure 12a and Equation 5) or creaming index (CI) (Figure 12b and Equation 6). The visual observation is cheap and straightforward method, and its main limitations rely on the subjective reading and time consumption of the procedure [146, 147].

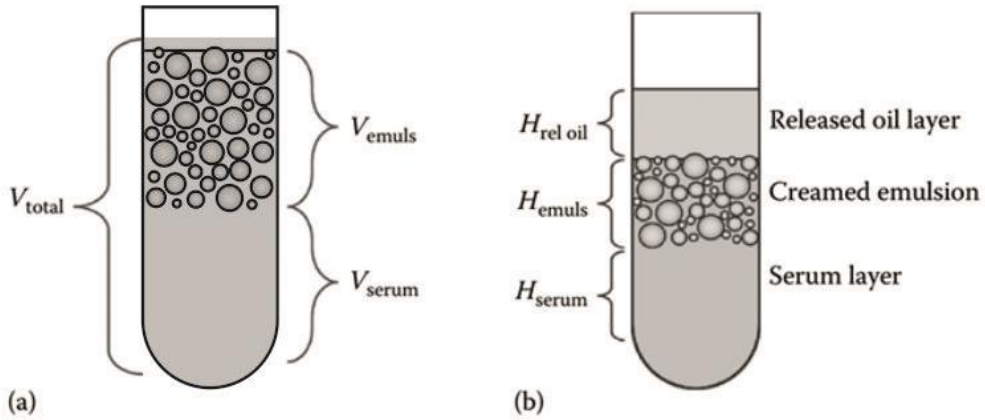

Figure 12 Test tubes showing principles for determination of (a) Emulsion index (EI); (b) Creaming index (CI) [146].

$$EI = \frac{V_{emuls}}{V_{total}} \cdot 100\% \qquad (5)$$

$$CI = \frac{H_{serum}}{H_{emuls}} \cdot 100\% \qquad (6)$$

5.4.2 **Laser dispersant tester (Turbiscan method)**

In order to avoid subjective interpretation of the visual assessments of the samples, various methods to determine the stability of emulsions have been developed. Many of the techniques are based on detection of light transmission, including a relatively recently developed technique, laser dispersant testing also called the Turbiscan method (Figure 13) [147]. This method uses a static multiple light scattering and allows to monitor the turbidity profile in various types of colloidal systems (e.g. emulsions, suspensions, foams), with concentration of dispersed phase up to 95% v/v, over a wide range of sizes (10 nm to 1 mm) as a function of time [147, 148]. During the test, a near infrared laser scanner (λ = 850 nm) moves vertically along the height of the test tube and analyses transmission and back scattering of light over the entire sample at different time intervals [147, 149]. As a result, the backscattering and transmission profiles are obtained, usually at ambient temperature. The stability profiles such as creaming, sedimentation, or phase separation rates, can be calculated over the whole sample height or in specific zones (bottom/middle/top), depending on the stability criteria [129, 148]. The main advantage of this method is providing a fast and accurate monitoring of processes related to aging of the samples in real conditions, as the measurement is performed without any mechanical or external stresses, and without any dilution. However, the measurement is limited to perform only one test at a time [147, 148].

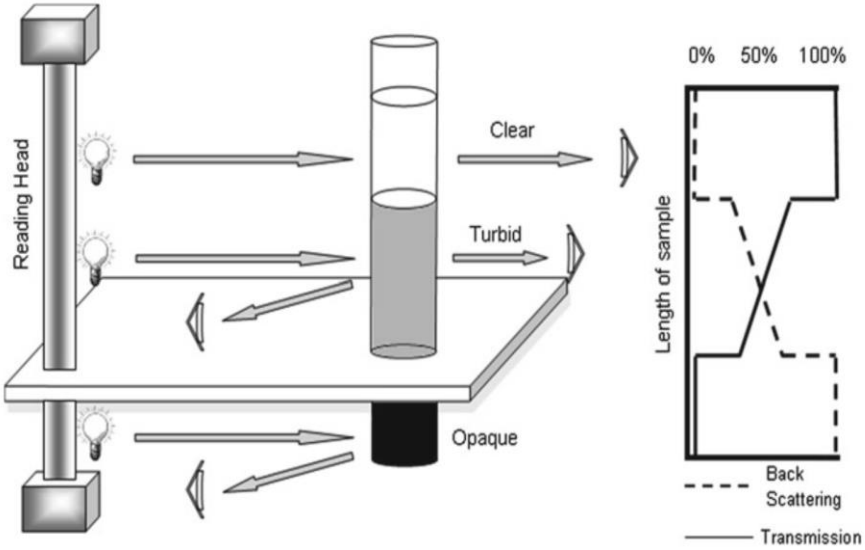

Figure 13 Scheme of the Turbiscan analyser [129].

5.5 Microscopic analysis

Microscopic analysis provides information on the size, morphology and organization of the particles within a colloidal system, which are important features in determining its physicochemical and functional properties. There are numerous microscopy techniques suitable for characterization of particles and structures in the colloidal size range, of which the optical, electron, and atomic force microscopy are most commonly used. The key parameters of any reliable microscopic analysis are resolution, magnification, and contrast [1, 127]. In the following section, optical microscopy and atomic force microscopy are discussed in more details.

5.5.1 Optical microscopy

Optical microscope (OM) remains still one of the most frequent instruments/devices used for observing the microstructure of colloids. Typically, it consists of a light source, a series of lenses, and an eyepiece or digital camera. The lenses direct the light waves through the sample and magnify the resulting image. Resolution of the image taken by microscope is governed by the wavelength of light used and the mechanical design of the device. In theory, the resolution of an optical microscope can be of around 200 nm. However, this is hardly to achieve in practice due to technical difficulties associated with the design and manufacture of the optical unit as well as Brownian motion of small particles. In fact, the resolution limit is often below approximately 1 μm. For this reason, optical microscopy has limited applications in case of visualization of colloidal systems containing relatively small particles. However, it can provide important information on shape, size and overall organization of particles within the systems containing larger particles [1, 127].

5.5.2 **Atomic force microscopy**

The atomic force microscopy (AFM), also referred to as the scanning force microscopy, is part of a large group of instrumental methods termed the scanning probe microscopy (SPM). The AFM is based on physical scanning of samples at submicron level by using a probe tip of atomic scale [150]. During the measurement, Coulomb, Van der Waals and other forces between the sample surface and a fine tip are detected to generate a high-resolution three-dimensional topographic image of the surface. In comparison with classical optical microscope, this technique possesses an extremely height sensitivity with reported resolution in the order of fractions of nanometre (~ 0.02 nm) [150-152]. Basic AFM set-up is illustrated in Figure 14.

Depending on the sample properties, contact or noncontact mode can be used for scanning. One of the major advantages of AFM is the ability to operate in air or fluid environment rather than in high vacuum, which allows the imaging of nanostructure and microstructure of biological and polymer samples in their native form. The other benefit of AFM technique is related to simple preparation of samples for imaging. In case of colloidal samples, one of the most common method involves a deposition of drops of colloids on a freshly cleaved mica surfaces and their drying under specific conditions [153]. In addition, the AFM can be also used for real time observations of processes *in situ*, for example, protein aggregation [127, 152]. In the study of colloidal dispersions, by using chemically functionalized probes, AFM techniques have been used to quantify surface forces from the interactions between particles and surfaces. Dependent on the experimental settings, a number of different types of interaction can be detected, including Van der Walls forces, electrical double layer forces, hydrophobic interactions, solvation forces, steric interactions, adhesion etc. [154, 155].

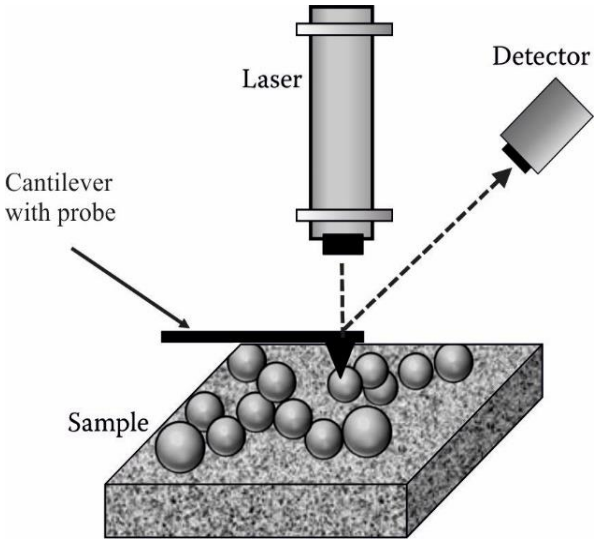

Figure 14 Basic simplified AFM set-up [127].

6. ANTIMICROBIAL TESTS

Various methods can be used to evaluate or screen the *in vitro* antimicrobial activity of bioactive compounds either in their virgin form or incorporated in emulsions. In this work, the disk-diffusion and broth dilution methods were used for antimicrobial evaluation of studied systems. The methods are briefly described in the following section.

6.1 Disk-diffusion method

The disk diffusion method (DDM) is a qualitative test which allows for classification of bacteria as susceptible or resistant to the tested sample. The antibacterial activity of the sample is determined according to size of diameter of the zone of inhibition formed after sample is in contact with bacterial species. This method has been widely used as a primary screening tool to assess the antimicrobial potential of a substances and it has been well-standardized [156]. Currently, accepted and approved standards for antimicrobial disk susceptibility test are published by European Committee on Antimicrobial Susceptibility Testing (EUCAST), which sets all the critical elements for the testing, such as the type and depth of a culture medium, density of inoculum, and incubation conditions (time, temperature and atmosphere) [157].

The test is performed on Mueller-Hinton (MH) agar plates inoculated with a standardized inoculum of microorganism at a density of McFarland turbidity standard being 0.5, which roughly corresponds to 1-2 $x10^8$ CFU/mL for *Escherichia coli*. Then filter paper discs (about 6 mm in diameter), containing the antimicrobial agent at a certain concentration, are placed on the agar surface [158, 159]. Alternatively, the antimicrobial compound may be applied to wells cut in the agar plate with a sterile cork borer [158, 160, 161]. The inoculated plates are incubated in aerobic conditions at 35±1°C for 16-20 hours. In general, antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganisms and then the diameters of inhibition growth zones are measured [158, 159].

The DDM is suitable for testing the majority of bacterial pathogens and antimicrobial agents, and absence of any special equipment needed for the tests have contributed to its common application in the antimicrobial screening of numerous substances, including plant extracts, essential oils and drugs [157, 158, 162-164]. However, the method provides only qualitative data and categorizes microorganisms as *susceptible, intermediate, and resistant* based on their susceptibility towards testing agent. The method has also another drawback. It is namely well documented that large molecules, such as vancomycin and highly lipophilic compounds such as triacylglycerols tend to diffuse poorly through solid media (agar plate), which could give the appearance of decreased antimicrobial action of such compounds [158, 165, 166].

6.2 Broth dilution method

Dilution methods are most suitable approaches for the determination of minimum inhibition concentration (MIC) values. The MIC is defined as the lowest concentration of the antimicrobial agent able to inhibit the visible bacterial growth, and it is usually expressed in mg/mL or mg/L [158]. In broth-dilution methods, turbidity is most frequently used for determination MIC endpoint, and can be estimated visually or measured spectrophotometrically [156]. There are many different guidelines approved for dilution antimicrobial susceptibility testing. The most generally accepted standards are those provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and International Standards Organization (ISO) [5, 167, 168].

Following these standards, the antibacterial agent is serially diluted and a single concentration is added either to culture tube (minimum volume of 2 mL – macrodilution) or 96-well microtitration plate (50 to 150 μL volume – microdilution) containing nonselective broth medium. Each tube or well is then inoculated in presence of standardized microbial suspension adjusted to 0.5 in the McFarland scale. After well-mixing, the inoculated tubes or microtitration plates are incubated under suitable conditions depending upon the tested microorganism [2, 158, 160].

The principal disadvantages of the macrodilution method is the manual task of preparing the antimicrobial agents solutions for each individual test, the possibility of errors in preparation of the antimicrobial agents' solutions, and the relatively large amounts of reagents and space required for single test. This could be overcome by using microdilution methods that utilize small volumes of reagents and allow relatively rapid testing of a large number of bacteria [2, 159].

7. EVALUATION OF THE STUDIED EMULSION SYSTEMS

In this work, two main types of emulsion-based systems, conventional and Pickering emulsions, have been prepared and studied. This part of the thesis will provide their brief and critical evaluation.

For a long time, conventional emulsions have dominated across the industries due to their easy use, relatively low costs and controlled preparation. However, in recent years, emulsions stabilized solely by solid particles, have attracted more attention. Micro- or nano-sized particles, as the main components of the Pickering emulsion, affect not only the preparation, but also the potential applications and properties of resulting formulations.

During research conducted within this doctoral thesis, surfactant-based and particle-based emulsions, each one consisting of antimicrobial oil of plant origin, were successfully prepared [110, 162]. Owing to different mechanism of stabilization, as described previously, these systems differ in various aspects such as type of stabilizing agent (surfactants *vs* particles) and its concentration needed for successful droplet stabilization, emulsifying properties, emulsification techniques needed for their preparation, encapsulated volume of oil phase, size of emulsion droplets, long-term stability, intended application etc. Some of these key features are discussed in the context of experimental results from the doctoral study.

To begin with, selection of suitable emulsifying agent required to produce stable classical emulsions is based on HLB system, whereas the crucial parameter for preparation of Pickering emulsions is the particle contact angle [52, 65]. In our work, classical o/w emulsions were formulated by employing pairs of non-ionic surfactants (Span, Tween) at HLB values ranging from 6 to 10, in order to find an optimum HLB for best performances in emulsification process and stability studies. In case of the study conducted on the emulsions stabilized by particles, naturally hydrophilic cellulose particles with water contact angle of approximately 50° favouring formation of o/w emulsions were selected [21]. The overall results revealed that type of the stabilizing agent, whether they are emulsifiers or particles, and its concentration play an important role in both conventional and Pickering emulsions. In particular, for classical emulsions the most efficient emulsification was observed at 10% w/w concentration of surfactants. By comparison, only relatively small particle concentrations were required to successfully stabilize Pickering emulsions. It has been shown that up to 40% w/w oil can be safely encapsulated by using of only 0.5% w/w of cellulose particles. This contrasted with classical emulsions, where at least 5% w/w of surfactants concentration was required for stabilization of 5% w/w oil content. Thought the antimicrobial oils used in both types of emulsions were of different origin, which will influence performance of resulting emulsions, this comparison indicates that particle stabilized emulsions might be of advantage with respect to

reduction of presence and amount of surfactants in cosmetic products, which are known for their irritation potential.

Regarding the long-term stability, particle-stabilized emulsions are considered to be of superior stability to classical emulsions, which has also been confirmed by our studies. Once prepared, Pickering emulsions showed superior stability irrespective of used type of cellulose particles and their concentrations. In case of conventional emulsions, storage stability period ranged from few days (10% w/w Tween 85/Span 85-based systems at HLB 7) to several months (10% w/w Tween 80/Span 80-based systems at HBL 9). Excellent long-term stability provided by Pickering emulsions is well-known and it is mainly attributed to the strong binding energies of the particle stabilizers with the interface, which arise from the relatively large size of solid particles (typically between 100 nm and 5 μm), when compared to surfactant molecules (0.4 to 1 nm) [10]. As demonstrated Binks et al. [52], surfactants adsorb at the interface less strongly then particles and produce emulsions with a reduced long-term stability.

Interestingly, conventional emulsifiers usually yielded smaller emulsion droplets compared to particles [9]. For example, in our studies, using Span–Tween systems resulted in droplet size ranging from 80 to 650 nm depending on the emulsification process. By comparison, the mean diameter of the droplets obtained when using cellulosic particles as stabilizer was of about 14–50 μm. This is, however, natural as stabilizing cellulose particle are notably bigger in their sizes in comparison with molecules of surfactants.

The other specific feature of the particles used as stabilizers is the fact that shape and size of particles influence the properties of the resulting emulsions [61]. For instance, in our study, emulsions prepared with smaller CNC particles afforded smaller droplets (14–34 μm) compared to those stabilized with larger MFC particles (27–51 μm). Additionally, the shape of the particles influenced the properties and performance of emulsions as well and contributed, for example, to antibacterial properties of the emulsions, especially at low content of antimicrobial oil. With this respect, CNC rod-shaped particles provided a more homogenous layer at the oil-water interface thus preventing oil leakage from the dispersed phase of emulsions into their continuous phase.

Another great advantage of using particles instead of surfactants is the possibility to modify the properties of particle surfaces and thus afford functionalities that are difficult to achieve in conventional emulsions. In this respect, stimuli-responsive systems, which properties can be altered by external stimuli, such as temperature, pH, ionic strength, electric/magnetic fields, or light, has become increasingly important [169]. The interest in such responsive systems is reflected, for instance, in our systematic study on the pH-dependent behaviour of emulsions stabilized with carboxylated cellulose nanocrystals (cCNC) [170]. The employed cCNC was prepared *via* a one-step oxidation procedure of cellulose by APS, thus introducing carboxylic acid groups on the particles surface. By this way, a certain pH responsiveness to the colloidal suspension can be imparted,

which in turn has a key impact on colloidal behaviour of cCNC in environment with various pH values and ionic strengths.

In conclusion, the presented studies unambiguously documented that Pickering emulsions are promising systems, which may exhibit some unusual, advantageous features compared to classical emulsions. From an application perspective, biopolymer-based particles used as stabilizing agents are of particular interest. These particles are cheap, abundant in nature and less harmful alternative to conventional surfactants. For these reasons, the field of Pickering emulsions is recently receiving much attention both from the research and also industrial community. On the other way, emulsion systems stabilized with classical emulsifiers are still prevailingly used in practice and probably will never be fully replace by Pickering emulsions. Both emulsion types have their advantages and disadvantages and users should always critically evaluate the choice of the stabilizer, which is suitable for given application.

8. AIMS OF THE DOCTORAL STUDY AND THEIR FULFILMENT

The doctoral study "Dispersion systems as carriers of active substances" is focused on the formulation, preparation and characterization of the suitable particular systems with the ability to carry, effectively encapsulate and release active substances. The main goals of the work have been subdivided into three following areas covering different types of systems addressed:

8.1 Emulsions stabilized with surfactants

a) Preparation of (nano)emulsion loaded with hemp seed oil by different emulsification methods (spontaneous emulsification *vs* high-intensity stirring) and comparison of the resulting emulsions in terms of their long-term stability and droplet size/distribution determined by photon correlation spectroscopy (PCS).

b) Simultaneous monitoring of the effect of different physicochemical parameters, such as oil type (refined, unrefined), o/w ratio, HLB value, surfactant type and concentration on the parameters given above.

c) Determination of antibacterial activity of the emulsions and hemp seeds oils used therein against common pathogenic bacteria.

This goal of the thesis was completed and results have been published in the paper Formulation, Characterization and Properties of Hemp Seed Oil and Its Emulsions in the Molecules in 2017.

8.2 Emulsions stabilized with particles

a) Preparation of cellulose nanocrystals (CNCs), intended for stabilization of emulsions, by using different procedures, concretely oxidation of microcrystalline cellulose (MCC) with ammonium persulfate or its hydrolysis with sulfuric acid**.** Comparison and characterization of prepared nanocrystals in terms of their morphology (AFM), particle size and zeta-potential (PCS) as well as particle charge density (titration, particle charge detector). Assessment of behaviour of the CNCs in aqueous suspensions in presence of salts or under different pH.

b) Formulation of Pickering emulsions containing three different antimicrobial oils (cinnamaldehyde, eugenol and limonene) stabilized with CNCs prepared by hydrolysis route and commercial MFC. Evaluation of the effect of oil type, o/w ratio, type and concentration of cellulose particles on the physicochemical characteristics of the emulsions, including their stability during storage. Testing of

antibacterial activity of the emulsions against common pathogenic bacteria.

c) Preparation of pH responsive triacylglycerol-in-water emulsions stabilized with carboxylated cellulose nanocrystals (cCNC) suited as carriers for lipophilic active ingredients in cosmetics and pharmacy. Physicochemical characterization of prepared emulsions by droplet size and zeta-potential analyses, phase behavior and determination of their pH responsiveness.

d) Formation of model Pickering emulsions stabilized with binary mixtures of two different types of cellulose particles, namely MFC and CNCs, and their comparison with systems where each of the particle types was used individually. Examination of the influence of different physicochemical parameters, including type and concentration of cellulose particles, mass ratio MFC/CNCs in the mixtures, and o/w ratio on the size and distribution of emulsion droplets (laser diffraction), encapsulation efficacy and phase stability (optical microscopy).

e) Introductory studies into biological properties of Pickering emulsions stabilized with CNCs or MFC and loaded with antibacterial oils (limonene, eugenol, cinnamaldehyde). Examination of transdermal absorption of these lipophilic active ingredients from MFC and CNCs emulsions by using the Franz diffusion cells on porcine skin permeation model. Examination of cytotoxicity of the emulsions on selected cell lines.

The tasks a) to c) were completed and resulted in two publications:
1)"On the preparation and antibacterial activity of emulsions stabilized with nanocellulose particles" published in the Food Hydrocolloids, 2016 and 2)"Pickering oil-in-water emulsions stabilized by carboxylated cellulose nanocrystals - effect of the pH" submitted to Carbohydrate Polymers.
In case of task d), results were obtained and publication is under preparation. Regarding the task e), work is in progress.

8.3 Emulsion-coated surfaces

a) Preparation of surfaces coated with emulsion droplets by employing soft surface treatment, with the first layer formed by chitosan and the second one consisting of cCNC-stabilized emulsions containing essential oils.

b) Real-time monitoring of adsorption processes by quartz crystal microbalance with dissipation monitoring (QCM-D). Characterization of prepared surfaces and determination of their antibacterial properties.

This goal of the thesis is still in progress.

LIST OF PAPERS

Publication I
MIKULCOVÁ, V., KAŠPÁRKOVÁ, V., HUMPOLÍČEK, P. and L. BUŇKOVÁ, Formulation, Characterization and Properties of Hemp Seed Oil and Its Emulsions. *Molecules*, 2017. **22(5)**: p. 700.

Publication II
MIKULCOVÁ, V., BORDES, R. and V. KAŠPÁRKOVÁ, On the preparation and antibacterial activity of emulsions stabilized with nanocellulose particles. *Food Hydrocolloids*, 2016. **61**: p. 780-792.

Publication III
MIKULCOVÁ, V., BORDES, R., MINAŘÍK, A. and V. KAŠPÁRKOVÁ, Pickering oil-in-water emulsions stabilized by carboxylated cellulose nanocrystals - effect of the pH. *Submitted to Carbohydrate Polymers*.

Publication IV
KEJLOVÁ, K., KAŠPÁRKOVÁ, V., KRSEK, D., JÍROVÁ, D., KOLÁŘOVÁ, H., DVOŘÁKOVÁ, M., TOMÁNKOVÁ, K. and V. MIKULCOVÁ, Characteristics of silver nanoparticles in vehicles for biological applications. *International Journal of Pharmaceutics*, 2015. **496(2)**: p. 878-885.

SUMMARY OF THE PAPERS

Paper I was focused on the formulation and characterization of hemp seed oil emulsions stabilized with pairs of non-ionic surfactants, prepared by low energy (emulsion inversion point) and high energy (high-intensity stirring) methods. The goal of this paper was to determine the influence of composition (HLB value, concentration of surfactants and content of encapsulated oil) and preparation procedure on properties of formulated emulsions, including their stability. In addition, the antibacterial potential of the hemp seed oil and prepared emulsions against common pathogens was evaluated.

It was found that the formation of stable emulsions of small, initial particle size was primarily dependent on the given method of preparation and the HLB value of the used surfactant pairs. In particular, the high-energy method led to efficient emulsification that afforded the emulsions with fine particles (151 ± 1 nm). Regarding the long-term stability, emulsions prepared at HBL 9 with 10% w/w concentration of surfactants performed best.

Testing of antibacterial properties of the oils using the disk diffusion and broth microdilution methods revealed the inhibition effects against *Micrococcus luteus* and *Staphylococcus aureus,* subsp. *aureus*. However, the emulsions formulated did not exhibit the antibacterial activity that had been anticipated.

In **Paper II**, Pickering emulsions containing antimicrobial oils (cinnamaldehyde, eugenol and limonene) stabilized with cellulose particles (CNCs and MFC) were successfully prepared and the effects of the oil type, o/w ratio, type and concentration of cellulose on the physicochemical characteristics of the emulsions, such se droplet size and phase behavior, were determined. The antibacterial activity of emulsions towards the most common gram positive and gram negative bacteria was investigated using agar diffusion and broth dilution methods.

The results showed that both CNC and MFC were capable to produce o/w Pickering emulsions with antimicrobial oil content as high as 40% w/w even at the lowest concentration of cellulose used 0.1% w/w. It was also found out that emulsion droplets stabilized with CNC particles were smaller than those stabilized with MFC. The prepared emulsions showed reasonably good stability during the 8-week storage in terms of changes in droplet size as well as occurrence of creaming or sedimentation.

Antibacterial testing revealed that the activity of emulsions was mainly influenced by the type and content of antibacterial oil loaded in emulsion droplets. The type of nanocellulose particles used for droplet stabilization showed, in this respect, only a minor contribution which was observed mainly at high concentrations of emulsions. In contrast, at low concentrations of emulsion, the effect of nanocellulose type appeared to be more important and MFC-stabilized emulsion exhibited better antibacterial activity.

Paper III was aimed at formulation of pH responsive triacylglycerol-in-water emulsions stabilized with carboxylated cellulose nanocrystals (cCNC). The effect of varying pH of dispersion phase (pH 2, 4, 7) on the particle characteristics and emulsion properties was studied and established. In the first step of the work, nanocrystals of carboxylated cellulose were prepared by oxidation of microcrystalline cellulose with ammonium persulfate and their physicochemical properties were determined, including behaviour in media with different pH and ionic strength, and visualization of particles by AFM.

In the next step, the emulsions were prepared. The results revealed, that using cCNC, stable Pickering triglyceride-in-water emulsions at pH of 2, 4 and 7 can be formulated. The size of emulsions droplets was influenced by oil and cCNC contents. Nevertheless, the most crucial parameter for emulsion formation was the pH value of the continuous phase and it was revealed that droplet size and stability of emulsions are governed just by the pH. At the same time, responsiveness of emulsions towards pH changes was not as dominant as expected, and pH variation did not trigger the release of oil from droplets. The strong adsorption of the cCNC onto the droplet surface, relatively polar triglyceride oil used in the droplets, and the limited ability to induce desorption of nanocrystals from oil surface could be possible explanation for this finding.

Paper IV

Main subject of this study was to assess behavior of silver nanoparticles (AgNPs) in various media used for testing of their biological properties. The behavior of AgNPs in terms of size and distribution was determined using two independent methods, photon correlation spectroscopy (PCS) and transmission electron microscopy (TEM). The particles were subjected to contact with culture medium with/without serum (DMEM) and phosphate buffered saline (PBS).

Comparison of PCS and TEM analyses showed that both techniques provided qualitatively similar information with respect to characterization of the tested AgNPs, however the size of particles measured by PCS was bigger in absolute values. The study revealed that during exposure of AgNPs to the PBS and DMEM without serum, the agglomeration process occurred leading to the growth of the particles. The effect was due to the presence of ions in the dispersion medium. Interestingly, although the average size of the particles increased, width of the size distribution was not substantially changed during on-going agglomeration and only negligible distribution broadening was observed. Behavior of AgNPs in both serum free and serum added DMEM exhibited comparable behavior in terms of particle size distribution. However, the impact of serum proteins as such cannot be omitted. This study contributed to increased knowledge of AgNPs behavior in contact with physiologically relevant liquids. This can serve as a supporting information when using the AgNP in dispersion formulations to enhance their antibacterial properties.

CONTRIBUTION TO SCIENCE AND PRACTICE

The most important contributions of the doctoral thesis to science and practice can be summarized as follows:

- Better understanding of the preparation of emulsions by low energy (emulsion inversion point) and high energy (high-intensity stirring) methods; developing of an optimized formulation possessing good long-term stability.
    - *The outputs gathered within this part of the thesis can be used under preparation of cosmetic and food formulations containing triacylglycerol-based oils.*

- The mastering of the preparation of surfactant-free emulsions stabilized by solid particles intended for cosmetic or food applications.

- The development of novel antibacterial Pickering emulsions containing essential oils and stabilized by different cellulosic nanoparticles.
    - *The work related to Pickering emulsion brings the increased knowledge within theory and understanding of these systems and provides evidence of their practical applications in antibacterial protection.*

- The successful preparation of carboxylated cellulose nanocrystals containing −COOH groups on the surface by low-energy one-step procedure; clarification of their colloidal behaviour at various pH and ionic strength.

- The formulation of pH responsive triacylglycerol-in-water emulsions stabilized with cCNCs suited as carriers for lipophilic active ingredients in cosmetics and pharmacy.
    - *This part of the work notably contributed to description of encapsulation systems which are responsive to external stimuli, in this case pH. In practice, the pH responsiveness can be used for cosmetic, pharmaceutical and food applications.*

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LIST OF ABBREVIATIONS

AFM	atomic force microscopy
AgNPs	silver nanoparticles
AGU	anhydrous *D*-glucose units
APS	ammonium persulfate
BNC	bacterial nanocellulose
cCNCs	carboxylated cellulose nanocrystals
CI	creaming index
CNCs	cellulose nanocrystals
DLS	dynamic light scattering
DMEM	Dulbecco's Modified Eagle's medium
DP	degree of polymerization
EI	emulsion index
HIPEs	high internal phase emulsions
HLB	hydrophilic-lipophilic balance
LAI	lipophilic active ingredients
LD	laser diffraction
MCC	microcrystalline cellulose
MFC	microfibrillated cellulose
OP	optical microscopy
PBS	phosphate buffered saline
PCS	photon correlation spectroscopy
PCD	particle charge density
PLA	poly(lactic acid)
PLGA	poly(lactic-*co*-glycolic acid)
QCM-D	quartz crystal microbalance with dissipation monitoring
SLNs	solid lipid nanoparticles
SPM	scanning probe microscopy
TEM	transmission electron microscopy
ZP	Zeta potential

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2. MIKULCOVÁ, V. AND V., KAŠPÁRKOVÁ Undecane-in-water Emulsions prepared by the Phase Inversion Temperature Method. 15th European Student Colloid Conference, Krakow, June 8–11, 2015.
3. MIKULCOVÁ, V., KAŠPÁRKOVÁ, V. AND A., HAMANOVÁ Behaviour of binary surfactant mixtures based on N-lauroylsarcosine sodium salt. 6th International Colloids Conference, Berlin, June 19–22, 2016.
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PAPER I

Formulation, Characterization and Properties of Hemp Seed Oil and Its Emulsions

Veronika Mikulcová, Věra Kašpárková, Petr Humpolíček, Leona Buňková

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Article

Formulation, Characterization and Properties of Hemp Seed Oil and Its Emulsions

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Abstract: The formulation, characterization, and anticipated antibacterial properties of hemp seed oil and its emulsions were investigated. The oil obtained from the seeds of *Cannabis sativa* L. in refined and unrefined form was characterized using iodine, saponification, acid values, and gas chromatography, and was employed for the preparation of stable oil-in-water emulsions. The emulsions were prepared using pairs of non-ionic surfactants (Tween, Span). The effects of the emulsification method (spontaneous emulsification vs. high-intensity stirring), hydrophilic lipophilic balance (HLB), type and concentration of surfactant, and oil type on the size and distribution of the emulsion particles were investigated. It was found that the ability to form stable emulsions with small, initial particle sizes is primarily dependent on the given method of preparation and the HLB value. The most efficient method of emulsification that afforded the best emulsions with the smallest particles (151 $\pm$ 1 nm) comprised the high-energy method, and emulsions stable over the long-term were observed at HBL 9 with 10 wt % concentration of surfactants. Under high-intensity emulsification, refined and unrefined oils performed similarly. The oils as well as their emulsions were tested against the growth of selected bacteria using the disk diffusion and broth microdilution methods. The antibacterial effect of hemp seed oil was documented against *Micrococcus luteus* and *Staphylococcus aureus* subsp. *aureus*. The formulated emulsions did not exhibit the antibacterial activity that had been anticipated.

Keywords: hemp seed oil; emulsion; particle size; fatty acid composition; antibacterial activity

1. Introduction

Hemp seed oil, obtained from the seeds of *Cannabis sativa* L., is known for its nutritive, health-enhancing properties and bioactivity. Compared to other vegetable oils, it is an especially rich source of both n-3 and n-6 essential fatty acids, namely linoleic acid (18:2 n-6, at 55 wt %) and alpha-linolenic acid (18:3 n-3, at 20 wt %). The content of gamma-linolenic acid (18:3 n-6) equals approximately 1–4 wt %, while that of stearidonic acids (18:4 n-3) ranges from 0.5–2 wt % [1]. Of no less importance is that hemp seed oil contains a moderate to high amount of tocopherols and tocotrienols (100 to 150 mg per 100 g of oil), phytosterols, phospholipids, carotenes, and minerals [2]. Interestingly, the aforementioned beneficial properties of hemp seed oil offer numerous potential applications, e.g., as components in functional foodstuffs and in treating various health problems. Here, the lowering of high cholesterol and high blood pressure can be named as examples [3,4]. The health benefits of

hemp seed oil are attributed mainly to its desirable n-3 and n-6 fatty acid ratio, 3:1, which is suggested as being optimal for human nutrition [5,6]. It has been shown that unbalanced intake of n-3 and n-6 fatty acids is associated with many diseases, e.g., diabetes, cardiovascular diseases, and cancer. The presence of gamma-linolenic acid in the oil, which is deficient in the average Western diet, is also noteworthy [6]. This unique composition of hemp seed oil differs from the other common seed oils and offers opportunities for the development of special nutritional formulations [7–9]. However, besides the well-documented health and nutrition effects, the antimicrobial and specifically the antibacterial effects of hemp seed oil still remain questionable [10,11].

Although many studies have focused on preparing oil-in-water (O/W) emulsions consisting of either mineral or synthetic oils, less attention has been paid to their formation in the presence of vegetable oils as a dispersed phase. Moreover, most studies and applications of vegetable oil-based emulsions involve one of the following commonly used oils; palm, soybean, rapeseed, sunflower, coconut, palm-kernel, cottonseed, groundnut, and olive [4,12,13]. However, with the development of the nutraceutical and functional foods market, more attention is now devoted to non-traditional vegetable oils and their encapsulation in emulsions. Examples include argan oil [14], pomegranate seed oil [15], and grapeseed and sesame oils [16]. In this context, the application of hemp seed oil in emulsions, which might further enhance its beneficial properties, has not been fully exploited. Due to reduced sizes of droplets and high surface to volume ratio, loading of the oil in nanoemulsions may improve the bioavailability of present unsaturated fatty acids [7], help to protect the oil against oxidation and interaction with other ingredients, and contribute as a dietary source of natural antioxidants for disease prevention and health promotion [9,17]. Only a few studies have actually dealt with hemp seed oil emulsions (water-in-oil) while, as far as the authors are aware, even less research has concentrated on oil-in-water emulsions [18,19].

The past few years have also witnessed a widening in the utilization of oils from plant sources into non-food areas, such as the pharmaceutical and cosmetics industries, due to their qualities of being non-toxic, biodegradable, and environmentally friendly. This is reflected in current research on the exploration of vegetable-oil-based emulsions [20,21]. The aim of this paper was to prepare stable oil-in-water emulsions based on hemp seed oil, and determine the influence of emulsion composition and preparation methods on their characteristics, including stability. In this regard, the quality of two brands of oil extracted from hemp seeds was investigated to describe differences between the properties of refined and unrefined oil when utilized in emulsions. Research was also carried out on the possible bioactivity of the hemp seed oils and their emulsions as pertaining to their antibacterial properties against common pathogens.

2. Results and Discussion

Prior to preparing the emulsions, basic characterization of the hemp seed oils was carried out in order to elucidate differences between their refined and unrefined types. The iodine values of 155.8 ± 1.9 and 167.4 ± 0.6 g iodine/100 g of oil were determined for refined and unrefined oil, respectively. Correspondingly, the saponification values of 197.6 ± 4.5 and 202.2 ± 3.9 mg KOH/g of oil, and acid values of 1.7 ± 0.6 and 0.7 ± 0.0 mg KOH/g of oil were measured. Comparing the obtained results with the data published by Anwar et al. [22] revealed reasonably good compliance. The published iodine values were quite similar to those herein, ranging from 154 to 165 g iodine/100 g oil and the saponification values were slightly lower (184–190 mg KOH/g of oil). The fatty acid composition of the oils is provided in Table 1. The main differences between the contents of particular fatty acids pertained to oleic and alpha-linolenic acids. Regarding the content of alpha-linolenic acid in unrefined and refined oils, values of 20.3 ± 0.03 wt % and 16.7 ± 0.04 wt %, respectively, were measured. The content of oleic acid was 12.1 ± 0.03 wt % and 9.0 ± 0.1 wt % for the unrefined and refined samples, respectively. However, the variations observed did not obviously deviate from the fatty acid contents reported for a range of hemp seed oils by different authors [22,23]. It is therefore apparent that oil composition prevailingly depends on its origin and/or the extraction procedure

used to obtain it. Unrefined hemp seed oil also possessed trace concentrations of eicosadienoic and myristic acids.

Table 1. Composition of fatty acids (g/100 g) in unrefined and refined hemp seed oils determined by gas chromatography (GC).

Fatty Acid	Concentration (g/100 g)	
	Unrefined Oil	Refined Oil
Myristic	0.04 ± 0.03	n.d.
Palmitic	5.9 ± 0.27	6.2 ± 0.13
Palmitoleic	0.1 ± 0.00	0.1 ± 0.02
Stearic	2.2 ± 0.04	2.4 ± 0.07
Oleic	9.0 ± 0.15	12.1 ± 0.03
Linoleic	55.3 ± 0.12	57.3 ± 0.03
gamma-linolenic	4.4 ± 0.03	3.0 ± 0.02
alpha-linolenic	20.3 ± 0.03	16.7 ± 0.04
Arachidic	1.7 ± 0.04	1.0 ± 0.04
Eikosanoic	0.7 ± 0.05	0.8 ± 0.00
Eikosenoic	0.4 ± 0.02	0.4 ± 0.02
Eikosadienoic	0.1 ± 0.01	n.d.

n.d.: not determined.

2.1. Formulation of Emulsions

The O/W emulsions were prepared by two different emulsification procedures; these also involved varying the types and concentrations of emulsifiers, with the hydrophilic-lipophilic balance (HLB) ranging from 6 to 10, as well as utilizing two types of hemp seed oil. Key characteristics of an emulsion are the size and distribution of the emulsion particles, as their changes indicate the stability of the formulation. As expected, it was discerned that particle size and distribution were notably influenced by the method of preparation. This is illustrated in Figure 1, which shows the particle size of emulsions and methods for preparing the same, relating to the samples based on Span 85/Tween 85.

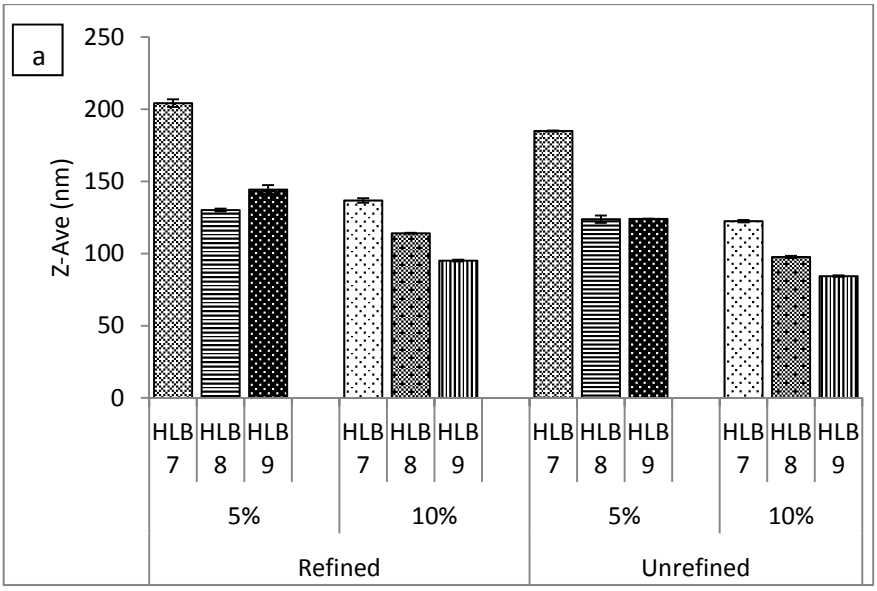

Figure 1. *Cont.*

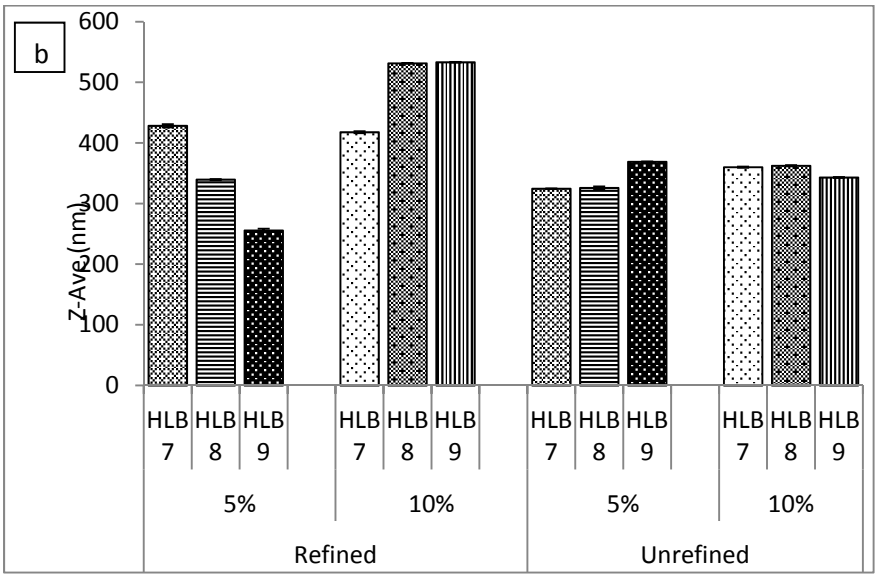

Figure 1. Effect of oil type (refined vs. unrefined), hydrophilic lipophilic balance (HLB), and surfactant concentration on the particle size of emulsions prepared by (**a**) high-energy method and (**b**) low-energy method. Tween 85/Span 85 ratios in the emulsions were 1.30 (HLB 7), 2.07 (HLB 8), and 3.60 (HLB 9).

As can be seen, the diameters of the particles present in all emulsions prepared by the high-energy method were significantly smaller ($p \leq 0.001$), regardless of HLB values, than the emulsions produced by the low-energy EIP (emulsion inversion point) method. This observation is in agreement with a study published by Ostertag et al. [24]. In this study, only relatively large droplets (>600 nm) were formulated by the EIP method using long-chain triglyceride oils (such as olive, grape seed, sesame, peanut, and canola oils). Similar findings were reported by Gullapalli and Sheth [25], who found that non-ionic emulsifiers reduced the particle size of hydrocarbon-in-water emulsions more effectively than triglyceride-in-water emulsions. Recently, exotic vegetable oil-in-water nanoemulsions with the addition of ethoxylated and acetylated lanolin have been obtained by using a low energy EIP method. It has been shown that the lanolin derivative addition caused alterations of droplet size and conductivity of the systems, however the droplet size remained still within the nanometer range (20–200 nm) [26]. Lane et al. [27] reported on flaxseed and algae nanoemulsions formulated with combinations of Tween 40 and lecithin. Stable emulsions were prepared up to 50 wt % of oil content with a droplet size of 192 nm for flaxseed-oil and 182 nm for emulsions loaded with algae oil. In the study of Krasodomska and Jungnickel [28], various seed oils (apple, strawberry, and raspberry) have been used as components of the oil phase in O/W emulsions. The best emulsion contained 4 and 5 wt % of seed oil together with other components of the oil phase and the O/W ratio was 20/80. The emulsification procedure also affected the particle size distribution of the emulsions, and homogenization via the high-energy method brought about emulsions with narrower distribution, in comparison with the EIP method. This is shown in Figure 2 for systems consisting of the Tween 80/Span 80 mixture (5 wt %) at HLB 9, where only the main particle population was observed when the high-energy method was utilized, while EIP afforded bimodal size distribution with the occurrence of two main droplet populations.

On average, emulsions prepared using the high-energy method contained considerably smaller droplets than systems prepared with the EIP technique. Immediately after preparation, the former of the two systems showed particles from 151 ± 1 nm to 209 ± 5 nm, whilst the particle sizes of the latter were significantly higher ($p \leq 0.001$) and ranged from 502 ± 22 nm to 1050 ± 29 nm. Correspondingly, the polydispersity index (PDI) of the high-energy samples was approximately 0.18 ± 0.01 to 0.26 ± 0.01, whereas the PDI of the emulsions prepared by EIP ranged from 0.26 ± 0.09 to 0.75 ± 0.44. It is known that the PDI is an estimate of the width of the droplet distribution in samples. The PDI values of

approximately 0.1 correspond to the polydispersity of monodisperse standards and values greater than 0.7 indicate that the sample has a very broad size distribution. The PDI values measured on the studied emulsions therefore clearly show that the high-energy method provides significantly narrower droplet distributions than the EIP technique.

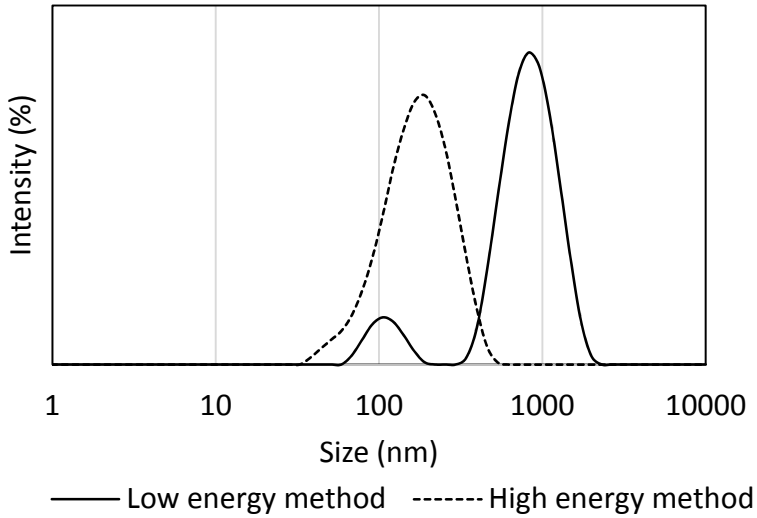

Figure 2. Particle size distribution recorded after emulsification with low-energy vs. high-energy conditions at emulsions with 5 wt % Tween 80/Span 80 and HLB 9. The Tween 80/Span 80 ratio was 0.78.

2.2. Influence of HLB and Oil Type

Finding the optimum HLB value required for the successful encapsulation of the hemp seed oil was based on the premise that at optimum HLB, the mean particle sizes of the emulsion droplets are at their minimum. This factor also influences, to a large extent, the stability of the emulsions produced. Another possible procedure to determine optimum HLB encompasses visual observation of the emulsions, and the system with minimal creaming and phase separation is deemed to possess the optimum HLB [29]. The values chosen for preparing the emulsions ranged from 6–10 for the pairs of Tween 80/Span 80 and 7–9 for Tween 85/Span 80. Figure 3 depicts the impact of the HLB value on the particle sizes of the emulsions, revealing the evolution of droplet size concurrent with changes in HLB for Tween 80/Span 80 under differing preparation conditions. This figure shows that the optimal HLB for hemp seed oil lies between 8 and 9, and is identified with affording the minimum average particle size, irrespective of the method used for producing the emulsions. In the case of the EIP method, a U-shaped curve is clearly visible, showing the strong correlation that exists between particle size and HLB. Unfortunately, despite finding the optimum HLB value for this system, it was not possible to formulate stable emulsions with EIP and emulsions prepared by this low-energy method became unstable or broke down within several minutes of preparation. On the contrary, for analogous emulsions prepared by the high-energy method, merely negligible changes in droplet size alongside changes in HLB were observed. Furthermore, the emulsions were reasonably stable, not exhibiting any sign of destabilization. In the literature, the aforementioned U-shape dependence of particle size on HLB is much less reported for the high-energy methods than the low-energy methods (such as EIP) [30]. Figure 3 also highlights the significant impact of HLB and the method of emulsion preparation on the PDI, which was systematically higher in emulsions prepared by EIP. Hence, the low-energy method utilized is far more sensitive to the proper choice of emulsion composition than the high-energy method. Of all the samples, the smallest particles (84 $\pm$ 1 to 122 $\pm$ 2 nm) were achieved at any given HLB with the Tween 85/Span 85-based emulsions (10 wt %) prepared by the high-energy method.

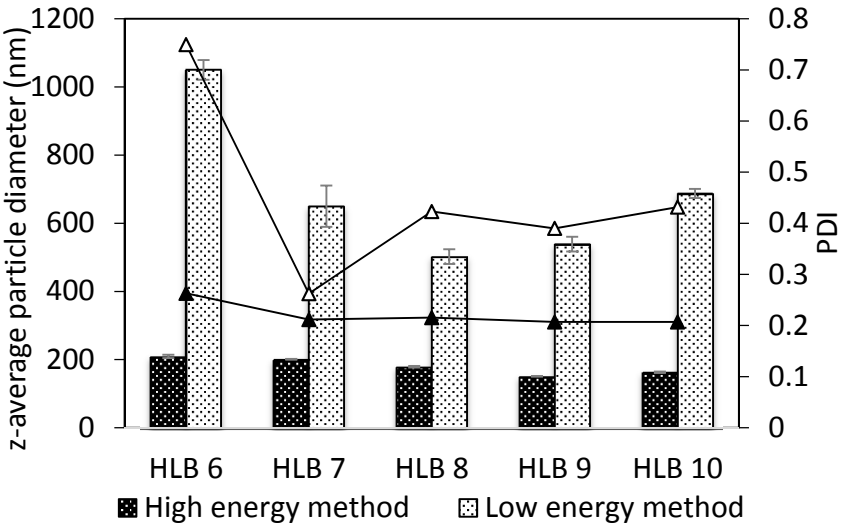

Figure 3. The influence of the HLB on particle sizes (columns) and polydispersity index (lines) of the nanoemulsions (5 wt % Tween 80/Span 80) prepared by the low-energy and high-energy methods using refined oil.

The above results demonstrated that emulsions with fine droplets could primarily be produced by the high-energy approach, but the question remained as to whether the type of oil (refined vs. unrefined) actually influenced the particle sizes of emulsions formulated through both approaches. Figure 1a clearly shows the similar behaviour exhibited by the Tween 85/Span 85 emulsions over the range of the HLB tested, irrespective of the type of oil used when applying the high-energy method. This contrasted with emulsions prepared by the EIP method (Figure 1b), which tended to vary and behave differently, even if they shared the exact same formulation. Here, a statistically significant difference ($p \leq 0.01$) was observed for particle sizes of the emulsions formulated with refined and unrefined oils, respectively. It was also seen that higher concentrations of surfactant provided more uniform systems across the HLB range examined and enabled the preparation of emulsions with significantly smaller particles ($p \leq 0.01$).

2.3. Emulsion Stability

The long-term stability of the hemp seed oil-in-water emulsions was assessed over a period of a maximum of 230 days by recording changes in their visual appearance during storage at 25 and4 °C. The best stability discerned for emulsions formed by the EIP method, regarding the visual assessment, was demonstrated by emulsions containing 10 wt % Tween 85/Span 85 at HLB 7. These emulsions were stable for 8 days and such early break-down was attributed to their relatively large sizes of particles (590 to 780 nm). However, emulsions prepared on the Ultraturrax device (IKA, Staufen, Germany), stabilized by Tween 80/Span 80, were stable for 24 days of storage, with no phase separation or creaming observed. Later, when creaming of the emulsions was recorded, no significant changes were observed in particle size, as measured by dynamic light scattering (DLS). The greatest stability was shown by Tween 80/Span 80-based systems at HBL 9 and 10 wt % surfactant (stable for 230 days), and the Tween 85/Span 85 system (200 days), stored at both temperatures. This long-term stability might be associated with the small, initial droplet size observed after preparation. According to study conducted by Rao and McClements [31], lemon oil nanoemulsions with initially smaller droplets were more stable against coalescence, aggregation, as well as flocculation. Similar findings were also reported in several other papers. For example, in recent works studying the effect of various processing factors on the formulation of vegetable oil-based nanoemulsions [32,33], the smaller droplets resulted in their better long-term stability, which also correlated well with values of droplet sizes predicted by using mathematical modelling.

Variations in mean particle sizes during the storage of emulsions formulated with Tween 85/Span 85 (10 wt %) at HLB 9 are illustrated in Figure 4, which documents only minor changes, regardless of the storage temperature. This finding is consistent with the study of Rebolleda et al. [34], in which wheat bran oil nanoemulsions stabilized by mixtures of Span 80/Tween 80 showed only negligible changes in the droplet size of the emulsions during storage.

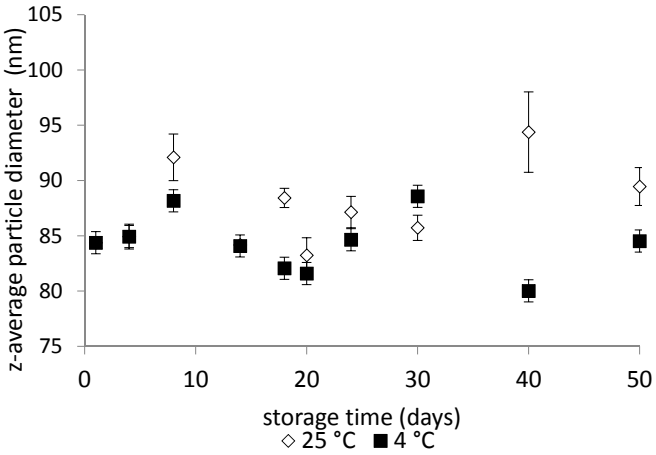

Figure 4. Changes in the z-average diameter of emulsion droplets as a function of storage time and temperature (4 and 25 °C) determined for emulsions with Tween 85/Span 85 (10 wt %) at HLB 9.

2.4. Antibacterial Activity

Both hemp seed oils and their emulsions were screened with respect to their antibacterial effects. Table 2 displays the results from the disc diffusion method as recorded for both oils, revealing their weak antibacterial activity against selected species. Using this method, the antibacterial activity was determined by the measurement of the diameter of the inhibitory zone (mm) formed around the discs soaked with the oil and the size of the disc was subtracted from the size of the inhibitory zone. The unrefined oil possessed inhibition to all strains, with inhibition zones ranging from 0.3 to 3.3 mm, and the effect was mainly dependent on the type of bacteria. These results were further supported by a broth microdilution method (data not presented). A previous report on the antimicrobial activity of hemp seed oil was published by Leizer et al. [11], who observed some bioactivity during primary screening. In past years, the antibacterial activity of essential oils extracted from *C. sativa* was, however, more extensively studied [35,36].

Table 2. Inhibitory effect of hempseed oils (mean ± SD, $n = 3$) against most common pathogenic bacteria, expressed as diameter of the inhibition zone in mm (diameter of the disc was subtracted from the total size of inhibition zone). Means within a line with the same superscript differ significantly ($p \geq 0.005$).

	Bacterial Strain	Inhibition Zone Size (Ø ± σ) mm	
		Unrefined Oil	Refined Oil
G^+	*Bacillus cereus* CCM 2010	2.3 ± 0.6 [a]	0.0 ± 0.0 [a]
	Bacillus subtilis subsp. *subtilis* CCM 2216	2.3 ± 1.8 [a]	0.0 ± 0.0 [a]
	Micrococcus luteus CCM 732	3.3 ± 1.8	2.7 ± 1.2
	Staphylococcus aureus subsp. *aureus* CCM 3953	3.0 ± 0.0	3.0 ± 1.2
G^-	*Citrobacter freundii* CCM 7187	2.3 ± 0.6 [a]	0.0 ± 0.0 [a]
	Enterococcus faecalis CCM 4224	2.3 ± 0.6	2.7 ± 0.6
	Escherichia coli CCM 3954	0.3 ± 0.6	0.0 ± 0.0
	Salmonella enterica subsp. *enterica* ser. Enteritidis CCM 4420	3.0 ± 1.8	2.0 ± 1.2
	Serratia marcescens subsp. *marcescens* CCM 303	2.7 ± 0.6	0.7 ± 1.2
	Pseudomonas aeruginosa CCM 395	1.7 ± 0.6	2.3 ± 0.6

In order to understand the difference in antibacterial activity of both types of oil, the results from antibacterial testing were compared to the fatty acid analyses. As stated earlier, the antibacterial action of fatty acids is usually recognized for long-chain unsaturated fatty acids, including oleic acid, linoleic acid, and linolenic acid [37]. The higher content of alpha-linolenic acid in the unrefined oil could explain the increase in antibacterial activity, compared with findings for the refined oil. Another possible explanation might pertain to the fact that during the refining process, minor components such as tocopherols and tetrahydrocannabinol are removed, which may contribute to the antibacterial activity of unrefined hemp seed oils [38].

As reported earlier [39], gram-positive bacteria were seen to be more sensitive to the unrefined oil than gram-negative strains, as a result of differences in the composition of the bacterial cell wall. In accordance with this fact, *E. coli* was the most resistant species, and *M. luteus* and *S. aureus* proved to be most sensitive to the oils tested. However, statistical analysis did not prove any significant difference between the group of gram positive and gram negative strains regarding the effect of refined and unrefined oils, respectively. Though the observed antibacterial activity of hemp seed oil was weak, it can be regarded as an added value to the main positive characteristics of hemp seed oil, namely the content of n-3 and n-6 fatty acids. Additionally, an investigation was conducted on the potential antibacterial effects of the formulated emulsions. Previously, a synergistic effect of antibacterial substances encapsulated in emulsions or nanoemulsions has been observed, as reported by Ghosh et al. [40]. The enhanced activity of emulsions against microorganisms is primarily explained by the presence of non-ionic surfactants in the formulations. Several studies [30,41] reported on increased antibacterial activity when non-ionic Tween 80 made up part of the formulation. Therefore, a similar effect was expected for the hemp seed oil emulsions studied herein. Nevertheless, the disk diffusion and broth dilution methods did not reveal the rise that had been anticipated in antibacterial activity by emulsions containing oil, relative to pure forms of oils alone.

3. Experimental Methods

3.1. Materials

Two types of cold-pressed hemp seed oil were employed: an unrefined oil was kindly donated by Míča a Harašta (Prague, Czech Republic), and a refined, commercially available oil was purchased from Cannaderm (Prague, Czech Republic). The non-ionic surfactants Span 80 (Sorbitane monooleate, HLB 4.3), Span 85 (Sorbitane trioleate, HLB 1.8), Tween 80 (Polyoxyethylenesorbitan monooleate, HLB 15), and Tween 85 (Polyoxyethylenesorbitan monooleate, HLB 11) were supplied by Sigma-Aldrich (Steinheim, Germany) and were used without further purification.

3.2. Microorganisms

The test microorganisms, including gram-positive and gram-negative strains, were obtained from the Czech Collection of Microorganisms (CCM, Czech Republic). The bacteria were selected to represent the major spoilage classes. Tests utilized gram-positive *Bacillus subtilis*, subsp. *subtilis* CCM 2216, *Bacillus cereus* CCM 2010, *Enterococcus faecalis* CCM 4224, *Micrococcus luteus* CCM 732, *Staphylococcus aureus*, subsp. *aureus* CCM 3953, and gram-negative *Citrobacter freundii* CCM 7187, *Escherichia coli* CCM 3954, *Proteus vulgaris* CCM 1799, *Pseudomonas aeruginosa* CCM 3955, and *Serratia marcescens*, subsp. *marcescens* CCM 303. All strains were maintained on nutrient agar (5 g L^{-1} peptone, 5 g L^{-1} NaCl, 1.5 g L^{-1} beef extract, 1.5 g L^{-1} yeast extract, 15 g L^{-1} agar; from Hi-Media Laboratories Bombay, India) and were sub-cultured onto fresh media every two weeks. The initial test inocula of the microorganisms were prepared from 24 h cultures. Each bacterial suspension was adjusted by dilution with a nutrient broth to 5×10^8 CFU mL^{-1}.

3.3. Characterization of Hemp Seed Oil

Basic characteristics in terms of iodine, saponification, and acid value were determined using respective methods described elsewhere [42]. Fatty acid methyl esters (FAME) were prepared by transesterification with KOH in methanol. In brief, oil was mixed with methanolic potassium hydroxide (1 M), boiled for 30 min and cooled to room temperature. Hexane and aqueous sodium chloride solution were added, and the organic, upper layer was separated for gas chromatography (GC) analysis. The composition of fatty acids was determined using GC on a Shimadzu GC-14A device equipped with an flame ionization detector (FID). A capillary DB-WAX column (30 m × 0.25 mm, Agilent, Santa Clara, CA, USA) was used. The temperature program employed was as follows: the column temperature was programmed at 110 °C and maintained for 3 min, then the temperature was raised to 220 °C at a rate of 15 °C/min, and an isothermal step followed at this temperature for 10 min. The temperatures for the injector and detector were set at 225 °C and 230 °C, respectively. Identification of the fatty acids present was carried out using the FAME SUPELCO 37 Component FAME Mix (Sigma Aldrich, Steinheim, Germany) standard. The content of the respective FAME in oil was expressed in percent by applying an internal normalization procedure.

3.4. Preparation of Emulsions

Two different methods were carried out to prepare the emulsions: high-energy emulsification and low-energy, phase-inversion emulsification (EIP). The oil-to-water (O/W) ratio of 5/95 (*w/w*) was employed. Suitable pairs of Spans and Tweens (Table 3) at the amounts of 5 and 10 wt % were used to formulate emulsions with a hydrophilic-lipophilic balance (HLB) ranging from 6 to 10. The required HLB was calculated using the following formula:

$$HLB = w_1 \times HLB_1 + w_2 \times HLB_2 \quad (1)$$

wherein w_1 and w_2 represent weight fractions of the emulsifiers utilized with HLB_1 and HLB_2, respectively. High-energy emulsification was performed on an Ultra Turrax T 25 device (IKA, Staufen, Germany). Appropriate amounts of hemp seed oil (5 wt %), a suitable pair of emulsifiers (5 or 10 wt %), and distilled water (add to 100 wt %) were heated in a test tube to 70 °C and homogenized immediately at 13,400 rpm for 15 min. The low-energy, phase-inversion procedure went as follows. Both the water and oil phases were heated to 70 °C and maintained at this temperature. The aqueous phase with a dissolved, water-soluble surfactant (Tween) was added drop-wise to the oil phase, which contained the oil-soluble surfactant (Span) and hemp seed oil. A constant stirring rate of 1050 rpm was utilized over a duration of 30 min. Homogenization was performed using an RZR Heidolph homogenizer (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany).

Table 3. Mixtures on non-ionic surfactants used for obtaining the chosen HLB values; design of the study used for the preparation of hemp seed oil emulsions. Amounts of surfactants are given for their 5 wt % contents in 100 g of emulsions.

Amount of Surfactants (g)				HLB of the Mixture (*Tween*/*Span Ratio*)
Tween 80 (HLB 15)	**Span 80 (HLB 4.3)**	**Tween 85 (HLB 11)**	**Span 85 (HLB 1.8)**	
0.794	4.206	-	-	6 *(0.19)*
1.262	3.738	-	-	7 *(0.34)*
-	-	2.826	2.174	7 *(1.30)*
1.729	3.271	-	-	8 *(0.52)*
-	-	3.370	1.630	8 *(2.07)*
2.196	2.804	-	-	9 *(0.78)*
-	-	3.913	1.087	9 *(3.60)*
2.664	2.336	-	-	10 *(1.14)*

3.5. Particle Size Measurements

Particle size, particle size distribution, and the polydispersity index (PDI) were determined by dynamic light scattering (DLS) on a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK). Measurements for the hydrodynamic radii of emulsion droplets were expressed as intensity-weighted z-average diameters (nm). Analyses were carried out at a scattering angle of 90° at the temperature of 25 °C. The stability of the emulsions was evaluated at regular time intervals by measuring the particle size under different storage conditions (25 and 4 °C).

3.6. Antibacterial Testing

The oil samples and their emulsions were screened for the antibacterial activity they exhibited against common pathogenic bacteria by utilizing the disk diffusion and broth microdilution methods.

Disk diffusion method: Suspensions of each tested microorganism (100 µL) were spread on Mueller-Hinton sterile agar plates (Hi-Media Laboratories, Bombay, India). Sterile paper discs of 6 mm diameter, soaked with 5 µL of sample (hemp seed oil or emulsions), were placed on surfaces of the agar plates. As a reference, sample discs soaked with emulsions absent of hemp seed oil were utilized. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone in mm after 24 h of incubation at 30 °C (*Bacillus subtilis* subsp. *subtilis*; *Pseudomonas aeruginosa*; and *Bacillus cereus*) or 37 °C (the remaining bacteria) and expressed in mm. In order to calculate the inhibition zone, the diameter of the paper disc was subtracted from the diameter of the inhibition zone.

Broth microdilution method: Under sterile conditions, 20 µL of bacterial suspension and 200 µL of a sample (containing hemp seed oil or a prepared emulsion) were pipetted into each well of the 96-well sterile microplate. Nutrient agar inoculated with bacterial suspension was employed as a positive reference. Emulsions, absent of hemp seed oil were used as a negative reference. Plates were then incubated for 30 min at 30 °C. After incubation, 100 µL of each individual suspension present in a respective well was spread over the surface of an agar plate and incubated again for 24 h at either 30 °C or 37 °C, depending on the bacteria used.

3.7. Statistical Analysis

The sizes of emulsion particles (DLS), iodine, saponification, and acid values as well as fatty acid composition (GC) of the hemp-seed oils were analysed at least in triplicate (n = 3); means and standard deviations were calculated in accordance with the Dean-Dixon method. Correspondingly, antibacterial testing (disk diffusion and broth microdilution methods) was conducted in triplicate and the Dean-Dixon method was utilized to calculate the means and standard deviations. The T-test was applied to determine statistical differences between the individual samples (Statistica, StatSoft, Inc., Tulsa, OK, USA). The p values of ≤ 0.05 were considered statistically significant.

4. Conclusions

This study has shown that emulsions of bioactive hemp seed oil can be formulated by employing pairs of non-ionic surfactants (Span, Tween) under appropriate conditions. It was found that the ability to form stable emulsions of small, initial particle size is primarily dependent on the given method of preparation and the HLB value. The low-energy method was suitable for producing emulsions without a high-energy input, but these systems turned out to be unstable due to the large, initial droplet sizes (502 ± 22 nm to 1050 ± 29 nm). However, high-energy homogenization produced nanoemulsions with fine droplets (151 ± 1 nm to 209 ± 5 nm), also supporting the stability of the emulsions. Regarding the influence of oil type on the formulations, emulsions containing refined and unrefined hemp seed oil performed similarly when using the high-energy method but differed when low-energy emulsification was employed. Testing for antibacterial properties by the disk diffusion and broth dilution methods confirmed the activity of the hemp seed oil utilized herein, although there was no sign of the enhancement that had been anticipated in the capability of the oil to act against bacteria

via encapsulation in the emulsions. Regardless of this fact, the results suggest that such formulated emulsions could serve to fortify foodstuffs, as hemp seed oil is an exceptionally rich source of essential fatty acids, and the n-6 to n-3 fatty acid ratio in the oil stands at 3:1, which is considered optimal for human dietary purposes.

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Author Contributions: Veronika Mikulcová: was responsible for the preparation and characterization of the emulsions, and she also partially performed the characterization of the hemp seed oil, and conducted and evaluated the results of the microbiological studies; Věra Kašpárková: performed the characterization of the hemp seed oil, designed and supervised the formulation of the emulsions; Petr Humpolíček: analysed the experimental data and conducted the statistical analysis; Leona Buňková: supervised and designed the microbiology studies; All authors contributed to the writing of the manuscript.

Conflicts of Interest: The authors declare that there is no conflict of interest.

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PAPER II

On the preparation and antibacterial activity of emulsions stabilized with nanocellulose particles

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On the preparation and antibacterial activity of emulsions stabilized with nanocellulose particles

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ABSTRACT

Formation, droplet size and antibacterial activity of emulsions stabilized with cellulose particles, namely cellulose nanocrystals (CNCs) and microfibrillated cellulose (MFC) were investigated. The effects of the oil-in-water ratio (10/90 to 40/60), type and concentration of cellulose (0.1–1.0%) on the physico-chemical characteristics of the emulsions containing three different antimicrobial oils (cinnamaldehyde, eugenol and limonene) were studied. Emulsions were characterized in terms of emulsification indexes, encapsulation efficiency and droplet size and distribution. Stability during the eight-week storage period and after mild centrifugation were evaluated. The results showed that it is possible to produce oil-in-water CNC and MFC-stabilized Pickering emulsions with antimicrobial oil content as high as 40 wt%. Furthermore, emulsions showed good stability during storage and towards mild centrifugation. Antibacterial activity of the emulsions investigated using agar diffusion and broth dilution methods was mainly influenced by the type and content of antibacterial oil. Type of nanocellulose particles used for stabilization of emulsion droplets showed only a minor contribution, when high concentrations of oil were used. Results also suggested that the antibacterial effect was dependent primarily on direct interaction between emulsion droplets and pathogens, while the impact of free, non-encapsulated oil was only marginal. However, when low concentrations of emulsion were used, the role of the nature of nanocellulose could be evidenced, and MFC-stabilized emulsions exhibited better antibacterial activities compared to CNC-stabilized.

1. Introduction

With a constantly increasing level of requirements of health public agencies, a strong demand is put on the food and packaging industries, among others, to provide secured goods with expanded shelf-life. These industries have therefore recently shown a growing interest for naturally occurring antimicrobial agents, and especially essentials oils (EO) and their active components. The reasons for this are multifold, and one of the strongest driving forces is the consumer preference for natural products, owing to the perception that these can be less harmful than their synthetic homologues (Weiss, Gaysinsky, Davidson, & Mcclements, 2009).

Despite good antimicrobial properties, these natural compounds are often fragile and susceptible to degradation, and can negatively interact with food components, thus compromising the flavour and the sensory of the products. With respect to these falls, encapsulation can provide an efficient protection towards for instance oxidation (Kargar, Fayazmanesh, Alavi, Spyropoulos, & Norton, 2012) and it can furthermore facilitate controlled delivery of encapsulated substance (Blanco-Padilla, Soto, Iturriaga, & Mendoza, 2014). Recently, there has been an increasing interest for the utilization of colloidal systems for their capability of decreasing volatility, improving the stability, water solubility, and efficacy of EO-based formulations (Bilia et al., 2014). While the vast majority of the work reported focuses on encapsulation of EO by conventionally-based systems (surfactants and polymers) (Donsì, Annunziata, Sessa, & Ferrari, 2011; Gaysinsky, Davidson, Bruce, & Weiss, 2005; Salvia-Trujillo, Rojas-Grau, Soliva-Fortuny, & Martin-

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Belloso, 2015; Terjung, Löffler, Gibis, & Hinrichs, 2012), limited attention has been paid to particle-stabilized emulsions.

Particle-stabilized emulsions, also referred to as Pickering emulsions, are not physically stabilized by any of the conventional emulsifiers (surfactants or polymers), but by solid particles wetted by the two phases (Berton-Carabin & Schroën, 2015; Binks, 2002). The stabilization mechanism is based on the accumulation of dispersed particles at the oil-water interface to form a mechanical (steric) barrier that protects the emulsion droplets against coalescence. Depending on whether the particles are preferentially hydrophilic or hydrophobic, the systems may be either oil-in-water (O/W) or water-in-oil (W/O) emulsions (Chevalier & Bolzinger, 2013; Dickinson, 2006; Leal-Calderon & Schmitt, 2008). The research on the food Pickering emulsions has primarily been focused on model systems stabilized by inorganic particles (silica nanoparticles), fat crystals, protein-based nanoparticles (Dickinson, 2012; Lam, Velikov, & Velev, 2014; Rayner et al., 2014) and chitin nanocrystals (Tzoumaki, Moschakis, Kiosseoglou, & Biliaderis, 2011). Several works have been also devoted to use of nanocellulose particles (Cunha, Mougel, Cathala, Berglund, & Capron, 2014; Zoppe, Venditti, & Rojas, 2012) with or without surface modification (Kalashnikova, Bizot, Cathala, & Capron, 2011a; Sébe, Ham-Pichavant, & Pecastaings, 2013; Winuprasith & Suphantharika, 2013) in preparation of oil-in-water emulsions. Recent research interest in Pickering emulsions has demonstrated the ability of particles derived from cellulose to be used as templates for novel colloidal systems, including foams or microcapsules (Campbell, Stoyanov, & Paunov, 2009; Jin et al., 2012; Nypelö, Rodriguez-Abreu, Kolen'ko, Rivas, & Rojas, 2014). In comparison with other types of particular stabilizers, cellulose nanoparticles have been found to be superior when it comes to biocompatibility, biodegradability, low density, thermo-mechanical behaviour and costs (Habibi, Lucia, & Rojas, 2010; Salas, Nypelö, Rodriguez-Abreu, Carrillo, & Rojas, 2014).

Previous works on Pickering emulsions stabilized by cellulose particles, such as cellulose nanocrystals (CNC) or microfibrillated cellulose (MFC), have been usually limited to the model systems containing synthetic oils as dispersed phase. Most of the studies worked with emulsions having a content of oil phase in the range of 10–30%. Trials to incorporate larger amounts of oil, high internal phase emulsions (HIPEs) with oil fractions higher than 90% stabilized by less than 0.1% CNC have been recently reported (Capron & Cathala, 2013). Particle wettability is of course of great importance, especially given the natural hydrophilic character of cellulose particles. Recent studies have demonstrated that hydrophobically modified MFC or CNC, without any dispersing agent, effectively stabilize water-in-oil emulsions (Lee, Blaker, Murakami, Heng, & Bismarck, 2014; Stenius & Andresen, 2007; Xhanari, Stenius, & Syverud, 2011). Multiple emulsions, particularly, oil-in-water-in-oil have also been prepared by using both native and hydrophobized NFC (nanofibrillated cellulose) and CNC (Cunha et al., 2014; Zoppe et al., 2012). In this context, the contact angle between a particle and a given liquid–liquid interface is an important parameter and can be successfully controlled for example by the degree and type of particle functionality, in order to prepare the emulsions with tailor-made properties for specific applications. For example, using multifunctional protein leads to a self-assembly properties of NFC (Varjonen et al., 2011), grafting of CNCs with poly(N-isopropylacrylamide) or poly[2-(dimethylamino)ethyl methacrylate], afforded temperature-sensitive or pH-responsive properties to emulsions, respectively (Tang et al., 2014; Zoppe et al., 2012). Recently, the study (Wen, Yuan, Liang, & Vriesekoop, 2014) on the formulation of cellulose particle-stabilized emulsions loaded with EO was reported and illustrated the potential of CNC particles to be used for this purpose. The stabilizing agent used to disperse D-limonene, a principal component of lemon EO, was CNC prepared from corncob cellulose hydrolyzed by ammonium persulfate. This type of CNC differs from the cellulose produced by the classic sulphuric acid route in the sense that it bears carboxylic groups on the surface instead of sulphate esters.

While many publications have been focused on studying antimicrobial activity of nanoemulsions, microemulsions, nanoliposomes, nanoparticles, or nanofibers (Blanco-Padilla et al., 2014), surprisingly none of published work has been, according to best authors knowledge, focused on antimicrobial activity of Pickering emulsions. Their stability or the absence of classical surfactants with potentially harmful health effect among other advantageous properties makes them, therefore attractive. Moreover, the combination of cellulose particles as a bio-stabilizer and EO (or their components) as natural antimicrobial agents would then exhibit an advanced, organic antimicrobial system with a wide application potential.

In this work, the preparation and evaluation of nanocellulose stabilized emulsions loaded with limonene, cinnamaldehyde, and eugenol, which are the principal components of essential oils, is reported. Both MFC and CNC were used without any surface modification and the antibacterial properties of the dispersions are presented against different bacterial species, representing a wide range of the major food-borne pathogens.

2. Materials and methods

2.1. Materials

Cellulose nanocrystals were obtained by acid hydrolysis of commercially available microcrystalline cellulose (Avicel PH101, FMC Biopolymer) according to procedure described in Leung et al., 2011. The average length of the fiber was 234 ± 66 nm while a diameter of 30 ± 7 nm was determined by atomic force microscopy (AFM). Microfibrillated cellulose was a gift of StoraEnso (Karlstad, Sweden), and was characterized by AFM. The system was strongly entangled with long fibers (>1 μm) of thickness ranging from 30 nm to larger values. Eugenol, cinnamaldehyde and limonene were obtained from Sigma-Aldrich Co. (Steinheim, Germany) and used without further purification. Ultra-pure water was from a Mili-Q filtration system (Merck, Darmstadt, Germany). Anhydrous calcium chloride was purchased from IPL (Uherský Brod, Czech Republic).

2.2. Microorganisms

The test microorganisms, including gram-positive and gram-negative strains, were obtained from the Czech Collection of Microorganisms (CCM, Czech Republic). The bacteria were selected to represent a wide range of major food-borne classes. Gram-positive bacteria *Bacillus cereus* CCM 2010 (BC), *Enterococcus faecalis* CCM 4224 (EF), *Micrococcus luteus* CCM 732 (ML), *Staphylococcus aureus* subsp. *aureus* CCM 3953 (SA) and gram-negative bacteria *Escherichia coli* CCM 3954 (EC), *Salmonella enterica* subsp. *enterica* ser. Enteritidis CCM 4420 (SE), *Pseudomonas aeruginosa* CCM 3955 (PA), *Serratia marcescens* subsp. *marcescens* CCM 303 (SM) were employed in the test.

All tested microorganisms were maintained on nutrient agar (5 g/L peptone, 5 g/L sodium chloride, 1.5 g/L beef extract, 1.5 g/L, yeast extract, 15 g/L agar; Hi-Media Laboratories, India) and subcultured onto fresh media every two weeks. The initial test inocula of the bacteria were prepared from the 24-h cultures.

2.3. Preparation of Pickering emulsions

The oil-in-water (O/W) Pickering emulsions were prepared with an O/W ratio ranging from 10/90 to 40/60 (wt%). The aqueous phase consisted of CNC or MFC suspensions with cellulose concentrations of 0.1, 0.3, 0.5, 0.7 and 1.0 wt%. To the CNC suspension, calcium chloride (3 mM) was added to improve the emulsifying capacity of the system. Emulsifications were carried out by mixing oil (eugenol, cinnamaldehyde or limonene) and aqueous phase with a high-speed homogenizer Heidolph DX900 (Heidolph Instruments, Germany) operating at 24,000 rpm for 5 min.

2.4. Emulsion characterization

2.4.1. Size of emulsion droplets

The droplet size and distribution of the emulsion droplets were measured using laser diffraction (Master Sizer 2000, Malvern instruments, UK) and also evaluated on the basis of microscopic images.

For diffraction measurements, the emulsion was sampled and suspended in the instrument flow system containing milliQ-water at a pump velocity of 2000 rpm. The refractive index was set to 1.421. The volume mean diameter D(4,3) corresponds to the mean diameter of spheres with the same volume as the analysed droplets and was calculated according to $D(4,3) = \Sigma n_i\, d_i^4 / \Sigma n_i\, d_i^3$, where n_i is the number of particles with diameter d_i (Barth, 1984).

2.4.2. Optical microscopy

Emulsion droplets were observed using Zeiss AxioCam MR 5 optical microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Prior to observation, tenfold diluted emulsions were placed onto a glass microscope slide and viewed under 10–100× magnification. Processing of microscopic images with at least of 150 emulsion droplets was conducted using the ImageJ software and sizes of the droplets were obtained.

2.4.3. Emulsion stability

Emulsion stability was assessed daily by visual observation during the first week after preparation and then at seven-days intervals for a period of at least 2 months. The emulsions were stored at ambient temperature. The gravitational stability was verified by centrifugation of emulsion aliquot of 10 mL for 2 min at 500 rpm (Hettich EBA 270, Tuttlingen, Germany).

2.4.4. Emulsification index

The emulsifying capacity of both cellulose types used and the stability of the emulsions were expressed as the volume of emulsion layer formed (V_{emuls}) relative to the total volume of the sample (V_{total}), referred to as the emulsion index (EI). The EI (Wahlgren, M., Bergenståhl, B., Nilsson, L., & Rayner, M., 2015) was calculated using the following equation:

$$EI = \frac{V_{emuls}}{V_{total}} \cdot 100\% \quad (1)$$

2.4.5. Encapsulation efficiency

The encapsulation efficacy (EE) was determined according to the method reported by (Sabliov, Chen, & Yada, 2015) from a volume fraction of the encapsulated oil (V_{encaps}) related to the total volume of the oil phase used (V_{total}). Volume fraction of non-encapsulated oil was determined firstly by visual monitoring of separate phases, after 2 months by using centrifugation of emulsion aliquot of 10 mL for 2 min at 500 rpm (Hettich EBA 270, Tuttlingen, Germany) and volume fraction of non-encapsulated oil was measured.

$$EE = \frac{V_{encaps}}{V_{total}} \times 100\% \quad (2)$$

2.4.6. HPLC

The quantification of non-encapsulated oil was performed using a modular high performance liquid chromatography (HPLC) system consisting of Waters 600E pump, UV-VIS detector (UV 200, DeltaChrom, Watrex) and the Clarity software. Samples were analysed on C18 X-SELECT column (4.6 × 250 mm, 5 μm particle size, Waters) at room temperature. The wavelength of 200 nm for limonene, 280 nm for eugenol and 285 for cinnamaldehyde was employed. The mobile phase consisted of methanol/water 90/10 (v/v) and 60/40 (v/v) for limonene and eugenol, respectively. Cinnamaldehyde was analysed using acetonitrile/0.1% phosphoric acid 48/52 (v/v). The flow rate was 0.8 mL/min. For analysis, the emulsions were centrifuged at 800 rpm for 1 min (MiniSpin, Eppendorf, Hamburg, Germany), separated non-encapsulated oil was carefully withdrawn using a syringe and diluted with respective mobile phase. Prior to analysis, the samples were filtered through a syringe filter (0.45 μm, Millipore, Merck KGaA, Darmstadt, Germany).

2.5. Antimicrobial activity assay

For antibacterial testing, both types of nanocellulose were first heat sterilized at 121 °C for 20 min. After preliminary testing, emulsions with O/W 10/90 containing 0.5 wt% of cellulose were prepared for antibacterial assay. Two different methods were used, the well diffusion assay (as prepared, concentrated emulsions) and the broth microdilution assay (dilution series of emulsions).

2.5.1. Well diffusion assay

Using a sterile borer, wells of 6 mm diameter were carved into Müller-Hinton agar surface (300 g/L beef infusion, 17.5 g/L casein acid hydrolysate, 1.5 g/L starch, 17 g/L agar; Hi-Media Laboratories, India) and seeded with the respective microorganism at the level 0.5 of the McFarland standard. Each of the well was then filled with 100 μL of undiluted emulsion with an oil content of 10 wt%. As a control, sterile distilled water was employed. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone in millimetres (mm) after 24 h of incubation at 30 °C (*Bacillus subtilis* subsp. *subtilis*, *Pseudomonas aeruginosa* and *Bacillus cereus*) or 37 °C (the remaining strains). Each experiment was done in triplicates. The Dean-Dixon method was used to calculate means and standard deviations.

2.5.2. Broth microdilution assay

Parent emulsion with limonene, cinnamaldehyde, eugenol at oil fraction of 10 wt% were further diluted to 10, 5, 2.5, 1.0, 0.5, 0.05 and 0.005 mg/mL with a sterile Müller-Hinton broth (MHB) (300 g/L beef infusion, 17.5 g/L casein acid hydrolysate, 1.5 g/L starch, 17 g/L agar; Hi-Media Laboratories, India; Hi-Media Laboratories, India) and employed for the tests. Prepared culture media (200 mL) and 5 mL of 24-h bacterial suspensions with the turbidity adjusted to 0.5 of the McFarland scale were dispensed into sterile microtiter 96-well plate (Promed®). To bacterial suspensions, emulsions with the concentration series given above were added. As a negative reference, 200 μL of emulsion with an appropriate dilution was employed. MHB inoculated with bacterial suspension without emulsion was used as a positive reference. The plates were incubated at the temperature of 30 °C for 24 h. Bacterial growth was measured at 655 nm with absorbance readings at 30-min intervals,

using Microplate reader Benchmark (Bio-Rad, Japan). The growth index (*GI*), expressed in %, was calculated according to equation $GI = (OD_{655}\ OD_N)/OD_P \times 100$, where OD_{655} is optical density of bacterial suspension recorded after 20 h incubation in the presence of emulsions, OD_N and OD_P are optical densities of negative and positive references, respectively. In order to correlate the antimicrobial activity with the oil solubility in water, all tested components, further referred to as antimicrobial oils, were individually assayed for their antimicrobial activities according the above-mentioned method. All tests were performed in triplicates; means and standard deviations (SD), according to Dean-Dixon method, were calculated.

3. Results and discussion

Three series of Pickering emulsions were prepared containing three different essential oils (eugenol, cinnamaldehyde or limonene) with content ranging from 10 to 40 wt%. For stabilization of emulsion droplets either CNC or MFC were used in concentrations between 0.1 and 1 wt%.

3.1. Physicochemical properties of emulsions

3.1.1. Droplet size

Size of droplets is an important parameter with an impact on behaviour and stability of the emulsions. Results recorded using light diffraction showed that emulsion droplet size, expressed as D(4,3), was significantly influenced by all studied variables, namely type of cellulose particles, their concentration as well as content and type of oil. It was found out that the droplets become smaller with increasing concentration of cellulose, which can be explained by the fact that more cellulose is available to stabilize a higher overall interfacial area. This is in agreement with several studies performed on particle-stabilized emulsions (Aveyard, Binks, & Clint, 2003; Frelichowska, Bolzinger, & Chevalier, 2010; Kalashnikova, Bizot, Cathala, & Capron, 2011b; Rayner et al., 2014). Particularly, the effect of cellulose concentration was critical over the lowest used concentrations (below 0.3%) and was only marginal provided concentration increased above 0.7%. Similarly, the size of the emulsion droplets was affected by the type of cellulose. This is illustrated in Fig. 1 which shows emulsions with O/W 10/90 prepared with CNC, which yielded smaller droplets (14–34 μm) compared to those stabilized with MFC (27–51 μm), irrespective of the oil and its content. This clearly relates to the particle size. CNCs lie as a flat monolayer on the surface (Cherhal, Cousin, & Capron, 2016), thus enabling a lower droplet size as its shorter length can accommodate a higher curvature. In comparison, MFC is longer, and less keen on promoting high curvature. Differences in droplet size distribution of the emulsions stabilized with CNC and MFC are depicted in Fig. 2 for cinnamaldehyde emulsions (O/W 10/90, 0.5% cellulose). The distribution curves, obtained by laser diffraction, confirm the presence of one main population together with a minor population constituted by the non-adsorbed nanocellulose. There is no significant difference in the shape of the distributions recorded for the emulsions prepared with CNC or MFC. The above conclusions regarding droplet sizes are also confirmed by photomicrographs comparing limonene emulsions stabilized with either CNC or MFC (Fig. 3).

These findings are consistent with the study reported by Cunha et al., 2014, in which hexadecane/water interface was stabilized by using CNC or NFC. It was concluded that droplet size distributions of both emulsion systems, assessed by optical microscope, were slightly polydisperse with droplets smaller for CNC-stabilized emulsions (2.6 ± 0.8 μm) compared to those with NFC (3.3 ± 1.2 μm).

Fig. 1 also reveals that, with respect to the droplet size, the character of the oil plays an important role. The smallest droplets were observed in case of limonene and cinnamaldehyde-loaded emulsions, whereas in case of eugenol, their sizes increased. This behaviour can be attributed to chemical character of the individual oils. As the oils used within the studied systems are moderately polar with the least polar being limonene with a required HLB of 6–7 and the most polar being cinnamaldehyde and eugenol, their ability to form stable O/W emulsion is more limited and tends to be challenging compared to non-polar oils. The polar character indeed influences the particles adsorption, leading to a modification of the droplet curvature and subsequently of the droplet size. The emulsion droplets were influenced by the oil volume fraction as well and their sizes increased with increasing O/W ratio. This feature might be attributed to the low cellulose particles concentration. As the oil content increases, there is not enough cellulose particles to cover the oil droplets surface resulting in an increase of the droplet volume in order to decrease the total interfacial area.

The emulsions were also followed during storage and the data revealed that the average droplet size of both MFC and CNC-based emulsions increased during the first month of storage.

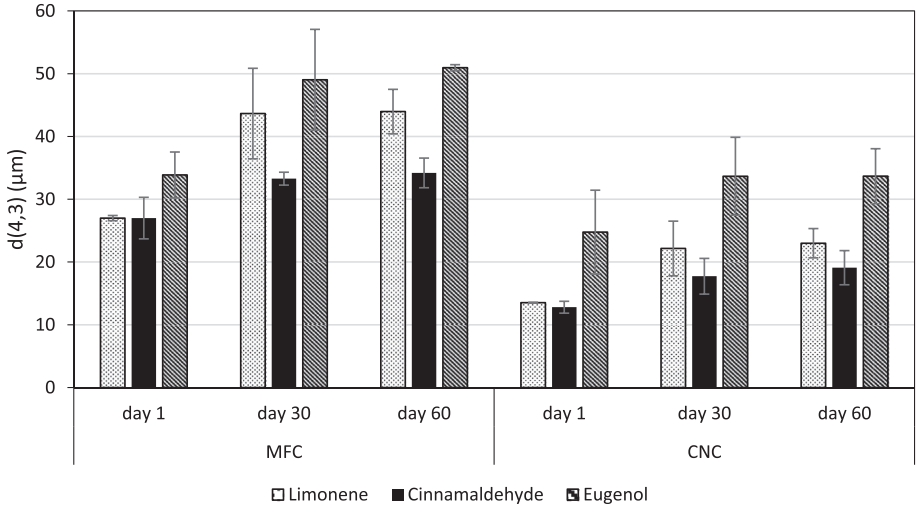

Fig. 1. Comparison of droplet size for O/W 10/90 emulsions stabilized with 0.5 wt% MFC or CNC assessed by laser diffraction.

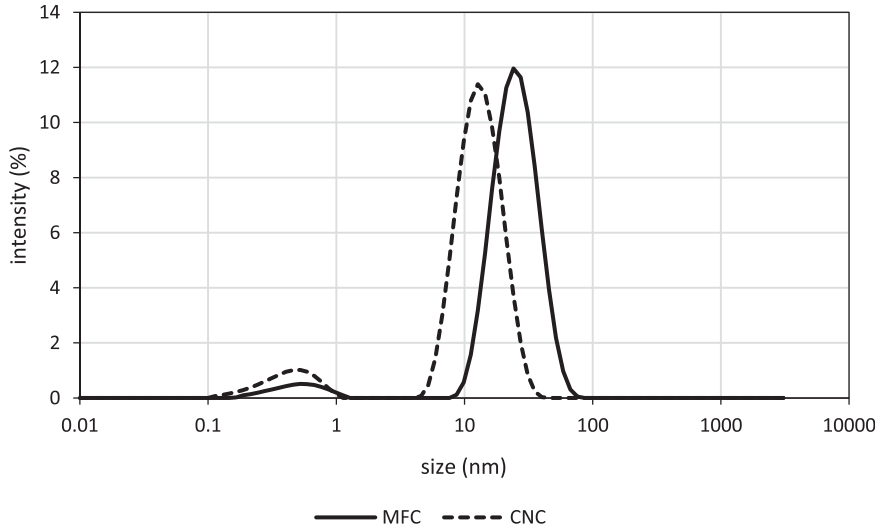

Fig. 2. Droplet size distribution of cinnamaldehyde-loaded emulsions prepared with 0.5 wt% CNC or MFC, obtained by laser diffraction.

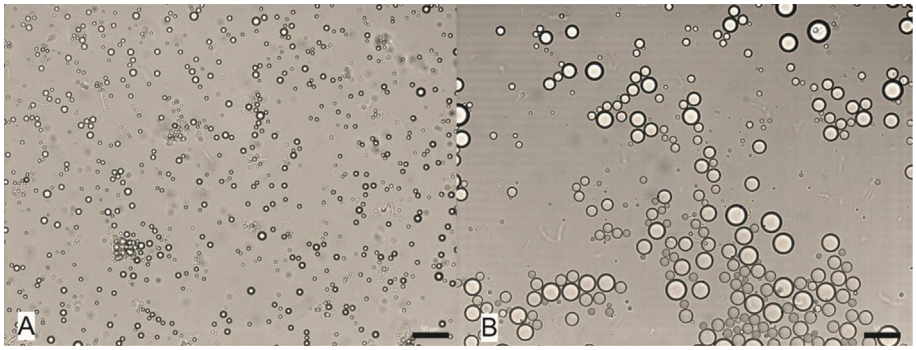

Fig. 3. Optical microscopy (Magnification 20×) of 10/90 limonene emulsions prepared with 0.5 wt% CNC (A) and MFC (B). Scale bar is 50 μm.

Interestingly, after two months no additional increase in droplet size was observed.

Excellent long-term stability provided by Pickering emulsions is well-known and could be attributed mainly to the strong binding energies of the particle stabilizers with the interface.

In practice, the particles used are considered as irreversibly attached to the interface, leading to the formation of highly stable emulsion droplet (Aveyard et al., 2003; Dickinson, 2010).

Publications dealing with emulsions stabilized with cellulose particles report on their reasonably good stability during storage (Cunha et al., 2014; Rayner, Sjöö, Timgren, & Dejmek, 2012). It should be however noted that the mentioned studies were conducted using model oils such as Miglyol® 812 (caprylic/capric triglyceride) or hexadecane and these model systems can hardly be compared with the oils used in our study. Essential oil components exhibit namely some water-solubility and the oil droplets in our emulsions are therefore prone to Ostwald ripening contrary to those containing completely water-insoluble oils (Chang, Chen, & Chang, 2001;; Ziani, Chang, McLandsborough, & McClements, 2011).

3.1.2. Encapsulation efficacy and emulsion index

The impact of the nature of the oil, the oil content and the amount as well as the type of nanocellulose on the emulsion properties was assessed by using two characteristics, namely encapsulation efficacy (EE) and emulsion index (EI). The encapsulation efficiency offers a mean to qualitatively assess the performances of a given system while the emulsion index reflects often the capacity of a given emulsifier/oil system to sustain the spontaneous phase separation.

3.1.2.1. Encapsulation efficiency (EE). Encapsulation efficiency was first assessed by visual observation and a complementary study was carried out after 2 months using centrifugation of prepared emulsions at 500 rpm for 1 min. In both cases the oiling-off was followed. During three days of preparation, an early oiling-off could be observed, that then stabilized. The influence of cellulose type on EE is illustrated by the behaviour of emulsions with 10% oil content (Fig. 4). In the case of MFC emulsions, the EE values varied from 47 to 100%, while for CNC emulsions ranged from 79 to 100%.

For CNC emulsions, minor phase separation could be noticed during the first two days after preparation and then no further separation was observed. Moreover, no considerable changes could be seen when submitting the emulsions to centrifugation compared with non-centrifuged ones, in terms of oil release. On the other hand, MFC emulsions were more susceptible to phase separation during centrifugation, as well as during the storage. The higher EE determined in CNC based emulsions can be attributed to the stabilizing effect of this cellulose type, as suggested by Ougiya, Watanabe, Morinaga, & Yoshinaga, 1997. As CNC consists of smaller and thinner particles than MFC, it can form a more densely packed layer at the oil-water interface thus forming a physical barrier

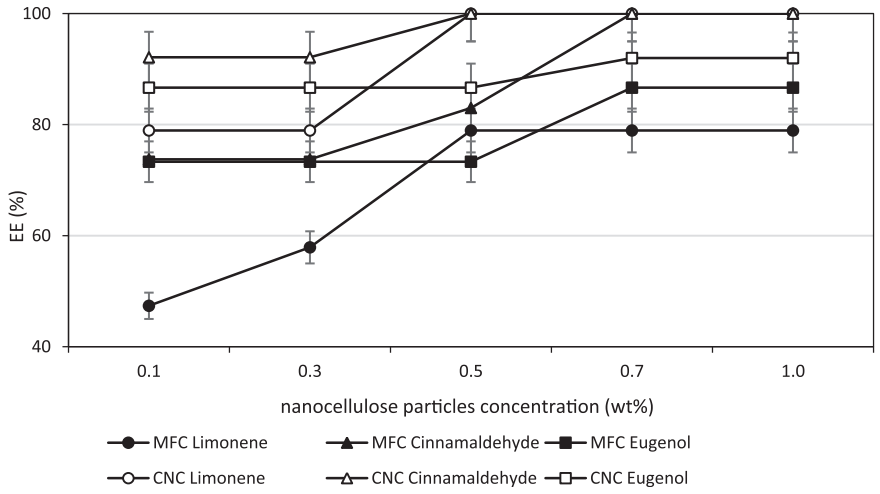

Fig. 4. Effect of cellulose particles concentration and oil type on EE of emulsions with O/W 10/90.

hindering oil leakage.

The cellulose particles concentration in emulsion sets the amount of stabilizer available to cover droplet surface (Paximada, Tsouko, Kopsahelis, Koutinas, & Mandala, 2016). It can be therefore assumed that increasing cellulose concentration will enhance the EE as illustrated in Fig. 4. It can be seen, that depending on the oil type, increase in cellulose concentration from 0.1 to 0.5% caused EE to gradually increase, especially for cinnamaldehyde and limonene emulsions. Content of cellulose higher than 0.7% did not further improve EE and values of 79–100% for MFC-based emulsions and 87–100% for CNC-emulsions were determined for 0.7–1.0% cellulose, irrespective of the oil used.

Effect of the oil content on EE is depicted in Fig. 5 showing emulsions stabilized with 0.3% CNC and MFC (wt%). Despite of increasing the oil fraction, EE remained unchanged until the oil content reached 30%; then EE for eugenol and limonene emulsions rapidly decreased. Interestingly, satisfactory encapsulation of cinnamaldehyde was observed also at 40% oil content without EE change. This behaviour was the most apparent in emulsions stabilized with low concentrations of cellulose (below 0.5%). Earlier studies have already shown that stability of the Pickering emulsions may be influenced, among others factors, by the oil polarity. If for example hydrophilic particles are used, the most stable O/W emulsions are obtained with the polar oils (Frelichowska et al., 2010). The effect of oil nature would be most noticeable at lower concentration of stabilizing particles when their layer on the droplets is insufficient to fully cover their surface. These results are in line with our observations. Whilst at low concentrations of cellulose particles oil polarity influenced emulsion stability, at higher concentrations the effect was not so clear.

3.1.2.2. Emulsion index (EI). Fig. 6 shows the correlation between the concentration of cellulose and emulsion index, three days after preparation. The emulsion index, expressed as the total volume of emulsion to the total volume of the system, is a measure of emulsion instability. As it can be seen, increased content of cellulose have led to higher values of EI, with the same trend observed for all oils and both cellulose types used. It is especially visible in the case of CNC based emulsions, in which already 0.5% of cellulose was capable of stabilizing oil droplets and EI of 95–100% was recorded. For MFC emulsions, EI values were lower in comparison with CNC, mainly at 0.3% cellulose content where differences between EI of

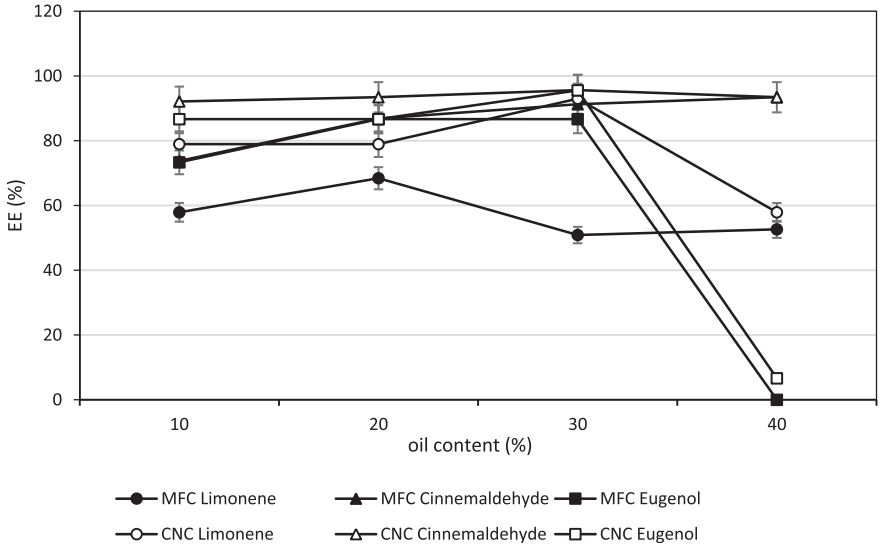

Fig. 5. Effect of oil content and oil type on EE of emulsions stabilized with 0.3 wt% CNC.

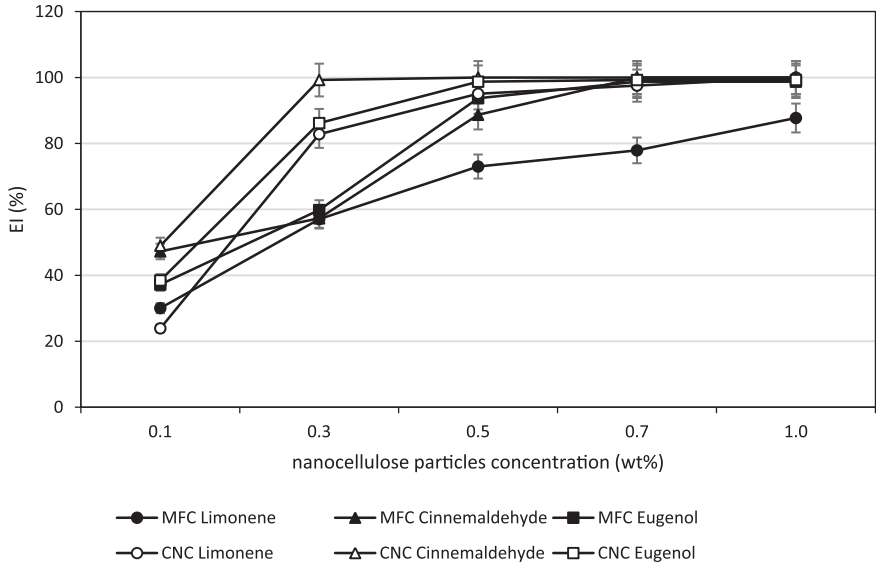

Fig. 6. The correlation between the concentration of cellulose particles and EI after 3 days of storage (emulsions with O/W 10/90).

CNC a MFC systems were the most evident.

Overall, with the emulsions stabilized by lower cellulose amounts (MFC $\leq$ 0.30%, CNC $\leq$ 0.50%), the EI decreased rapidly after emulsification, and creaming (limonene emulsions) or sedimentation (cinnamaldehyde and eugenol samples) occurred within several minutes after their preparation. This effect, however, stabilized after one to two days. On the contrary, the systems prepared with higher tested concentrations of cellulose (MFC $\geq$ 0.50%, CNC $\geq$ 0.70%) have not developed creaming or sedimentation and remained unchanged over a storage period of 2 months.

MFC ability to form more stable emulsions without creaming or sedimentation during the storage may be attributed to the formation of a strong three-dimensional fibril-droplets network in the emulsions; the droplets are thus stabilized by the long MFC particles thanks to their high aspect ratio (~50–100). Conversely, CNC particles having lower aspect ratio (~10–70) can form only a weak bonded network and their capability to prevent creaming is lower (Cunha et al., 2014; Winuprasith & Suphantharika, 2015).

Furthermore, as expected, EI increased with growth of the oil fraction. Moreover, EI was influenced by the nature of the encapsulated oil, with cinnamaldehyde emulsions having the highest EI values, which corresponds to most stable emulsions.

3.2. Antibacterial screening

3.2.1. Well diffusion assay

Antibacterial activity of oil-loaded emulsions stabilized by nanocellulose particles with a concentration of 0.5 wt% was determined using the well diffusion method and expressed in terms of the size of the inhibition zone (mm).

Among the samples tested, emulsions containing eugenol and cinnamaldehyde both with O/W ratio of 10/90 and 40/60, exhibited inhibitory activity against all the test strains, in contrast to limonene emulsions, where no antimicrobial effect was recorded. Emulsions with cinnamaldehyde showed the strongest antibacterial activity with a size of the inhibition zones ranging from 20.0 ± 0.9 mm (*P. aeruginosa*) to 55.0 ± 0.9 mm (*M. luteus*). Concerning the emulsions with eugenol, they inhibited bacterial growth to a lesser extent resulting in zones of diameter from 11.5 ± 0.9 for *S. marcescens* to 20.5 ± 0.9 mm for *S. aureus*. These results comply with findings reported by Sanla-Ead, Jangchud, Chonhenchob, & Suppakul, 2012, who showed that eugenol possesses moderately–strong inhibitory activity, whilst inhibitory activity of cinnamaldehyde was assigned as strong to highly strong.

As expected (Brenes & Roura, 2010; Burt, 2004; Canillac & Mourey, 2001; Mejlholm & Dalgaard, 2002), the emulsions showed better activity against gram positive than gram negative bacteria as a result of differences in their cell walls. The reason of higher resistance observed on gram negative species is the presence of an outer membrane containing lipopolysaccharides, which protects the bacteria more efficiently from disruption caused by antimicrobial oils. In accordance with this fact, *P. aeruginosa* and *S. marcescens* were found to be the most resistant species. On the contrary, *M. luteus* and *S. aureus* were the most sensitive bacteria to antibacterial activity of the emulsions tested. Interestingly, increase of O/W ratio from 10/90 to 40/60 did not result in enhancement or improvement of the overall antibacterial activity of the emulsions. There seems to be a limiting factor that will be discussed further.

Well diffusion assays further revealed that the chemical nature of antibacterial oils is solely responsible for the antibacterial activity of the tested emulsions, as 0.5% solution of nanocellulose particles (negative control) did not cause inhibition of any of the tested strains. It can be seen that the tested bacteria responded differently to the individual emulsions, which indicates that 1) different antibacterial oils may have different modes of action or 2) the metabolism of some of the bacterial species is able to better overcome the effect of the antibacterial oils or adapt to them. The differences between the antibacterial efficiency of formulated emulsions might be explained in the light of the mentioned differences in oil type used. It is known that hydrocarbon monoterpenes show the lowest antibacterial activity, while antibacterial potential of oxygenated compounds, especially phenol-type compounds is higher (Knobloch, Weigand, Weis, Schwarm, & Vigenschow, 1986). Hydrocarbons such as limonene then show even lower antibacterial properties, as the low water solubility limits their diffusion through the growth medium used in antibacterial tests (Griffin, Markham, & Leach, 2000). On the other hand, cinnamaldehyde and eugenol incorporated in emulsions displayed strong antibacterial activity, which can be related to the presence of the respective functional groups (aldehyde, phenol) in the oil and to the solubility and diffusibility of antibacterial oils into the agar.

3.2.2. Broth microdilution assay

Simultaneously with the well-diffusion assay, broth microdilution method was used in order to provide better insights into antibacterial potential of studied emulsions. The possible effect of parent nanocellulose particles, free (non-encapsulated) oil, and emulsion storage time on their antibacterial activity was also evaluated. As the emulsions both with O/W 10/90 and 40/60 possessed similar inhibitory effects in an initial screening, only formulation with O/W 10/90 was employed for the tests.

3.2.2.1. Effect of antibacterial oil on antibacterial activity of emulsions. Similarly to the results from the well diffusion method, the antibacterial potential of the studied samples decreased in the following order: emulsions with cinnamaldehyde > eugenol > limonene. Representative results are provided in Fig. 7 showing the values of growth index (GI) of *P. aeruginosa*, one of the leading, resistant foodborne pathogen.

As assumed, emulsions were less effective in inhibiting the growth of gram negative bacteria when compared with gram positive species. Similarly to well diffusion assay, the antibacterial activity of emulsions was mainly governed by oil type, its composition/chemical nature and oil fraction.

3.2.2.2. Cinnemaldehyde emulsions. In line with results from well diffusion assay, the cinnamaldehyde-loaded emulsions were proved to be the most active in the growth suppression of tested bacteria (Fig. 8). The total growth inhibition of all tested bacteria was observed at emulsions with an oil fraction of 0.5–10 mg/mL. These results are consistent with observations reported for pure cinnamaldehyde, as its minimal inhibitory concentration (MIC), i.e. the lowest concentration at which no growth occurs, was reported to lie from 0.25 to 1 mg/mL (Chang et al., 2001) and 0.5–1 mg/mL (Domadia, Swarup, Bhunia, Sivaraman, & Dasgupta, 2007) for a series of bacteria including *E. coli*, *S. aureus*, *B. subtilis* and *Streptococcus* spp. After the application of cinnamaldehyde emulsions with oil fraction lower than 0.5 mg/mL, the bacterial growth gradually enhanced. Emulsions with 0.05 mg/mL oil exhibited inhibitory effects against gram-positive bacteria causing more than 55% reduction of their growth regardless of type of nanocellulose used. At the lowest tested fraction of oil (0.005 mg/mL), notable bacterial growth was detected, however.

Gram negative species were more resistant to cinnamaldehyde-emulsion. At oil fraction of 0.05 mg/mL GI appeared to be higher than 65%, irrespective of type of nanocellulose particle used. The presence of emulsions with the lowest fraction of oil (0.005 mg/mL) led to more significant increase in the GI compared to gram positive strains.

3.2.2.3. Eugenol emulsions. In comparison with cinnamaldehyde emulsions, the antibacterial activity of those based on eugenol were lower (Fig. 9). However, complete inhibition of all bacteria was also observed after application of samples with oil content ranging from 1 to 10 mg/mL. The results generally agree with those obtained by (Walsh, Maillard, & Russell, 2003; Van Zyl, Seatlholo, & van Vuuren, 2006) who reported MIC ranging from 0.5 to 1 mg/mL for eugenol when applied on *S. aureus* and *E. coli* and 2 mg/mL for *B. cereus* and *E. coli*. At oil fraction of 0.5 mg/mL, a low growth activity of gram positive bacteria was recorded (GI below 20%). Moreover, in the presence of emulsions stabilized by MFC, no growth was detected. However, when the fraction of eugenol in emulsions decreased to 0.05 and 0.005 mg/mL, antibacterial activity was rapidly reduced. In contrast with gram positive bacteria, complete growth inhibition of gram negative species was not observed, even for the highest concentration of emulsions. Acceptable reduction in growth of gram negative species (GI $\leq$ 50%) was, however, observed at oil fraction of 0.5 mg/mL. Correspondingly to the previous results, additional decrease of oil amount in emulsion (0.05–0.005 mg/mL) caused an increase in bacterial growth.

3.2.2.4. Limonene emulsions. Within the set of emulsions tested, those containing limonene showed the lowest antibacterial activity and their behaviour was more complicated to rationalize than that of the above discussed samples. Interestingly, compared with well diffusion assay which did not prove any antibacterial properties of these emulsions, broth microdilution method revealed their activity. The reason for the discrepancy between these methods can be related to poor availability of limonene to bacterial species, originating from its low polarity and hence low ability to penetrate/diffuse through a hydrophilic, stiff and jelly agar used as a growth medium (see Table 1). Hence, the broth dilution method appeared to be more suitable for the quantitative determination of antibacterial activity of emulsions in the test.

With respect to gram positive bacteria no growth was apparent in the presence of limonene emulsions with oil fraction of 10 and 5 mg/mL stabilized by CNC. The only exception was *E. faecalis* with GI of 11%. Similarly, total inhibition was detected at oil fraction of 2.5 mg/mL for CNC emulsions with exception of *B. cereus*. In contrast, total inhibition of gram negative bacteria was not

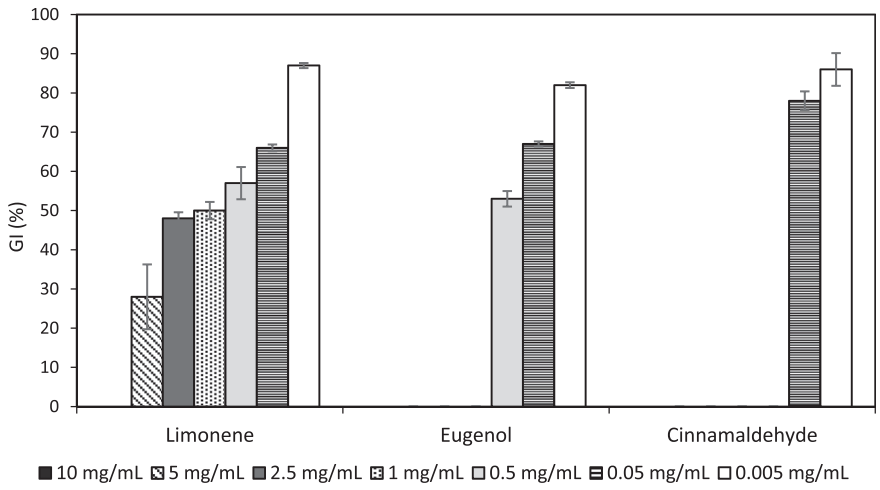

Fig. 7. The effect of O/W 10/90 emulsions stabilized by 0.5 wt% CNC loaded with limonene, eugenol and cinnamaldehyde, on the growth of *P. aeruginosa*.

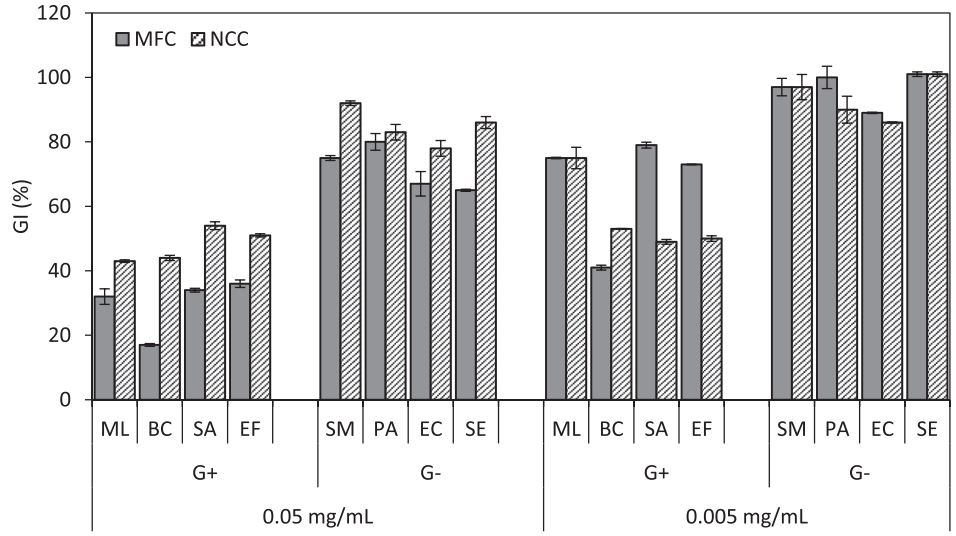

Fig. 8. The effect of cinnemaldehyde loaded emulsions (O/W 10/90) at the two lowest oil concentration used on the growth of all bacteria tested. Higher concentration than 0.05 mg/mL caused total growth inhibition.

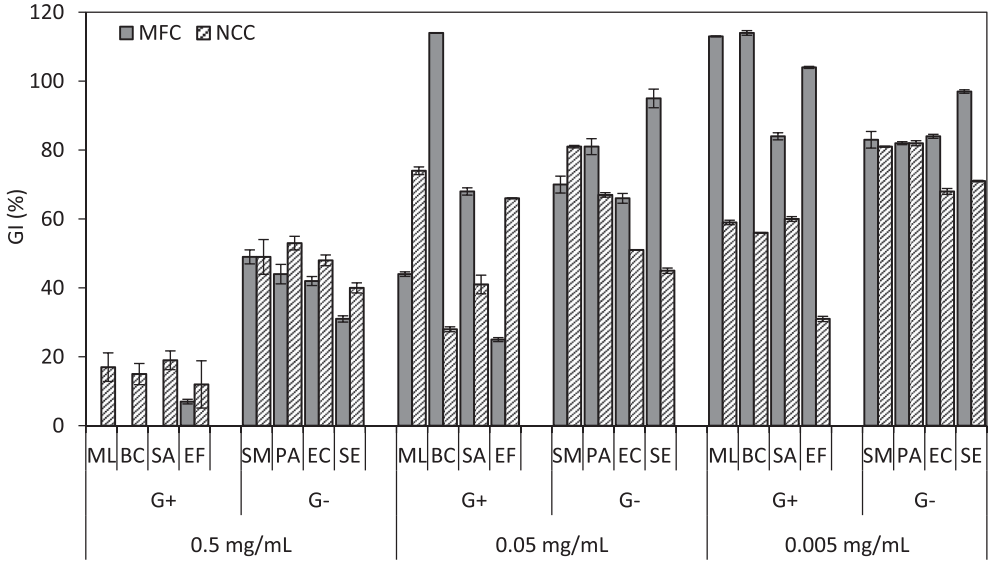

Fig. 9. The effect of eugenol emulsions on the growth of all bacteria tested. Higher concentration than 0.5 mg/mL caused total growth inhibition.

Table 1
Concentration of free oil and oil entrapped in emulsions determined using HPLC. Solubility of respective oil in water (25 °C) is also provided [HSDB, 2012].

Oil	Solubility in water (mg/mL)	Theoretical amount of oil in emulsion (mg/mL)	Oil in emulsion droplets (mg/mL)		Free oil (mg/mL)	
			CNC	MFC	CNC	MFC
Eugenol	2.46	100.50	97.15	78.03	3.35	22.47
Cinnamaldehyde	1.42	100.50	100.50	89.08	n.d.	11.42
Limonene	0.014	98.14	98.14	88.00	n.d.	10.14

recorded until the highest oil fraction of 10 mg/mL was applied. The results generally comply with those obtained in study of van Vuuren, & Viljoen, 2007, who reported the MIC for limonene ranging from 2 to 13 g/mL for *S. aureus*, *B. cereus*, *E. coli* and *P. aeruginosa*, depending on the bacteria studied. As it can be seen in Fig. 10, at oil fractions lower than 0.5 mg/mL bacterial growth increased concomitantly. In contrast, MFC based limonene emulsion performed worse.

3.2.2.5. Effect of cellulose type on antibacterial properties of emulsions. In order to assess the effect of pristine cellulose particles on the antibacterial activity of emulsions, 0.5% MFC or CNC dispersions without antibacterial oil were tested. These reference samples exhibited no antibacterial activity (GI = 100%) during the trial. In

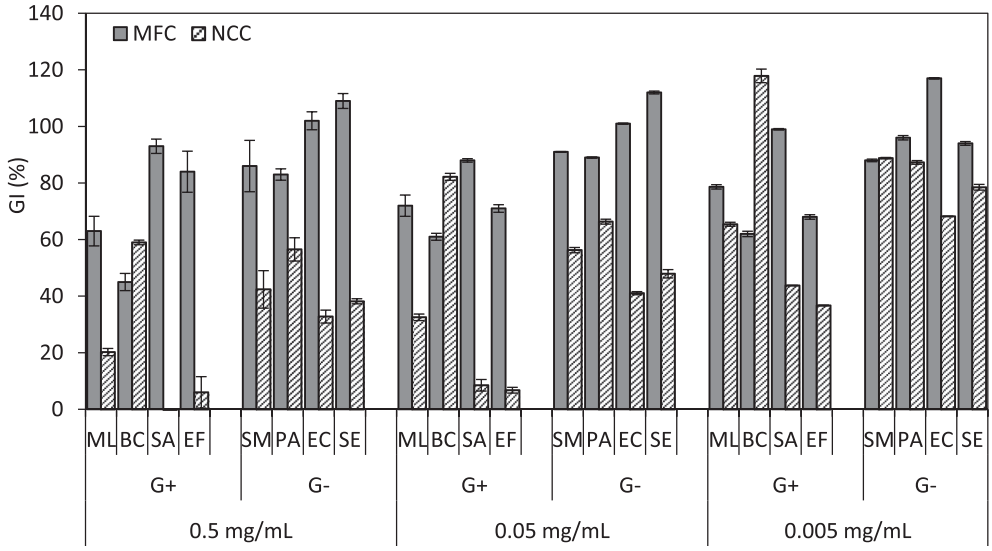

Fig. 10. The effect of limonene emulsions at O/W 10/90 on the growth of all tested bacteria.

addition, no differences in the bacterial growth caused by the presence of MFC or CNC dispersions appeared.

Both CNC and MFC emulsions with oil fractions higher than 0.5 mg/mL (eugenol) and 0.05 mg/mL (cinnamaldehyde) performed equally and caused total inhibition of all bacteria tested. The situation was, however, different when it comes to the oil content below these limits. Here, the emulsions stabilized by MFC differed in antibacterial activity from emulsions stabilized by CNC (Figs. 8–10). The figures illustrate that this activity was affected by several parameters, namely by the type of cellulose particles, the oil fraction and also by the type of bacterial species used in the test. This is mostly evident for cinnamaldehyde emulsions stabilized by MFC (oil fraction of 0.05 mg/mL) and eugenol emulsions (0.5 mg/mL), which both possessed higher antibacterial properties compared to corresponding samples stabilized with CNC, especially in the case of gram positive bacteria. As the concentration further decreased, the "cellulose" effect was lost and at even lower oil content in emulsions, no systematic trend in antibacterial effect of CNC or MFC emulsions was observed. Due to these variations, it is not possible to unambiguously conclude, at higher concentration, whether emulsions with MFC or CNC performed better. Due to the fact that nanocellulose itself has no antibacterial activity, the better effect of some MFC emulsions might be attributed to the higher amount of free oil which is not entrapped into emulsions droplets. As it was already mentioned, emulsions containing limonene showed more complex behaviour and their antibacterial activity was strictly dependent on emulsion concentrations applied and bacterial species; and as such, the effect of cellulose type on antibacterial properties can be neglected.

3.2.2.6. Effect of storage on antibacterial activity. In order to assess the effect of aging on the antibacterial activity, emulsions loaded with cinnamaldehyde and eugenol stored at 25 °C were re-tested against selected, most common bacterial pathogens, *E. coli and S. aureus*, two months after the first testing. The microdilution assay showed the decrease of antibacterial effect towards both bacterial species over storage time. The loss of activity was recorded for both cinnamaldehyde and eugenol emulsions, irrespective of type of nanocellulose used. The reduction of activity can be observed for example on cinnamaldehyde emulsions; whereas in the initial test cinnamaldehyde emulsions induced the total inhibition of *S. aureus* at oil fraction of 0.5 mg/mL (GI = 0), antibacterial assay conducted later on showed decrease in activity, with GI values increasing to 7% for MFC and 39% CNC emulsions, respectively. Regarding eugenol, the antibacterial activity decreased as well resulting in increase of GI value to 26% for CNC and 70% MFC based emulsions.

The loss of antibacterial activity of emulsions over time may be caused by various factors. Upon aging, eugenol and cinnamaldehyde have proved to be oxidized or decomposed to reactive intermediates when exposed to the air, light or heat (Turek & Stintzing, 2013), which can accelerate the oxidation in emulsions. The effect can be enhanced especially in water rich- o/w emulsions containing essential oils or their components with some solubility in water. In these systems oils come into a close contact with the water phase and oxygen dissolved in water. Also, the emulsification process as such promotes oxidation through incorporation of oxygen into emulsion during its preparation (Berton-Carabin & Schroën, 2015). The oxidation could therefore result in the formation of degradation products with lower antibacterial activity compared to original cinnamaldehyde or eugenol. This process is well-described, especially in the case of cinnamaldehyde, with its potential to be oxidized to a cinnamic acid of weaker antibacterial activity (Hawkins, 2014; Horuz & Medeni, 2015).

3.2.2.7. Non-encapsulated oil and oil dissolved in water phase of emulsions. When discussing the antibacterial properties of tested emulsions in a wider context, in addition to activity of oil encapsulated in emulsion droplets, two more aspects have to be considered, namely contribution of 1) free, non-encapsulated oil and 2) oil dissolved in water phase of the emulsions. By comparing the amounts of non-encapsulated oil (Table 1) it is seen that MFC emulsions are more prone to oil leakage and contain higher amount of free oil, as determined in the stress test. This finding is also in accordance with the results from the determination of encapsulation efficacy. Presence of oil leaked from MFC stabilized droplets thus results in more oil available for a direct contact with bacteria compared to CNC emulsions. However, the effect of free oil contributed to the final antibacterial effect of emulsions only to minor extent. This is documented on the antibacterial activity of cinnamaldehyde-emulsions, which exhibited the strongest activity among the tested samples together with the best encapsulation efficiency.

Table 2
Minimal inhibitory concentration (MIC) in mg/mL of pristine oils dissolved in water at maximum solubility reported in literature (HSDB, 2000). In parenthesis, oil concentrations causing 50% growth reduction of bacteria is reported.

MIC (mg/mL)		
	Eugenol	Cinnamaldehyde
G^+ bacterial strains		
M. luteus	1 (*0.5*)	0.5 (*0.5*)
B. cereus	1 (*0.5*)	0.5 (*0.5*)
S. aureus	2.5 (*1*)	0.5 (*0.5*)
E. faecalis	2.5 (*1*)	0.5 (*0.5*)
G^- bacterial strains		
S. marcescens	2.5 (*1*)	0.5 (*0.5*)
P. aeruginosa	2.5 (*1*)	0.5 (*0.5*)
E. coli	1 (*1*)	0.5 (0.5)
S. enteritidis	1 (*0.5*)	0.5 (*0.5*)

It should be also noted that antibacterial oils dissolved in aqueous phase of emulsions might theoretically be responsible for the antibacterial activity of the emulsions. In order to verify the effect of dissolved oil, the broth microdilution assay was performed with aqueous solutions of each of the tested oils. Both cinnamaldehyde and eugenol solutions prepared at the concentrations corresponding to their maximum solubility in water at 25 °C, showed antibacterial activity, with MIC values reported in Table 2. The obtained MIC values corresponded to those found in literature (Carson & Hammer, 2011).

However, as regards the contribution of dissolved oil to total antibacterial activity of the emulsions, its maximum content in the water phase refers only to concentrated emulsions. During dilution performed for antibacterial testing, the concentration of dissolved oil gradually decreases. For example, in case of cinnamaldehyde emulsions, in ten times diluted parent emulsion (dilution corresponds to the highest tested fraction of encapsulated oil 10 mg/mL), the dissolved oil represents amount of only 0.142 mg/mL, which is well below the MIC determined in our test and reported in Table 2. Solubility of eugenol in water is somewhat higher and its content after dilution for antibacterial testing is of 0.246 mg/mL, which is also below the MIC determined for this substance (Table 2). The above discussion lead therefore to natural conclusion, that at the most concentrated emulsions tested for antibacterial assay, the oil dissolved in water can to some minor extent contribute to their total antibacterial activity, however the main action consists in encapsulated oil and its direct interaction with microorganisms. When the emulsions are further diluted, content of dissolved oil decreases and thus does not contribute to the antibacterial activity of the emulsions. MIC values for emulsions stabilized by MFC and CNC are shown in Table 3. This stresses the importance of the interfacial behaviour of the oils, and directly relates to the particles at the interface, and how crucial it is for the antibacterial properties. It correlates with the earlier observation that the type of nanocellulose plays a role for the interfacial passage, thus influencing the bulk availability and the overall activity, when the concentration is close to the threshold of activity of the dissolved oil.

4. Conclusion

In this work, novel emulsion systems with antibacterial properties have been developed and characterized. The emulsions containing cinnamaldehyde, eugenol or limonene were prepared without classical surfactants, using stabilization effect of cellulose nanocrystals and microfibrillated cellulose, respectively. Influence of the oil type and content as well as the type and concentration of cellulose particles on the physico-chemical properties of the emulsions was investigated and their antibacterial activity towards the most common gram positive and gram negative bacteria was determined. The analyses revealed that emulsion droplets stabilized with CNC were smaller than those with MFC. Concerning the effect of cellulose concentration, both CNC and MFC were capable to produce oil-in-water Pickering emulsions with antimicrobial oil content as high as 40 wt%, even at the lowest concentration of cellulose used 0.1 wt%. Despite the fact that the formulation of emulsions using antimicrobial oils is challenging, the prepared emulsions showed reasonably good stability during the 8-week storage, both in terms of droplet size changes and creaming or sedimentation.

Antibacterial testing revealed that the activity of emulsions tested was mainly controlled by the type of antibacterial oil and its content. The type of nanocellulose particles used for droplet stabilization showed, with this respect, only a minor contribution for high emulsion concentration. At low emulsion concentration, the effect of nanocellulose appeared to be more important and MFC-stabilized emulsion exhibited better antibacterial activity.

Although the nanocellulose-stabilized emulsions demonstrated antibacterial effects in general, variations could be identified in sensitivity of individual bacterial strains towards their action. In that sense, gram positive bacteria were found to be more susceptible to formulated emulsions in comparison with gram-negative species. It was also found out that the overall antibacterial effect is controlled mainly by the oil encapsulated in droplets, the contribution of free, non-encapsulated contributed only to minor extent. The best antibacterial activity against both bacterial types was observed in cinnamaldehyde emulsions with minimum inhibitory concentration of 0.5–1.0 mg/mL.

Table 3
Minimal inhibitory concentration (MIC) in mg/mL of MFC and CNC emulsions. In parenthesis, oil concentrations causing 50% growth reduction of bacteria is reported.

MIC (mg/mL)				
	Eugenol		Cinnamaldehyde	
	CNC	MFC	CNC	MFC
G^+ bacterial strains				
M. luteus	1 (*0.5*)	0.5 (*0.05*)	0.5 (*0.05*)	0.5 (*0.05*)
B. cereus	1 (*0.05*)	0.5 (*0.5*)	0.5 (*0.05*)	0.5 (*0.05*)
S. aureus	1 (*0.05*)	0.5 (*0.5*)	0.5 (*0.5*)	0.5 (*0.05*)
E. faecalis	1 (*0.5*)	1 (*0.05*)	0.5 (*0.5*)	0.5 (*0.05*)
G^- bacterial strains				
S. marcescens	1 (*0.5*)	1 (*0.5*)	0.5 (*0.5*)	0.5 (*0.5*)
P. aeruginosa	1 (*0.5*)	1 (*1*)	0.5 (*0.5*)	0.5 (*0.5*)
E. coli	1 (*0.5*)	1 (*0.5*)	0.5 (*0.5*)	0.5 (*0.5*)
S. enteritidis	1 (*0.5*)	1 (*1*)	0.5 (*0.5*)	0.5 (*0.5*)

The developed emulsions comply with a growing interest of the food industry and consumers for using of the products formulated on the basis of natural substances. Therefore, the results presented might have important implications for formulation of particle-stabilized antimicrobial emulsions with potential food applications.

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PAPER III

Pickering oil-in-water emulsions stabilized by carboxylated cellulose nanocrystals - effect of the pH

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Manuscript

Pickering oil-in-water emulsions stabilized by carboxylated cellulose nanocrystals – effect of the pH

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ABSTRACT

Carboxylated cellulose nanocrystals (cCNC) were prepared by oxidation of microcrystalline cellulose with ammonium persulfate and characterized by AFM. Zeta potential was measured at different pH and ionic strength, in presence of mono- and divalent cations. With a length ranging from 50 to 450 nm and a thickness varying between 20 and 60 nm, the cCNC had a surface charge that appeared to be more sensitive to the presence of divalent cations and exhibited a strong pH dependence. The nanocrystals were capable of forming stable oil-in-water emulsions at three different pH of 2, 4 and 7 with a triglyceride oil. The sizes of emulsion droplets were dependent on oil and cCNC contents. Emulsification was, however, mainly influenced by the pH of the continuous phase, which can be related to reduction of charge on the cCNC surface with decreasing pH. Responsiveness of emulsions towards pH changes was not as dominant as expected, and lowering of pH did not trigger the release of oil from droplets. This can be explained by the strong adsorption of the cCNC, relatively polar

triglyceride oil and the limited possibility to induce desorption of nanocrystals from oil surface.

Key words: Pickering emulsions, carboxylated cellulose nanocrystals, pH responsiveness, stability, triglyceride oil.

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Highlights

• Nanocrystals of carboxylated cellulose (cCNC) were prepared and characterized.

• Using cCNC, stable Pickering triglyceride-in-water emulsions were formulated.

• pH was a crucial parameter which influenced emulsification process.

• pH variation did not trigger the release of oil from droplets.

1. Introduction

Over the past decades, an increasing interest in the development and application of particle-stabilized emulsions has appeared (Aveyard, Binks, & Clint, 2003; Binks, 2002), driven by the effort to replace, at least partially, synthetic surfactants known for their topical toxicity (Lémery et al., 2015). Various types of synthetic particles, whether organic or inorganic, have been used as emulsion stabilizers (Lam, Velikov, & Velev, 2014; Wu & Ma, 2016; Xiao, Li, & Huang, 2016). However, the call for using environmentally sustainable resources has oriented the interest towards materials of natural origin, and among such type of materials cellulose particles have gained a special interest (Mikulcová, Bordes, & Kašpárková, 2016; Wang et al., 2016; Winuprasith & Suphantharika, 2013). There is a variety of suitable types of cellulose-based particles for the stabilization of emulsions, and cellulose nanocrystals (CNC) have proven to be very efficient in stabilizing interfaces (Capron, Rojas, & Bordes, 2017). CNC is obtained from a top-down preparation route that allows the removal of the amorphous part of cellulose fibres to extract the more crystalline portions (Habibi, Lucia, & Rojas, 2010; Peng, Dhar, Liu, & Tam, 2011; Zhang et al., 2013). The removal of the amorphous part can be carried out by several procedures, all of which significantly influence the surface properties and the applicability of the final CNC. The more common methods employ treatments of cellulose containing materials with acids, enzymes, oxidation agents, mechanical stress or a combination of them (Cao, Dong, & Li, 2007; Filson, Dawson-Andoh, & Schwegler-Berry, 2009; Jonoobi et al., 2015; Montanari, Roumani, Heux, & Vignon, 2005; Revol, Bradford, Giasson, Marchessault, & Gray, 1992; Sacui et al., 2014). In contrast to the well-established hydrolysis of cellulose by sulfuric acid that gives stable colloidal suspensions of cellulose nanocrystals which exhibit a low pH dependence (Irina Kalashnikova, Bizot, Cathala, & Capron, 2011; Wang et al., 2016), alternative routes have been developed for preparing pH responsive CNC. For instance, by treating a nanocellulose with the so-called

TEMPO oxidant several groups obtained highly crystalline CNCs bearing carboxylic groups on the surface, referred to as carboxylated cellulose nanocrystals (cCNC). (Isogai, Saito, & Fukuzumi, 2011; Jia et al., 2016; Shimizu, Fukuzumi, Saito, & Isogai, 2013) More recently, Leung et al. (2011) reported an original and simple method for the direct preparation of cCNC by employing a strong oxidizing agent, ammonium persulfate (APS). This procedure could be used for the processing of variety of native plant fibres and other cellulose sources.

Whereas several studies report on the application of bare nanocellulose for stabilizing oil-in-water emulsions (see for instance the pioneering work by Irina Kalashnikova et al., 2011; I. Kalashnikova, Bizot, Cathala, & Capron, 2012; Wang et al., 2016, that has been followed by others Hu et al. 2015a; Hu et al. 2015b) or after hydrophobization, water-in oil emulsions (Lee, Blaker, Heng, Murakami, & Bismarck, 2014; Saidane, Perrin, Cherhal, Guellec, & Capron, 2016), much less work has been dedicated to the emulsification performances of cCNC, especially in relation to the pH responsiveness. In 2014, Wen et al. studied the emulsification of d-limonene by carboxylated cellulose nanocrystals prepared *via* APS treatment of corncob cellulose. They obtained system with double responsiveness, both towards temperature and pH; stability of the emulsions was improved by increased temperature, whilst it was reduced at low pH or high salt concentration due to electrostatic screening of the negatively charged cCNC particles. However, in this unique example no systematic study on the pH dependent behaviour of cCNC stabilized emulsions was reported.

The present work, therefore, focuses on the formulation of cCNC stabilized Pickering emulsions prepared at three different pH, and on the characterization of their behaviour in terms of particle size, zeta potential and phase behaviour. The oil phase of the emulsions was triglyceride oil, which is commonly used as a neutral carrier for various lipophilic bioactive substances. The employed carboxylated nanocrystalline cellulose was prepared *via* a one-step oxidation procedure by APS. The nanocrystals were characterized using atomic force

microscopy (AFM) and dynamic light scattering (DLS). Behaviour of cCNC under different pH and ionic strength was studied by zeta potential measurements.

2. Materials and methods

2.1 Materials

Microcrystalline cellulose Avicel® PH-101 and ammonium persulfate both supplied by Sigma Aldrich (Germany) were used for the preparation of cellulose nanocrystals. The nanocrystals were utilized for the preparation of emulsions containing tricaprylin/tricaprin oil (Tegosoft ®CT, Evonik Industries AG, Germany). Water was purified by reverse osmosis (0.06 μS/m). Tegosoft composition, according to Ph.Eur. was as follows: caproic acid ≤ 2%, caprylic acid 50 – 65 %, capric acid 30 – 45 %., lauric acid ≤ 2 % and myristic acid ≤ 1 %. Hydrochloric acid, sodium hydroxide, calcium chloride, sodium chloride (IPL Petr Lukeš, Czech Republic) were used without purification.

2.2 Preparation of cellulose nanocrystals

Cellulose nanocrystals were prepared by adapting a procedure inspired by Leung et al. (2011). To 10 g of microcrystalline cellulose was added 1 L of 1 M APS solution. The suspension was first heated at 50 °C for 15 min and temperature was then increased to 70 °C. The stirring of the solution was kept at this temperature for 24 h. The suspension was centrifuged (7000 rpm) for 10 min using Superspeed Centrifuge Sorvall Lynx 4000 (Thermo Scientific, USA). After each centrifugation, supernatant was decanted and replaced with purified water. The centrifugation/washing cycle was repeated until conductivity of suspension reached 3 μS/cm. The suspension was then sonicated for 30 min at an amplitude of 40 % using a UP400S sonicator (Heielscher, Germany) after adjusting the pH to 7 with NaOH (~1M). Finally, the

concentration of cCNC dispersion was adjusted to 2 % wt by removing the water with a rotary evaporator.

2.3 Characterization of nanocrystals

Atomic force microscope (AFM) PeakForce TUNA module on Dimension ICON (Bruker Corporation, USA) was utilized for the characterization of the cellulose nanocrystals. The measurements were conducted at normal RH and room temperature in semi-contact mode. A silicon nitride probe (Bruker Corporation, USA) with a spring constant of 5 N/m and resonant frequency of 150 ± 50 kHz was employed. The image was recorded at a scanning rate of 0.5 Hz.

Particle size, particle size distribution, polydispersity index (PDI) and zeta potential were determined by dynamic light scattering (DLS) carried out on a Zetasizer Nano ZS90 instrument (Malvern Instruments, Malvern, UK). The analyses were carried out on samples diluted in water at a scattering angle of 90° and temperature of 25 °C. Prior to the measurements, the suspension was filtered with a hydrophilic 800 nm syringe filter (Sartorius). Zeta potential of cCNC particles was measured over a pH range of 2-10. The cCNC suspensions were diluted (0.35 %) in pH-adjusted water. Correspondingly, zeta potential at different ionic strengths of dispersion media was determined using series of NaCl and $CaCl_2$ solutions with concentrations ranging from 0 to 100 and 0 to 10 mM, respectively. The ionic strength was calculated using equation $I = ½ \Sigma c_i z_i^2$, where c_i represents the molar concentration of the ion and z_i is the charge number of that ion. All sizing and zeta potential measurements are reported as means and standard deviations was calculated on the basis of at least three repeated measurements.

2.4 Stability of oil under emulsification

In order to verify the stability of the triglyceride oil during the emulsification, acid value of the oil was determined prior and after 1 and 10 min sonication according to a standard procedure described elsewhere (AOCS & Firestone, 2011).

2.5 Preparation of emulsions

Prior to emulsification, the cCNC suspension was sonicated for 5 min. using a UP400S sonicator (Heielscher, Germany) in order to disintegrate agglomerates possibly formed during storage. Individual components of each of the emulsion (oil, water and cCNC) were, in pre-calculated amounts, weighed directly into the glass vial. The aqueous phase containing cCNC was left either native (pH of 7) or adjusted using HCl to pH of 4 and 2. Oil to water (O/W) ratios of 10/90, 20/80, 30/70 and 40/60 were used. Four different concentrations of cCNC of 0.01, 0.05, 0.1, and 0.3 wt. % were applied. The cellulose mass content refers to its content in the total emulsion. Emulsifications were carried out using sonication (UP400S sonicator), (Heielscher, Germany) for 1 minute at 100 % amplitude. During the preparation, the samples were cool down with an ice bath.

2.6 Characterization of emulsions

The size and distribution of the emulsion droplets were determined using laser diffraction on Master Sizer 3000 (Malvern instruments, UK). For diffraction measurements, the emulsions were sampled and suspended in the instrument flow system containing milliQ-water at a pump velocity of 2200 rpm. The refractive index of cCNC was set to 1.421. The volume mean diameter D(4,3) corresponding to the mean diameter of spheres with the same volume as the analysed droplets was calculated according to $D_{(4,3)} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$, where n_i is the number of particles with diameter d_i (Barth, 1984) Data reported as a mean and standard deviations was calculated on the basis of at least three repeated measurements.

Zeta potential of c-CNCs stabilized emulsions was measured using a Zetasizer Nano ZS90 (Malvern Instruments, UK). Prior to measurements, freshly prepared emulsions were diluted using deionized water and measured for the zeta-potential, which was reported as a mean and standard deviation of three measurements.

cCNC-stabilized Pickering emulsions were imaged by an Olympus CX41 optical microscope fitted with a digital camera (Canon E05 1100 D, Japan). Emulsion droplets were placed directly onto a glass microscope slide and viewed under 40x magnification.

The stability of emulsions in terms of creaming/sedimentation was evaluated by visual observation of the emulsions placed in closed glass tubes stored at rest under ambient conditions. At regular time intervals, the thickness of the creaming/serum layers was measured and the index of creaming (CI) was calculated using the equation $CI = (H_S/H_E)x100\ \%$, where H_E is total height of the emulsion in tube and H_s represents the height of the transparent serum layer (Keowmaneechai & McClements, 2002). The ability of emulsion to sustain gravitational forces was also determined. For this purpose, a centrifugation test was used. Emulsion aliquots were transferred to centrifugation tubes and treated for 2 min at 750 rpm on a Hermle Z 300 K (Hermle, Germany) centrifuge. Encapsulation efficacy (EE) was determined according to equation $EE = (V_E)/(V_T)x100\ \%$, *where* V_E is the volume fraction of the encapsulated oil and V_T is the total volume of the oil phase used in the corresponding sample (Wahlgren, Bergenstahl, Nilsson, & Rayner, 2015).

The stability of the emulsions was assessed with the help of particle size measurements (Master Sizer 3000, Malvern Instruments, UK) performed 7 and 14 days after preparation.

3. Results and discussion

3.1 Characterization of cCNC nanocrystals

The morphology of the carboxylated cellulose nanocrystals prepared via oxidative treatment of microcrystalline cellulose was investigated by AFM. It confirmed the formation of elongated, rod-like colloids with length ranging from 50 to 450 nm and height values were around 5 nm, hence with a relatively high aspect ratio (Fig. 1). The width was between 20 and 60 nm. These dimensions were in agreement with previous report regarding cCNC (Bai, Holbery, & Li, 2009; Elazzouzi-Hafraoui et al., 2008; Habibi et al., 2010).

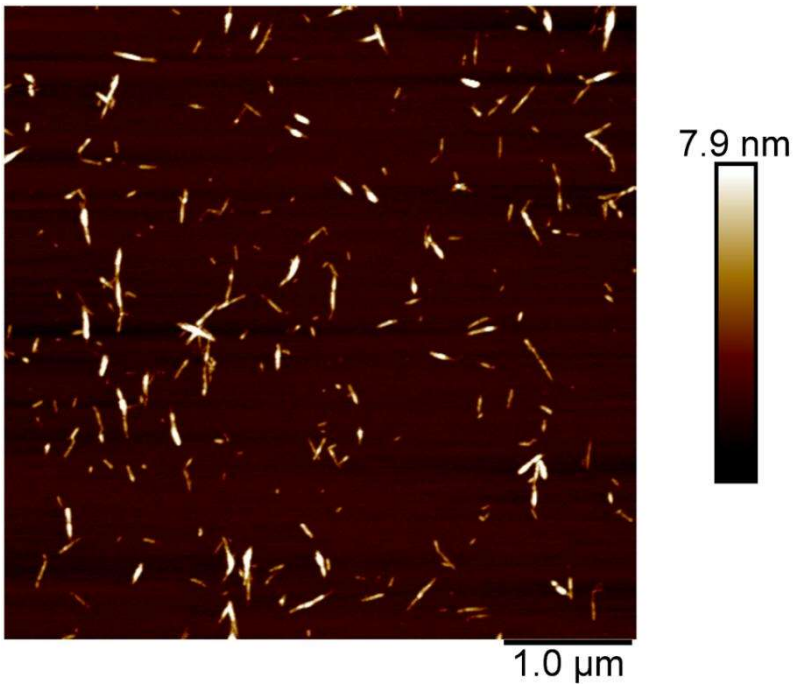

Figure 1. AFM of cellulose nanocrystals obtained from microcrystalline cellulose via APS oxidation

The cCNC suspension were also analysed by DLS. The determination of the exact parameters of rod-like particles by light scattering usually requires the use of depolarized dynamic light scattering by which the dynamic properties of the CNC suspension can be investigated in VV mode (vertical – vertical mode) as well as in VH mode (vertical – horizontal mode). This allows the determination of the rotational and translational motion which is needed to

determine the length and width of the rods (Phan-Xuan et al., 2016). In the present study the aim of the DLS experiments was to briefly evaluate the average dimension of the particles assuming a spherical geometry. The intensity based z-average particle diameter of diluted dispersions was found to be about 117 ± 4 nm with a polydispersity index PDI = 0.44 ± 0.04 based on the fitting by a cumulant model of the autocorrelation function. In fact, the dimensions of cCNC crystals determined by AFM and DLS can hardly be compared. This method, however, constitutes a rapid and straight–forward technique for easy control of particle size and distribution of cCNC nanocrystals.

The cellulose nanocrystals obtained by the treatment of a cellulose source with a strong oxidizing agent are expected to bear carboxylic acid groups on the particles surface (Leung et al. 2011). This way, we can impart a certain pH responsiveness to the colloidal suspension which in turn has a key impact on colloidal behaviour of cCNC in environment with various pHs and ionic strengths. According to Boluk, Lahiji, Zhao, and McDermott (2011) suspensions of nanocellulose particles prepared using APS exhibited a very good stability above the pKa of the carboxylic groups. This stability is lost when the pH is decreased, as results of the surface charge density, leading to aggregation. In the current work, the behaviour of cCNC was investigated within a pH range of 2 to 10 and the corresponding zeta potential values are shown Fig. 2. As expected, starting from a value of −41 ± 4 mV above pH=7, the zeta potential increased when the pH was lowered, to reach values close to zero, at pH=2. Under these conditions, the nanocrystals were not stable, and tended to strongly aggregate, which affected the zeta potential measurements.

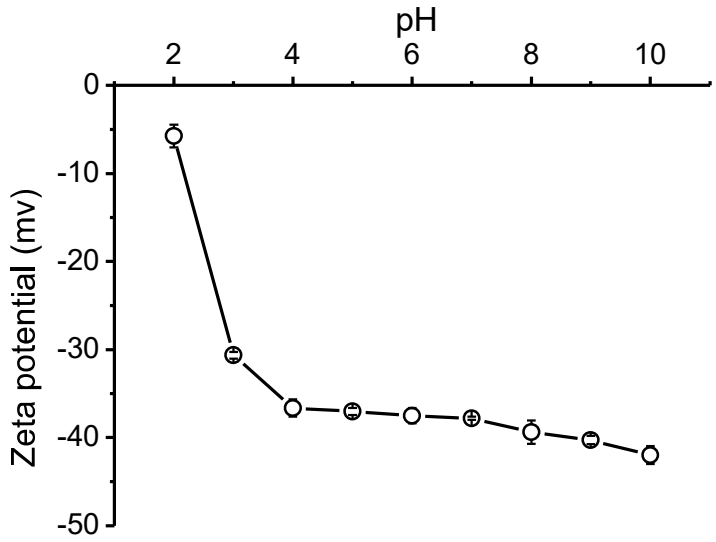

Figure 2. Zeta potential of cCNC suspension in NaCl (1mM) as a function of pH

These changes clearly illustrated the protonation-deprotonation process taking place and the possibility to control the particles charge density. In practice, the colloidal behaviour of the particles is crucial when it comes to the emulsion stability. Counter-ions present in the dispersion medium may associate with the charged particles and play thus an important role in determining the inter-particle forces, and by extension the droplet-droplet interactions. The zeta potential of cCNC particles was therefore determined as function of the ionic strength, with NaCl and $CaCl_2$, see figure 3, at pH=6.9. The zeta potential increased with increasing salt concentration. The dependencies were, however, different and reflected the effect of the valence of the cation. Whilst after NaCl addition zeta potential increased smoothly and reached a plateau at ionic strength of about 60 mM (−12 mV), the increase of zeta potential of nanocellulose particles by $CaCl_2$ was abrupt and a steady-state value was achieved at notably lower ionic strength, i.e. 3 mM. It implies that beyond simple charge screening, the carboxylate groups might have a higher affinity for the calcium cation than for sodium. The consequences of this behaviour on the emulsions will be further discussed.

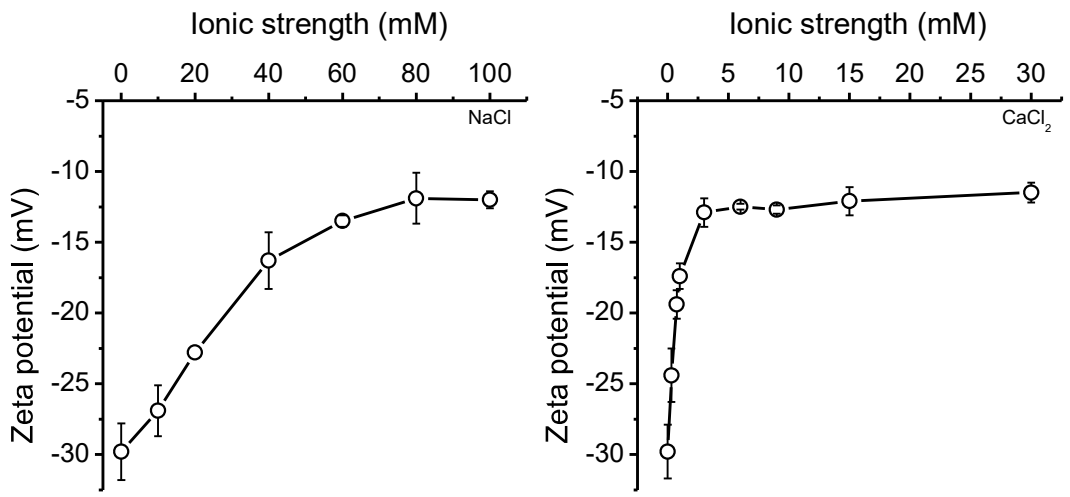

Figure 3. Zeta potential of cCNC suspensions determined at pH 6.9 as a function of ionic strength; left: NaCl, right: $CaCl_2$

3.2 Size and distribution of emulsion droplets

The formulation of the Pickering emulsions in this work utilized medium-chain triglycerides (tricaprine/tricapryline) as oil phase, which is a neutral and efficient carrier of lipophilic actives of various origin. We also investigated the possible degradation of the oil by the emulsification, during which, when performed using sonication, temperature might locally have increased strongly. Even though the sonication of emulsions was conducted in an ice bath, the stability of triglyceride-based oil was verified by determination of the acid value. This value provides the concentration of free fatty acids in the sample, the presence of which is a clear sign of triglyceride hydrolysis. The analyses, however, proved that after sonication, the acid value was lower than 0.2 mg/g, which was well within the specifications given by supplier.

Emulsification tests were carried out at pH 2, 4 and 7, while the cCNC content varied between 0.01 wt% and 0.3 wt%. Under these conditions, for oil contents ranging from 10 to 40 wt%,

oil-in-water emulsions were obtained. Fig. 4 shows the size of the droplets, expressed as $D_{(4,3)}$, as a function of the cCNC and oil contents, and at the different tested pH values. In addition to the pH of continuous phase, the concentration in cCNC appeared to be the parameter influencing the most significantly the emulsification and the final size of the droplets.

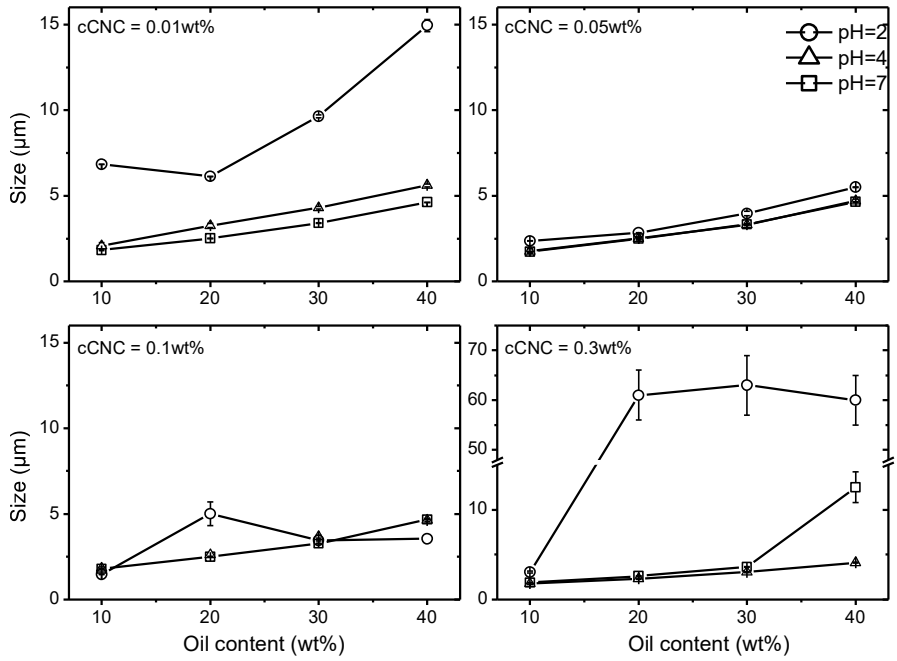

Figure 4. Effect of cCNC and oil content, and pH on the size of the emulsion droplets $D_{(4,3)}$ determined by laser diffraction. Note the break in the y-scale for the cCNC content of 0.3 wt%.

At very low cCNC content, emulsions prepared at pH 4 and 7 had sizes ranging from about 2 to 5 µm, i.e. slightly increasing with the oil content, as one would expect. The decrease of the pH below the apparent pKa of cCNC led to an important decrease of the efficiency of the particles to stabilize the interface which translated by droplet sizes above 5 µm, and a much larger dependence to the oil content. For cCNC contents of 0.05 and 0.1 wt%, the influence of pH could not be noticed and the sizes of the droplets remained below 5 µm in average. Again, the size dependence with oil content was observed. At the highest cCNC content studied, i.e.

0.3 wt%, the situation is more complex. Whereas the emulsion prepared at pH 4 and 7 behave similarly to lower particles contents, the emulsion prepared at low pH showed the dramatic loss of efficiency of cCNC, as demonstrated by the important size increase with the oil content, from 3 µm for 10 wt% oil content up to 60 µm for 40 wt%. This catastrophic loss of efficiency of cCNC particles to act as a stabilizer was indeed unexpected, especially given the size yielded by lower cCNC concentration. A tentative explanation to this observation can be done accounting for the colloidal behaviour of the cCNC at pH below the apparent pKa. As demonstrated by the zeta potential measurement a decrease of the pH tended to reduce the apparent surface charge, which led to a lower colloidal stability of the system resulting finally in aggregation at pH 2. However, the aggregation rate is highly depending on the concentration, especially for anisotropic particles. At very low concentration, the aggregation takes place at a low rate, leading to particles still capable of efficiently stabilizing the oil-water interface. The situation is very different at higher concentration, and at 0.3 wt% the aggregation occurs rapidly, leading to large aggregates that are not able to properly adsorb and stabilize the interface. This situation is amplified when the oil content increases, as more particle are needed to maintain the droplet size.

Interestingly, with the emulsions prepared at pH 4 or 7, variations of pH to 2 had only a minor effect on the stability, and no major changes could be macroscopically observed. This was indeed not expected and tend to illustrate that once cCNC is adsorbed at the interface, its local behaviour, and in particular its desorption, can not be triggered by variation of its charge density. This is supported by the fact that the surface jamming of rod-like particle is part of the mechanism of stabilization of Pickering emulsion prepared with anisotropic particles.

The changes in droplet size distribution was also evaluated using laser diffraction and Fig. 5 shows the effect of the oil content on the size distributions of emulsion droplets in samples prepare at pH 4, stabilized with 0.01 wt% nanocellulose. An increasing oil content triggered a

shift of the distribution towards the larger droplets. The distribution was originally monomodal, though the main peak was split, but developed into a bimodal distribution with the presence of two distinct droplet populations. This effect was more pronounced with emulsions containing 40 w% oil. Similar behaviour was also observed for emulsions prepared at pH of 7. Size distribution of droplets recorded for emulsions prepared at pH 2, however, differed and showed prevailingly the presence of large droplets, see inset Fig. 5. The fact that this population actually consisted of droplets and not agglomerates or small flocks was verified *via* microscopy observations of the emulsions, which clearly showed absence of other structures than round-shaped oil droplets.

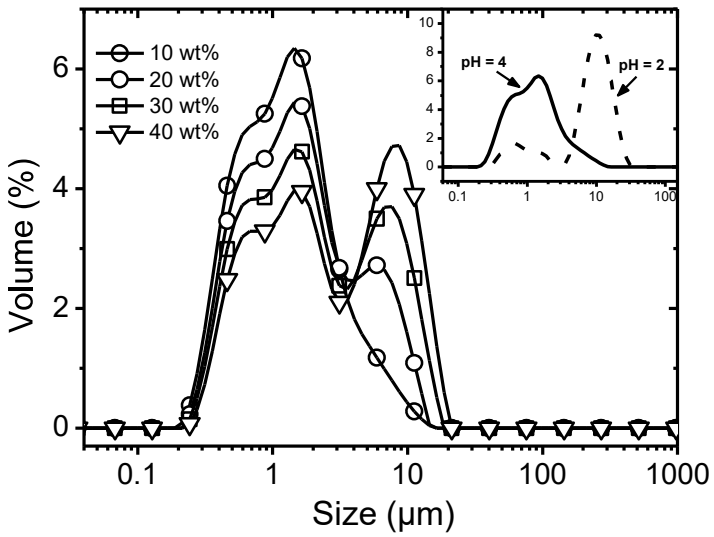

Figure 5. Size distribution of emulsion prepared at pH 4 with varying oil content (cCNC concentration 0.01 wt%. Inset: comparison with an emulsion prepared at pH 2 (oil content 10 wt%).

3.3 Zeta potential

Zeta potential was determined for the emulsions in order to correlate the observations done in relation with the stability of the emulsion. Zeta potential provides a simple mean to evaluate the electrostatic contribution of the adsorbed particles in the mechanism of stabilization. Such approach assumes that most of the cCNC was adsorbed and that the contribution of free

particles to the measurements could be neglected. Generally, a high zeta potential implies a good stability whereas as coalescence, flocculation or aggregation can be occur with lower values. Typically, the limit between a stable and an unstable emulsion is around 30 mV; systems with zeta potential larger than that are regarded as stable (Albright, 2008). Zeta potential determined on emulsions prepared at pH 2, 4 and 7 are compared in Fig. 6.

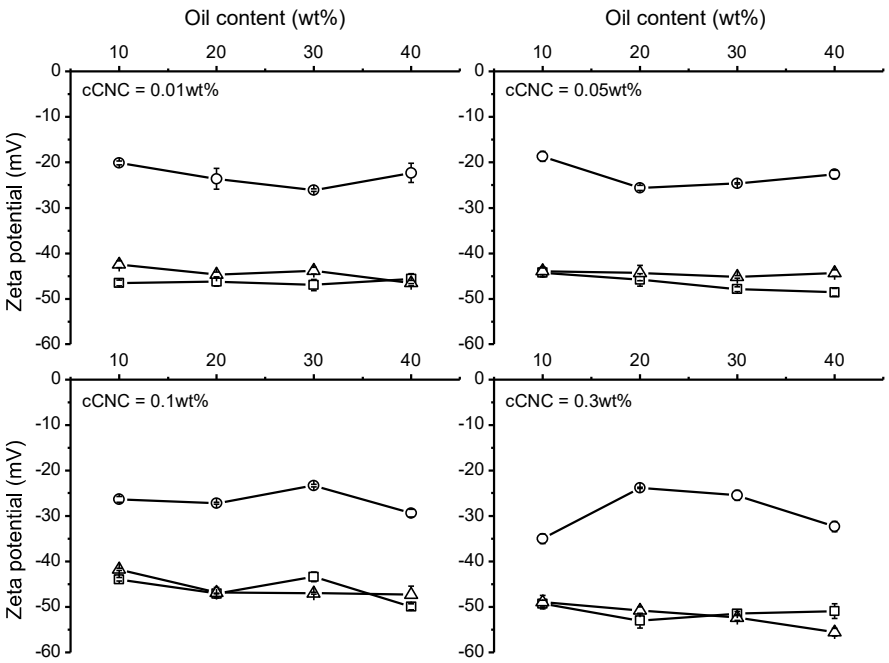

Figure 6. Zeta potential of the emulsions prepared at pH of 2, 4 and 7 and with different cCNC and oil contents.

As expected, the zeta potential of the droplets was strongly dependent on the pH of the continuous phase, and the values somewhat followed the trend observed for cCNC dispersions in water, with zeta potential values being lower for the systems at pH 2. This lower surface charge of the emulsions was in agreement with the lower stability observed of the emulsion prepared at pH 2. This will be discussed further. Interestingly, the emulsions prepared at pH 2 had a zeta potential values around -30 mV, which is significantly lower than for the simple suspension. A partial explanation can lie in the fact that it is commonly admitted that oil-water interface can have a negative potential, rarely to that extent though.

3.4 Emulsion stability

The stability of emulsions was evaluated by simple visual inspection of their appearance. Typically, emulsions with dispersion media of pH 7 and 4 exhibited reasonably good homogeneity and stability with absence of notable creaming or phase separation. Emulsion prepared at pH exhibited more pronounced changes. All emulsions were assessed on their seventh day after storage at room temperature. A distinct separation of a serum layer from the remaining emulsion was clearly visible and its thickness was governed by the cellulose concentration as well as oil content. The best performance in terms of phase separation was surprisingly observed in emulsions containing the highest cellulose (0.3 %) and oil (40 %) contents.

Visual observation served also for the assessment of the emulsion stability by determination of the creaming index (*CI*). The *CI* is reported only for emulsions prepared at pH of 2 (Fig. 7). Emulsions with dispersion medium of pH 4 and 7 were visually stable and no separation between transparent serum and emulsion layer was observed in these samples. Their stability was determined in more details using laser diffraction measurements (see below). *CI* values calculated from data recorded during storage of emulsions showed that the separation of the emulsion phase and serum started soon after preparation for all formulations, and independently of the amount of cCNC used for emulsion stabilization. Changes in creaming were observed mainly in the very beginning of storage and *CI* then only slowly increased or remained stable throughout the testing period. Visually, the most stable emulsions were those containing 0.3 % cellulose. The influence of the cellulose amount on *CI* was more notable in case of lower 10 % oil content. At the same time, it is clear that an emulsion with a lower amount of oil and a lower content of cellulose (0.01 and 0.05 %) showed more creaming than

emulsions in which the oil and cellulose contents were higher. Most likely this can be attributed to the higher viscosity of the continuous phase of the latter ones.

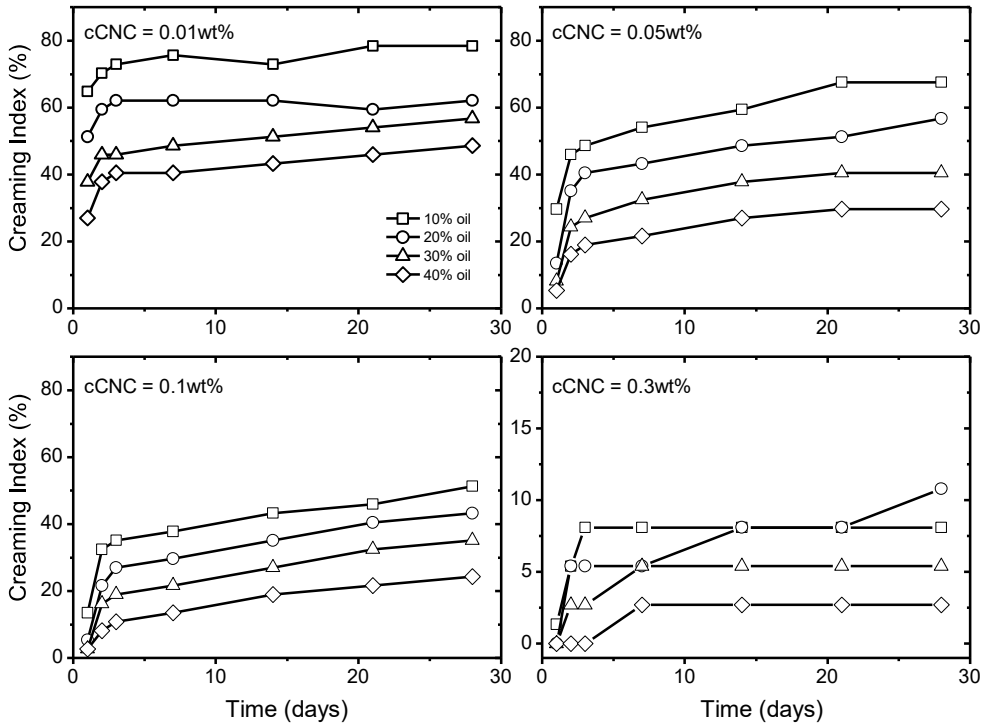

Figure 7. CI of emulsions prepared at pH 2 with different cCNC and oil contents.

The differences in stability of emulsions prepared at different pH were corroborated by centrifugation tests (2 min, 750 rpm). After the treatment, a layer of oil released was observed in some of the samples and was most notable in emulsions prepared at pH 2. Encapsulation efficacy (*EE*) is displayed Fig. 8. for emulsions stabilized with 0.3 wt% cellulose. The centrifugation tests confirmed that the extent of the oiling-off clearly depended on the pH of the continuous phase. At the highest concentration of cCNC used, the amount of released oil obviously increased with decreasing pH. Minimum or absence of oiling-off was observed in emulsions stabilized with 0.1 and 0.05 % cellulose. At 0.01 wt% the tendency of emulsions

towards breaking and releasing the oil increased again. The latter effect was observed irrespective of pH of dispersion phase.

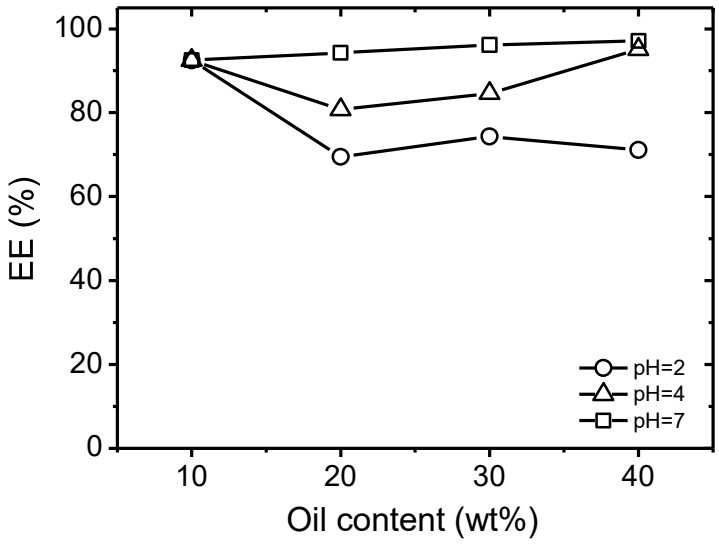

Figure 8. Correlation between oiling-off expressed as emulsification index (EE) and oil content in emulsions prepared at various pH, after centrifugation (0.3 wt% cCNC).

Changes taking place in emulsions during storage were also assessed *via* evaluation of sizes of emulsion droplets measured by laser diffraction. Analyses showed that the droplet sizes in the samples with a continuous phase adjusted to pH of 4 and 7 had not notably changed for 14 days of storage, irrespective of the O/W ratio and cellulose particles contents. For example, in the emulsion with 0.1 wt% c-CNC and O/W 40/60, the droplet diameter increased from 4.7 μm to 5.1 μm during the first seven days of storage and further to 6.3 μm in the following storage period. Surprisingly, only minor changes in droplet diameter were also observed in case of emulsions with dispersion phase adjusted to pH=2, and samples with initially large droplets sizes (above 60 μm) have not notably grown in size during the 14 days storage. As regards stability of Pickering emulsions, published studies have suggested that it can be influenced by polarity of dispersed oil and in case hydrophilic stabilizing particles were used, the most stable O/W emulsions were obtained with polar oils (Frelichowska, Bolzinger, &

Chevalier, 2009). These conditions were actually fulfilled in case of the used oil phase composed of tricaprine/tricapryline, which could be considered as relatively polar due to the presence of short chains.

4. Conclusion

Cellulose nanocrystals with carboxylic groups on the surface were prepared *via* oxidation of microcrystalline cellulose with APS. The cellulose nanocrystals were characterized by atomic force microscopy and zeta potential measurements. AFM analyses confirmed the presence of nanocrystals with an average length and diameter of 50-450 and 20-60 nm, respectively. Zeta potential of nanocrystals, determined in aqueous media with different ionic strength, was notably dependent on the character of the salt used with a stronger tendency for divalent species to induce potential variations. The surface charge was also reduced with decreasing pH and this effect was most noticeable between pH=2 and 4, leading to a loss of the good colloidal stability of the cellulose nanocrystals observed at pH above 4. The prepared cCNC was capable of forming stable oil-in-water emulsions with triglyceride (tricaprylin/tricaprin) as an oil phase. Without any further modification of the nanocrystals, the emulsions were successfully formulated at three different pH=2, 4 and 7. The prepared emulsions were characterized in terms of size, zeta potential and stability by creaming index measurement and evaluation of the oiling off. In general, the size of the emulsion droplets and their stability were dependent on oil and cCNC contents. More interestingly, we could show that the emulsification was mainly influenced by the pH of the continuous phase and the biggest droplets were found for the emulsions prepared at pH=2. This was related to the reduction of the charge on the cCNC with decreasing of pH. However, response of emulsions towards changes in pH was not as dominant as expected, and lowering of pH did not allow to induce release of oil from droplets. This can be explained by the fact that adsorption of cCNC on

used, relatively polar triglyceride oil can be so strong that changes in pH did not induce desorption of nanocrystals from oil surface, while it was capable of reducing the charge density on the particles/droplets. The presented results contribute to deeper understanding of the currently growing interest in developing stimuli-responsive or switchable emulsion systems based on anisotropic particles.

Acknowledgments

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PAPER IV

Characteristics of silver nanoparticles in vehicles for biological applications

Kristina Kejlová, Věra Kašpárková, Daniel Krsek, Dagmar Jírová, Hana Kolářová, Markéta Dvořáková, Kateřina Tománková, Veronika Mikulcová

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Characteristics of silver nanoparticles in vehicles for biological applications

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ABSTRACT

Silver nanoparticles (AgNPs) have been used for decades as anti-bacterial agents in various industrial fields such as cosmetics, health industry, food storage, textile coatings and environmental applications, although their toxicity is not fully recognized yet. Antimicrobial and catalytic activity of AgNPs depends on their size as well as structure, shape, size distribution, and physico-chemical environment. The unique properties of AgNPs require novel or modified toxicological methods for evaluation of their toxic potential combined with robust analytical methods for characterization of nanoparticles applied in relevant vehicles, e.g., culture medium with/without serum and phosphate buffered saline.

1. Introduction

Silver nanoparticles (AgNPs) have been used for decades as anti-bacterial agents in cosmetics, health industry, food storage, textile coatings and a number of environmental applications, although there is still insufficient information on their toxicity and unambiguous opinion on behaviour in vivo. The issues related to synthesis, properties and characterization of AgNPs have been addressed in many publications and reviews (Reidy et al., 2013) where it was clearly stated that antimicrobial and catalytic activity of AgNPs depend on size and size distribution as well as their structure, shape, and physico-chemical environment.

Particle size is one of the most important parameters not only for description of fundamental properties of materials, but also within the biological systems as it can affect a number of key features and processes, such as drug targeting, delivery or distribution. Regarding nanosized particles, ISO standard ISO/TS 27687:2008 (ISO, 2008) provides their definition as an object with a size between 1 and 100 nm. In the EU, the respective Commission Recommendation (EC, 2011) defines a "nanomaterial" to be a "natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1–100 nm". In an ideal case, the particles that are subject of characterization would be all homogeneous in shape and size with uniform properties. In this situation, any method measuring particle size would provide the same values of their diameters and the same particle size distribution, regardless of the principle of the measurement technique used. In the real world, however, most of the particles are non-spherical with different shapes that would undoubtedly influence their diameter determined using different methods (Merkus, 2009; Barth, 1984). Techniques used for the particle size measurements are based on different principles. Here, visual or microscopic observation, the light scattering, ultrasound absorption, sedimentation velocity or Brownian motion can be named. One of the most relevant methods used is dynamic light scattering (DLS) as it provides measurements of particle sizes from the nanometer up to a few microns. This technique measures scattered light fluctuations caused by the Brownian motion which are then related to the size of the particles via translational diffusion coefficient *D*. Particle diameter thus obtained is referred to as a hydrodynamic diameter and stands for the diameter of a sphere that has the same translational diffusion coefficient as the particle. It is worth mentioning that the hydrodynamic diameter measured by DLS corresponds to the diameter of its dense core

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Table 1
AgNPs used in the study.

Sample	Ag content[a] (ppm)	Date of manufacture
S9	20	03/2013
S11	20	05/2013
S29	20	02/2014

[a] Information given by manufacturer.

increased by the thickness of a layer of molecules adsorbed on its surface (for example surfactants) plus the thickness of the solvation, counter ion layer. The size of particles determined by DLS is z-averaged according to the scattering intensity of each particle fraction present in the sample. In practice relevant to biological applications, volume and even number distribution are more appropriate and they are smaller than z-averages.

Another group of methods commonly applied for AgNPs characterization is a family of microscopic methods. As they facilitate direct observation of the measured objects, they are probably the first-choice techniques to be used. However, the drawback related to their application is the time-consuming sample preparation and necessity to collect sufficient number of images to obtain reliable data. Transmission electron microscopy (TEM) is a technique reporting particle size as an equivalent diameter of a sphere that has the same projected areas as the projected image of the particle. Statistical analysis of the data enables to obtain number based particle distribution as primary data which can be further transformed to volume distribution (Reetz et al., 2000; Rubin, 2004). TEM analysis and light scattering technique DLS are the methods suggested for measurement of size and distribution of nanosized particles in the current study. As the methods work on different principles, their comparison is of interest and is one of the subjects of this study.

When applying AgNPs in biological systems, their bioavailability and cytotoxicity are governed by colloidal stability in respective environment, which may be influenced by several variables, such as pH, ionic strength, and the type of electrolyte present (El Badawy et al., 2010; Römer et al., 2011; Prathna et al., 2011). A number of studies dealing with the aggregation of nanoparticles in various environments has been conducted and reported (Cumberland and Lead, 2009; Römer et al., 2011), however, only limited information can be found in literature dealing with the influence of the above listed variables on the changes of particles used for biological studies. The presented study was therefore focused on the determination of size and distribution of AgNPs using the two above mentioned, independent methods, dynamic light scattering and transmission electron microscopy. Behaviour of the particles was assessed in more details after their contact with vehicles simulating human body fluids such as culture medium with/without serum (DMEM) and phosphate buffered saline (PBS).

2. Materials and methods

2.1. Tested materials

Aqueous dispersions of colloidal silver nanoparticles (20 ppm Ag) prepared by electrolysis were kindly provided by Petr Rulc—KC (Děčín, Czech Republic). Specification of the samples used in the study is provided in Table 1.

Dulbecco's Modified Eagle's Medium DMEM containing 4.5 g/L glucose with L-glutamine (LONZA, Cat. No. BE12-604F), new-born calf serum (LONZA, Cat. No. D/14-417F) and phosphate buffered saline PBS (LONZA, Cat. No. D/17-516F) were purchased from P-LAB, Czech Republic.

2.2. Methods for characterisation of the tested materials

2.2.1. Dynamic light scattering (DLS)

Size and distribution of the AgNPs were determined by DLS using a Zetasizer Nano ZS instrument (Malvern Instruments, UK). Measurements of the hydrodynamic radii of colloidal particles, expressed as z-average particle diameter, were performed at 25 °C. The intensity of scattered light ($\lambda = 633$ nm) was observed at a scattering angle of 90°. The polydispersity index (PDI), describing distribution width, was evaluated by assuming log-normal distribution of particle sizes. Prior to measurements,

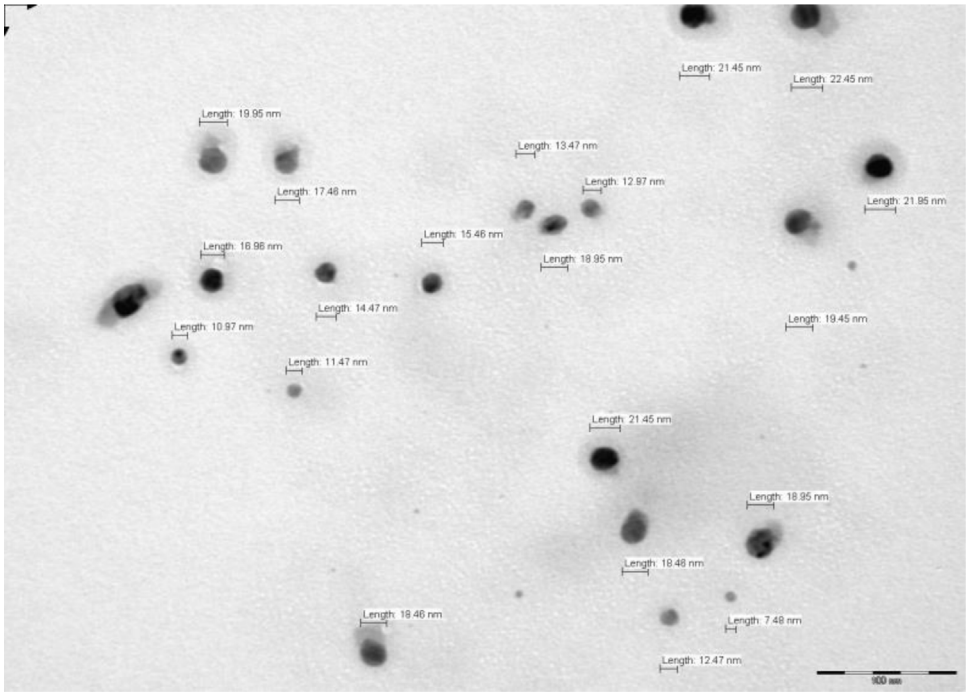

Fig. 1. TEM microphotogram of AgNPs, sample S29.

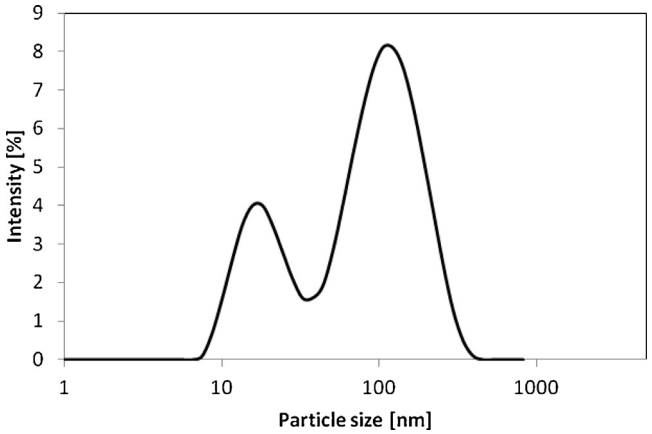

Fig. 2. Particle size and distribution, sample S29.

the performance of the instrument was verified by using polystyrene latex nanoparticles with the nominal size of 92 ± 3 nm (Thermo Scientific, Germany).

2.2.1.1. Sample preparation for DLS. The samples of AgNPs were either analyzed as delivered (pristine) or homogenized for 30 min by sonication in an ultrasonic bath. Measurement of the particle size was then carried out using (1) the non-diluted sample (both pristine and sonicated), (2) the sonicated AgNPs diluted 1:1 with demineralized water, (3) the sonicated AgNPs diluted 1:1 with serum free DMEM, (4) the sonicated AgNPs diluted 1:1 with DMEM with added 10% newborn calf serum and (5) the sonicated AgNPs diluted 1:1 with PBS.

2.2.2. Transmission electron microscopy (TEM)

Samples of AgNPs were examined under the transmission electron microscope Philips Morgagni 286 (FEI, USA). Formvar/carbon coated 400 mesh copper grids were floated in a drop of each sample for 20 min, left to dry and observed at magnification of 52,000×. The shape and size of AgNPs were recorded by means of electron microphotography using side-mounted CCD camera MegaView II (Olympus, Germany). Diameter of AgNPs was measured by iTEM image analysis platform (Olympus, Germany).

2.2.2.1. Sample preparation for TEM. The samples of AgNPs were analyzed as delivered (pristine), slightly shaken before preparation. Measurement of the particle size was then carried out using (1) the non-diluted sample, (2) AgNPs diluted 1:1 with serum free DMEM, (3) AgNPs diluted 1:1 with DMEM with added 10% newborn calf serum and (4) AgNPs diluted 1:1 with PBS.

3. Results and discussion

3.1. Characterization of AgNPs

TEM analysis revealed that the size of the majority of Ag nanoparticles detected in the sample S29 was around 20 nm in diameter with sporadic occurrence of particles above 100 nm (Fig. 1).

Results from DLS correlate reasonably well with the TEM findings and show z-average particle diameter of 48 ± 2 nm (measured both for non-diluted sample and the sample diluted 1:1 with demineralized water). In more detailed evaluation, two particle populations were observed, the first fraction with a size of 15 ± 2 nm and a second population with a size of 101 ± 13 nm in diameter. On the intensity basis, the 15 ± 2 nm fraction accounted for approximately 30% of all the particles; however, when the size of particles was determined on the volume basis, which is more relevant for comparison with TEM, this population represented 98–99% of all particles (Fig. 2).

TEM analysis of the sample S9, which was manufactured by the same procedure but using a different batch of water (Fig. 3), showed the presence of loose aggregates of AgNPs predominantly with a diameter of around 20 nm.

Sporadic occurrence of larger particles up to 50 nm was observed. In this case, the DLS measurements revealed z-average particle diameter of 91 ± 2 nm comprising two distinct particle populations, the major fraction with a size of 93 ± 3 nm

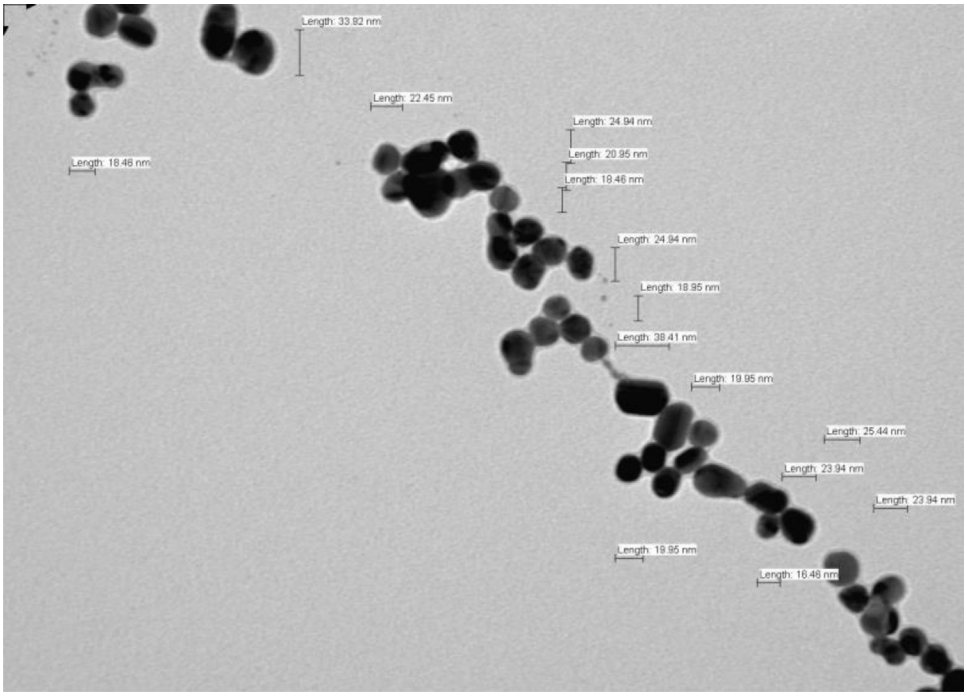

Fig. 3. TEM microphotogram of AgNPs, sample S9.

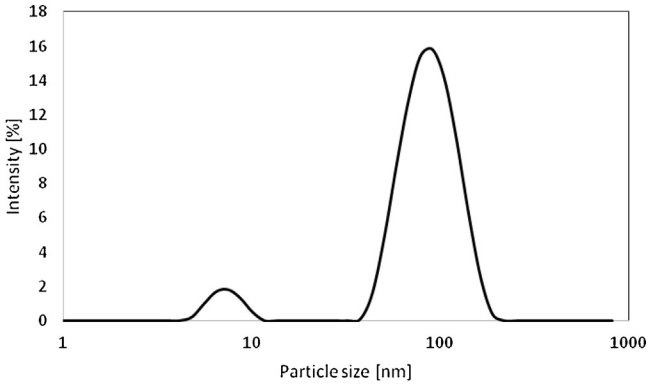

Fig. 4. Particle size and distribution, sample S9.

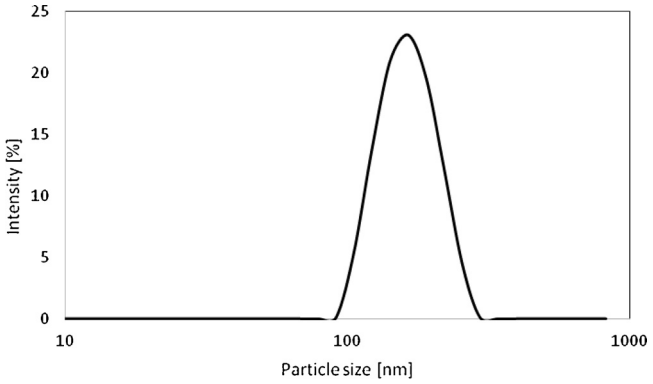

Fig. 6. Particle size and distribution, sample S11.

representing 93% of all particles contained in the sample and a minor fraction with a size of around 8 nm. Only negligible difference was observed between pristine samples and AgNPs treated with sonication prior to DLS analysis (Fig. 4).

In contrast to samples S9 and S29, TEM analysis of S11 revealed that this sample consisted mainly of needle-shaped, compact aggregates up to several micrometers long. Nanoparticles of around 10 nm in diameter were also present, albeit only in a small amount (Fig. 5a and b).

TEM experiments conducted on this sample pointed to a weakness of the DLS method observed in distinct cases. It is known that DLS is unable to reasonably cope with highly anisotropic particles; hence the needle-like particles observed in the sample by TEM would theoretically be in DLS approximated with a hydrodynamic sphere having a diameter equal to the longer dimension of the particle. Using DLS, z-average particle size of sample S11 was measured to be 198 ± 4 nm (Fig. 6).

Needle-like structures with the size detected by TEM lie well above the measuring range of the DLS instrument (4 μm) and were not observed by light scattering at all. The only indication of their presence in the sample while using DLS might be the unsatisfactory quality of measurements reported by the instrument in-build quality system, informing on the presence of "large sedimenting particles". It can be hence concluded that this sample was not suitable for DLS analyses and when measured, incorrect and false results on size of such particles were obtained.

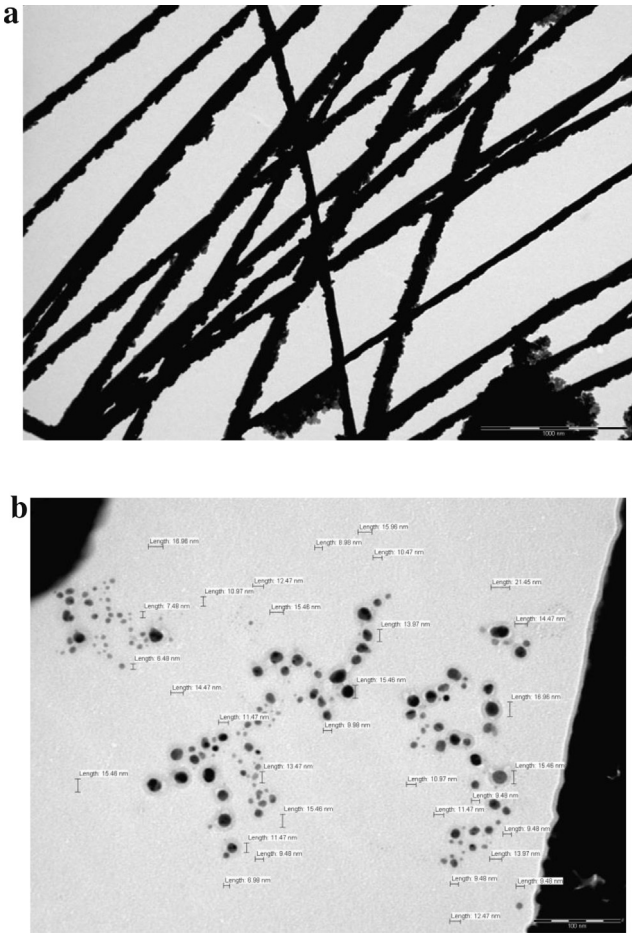

Fig. 5. (a and b) TEM microphotogram of AgNPs, sample S11.

3.2. Behavior of AgNPs in biologically applicable vehicles

In the sample S29 diluted 1:1 (v/v) with PBS the TEM analysis revealed presence of rounded compact particles mostly larger than 100 nm (Fig. 7).

A small fraction of particles around 50 nm was also observed, however, in relation to the overall number of AgNPs this fraction was negligible. The data illustrate a clear increase of the particle size after dilution with PBS when compared to data obtained on particles dispersed in water. These outcomes can also be supported by light scattering analyses. DLS measurements confirmed that z-average diameter of AgNPs diluted with PBS grew from 48 ± 2 nm measured in water to sizes ranging from 122 to 202 nm. The increase of z-average diameter was recorded immediately after PBS addition and interestingly, at three successive measurements performed at different time intervals following the sample dilution, the particle diameter further gradually increased (Fig. 8).

This is an evidence of time-dependent agglomeration induced by the presence of ions of salts present in PBS. This aggregation was clearly demonstrated for all the tested AgNPs.

With regard to analysis of particles diluted with serum free DMEM and DMEM containing serum recorded on sample S29, an analogous behavior to what has been observed on AgNPs diluted with PBS was noticed. The results showed that immediately after AgNPs dilution with both DMEM with and without serum, the particle sizes increased. Bigger particles were detected after dispersion in serum free DMEM (176 nm) in comparison with DMEM enriched with 10% newborn calf serum (144 nm). Moreover, in the serum free DMEM, the time-dependent particle size increase, similar to that in PBS, was observed. The AgNPs diameter has grown from 176 to 265 nm in three successive measurements. Correspondingly to DLS, also TEM microphotograms presented in Fig. 9a and b revealed changes in particle size and proved that dilution of the sample S29 in DMEM led to the formation of star-shaped compact aggregates with more than 200 nm in diameter

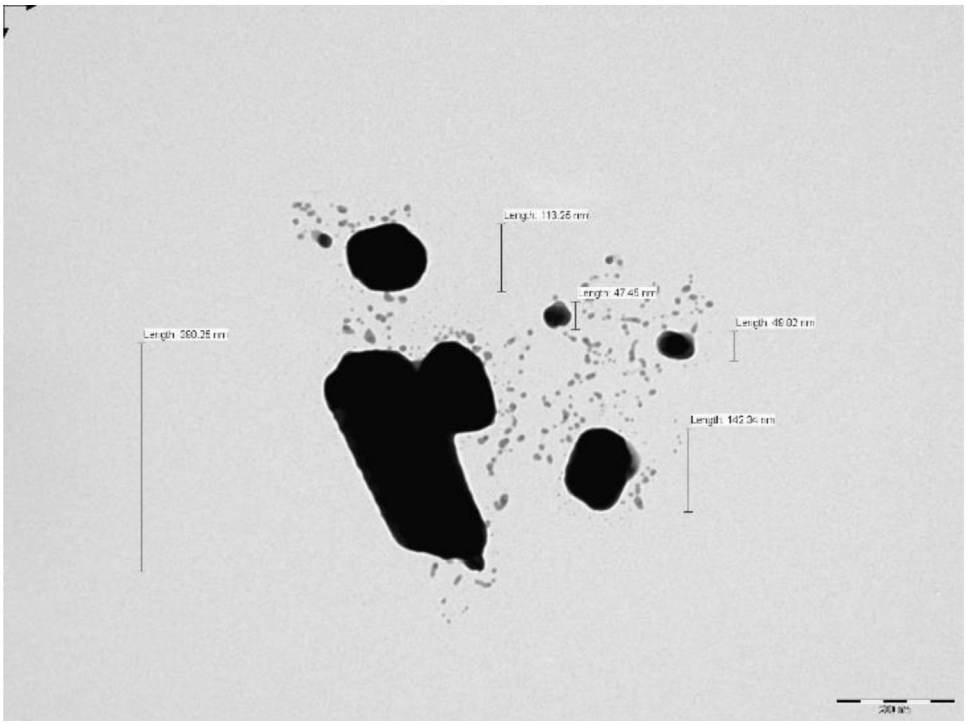

Fig. 7. TEM microphotogram of AgNPs (sample S29) after dilution with PBS 1:1.

accompanied with a minor proportion of particles below 100 nm in diameter.

An interesting situation was encountered after dilution of sample S29 with DMEM containing serum. In this case, the increase in particle size immediately after dilution was observed as well, however, not as pronounced as after dilution with serum free DMEM. Numerically expressed, z-average diameter of AgNPs grew from 144 to 153 nm in three successive measurements. Unlike DLS measurements, on TEM images none large AgNPs aggregates were observed in the sample diluted in DMEM with serum. Free, discrete nanoparticles in diameter of around 20 nm were observed, however, a significant fraction of nanoparticles was attached most probably to serum proteins. Inspecting TEM microphotograms in details revealed similar increase in size of observed particles seen in serum free DMEM (overall diameter of agglomerates about 200–300 nm), however, the appearance of these agglomerates was totally different. Unlike in the case of sample S29 diluted in serum

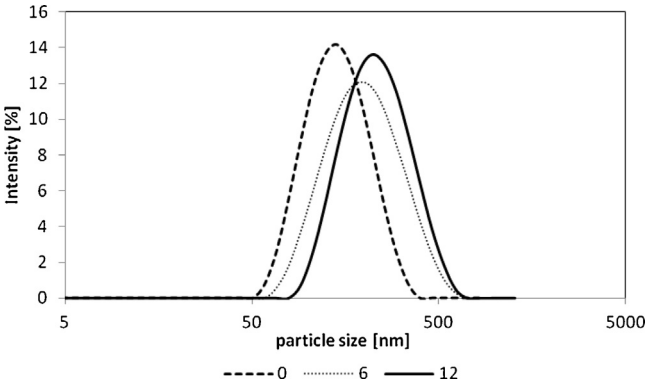

Fig. 8. Intensity based particle size distributions of sample S29 after dilution with PBS 1:1. Comparison of three measurements performed at different time intervals after AgNPs dilution.

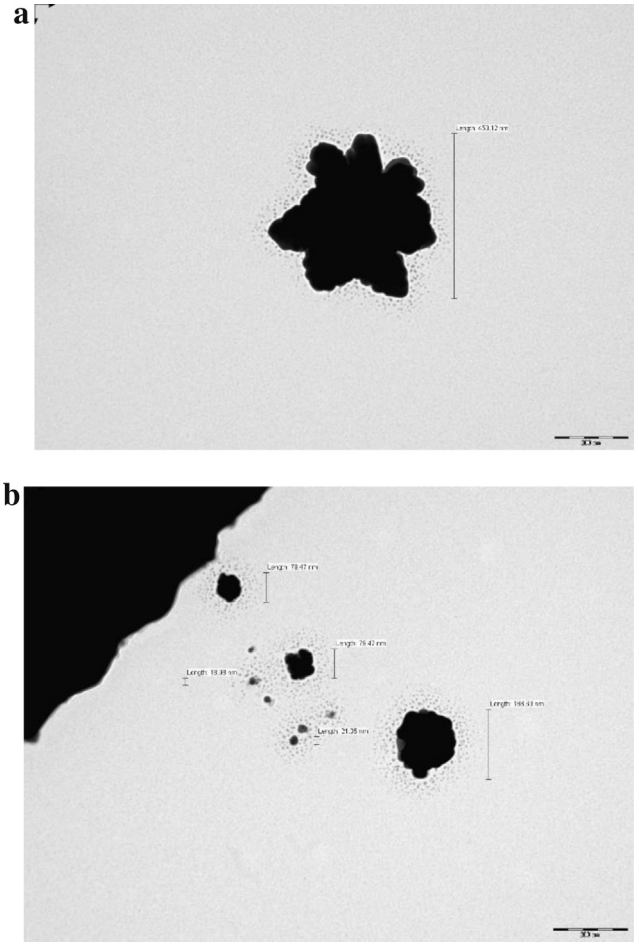

Fig. 9. (a and b) TEM microphotograms of AgNPs (sample S29) after dilution with DMEM.

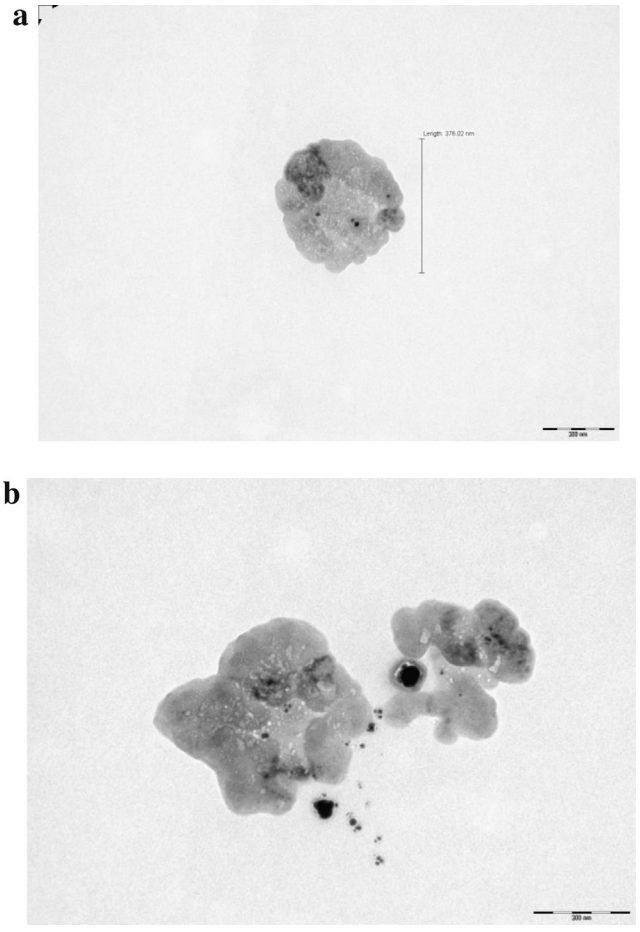

Fig. 10. (a and b) TEM microphotograms of AgNPs (sample S29) after dilution with DMEM with 10% newborn calf serum.

free DMEM, AgNPs diluted in DMEM containing serum formed AgNPs-protein-like clusters, where the prevailing volume of these agglomerates was comprised probably of proteins and/or bounded inorganic ions. Regarding to particle size increase, these results conform to DLS results, however, the average size of agglomerates observed in TEM was larger in the sample diluted in DMEM containing serum (overall diameter of about 300–500 nm) (Fig. 10a and b).

4. Conclusions

Comparison of DLS and TEM analyses shows that both techniques provide qualitatively similar information with respect to characterization of the tested AgNPs, although in absolute values the particle sizes measured by DLS are bigger. This results from the light scattering theory assuming that the z-average diameter is weighed by the particle scattering intensity. Accordingly, size distribution generated by DLS is intensity based distribution. As the intensity of scattered light is proportional to the sixth power of the particle diameter (d6), this technique significantly overestimates larger particles relatively to small ones. However, using Mie theory the scattering-based distribution can be converted to a volume distribution (d3) where the contribution of large particles is reduced. Using further assumptions related to particle shape, a number distribution can be also obtained, which is however considered as rather inaccurate (Coelho, 2013).

The agglomeration process observed on AgNPs diluted in PBS and DMEM, respectively resulting in growth of the particles is induced by increase of the ionic strength in the nanoparticle dispersion. This is a known form of dispersion instability, which is explained through DLVO theory (Hiemenz and Rajagopalan, 1997; Adamczyk and Weronski, 1999). This theory describes the aggregation of aqueous dispersions and identifies the forces between charged particles interacting via a liquid medium. Explanation of the observed AgNPs agglomeration comprises the fact that the growth of ionic strength reduces thickness of the diffuse layer around each of the particle and as a consequence, the particles can approach sufficiently close to each other and start to aggregate.

Another noteworthy effect can be observed with respect to changes of particle size distribution of studied samples after dilution with physiologically relevant media. From Fig. 8 and from numerical values of polydispersity indexes (not reported) it is seen that, although the average size of the particles increased, width of the particle size distribution was not substantially changed during on-going agglomeration and only negligible distribution broadening was observed.

Based on the TEM results, question may arise how the composition of DMEM and DMEM with serum influences the interpretation of DLS measurements of AgNPs. Taking into account medium constituents comprising inorganic salts, vitamins and amino acids, which all are low-molar-mass substances, intensity of scattered light detected from DMEM (expressed in kilo counts) is similar to that recorded from water and no information about particle size in such sample can be obtained as no particles are present. Size distribution of AgNPs recorded after dilution with DMEM can not hence be influenced by any of the medium components.

The scattering behavior of DMEM with added serum was however different. As a major component of serum is a globular protein, bovine serum albumin (BSA), it is obvious that this system will express a scattering behavior. This is documented in Fig. 11, depicting particle size distribution recorded on this medium.

It is apparent that DMEM with serum shows bimodal distribution with z-average particle diameter of 43 nm and two particle populations with a size of about 20 and 180 nm. In such situation, the presence of BSA peak from the serum might theoretically interfere with size distribution measured on AgNPs. In this context valuable information can be gathered from comparison of intensity based size distributions of DMEM with serum and AgNPs diluted with DMEM with and without serum added (Fig. 12).

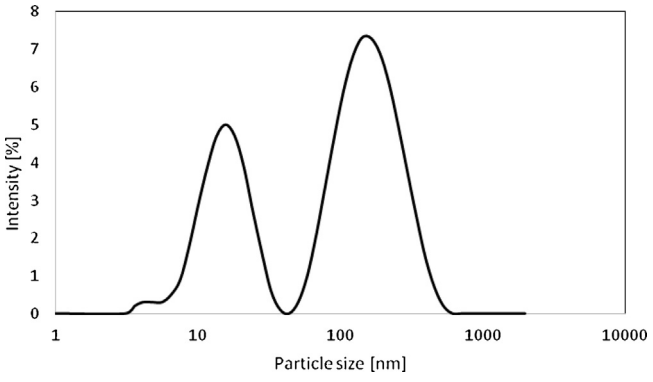

Fig. 11. Particle size and sistribution, DMEM with 10% newborn calf serum.

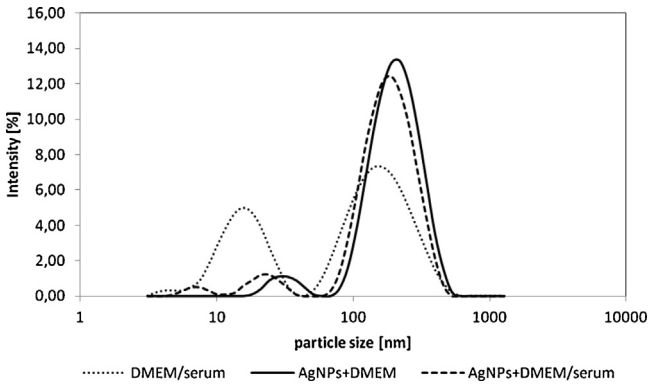

Fig. 12. Intensity based particle size distributions of AgNPs (sample S29) after dilution with DMEM and DMEM/serum. Comparison with dilution medium—DMEM/serum.

It is seen that distributions of DMEM with serum and AgNPs diluted with this medium overlapped and DLS was not capable to distinguish components of these dispersions. At the same time, AgNPs diluted with DMEM with and without serum exhibited comparable behavior in terms of particle size distribution. This might lead to conclusion that the aggregation pattern of AgNPs in both serum free and serum added DMEM is similar and is preferentially influenced by the presence of ions of salts; however, the impact of serum proteins as such cannot be omitted. A slightly different situation occurred when comparison of these samples was performed based on volume distributions derived from intensity distribution with the aid of Mie theory (Fig. 13).

Under these conditions, distributions of DMEM with serum as such and AgNPs diluted with this medium can be differentiated showing only partial overlap.

Taking into account TEM microphotograms, the conclusions that AgNPs form aggregates together with serum proteins under support of ions can be plausible. Nanoparticle-protein complexes are commonly referred to as the nanoparticle-protein corona (NP-PC) and formation of these complexes can affect bioavailability of nanoparticles in biological systems (Ge et al., 2011). Adsorption of proteins to nanoparticle surface could also modify the structure and consequently the function of attached proteins (Worrall et al., 2006). Protein-independent formation of nanoparticle bio-complexes, where inorganic ions are involved, is also known from biological solutions (Xu et al., 2012). However, no agglomerates similar to particles observed during TEM analysis of AgNPs diluted with DMEM containing serum were observed in serum free DMEM. Therefore participation of proteins in formation of these aggregates can be anticipated.

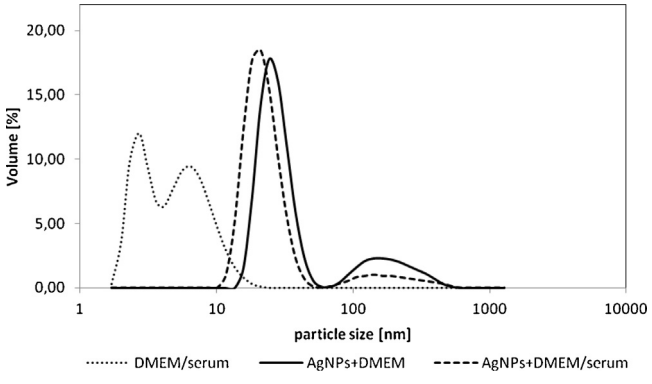

Fig. 13. Volume based particle size distributions of AgNPs (sample S29) after dilution with DMEM and DMEM/serum. Comparison with dilution medium—DMEM/serum.

It has already been outlined that a basic and routine characterization of AgNPs in pristine form using different methods is a complex process. Prediction and anticipation how AgNPs will behave in real biological systems due to changes in their size is even more challenging. Practical application of AgNPs necessitates the use of systems with stable particle parameters, regardless of the environment in which these particles are present. However, it is often possible to meet the situation when the dispersions of AgNPs are unstable under specific conditions. Changes in pH, the presence of electrolytes or certain organic compounds can lead to destabilization of the dispersions followed by particle aggregation, coagulation or other forms of instability. As all these variables concern physiological environment of the human body, it also might be the case of AgNPs applied in vivo or in vitro under physiologically relevant conditions. Then, in principle, AgNPs in human body fluids might possess bigger sizes than originally applied and might behave differently. In order to approach the real situation, the performance of S9, S11 and S29 samples was therefore assessed by measuring the size of the particles in dilutions with media simulating body fluids. The study confirmed that the results are completely different compared to those recorded for AgNPs dispersed in demineralized water. This fact might significantly change the view on the behaviour of nanoparticles in biological systems, where the presence of salts and proteins is quite natural. When biological effects of AgNPs are evaluated using methods in vitro, the nanoparticle characterisation should be performed in foreseen product application form. However, relevant media simulating human body fluids should be employed to monitor nanoparticle behaviour and transformation under relevant biological conditions.

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