Tomas Bata University in Zlín
Faculty of Technology

Ing. Petra Ševčíková

A study of the formation and characterization of a cosmetic emulsion system

Studium tvorby a vlastností kosmetických emulzních systémů

Doctoral Thesis

Doctoral study programme: P 2808 Chemistry and material technology
Course: 2808V009 Chemistry and material technology

Supervisor: doc. Ing. Věra Kašpárková, CSc.
Consultant: Ing. Pavlína Vltavská, PhD.

Zlin, August 2014

ABSTRACT

The need to stabilize active compounds has notably increased in recent years. The reason is to better protect the sensitive, reactive and short shelf-life ingredients contained in final products used in many areas of industry. The protection process is influenced by a variety of factors, such as interaction with other components and the volatility or toxicity of active ingredients. Therefore, modern technology has led to the development of a variety of delivery systems that effectively address these important issues. By definition, a delivery system is any type of vehicle that makes an active substance available to a target site and that can provide beneficial properties from the cosmetic or therapeutic points of view.

The main objective of this doctoral thesis is to introduce some of the types of vehicles which may be used for transport of active substances in the cosmetics, pharmaceutical or food industries.

This work is divided into two main parts. The first part is focused on a description of the various types of particulate systems serving as vehicles, such as emulsions, nanoemulsions, microemulsions and microparticles, wherein each mentioned system is discussed in a separate chapter in terms of their formation, characterization and properties. The work also contains a chapter that provides a mutual comparison as well as an overview of the main advantages and disadvantages of these systems and possibilities for their practical applications. The last chapter of the theoretical part of this thesis is devoted to the methods used for their characterization.

In the second part of the thesis, results obtained during the doctoral work are reported in short summaries of four research papers that document the current status of the problem. At the end of the work, full-texts of the research papers are enclosed.

Key words: dispersion system, emulsion, nanoemulsion, microemulsion, microparticle, vehicle

ABSTRAKT

Potřeba stabilizace aktivních látek se v poslední době výrazně zvýšila. Důvodem je snaha ochránit citlivé a reaktivní látky, jakož i látky s krátkodobou životností přítomné ve výrobcích používaných v řadě průmyslových oblastí. Proces spojený s touto ochranou je ovlivněn řadou faktorů, jako jsou interakce s jinými složkami, těkavost nebo toxicita aktivních složek. Moderní technologie proto vedou k rozvoji celé řady systémů, které účinně řeší tyto důležité otázky. Systémem vhodným pro transport aktivních látek je jakékoliv vehikulum, které je schopno je doručit do cílového místa a které poskytuje výhodné vlastnosti z kosmetického nebo terapeutického hlediska.

Hlavním cílem této dizertační práce je představit různé typy vehikul, které mohou být použity jako vhodné nosiče aktivních látek v kosmetickém, farmaceutickém nebo potravinářském průmyslu.

Předložená práce je rozdělena do dvou hlavních částí. První, teoretická část práce, je zaměřena na popis různých typů částicových systémů, jako jsou emulze, nanoemulze, mikroemulze a mikročástice, přičemž každý ze zmíněných sytémů je představen v samostatné kapitole, kde je uvedena jejich stručná charakterizace, vlastnosti a příprava. Práce také obsahuje kapitolu týkající se vzájemného porovnání těchto systémů včetně přehledu jejich hlavních výhod a nevýhod a možností praktických aplikací. Kromě toho je poslední oddíl teoretické části práce věnován metodám, které slouží k jejich charakterizaci.

V druhé části práce jsou formou čtyř publikací souhrnně prezentovány konkrétní, praktické výsledky získané v průběhu doktorského studia. Obsahy jednotlivých článků jsou uvedeny vždy krátkým shrnutím, které se týká řešeného problému. V závěru práce jsou pak přiloženy plné texty výzkumných prací.

Klíčová slova: disperzní systém, emulze, nanoemulze, mikroemulze, mikročástice, vehikula

ACKNOWLEDGMENTS

First of all I would like to express my deepest gratitude to my supervisor, doc. Ing. Vera Kasparkova, CSc., for her patient guidance, enthusiastic encouragement, and for her constant support and motivation, all of which contributed to the completion of my doctoral thesis.

My great thanks are also extended to Ing. Jiri Krejci, CSc. for his editing skills and for his useful critiques and comments.

I would also like to extend my thanks to the technicians of the laboratory of the Fat, Surfactants and Cosmetics Technology Department for creating a friendly working environment and for their valuable technical support.

And finally, I wish to thank my parents and boyfriend for their support, encouragement and understanding throughout my Ph.D. studies.

CONTENT

LIST OF FIGURES AND TABLES

LIST OF SYMBOLS AND ACRONYMS

BSA	bovine serum albumin
CMC	critical micellar concentration
CO_2	carbone dioxide
CPI	catastrophic phase inversion
DSC	different scanning calorimetry
EIP	emulsion inversion point
GML	glycerolmonolaurate
HLB	hydrophile-lipophile balance
HPLC	high-performance liquid chromatography
HPMC	hydroxymethylcellulose
LSW	Lifshitz-Slyozov-Wagner
MAG	monoacylglycerol
MCT	medium-chain triacylglycerol
O/W	oil in water
O/W/O	oil in water in oil
PGSS	particle generation from gas saturated solutions
PIT	phase inversion temperature
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
PVA	poly(vinylalcohol)
RESS	rapid expansion of supercritical solutions
SAA	supercritical assisted atomization
SAS	supercritical antisolvent precipitation
SC-CO_2	supercritical carbone dioxide
SMDNS	sodium mono and dimethylnaphthalene sulfonate
STP	standard temperature and pressure
TPI	transitional phase inversion
W/O	water in oil
W/O/W	water in oil in water
XRD	x-ray diffractometry

THEORETICAL BACKGROUND

1 INTRODUCTION TO COSMETICS VEHICLES

Recently, there has been a growing interest in the cosmetics, food, pharmaceutical and other industries in using different types of vehicles, for example colloidal dispersions or microparticles, capable of the encapsulation, protection and delivery of active components, such as drugs, antioxidants, vitamins, antimicrobials and nutraceuticals [1][2][3][4][5].

Cosmetic vehicles are used as carriers for active substances, delivering them to specified targets. This is allowed only if no systemic, physiological or pharmacological effect is achieved and the product is shown to be safe. Delivering active substances to these targets requires the formulation of the appropriate concentration of actives to achieve the optimal release rate and desired distribution of active substances between the vehicle and the target. It follows that the vehicle should penetrate into the stratum corneum and release the active component at the target where the desired effect is achieved [6].

The efficacy of dermatological and cosmetic products is influenced by the type of vehicle, its active composition and mechanism of action. Hence, the correct selection of a suitable vehicle plays an important role during the development of the product [7]. The type of vehicle can already be determined by the product target profile. Important selection criteria include the desired effect of the vehicle on the skin, ease of formulation, and physical and chemical stability [8].

Despite increasing technological efforts in the field of skin care, no universal vehicle has yet been identified. Each active compound requires a different type of vehicle for an optimized treatment [9]. The literature sources document many types of vehicles which can be classified according to various principles. However, cosmetic preparations are complex systems, making it difficult to find a universal classification system [7]. A simple classification may be performed according to the appearance of the vehicles: liquid, semisolid and solid systems (Fig. 1). However, this classification is not important for rational formulation design and development [6].

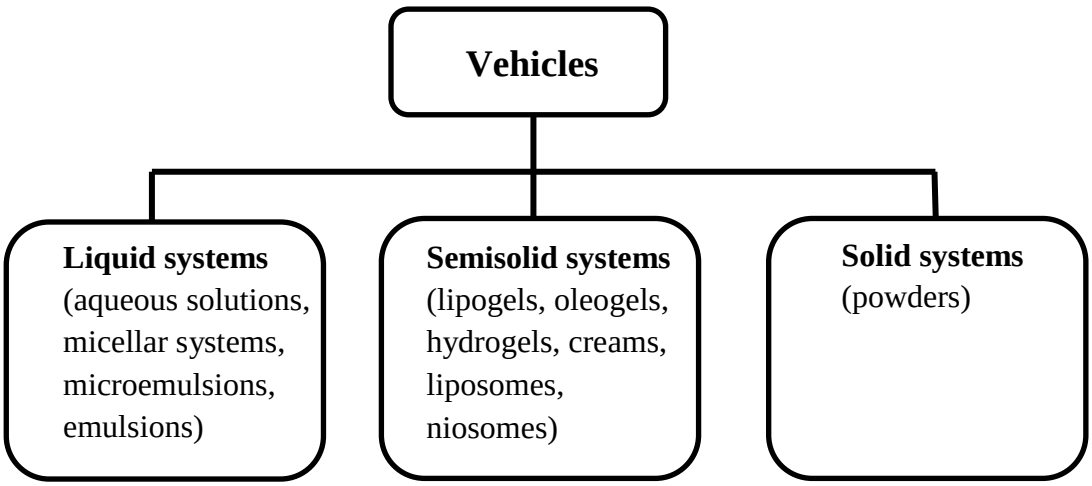

Figure 1 Classification of vehicles according to appearance.

Another classification system involves the physical state and optical divergances of the system. Vehicles can be classified into monophasic, isotropic systems or anisotropic heterophasic systems [6].

The classification of vehicles may also be performed as a function of their use and application site, i.e., vehicles for hairs (shampoo, depilatory agents, hair colorant), nails (polish), mouth (toothpaste, lipstick) and skin (body lotion, deodorant, antiperspirant, etc.) [6].

In the development of cosmetic products, a physical-chemical classification system is often preferred since this classification determines the principal properties and matrix character of vehicles. In a physical-chemical classification system, various characterization critieria are used [6]:

- Polarity (hydrophilicity, lipophilicity),
- Physical state (solid, semisolid, liquid, gaseous),
- Size/dimension of dispersed particulates,
    - True solution, molecular dispersions: particles size < 1 nm,
    - Colloidal dispersions: particle size 1–500 nm,
    - Coarse dispersions: particle size > 500 nm,
- Solubility characteristics,
- Rheology,
- Composition and physical-chemical characteristics.

From this short summary of different possibilities of classifications and types of delivery systems it follows that there is a large number of potential vehicles for transporting active compounds. Therefore, this doctoral thesis is focused only on some of them, namely on the dispersion systems (emulsions, microemulsions, nanoemulsions) and particulate systems (microparticles).

2 DISPERSION SYSTEMS

Dispersion systems are two phase systems, consisting of small particles or droplets of dispersed phase (discontinuous phase) distributed uniformly in a dispersion medium (continuous phase) [10][11]. As already mentioned, they can be classified according to their particle size into true, colloidal and coarse dispersions [6]. This chapter concerns one of the groups, colloidal dispersions.

2.1 Colloidal dispersion systems

The term colloid was first introduced by Graham in 1861. Since then, the language of colloidal science has developed and distinguished three terms: lyophilic (surfactant micelles, protein solutions), lyophobic (emulsions, foams, suspensions) and association colloidal dispersion systems. Lyophilic colloids are formed spontaneously by dissolving the material in the solvent (thermodynamically stable). The second type of colloid is not formed spontaneously and can be only formed with mechanical energy input [11]. The formation of the association colloids is also spontaneous but the concentration of amphiphile in the solution exceeds the CMC (critical micellar concentration) [12]. An overview of different types of colloidal dispersions is provided in Table 1. A typical example of lyophobic dispersions are emulsions, which are considered as the most important delivery systems [13] and are therefore discussed next.

Table 1 Types of colloidal dispersion systems [12]

Dispersed phase	Dispersion medium	Name
Liquid	Gas	Liquid aerosol
Solid	Gas	Solid aerosol
Gas	Liquid	Foam
Liquid	Liquid	Emulsion
Solid	Liquid	Sol, suspension
Gas	Solid	Solid foam
Liquid	Solid	Solid emulsion
Solid	Solid	Solid suspension

2.1.1 Emulsions

Emulsions are colloidal dispersions composed of at least two immiscible liquids (water and oil), one of which is usually dispersed as fine droplets throughout the other liquid phase. The phase existing as droplets is called the dispersed phase and the liquid surrounding them is known as the continuous phase [11]. Emulsions can be distinguished according to different criteria.

Firstly, they can be distinguished according to the polarity of dispersed and continuous phase on water in oil (W/O) and oil in water (O/W) emulsions depending on whether the dispersed phase is water or oil [14][15][16]. Moreover, it is also possible to prepare special type of emulsions, so-called multiple emulsions of oil in water in oil (O/W/O) and water in oil in water (W/O/W), in which a primary emulsion is dispersed in an external phase [6][15]. Besides this basic classification, the emulsions can be categorized according to the concentration of the dispersed phase into diluted, concentrated and gel emulsions [17]. Finally, there is a classification of emulsions according to the size of dispersed phase into microemulsions, nanoemulsions and macroemulsions.

3 MICROEMULSIONS

Microemulsions are the spontaneously formed, clear, thermodynamically stable homogenous dispersion of two immiscible liquids containing appropriate amounts of surfactants and co-surfactants [18]. The microemulsion concept was firstly introduced by Hoar and Schulman [19] in the 1940s, who found that the addition of a fourth component (often an alcohol) to an emulsion containing oil, water and a surfactant led to the formation of a clear, apparently homogenous phase. This additional component is usually called the co-surfactant [10]. These systems normally have droplet diameters of 100 nm or less, and many of them contain droplets only slightly larger than a simple micellar system [20]. As those particles are much smaller than the wavelength of visible light, microemulsions are transparent or translucent in appearance [21][22] and their structure cannot be observed through an optical microscope [23]. Real microemulsions behave as Newtonian liquids and their viscosity is not shear-dependent [24][25].

3.1 Formation of microemulsions

3.1.1 Theories of microemulsion formation

There are different theories relating to the formation of microemulsions, such as interfacial or mixed film theories [26][27], solubilisation theories [28][29][30], and thermodynamic treatments [31][32][33]. As an example, the simplified thermodynamic model, which is presented below, has been proposed to explain the formation of microemulsion systems. The free energy of microemulsion formation is considered to depend on the extent to which surfactant decreases the interfacial tension, hence on the interfacial energy ($\gamma\Delta A$) of the oil-water interface and on the change in entropy of the system upon droplet formation ($T\Delta S$). The following equation then applies (1)

$$\Delta G_f = \gamma \Delta A - T\Delta S \,, \qquad (1)$$

where ΔG_f is the free energy of microemulsion formation, γ is the interfacial tension of the oil-water interface, ΔA is the change in interfacial area upon microemulsification, ΔS is the change in entropy of the system, and T is the temperature [21].

When microemulsion is formed, the change in ΔA is very large because of the large number of very small droplets formed, which dictate large amounts of surfactant that will be needed to cover all the interface, γ is very small (of the order of fractions of mN/m but not zero), and the entropic component is the major factor governing spontaneous droplet formation and thermodynamic stability. The component that dominates $T\Delta S$ is the dispersion entropy, arising from the mixing of one phase in the other in the form of large numbers of small droplets. However, there are also expected entropic contributions originating from other dynamic processes, such as surfactant diffusion in the interfacial layer. Thereby, a negative free energy of formation is achieved when large reductions in surface tension are accompanied by a significant favourable entropic change, and the production of microemulsion is spontaneous [21].

3.1.2 Phase behaviour of microemulsions

The correlation between the phase behaviour of a mixture and its composition can be obtained with the help of phase diagrams [21]. The construction of phase diagrams is a useful way to study the complex series of interactions that can occur when different components are mixed. Microemulsions might be formed together with various association structures, including emulsions, micelles, lamellars, hexagonals, cubic structures, gels and/or oily dispersions, depending on the chemical composition and concentration of each of the components. Therefore, an understanding of their phase equilibrium and a determination of the phase boundaries are essential aspects of their study [25]. The phase behaviour of simple microemulsion systems consisting of oil, water and surfactant can be studied with the aid of a ternary phase diagram in which each corner represents 100% of that particular component. However, more commonly, the microemulsion contains an additional component, a co-surfactant. In the case of four-component systems, pseudoternary phase diagrams are used in which one of the corners represents a binary mixture of two components and the remaining two corners are assigned to the pure components. Nevertheless, the construction of phase diagrams is time consuming, especially when the goal is to accurately delineate phase boundaries among the individual phases formed [21].

The phase diagram approach to microemulsions was introduced decades ago by Gilbert and co-workers [34]. The construction of a phase diagram is generally performed for the determination of microemulsion (homogeneous)

and multiphase (heterogeneous) areas. In practice, the microemulsion area is determined through the titration method. The water is added dropwise with a micropipette to the mixture of oil/surfactant (co-surfactant), and after the addition of each drop, the mixture is homogenized (usually by vortex) and examined visually and with a crossed polarized filter [6][35]. The addition of water continues until the addition of one more drop produces turbidity [25]. The appearance (transparency, opalescence or isotropy) is recorded, along with the number of phases formed. In this way, the boundaries of each area (microemulsion region and multiphase regions) can be determined [6][35], which corresponds to the chosen content of oils as well as the surfactant or co-surfactant mixing ratio [25].

A schematic representation of different, thermodynamically stable single phase as well as multiphase regions for a system of water, surfactant and oil is presented in a ternary phase diagram (Fig. 2). These multiphase systems with the following distinct features were firstly described by Winsor [36]. According to his theory, the following phases can be distinguished:

- Winsor type I: two phases, the lower microemulsion (O/W) phase is in equilibrium with the upper excess oil phase,
- Winsor type II: two phases, the upper microemulsion (W/O) phase is in equilibrium with the excess water phase,
- Winsor type III: three phases, the middle bicontinuous phase containing microdomains of O and W is in equilibrium with the upper excess oil phase and lower excess water phase, and
- Winsor type IV: a single phase, where oil, water and surfactant are homogenously mixed [37][38].

The transition between microemulsion phases can be achieved in several ways, depending on the type of surfactants used. For ionic surfactants, a microemulsion type I-II-III transition can be obtained by increasing the electrolyte concentration, whereas for non-ionic surfactants it is accomplished by increasing the temperature. Increasing the electrolyte concentration or temperature causes the non-ionic surfactant solution to become more hydrophobic and segregate more towards the oil-water interface, thus reducing the surfactant film curvature and interfacial tension. At net zero curvature, Winsor type III is formed [39]. Winsor type IV occurs when surfactant concentration is increased in Winsor type III, thereby increasing the volume of the middle phase until it becomes a single phase [40]. Figure 2 also illustrates the composition dependent internal structures (microstructures) of microemulsion systems. In the water-rich corner, L_1 – a single phase region of normal micelles or oil-in-water microemulsion is formed. In the oil-rich domain, formation of L_2 – reverse micelles or water-in-oil microemulsion is preferred [37].

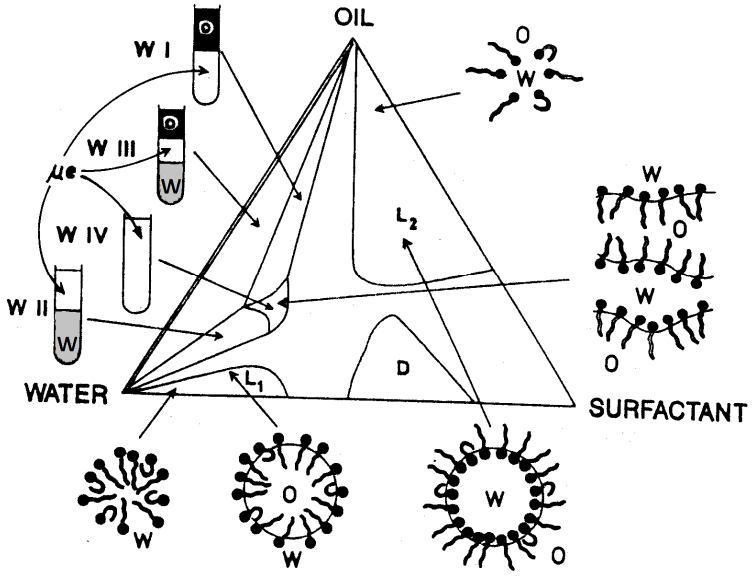

Figure 2 Schematic ternary phase diagram of water-oil-surfactant mixtures illustrating Winsor classifications and probable internal structures. L_1, a single phase region of normal micelles or (O/W) microemulsion; L_2, reverse micelles or (W/O) microemulsion; D – anisotropic lamellar liquid crystalline phase. The microemulsion is marked by μe, oil by O and water by W [37].

3.1.3 Factors affecting phase behaviour

Salinity

The droplet size of O/W microemulsion increases at low salinity. This fact corresponds to growth in the solubilisation of oil. When salinity rises, the system becomes bicontinuous with reduction in particle size over an intermediate salinity range. A further increase in salinity finally results in complete phase separation [25].

Alcohol concentration

The phase transition from W/O to bicontinuous and ultimately to O/W microemulsion type occurs by increasing the concentration of low molecular weight alcohol (co-surfactant). The opposite type of phase transition is observed in the case of high molecular weight alcohols [25].

Surfactant hydrophobic chain length

An increase in the length of the hydrophobic chain of the surfactant causes the O/W microemulsion to invert to type W/O *via* the bicontinuous phase [25].

pH

Changes in pH affect the microemulsion systems contaning pH sensitive surfactants. The effect is more pronounced when using acidic or alkaline surfactants. Carboxylic acids and amines change the microemulsion type from W/O to O/W by raising the pH [25].

Nature of oil

Increase in the aromaticity of oil leads to phase transitions from O/W to W/O microemulsion and is opposite to that of the increase in the oil alkane carbon number [25].

Ionic strength

When the ionic strength increases, the microemulsion system passes from O/W microemulsion in equilibrium with oil excess to the middle phase and finally to W/O microemulsion in equilibrium with water excess [25].

3.1.4 Factors affecting the microemulsion type

The microemulsion type depends on the following factors:

Critical Packing Parameter (CPP)

Changes in film curvature and microemulsion type can be addressesd quantitatively in terms of geometric requirements. This concept was introduced by Israelachvili et al. [41] and is widely used to relate surfactant molecular structure to interfacial topology. The preferred film curvature of surfactant is governed by the relative values of the head group (a_o) and tail effective area (v/l_c) where v is the partial volume of the hydrophobic part of surfactant and l_c is the effective length of the surfactant hydrocarbon chain (Fig. 3) [38].

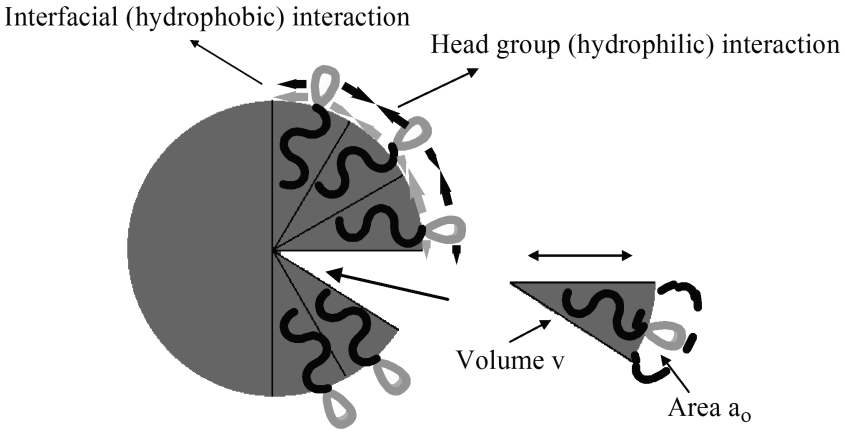

Figure 3 The critical packing parameter relates the head group area, the length and the volume of the carbon chain to a dimensionless number [38].

The critical packing parameter is expressed by equation (2)

$$CPP = v/l_c a_o , \qquad (2)$$

Generally, if a CPP value lies between 0–1, the interface curves towards water (positive curvature) and O/W systems are favoured. When the CPP is greater than 1, the interface curves spontaneously towards oil (negative curvature) and W/O microemulsions are formed. At zero curvature, when the HLB value is balanced, either bicontinuous or lamellar structures may form according to the rigidity of the film [25].

Hydrophilic-Lipophilic Balance (HLB)

Another concept relating molecular structure to interfacial packing and film curvature is the HLB value concept. The HLB takes into account the relative contribution of hydrophilic and hydrophobic parts of a surfactant molecule. The HLB concept was firstly introduced by Griffin in 1949 [42]. He suggested a semi-empirical HLB scale for predicting emulsion type from a surfactant molecular composition. Non-ionic surfactants are sorted from lipophilic to hydrophilic on a scale of 1 to 20 at 25 °C [43]. HLB and packing parameter describe the same basic concept although the latter is more suitable for microemulsions.

Characteristics of surfactant and oil phase

A surfactant molecule consists of a hydrophilic head group and lipophilic tail group. The areas of these groups, which are a measure of the differential tendency of water to swell the head group and oil to swell the tail, are important for specific formulation when assessing the surfactant HLB in a particular system. When a high surfactant concentration is used or the surfactant is in the presence of salt, then the degree of dissociation of polar groups in the surfactant decreases and the resulting system may be of W/O type. Diluting with water then increases dissociation and leads to an O/W system.

The character of the oil phase also affects interfacial curvature (type of emulsion formed) due to its ability to penetrate into the surfactant monolayer and swell the lipophilic tail group region of the surfactant. As a consequence of the oil penetration into the surfactant lipophilic region, the negative curvature increases, the surfactant monolayer is more convex towards the oil phase and W/O emulsions are preferably formed [44].

Temperature

Temperature is particularly important for non-ionic surfactants in determining their effective head group size. At low temperatures, they are hydrophilic and form normal O/W systems, while at higher temperatures, they

are lipophilic and form inverse systems of W/O. Moreover, at an intermediate temperature, microemulsion also coexists with excess water and oil phases when a bicontinuous structure is formed [44].

The chain length, type and nature of the co-surfactant

The addition of a co-surfactant with a shorter chain, for example alcohol, produces a positive curvature effect as alcohol swells the head region of the surfactant more than the tail region. As a result, the surfactant becomes more hydrophilic and an O/W microemulsion type is formed. Conversely, a co-surfactant with a longer chain favours a W/O microemulsion type, with alcohol swelling more in the tail region [44].

3.2 Properties of microemulsions

The main characteristics of microemulsions are a low viscosity associated with a Newtonian-type flow [6][35], an ultralow interfacial tension, a large interfacial area [45] and the ability to solubilize both water-soluble and oil-soluble compounds [45][46]. Moreover, they are also characterized by excellent stability, easy preparation, optical clarity [46] and, in particular cases, antimicrobial properties [47]. All of these favourable properties make them desirable in a variety of applications including the food industry, cosmetics, pharmaceuticals [48], cleaning technologies and soil remediation [40]. Microemulsions have also been utilized as templates for the synthesis of polymer dispersions and nanoparticles for a broad range of technological applications [49]. On the contrary, a major practical drawback of microemulsions is usually connected with their composition. Since microemulsions represent droplets with very small diameters, they form very high surfaces requiring a higher surfactant concentration compared to classical emulsions, typically 10–30 wt.% [50][51]. This leads to high residues, contaminant levels, formulation costs [49] and limitations of their widespread use in some practical applications [18].

3.3 Formulation of microemulsion systems

Generally, microemulsions are systems composed of four components: water, oil, a surfactant and a co-surfactant [52][53][54][55]. These systems can be stabilized by non-ionic and ionic surfactants and mixtures thereof [56]. The chosen surfactant must be able to lower the interfacial tension to an extremely small value, allowing dispersion of the oil phase during the production of the microemulsion and providing a flexible film that can readily deform the droplets and be of suitable liophilic/hydrophilic character to ensure the correct curvature at the interfacial region [57]. It is well-known that microemulsions prepared using ionic surfactants require a relatively high

ratio of surfactant in the dispersed phase or even the addition of a co-surfactant to attain the required interfacial properties leading to the spontaneous dispersion of one phase in the other. On the other hand, some non-ionic surfactants have been found to produce microemulsions with lower levels of surfactant (~10 wt.%) and without the addition of a co-surfactant [20]. Therefore, non-ionic surfactants are particularly interesting in relation to microemulsion formation due to the absence of repulsive electrostatic interactions [46]. Yet, in microemulsions stabilized by non-ionic surfactants, the temperature is the decisive parameter, and the microemulsion phase is stable only in a certain, limited temperature range [58].

Short-chain alcohols (ethanol, propanol, butanol, pentanol and hexanol, etc) [19][55] were originally used as co-surfactants, but nowadays polyols, esters of polyol, derivates of glycerol, and organic acids are often included [55]. The purpose of their application is to sufficiently reduce the interfacial tension between oil and water in order to achieve almost spontaneous microemulsion formation [45]. The effectiveness of alcohol as a co-surfactant depends decisively on its chain length, which was mentioned in section 3.1.3 [59].

The effects of short-chain alcohols (such as ethanol), polyols (propylenglycol or glycerol) and salts (organic and inorganic) on the phase behaviour of food-grade glycerol monolaurate microemulsions have recently been discussed [48][60][61]. It was found that the solubilisation of water and oil with ethoxylated non-ionic surfactants was dramatically improved in the presence of short-chain alcohols and polyols, and organic salts contributed to the improved oil solubilisation as hydrotropes. Moreover, the study [62] showed that the solubilisation of water-soluble salts with antimicrobial properties in microemulsions enhanced their antimicrobial effects. Aboofazeli et al. [63] examined the oil effect on the phase behaviour of microemulsions. The pseudoternary phase of systems composed of water, lecithin, propanol (or butanol) and oils with different polarity (soybean oil, ethyl oleate, oleic acid, ethyl octanoate, octanoic acid, butoxyethanol) were reported. It was found that the phase behaviour of these systems was largely dependent upon the nature and mixing ratio of the consurfactant as well as the length of the oil chain, rather than the polarity of the oils used in this study.

The effect of alcohol was also studied by Leser et al. [64], who examined the phase diagrams of phospatidylcholine, medium-chain triacylglycerol (MCT) and alcohols (propanol, butanol and pentanol) with different chain lengths. They concluded that propanol disorders the liquid crystalline structure, enhances the flexibility of the surfactant film and enables the MCT to penetrate the surfactant. Another study [65] has demonstrated that the formation of triacylglycerol microemulsion can be achieved by incorporating sucrose and short-chain alcohol (ethanol). Their synergistic interactions result

in the destabilization of liquid crystalline mesophases and lead to triacylglycerol microemulsion formation.

Graciaa and co-workers [66] first introduced the lipophilic linker (dodecanol) concept, which improves the solubilisation capacity of hydrocarbon and polar oils. These lipophilic linkers (long-chain alcohols) have a tendency to segregate near the oil side of the oil-water interface, very close to the tails of the surfactants. Conversely, Acosta et al. [67] found that hydrophilic linkers (sodium mono and dimethylnaphthalene sulfonate – SMDNS) can also increase solubilisation because they provide more space for lipophilic linkers to segregate and further enhance the solubilisation ability. Recently, Komesvarakul et al. [68] published a modified approach regarding the formation of sebum microemulsions using linker molecules with the addition of co-oil to improve sebum solubilisation. They used a combination of a lipophilic linker (sorbitan monooleate), a hydrophilic linker (hexylglucocide), a main surfactant (sodium dioctyl sulfosuccinate), a co-oil (squalene, squalane, isopropyl myristate and ethyl laurate), and artificial sebum for microemulsion formation. They have found that using co-oil helps the solubilization of triacylglycerols at lower surfactant concentrations and reduces the amount of surfactant necessary to form a single-phase microemulsion. These formulations have a major advantage since they can reduce the amount of surfactant and completely remove alcohol from the formulation. Since alcohols are potentially irritating, alcohol-free formulations could be useful especially for consumers with sensitive skin.

Recently, several studies have investigated the antimicrobial properties of microemulsions containing different types of monoacylglycerols (MAG) as an oil phase. For example, the testing of the antimicrobial activity of a microemulsion system with monolauroylglycerol was published in [69][70][71][72]. In the publication of Fu et al. [73], the enhancement of the inhibition ability of this MAG towards bacteria *via* loading in microemulsions in comparison with the antimicrobial activity of corresponding MAGs was reported.

4 NANOEMULSIONS

Nanoemulsions are defined as a class of emulsions with a uniform and small droplet size, typically in the range of 50–200 nm [74][75] or 50–500 nm, as given in publications [76][77][78][79]. In the literature they are often referred to as miniemulsions [80][81], sub-micron emulsions [82] or ultrafine emulsions [18]. The term nanoemulsions has been increasingly adopted because it signifies the nanoscale size range of the droplets and avoids confusion with other kinds of dispersions such as microemulsions

[58][74]. They are intermediates between classical emulsions and microemulsions [83].

4.1 Preparation of nanoemulsions

Although the preparation of nanoemulsions is more complex than that of microemulsions, from a practical view point they possess an important advantage stemming from the reduced amounts of surfactants needed for their formation [58][90]. Conversely, nanoemulsions cannot be formed spontaneously like microemulsions, and energy input, generally from mechanical devices or from the chemical potential of the components, is required [50][85][91][92]. According to the literature, nanoemulsions can be prepared by high-energy [93][94][95] and low-energy [75][96][97] emulsification methods.

4.1.1 High-energy emulsification method

High-energy emulsification methods are extensively used in industries to produce emulsions with small and uniform droplet sizes [97]. These techniques require high mechanical energy, which is achieved by applying high-shear stirring, high-pressure homogenizers or ultrasound generators [78].

During emulsification, various processes take place including the break up of droplets, adsorption of surfactant molecules, and droplet collision, which may lead to coalescence and the formation of larger droplets. These processes may occur simultaneously during emulsification, as the time scale for each process is extremely small (miliseconds). The breaking of droplets occurs when the deforming force exceeds the Laplace pressure, p_L, which is the interfacial force that acts against droplet deformation. It follows that Laplace pressure is the difference in pressures between two sides of a curved interface and is defined by equation 3

$$p_L = \gamma \left(\frac{1}{R_1} + \frac{1}{R_2}\right), \qquad (3)$$

where R_1 and R_2 are the principal radii of curvature and γ is the interfacial tension. From equation 3 it is clear that the smaller the droplet size for a given system, the more energy input and/or surfactant is required [58].

4.1.2 Low-energy emulsification method

In contrast, low-energy emulsification methods can use the advantage of the chemical energy of components to produce nanoemulsions almost spontaneously. These techniques are based on the phase transitions taking place during emulsification [85][98]. These phase transitions result from

changes in the spontaneous curvature of the surfactant [99]. As defined, phase inversion is a process by which water-in-oil (W/O) emulsion can be inverted to an oil-in-water (O/W) emulsion or *vice versa* [100]. This phenomenon can be induced either by increasing the volume fraction of the dispersed phase (*catastrophic phase inversion*) or by changing the affinity of the surfactants towards the two phases (*transitional phase inversion*) [101][102]. Both types of phase inversion can be seen in Figure 4 [51]. A catastrophic phase inversion occurs between a normal and an abnormal emulsion, while a transitional inversion is a process that occurs between two normal emulsions [103]. Emulsions are deemed normal when the surfactant is more soluble in the continuous phase and abnormal when the surfactant is more soluble in the dispersed phase. These abnormal emulsions are highly unstable and can only be maintained under vigorous mixing for a short period of time [101]. Generally, factors that affect the phase inversions of O/W emulsions are the nature of the oil phase, the surfactant type and its concentration, the temperature of the system, the O/W ratio, the presence of other components in the oil or water phase, the process conditions and the rate and order of addition of the different components [102].

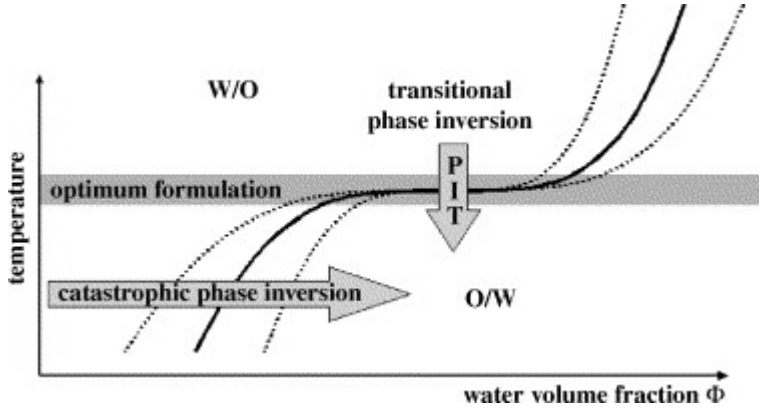

Figure 4 Schematic illustration of both catastrophic and transitional phase inversion for the preparation of O/W emulsions [76].

The **Phase inversion temperature (PIT) method**, first introduced by Shinoda and Saito [104][105], is widely used in industry. This transitional phase inversion is based on the temperature dependent changes in the spontaneous curvature of polyoxyethylene type non-ionic surfactants in the emulsion system with a constant composition [106]. These types of surfactants become lipophilic with increasing temperature due to the dehydration of the polyoxyethylene chains. The curvature of the surfactant monolayer is more convex (has a large positive spontaneous curvature) towards water at low temperatures [106][107], and a ternary mixture of oil, water and surfactant will be phase separated into a surfactant-rich aqueous

phase and almost pure oil (Winsor I). The surfactant-rich aqueous phase, also reffered to as L_1, consists of direct micelles swollen by oil. At high temperatures, the curvature of the surfactant is more concave (curvature becomes negative) towards water due to the dehydration of the hydrophilic tail in a non-ionic surfactant, and it self-assembles into reverse micelles. A ternary mixture of oil, water and surfactant will separate into an oil phase containing the surfactant, referred to also as L_2, in coexistence with almost pure water (Winsor II) [108]. It follows that O/W emulsions are preferably formed at low temperatures while W/O emulsions form better at high temperatures [106][107]. At an intermediate temperature, a so-called PIT temperature, the surfactant is "equilibrated" and its spontaneous curvature is close to zero. At this temperature, a bicontinuous or liquid crystalline phase is in equilibrium with oil and water phases (Winsor III) and the transition from O/W to W/O emulsions takes place [108]. During the emulsification process, the interfacial tension between oil and water in the presence of non-ionic surfactants decreases with temperature and reaches a minimum at a PIT temperature before it starts to increase again if temperatures are further increased [106][107]. Therefore, emulsions with very small droplets can be formed at this PIT temperature. However they are highly unstable, and a rapid cooling of the systems well below the PIT temperarure is necessary to obtain fine and stable O/W nanoemulsions [105]. If the cooling process is slow, coalescence predominates and coarse emulsions are formed [109]. To prolong the stability of nanoemulsions, a storage temperature of about 20 to 65 °C lower than the PIT temperature is suggested [105].

The PIT temperature can be determined by conductivity measurements, after a small amount of sodium chloride is added. At low temperatures, where O/W nanoemulsions are present, a high conductivity is measured. With increasing temperature, conductivity decreases. Low conductivity values are obtained at temperatures above PIT where W/O nanoemulsions are present. The PIT temperature is then calculated as an average temperature between the temperatures at the highest and lowest conductivities [90]. The phase inversion temperature can also be observed by the naked eye. With this method, the PIT is determined by slowly heating the emulsion and visually observing the start of turbidity. Below the PIT, the emulsion is white, opaque, glossy and thickened, while above the PIT, it is thin and watery [110]. Instead of a visual observation, turbidity can be detected using spectrofotometry.

The practical application of the PIT technique is documented by the following examples. Morales and co-workers [111] prepared O/W nanoemulsions with narrow size distributions and droplet sizes around 40 nm in the system water/hexaethylene glycol monohexadecyl ether ($C_{16}E_6$)/mineral oil. Nanoemulsions with a droplet size in the range of 30–130 nm have been investigated by Izquierdo et al. using technical-grade non-ionic surfactants ($C_{12}E_4$ and $C_{12}E_6$) and hexadecane [89][90] and isohexadecane [106] as an oil

phase. These O/W nanoemulsions consisted of 20 wt.% of the oil phase and 4 to 8 wt.% of the surfactant. Inverted, W/O nanoemulsions have been prepared by the same method and using the same components and studied by Peng et al. In this case, the optimum composition in terms of stability and particle size was isohexadecane/polyoxyethylene 2-lauryl ether ($C_{12}E_2$)/polyoxyethylene 4-lauryl ether ($C_{12}E_4$)/water mixed in the ratio of 70:6:4:20 (wt.%). These nanoemulsions were transparent with a droplet size of 21 nm and showed stability for more than 200 days of storage without phase separation [51]. Interesting contributions concerning the PIT emulsification process were made by Roger et al. [112], who re-examined this process in a water/$C_{16}E_8$/hexadecane system. They found that successful emulsification is ensured by gently stirring the mixture throughtout the process. Moreover, the stirred mixture must be heated above the PIT temperature and then fast quenched to lower temperatures. These conditions lead to emulsions that have excellent metastability and a narrow size distribution with a particle size in the range of 20–100 nm.

The second type of transitional phase inversion, **Emulsion Inversion Point (EIP) technique,** can be induced by the gradual changing of the emulsion composition at constant temperature. By adding water to the oil/surfactant mixture, the water droplets are first produced in a continuous oil phase. An increase in the water volume fraction induces changes in the spontaneous curvature of the surfactant from initially stabilizing a W/O emulsion to an O/W emulsion at the inversion point. At this point, the affinity of the surfactant towards both phases is balanced, and a microemulsion phase or lamellar liquid phase with excess water and oil forms. During the transition, the system crosses the point of zero spontaneous curvature, and the interfacial tension reaches a minimum, supporting the formation of emulsions with small droplet sizes [76]. When the system is further diluted with water, this structure breaks up into an O/W nanoemulsion.

The formation and stability of nanoemulsions prepared by the EIP technique is highly dependent on the hydrophilic-lipophilic balance (HLB) number of non-ionic surfactants [113]. Surfactants with a higher HLB value are hydrophilic and act as solubilising agents, detergents and O/W surfactants. On the contrary, surfactants with a lower HLB value are lipophilic and stabilize W/O emulsions [114]. Using EIP, emulsions with submicrometer-sized droplets are only obtained if the water to oil ratio is over a certain value. Above this value, an excess of water may be added without any affect on droplet size. A critical surfactant concentration is also necessary for their preparation, as the oil has to be solubilised completely at the EIP. Besides, the resulting size distribution mainly depends on the surfactant/oil ratio [76] and on the order of mixing of the individual components used in the nanoemulsion formulation.

This was illustrated by Forgiarini et al. [115], who have studied the preparation of water/Brij30/decane nanoemulsions using three different low energy emulsification methods. The nanoemulsions with droplet sizes of 50 nm were formed only when water was added to mixtures of oil and surfactant. The formation of water/Cremophor EL/Miglyol 812[1] nanoemulsions with particle sizes from 14 to 39 nm has been reported by Sadurní et al. [97].

Following the same method, a considerable number of research studies have been devoted to the formation of nanoemulsions of cyclohexane [116][117], xylene [118] and polyisobutylene [119] using a mixture of high- and low-HLB surfactants. Liu et al. [96] made paraffin O/W nanoemulsions stabilized by two non-ionic surfactants Tween 80 and Span 80 using the emulsion inversion point method. Nanoemulsions with a droplet size below 200 nm were obtained above the critical surfactant-to-oil ratio of 0.20 at 50 °C. Surfactant combination seems to be also advantageous, as it is well-known that for a wide range of applications, the mixtures of surfactants with a high HLB and low HLB give more stable emulsions than the individual surfactants [43][120].

The EIP method is also applicable in the production of W/O nanoemulsions. This type of nanoemulsion with droplet sizes between 60 and 160 nm was produced by Uson and co-workers, using a stepwise addition of oil into a mixture of water and surfactant (Cremophor EL/Cremophor WO7[2]). Stable W/O nanoemulsions are usually produced only with a very low water concentration (max 4.5 wt.% of the dispersed phase) using a mixed surfactant system with a low HLB value [121]. In addition, W/O nanoemulsions have been formed by Porras et al. in water/mixed non-ionic surfactants/decane. They prepared nanoemulsions by adding water to a mixture of decane and non-ionic surfactants (Span 20, Span 80, Tween 20 and Tween 80), thus obtaining particles with sizes between 30 and 120 nm [50].

4.2 Properties of nanoemulsions

Nanoemulsions are non-equilibrium systems with a spontaneous tendency towards phase separation [84]. However, due to the extremely small initial size of their droplets, nanoemulsions may appear transparent or translucent [85]. Their kinetic stability is also typical [75]. The small droplet size in nanoemulsions makes them resistant to creaming or sedimentation because their Brownian motion is enough to overcome gravitational separation.

[1] Cremophor EL is obtained by reacting castor oil with ethylene oxide in a molar ratio of 1:35. Its HLB lies between 12 and 14. Mygliol 812 is a commercial, medium-chain triglyceride.

[2] Cremophor WO7 is non-ionic surfactant, manufactured by reacting hydrogenated castor oil with 7 moles of ethylenoxide. Its HLB is 5 ±1.

Moreover, they are also resistant to the flocculation or coalescence of droplets because of their efficient steric stabilization [86]. Additionally, a significant surfactant film thickness (relative to droplet radius) prevents thinning or disruption of the liquid film between droplets [102]. The principal destabilization mechanism of their breakdown is the Ostwald ripening [87]. This instability is governed by the molecular diffusion of oil from small to large droplets as a result of the difference in solubility between droplets of different sizes and will be described in greater detail in part 4.3 [88].

The attractive properties of nanoemulsions, including small droplet size, high kinetic stability, optical semi-transparency and low viscosity have garnered considerable interest for industrial applications, such as pharmaceuticals and cosmetics, where they can serve as drug delivery systems, agrochemistry for pesticide delivery, the chemical industry, etc. [51][89]. The main limitation for using nanoemulsions is their non-straightforward preparation procedure [87].

4.3 The instability of nanoemulsions

It is commonly reported, that the principal mechanisms of emulsion instability, leading to complete phase separation, are creaming, sedimentation, flocculation, coalescence and Ostwald ripening [91]. Compared to classical emulsions, the main destabilizing process of nanoemulsions is the Ostwald ripening mechanism. Regardless of the production method, the ripening rate increases with increasing oil concentration and a reduction of the surfactant amount [78][96]. In this process, the larger droplets grow at the expense of the smaller ones. As a consequence, the more monodispersed the droplets are, the less the nanoemulsions will be affected by Ostwald ripening. Therefore, it is important to control not only the particle size of nanoemulsions but also to prepare particles with a narrow size distribution [74].

There are several methods to reduce the ripening rate. This can be, for example, performed by the addition of a second, less soluble oil [90] or by using a surfactant that forms monolayers less permeable to the oil (an ionic surfactant) [122].

Ostwald ripening is described by the LSW theory, formulated by Lifshitz and Slezov [123] and independently by Wagner [124]. Generally, LSW theory is valid in the case of immobile particles and assumes that the droplets are separated by distances much larger than their diameters and that the transport of a dispersed component is only due to molecular diffusion [125][126]. These assumptions may not be completely valid for nanoemulsions because of their strong Brownian motion, which may induce convective diffusion, accelerating the diffusion rate [126]. Therefore, this theory is not suitable for predicting the Ostwald ripening rate in

nanoemulsions, but it is a good tool for identifying the Ostwald ripening as the main destabilizing factor in nanoemulsions [91]. The Ostwald ripening rate, ω, is expressed as follows:

$$\omega = \frac{dr_c^3}{dt} = \frac{8c(\infty)\gamma V_m D}{9RT}, \quad (4)$$

where r_c is the critical radius of droplets that are neither growing nor decreasing in size, $c(\infty)$ is the bulk phase solubility, γ is the interfacial tension, V_m is the solute molar volume, D is the diffusion coefficient, R is the gas constant, and T is the temperature. This equation shows that r^3 varies linearly with time. Therefore, plotting a linear graph of r^3 versus time makes it possible to determine Ostwald ripening rates [58].

4.4 The formulation of nanoemulsion systems

The procedure for encapsulating essential oils into soya lecithin-based nanoemulsions was published in [127]. This study was done in order to enhance the antimicrobial activity of essential oils while minimizing their impact on the quality of the final product (fruit juices). Nanoemulsion formation and its use as a vehicle for skin-care products have been described in publication [128]. These systems were evaluated and compared with two other products (body milk and body lotion) in terms of hydratation and the satisfaction of volunteers. It was found that nanoemulsions showed higher hydratation power compared with the two other products, and 80% of the volunteers preferred the nanoemulsion to their usual product. Another study [129] concentrated on the formation and stability of O/W nanoemulsions containing rice bran oil and their usage in the cosmetic industry. The studied nanoemulsions were applied to the skin of volunteers with normal and diseased skin types. It was found that these systems increased the relative skin hydration and skin oiliness, and maintained the normal skin pH. Hence, these nanoemulsions could serve as an alternative treatment for skin diseases (atopic dermatitis or psoriasis). Rao et al. [130] and Ziani et al. [131] dealt with the formation of flavoured oil microemulsions and investigated the influences of composition and preparation method on their stability. They used food-grade non-ionic surfactant Tween 80 and lemon oil. This study provides useful information on the development of stable food-grade delivery systems for incorporating flavour oils or other lipophilic components (colors, micronutrients and antimicrobials) into foods and beverages.

5 MICROPARTICLES

The emergence of microparticles with the potential application as carrier system dates back to 1930s when Bungenberg de Jong et al. [132][133] published studies dealing with the formation of gelatine particles by the coacervation method. The pharmaceutical applications of microparticles were recognized 30 years later [134]. They can be used to mask the taste of a drug, to protect the drug against undesired environmental effects, to incorporate volatile drugs, such as essential oils and mainly to control the release of the drug [135][136]. Nowadays, the production of microparticles with controlled particle sizes and particle size distribution is of significant interest in several fields, such as chemistry and material science, biotechnology, medicine, pharmacy or agriculture [137].

Microparticles are defined as particulate dispersions or solid particles with a size ranging between 1–1000 μm [137][138][139]. Strictly speaking, they can exist in two forms, namely as microcapsules (micrometric reservoir systems) and microspheres (micrometric matrix systems) [6]. While microcapsules are systems in which the drug is cased in a cavity surrounded by a polymer membrane, microspheres are matrix systems in which the drug is physically and uniformly dispersed [138].

5.1 Composition of microparticles

Microparticles (microcapsules and microspheres) consist of two major parts (Fig. 5); 1) the core material containing one or more active ingredients, which could be solids, liquids or gases and 2) the coating (wall) material, which can be selected from a wide variety of polymers, depending on the core material to be encapsulated and the desired characteristics of the particles [6][140]. The wall materials can be of natural or synthetic origin and polymers, such as gelatine, dextrin, chitosan, hyaluronic acid, dextran, poly(vinylalcohol) (PVA), poly(vinylpyrrollidone) (PVP), poly(lactic acid) (PLA), and poly(lactic-co-glycolic acid) (PLGA) can be applied. The coating material must be nonreactive to the core material, preferably biodegradable and nontoxic. Other components, for example surfactants (poloxamers, polysorbates, lecithins), organic solvents (ethanol, isopropanol, ethyl acetate, propylene carbonate, benzyl alcohol), co-surfactants (glycofurol, ethanol, isopropanol) and additives (buffers, salts, polyols) necessary for microcapsule formation may also be added [138].

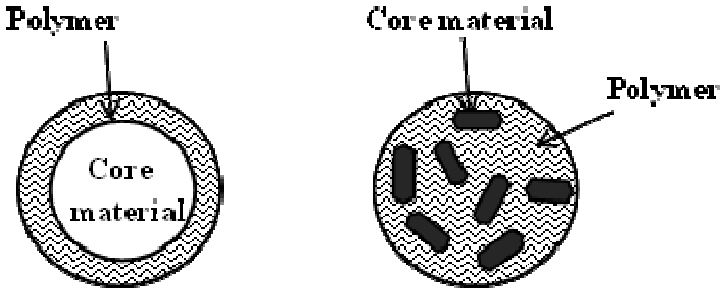

Figure 5 Schematic representation of two different types of microparticles; a) microcapsules b) microspheres [6].

5.2 Microencapsulation

Microencapsulation is a process involving surrounding the core with inert natural or synthetic polymeric materials [6]. Its main role is to produce a system capable of delivering, protecting, stabilizing and controlling the release of the core. Encapsulated ingredients include adhesives, drug substances, pigments, fragrances, flavors, agricultural chemicals, solvents or oils [141].

For microparticle production, different traditional techniques have been used including spray drying and spray freeze drying [142][143], jet milling [144], dispersion polymerization [145], nanoprecipitation [146] and solvent evaporation [147]. However, all of them have specific disadvantages. For example, spray drying and jet milling are not suitable for the treatment of thermolabile compounds because their operating temperatures are too high and because they are not able to produce a powder with narrow and controlled particle size distributions [148][149]. The remaining techniques suffer from common drawbacks, namely the need of organic solvents in at least one of the production steps, which have to be removed, and low encapsulation efficiency [150]. Therefore, supercritical fluid-based technologies have been proposed to overcome the limitations of these traditional encapsulation methods [151][152]. The use of supercritical fluids offers potential advantages related to the efficient extraction of solvents, the low amount of impurities and substantial control of the particle size. Effective control of the particle precipitation is possible by changing the mass transfer conditions and the phase behaviour of the ternary mixture solvent-antisolvent-solute. The most commonly used supercritical fluid is carbone dioxide (SC-CO_2). Normally, at standard temperature and pressure (STP), carbone dioxide behaves as a gas; when frozen, it turns to solid (dry ice). However, when the temperature and pressure increase from STP to or above the critical point for CO_2, it can adopt

properties between a gas and a liquid. It follows that it behaves like a supercritical fluid above its critical temperature (31.1 °C) and critical pressure (7.39 MPa). SC-CO_2 is very often used as a commercial and industrial solvent due to its low price, low toxicity and minimum environmental impact. The relatively low temperature of the micronization process and the stability of CO_2 allow most compounds to be micronized with only little to no damage [153].

Generally, micronizing techniques based on supercritical fluids include the rapid expansion of supercritical solutions (RESS) [154][155], particle generation from gas saturated solutions (PGSS) [156], supercritical antisolvent precipitation (SAS) [157] and supercritical assisted atomization (SAA) [158][159]. Of these techniques, SAA will be discussed in more detail, as it was applied in the Ph.D. experiments.

5.2.1 Supercritical Assisted Atomization – SAA

A major advantage of the SAA process is the opportunity to use either organic solvents or aqueous solutions which results in the possibility to process both water-soluble as well as water-insoluble compounds [160]. Moreover, this technique provides good particle size and good particle size distribution control (between 0.05 and 5 µm) [161]. These unique properties together with its environmentally friendly behaviour and non-aggressive processes with respect to the substances treated, allowed this technique to effectively and successfully micronize a wide range of compounds [162][161].

The supercritical assisted atomization process developed by Reverchon and co-workers [158][162] is based on both the solubilisation of controlled quantities of supercritical SC-CO_2 in a liquid solution containing a solid solute and the subsequent atomization of the ternary solution through an injector [162][163]. Carbon dioxide is used as a co-solute and the pneumatic agent responsible for the atomization [158]. The solubilisation is obtained in a high pressure vessel called a saturator, which is characterized by a high specific surface and a large residence time thus ensuring contact between the solution and CO_2. The prepared solution is sprayed through an injector into precipitator operating at atmospheric pressure to obtain droplets [164]. The forming of particles is characterized by a two-step atomization process. The first step represents the formation of primary droplets at the exit of the injector, which is caused by a pneumatic effect. The second step, called decompressive atomization, is induced by the quick release of CO_2 from primary droplets, giving rise to smaller ones [152]. Microparticles are obtained by droplet evaporation using nitrogen heated to 120 °C. The evaporation should be fast to avoid the coalescence phenomena and the

sticking of the droplets on the precipitator's inner surface [165]. The SAA apparatus is schematically sketched in Figure 6.

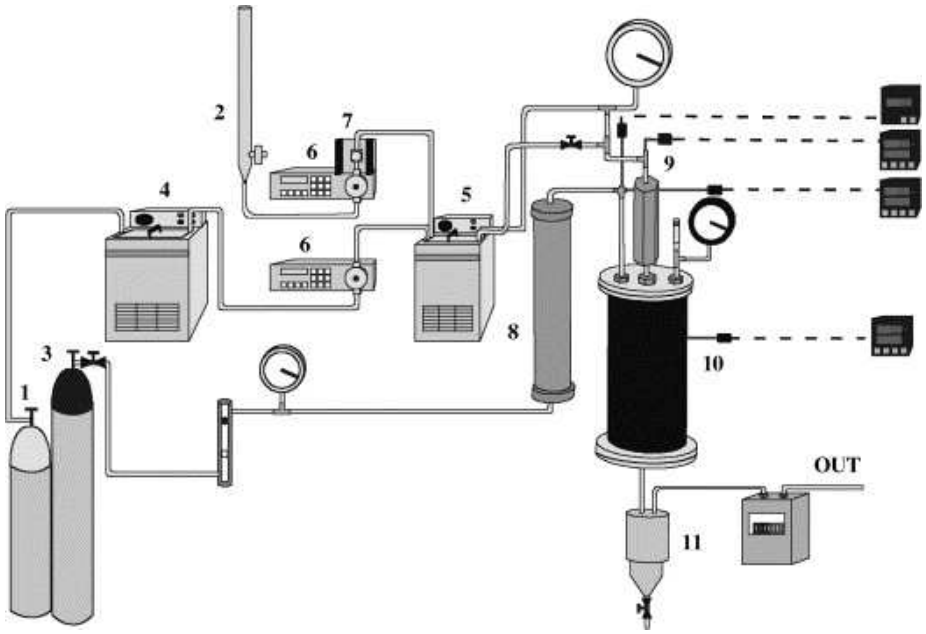

Figure 6 Schematic representation of the SAA apparatus: 1. CO_2 cylinder; 2. liquid solution; 3. N_2 cylinder; 4. cooling bath; 5. heating bath; 6. high pressure pumps; 7. dampener; 8. heat exchanger; 9. saturator; 10. precipitator and 11. condenser [148].

SAA process parameters

Process parameters influencing the efficiency of the atomization process, the particle size and particle size distribution include the following variables:

- Gas/liquid ratio – (the mass flow ratio) between CO_2 and the liquid solution,
- The operating pressure and temperature in the saturator,
- The concentration of solute in the liquid solution,
- The temperature in the precipitator, which should be high enough to allow the solvent to evaporate from the droplets, but lower than the degradation temperature of the core material to be encapsulated [153].

The SAA technique has been successfully used in the micronization of pharmaceuticals, polymers, copolymers or proteins. For example, it has been tested on various drugs, such as terbutaline [148], rifampicin and tetracycline [149] and particles were produced in the micron size-range with controlled distributions of compounds precipitated from water, methanol or acetone. The atomization of ampiciline microspheres with a wall created by

hydroxymethylcellulose (HPMC) or chitosan has been studied by Reverchon et al [161][166]. In both cases, spherical or doughnut microparticles with uniform morphology and a narrow distribution were formed. The size of obtained spherical particles was between 0.1–6 µm. It was determined that the wall-forming polymers protected ampicillin from thermal degradation and prolonged the release of the drug.

The SAA technique has been also studied for protein micronization. Adami et al. [167] micronized lysozyme using water, buffered water at a pH of 6.2 and water-ethanol mixtures at different volume ratios. The obtained lysozyme microparticles were spherical with a narrow size distribution ranging from 0.1 to 4 µm. Moreover, this study was conducted as a demonstration of lysozyme encapsulation as an efficient tool against denaturation. Good results in terms of stability and the activity of this enzyme after micronization were detected. Depending on process conditions, lysozyme retained 95 to 100% of the biological activity compared to the untreated enzyme. Wang et al. [168] applied an SAA equipped with a hydrodynamic cavitation mixer (SAA-HCM) to the micronization of bovine serum albumin (BSA). When the precipitator temperature was set at 70 °C, spherical microparticles with the size distribution of 0.3–5 µm were obtained using water as a solvent. It was found that an SAA-HCM offers a promising technique for micronizing bioactive macromolecules without any organic solvents, for effective drug delivery. Another work published by Porta et al. [169] dealt with the production of BSA heat denatured microspheres loaded with gentamicin sulphate. All microspheres produced with the SAA technique exhibited high drug content and good encapsulation efficiency leading to a significant reduction of the water content inside of these systems and resulting in the increased stability of the drug.

6 A COMPARISON OF THE DISCUSSED PARTICULATE SYSTEMS

All delivery systems have their own advantages and disadvantages related to the purpose of their use. It follows that various types of cosmetic vehicles are more suitable for a certain application area, and the right choice of the system is in many cases the decisive parameter for the efficient delivery and release of active compounds in the target location. Microemulsions are effective vehicles for dermal as well as transdermal compound delivery due to their small size and high-loading capacity. Besides, their thermodynamic stability and simple preparation process make them favoured vehicles for skin applications [6]. They are able to solubilize both hydrophilic and hydrophobic compounds. Because of the low interfacial tension between the oil and water phases, microemulsions exhibit excellent wetting and penetrating properties [9]. Despite all the benefits mentioned above, microemulsions possess some

drawbacks. They have limited solubizing capacity for high-melting substances, and their stability is influenced by environmental parameters such as temperature, pH and pressure [45]. Moreover, they are restricted in their usage because of the need for a relatively high surfactant concentration for their preparation (10–30 wt.%) [90], which might be a problem with respect to allergic and sensitization adverse reactions.

Conversely, nanoemulsions can be prepared using moderate surfactant concentrations, typically between 3 and 10 wt.% [92]. Their small particle size, relatively high kinetic stability and large surface area makes them suitable for the efficient delivery of active compounds and for their penetration through the skin [90][92]. Nanoemulsions can deliver both hydrophilic and hydrophobic compounds. They are non-toxic and commonly non-irritant, protect the drug from degradation caused for example by hydrolysis or oxidation, and can also provide taste masking [170]. However, the main limitation for their application is their non-straigtforward preparation procedure and limited long-term stability [84].

The previously mentioned systems are microparticles that exhibit long-term stability during storage. They can be stored in dry powder or in suspension with a little or no loss of activity over an extended storage period [140]. They provide highly reproducible formulations and along with emulsion systems can encapsulate both hydrophilic and hydrophobic compounds, making them widely applicable [171]. Microparticles also offer improved control over the release rate of the drug, and their particle size gives them the potential to enhance the solubility and bioavailability of the encapsulated substance. In spite of these advantages, microparticles have also some limitations. Their small particle size together with large surface area can lead to particle aggregation, which may significantly change the properties and performance of these systems [138].

7 CHARACTERIZATION METHODS OF PARTICULATE SYSTEMS

Dispersion systems can be characterized by the combination of a variety of techniques. For example, the macroscopic properties, such as viscosity, interfacial tension and conductivity can be determined by rheometry, conductometry and tensiometry. The size and shape of the particles can be measured by photon correlation spectroscopy (PCS), light diffraction or by electron microscopy, and the stability of these systems can be easily evaluated by visual observation. Moreover, the solid state properties and structure of particles can be measured by differential scanning calorimetry (DSC) or X-ray diffraction (XRD) [172].

The characterization of the particular systems is rather complicated due to their complexity, namely due to the series of structures and components that

may be involved in the systems. Therefore, studies covering a variety of techniques are required to gain a general overview of the physicochemical properties and structures of the dispersion systems [16]. Some of the most frequently used characterization techniques will be briefly mentioned in the next section of the doctoral thesis.

7.1 Visual observation

Emulsion systems are not thermodynamically stable and undergo a continuous destabilization process until one macroscopic water phase and one macroscopic oil phase occur [46].

Visual observation is an old, simple method still used to check emulsion stability. The emulsions instability is investigated by placing the samples in test tubes and then storing them under chosen conditions. The destabilization process is commonly reflected by the separation of the water phase at the bottom of the tube or by a complete breakdown into two or more phases [172].

7.2 Microscopic analysis

Microscopy techniques (optical microscopy, scanning and transmission electron microscopy) are able to provide information about the structure, dimensions and organization of the emulsion droplets. Each microscopic technique works on different physicochemical principles and can be used to examine different levels and types of structural organization. Nevertheless, any type of microscopic method must have three qualities: resolution, magnification and contrast [15].

7.2.1 Conventional optical microscopy

An optical microscope is still one of the most valuable tools for observing the microstructure of emulsions. It consists of a series of lenses which direct the light through the sample and magnify the resulting image. The resolution of the microscope is determined by the wavelength of light used and the basic structure of the device. Generally, the theoretical limit for resolution of an optical microscope is about 0.2 μm, but in fact the limit is below about 1 μm due to the difficulties arising from the design of the device and also mainly due to Brownian motion of small particles in the sample causing blurred images. Therefore, the use of this type of microscope is limited, especially in systems containing particles with sizes below the measuring limit of the instrument. However, the advantage of this technique over light scattering or ultrasonic methods is that it provides information not only about the particle

size but it can also be applied to detection of the flocculation or coalescence and the distinction between them [15].

7.2.2 Electrone microscopy

Electron microscopes use electron beams to obtain data on the structure of the samples. These beams are directed through the microscope using a series of magnetic fields. Electron microscopes can be used to measure much smaller objects (about 1 nm) because the electron beams have much smaller wavelengths than the light used in conventional microscopes. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are the most common types of electron microscopes used in practice. In both of these techniques, the microscope must be maintained under a high vacuum, otherwise the electrons would be scattered by atoms and molecules in a gas, causing deterioration in the image quality [15].

Scanning electron microscopy (SEM) is used to provide images of the surface topography of the samples in the micro- to nanometer range with relatively lower diffraction patterns. It is composed of an electron gun that generates the electron beams and electromagnetic optics which guide the beam and focus it. Moreover, there are the detectors collecting the electrons (direct scattering or emitted from the sample) that come from the sample, the energy of the detected electrons together with their intensity and location of the emission [173].

SEM is based on the measurement of secondary electrons generated by the sample, which is bombarded by an electron beam. A focused electron beam is directed at a certain point on the surface of the sample. Some of the energy associated with the electron beam is adsorbed by the sample, which leads to the formation of secondary electrons. These electrons, after leaving the surface of the sample, are recorded by the detector. An image of the sample is obtained by scanning the electron beam in an x-y direction over its surface and recording all electrons generated at each position. Additionally, the intensity of electrons at each location depends also on the contact angle between the electron beam and the surface. Therefore, the electron micrograph appears in a three dimensional arrangement. In comparison with TEM, the SEM sample preparation is easier and tends to produce fewer artefacts. This is because an image is generated by secondary electrons produced on the surface of the sample, rather than by an electron beam that travels through a sample, and thus it is not necessary to prepare ultrathin samples. On the other hand, the resolution power of SEM is of about 3–4 nm, which is an order of magnitude worse than when using TEM. However, compared to an optical microscope, SEM has about three orders of magnitude better resolution power and a larger depth of field, which means that the images of relatively large structures are all in focus [15].

7.3 Photon correlation spectroscopy (PCS)

PCS is a non-invasive technique used for characterizing macromolecules in solutions and particles in suspensions. It is based on the measurements of the time-dependent fluctuations in the intensity of scattered light that occur because the particles undergo Brownian motion [174]. Brownian motion is the random movement of particles due to attacks by the solvent molecules that surround them. The velocity of Brownian motion depends on the particles sizes (the larger the particle, the slower the Brownian motion), solvent viscosity (the higher the viscosity, the slower the motion) and temperature (the higher the temperature, the more motion). The Brownian motion velocity is defined by a variable known as the translational diffusion coefficient (*D*). The size of a particle is then calculated from *D* by using the Stockes-Einstein equation, as follows:

$$d(H) = \frac{kT}{3\pi\eta D}, \qquad (5)$$

where *d(H)* is the hydrodynamic diameter, *D* is the translation diffusion coefficient, *k* is Boltzmann's constant, *T* is absolute temperature and η is viscosity [175].

Like other particle sizing techniques, PCS has its advantages and disadvantages. The indisputable benefits of this technique are the fast and easy measurements lasting from a few seconds to a few minutes. The method is absolute, and thus the calibration with particles of a known size distribution is not necessary. Additionally, very small quantities of the sample are sufficient for measurements, and the instrumentation is commercially available with automation including data analysis [176][177]. The major advantage of this technique is its applicability to systems with sizes from a few nanometers to several microns (1–2 μm) [177]. On the other hand, among its main disadvantages are cleaning and filling the cells, which is the critical point of the PCS application. If the solution to be measured contains dust or micro-bubbles, they will disrupt or overlap the signal of analyte (sample) and the measurement can be affected by artefacts [178]. Moreover, this method requires highly transparent and in general highly diluted samples [172].

PCS is a suitable method for the characterization of the particle size of latexes, pigments, emulsions, micelles, and liposomes. Moreover, another application for this method is in biology and microbiology, in determining the sizes of bacteria, viruses and DNA [177].

7.4 Rheology

The application of a force on a material causes its deformation or flow. Rheology is connected with the relationship between applied forces and the deformation and flow of matter. Most rheological tests involve the application of force to a material and a measurement of the resulting flow or change in its shape [15].

Generally, the rheological properties of emulsions are influenced by a number of factors, such as the nature of the continuous and dispersed phases, the phase volume ratio, and to a lesser extent particle size distributions. The overall consistency of emulsions of low dispersed phase volume is commonly similar to that of the continuous phase. It follows, that W/O emulsions are usually thicker than O/W emulsions [16].

7.4.1 Viscosity measurements

The viscosity of an ideal liquid (also referred to as a Newtonian liquid) is a measure of its resistance to flow. The higher the viscosity, the greater the resistance. When a shear stress is applied to an ideal liquid, it continues to flow as long as the stress is applied. When the stress is removed, there is no elastic recovery of the material, i.e., it does not return to its original shape.

Many instruments have been designed to measure the shear properties of liquids, viscoelastic materials, plastics and solids. The instruments are usually controlled by a computer and asked to perform sophisticated test procedures as a function of time, temperature, shear rate, etc. Generally, the sample to be analysed is placed in a thermostated measurement cell where it is subjected to a controlled shear stress (strain). The resulting shear (stress) is detected by the rheometer, and it follows that the rheological properties of the tested sample can be determined from the strain-stress relationship. There are two different types of rheological instruments: *constant stress instruments*, which apply a constant torque to the sample and measure the resulting strain or rate of strain; and *constant strain instruments*, which apply a constant strain or rate of strain and measure the torque generated in the sample. Constant stress instruments include for example, a parallel plate, cone-plate and concentric cylinder. The latter rheometer consists of two concentric cylinders, and the sample is placed in the gap between them. The inner cylinder is driven at a constant torque (angular force) and the resulting strain (angular deflection) or rate of the strain (speed at which cylinder rotates) is determined, depending on the physical state of the analysed sample (liquid or solid). For emulsions (liquids), the speed at which the inner cylinder rotates is governed by the viscosity of the fluid between the walls. The faster it spins at the given torque, the lower the viscosity of the liquid. Moreover, the torque can be varied in a controlled way and so the elastic modulus or viscosity can be measured as a function of shear stress [15].

7.5 Thermal analysis

Differential scanning calorimetry (DSC) and differential thermal analysis (DTA) are the most common thermal analysis techniques used to record the melting and crystallization of substances. They measure the heat released or adsorbed by a sample when exposed to a controlled temperature programme. The samples release heat when they crystallize and absorb heat when melting. The main difference between these two techniques is the system used to measure the heat released or adsorbed by the sample [15].

7.5.1 Differential scanning calorimetry (DSC)

DSC is a technique in which the difference in heat flow rate (or power) to the sample and to the reference sample is monitored over time while the samples are exposed to a temperature programme [179]. Thermocouples continuously measure the temperature of each of the two pans, and heaters below the pans deliver heat to one of the pans. When the sample undergoes a transition, it either absorbs or releases heat. To maintain the same temperature of the both pans, the same amount of heat (power) must be delivered to either the test or reference cells. The amount of the heat supplied is usually measured by special electrical circuitry. Therefore, data from DSC are reported as the rate of heat absorption (Q) by the tested sample relative to the reference sample as a function of temperature [15].

Thermal events in the sample appear as deviations from the baseline, i.e., as endothermic or exothermic responses (Fig. 7). Their sizes depend on whether more or less heat was supplied to the tested sample in comparison with reference. Generally, endothermic responses are usually situated above the baseline (positive), which corresponds to an increased heat transfer to the sample compared to the reference and leads to the melting of the sample. Conversely, exothermic responses are located under the baseline and describe the crystallization [180]. The major advantage of DSC is that samples are easily prepared, and it follows that measurements can be conducted quickly and simply [181].

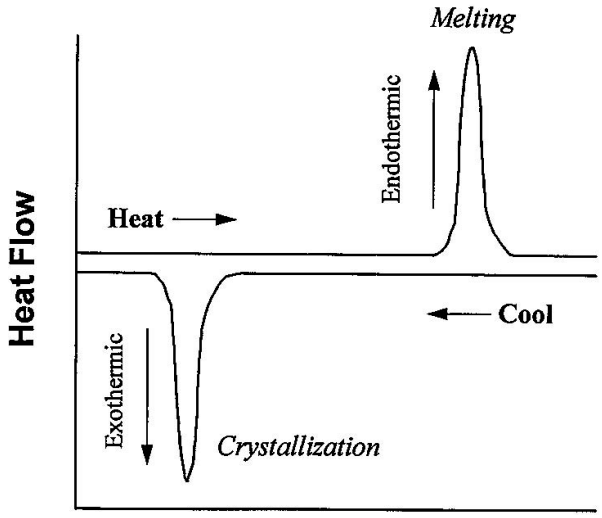

Figure 7 DSC analysis showing basic enthalpy changes during the heating and cooling of the sample [15].

7.6 High performance liquid chromatography (HPLC)

HPLC is an analytical technique used to separate chemical compounds present in mixtures. It is based on the separation of molecules due to differences in their structure or composition [182].

Components in the mixture are separated on a column that contains packing material (referred to as stationary phase) by pumping a solvent (referred to as mobile phase) through the column [183]. The components (referred to as analytes) in the sample have different affinities and interactions with the mobile and stationary phases, and it follows that each analyte moves along the column at different speeds and emerges from the column at various times, thus separating the mixture components. Analytes with a higher affinity for the mobile phase move faster through the column whereas those with the higher affinity for the stationary phase move slower [182]. The time at which the analyte elutes (comes out of the end of the column) is called the retention time and can be used to identify the components. Commonly used solvents include combinations of water or organic liquids (methanol, acetonitrile) [183].

The successful implementation of HPLC measurements requires a combination of a variety of operating conditions, e.g., column type and parameters (length, diameter, temperature), mobile phase used (type, flow rate) and sample size [184].

As shown in the schematic diagram in Fig. 8, the HPLC instrument consists of a mobile phase reservoir, a degasser for the mobile phase, a high-pressure pump, injection valve, waste reservoir, column, detector and data acquisition and display system [184].

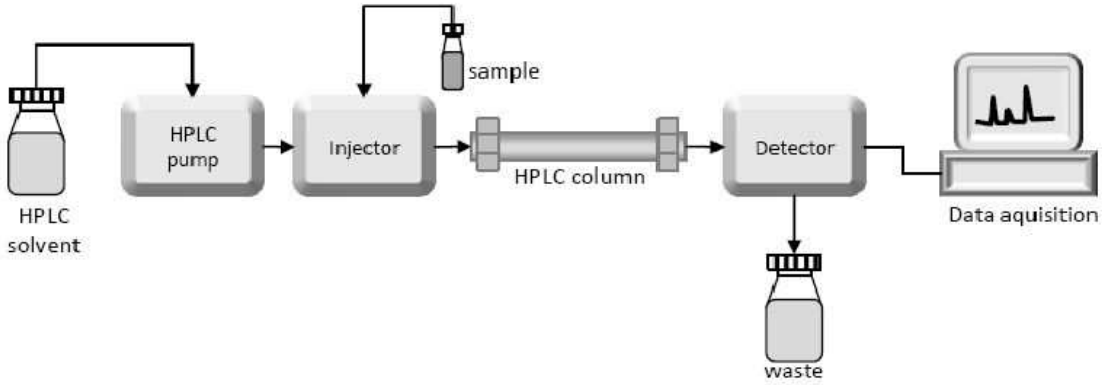

Figure 8 Schematic diagram of a high performance liquid chromatography (HPLC) system [185].

7.7 X-ray diffractometry (XRD)

X-ray diffraction is a universal analytical technique for the examination of crystalline materials such as ceramics, metals, electronic and geological materials, organics and polymers [184].

In the diffractometer, an X-ray beam of a single wavelength is used to examine crystalline samples. By continuously changing the incident angle of the X-ray beam, a spectrum of diffraction intensity versus the angle between incident and diffracted beam is recorded. This technique enables the identification of the crystal structure and an assessment of its quality by analysing and then comparing the spectrum with a database containing more than 60,000 diffraction spectra of known crystalline substances [186].

The diffractometer is based on the detection of X-ray diffractions from materials and recording the diffraction intensity as a function of the diffraction angle (2θ). Figure 9 describes the geometrical arrangement of X-ray source, sample and detector. The X-ray radiation generated by an X-ray tube passes through special slits which collimate the X-ray beam. These slits are made of a set of closely spaced thin metal plates to prevent beam divergence in the direction perpendicular to the figure plane. A divergent X-ray beam passing through the slits strikes the sample. The X-rays are diffracted by the sample and form a convergent beam at receiving slits before they enter a detector. The diffracted X-ray beam needs to pass through a monochromatic filter (monochromator) before being received by a detector [186].

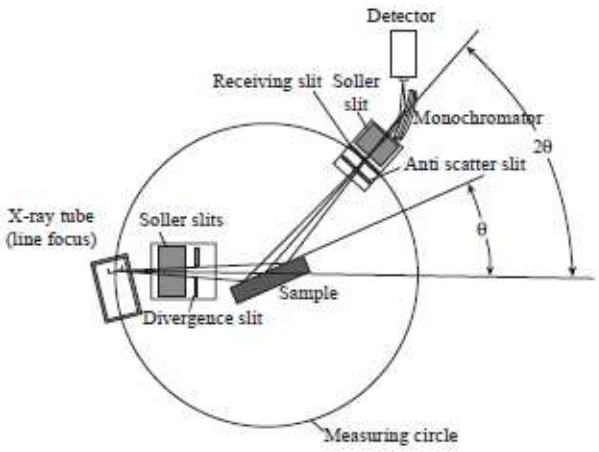

Figure 9 Geometric arrangement of an X-ray diffractometer [186].

The major advantage of this non-destructive technique is that only a small amount of the sample is needed for the measurement and easy and fast phase identification due to the existence of the comprehensive databases. The main disadvantage of the method is that it gives only very limited information about amorphous or low-crystalline materials [187].

AIMS OF THE DOCTORAL STUDY

The main goals of the present work have been focused on deeper understanding of the suitable carrier systems for cosmetics and have been subdivided into the following points that may contribute both to theoretical research and to practice:

- Preparation of classical (nano)emulsions and (nano)emulsions with the addition of different types of hydrocolloids by Emulsion inversion point technique (EIP) and their mutual comparison in terms of stability, particle size, etc.

- Monitoring of the effects of different O/W ratios, HLB values, surfactant types and concentrations on the particle size, distribution and viscosity of the tested emulsions by Photon correlation spectroscopy (PCS) and viscometry.

- Evaluation of the stability of (nano)emulsions stored at various conditions (4 °C, 25 °C, 35 °C) using visual observation, optical microscopy, viscometry and PCS.

- Formation of stable microemulsion systems based on 1-monoacylglycerols (1-MAG) as suitable carriers of antimicrobial agents.

- Testing of microemulsion cytotoxicity and antimicrobial activity against the most common gram-positive and gram-negative microorganisms. Monitoring their stability as well as the time development of their particle sizes by PCS.

- Exploration of a new modification of Supercritical Assisted Atomization process, the micronization of 1-MAG/PVA microparticles from O/W emulsion systems.

- Investigation of the effect of different 1-MAG/PVA ratios and the emulsion preparation method (homogenization, sonication) on the particle size, stability, and morphology by optical and electron microscope, PCS, DSC, X-ray and HPLC.

LIST OF PAPERS

The following papers have resulted from the doctoral research and are available in full-texts in this doctoral thesis:

Publication I
SEVCIKOVA P., KASPARKOVA V., VLTAVSKA P., KREJCI J., On the preparation and characterization of nanoemulsions produced by phase inversion emulsification. *Colloids Surf. A: Physicochem. Eng. Aspects.* 2012, vol. 410, p. 130–135.

Publication II
SEVCIKOVA P., KASPARKOVA V., KREJCI J., VLTAVSKA P., Dynamický rozptyl světla v analýze koloidních systémů. *Chem. Listy*. 2014, vol. 108, p. 479–482.

Publication III
SEVCIKOVA P., KASPARKOVA V., HAUERLANDOVA I., HUMPOLICEK P., KUCEKOVA Z., BUNKOVA L., Formulation, antibacterial activity, and cytotoxicity of 1-monoacylglycerol microemulsions. *Eur. J. Lipid Sci. Tech*. 2014, vol. 116, p. 448–457.

Publication IV
SEVCIKOVA P., ADAMI R., KASPARKOVA V., REVERCHON E., SEDLACEK T., PASTOREK M., Supercritical Assisted Atomization of 1-MAG/PVA microparticles . *J. Supercrit. Fluids* (in review process).

SUMMARY OF THE PAPERS

The presented doctoral thesis is focused on the preparation and characterization of different types of cosmetic vehicles, i.e., nanoemulsions, microemulsions and microparticles as promising transport systems in a variety of practical areas. The key findings are highlighted as summaries of each of the four scientific papers presented below.

Paper I was focused on the preparation and characterization of O/W nanoemulsions produced by a low-energy emulsification method (Emulsion inversion point technique – EIP) in the presence of two types of non-ionic surfactants (Igepal, Brij). The work was aimed at finding a suitable composition of emulsions and preparation conditions in terms of HLB value, O/W ratio, surfactant type and its concentration as well as an assessment of the impact of individual variables on particle size, viscosity and stability of these systems.

According to obtained data from the PCS and viscosity measurements, all variables mentioned above considerably influenced both the particle size and viscosity.

The overall results state that emulsion droplets produced with the mixture of the Igepals were mostly bigger in all studied O/W ratios and almost at all HLB values compared to droplets obtained with Brijs. The only exception were emulsions prepared at the HLB value 10.5, which exhibited similar sizes for both types of surfactants. In addition, these systems possessed the smallest particle sizes, being in the range of 50–200 nm for all studied O/W ratios. Further, almost all emulsions with O/W ratio 5/95 were semitransparent in appearance, indicating the formation of small particles. With an increasing oil content (10/90; 15/85; 20/80; 25/75 and 30/70) the particle size of the emulsions increased, which can be attributed to a rise in the volume fraction of oil in the emulsions.

Moreover, composition and preparation conditions also affected the stability of the studied systems. The emulsions prepared at HLB value 10.5 were chosen for the examination of the stability because at this HLB the smallest particles were obtained and hence the best stability was expected. The best long-term stability in terms of visual observation was found in emulsions with O/W ratios 5/95 and 10/90 prepared with Brijs, which were stable for more than 2 months. However, the samples produced using Igepals also showed sufficiently good stability and remained unchanged at least for one month.

Generally, the Igepal surfactants were more suitable for the preparation of nanoemulsions within a broader range of HLB values (9.5–11.5) in comparison with Brij surfactants. On the contrary, the Brij surfactants provided more time-stable nanoemulsions.

Paper II briefly described the principle of the Photon correlation spectroscopy (PCS) method and pointed out the main advantages and the possibilites of its usage in measuring the particle sizes and distributions of dispersion systems.

The applicability of the method for an analysis of surface active agents was tested by measuring the micelle sizes of ionic (SDSa) and non-ionic surfactants (Tweens, Igepal) as well as their mixtures. The results showed that this technique is capable of reliably distinguishing the micelle sizes of tested non-ionic surfactants (Tween 20, Tween 60) and also determining the sizes of mixed micelles formed by mixing both surfactants in the ratio of 50/50.

PCS also performed well in the characterisation of microemulsions and nanoemulsions. An analysis of 1-MAG microemulsions confirmed the presence of particles in the range of 11–24 nm, which corresponds to the literature findings for the range expected from these systems (10–50 nm). Moreover, almost all the 1-MAG microemulsions showed the presence of narrow monomodal distribution indicating the presence of a one particle population.

Results from the PCS characterization of the nanoemulsions revealed that their sizes were significantly affected by the composition, namely by HLB value as well as O/W ratio. It was found that the optimal HLB value in terms of the size (<200 nm) and stability of emulsion droplets was 10.5, irrespective of the O/W ratio. Additionally, differences in particle size were also observed when various amounts of surfactants (3 and 5 wt.%) were used. According to theoretical assumption, the particle size should decrease with an increasing surfactant concentration, which was confirmed by the finding presented in this paper.

From the experiment it was clearly visible, that the PCS technique is suitable for the evaluation of the stability of dispersion systems through the monitoring of the time changes in particle size and distributions. The unconsidered information potential of the method lies also in its possibility to determine the presence of one or more particle populations by an assessment of the distribution curves.

Based on the results mentioned above it can be concluded that the PCS technique has broad potential for the characterization of particulate systems in a variety of application areas, such as medicine, pharmaceuticals, comestics, food technology and the polymer industry.

Paper III was concentrated on the formulation of long-term stable 1-MAG microemulsions, their characterization by PCS as well as the monitoring of their cytotoxicity and antibacterial activity against the most common pathogenic microorganisms. Another goal of the paper was to compare the inhibition effect of microemulsions with that of 1-MAGs alone (not encapsulated in microemulsion) and suggest the mechanism of action of 1-MAGs and microemulsions.

Pseudo-ternary phase diagrams were constructed in order to determine the area of true microemulsions, which is characterized by the unlimited miscibility of individual components and the area of their limited miscibility, i.e., the classical emulsion region. The influence of different types of co-surfactants (ethanol, propanol, butanol and pentanol) on the phase behavior of 1-MAG microemulsions (ME) was also studied. The results confirmed that the size of both areas is strongly dependent on the type of alcohol used. It was determined that the region of limited miscibility in pseudo-ternary phase diagrams rises with an increasing chain length of alcohol. The 1-MAG microemulsions used in further tests were composed of ethanol, 1-MAG C10:0 (C11:0, C12:0 and C14:0) and surfactant Tween 80. They were transparent in appearance, stable more then 6 months and their sizes were between 12–20 nm.

The antibacterial effects of all 1-MAG MEs and 1-MAGs alone on the growth of gram-positive and gram-negative bacteria were investigated using the dilution method by measuring the optical density of cell suspensions growing in the presence of 1-MAG MEs. The tests revealed that 1-MAG MEs at the higher concentrations (>1000 mg/L) possessed better inhibition effects against the growth of gram-negative bacteria compared to the corresponding concentrations of 1-MAGs alone. At the lower concentrations, the antibacterial activity of both systems was comparable. On the other hand, the inhibition ability of 1-MAG MEs in the case of gram-positive bacteria was lower than the antibacterial activity of 1-MAGs alone. Generally, the best reduction of the bacteria growth was observed after the application of 1-MAG MEs of lauric acid.

Additionally, the antibacterial activity of MEs without 1-MAG was also observed, resulting from the combined effect of surfactant Tween 80

(membrane disrupter) and ethanol (an antimicrobial agent). The presence of 1-MAG in MEs then can act in synergy with these two components and further enhance their antibacterial effect.

The cytotoxicity measurement revealed the significant cytotoxicity of all MEs, and the threshold of 10 mg/L was detected as the limit for moderate toxicity, which corresponds to 40–60% cell survival. However, it is worth noting that, similar to bacteria cells, the cytotoxic effect of ME is not resting only on 1-MAGs but is also influenced by other components (Tween 80, ethanol), which can be also responsible for a high cytotoxicity of MEs.

Paper IV was aimed at the optimization of the composition and properties of emulsion systems (O/W) for the production of 1-MAG/PVA microparticles using supercritical assisted atomization (SAA). The prepared polymer microparticles containing 1-MAG as an antimicrobial agent were characterized by electron microscope, photon correlation spectroscopy (PCS), different scanning calorimetry (DSC), X-ray diffraction (XRD), and high pressure liquid chromatography (HPLC) in order to determine the effect of the emulsion composition and preparation method on the final particle sizes, structure or encapsulation efficacy.

Generally, the 1-MAG/PVA microparticles obtained by SAA were spherical in appearance with a diameter in the range of about 0.5–3 µm. The size of these microparticles was affected by the composition of emulsions, namely by the 1-MAG/PVA ratio as well as by the emulsification method. In the first case, there was the same trend, i.e. an increase in particle sizes and a shift of distributions towards larger particle diameters for both components used (PVA and 1-MAG), with their growing concentrations regardless of the emulsion preparation method applied. Moreover, the 1-MAG concentration in emulsion also influenced the recovery of the obtained powder. The emulsions containing 20 or 30 mg/ml of 1-MAG provided the highest recovery of the obtained microparticles with yields ranging between 50–60%. The emulsification method was another important parameter affecting the particle size and PDI, and it was found that the microparticles micronized by SAA from sonicated emulsion were smaller in their sizes and showed narrower distributions than those prepared using a high-speed stirrer.

It is worth noting, that the tested 1-MAG/PVA microparticles possessed a semi-crystalline structure similar to the structure of not micronized, raw PVA. Nevertheless, the crystallinity of microparticles was higher in comparison with raw PVA. Additionally, the DSC measurements also confirmed the occurrence of this type of structure in studied systems.

CONTRIBUTION TO SCIENCE AND PRACTICE

The main contributions of this doctoral thesis to science and practice can be summed in the following points:

- The mastering of the preparation of classical (nano)emulsions and (nano)emulsions with the addition of different types of hydrocolloids by Emulsion Inversion Point technique (EIP), optimizing preparation conditions and finding a suitable composition that provides long-term stable delivery systems.

- The understanding of the phase behaviour of microemulsion systems *via* studying the pseudo-ternary phase diagrams, a determination of the effect of all used components on the size of miscibility area (i.e., microemulsion region) and the establishment of model formulations.

- The successful optimization of an O/W emulsion system for the production of microparticles suitable for the encapsulation of active substances, in this case with antibacterial properties.

- The development of new application possibilities for the Supercritical assisted atomization method for the production of microparticles, consisting of a hydrophobic active agent and a hydrophilic polymeric carrier from the emulsion systems (O/W). The microparticles definitely belong to carrier systems, which may contribute to the increased bioavailability and stability of pharmaceutical compounds.

- The finding, formulation and characterization of promising transport systems capable of encapsulating active substances (microemulsions, nanoemulsions and microparticles), which can be used in the cosmetics, pharmaceutical and food industries.

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PUBLICATION I

On the preparation and characterization of nanoemulsions produced by phase inversion emulsification

Sevcikova Petra, Kasparkova Vera, Vltavska Pavlina, Krejci Jiri
Colloids and Surfaces A:Physicochemical Engineering Aspects, 2012, vol. 410, p. 130–135.

Colloids and Surfaces A: Physicochem. Eng. Aspects 410 (2012) 130–135

Contents lists available at SciVerse ScienceDirect

Colloids and Surfaces A: Physicochemical and Engineering Aspects

journal homepage: www.elsevier.com/locate/colsurfa

On the preparation and characterization of nanoemulsions produced by phase inversion emulsification

Petra Ševčíková[a,*], Věra Kašpárková[a,b], Pavlína Vltavská[a], Jiří Krejčí[a]

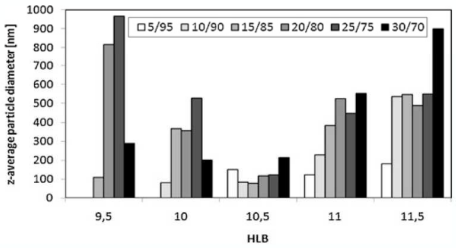
[a] Department of Fat, Surfactant and Cosmetics Technology, Faculty of Technology, Tomas Bata University in Zlin, nam. T. G. Masaryka 275, 762 72 Zlin, Czech Republic
[b] Centre of Polymer Systems, University Institute, Tomas Bata University in Zlin, Nad Ovcirnou 3685, 760 01 Zlin, Czech Republic

HIGHLIGHTS

- n-Undecane/water nanoemulsions were prepared using phase inversion.
- Nonionic surfactants (Igepal, Brij) were used at the O/W range of 5/95 to 30/70.
- The smallest emulsion droplets arose near the transition point, at HLB of 10.5.
- Igepals provided bigger droplets than Brijs in all studied O/W ratios and HLBs.
- Nanoemulsions based on Brijs were more time stable, for 121 days at room temperature.

GRAPHICAL ABSTRACT

Particle sizes of O/W (nano)emulsions determined immediately after the preparation. Influence of the HLB value (3% Igepal surfactant). Absent values of *z*-average particle diameter at HLB 10 (O/W 5/95) and 9.5 (O/W 5/95, 10/90) indicate impossibility to form the O/W emulsions under these conditions.



ARTICLE INFO

Article history:
Received 30 April 2012
Received in revised form 25 June 2012
Accepted 28 June 2012
Available online 5 July 2012

Keywords:
Emulsion
Hydrophile–lipophile balance
Particle size
Surfactant
Viscosity

ABSTRACT

Formation, appearance, particle size and viscosity of n-undecane in water (nano)emulsions prepared by the phase inversion method using non-ionic surfactants (Igepal, Brij) were investigated. The effects of the HLB value, oil-in-water ratio (5/95 to 30/70), surfactant type and its concentration (3 and 5%) on the physico-chemical characteristics of emulsions were studied. Emulsions were characterized by visual and microscopic observations and the particle size and distribution as well as emulsion viscosity were determined. The results showed that all the variables mentioned above considerably influenced both the particle size and the viscosity. The smallest particle sizes (lower than 250 nm) and the best emulsion stability were determined at optimum HLB of 10.5 for all the studied oil-in-water ratios, both types and concentrations of surfactants. Typically, the emulsion droplets prepared with Igepals were bigger than those prepared with Brij systems.

1. Introduction

In recent years, nanoemulsions have been extensively studied due to their potential use in various industrial applications. They can be, for example used in polymer synthesis, foodindustry, pharmacology or agriculture. The high kinetic stability, low viscosity and optical transparency make them also attractive for practical applications in pharmaceutical industry and cosmetics [1]. Nanoemulsions belong to a class of emulsions with a particle size ranging from 20 to 500 nm [2–4], or 50–200 nm, as given in publication [5], and are considered as intermediates between classical emulsions and microemulsions [6]. Due to their small particle size, nanoemulsions may appear transparent and are considered stable against sedimentation or creaming [7,8]. They can also be stable

* Corresponding author. Tel.: +420 576 031 535; fax: +420 577 210 172.
E-mail address: psevcikova@ft.utb.cz (P. Ševčíková).

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http://dx.doi.org/10.1016/j.colsurfa.2012.06.031

against flocculation and coalescence and break down through Ostwald ripening. One important advantage of nanoemulsions over microemulsions is a lower surfactant concentration required for their formation [1].

The preparation of nanoemulsion with a sufficiently low particle size can be achieved either by high-energy or by low-energy emulsification methods [9]. High-energy emulsification methods used in the formation of nanoemulsions require high input of mechanical energy. For this purpose, the three main types of devices can be used; ultrasound generators, rotor/stator type and high-pressure homogenizers. On the contrary, low-energy emulsification methods require only a low quantity of the applied energy and are based on the phase transitions taking place during the emulsification process and make use of changing the spontaneous curvature of the surfactant [3]. The phase inversion is a process by which water-in-oil (W/O) emulsion can be inverted to oil-in-water (O/W) emulsion or vice versa [10,11] and at least two types of phase inversions – the catastrophic phase inversion (CPI) and the transitional phase inversion (TPI) have been described [5,10,12]. The best known low-energy emulsification method is called phase inversion temperature (PIT) [3]. This concept, introduced by Shinoda and Saito [13,14], uses the specific ability of surfactants, usually nonionic, such as polyethoxylated surfactants, to modify their affinities to water and oil as a function of the temperature and thus undergo a phase inversion [3]. Transitional phase inversion can be also induced by changing the HLB number of the surfactant at the constant room temperature using the surfactant mixtures of high and low HLB [5]. It is well-known that for a wide range of applications, a certain mixture of surfactants can provide better performance than the pure surfactants [2,15]. Although a considerable number of research studies have been devoted to the formation of nanoemulsions of cyclohexane [16,17], xylene [18] and polyisobutylene [19] by the TPI process using a mixture of high- and low-HLB surfactants, less attention has been paid to their preparation by using n-undecane as the oil phase. Due to its favorable properties, namely well-defined structure and relatively low volatility, this oil phase can be suitable as a model system for the formation of stable nanoemulsions.

The main objective of this study is hence focused on the formation, appearance, particle size and viscosity of n-undecane in water nanoemulsions prepared by the transitional phase inversion. For this purpose, two different surfactant groups, Igepal and Brij, were used. Required HLB values were obtained via mixing Igepal 720, Igepal 520 and Igepal 210 or Brij 92, Brij 30 and Brij 98. In addition, the influence of emulsion composition in terms of different oil to water ratio, the surfactant concentration and the HLB value, on particle size and viscosity of prepared nanoemulsions were investigated using Photon correlation spectroscopy and rheological measurements.

2. Experimental

2.1. Materials

The n-Undecane (Sigma–Aldrich) and purified water were used as oil and water phases, respectively. Three grades of nonionic Igepal (polyoxyethylene nonylphenylether, NPE) and three grades of nonionic Brij (polyoxyethylene lauryl ether, polyoxyethylene oleyl ether) surfactants, supplied by Sigma–Aldrich were used as delivered, without additional purification. Specification of surfactants is as follows: Igepal CA 210 (HLB 4), Igepal CA 520 (HLB 10) and Igepal CA 720 (HLB 14.2) with nonyl phenol ethoxylate chain length of 2 (NPE2), 5 (NPE5), 12 (NPE12); Brij 92 (HLB 4,9), Brij 30 (HLB 9,7) and Brij 98 (HLB 15,3) with an average of 2 (C18E2), 4 (C12E4) and 20 (C18E20) mol of ethylene oxide (EO) per surfactant molecule.

2.2. Preparation of nanoemulsions

Nanoemulsions were prepared by the phase inversion emulsification. Emulsifications were carried out using simple laboratory equipment consisting of an RZR 2020 stirrer (Heidolph), a glass vessel and a buret. The target surfactant mixtures of required HLB values were blended from the suitable pairs of nonionic surfactants (Table 1). In the first sample series, Igepals with different HLBs were used. The second series employed surfactant mixtures blended from pairs of suitable Brijs. Resulting HLBs of surfactant mixtures were calculated with the HLB values given by surfactant supplier using equation $HLB = x_1 \times HLB_1 + x_2 \times HLB_2$, where x_1 and x_2 are volume fractions of surfactants with HLB_1 and HLB_2. Depending on the required HLB, low- and high-HLB surfactants were pre-dissolved in the calculated ratios in the oil and water phase, respectively. The surfactant containing water phase was added drop-wise at the rate of 1 ml/min to the surfactant containing oil phase under continuous stirring. During emulsifications, the stirring rate was controlled at 1050 rpm and all experiments were run at the room temperature (25 °C). By the dissolving of surfactants in either of phases, their diffusion through the interface during emulsification is minimized, allowing thus a rapid establishment of equilibrium. Oil to water (O/W) ratios of 5/95, 10/90, 15/85, 20/80, 25/75 and 30/70 were used. Two different concentrations of surfactants of 3 and 5 wt% and HLB values of 9.5, 10, 10.5, 11 and 11.5 were applied. The choice of HLB range was motivated by the effort to thoroughly investigate the behavior and especially size of emulsion droplets in the vicinity of an optimum, which is, for oil phase based on hydrocarbons, located between HLB 10 and 11 [20]. Surfactant amounts of 3 and 5% were used on the basis of literature study, where a wide range of concentrations, typically from 1.5 to 10 wt%, is used. However, application of emulsions produced with the higher concentrations (8–10 wt%) can be limited for the reason of the potential harmful effects and the 5% content is the most frequently reported as appropriate and sufficient for the emulsions production. Therefore, 5 and 3 wt% were chosen to compare their ability to form a stable nanoemulsion system.

2.3. Emulsion appearance and stability

In addition to appearance of emulsions assessed immediately after preparation, their stability was also evaluated by visual observation. Emulsions stored at room temperature (25 °C) were observed daily at regular time intervals and the stability was assessed by measuring the height of the phase separated by creaming in centimeters as a function of time. Stability was also assessed from the time changes of particle sizes measured by photon correlation spectroscopy. Samples prepared at HLB of 10.5 with all studied O/W ratios, in the presence of Brijs or Igepals, both with 3 and 5% content, were followed.

2.4. Measurement of particle size distribution

Particle size and particle size distribution of nanoemulsions were determined by photon correlation spectroscopy (PCS). Zetasizer Nano ZS instrument (Malvern Instruments, UK) was used for this purpose. Measurements of hydrodynamic radius of emulsion droplets, expressed as intensity-weighed z-average particle diameter, was performed at the constant temperature of 25 °C. The intensity of scattered light was observed at 173°.

2.5. Measurement of viscosity of nanoemulsions

Viscosity measurements were performed using a concentric cylinder type of geometry with the viscometer Brookfield VD-III ULTRA and spindle SC4-18 at 25 °C over the range of 1–100 rpm. The

Table 1
Composition of mixed surfactants providing required HLBs used for preparation of nanoemulsions.

HLB of surfactant mixture	Igepal systems			Brij systems		
	Igepal 720 (HLB 14.2)	Igepal 520 (HLB 10)	Igepal 210 (HLB 4.0)	Brij 98 (HLB 15.3)	Brij 30 (HLB 9.7)	Brij 92 (HLB 4.9)
9.5	x	–	x	x	–	x
10.0	x	–	x	x	–	x
10.5	x	x	–	x	x	–
11.0	x	x	–	x	x	–
11.5	x	x	–	x	x	–

HLB values of surfactants as given by supplier.

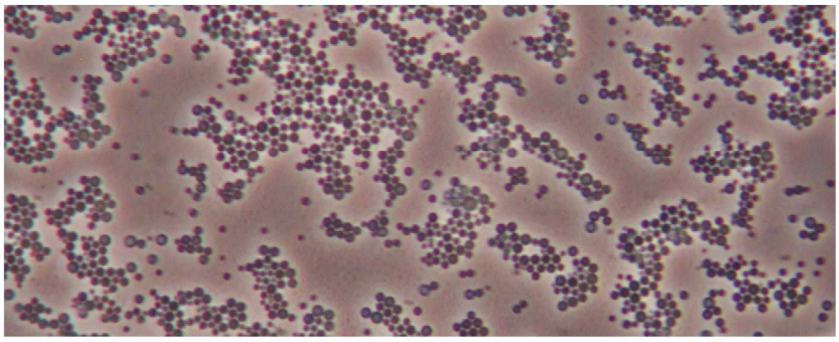

Fig. 1. Photomicrograph of O/W ration of 25/75 emulsion prepared with 5% of Igepal surfactants and HLB value of 11.

Rheocalc V3.3 Build 49-1 software was used for the calculations. The analyses were carried out 1 h after sample preparation.

3. Results and discussion

3.1. Appearance of emulsions

Visual assessment of emulsions with respect to appearance and potential phase separation was performed immediately after their preparation. Already this simple test indicated that the HLB value and the oil phase content influence their properties. Using Igepals, emulsions with O/W of 5/95, were mostly semi-transparent indicating presence of small oil droplets, irrespective of HLB value. Appearance of emulsions with higher oil content (O/W of 10/90 to 30/70) was more varying, reflecting differences in emulsion droplet sizes. The prepared samples were mostly homogeneous, with sparsely observed creaming characterized by typical accumulation of the oil droplets on the top of the vial. This was detected for example for O/W of 20/80, 25/75 and 30/70 at HLB 11.5 shortly after emulsifications. At HLBs equal and below 10, which are less-suitable for the formation of O/W emulsions, this emulsion type developed, however, at the low oil content (5/95 and 10/90) and 3% surfactant, the inverted water in oil (W/O) emulsions arose.

In contrast to emulsions with Igepals, the Brij-based emulsions with higher O/W content of 30/70 and HLB 11 and 11.5 were more unstable and broke down soon after preparation to oil and water phases. Using the Brijs at HLB 10, O/W emulsions were obtained only for the 5% surfactant content and O/W of 20/80, 25/75 and 30/70. Nevertheless, they were highly unstable with phase separation occurring only a few minutes after preparation. At the lower oil content (5/95, 10/90 and 15/85), inverted W/O emulsions were again obtained. Preparation of O/W emulsions at HLBs below 10 was not possible at all.

Simultaneously with the visual description, a microscopic observation of emulsions with droplet size accessible via optical microscopy was carried out, which corroborated the visual assessment of the destabilization of particular emulsions. For rapidly separating emulsions, creaming, changes in particle sizes or flocculation were observed, as a mechanism of emulsion break-out. The last mentioned process is illustrated in Fig. 1.

3.2. Particle size

As the particle size of the emulsion droplets is the crucial parameter in defining their application and stability, all the prepared emulsions were carefully characterized in terms of their particle size measured as a *z*-average particle diameter using PCS. It was found out that particle size was notably affected by O/W ratio, surfactant pair (Brij vs Igepal) and HLB.

This is illustrated in Fig. 2 showing example of the relationship between O/W ratio and the particle size for the Igepal and Brij based emulsions determined immediately after their preparation at the HLB value of 11 and the surfactant concentration of 3%. As it can be seen, the particle size of emulsions was, in general, growing with an increasing amount of oil phase. This figure also proves that the particle size and the particle size distribution were influenced by the surfactant type, showing *z*-average diameter in

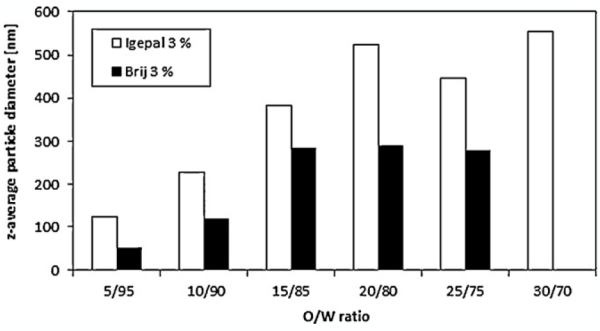

Fig. 2. *z*-Average particle diameter of emulsions determined immediately after the preparation. Influence of O/W ratio in the presence of both type surfactants and HLB value of 11.

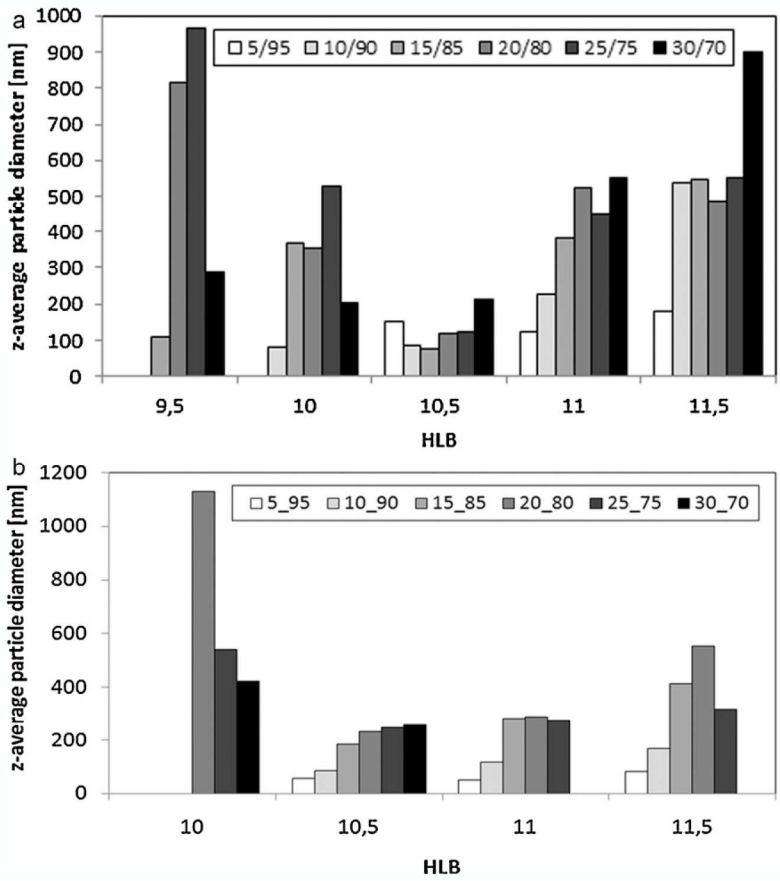

Fig. 3. (a) Influence of the HLB value on particle sizes of O/W (nano)emulsions determined immediately after the preparation (3% Igepals). Absent values of *z*-average particle diameter at HLB 10 (O/W 5/95) and 9.5 (O/W 5/95, 10/90) indicate impossibility to form the O/W emulsions under these conditions. (b) Influence of the HLB value on particle sizes of O/W (nano)emulsions determined immediately after the preparation (3% Brijs). Absent values of *z*-average particle diameter at HLB 9.5, 10 (O/W 5/95, 10/90, 15/85), 11 (O/W 30/70) and 11.5 (O/W 30/70) indicate impossibility to form the O/W emulsions under these conditions.

Igepal emulsions systematically higher in all studied O/W ratios, HLB values of 10, 11 and 11.5 and 3% surfactant compared with Brij-based emulsions. Exception here was the HLB of 10.5 providing emulsions with similar particle sizes irrespective of used surfactant (data not presented). Their particle sizes ranged mostly from 50 to 200 nm indicating thus nanoemulsion formation. Corresponding results, namely increase of particle size with increase of oil content and formation of bigger particles with Igepals, were also observed for emulsions prepared with 5% surfactant. In absolute values and within each of the used surfactant systems, differences in particle sizes were also observed when using different amount of the surfactants. Lower surfactant concentration of 3% gave bigger emulsion droplets in contrast with 5% surfactant content. Influence of the surfactant concentration can be illustrated on Brij–based emulsions with O/W 30/70, which were extremely unstable at 3% surfactant content, breaking down immediately after the preparation. However, increasing the Brij content to 5% provided stable emulsions with droplet size of about 260 nm (HLB 11) and 460 nm (HLB 11.5). The reasons for the interesting phenomenon showing that Brijs provided, at the analogous conditions, smaller particles compared to Igepals can likely be seen in different structures of both surfactants. Hydrophobic part of Brijs, which afforded smaller particles, is composed of flexible lauryl or oleyl chains which can arrange tightly around the oil droplets. Conversely, the hydrophobic part of Igepals is, due to the presence of benzene ring, more bulky and rigid. It can be hence assumed that its arrangement around the oil droplets is due to sterical conditions looser, forming thus bigger particles.

The significant impact of HLB on the emulsion particle size is depicted in Fig. 3a presenting the evolution of the droplet size with changing HLB (3% of Igepal). The figure demonstrates that HLB of 10.5 is an optimum for this system, in which the particle size is the smallest in all O/W ratios and increases with both HLB higher or lower than the optimal one. The observed optimum value is in agreement with the published studies, where the HLB between 10 and 11 was recommended to achieve the formation of stable O/W emulsions based on hydrocarbon oil phase [20–22]. In addition to observed increase in size, the emulsions prepared at lower than optimum HLBs (10 and 9.5) became unstable or broke down soon after their preparation as a natural consequence of low HLBs unsuitable to form O/W emulsions. It is reported [11] that the droplet sizes decrease to reach the minimum in the vicinity of the inversion point and increase again when moving away from this point, which is clearly illustrated with current results. Similar results were observed in emulsions prepared with the Igepals at concentration of 5%. Also in this case, the particle size in emulsions was increasing on either side of the optimum HLB, determined at HLB of 10.5. In accordance with size variation, the size distribution of droplet became broader at both sides to minimum. Although the optimum HLB values were identified, it was also possible to formulate Igepal nanoemulsions with mixtures of surfactants below as well as above the optimum. Nanoemulsions with particle size below 200 nm were obtained for example for O/W ratio of 5/95 at HLB 11 (64 nm) and HLB 11.5 (43 nm) with 5% surfactant or O/W ratio 30/70 at HLB 11.5 (140 nm), with the same surfactant amount.

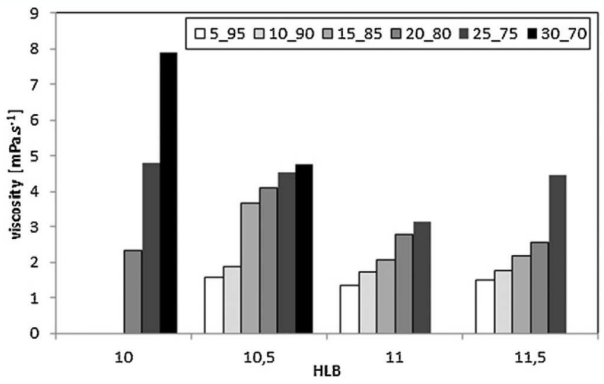

Fig. 4. Viscosity of O/W emulsions determined immediately after the preparation. Influence of the HLB value (3% Brij surfactant).

Emulsions prepared using Brijs showed the same optimum HLB with respect to used oil phase, providing the smallest particle sizes in the range of 56–260 nm (3%) and 44–200 nm (5%). Fig. 3b depicts the droplet size dependence on HLB for emulsions prepared with 3% Brij. With a decreasing HLB, emulsions were highly unstable and broke down after 24 h of their formation. At HLB of 10, O/W emulsions could be prepared at oil contents of 20/80 to 30/70. However, limited emulsion stability was also observed at the HLB of 11, 11.5, 3% surfactant and O/W 25/75 which broke down immediately after their formation. On the contrary, analogous emulsions prepared at the same conditions but with 5% of the surfactant were stable in all studied O/W ratios.

From Fig. 3 it is clearly visible the U-shape of the curve, showing the correlation between the particle size and the HLB. This shape of curve is more notable in emulsions prepared with Igepals than with Brijs. The typical shape of the curve was also reported in literature [21,23]. For example, variation of mean particle size in a mineral oil-in-water emulsion as a function of the HLB was published in [21]. Here, the data were recorded for 2.5% mixtures of Brij 92 and Brij96. In this particular case the emulsion droplets were in micrometer size and ranged from approximately 2 μm recorded for optimum HLB of 7–8 to 14 μm and 20 μm obtained for the outmost HLBs of 6 and 11, respectively. In publication [23] modeled U-shape dependence of particle diameter on HLB for paraffin oil–in-water emulsions with Tween20/Span60 mixtures was presented with minimum particle size at HLB of 10 ± 0.3. However, also in this case the emulsion droplet sizes were in micrometer region. Behavior of current Brij-based systems differs, in some aspect, when compared with systems where Igepals were used. Nanoemulsions prepared with Brijs showed more uniform and consistent behavior with a steady increase of particle size with increased oil content within particular HLB value. Interestingly, in the case of 5% Brijs nanoemulsions mean droplet size, recorded over the entire tested HLB and oil content ranges, laid below the limit of 500 nm. The particle size measurements revealed that a phase inversion emulsification achieved through a variation of HLBs provides nanoemulsions with the particle size smaller than 200 nm within the ranges of O/W ratios 5/95 to 30/70, at HLB 10.5 in both surfactant systems and their concentrations of 3 and 5%.

3.3. Viscosity

Relation between the HLB and the emulsion viscosity (3% Brij) is shown in Fig. 4. As it can be seen, there is a general trend showing a viscosity decrease with an increase of the HLB value. This is especially notable for viscosity drop between the HLB 10 and 10.5 and O/W 30/70. For even higher HLBs (11 and 11.5), only minor changes in viscosity were observed. The figure also demonstrates viscosity increase with an increasing content of oil phase at each of the HLB values tested, which can be attributed to increase in volume fraction of oil in emulsion and which also correspond to changes in particle size showing similar trend. All tested emulsions, irrespective of HLB and oil content, showed Newtonian behavior. Mutual correlation between particle size, viscosity and HLB was studied by Prinderre et al. [23]. The authors presented the modeled emulsion system water/paraffin/Tween20–Span60 with droplet sizes ranging from 3.5 to 9 μm, hence significantly higher compared to nanoemulsions. They concluded that the best emulsion stability corresponded to the smallest particle size, occurring at a minimum HLB = 10 ± 0.3. In accord with present study, they also observed that viscosity of the emulsions decreased as HLB rose. In the range with HLBs lower than optimum, a rapid drop in viscosity was revealed as HLB increased. On the contrary, above the optimum the viscosity was HLB independent.

3.4. Emulsion stability

Long term stability of nanoemulsions was determined on samples prepared at the optimum HLB value of 10.5. These systems provided smallest particles and hence the best stability was assumed. It was found that emulsions with lower oil content of 5/95 and 10/90 were more stable over time. The best stability, in terms of visual assessment, showed emulsions with O/W 5/95, prepared with 3% Brijs concentration. These nanoemulsions were homogenous, bluish in appearance and showed a negligible creaming for 121 days. Corresponding behavior was detected on the samples prepared using the same type of surfactant (Brij) with O/W ratio of 10/90 (3%) and 5/95 (5%), which were stable for 50 days. Samples prepared using Igepals showed also sufficiently good stability under followed conditions. For example, emulsion with O/W 5/95 and 3% surfactant remained visually unchanged for 40 days, as well as, emulsions with O/W of 10/90 and 5% surfactant which were stable for more than 20 days. Fig. 5 illustrates dependence of *z*-average particle diameter on storage time recorded for nanoemulsions with O/W 5/95 and 3% Igepal content. The samples were observed for 40 days and the figure confirms that during the first 25 days, droplet size has not changed showing only minimal variations. Only after 28 days, particle sizes increased from 140 nm (25th day) to 250 nm (28th day) and continued growing during the storage. However, the observed increase has not resulted in phase separation, which was confirmed by visual observation showing homogeneous samples.

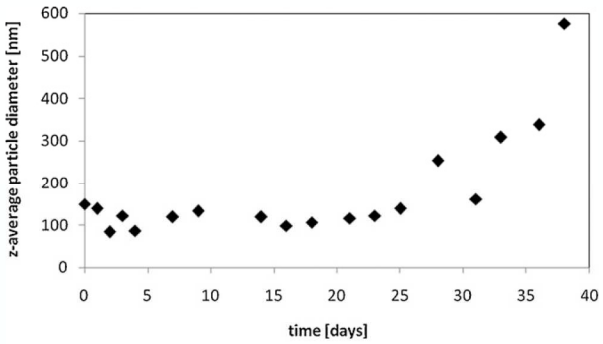

Fig. 5. Dependence of *z*-average particle diameter on storage time. Data recorded for nanoemulsions with O/W 5/95 and 3% Igepals stored at 25 °C.

4. Conclusion

In this study phase inversion technique was chosen for production of n-undecane/water emulsions and nanoemulsions employing the pairs of nonionic surfactants (Igepal, Brij). The effect of O/W ratio, HLB value, surfactant type and its concentration on the formation and appearance of emulsions as well as on the particle size and the viscosity was investigated.

The overall results state that Igepal surfactants were more suitable to prepare emulsions within a broader range of HLBs compared to the Brij surfactants. Almost all emulsions with O/W ratio of 5/95, HLB values (10.5–11.5) regardless of the type and concentration of the surfactant were semitransparent in appearance indicating formation of small particles at the vicinity of the phase transition point. With increased oil content (O/W10/90, 15/85, 20/80, 25/75 and 30/70) particle size increased. At low HLB and low oil content O/W 5/95 and 10/90, the formation of the inverted W/O emulsions was typically present.

Generally, the oil droplets in emulsions prepared with mixtures of Igepal surfactants were mostly bigger in all studied O/W ratios and almost at all HLB values (10, 11 and 11.5) compared to Brij systems. Exceptions were emulsions prepared at the HLB of 10.5 showing similar particle sizes for both types of surfactant mixtures. Furthermore, these emulsions showed the smallest particle sizes, being in the range of 50–200 nm for all studied O/W ratios. From the results it is clear that viscosity and the particle size of emulsions prepared with Brij surfactant system increased with an increasing content of oil phase at all tested HLB values and both surfactant concentrations. The higher viscosity was observed in emulsions with the HLB values (10 and 10.5) where the emulsion droplets were small in contrast to emulsions which were prepared with the HLBs (11 and 11.5). The study proved successful formation of stable nanoemulsions using emulsion inversion point method at optimum HLB of surfactant mixtures for rather broad range of O/W ratios. The nanoemulsions were in addition prepared with reasonably low concentration of surfactants and showed good stability at room temperature. Some of them were stable for more than a month and absence of creaming or other destabilization mechanisms were observed. Comparing the both surfactant systems used under optimal conditions, the Brijs provided more time-stable nanoemulsions.

Acknowledgements

This article was written with support of Operational Program Research and Development for Innovations co-funded by the European Regional Development Fund (ERDF) and national budget of Czech Republic, within the framework of project Center of Polymer Systems (reg. number: CZ.1.05/2.1.00/03.0111). This article was also supported by an internal grant of TBU in Zlín IGA/FT/2012/036 financed from funds of specific academic research.

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PUBLICATION II

Dynamický rozptyl světla v analýze koloidních systémů

Sevcikova Petra, Kasparkova Vera, Krejci Vera, Vltavska Pavlina
Chemické Listy, 2014, vol. 108, p. 479–482.

DYNAMICKÝ ROZPTYL SVĚTLA V ANALÝZE KOLOIDNÍCH SYSTÉMŮ

PETRA ŠEVČÍKOVÁ[a], VĚRA KAŠPÁRKOVÁ[a,b], JIŘÍ KREJČÍ[a] a PAVLÍNA VLTAVSKÁ[a]

[a] Ústav technologie tuků, tenzidů a kosmetiky, Fakulta technologická, Univerzita Tomáše Bati ve Zlíně, nám. T. G. Masaryka 275, 760 01 Zlín, [b] Centrum polymerních systémů, Univerzitní institut, Univerzita Tomáše Bati ve Zlíně, Nad Ovčírnou 3685, 760 01 Zlín
psevcikova@ft.utb.cz, vkasparkova@ft.utb.cz

Došlo 18.4.13, přijato 14.6.13.

Klíčová slova: dynamický rozptyl světla, mikroemulze, nanoemulze, povrchově aktivní látka

Úvod

V posledních letech došlo k výraznému rozvoji metod zabývajících se problematikou měření velikosti částic disperzních soustav a tím i k jejich rozšíření především do průmyslové praxe. Jednou z těchto metod, která je rutinním způsobem stále častěji využívána v řadě průmyslových odvětví, je metoda dynamického rozptylu světla[1].

Dynamický rozptyl světla (DLS), rovněž nazývaný jako kvazielastický rozptyl světla (QELS) nebo fotonová korelační spektroskopie (PCS), je metoda vhodná pro měření velikosti částic v submikronové oblasti[2,3,4]. Základem této neinvazivní techniky je měření fluktuace intenzity rozptýleného světla z laserového zdroje okolo její průměrné hodnoty. Tyto fluktuace souvisí s interferenčním zeslabováním a zesilováním světla rozptýleného na nestacionárních částicích disperzní fáze, podléhajících Brownovu pohybu[1]. Čím rychleji se částice pohybují, tím rychleji se intenzita rozptýleného světla mění. Rychlost těchto změn je tudíž přímo závislá na pohybu molekuly[5]. Okamžitou hodnotu fluktuace intenzity v čase τ popisuje pro monodisperzní systém autokorelační funkce $g(\tau)$ definovaná vztahem:

$$g(\tau) = \exp[-\tau/\tau_C]$$

kde τ_C je relaxační čas, který je charakterizován jako doba potřebná k návratu fluktuace k průměrné hodnotě intenzity rozptýleného světla. Hodnota relaxačního času je v úzkém vztahu k difuznímu koeficientu D rozptylujících částic, který lze vyjádřit pomocí rovnice:

$$\tau_C = 1/Dq^2$$

kde q je absolutní hodnota vlnového vektoru, daná vlnovou délkou primárního světelného paprsku λ a úhlem θ, pod nímž je měřena intenzita rozptýleného světla: $q = (4\pi/\lambda)\sin(\theta/2)$ (cit.[1]).

Velikost částic lze pak vypočítat pomocí translačního difuzního koeficientu D prostřednictvím Stokesovy-Einsteinovy rovnice:

$$d(H) = kT/3\pi\eta D$$

kde $d(H)$ je hydrodynamický průměr částice, D je translační difuzní koeficient, k je Boltzmannova konstanta, T je absolutní teplota a η je viskozita disperzního prostředí[2,6,7].

Nespornými výhodami této metody je rychlý a jednoduchý průběh měření, které trvá obvykle od několika sekund do 10 minut (cit.[3,8,9]), metoda je absolutní (kalibrace pomocí standardů o známé velikosti a distribuci není nutná), analýza velikosti částic vyžaduje jen velmi malé množství vzorku a měřící zařízení je komerčně dostupné s automatizací včetně analýzy dat[8,9]. Jednou z hlavních výhod této techniky je její využití při analýze velikosti částic s rozměry od několika nanometrů až do 1 až 2 μm (cit.[8,10]).

Kritickým krokem u většiny aplikací DLS je čistění a plnění měřicí kyvety, do které přichází vzorek. Pokud se v roztoku objeví prach nebo mikrobublinky, může dojít k narušení nebo překrytí signálu analytu a měření pak může být zatíženo artefakty[5]. Z tohoto důvodu je nezbytná filtrace všech používaných kapalin, zvláště vody, a důkladné čištění kyvet, pipet a stříkaček[8].

Dynamický rozptyl světla je metoda, která je vhodná pro přesné stanovení velikosti částic v suspenzích[11]. Lze ji využít při charakterizaci emulzí, micel, liposomů, latexů a pigmentů. Velmi zajímavé uplatnění si tato metoda našla také v biologické a mikrobiologické oblasti pracující s materiálem, jako jsou bakterie, viry, proteiny a DNA[8].

Cílem tohoto příspěvku bylo prakticky dokumentovat širokou využitelnost metody dynamického rozptylu světla, a to jak při měření velikostí částic povrchově aktivních látek (PAL), tak i při charakterizaci relevantních emulzních systémů, mikroemulzí, nanoemulzí a klasických emulzí s velikostí částic v měřicím rozsahu přístroje.

Experimentální část

Příprava vzorků

Povrchově aktivní látky (PAL): Anionická PAL lauroyl sarkosinát sodný (SDSa) (Merck) a neionické PAL Igepal CA 720, Tween 20 a Tween 60 (Sigma-Aldrich) byly rozpuštěny v demineralizované vodě v koncentracích, které ležely vždy nad hodnotou jejich kritické micelární koncentrace (CMC).

Mikroemulze: Stabilní mikroemulze 1-monoacylglycerolů (1-MAG) byly připraveny na základě předem sestrojených fázových diagramů. Olejovou složku mikroemulze tvořil roztok 1-MAG v ethanolu (1-butanolu) v poměru 1:2 (hm.%). Tyto dva alkoholy s krátkou délkou řetězce sloužily zároveň jako kosurfaktanty. Byly použity monoacylglyceroly s nasycenou mastnou kyselinou o různé délce uhlovodíkového řetězce, C10, C11, C12 a C14, které byly připraveny na Ústavu technologie tuků, tenzidů a kosmetiky Fakulty technologické, UTB ve Zlíně postu-

pem popsaným v publikaci[12]. Jako PAL byl zvolen Tween 80 (Sigma-Aldrich). Jelikož mikroemulze vznikají spontánně, byly vodná a olejová složka v předem stanoveném poměru smíchány s PAL a kosurfaktantem a systém byl krátce homogenizován.

Nanoemulze: Nanoemulze undekanu ve vodě byly připraveny metodou fázové inverze. Na přípravu směsi o požadované hodnotě HLB byla použita vhodná dvojice neionických PAL Igepal 210 (HLB = 4), Igepal 520 (HLB = 10) a Igepal 720 (HLB = 14,7). Hodnota HLB vyjadřuje hydrofilně-lipofilní rovnováhu mezi polární a nepolární částí molekuly tenzidu. Výsledné HLB hodnoty směsí PAL byly vypočítány z individuálních hodnot HLB udaných dodavatelem pomocí rovnice $HLB = x_1 \cdot HLB_1 + x_2 \cdot HLB_2$, kde x_1 a x_2 jsou hmotnostní zlomky použitých PAL s hodnotami HLB_1 a HLB_2. Emulgace probíhala při laboratorní teplotě (25 °C) pomocí míchadla RZR 2020 (Heidolph), při rychlosti míchání 1050 ot./min. Byly připraveny nanoemulze s následujícími poměry olej/voda: 10/90, 15/85, 20/80, 25/75 a 30/70. Celkové množství PAL v emulzi činilo 3 nebo 5 hm.%. Na základě předběžných studií[13] byly použity PAL o HLB hodnotách 9; 5; 10; 10,5; 11 a 11,5.

Úprava vzorku pro DLS

Měření velikosti částic pomocí DLS probíhalo u PAL a mikroemulzí bez dalšího ředění vzorku, a to tak, že 1 ml dané suspenze byl pipetován do plastové kyvety a použit pro analýzu. U nanoemulzí bylo vždy nutno vzorky zředit. Pro měření bylo použito 5 μl vzorku dané emulze v 1 ml sterilní, demineralizované vody, která byla dvakrát filtrována. Všechna měření velikosti částic byla prováděna při teplotě 25 °C a rozptylovém úhlu 173°. Vyhodnocení výsledků bylo zpracováno na přístroji Zetasizer Nano ZS (Malvern Instruments, UK).

Kontrola přístroje Zeta Nano ZS

Správnost měření velikosti částic byla prověřena analýzou suspenze monodisperzního standardu polystyrénového latexu o nominálním průměru velikosti částic 60 nm (Thermo Scientific). Jako disperzní médium byl použit roztok NaCl o koncentraci 9 mg ml^{-1}. Pro vlastní měření byl použit 1 ml roztoku NaCl a 5 μl standardu. Získané výsledky analýzy prokázaly, že hodnoty velikosti částic polystyrénového latexu ležely v rozmezí 59±2,5 nm, udávaném pro tuto nominální velikost částic standardu dodavatelem.

Jako součást kontroly měření byla stanovena i opakovatelnost měření velikosti částic emulzí (z-průměr), která byla vyjádřena pomocí směrodatné odchylky devíti analýz. Při z-průměru 149,1 nm byla směrodatná odchylka 0,76 nm.

Výsledky a diskuse

Velikost částic

Micely ionických i neionických PAL lze studovat řadou metod. Dynamický rozptyl světla je jednou z nich a vzhledem k sub-mikronovému pracovnímu rozsahu je metodou velmi vhodnou. Aplikovatelnost metody pro analýzu micel byla testována měřením velikosti micel vybraných ionických i neionických PAL a jejich směsí. Výsledky zahrnující hodnoty z-průměru velikosti částic jsou shrnuty v tab. I.

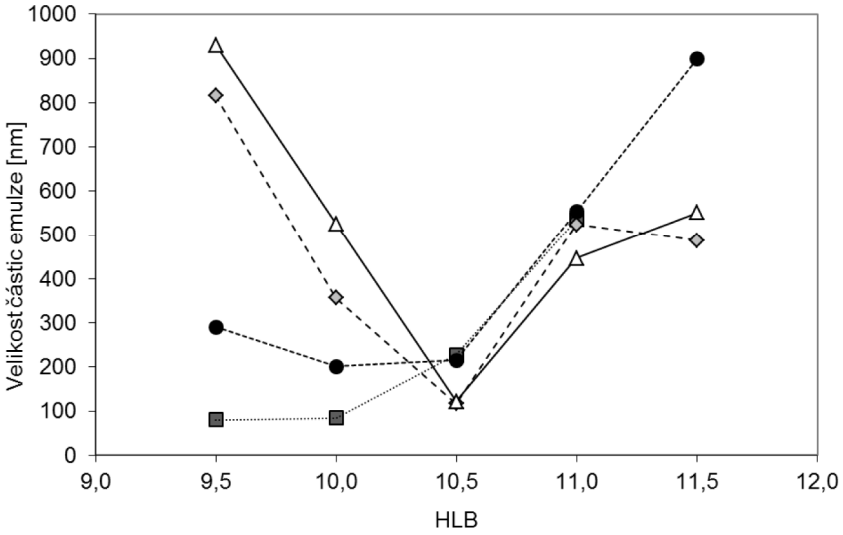
Z tabulky je zřejmé, že v případě PAL Tween 20 a Tween 60 dokáže metoda spolehlivě rozlišit velikosti micel obou neionických PAL i stanovit velikost směsné micely vzniklé po smísení obou PAL v poměru 50/50. Micely Tweenu 60 (13±0,1 nm) jsou větší než Tweenu 20 (8,4±0,1 nm), což je v dobré shodě se strukturou obou PAL. Analýza ionické PAL laurylsarkosinátu sodného, pro který byla publikována velikost micely 2,2 nm (cit.[14]), byla stanovena pomocí DLS na 2,7 nm. Velikost micel PAL Igepal 720 stanovená v této práci je však větší (7,1±0,2 nm) než její rozměr získaný touto metodou v práci[15] (4,5 nm).

Analýza 1-MAG mikroemulzí prokázala přítomnost částic s velikostí v rozmezí 11 až 24 nm, což dle publikací[16,17] odpovídá rozsahu očekávanému pro tyto emulzní systémy, tedy 10 až 50 nm. Výsledky získané z měření distribuce velikosti částic ukázaly u většiny vzorků na přítomnost úzké monomodální distribuce, svědčící o výskytu jedné populace částic.

Výsledky analýzy nanoemulzí ukazují, že velikost jejich částic je významně ovlivněna jak hodnotou HLB použitých PAL, tak také poměrem olejové a vodné (O/V) fáze. Obr. 1 znázorňuje vztah mezi HLB a velikostí částic pro sledované poměry O/V a 3% koncentraci povrchově aktivních látek. Z obrázku je patrná optimální hodnota HLB 10,5, při které byly u všech studovaných poměrů O/V připraveny nanoemulze, tedy emulze s velikostí částic menší než 200 nm. Toto zjištění je v souladu se závěry práce[13], která pro úspěšnou tvorbu stabilních emulzních systémů s undekanem jako olejovou fází doporučuje pou-

Tabulka I
Velikost micel studovaných povrchově aktivních látek stanovená jako průměr sedmi měření a směrodatná odchylka σ stanovení

PAL	z-průměr ± σ [nm]
Tween 20	8 ± 0,1
Tween 60	13,0 ± 0,1
Tween 20/Tween 60 v poměru 50/50	9,0 ± 0,1
SDSa	2,7± 0,1
Igepal CA 720	7,1 ± 0,2



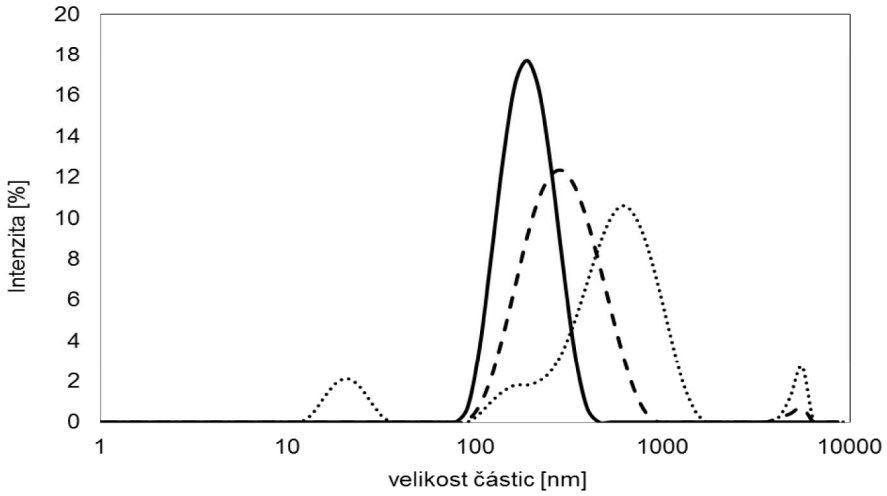
Obr. 1. **Velikost částic emulzí změřená ihned po jejich přípravě.** Vliv hodnoty hydrofilně-lipofilní rovnováhy (HLB) a poměru olej/voda (3 % povrchově aktivních látek); ■ 10/90, ◆ 20/80, Δ 25/75, ● 30/70

žít PAL v rozmezí HLB hodnot 10 až 11. V přítomnosti PAL s hodnotami HLB vyššími i nižšími než je tato hodnota, lze pozorovat vzrůstající velikost částic po obou stranách optima HLB. S klesající hodnotou HLB byla rovněž zjištěna značná nestabilita připravených emulzních systémů, zejména při poměru O/V 10/90 a HLB 9,5. Z obr. 1 je dále zřejmý charakteristický tvar závislosti mezi velikostí částic a HLB, který byl rovněž uveden v pracech[18,19], zabývajících se přípravou emulzních systémů typu O/V pomocí neionických PAL.

Odlišnosti ve velikosti částic byly dále pozorovány při použití rozdílného množství PAL (3 a 5 hm.%). Podle Chanada a spol.[20] by měla velikost částic s rostoucí koncentrací povrchově aktivní látky klesat. Tento předpoklad byl v převážné většině studovaných nanoemulzních systémů potvrzen.

Stabilita nanoemulzí

Stabilita nanoemulzí byla hodnocena prostřednictvím změn velikostí částic a jejich distribuce v čase. Výsledky prokázaly, že stabilita je ovlivněna nejen jejich složením a způsobem přípravy, ale především dobou a teplotou jejich skladování. Obr. 2 znázorňuje průběh a změny v distribuci velikostí částic nanoemulze během skladování při teplotě 25 °C. Ihned po výrobě vykazovala tato nanoemul-



Obr. 2. **Změny distribuce velikostí částic nanoemulze během skladování při teplotě 25 °C.** Ihned po výrobě (plná čára), po 4 dnech (přerušovaná čára) a po 11 dnech (tečkovaná čára)

ze úzkou monomodální distribuci částic se z-průměrem 193 nm. Po 4 dnech skladování se velikost částic zvýšila na 228 nm za současného vzniku další populace částic, pohybující se v limitní oblasti přístroje udávanou výrobcem na 6 μm. Na distribuční křivce zaznamenané po 11 dnech skladování je pak patrný i výskyt třetí populace částic s velikostí desítek nanometrů a vzrůst z-průměru částic na 398 nm.

Závěr

Výsledky studie, která se zabývá stanovením velikostí a distribuce částic povrchově aktivních látek, jejich směsí a emulzních systémů pomocí metody dynamického rozptylu světla potvrdily, že metoda je schopna relativně snadno a rychle stanovit velikosti částic disperzních systémů, které se liší jak svou velikostí (micely < 10 nm, mikroemulze 10–50 nm, nanoemulze 50–200 nm a emulze >500 nm), tak také postupem přípravy. Z výše uvedených poznatků tedy plyne možnost využít DLS k základní charakterizaci částicových systémů v řadě aplikačních oblastí, jako jsou např. medicína, farmacie, kosmetika, potravinářství, polymerní průmysl nebo průmysl barev a nátěrových hmot. Z experimentů je zřejmá i vhodnost této techniky při sledování stability disperzních systémů, s částicemi v submikronové oblasti, prostřednictvím sledování změn distribuce velikostí částic v čase, a dále také její informační potenciál při stanovení přítomnosti jedné nebo více populací částic zhodnocením průběhu distribučních křivek.

Tento článek byl vytvořen za podpory Operačního programu Výzkum a vývoj pro inovace, jenž je spolufinancován Evropským fondem pro regionální rozvoj (ERDF) a státním rozpočtem České republiky, v rámci projektu Centra polymerních systémů (CZ.1.05/2.1.00/03.0111). Tato práce vznikla také za podpory interního grantu UTB ve Zlíně č. IGA/FT/2013/016 financovaného z prostředků specifického vysokoškolského výzkumu.

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P. Ševčíková[a], V. Kašpárková[a,b], J. Krejčí[a], and P. Vltavská[a] *([a] Department of Fat, Surfactant and Cosmetics Technologies, Faculty of Technology, [b] Centre of Polymer Systems, Tomas Bata University, Zlín):* **Dynamic Light Scattering in Analysis of Colloidal Systems**

Dynamic light scattering (DLS) is a non-invasive technique, capable of measuring the size of submicron particles in the submicron range. The study was focused on the applicability of DLS in measurement of colloidal particles of different types. The micelle sizes of surfactants (Tween 60, Tween 20, Igepal CA 720 and Na lauryl sarcosinate) and particle sizes of micro- and nanoemulsions were measured. The diameters of surfactant micelles were 13.0±0.1 nm, 8.4±0.1 nm, 7.1±0.2 nm and 2.7±0.1 nm for Tween 60, Tween 20, Igepal CA 720 and Na lauryl sarcosinate, respectively. Stability of nanoemulsions as a consequence of the changes of particle size distributions in storage was monitored. The results revealed the presence of uni-, bi- and trimodal distributions reflecting the occurrence of various particle populations as well as a shift of distribution curves due to the particle growth. It was confirmed that DLS can provide reliable and rapid determinations of particle sizes of micro-, nano- and classical emulsions.

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PUBLICATION III

Formulation, antibacterial activity, and cytotoxicity of 1-monoacylglycerol microemulsions

Sevcikova Petra, Kasparkova Vera, Krejci Vera, Hauerlandova Iva, Humpolicek Petr, Kuceková Zdenka, Bunkova Leona

European Journal of Lipid Science and Technology, 2014, vol. 116, p. 448–457.

Research Article

Formulation, antibacterial activity, and cytotoxicity of 1-monoacylglycerol microemulsions

Ševčíková Petra[1], Kašpárková Věra[1, 2], Hauerlandová Iva[1], Humpolíček Petr[2, 3], Kuceková Zdeňka[2, 3] and Buňková Leona[4]

[1] Department of Fat, Surfactant, and Cosmetics Technology, Faculty of Technology, Tomas Bata University in Zlin, Zlin, Czech Republic
[2] Centre of Polymer Systems, University Institute, Tomas Bata University in Zlin, Zlin, Czech Republic
[3] Polymer Centre, Faculty of Technology, Tomas Bata University in Zlin, Zlin, Czech Republic
[4] Department of Environmental Protection Engineering, Faculty of Technology, Tomas Bata University in Zlin, Zlin, Czech Republic

Antibacterial activity of stable 1-monoacylglycerol (1-MAG) microemulsions (MEs) of capric (C10:0), undecanoic (C11:0), lauric (C12:0), and myristic (C14:0) acids against the growth of gram-positive and gram-negative bacteria was tested and their inhibition effect was compared to that of neat 1-MAG. To reveal their behavior with respect to eukaryotic cells, the cytotoxicity on mouse fibroblasts was also determined. The MEs were studied via pseudo-ternary phase diagrams in order to find out the miscibility areas of all components used. The results indicated that at 1-MAG concentrations of 1000–1900 mg/L, an inhibition of gram-negative strains was improved compared to neat 1-MAG and depended on the fatty acid type; however, at lower 1-MAG concentrations, both systems showed comparable effect. The opposite trend was detected on gram-positive bacteria, where 1-MAGs showed better performance compared to corresponding MEs. The best antibacterial activity against both bacterial types was observed in 1-MAG C12:0 MEs. MTT assay revealed significant cytotoxicity of all MEs. The threshold of 10 mg/L was determined as the limit for moderate toxicity, which represents 40–60% cell survival. All prepared MEs were transparent, stable during the storage period of 6 months and their particle sizes were between 15 and 20 nm.

Practical applications: 1-MAGs belong to safe and efficient antimicrobial agents and their application may be a convenient alternative to usage of synthetic antimicrobials; however, one of their limitations for certain applications is their insolubility in aqueous systems. This drawback can be reduced by encapsulation of 1-MAGs in suitable ME system. The presented study has confirmed that 1-MAG MEs possess suitable inhibitory activity against both gram-negative and gram-positive bacteria. Efficacy of MEs against gram-negative strains was enhanced compared to 1-MAG alone. Although the growth of gram-positive bacteria was less affected by MEs when compared to neat 1-MAG, the results can be closely related to potential industrial applications of the MEs as effective agents for cosmetics, dermatology, food industry, paint or coatings where water-based systems are frequently used and desired. The application of studied systems might be to some extent limited due to the potential health risk caused by the observed cytotoxicity.

Keywords: Bacteria / Cytotoxicity / Inhibition effect / Microemulsion / Monoacylglycerol

Received: May 15, 2013 / Revised: December 27, 2013 / Accepted: January 14, 2014

DOI: 10.1002/ejlt.201300171

Correspondence: Petra Ševčíková, Department of Fats, Surfactant and Cosmetics Technology, Faculty of Technology, Tomas Bata University in Zlin, T. G. Masaryk Sq. 275, 762 72 Zlin, Czech Republic
E-mail: psevcikova@ft.utb.cz
Fax: +420 577 210 172

Abbreviations: GI, growth index; **ME,** microemulsion; **MPB,** meat-peptone broth; **PCS,** photon correlation spectroscopy; **PDI,** polydispersity index; **MAG,** monoacylglycerol

1 Introduction

Due to their unique properties, microemulsions (MEs) have attracted considerable attention in many applications, such as food industry [1], cosmetics, agriculture [2], and coating materials [3]. The ultralow interfacial tension, the ultrahigh solubilization, the low viscosity and transparency make them desirable also in pharmaceutical formulations for improved

drug solubilization [4], cleaning technologies and soil remediation [5].

MEs are thermodynamically stable, isotropic solutions formed of oil, water, and surfactants [6] with the particle size in the range of 10–100 nm [7, 8]. They were first discovered by Schulman, who found that the addition of a fourth component (often an alcohol) to an emulsion containing oil, water, and a surfactant led to the formation of a clear, apparently homogenous phase. The additional component is usually called the *co-surfactant* [9]. A major practical disadvantage of MEs is usually connected with their preparation, requiring higher surfactant concentration than classical emulsions, typically of about 30% w/w [10–13]. It has been suggested that MEs can be self-preserving antimicrobials in their own and can be potentially antimicrobial [14].

1-MAGs are partial esters of glycerol and medium- to long-chain fatty acids [15–17]. Behavior of 1-MAGs is governed by their amphiphilic structure [18] suggesting their usage as non-ionic surfactants with a low HLB value, lying between 3 and 6 [19, 20]. Owing to these properties, 1-MAGs are used in drug delivery systems, production of antimycotic materials getting into touch with skin, and products preventing fermentation [21]. Generally, antimicrobial effect of 1-MAG depends on the fatty acid carbon chain length and its saturation or unsaturation. Gram-positive bacteria are sensitive to fatty acids with a short and medium-carbon chain and their esters [22] and this sensitivity is associated with the absence of lipopolysaccharides in their cell wall [23]. On the other hand, gram-negative bacteria are reported to be resistant against inhibitory effects of 1-MAG [22].

Recently, several studies have been focused on the investigation of antimicrobial properties of 1-MAG formulated in MEs. For example, the testing of antimicrobial activity of ME system with 1-MAG of lauric acid was published in ref. [1, 24–26]. Also, the publication [2] dealt with the enhancement of inhibition ability of monolaurin through loading in MEs and their comparing with the antimicrobial activity of corresponding 1-MAG. Nevertheless, only limited attention has been paid to the comparison of inhibition effects of different 1-MAGs in MEs, especially those with odd number of carbons in molecule, which are not common in nature.

The study was hence focused on the preparation of stable ME systems based on 1-MAGs, containing fatty acids with chain length varying from 10 to 14 carbons including undecanoic acid (C11:0) with odd number of carbons, monitoring their antibacterial activity against the most common pathogenic microorganisms and evaluation of their inhibition effect in comparison with 1-MAGs. Additionally, cytotoxicity of MEs was determined on mouse fibroblasts using MTT assay, which reflects the influence of MEs on eukaryotic cells. Particle size and stability of these systems was verified by photon correlation spectroscopy (PCS).

2 Materials and methods

2.1 Microorganisms

The tested gram-positive bacteria: *Bacillus cereus* (CCM 2010), *Bacillus subtilis* subsp. *subtilis* (CCM 2216), *Enterococcus faecalis* (CCM 4224), *Staphylococcus aureus* subsp. *aureus* (CCM 3953), *Micrococcus luteus* (CCM 732) and gram-negative bacteria: *Citrobacter freundii* (CCM 7187), *Escherichia coli* (CCM 3954), *Pseudomonas aeruginosa* (CCM 3955), *Salmonella enterica* subsp. *enterica* ser. *Enteritidis* (CCM 4420), and *Serratia marcescens* subsp. *marcescens* (CCM 303) were selected as the representatives of the common pathogenic microorganisms. All bacterial strains were obtained from the Czech Collection of Microorganisms (*CCM, Czech Republic*).

Each suspension was prepared by inoculating 5 mL of commercial culture medium with 24-h bacterial inoculum.

2.2 Materials

Monoacylglycerols of capric (1-MAG C10:0), lauric (1-MAG C12:0), undecanoic (1-MAG C11:0), and myristic (1-MAG C14:0) acid were prepared according to procedure described in ref. [27]. Due to chosen method of synthesis, all MAGs were racemic mixtures of 1- and 3-MAGs. For simplification, 1-MAG denomination is used hereinafter. The non-ionic surfactant Tween 80 (polyoxyethylene (20) sorbitan monooleate) with the HLB value of 15, supplied by *Sigma–Aldrich* (*Germany*) was used throughout the study. Four types of alcohols, ethanol (*Penta, Czech Republic*), propanol, butanol, and pentanol (*IPL Petr Lukeš, Czech Republic*) were used as co-surfactants in the formulations.

2.3 Formulation of stable microemulsions

MEs were prepared with the help of pseudo-ternary phase diagrams. The alcohols with short and medium chain length (ethanol, propanol, butanol, and pentanol) were used as co-surfactants and Tween 80 was applied as a surfactant. Oil phase was always composed of 1-MAG solution in respective alcohol mixed in the ratio of 1:2 w/w. For construction of phase diagrams, series of solutions containing oil phase and Tween 80 in pre-defined weight ratio was prepared at 25°C, which were then titrated with distilled water under vigorous stirring. MEs were present in the region of clear and isotropic solutions, whereas classical emulsions were observed in the region of visibly cloudy dispersions.

For testing of antibacterial efficacy and cytotoxicity, the following composition and preparation of MEs was chosen. Oil phase, 250 μL of 1-MAG ethanolic solution (concentration of 300 mg/mL), was pipetted into plastic tube together with 2.25 g of Tween 80 and filled to volume with 20 mL distilled water. The resulting ME was homogenized using Wortex mixer and sterilized by filtration through a sterile

syringe filter with a pore size of 0.22 μm (*Millipore, Germany*). Parent ME with 1-MAG concentration of 3800 mg/L was further diluted to 2300, 1900, 1000, 500, 250, 100, 50, 10, 5, 1, and 0.5 mg/L and employed for the tests. Dependent on fatty acid type in the 1-MAG, the samples were assigned 1-MAG C10:0 ME (capric acid), 1-MAG C11:0 ME (undecanoic acid), 1-MAG C12:0 ME (lauric acid), and 1-MAG G CC14:0 ME (myristic acid).

The reference MEs containing only ethanol and Tween 80 were prepared via the same procedure described above, assigned ME_E and tested for antibacterial effect and cytotoxicity. Ethanol and surfactant concentrations in the ME_E corresponded to those in 1-MAG MEs.

2.4 Monitoring the effect of 1-MAG microemulsions on selected microorganisms

MEs were dosed into the tubes with sterile meat-peptone broth (MPB) (*Mueller-Hinton Broth, HiMedia, Bombai, India*) obtaining thus cultivation media with 1-MAG ME in concentrations ranging from 1900 to 100 mg/L. Prepared cultivation media (200 μL) and 5 μL of 24-h bacterial suspensions hundredfold diluted to 2×10^4 CFU/mL were pipetted into microplate wells. As a positive reference, MPB inoculated with bacterial suspension without ME was used. MPB containing MEs, but not bacteria, served as a negative reference. Tested bacterial suspensions were cultivated at the temperature of 25 ± 1°C for 15 h, and the optical density of cell suspension was measured at 30-min intervals at the wavelength of 655 nm. Measurements were carried out on a 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 15 h incubation in the presence of 1-MAG ME, OD_N and OD_P are optical densities of negative and positive references, respectively. All tests were performed in triplicates; means and SDs, according to Dean-Dixon method, were calculated.

2.5 Cytotoxicity

Cytotoxicity testing was accomplished with a mouse embryonic fibroblast cell line (ATCC CRL-1658 NIH/3T3). The ATCC-formulated Dulbecco's Modified Eagle's Medium (catalog No. 30-2002), with added bovine calf serum to a final concentration of 10% and penicillin/streptomycin, 100 U/mL, was used as the culture medium.

Tested samples were diluted to concentrations of 2300, 1900, 1000, 500, 250, 100, 50, 10, 5, 1, and 0.5 mg/L in the culture medium. Cytotoxicity testing was conducted according to the EN ISO 10993-5 standard procedure, with modification. Cells were pre-cultivated for 24 h and the culture medium was subsequently replaced with dilutions of 1-MAG MEs. As a reference, cultivation in a pure medium without tested compound was used. To assess cytotoxic effect, a MTT assay (*Invitrogen Corporation, USA*) was carried out after 1-day cell cultivation in the presence of 1-MAG ME. All the tests were performed in quadruplicates. The absorption was measured at 570 nm with an Infinite M200 Pro NanoQuant (*Tecan, Switzerland*). Dixon's Q test was used to remove outlying values, and mean values were calculated. The cell viability is reported in two ways in order to provide a comprehensive view of the results: (i) as averages and theirs SDs (ii) as a percentage of cells present in the respective extract relative to cells cultivated in a pure extraction medium without MEs (100% viability).

2.6 Measurement of particle size distribution

The particle size and particle size distribution of 1-MAG ME were determined by PCS. The hydrodynamic radius, expressed as intensity weighted *z*-average particle diameter was measured using Zetasizer Nano ZS instrument (*Malvern Instruments, UK*) at a scattering angle of 173° and at a constant temperature of 25°C.

3 Results and discussion

3.1 Influence of alcohol type on phase behavior of 1-MAG microemulsions

The phase behavior of 1-MAG MEs was studied with the help of pseudo-ternary phase diagrams, which were used to determine a target composition of 1-MAG MEs utilized for antibacterial and cytotoxicity tests. Figure 1 shows the phase diagrams of the system containing 1-MAG C10:0, where alcohols with different chain lengths were used. As it can be seen, the phase diagrams are divided by binodal curve into two areas, the homogenous (labeled white) and the heterogeneous (labeled grey). The heterogeneous area is characterized by limited miscibility of all components and by the presence of classical emulsion. On the contrary, within the homogenous area MEs are present.

According to the literature [28, 29], the phase behavior of MEs is influenced by the presence of surfactants, salts and co-surfactants. This is clearly confirmed by the present data (Fig. 1) showing that the size of both homogeneous and heterogeneous areas depends on the type of co-surfactant. Figure 1a illustrates the system, in which ethanol is used as co-surfactant, with only a small heterogeneous area of the limited miscibility in comparison with the systems utilizing propanol, butanol, and pentanol (Fig. 1 b–d). The results clearly showed that the largest area of limited miscibility was observed with co-surfactant pentanol. The presence of butanol, ethanol, and propanol as co-surfactants then caused gradual reduction of limited miscibility area. It follows, that the area of limited miscibility increased with an increasing chain length of the alcohol. Exception from this rule was propanol (Fig. 1b) showing the smallest area of limited

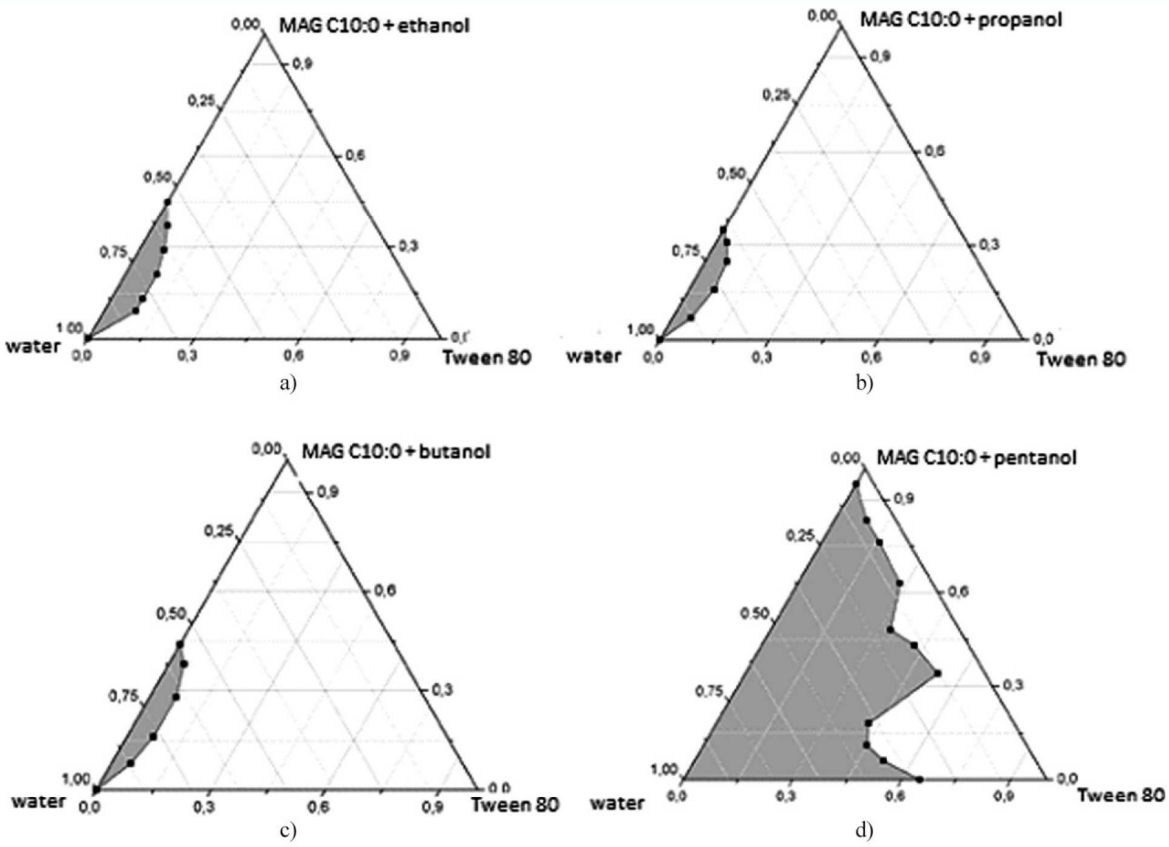

Figure 1. Ternary-phase diagrams of water-oil-surfactant with oil phase composed of: (a) MAG C10:0 + ethanol, (b) MAG C10:0 + propanol, (c) MAG C10:0 + butanol, (d) MAG C10:0 + pentanol, constructed at 25°C.

miscibility. This finding is consistent with the results of study [29] reporting that replacement of ethanol with propanol has led to reduction of the region of limited miscibility.

Similar influence of co-surfactant chain lengths on size of the region with limited miscibility was also found in MEs composed of 1-MAGs with longer fatty acids (C11:0 and C12:0). Also in these cases, area of limited miscibility grew gradually with increasing chain length of alcohol (data not presented).

Phase behavior of MEs based on 1-MAG of myristic acid was studied only with ethanol and butanol as co-surfactants. The reason was a poor solubility of this 1-MAG in remaining alcohols. However, also in this case MEs prepared with ethanol showed a smaller area of limited miscibility in comparison with butanol. The obtained phase diagrams served as a background for formulation of final MEs for antibacterial and cytotoxicity testing.

3.2 Particle size of 1-MAG microemulsions

Droplet size of 1-MAG MEs prepared with ethanol as co-surfactant was verified with PCS. It was found that the particles were in the size range of 15–20 nm, which corresponded to the sizes given for these systems in literature [7, 30]. The results revealed the presence of relatively narrow particle size distribution which indicated the occurrence of one main particle population, occasionally accompanied by minor population of particles at the size range of around 50–80 nm. This population, however formed only of about 5% of the particles (Fig. 2). Distribution width was described by polydispersity index (PDI), a dimensionless quantity being of around 0.1 for near-monodisperse samples and >0.7 for the samples with a very broad size distribution. Prepared MEs showed PDI ranging from 0.27 to 0.35. The measurements also confirmed a long-term stability of 1-MAG MEs, which were stable for more than 6 months and showed only minor changes in the droplet size (Table 1). All samples were visually transparent.

3.3 The influence of 1-MAG microemulsions on gram-negative bacteria

Antibacterial effect of MEs based on 1-MAGs was determined and the results were compared to the study [31]

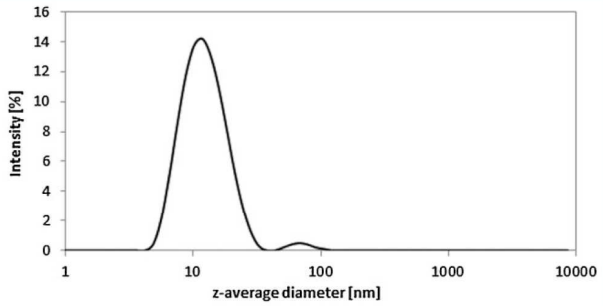

Figure 2. Particle size distribution of freshly prepared 1-MAG C12:0 microemulsion.

Table 1. Droplet size of the reference microemulsion ME_E and 1-MAG microemulsions with ethanol as cosurfactant expressed as intensity weighted *z*-average diameter (nm) determined immediately and 6 months after preparation

	Droplet size (nm)	
1-MAG ME	After production	After 6 month
C10:0	20 ± 5	15 ± 0
C11:0	14 ± 2	n.d.
C12:0	18 ± 4	11 ± 4
C14:0	17 ± 4	10 ± 1
ME_E	15 ± 1	n.d.

n.d., not determined.
The results are expressed as means ± SDs ($n = 3$).

describing antibacterial activity of 1-MAGs applied in the form of ethanol solutions. Within the tested samples, 1-MAG C12:0 ME and 1-MAG C14:0 ME exhibited the strongest inhibitory effect on the growth of the gram-negative bacteria at the concentration of 1900 mg/L. In the presence of both MEs, total growth inhibition of all tested bacteria was observed. Moreover, in the presence of 1-MAG C12:0 ME, significant reduction of gram-negative bacteria was also detected at the concentration of 1000 mg/L, which totally inhibited *C. freundii*, *E. coli* and *S. enterica*. Conversely, Buňková et al. [31] who tested antibacterial properties of MAG C8:0 to C16:0 against gram-negative bacteria observed the best inhibition effect for MAG C10:0 at the concentration of 1500 mg/L with no bacterial growth detected. The results revealed that *P. aeruginosa* was the least resistant to the inhibitory effects of 1-MAG MEs. As it can be seen in Fig. 3, more than 50% inhibition occurred at all tested concentrations of 1-MAG C12:0 ME and 1-MAG C14:0 ME. In addition, total growth inhibition was found at the highest concentrations of 1900 mg/L for 1-MAG C10:0 ME, 1-MAG C12:0 ME and 1-MAG C14:0 ME. On the contrary, *S.*

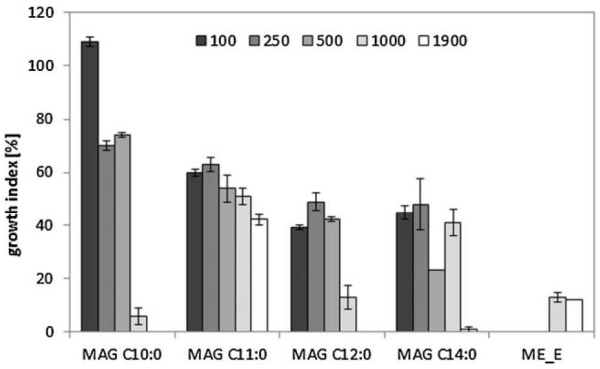

Figure 3. Values of GI of *Pseudomonas aeruginosa* CCM 3955 cultivated in the presence of the reference microemulsion ME_E and 1-MAG microemulsions (MAG C10:0, MAG C11:0, MAG C12:0, and MAG C14:0) at the concentrations of 100–1900 mg/L. The results are expressed as means ± SDs ($n = 3$).

marcescens subsp. *marcescens* was identified as the most resistant to antibacterial activity of tested MEs; it was only inhibited by 1-MAG C10:0 ME with concentrations above 1000 mg/L, causing 80% decrease in bacterial growth (Fig. 4). Additionally, the presence of 1-MAG C11:0 ME, 1-MAG C12:0 ME, and 1-MAG C14:0 ME at the concentrations ranging from 100 to 250 mg/L even increased GI compared to the positive reference. Altieri et al. [32] state that this effect is not rare and can be caused by increasing the permeability of cell membrane with a subsequent acceleration of exchange of substances and nutrients between the external environment and bacterial cells.

In summary, antibacterial activity higher than 60% was recorded for 1-MAG C10:0 ME, 1-MAG C12:0 ME, and 1-MAG C14:0 ME at concentrations ≥1000 mg/L against all

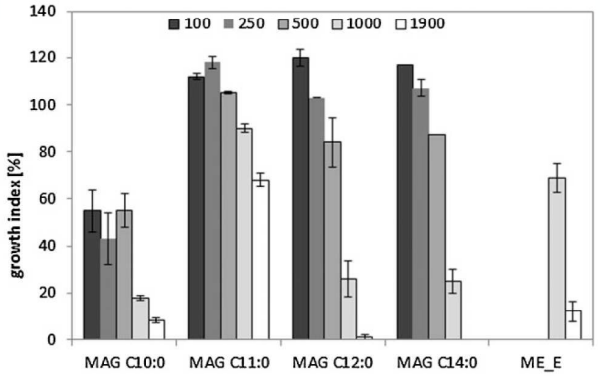

Figure 4. Values of GI of *Serratia marcescens* subsp. *marcescens* CCM 303 cultivated in the presence of the reference microemulsion ME_E and 1-MAG microemulsions (MAG C10:0, MAG C11:0, MAG C12:0, and MAG C14:0) at the concentrations of 100–1900 mg/L. The results are expressed as means ± SDs ($n = 3$).

studied bacteria. In comparison with study [31] where neat 1-MAGs were used, the antibacterial effect of 1-MAG MEs on gram-negative bacteria was improved.

3.4 The influence of 1-MAG microemulsions on gram-positive bacteria

As expected [32, 33], gram-positive bacteria were more sensitive to 1-MAG ME than gram-negative strains. This behavior is due to the differences in the structure of their cell wall [34]. In the study published by Růžička et al. [35] it has been reported that 1-MAG C10:0 and 1-MAG C12:0 were more efficient against gram-positive bacteria compared to gram-negative ones. This finding is consistent with results of the current study showing better inhibition of gram-positive bacteria in the presence of the 1-MAG ME. Figure 5 describes the values of GIs of *M. luteus* CCM 732. In this case, "no growth" was detected after the application of 1-MAG C10:0 ME and 1-MAG C11:0 ME at the concentrations down to 250 mg/L. Total growth inhibition was also observed when 1-MAG C12:0 ME was applied at the concentrations higher than 1000 mg/L.

Both species of the genus *Bacillus* showed similar behavior with respect to tested 1-MAG ME and total inhibitions were mostly detected in the presence of 1-MAG C12:0 ME; *B. cereus* CCM 2012 was inhibited even at the lowest tested concentration of 100 mg/L (Fig. 6). Correspondingly, 1-MAG C12:0 ME entirely inhibited *E. faecalis* CCM 4224. Generally, monolaurin ME showed satisfactory inhibition effect against almost all tested bacteria causing more than 80% reduction of their growth (Fig. 7). The only exception was *S. aureus* subsp. *aureus* CCM 3953 with a high growth activity (≥60%) after treatment with 1-MAG C12:0 ME in concentrations of 100, 250, and 500 mg/L. It follows that *S. aureus* subsp. *aureus* CCM 3953 was the most

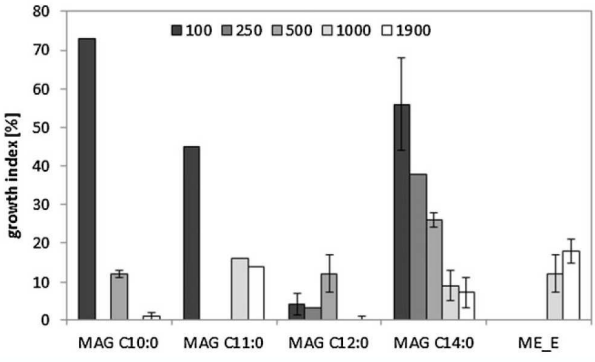

Figure 5. Values of GI of *Micrococcus luteus* CCM 732 cultivated in the presence of the reference microemulsion ME_E and 1-MAG microemulsions (MAG C10:0, MAG C11:0, MAG C12:0, and MAG C14:0) at the concentrations of 100–1900 mg/L. The results are expressed as means ± SDs ($n = 3$).

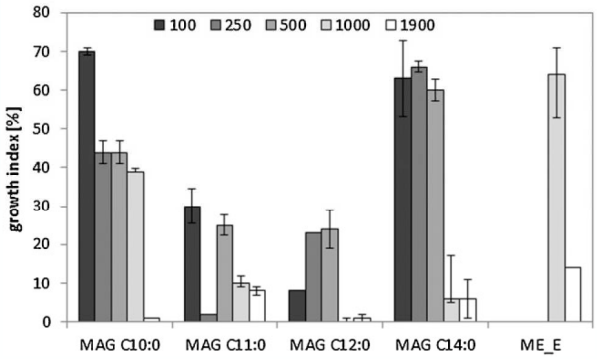

Figure 6. Values of GI of *Bacillus cereus* CCM 2012 cultivated in the presence of the reference microemulsion ME_E and 1-MAG microemulsions (MAG C10:0, MAG C11:0, MAG C12:0, and MAG C14:0) at the concentrations of 100–1900 mg/L. The results are expressed as means ± SDs ($n = 3$).

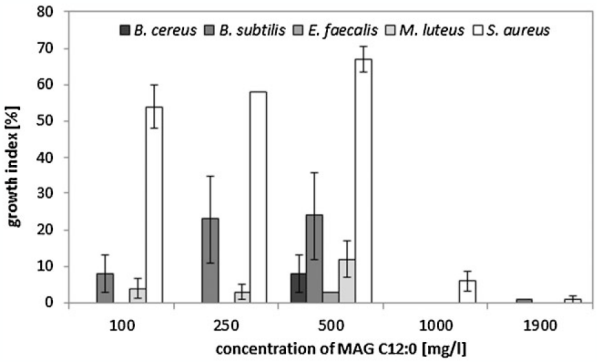

Figure 7. The effect of microemulsion of 1-MAG C12:0 on the growth of all tested gram-positive bacteria. The results are expressed as means ± SDs ($n = 3$).

resistant bacteria, which corresponds to conclusions from publication [31].

Although satisfactory antibacterial activity of 1-MAG ME with respect to gram-positive bacteria was observed in current work, 1-MAG solutions studied in ref. [31] were more efficient even at the concentrations down to 25 mg/L. In comparison with 1-MAG ME, application of 1-MAGs showed stronger inhibitory effect on the growth of *B. cereus* CCM 2012 and *B. subtilis* subsp. *subtilis* CCM 2216 (1-MAG C12:0 at concentration 25 mg/L, 1-MAG C11:0 at concentrations of 50–100 mg/L and 1-MAG C10:0 at 100–250 mg/L) as well as *E. feacalis* CCM 4224 (1-MAG C10:0 and 1-MAG C11:0 at concentrations ≥50 mg/L) [31]. Also Thormar et al. [34] concluded in their paper that all of saturated fatty acids with chain length in the range from C6:0 to C18:0, lauric acid (C12:0) had the highest antibacterial activity against the gram-positive bacteria.

Several studies [1, 29, 36] have reported that 1-MAG MEs enhanced the inhibition effect against microorganisms compared to 1-MAGs alone. However, in case of antibacterial efficacy of MEs against gram-positive bacteria, this general trend was not fully confirmed. The obtained results showed that the antibacterial activity of neat 1-MAGs is higher than the effect of 1-MAG MEs.

The antibacterial activity of tested 1-MAG ME is summarized in Table 2 in terms of minimal inhibition concentrations (MIC) and concentrations causing 50% growth reduction of bacterial cells. This overview confirms the best performance of 1-MAG C12:0 ME against both gram-negative and gram-positive bacteria.

The efficacy of formulated 1-MAG ME have to be discussed in the light of the fact that MEs alone can also possess antibacterial properties. The tests performed on ME_E revealed that MEs without 1-MAGs showed also inhibitory activity, especially at the highest used concentrations of ethanol. This can be mainly attributed to the fact that in MEs, ethanol is entrapped in surfactant capsule and is targeted to microorganisms in different physical form compared to ethanol in solution. It was reported [31] that ethanol solutions with concentrations up to 7% had no inhibition effect on the bacterial growth. As the ethanol content of 1.25% present in tested MEs is well below this limit, the antibacterial effect of ME_E is likely induced not only by ethanol and surfactant, but also by the particular character of MEs.

3.5 Mechanism of antibacterial activity of 1-MAGs and microemulsions

Monoacylglycerols are lipophilic substances and a primary target of their attack is the cytoplasmatic membrane of the cells. Although, antibacterial effects of 1-MAGs are well-known for a long time, the exact mechanism of their activity has not been fully recognized. Therefore, the several hypotheses have been suggested in order to explain this process. One of the theories describes 1-MAGs as the surface active substances which penetrate and incorporate into the cell membrane used as a permeable barrier. In this way, they cause membrane destruction and inhibit the transport of amino acids into the cells [34, 37, 38]. Using the electron microscope, Bergsson et al. [37] found out the disintegration of cytoplasmatic membrane and disruption of integrity of bacterial cell walls exposed to 1-MAGs. Another hypothesis suggests that 1-MAGs with a short and medium chain penetrate into bacterial cells where dissociate and cause the acidification of the cell contents. Reduction in the intracellular pH can lead to inactivation of the intracellular enzymes and inhibition of nutrients transport [39, 40]. The inhibition activity of 1-MAGs can be also influenced by other substances. Some compounds, namely albumin, phospholipids, starches or cholesterol can form the complexes with the MAGs and block their activity [38, 40]. Conversely, the inhibition effect of 1-MAGs can be increased using organic acids [41], high temperatures or chelating agents [38, 42].

Regarding the antibacterial activity of MEs as such, it was suggested that the structure of MEs is damaging to the bacterial cells and it adversely influences the function of the bacterial membranes [14]. Recently, the study was performed with the aim to clarify the kinetics of bacteria killing (*Candida albicans*, *Aspergillus niger*, *Schizosaccharomyces pombe* and *Rhodotorula spp.*) with MEs composed of Tween-80, *n*-pentanol, isopropyl myristate, and water. The results proved that the MEs effectively destroyed all the tested bacterial species. The mechanism of action was proposed relying in significant anti-membrane activity, causing disturbance and dysfunction of the cytoplasmic membrane, followed by cell wall changes, cytoplasmic coagulation, disruption of intracellular metabolism and cell death. However, the antibacterial effect of MEs cannot be separated from the effect of their individual components [43].

Table 2. Minimum inhibition concentrations of 1-MAG microemulsions and their concentrations causing 50% growth reduction of bacteria

	c (mg/L)			
	1-MAG C10:0	1-MAG C11:0	1-MAG C12:0	1-MAG C14:0
Gramnegative species				
Citrobacter freundii	1000 (*1000*)	>1900 (*1000*)	1000 (*1000*)	1900 (*1000*)
Escherichia coli	>1900 (*1000*)	>1900 (*>1900*)	1000 (*1000*)	1900 (*1000*)
Pseudomonas aeruginosa	1000 (*1000*)	>1900 (*500*)	1900 (*100*)	1900 (*100*)
Salmonela enterica	1900 (*1000*)	>1900 (*100*)	1000 (*1000*)	1900 (*1900*)
Serracia marcescens	1900 (*100*)	>1900 (*>1900*)	1900 (*1000*)	1900 (*1000*)
Grampositive species				
Bacillus cereus	1900 (*250*)	1000 (*100*)	1000 (*100*)	1000 (*1000*)
Bacillus subtilis	1900 (*1000*)	>1900 (*250*)	1000 (*100*)	>1900 (*500*)
Enterococcus faecalis	1900 (*1900*)	1900 (*1000*)	100 (*100*)	1900 (*1000*)
Micrococcus luteus	1000 (*250*)	250 (*100*)	100 (*100*)	1000 (*250*)
Staphylococcus aureus	1900 (*1000*)	>1900 (*1000*)	1000 (*1000*)	>1900 (*1000*)

In current work, the synergic effect of 1-MAG dissolved in ethanol encapsulated in surfactant was observed as well. Surfactant Tween 80, used for ME formation, was reported to enable inhibition of *P. aeruginosa* [44]. Although Tween 80 exhibits limited antimicrobial activity as such, as to mechanism of action, it can increase cell permeability and by this way enhance the antimicrobial action of accompanying antimicrobial agents [45]. Hence, the antibacterial activity of ME_E without 1-MAG can combine effect of Tween 80 as a membrane disrupter and ethanol as an antimicrobial agent. The 1-MAGs present in ME then act in synergy with the other two components and further enhance their antibacterial effect.

3.6 Cytotoxicity of 1-MAG microemulsions

The results presented in Table 3 clearly show that tested MEs can be cytotoxic to the NIH/3T3 cells. As this cell line is one of the most frequently used lines for the purposes of cytotoxicity determination, the results can be also generalized to other cell lines. There are two important outcomes of the study, which can be summarized as follows: (i) The cytotoxicity was dose dependent and all MEs with 1-MAG contents above 10 mg/L showed similar cytotoxicity, which can be classified as *severe*. At concentrations lower than 10 mg/L, the cytotoxicity decreased, MEs exhibited *moderate cytotoxicity* and at concentration of 0.5 mg/L, 1-MAG C10:0 ME a 1-MAG C11:0 ME showed even *mild cytotoxicity*, corresponding to higher than 60% cell survival. (ii) The cytotoxicity can be prevailingly assigned to the effect of MEs formulated as particular systems but not to influence of 1-MAGs alone. This is obvious from the behavior of reference ME_E containing only ethanol and Tween 80, which showed similar cytotoxicity profile as 1-MAG ME. These results, together with the information about the MEs particle size lower than 25 nm are crucial in explanation of the observed effects. It is well known, that ethanol has significant impact on the fibroblast metabolism and cell death. Its effect is time and concentration dependent [46]. Tween 80 is considered as low-cytotoxic, however the concentration of 210 mg/L was reported to cause the 50% inhibition of the cell growth as find out by the MTT assay [47]. Similarly as for bacterial cells, 1-MAGs together with ethanol encapsulated in the nano-sized particles can be responsible for cytotoxicity of MEs and can be a reason for their limited applications in tested concentration range.

4 Conclusions

The study was focused on the preparation of stable 1-MAG MEs and testing their antibacterial activity against the growth of gram-positive and gram-negative bacteria. The obtained data were compared to the results of study dealing with the inhibition effects of 1-MAGs alone, which were not entrapped in ME droplets. Additionally, the study was also aimed at the determination of cytotoxicity of MEs on NIH/3T3 mouse fibroblast cell line.

In the first step, pseudo-ternary phase diagrams were constructed using various types of co-surfactants (ethanol, propanol, butanol, and pentanol) and different oil/surfactant ratios in order to determine the area of miscibility of all components, oil (1-MAG solution in alcohol), surfactant (Tween 80) and water. It was found that the area of limited miscibility in pseudo-ternary diagrams increased with an increasing chain length of alcohol, used as the co-surfactant. Testing of antibacterial efficacy revealed that 1-MAG ME at

Table 3. Cytotoxicity of the reference ME, ME_E and MAG MEs reported as means ± SDs ($n = 4$) and according to requirements of ISO 10993-5

	1-MAG C10:0		1-MAG C11:0		1-MAG C12:0		1-MAG C14:0		ME_E	
c (mg/L)	Mean ± SD	ISO	Mean ± SD	ISO	Mean ± SD	ISO	Mean ± SD	ISO	Mean ± SD	ISO
2300	0.17 ± 0.01	23	0.19 ± 0.01	26	0.17 ± 0.01	24	0.18 ± 0.00	25	0.17 ± 0.02	23
1900	0.18 ± 0.01	25	0.18 ± 0.02	25	0.18 ± 0.01	25	0.19 ± 0.02	26	0.20 ± 0.00	27
1000	0.18 ± 0.01	25	0.18 ± 0.00	25	0.18 ± 0.01	24	0.19 ± 0.02	27	0.21 ± 0.02	29
500	0.19 ± 0.01	26	0.19 ± 0.01	25	0.19 ± 0.01	25	0.19 ± 0.01	27	0.21 ± 0.03	30
250	0.20 ± 0.01	27	0.19 ± 0.01	26	0.19 ± 0.02	26	0.19 ± 0.01	26	0.21 ± 0.02	29
100	0.20 ± 0.01	27	0.21 ± 0.02	29	0.21 ± 0.02	29	0.19 ± 0.02	26	0.19 ± 0.01	26
50	0.25 ± 0.03	34	0.23 ± 0.01	31	0.24 ± 0.02	32	0.23 ± 0.02	32	0.32 ± 0.07	43
10	0.37 ± 0.06	50	0.34 ± 0.04	46	0.41 ± 0.03	55	0.37 ± 0.04	51	0.38 ± 0.05	51
5	0.42 ± 0.06	57	0.47 ± 0.07	64	0.32 ± 0.04	43	0.42 ± 0.08	57	0.46 ± 0.05	63
1	0.39 ± 0.04	53	0.52 ± 0.03	70	0.35 ± 0.07	48	0.43 ± 0.01	58	0.48 ± 0.01	65
0.5	0.44 ± 0.04	60	0.46 ± 0.03	63	0.36 ± 0.01	49	0.36 ± 0.02	49	0.48 ± 0.07	65
Reference	0.73 ± 0.12	100	0.73 ± 0.12	100	0.73 ± 0.12	100	0.73 ± 0.12	100	0.73 ± 0.12	100

According to requirements of ISO 10993-5: cytotoxicity equal to 100 corresponds to 100% cell survival values of >80 are assigned to no cytotoxicity, 60–80 mild cytotoxicity, 40–60 moderate toxicity, 40 severe cytotoxicity.

the concentrations between 1000 and 1900 mg/L improved inhibition effects against gram-negative bacteria compared to the corresponding concentrations of 1-MAGs alone. At the concentrations below 1000 mg/L, the antibacterial activity of both systems was comparable. Conversely, the inhibition effect of 1-MAG MEs on more sensitive gram-positive bacteria was lower than in case of 1-MAGs alone. The best antibacterial activity, i.e. the most effective restriction of bacterial growth, was observed after application of 1-MAG C12:0 MEs. The antibacterial effect of MEs can be assigned to synergic action of Tween 80, ethanol and 1-MAGs and adjustment of formulation may further improve their current efficacy. MTT assay revealed significant cytotoxicity of all studied MEs including reference sample, in which 1-MAG was absent. For all tested MEs, the 1-MAG concentration of 10 mg/L can be considered as a limit for *moderate cytotoxicity*, which represents 40–60% cell survival.

The particle size of 1-MAG MEs measured by PCS was in the range of 12–20 nm. All prepared samples showed optical transparency, long-term stability and the presence of one particle population.

This article was written with support of Operational Program Research and Development for Innovations co-funded by the European Regional Development Fund (ERDF) and national budget of Czech Republic, within the framework of project Centre of Polymer Systems (CZ.1.05/2.1.00/03.0111). The support of an internal grant of TBU in Zlin, IGA/FT/2013/016 financed from funds of specific academic research is also appreciated. Authors would like to express their gratitude to Mrs. Markéta Křížová for technical assistances.

The authors have declared no conflict of interest.

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PUBLICATION IV

Supercritical Assisted Atomization of 1-MAG/PVA microparticles

Sevcikova Petra, Adami Renata, Kasparkova Vera, Reverchon Ernesto,
Sedlacek Tomas, Pastorek Miroslav
Journal of Supercritical Fluids (under review)

Supercritical Assisted Atomization of 1-MAG/PVA microparticles

Ševčíková Petra [a*], Adami Renata [b], Kašpárková Věra [a,c], Reverchon Ernesto [b], Sedláček Tomáš [c], Pastorek Miroslav [d]

[a] Department of Fat, Surfactant and Cosmetics Technology, Faculty of technology, Tomas Bata University in Zlin, nám. T. G. Masaryka 275, 762 72 Zlin, Czech Republic

[b] Department of Industrial Engineering, University of Salerno, Via Ponte Don Melillo, 84084 Fisciano (SA), Italy

[c] Centre of Polymer Systems, University Institute, Tomas Bata University in Zlin, Nad Ovcirnou 3685, 760 01 Zlin, Czech Republic

[d] Department of Polymer Engineering, Faculty of technology, Tomas Bata University in Zlin, nám. T. G. Masaryka 275, 762 72 Zlin, Czech Republic

Abstract

The optimum composition of O/W emulsions, consisting of 1-monoacylglycerol (1-MAG) and polyvinylalcohol (PVA) for the production of microparticles by means of supercritical assisted atomization (SAA) were studied, as well as the micronization conditions were established. The effect of the different 1-MAG/PVA ratio and the emulsion preparation method on the size, stability, and morphology of microparticles was investigated by microscopy, photon correlation spectroscopy, different scanning calorimetry and X-ray diffraction. The results showed that the emulsion composition and emulsification method

* Corresponding author. Tel: +420 576 031 535; fax: +420 577 210 172.
E-mail address: psevcikova@ft.utb.cz (P. Sevcikova)

considerably affected the sizes of microparticles. The smallest 1-MAG/PVA microparticles were determined at lower 1-MAG (<30 mg/ml) and PVA (6 mg/ml) concentrations which were prepared from sonicated emulsions. The emulsions containing 20 or 30 mg/ml 1-MAG provided the highest recovery of the microparticles with the yields of 50–60 %. All prepared 1-MAG/PVA microparticles were spherical with the diameter from 0.5 to 3 μm and had a semi-crystalline structure.

Keywords: emulsion; microparticle; monoacylglycerol; polyvinylalcohol; supercritical assisted atomization.

1. INTRODUCTION

Recently, a growing interest in the application of 1-monoacylglycerols in variety industrial fields, such as food and cosmetic industry [1,2], textile, packaging, plastic processing and construction materials has appeared [3]. MAG have attracted considerable attention due to their simple structure, availability and chemical stability [4] as well as their biocompatibility, biodegradability and absence of irritation potential [5,6]. They are also characterized by a relatively high antimicrobial activity, mainly against gram-positive bacteria and fungi [4]. Their inhibition effect depends on the number of carbon atoms and on the presence of double bonds in their fatty acid chain [7]. Moreover, owing their amphiphilic properties they are well-known emulsifiers and stabilizers of water in oil (W/O) emulsions [8]. Further applications include their use in pharmaceutical industry as drug delivery systems, solubilizers and absorption enhancers [9]. Nowadays, advanced drug delivery requires control of physicochemical properties of these systems including the particle size, shape, density, surface properties and crystalline purity. All these characteristics are the key factors to achieve the bioavailability and effectiveness of pharmaceuticals. For this reason, enormous

research efforts have been devoted to develop efficient micronization technologies [10]. Traditional techniques used for the microparticle production include for example, spray drying, spray freeze drying and jet milling [11,12]; however, all these methods show some drawbacks. Because of too high operating temperatures, spray drying and jet milling are not suitable for treatment of thermolabile compounds and moreover they are not able to produce powders with narrow and controlled particle size distributions [13,14]. Due to lipidic nature causing their easy decomposition at increased temperatures, also 1-MAGs belong to substances not suited for the encapsulation with the above mentioned methods. Therefore, another possible micronization processes capable of 1-MAG treatment were studied by Chiewpattanakul et al. [15]. In their work, dextran-covered nanoparticles encapsulating monomyristin (1-MAG C14:0) were produced by emulsion/solvent evaporation method and by nanoprecipitation. While the first procedure can be considered as a rather general method, nanoprecipitation needs specific conditions in terms of solvent temperature and concentration to provide satisfactory results in terms of particle size and distribution [15]. Problems faced by traditional processes can be overcome by supercritical fluid based technologies, which have been proposed as an alternative to conventional microencapsulation technologies [16,17]. Among them, the rapid expansion of supercritical solutions (RESS) [18,19], the particles generation from gas saturated solutions (PGSS) [20], the supercritical antisolvent precipitation (SAS) [21] and the supercritical assisted atomization (SAA) are included [22,23].

Supercritical Assisted atomization (SAA) developed by Reverchon [22] is a method based on the solubilisation of controlled quantities of supercritical carbon dioxide (SC-CO_2) in liquid solutions [24,25]. The prepared solution is atomized through an injector in precipitator to obtain droplets [26]. The following two-step atomization process is used. In the first step, the formation of the primary droplets at the exit of the injector is realized, which is induced by

pneumatic effect. During the second stage, decompressive atomization takes place, caused by fast expansion of the SC-CO_2 from the inside of the primary droplets under forming the secondary ones [27]. In this way, smaller droplets are obtained compared to traditional spray drying techniques [28]. Microparticles are obtained by droplet evaporation using warm nitrogen [29]. One of the major advantages of this technique is the possibility to use organic or inorganic solvents which results in possibility to process both water-soluble as well as non-water-soluble systems [24]. In addition, this process is capable of providing micron- and submicron-sized particles with controlled particle size ranging between 0.05 and 5 μm. These characteristics, joined to the green and not aggressive processes with respect to the substances treated, allowed this technique effectively and successfully micronize a wide range of compounds [24,30]. SAA technique has been successfully used in micronizing of pharmaceuticals [13,14,26,30,31], polymers and copolymers [32–34]. Moreover, this technique has been studied for the production of protein microparticles containing the bovine serum albumin [29] or lysozymes [35]. Although several studies have been reported on application of SAA in the preparation of microparticles from various types of solutions and coprecipitates containing drugs, polymers and proteins, only small attention has been devoted to the production of microparticles from emulsion based systems.

In order to expand knowledge in this field of interest, the primary aim of this work was to prepare 1-monoacylglycerol/polyvinyl alcohol (1-MAG/PVA) microparticles using SAA from O/W emulsions. The conditions for SAA process were established and prepared 1-MAG/PVA microparticles were thoroughly characterized using relevant analytical methods. The effect of different 1-MAG/polymer ratio and the emulsion preparation method on the particle size, stability, morphology and loading of obtained powders were examined using optical and electron microscope, photon correlation spectroscopy (PCS), different scanning calorimetry (DSC), X-ray diffraction (XRD), and high pressure liquid chromatography (HPLC).

The potential of this new approach is the possibility to obtain microparticles of coprecipitates of hydrophobic active substances and hydrophilic polymer carrier. This is a great advantage to increase the bioavailability of pharmaceutical compounds.

2. EXPERIMENTAL

2.1 Materials and methods

Polyvinyl alcohol (PVA, MW: 30.000–70.000, hydrolysis of 87–90%) and ethylacetate (EA, purity 99.8%) were purchased by Sigma-Aldrich (Milan, Italy). Monoacylglycerol of capric acid (1-MAG C10:0) was obtained from Tomas Bata University in Zlin and used as supplied [36]. Carbon dioxide (CO_2, purity 99 %) was supplied by SON (Naples, Italy) and nitrogen (N_2, purity 99 %) by SOL (Caserta, Italy).

2.2 Emulsion preparation

Emulsions of O/W (ratio 10/90 w/w) were prepared using two different emulsification methods. In the first one, emulsifications were carried out using high-speed stirrer (mod. L4RT, Silverson Machines Ltd., Waterside, Chesham Bucks, UK). The known amount of 1-MAG C10:0 was dissolved in EA to form an oil phase. Then, the oil phase was added drop-wise to the EA-saturated aqueous PVA solution (0.9% w/w EA in water) under continuous stirring and the emulsion was formed. All experiments were run at the room temperature (25 °C) and the stirring rate was controlled at 4000 rpm for 4 min.

For comparison, emulsions were also produced *via* sonication using the Digital Sonifier (model S-450D, Branson Ultrasonic Corporation, Danbury, Connecticut, USA). Water phase and oil phase were poured into the vessel and sonicated at 20 kHz for 2 minutes. The emulsions prepared using sonication were studied and compared with those formed by stirrer.

Two different concentrations of PVA (6 and 10 mg/ml) and five various concentrations of 1-MAG C10:0 (0, 10, 20, 30 and 40 mg/ml) were applied through the study. As PVA and 1-MAG are, by their nature, surface active agents, resulting values of hydrophilic lipophilic balance (HLB) of surfactant mixtures were calculated using equation $HLB = x_1 \times HLB_1 + x_2 \times HLB_2$, where x_1; x_2 are volume fractions of surfactants with HLB_1 and HLB_2. HLB values of 18 and 6.5, given by surfactant suppliers, were used for PVA and 1-MAG C10:0, respectively [37, 38].

2.3 Supercritical Assisted Atomization (SAA) process

Experiments leading to microparticle production were performed on SAA in-house apparatus (University of Salerno, Italy) which is schematically describe in details in [31]. It consists of three feed lines, the first for delivering supercritical carbon dioxide (SC-CO_2), the second for liquid solution to be micronized and the third for inert gas (warm nitrogen), as well as of three main vessels: saturator (Sa), precipitator (Pr) and condenser (Co). Firstly, the liquid CO_2 is taken from a cylinder and sent to the high-pressure pump where it is pressurized. Then it continues to a heated bath and finally to the saturator where it solubilises into the liquid solution. The saturator is a high pressure vessel (internal volume of 0.05 dm^3) loaded with a stainless steel perforated saddles, which provides a large contact surface and an adequate residence time allowing the dissolution of SC-CO_2 in the liquid solution. Then, the expanded liquid solution obtained in saturator is sprayed through a thin wall (80 μm internal diameter) injection nozzle into the precipitator operating at near atmospheric pressure. To facilitate the evaporation of liquid droplets, a controlled flow of N_2 is heated in electric heat exchanger (S1) and delivered into the precipitator. The saturator and the precipitator are electrically heated by thin band heaters. The dry powder is collected with a 0.1 μm pore size stainless steel filter located at the bottom of the drying chamber, whereas the gaseous stream passes

through the filter and reaches the condenser where the liquid is separated from the gas mixture (CO_2 and N_2).

The list of micropaticle samples prepared by SAA micronization process is provided in Tab. 1 and the samples are denoted through their 1-MAG and PVA content as follows.

2.4 Emulsion droplets and microparticle morphology

Microscopic observation of emulsion droplets was performed using an optical microscope Olympus (model BX 50, Tokyo, Japan) immediately after their preparation. The morphology of the produced 1-MAG/PVA microparticles was evaluated by a scanning electron microscope (SEM, mod. LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany). The powders were dispersed on a carbon tap previously stuck to an aluminum stub. Samples were coated with gold (layer thickness of 25 nm) using a sputter coater (mod. 108A, Agar Scientific, Stansted, UK).

2.5 Measurement of particle size distribution

The particle size, particle size distribution (PSD) and polydispersity indexes (PDI) of emulsions used for micronization were determined by photon correlation spectroscopy (PCS) immediately after their preparation. For this purpose, Nanosizer Nano ZS instrument (Malvern Instruments, UK) equipped with a He-Ne laser operating at 4.0 mW and 633 nm was used. The measurements were carried out at a scattering angle of 173 ° and at the constant temperature of 25 °C.

The obtained 1-MAG/PVA microparticles were characterized by PCS as well. Samples were prepared by dispersion of 2 mg micronized powder in 1 ml ethylacetate followed by 30 min sonication.

2.6 Solid state analysis

DSC measurements were performed using a differential scanning calorimeter (model DSC1-Star^e System, Mettler Toledo). Samples of 1-MAG/PVA microparticles (2–3 mg) were accurately weight, placed in aluminium pans, sealed and analyzed using the following temperature program: 1) cooling down to –40 °C with an isothermal hold for 5 minutes 2) heating from –40 °C to 240 °C with hold for 3 minutes 3) cooling from 240 °C to –40 °C with hold for 5 min at the temperature and 4) repeated heating from –40 °C to 240 °C. Heating rate of 20 K/min under nitrogen purge of 40 ml/min was applied. Instrument calibration was done with indium. The resulting DSC data were evaluated and peak melting temperature (T_m), peak onset temperature (T_{onset}), heat of fusion ΔH_m and glass transition temperature (T_g) were recorded.

2.7 X-ray analysis

Wide-angle X-ray diffraction patterns were obtained using PANalytical X´Pert PRO (Holland) with theta-theta goniometer, Ni-filtered CuKα radiation (0.1542 nm) and PIXcel detector. All the measurements were operated at ambient temperature in reflection mode, at 40 kV and 30 mA with automatic divergence and anti-scatter slits in the range of angles from 5 to 95° (2θ) and step size 0.0263°. Counting time 42.8 s per step was used. Measurements of studied 1-MAG/PVA microparticles were performed on two samples with the same composition but prepared by two independent atomization processes to evaluate the reproducibility of the production method.

2.8 Loading of 1-monoacylglycerols in microparticles

The quantification of 1-monoacylglycerol in 1-MAG/PVA microparticles was performed using a modular high performance liquid chromatography system consisting of Waters 600E

pump, UV-VIS detector (UV 200, DeltaChrom) 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 205 nm was employed. Two different methods were used in order to properly determine 1-MAG concentration in microparticles and assure complete dissolution of the sample; 1) A known quantity of the tested sample (5 mg/ml) was dissolved for 20 h at 50 °C in ethanol/water (2/1) mixture. The mobile phase consisting of acetonitrile/water/acetic acid (90/10/0.1) at a flow rate 1ml/min was used for each analysis. 2) The powder was dissolved in dimethylsulfoxide/water (1/4) mixture under above given conditions and the HPLC analysis was conducted with the mobile phase containing acetonitrile/water/acetic acid (70/30/0.1).

3. RESULTS AND DISCUSSION

3.1 Optimization of emulsions prior to micronization

Two different emulsification methods (high-speed stirring and sonication) were used to prepare stable O/W emulsions and compare their characteristics such as appearance, particle size, particle size distribution and stability with the goal to find out an optimal emulsion system for the micronization by SAA.

The results showed that the emulsions prepared by the sonication appeared to have more favourable characteristics, such as smaller dispersed oil droplets that were distributed more uniformly in the continuous water phase of the emulsion compared to those produced by stirring (Fig. 1). These data correspond to the results obtained by Lin et al. [39] who prepared two-phase W/O and three phase O/W/O emulsions using ultrasonically vibrating and mechanically homogenizing methods and compared them in terms of particle size, viscosity and stability. They found out that the ultrasonically vibrating method is more efficient for the preparation of both types of emulsions and provides systems with more suitable

characteristics in terms of smaller droplets and more uniform droplet distribution. In addition, the sonicated emulsions studied in current work possessed a grey colour in appearance, indicating presence of small droplets, without foam on the top of the emulsion. All the produced emulsions were stable at least one hour with non-coalescing droplets, as illustrated by two optical microscope images reported in Fig. 1a, and 1b.

3.2 SAA micronization

Emulsions containing different ratios of 1-MAG/PVA prepared by stirring and sonication were micronized using SAA under identical process parameters. The experimental conditions of SAA as well as the parameters of emulsions containing 10 mg/ml of PVA and different 1-MAG concentrations produced by high-speed stirring can be seen in Tab. 2. From the table it is clearly visible that the yield of microparticles is affected by the 1-MAG/PVA ratio. The lowest microparticle recovery of 18 % was detected in the sample denoted M0/P10, containing only pure PVA without addition of 1-MAG. Conversely, the highest yields ranged between 50–60 % and were observed in samples containing 20 or 30 mg/ml of 1-MAG. Further, a slight decrease in powder recovery to 40 % is seen with an increasing 1-MAG concentration to 40 mg/ml. The identical trends in terms of powder recovery were also found after micronization of emulsions prepared by the same emulsification method (homogenization) with lower PVA concentration (6 mg/ml) as well as emulsions produced by sonication. (data not presented)

3.3 1-MAG/PVA microparticles

Spherical 1-MAG/PVA microparticles with the diameters ranging between 0.5–3 μm having well-defined boundaries were obtained in all experiments, which is qualitatively shown in the SEM image reported in Fig. 2. Despite using the same magnification (10 K), a quantitative

evaluation of the change in particle size with the various concentrations of 1-MAG and PVA wasn't possible. In order, to investigate the effect of PVA and 1-MAG concentrations as well as the different emulsification method on the size and distribution of the prepared microparticles, systematic measurements were performed using PCS after particle re-dispersion in ethylacetate. The corresponding distribution curves, expressed in terms of number of particles or particle volume, are given in Figs. 3 and 4, respectively.

3.3.1 Effect of PVA and 1-MAG concentration

The influence of various 1-MAG/PVA ratios on the particle size and PDI is clearly visible in Fig. 3 and 4. The similar trends in changes, i.e., increase of particle size and PDI, was observed with growing concentrations of both components, regardless of the preparation method of emulsions. The concentration effect of 1-MAG is more evident in Fig. 3 depicting a shift of distributions towards larger particle diameters when 1-MAG concentration increased. It is also remarkable that the size distributions became broader as the 1-MAG concentration grew, which is especially seen on 1-MAG with concentration of 40 mg/ml. Conversely, when less amount of 1-MAG was processed, the number of small particles increased, while a few large particles were produced under micronization.

Similar results in terms of increasing of particle sizes and broadening distributions were detected at 1-MAG/PVA microparticles produced at various PVA concentrations. Fig. 4 gives the particle size distributions calculated on an integral basis and shows the data regarding the volume percentage of the particles produced. Volume based curves illustrates that the microparticles with lower PVA concentration (6 mg/ml) were smaller in their sizes and that their distributions were narrower in comparison with those containing the higher PVA concentration of 10 mg/ml, irrespective of the emulsification method used.

The compositional change in particle sizes and distributions during micronization is a typical phenomenon observed in various materials processed by SAA [24,28,40,41]. This fact can be explained considering physical characteristics of parent solution undergoing micronization process, such as its viscosity and surface tension. An increase of solution concentration causes a rise of the viscosity as well as surface tension of emulsions, resulting in the formation of larger primary particles, which subsequently influences the dimension of the secondary particles.

3.3.2 Effect of the emulsification method

The emulsion preparation method was evaluated as another important parameter affecting the particle size, particle size distribution and polydispersity index of resulting microparticles. It was found that the 1-MAG/PVA microparticles micronized by SAA from O/W emulsions prepared by sonication were much smaller in their sizes than those produced using a high-speed stirrer (Fig. 5). In addition, they also possessed narrower particle size distribution with PDI in the range of 0.24–0.32 compared to the microparticles produced by stirring (PDI = 0.34–0.69). However, these results could be expected due to the results obtained from the optical microscopy which was performed on freshly prepared emulsions prior their micronization.

These data correspond to the conclusions presented in publication [42] saying that the use of the sonicator for emulsification leads to the smaller droplet sizes while obtaining more homogenous samples in comparison with the conventional mechanical processes.

3.4 Solid state characterization

3.4.1 X-ray

X-ray analysis provided information about crystallinity of the obtained powders. Fig. 6 illustrates X-ray spectra of raw materials (1-MAG, PVA) as well as 1-MAG/PVA

microparticles processed by SAA. The presence of sharp peaks, clearly notable in the spectra, indicate the crystalline structure of raw 1-MAG C10:0 (Fig. 6, curve labelled a). This is not surprising, as crystalline properties of 1-MAG are known and the presence of soft, layered crystals is typical in solid state. From the same figure, it is obvious that raw PVA has semi-crystalline structure (curve labelled b). Correspondingly, the microparticles consisting of PVA and 1-MAG (curves labelled c and d), prepared by SAA under the same conditions but with different monoacylglycerol/polymer ratio (1/2 and 1/4), possess also semi-crystalline structure similar to that of raw PVA. Nevertheless, X-ray data proved that the 1-MAG/PVA microparticles show higher crystallinity than the raw PVA, which is clearly visible from the Tab. 3, as well as from the shape of the peaks in XRD spectra. The X-ray analyses therefore indicate that the 1-MAG/PVA microparticles preserved the semi-crystalline structure of the parent PVA. These results are not quite common with respect to solid-state properties of particles conventionally obtained by SAA process, which are typically amorphous [28–30, 41]. Only rarely, formation of crystalline particles *via* SAA micronization was reported [32, 43]. This phenomenon might be explained by very fast crystallization rate, being much higher than the rate of SC-CO_2 evaporation; therefore, the molecules are allowed to arrange their structure before the drying process is completed. Reproducibility of SAA micronization process was very good because the samples of the same composition, produced by two independent atomization processes exhibited almost the same crystallinity, differing only in the order of tenths of a percent (Tab. 3, values given in parentheses).

3.4.2 Different scanning calorimetry

Different scanning calorimetry (DSC) serves as an efficient tool for the characterization of solid state of materials. Therefore, 1-MAG/PVA microparticles were also analysed by DSC to gain further insight in structure of prepared powders, which may supplement the XRD results. The DSC thermograms recorded during the first heating on melting of 1-MAG/PVA

microparticles after micronization are reported in Fig. 7. It is seen that the DSC curves exhibit two notable thermal events, similar for all tested samples. The first one, positioned at the temperature range of 40–65 °C can be assigned to glass transition (T_g) and the second, a broad endothermic peak present at about 189 °C, can be considered as the melting temperature (T_m) of the tested samples. Though the peak melting temperature is roughly similar for all the samples, the heat of fusion (ΔH_m) of the obtained microparticles is changing. While the microparticles containing only PVA show $\Delta H_{m(H\ M0/P10)}$ = –91.7 J/g, the 1-MAG/PVA microparticles have heat of fusion lower, being $\Delta H_{m(H\ M10/P6)}$ = –70.2 J/g and $\Delta H_{m(H\ M30/P6)}$ = –63.1 J/g. It is hence obvious that the ΔH_m values decrease with an increasing 1-MAG content. This observation suggests that 1-MAG can act as a plasticizing agent, disturbing the crystalline structure of polyvinylalcohol. The same phenomenon is also observed during the second heating and both cooling steps, as can be seen in Tab. 4. As already mentioned above, the DSC measurements performed on SAA processed samples revealed the presence of glass transitions (T_g). Their occurrence is typical for semi-crystalline materials due to the certain content of amorphous phase in their structure. The T_g values are at around 45 °C during the first heating and 57 to 70 °C during the repeated heating, here showing a slight shift towards higher temperatures with an increasing 1-MAG concentration.

However, the melting processes occurring during the second heating step are difficult to assign the properties of micronized powder, as during the first heating to temperatures above the melting point of both 1-MAG and PVA, the particular structure is destroyed.

X-ray data illustrates slight increase in crystallinity of PVA particles after 1-MAG was added. This was not fully confirmed by DSC measurements, as the highest 1-MAG content provided the lowest recorded heat of fusion ΔH_m. This discrepancy can be caused by the different treatments of the samples prior to analyses. Whilst XRD analysis was carried out at ambient

temperature, the DSC measurements started first after cooling the sample down to –40 °C followed with annealing for 5 minutes at this temperature. However, both techniques show that the 1-MAG/PVA microparticles are semicrystalline with the most of the crystallinity retained from PVA polymer.

3.5 1-monoacylglycerol loading and encapsulation efficiency

Two different HPLC methods were used to detect the amount of loaded 1-MAG in micronized samples. On the basis of experimental data it was found that both techniques are able to reliably determine the quantity of 1-MAG in microparticles, which was mostly lower than 15% in all studied systems. These results show a relatively low encapsulation efficiency of tested systems, nevertheless since it was a pilot study dealing with the new modification of SAA process, the micronization of 1-MAG/PVA microparticles from O/W emulsion systems, the results can be further improved.

4. CONCLUSION

The main purpose of the study has been to optimize composition and properties of emulsion systems (O/W) for the production of 1-MAG/PVA microparticles using supercritical assisted atomization (SAA) and to establish the suitable conditions for the micronization process. The obtained 1-MAG/PVA microparticles were then characterized by common analytical techniques, such as optical and electron microscopy, photon correlation spectroscopy (PCS), different scanning calorimetry (DSC), X-ray diffraction (XRD), and high pressure liquid chromatography (HPLC) in order to determine the effect of the emulsion composition and preparation method on the final particle sizes, structure or encapsulation efficacy.

The overall results state that the sonication was more suitable emulsification method for the preparation of O/W emulsions intended for micronization by SAA than the high-speed

stirring. The main reasons were their more favourable properties in terms of smaller dispersed oil droplets and uniform droplet distribution in aqueous phase.

Generally, the 1-MAG/PVA microparticles obtained by SAA possessed spherical shape with the diameter in the range of about 0.5–3 μm. The size of the microparticles was affected by properties of parent emulsions, namely by the 1-MAG/PVA ratio as well as by the emulsification method. Regarding influence of composition, the similar trend, i.e., an increase in particle sizes and a shift of distributions towards larger particles was observed with growing concentrations of both PVA and 1-MAG, regardless of the emulsion preparation method applied. Moreover, the 1-MAG concentration in emulsion also influenced the recovery of the obtained powders. The highest yields, ranging between 50–60 %, were found in samples containing 20 or 30 mg/ml of 1-MAG. The emulsification method was another important parameter influencing the particle size and PDI and it was found that the microparticles micronized from sonicated emulsions were smaller in their sizes and showed narrower distributions than those prepared from homogenized emulsions.

It is worth noting, that the tested 1-MAG/PVA microparticles had semi-crystalline structure similar to the structure of not micronized, raw PVA. However; the crystallinity of prepared microparticles was higher compared to raw PVA. This effect was especially observed in particles with the higher 1-MAG concentrations. Additionally, the DSC measurements also confirmed the occurrence of this type of structure in microparticles *via* determining their glass transitions which are typical for semi-crystalline materials.

Acknowledgement:

This article was written with support of an internal grant of TBU in Zlin, IGA/FT/2014/004 financed from funds of specific academic research.

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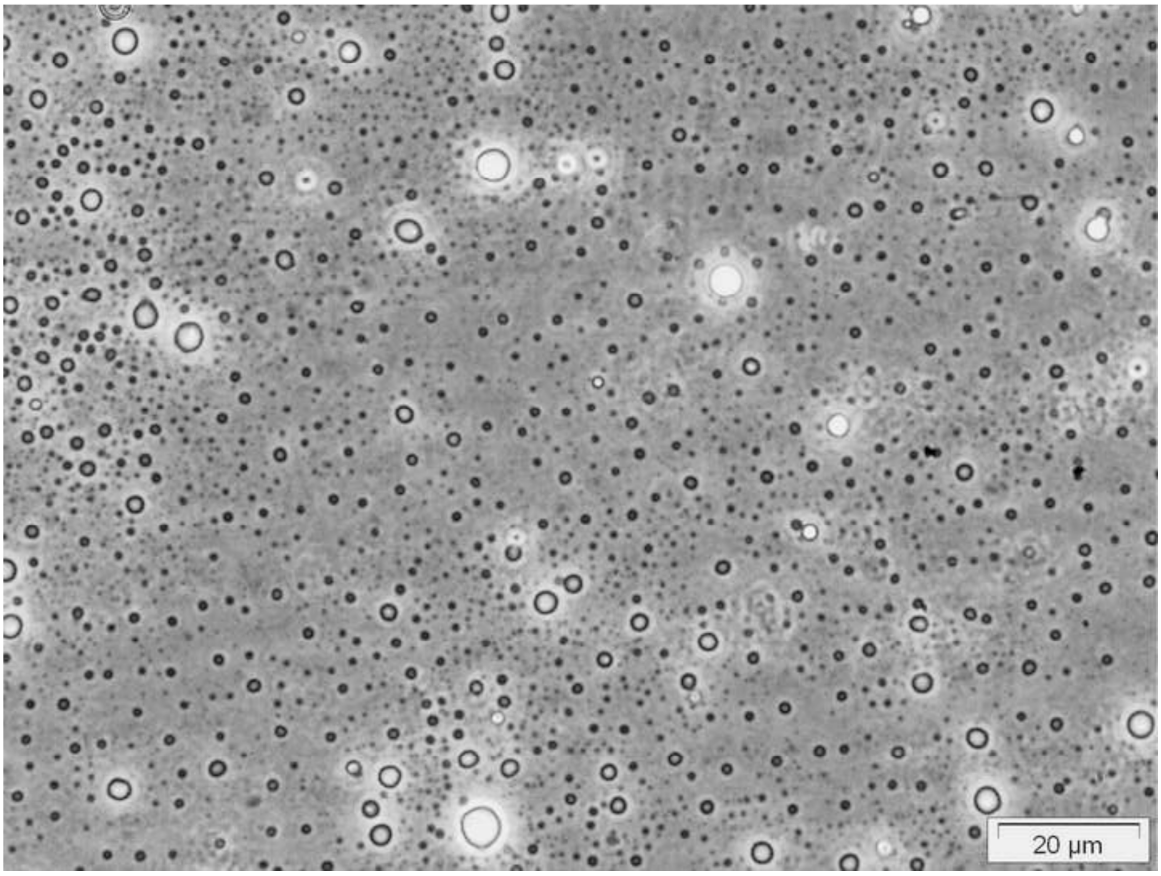

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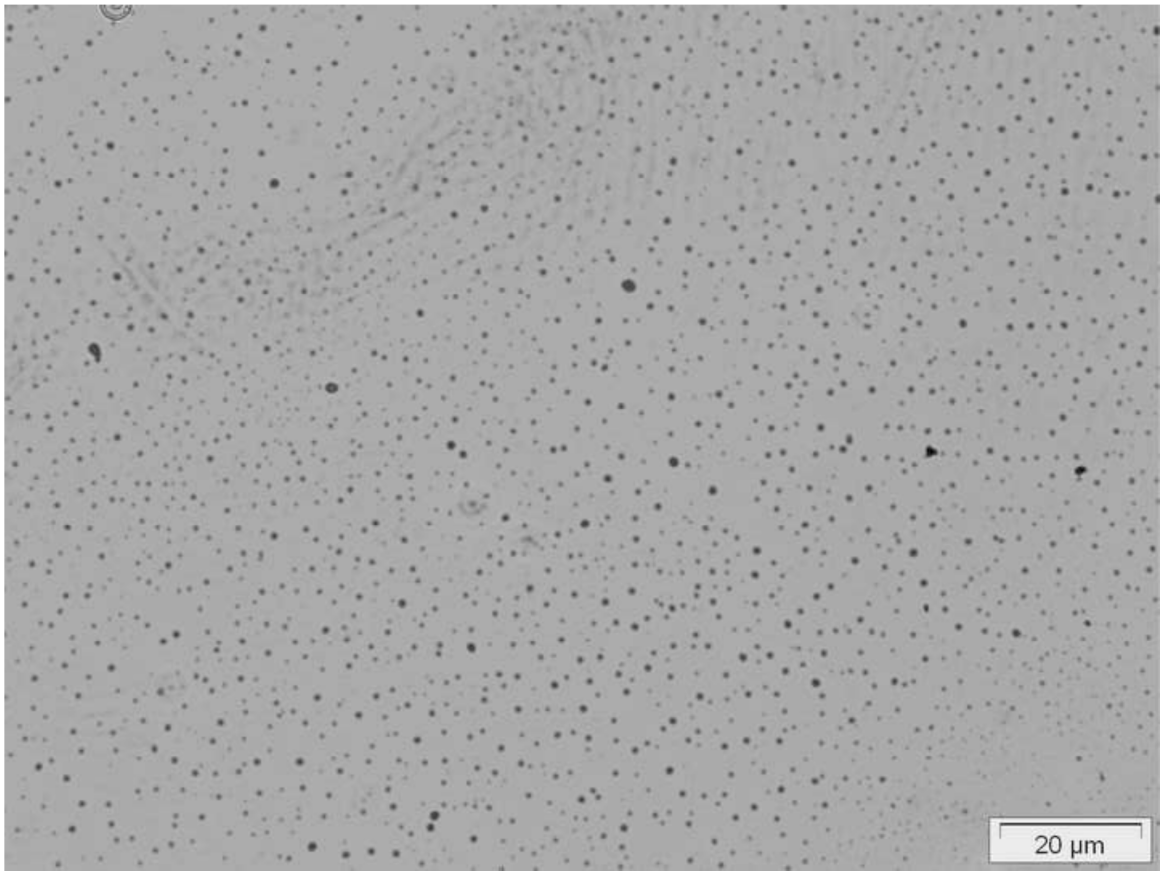

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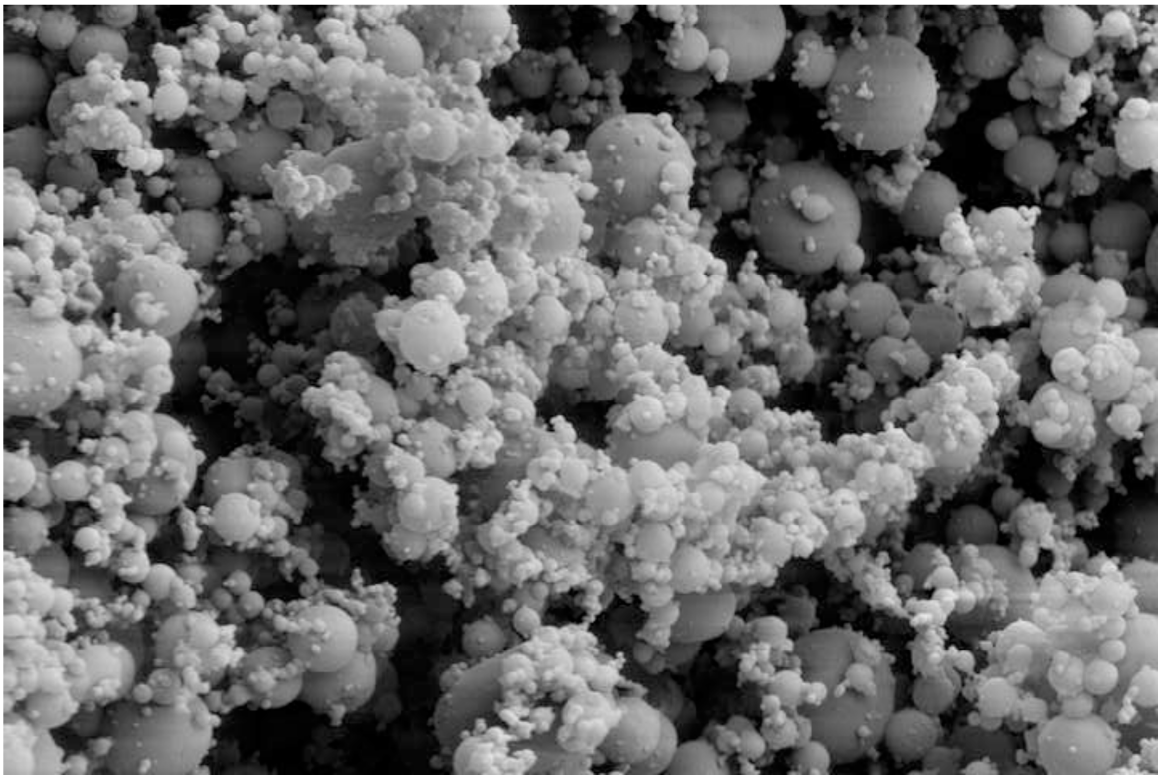

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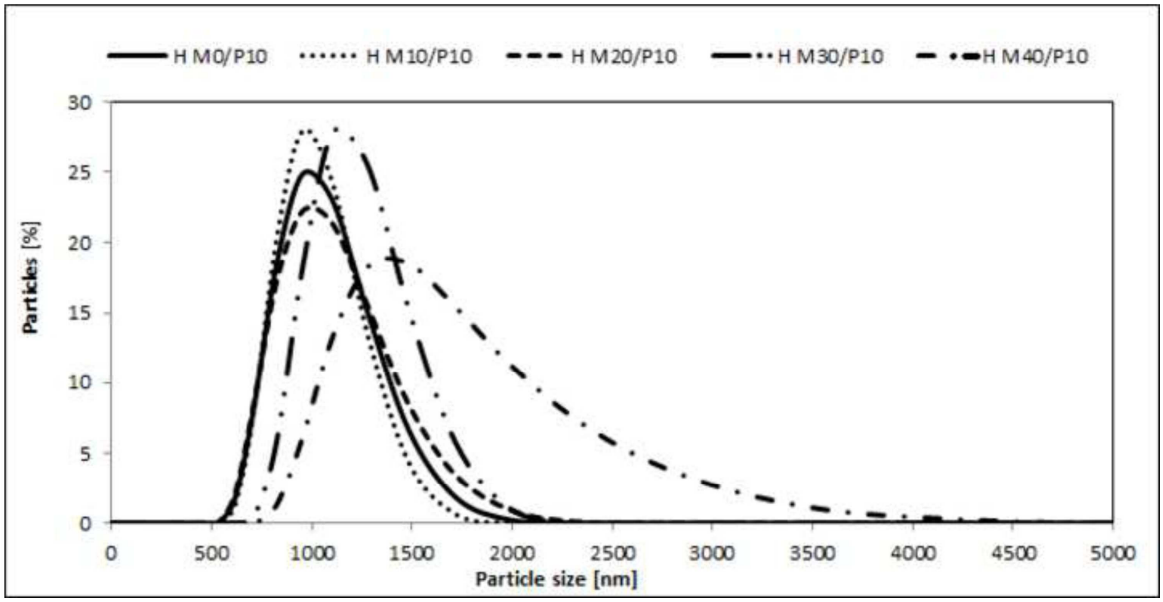

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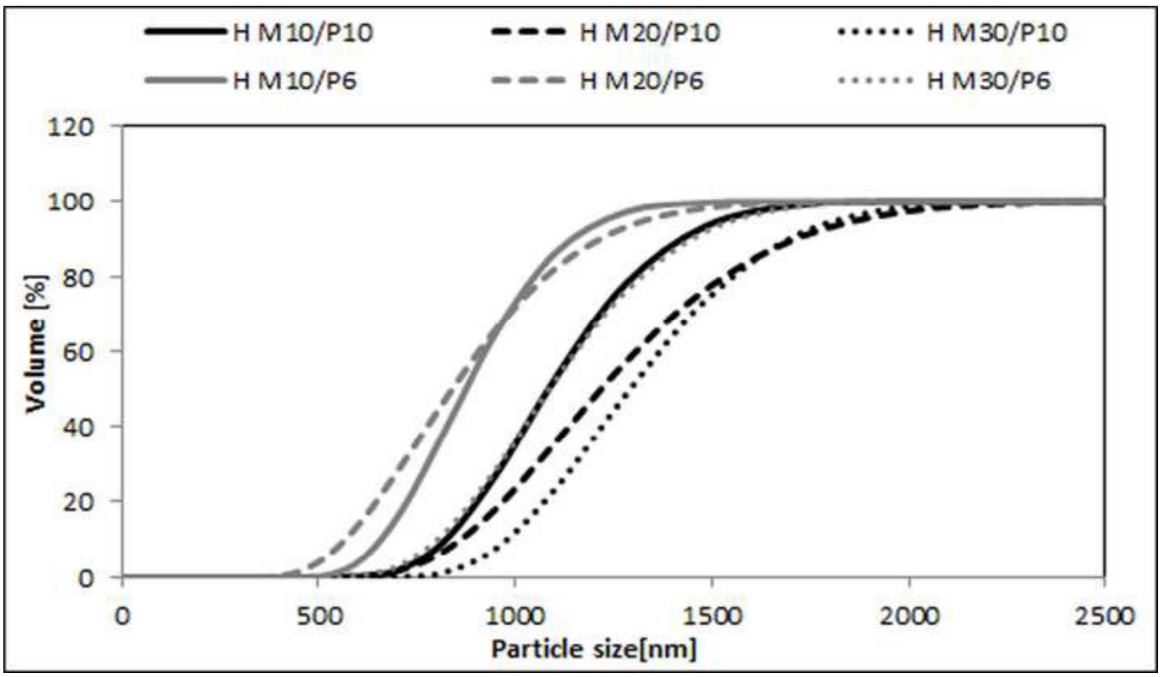

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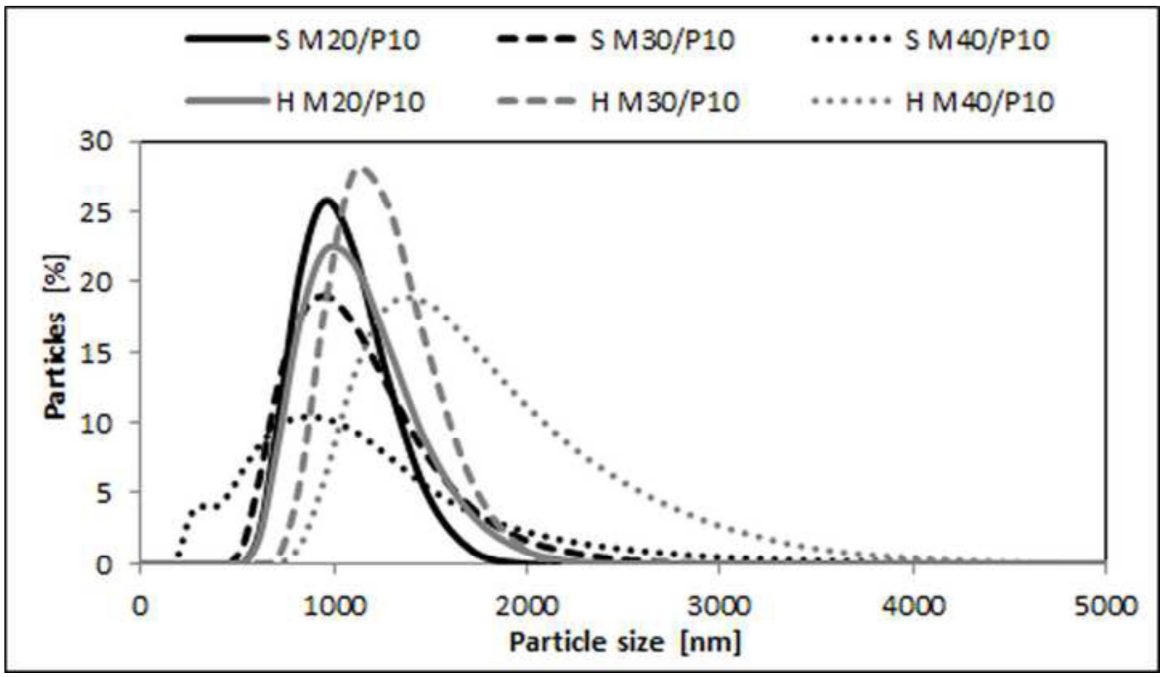

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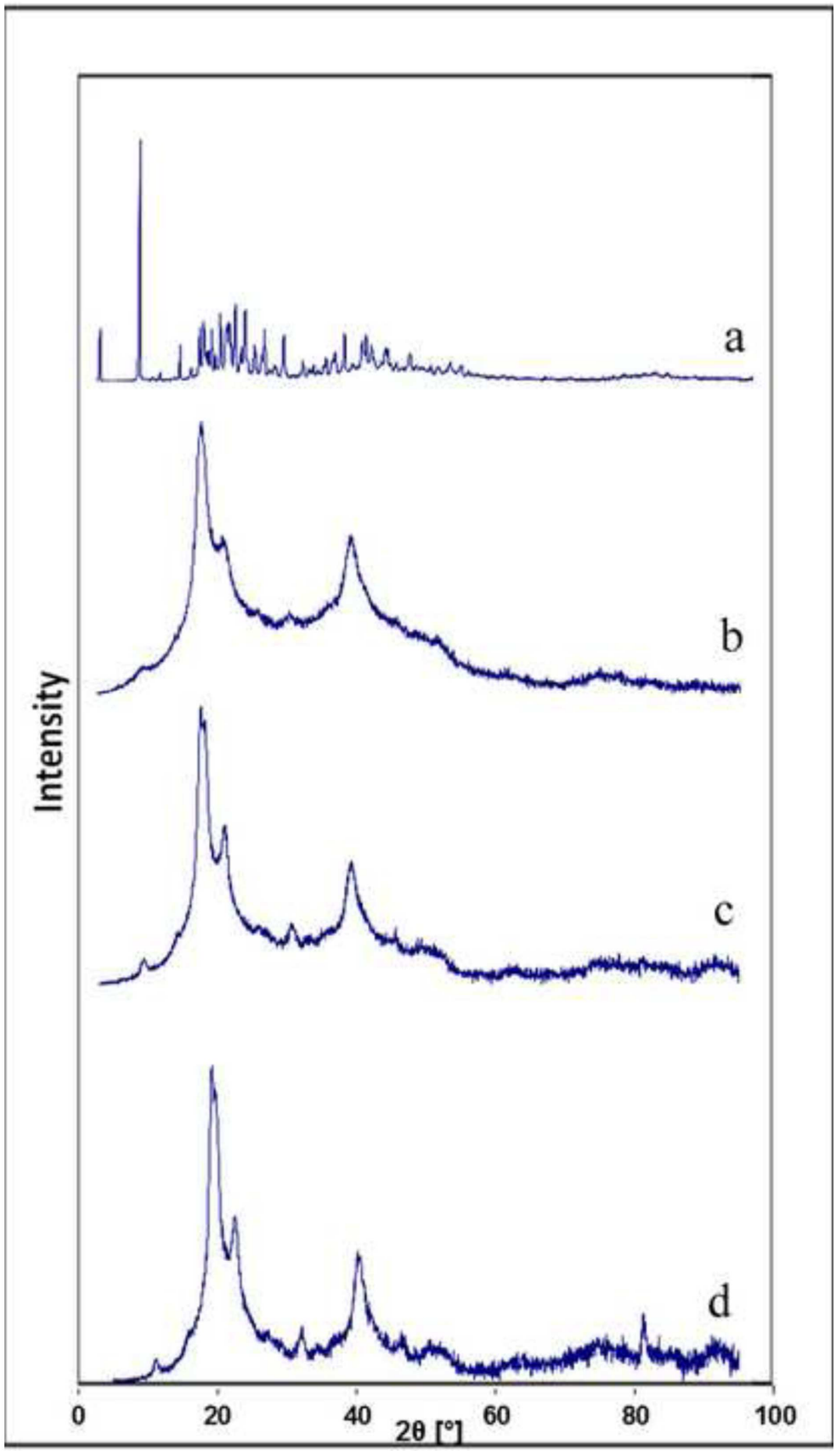

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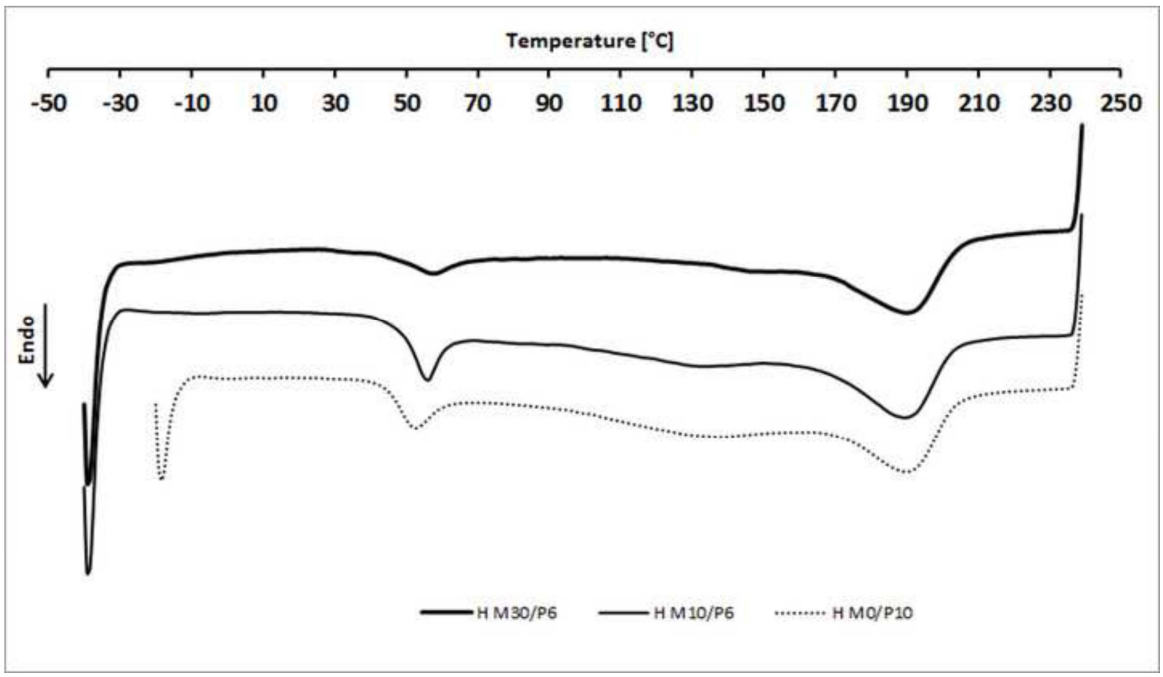

Figure captures

Fig. 1 Optical microscope images of emulsion with 10 mg/ml of PVA and 10 mg/ml of 1-MAG prepared by: a) high-speed stirrer; b) sonication.

Fig. 2 SEM image of 1-MAG/PVA microparticles produced by SAA.

Fig. 3 Number of 1-MAG/PVA particles % distributions produced by SAA from O/W emulsions prepared by stirring with different 1-MAG concentrations (in mg/ml).

Fig. 4 Cumulative distribution curves of 1-MAG/PVA microparticles produced by SAA from O/W emulsions prepared by stirring with various PVA and 1-MAG concentrations (in mg/ml).

Fig. 5 Number distribution of 1-MAG/PVA particles produced by SAA from O/W emulsion prepared using different emulsification methods; sonication (S); or high-speed stirrer (H).

Fig. 6 X-ray diffraction patterns of: a) raw 1-MAG; b) raw PVA; c) S M20/P10 microparticles with the ratio 1/4; d) S M40/P10 microparticles with the ratio 1/2.

Fig. 7 DSC thermographs of H M0P10, H M10/P6 and H M30/P6 microparticles recorded during the first heating.

Table(s)

Tab. 1 List of micronized 1-MAG/PVA microparticles.

Sample 1-MAG/PVA	1-MAG C10:0 [mg/ml]	PVA [mg/ml]	1-MAG/PVA ratio	HLB value	Emulsification method	
					High-speed stirring (H)	Sonication (S)
H M0/P10	0	10	n.a.	18	X	n.a.
H M10/P10	10	10	1/9	16.95	X	n.a.
H M20/P10	20	10	1/4	15.78	X	n.a.
H M30/P10	30	10	1/2.5	14.99	X	n.a.
H M40/P10	40	10	1/2	14.35	X	n.a.
H M10/P6	10	6	1/4.15	15.96	X	n.a.
H M20/P6	20	6	1/2.25	14.77	X	n.a.
H M30/P6	30	6	1/1.5	13.8	X	n.a.
S M20/P10	20	10	1/4	15.78	n.a.	X
S M30/P10	30	10	1/2.5	14.99	n.a.	X
S M40/P10	40	10	1/2	14.35	n.a.	X

n.a. not applicable

Tab. 2 Experimental conditions for SAA for micronization of emulsions produced by high-speed stirring.

Sample 1-MAG/PVA	SAA conditions				Yield [%]
	T Sa [°C]	T Pr [°C]	P Sa [bar]	P Pr [bar]	
H M0/P10	80	100	110	1.30	18
H M10/P10	80	100	115	1.30	40
H M20/P10	80	99	110	1.30	49
H M30/P10	80	99	103	1.30	59
H M40/P10	80	99	107	1.38	37

Tab. 3 Values of the crystallinity of the 1-MAG/PVA microparticles.

Sample 1-MAG/PVA	1-MAG/PVA ratio	Crystallinity [%]
raw PVA	n.a.	16.6
S M20/P10	1/4	23.1 (24.1)
S M40/P10	1/2	27.9 (27.6)

n.a. not applicable

Tab. 4 DSC results obtained by heating and cooling of tested 1-MAG/PVA microparticles.

		1. Heating		Glass transition T_g	
Sample	**ΔH [J.g^{-1}]**	**peak [°C]**	**onset [°C]**	**onset [°C]**	**midpoint [°C]**
H M0/P10	-91.7	189.5	157.4	43.3	46.1
H M10/P6	-70.2	189.5	157.8	46.8	45.4
H M30/P6	-63.1	190.5	153.6	46.4	46.8
		2.Heating			
H M0/P10	-22.3	166.2	133.7	65.75	70.0
H M10/P6	-21.3	164.2	135.3	61.30	65.6
H M30/P6	-18.1	161.2	134.1	52.09	57.2
		1.Cooling			
H M0/P10	17.1	113.8	122.0	75.7	68.6
H M10/P6	19.7	114.1	122.4	71.5	63.6
H M30/P6	17.6	111.8	123.2	62.0	55.4
		2.Cooling			
H M0/P10	6.3	99.8	112.2	74.8	67.8
H M10/P6	10.3	100.4	112.9	71.2	62.3
H M30/P6	10.4	101.1	112.6	63.2	55.9

CURRICULUM VITAE

Name:	Petra Ševčíková
Date of birth:	1985, April 23
Place of birth:	Zlin
Permenent address:	Šanov 175, Slavičín 763 21
Nationality:	Czech
Affilation:	Department of Fat, Surfactnats and Cosmetics; Faculty of technology; Tomas Bata University in Zlin; nam. T. G. Masaryka 275; 762 72 Zlin; Czech Republic Phone: +420 576 031 535 e-mail: psevcikova@ft.utb.cz

Education:

September 2010 – to date	Tomas Bata University in Zlin, Faculty of technology Chemistry and Materials Technology
Doctoral studies	Doctoral topic: Study of formation and characterization of cosmetic emulsion systems (Studium tvorby a vlastností kosmetických emulzních systémů)
2008 – 2010	Tomas Bata University in Zlin, Faculty of technology Chemistry and Food Technology
Master's degree	Master thesis: Preparation of emulsions (Příprava emulzí)
2005 – 2008	Tomas Bata University in Zlin, Faculty of technology Chemistry nad Food Technology
Batchelor's degree	Batchelor thesis: Toxicants in the foods of base of vegetable origin (Toxické látky v potravinách rostlinného původu)

Work experience:

August 2013 to May 2014	Assistant, Department of Food Technology, Tomas Bata University in Zlin

Training abroad:

Date (duration)	2012 (3 months)
Place	Italy, Salerno
Organization	University of Salerno, Faculty of Industrial Engineering

Training courses:

Date	26. 5. 2011
Principal subject	Photon correlation spectroscopy (PCS) and Size exlusion chromatography (SEC)
Organization providing education and training	Anamet s.r.o, Brno
Date	26. 4. 2012
Principal subject	Fundamentals of rheology and rheometry
Organization providing education and training	Pragolab, Praha
Date	14. 3. 2013
Principal subject	Rheology in theory and practice
Organization providing Education and training	Anamet s.r.o, Brno

Work on projects:

2011	Grant UTB IGA/15/FT/11D The in vitro assessment of transepidermal absorption of nanoparticles (Stanovení transepidermální absorpce nanočástic metodou in vitro) - Project leader
2012	Grant UTB IGA/FT/2012/036 The influence of model cosmetics on protective barrier function of the skin (Vliv modelových kosmetických prostředků na ochrannou bariérovou funkci kůže) - Member of research team
2013	Grant UTB IGA/FT/2013/016 Particle systems as carriers of active compounds in cosmetic and pharmacy (Částicové systémy jako nosiče aktivních látek v kosmetice a farmacii) - Project leader

2014	Grant UTB IGA/FT/2014/004 The barrier function of the skin and assessment of transepidermal water loss (Bariérová funkce kůže a hodnocení transepidermální ztráty vody) - Member of research team

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1. SEVCIKOVA P., KASPARKOVA V., VLTAVSKA P., KREJCI J., On the preparation and characterization of nanoemulsions produced by phase inversion emulsification. *Colloids Surf. A:Physicochem. Eng. Aspects.* 2012, vol. 410, p. 130–135.

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