Chapter Four

Nanoemulsions as carriers for natural antioxidants: formulation development and optimisation

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Abstract In the era of returning to the compounds derived from natural sources, antioxidants and other botanical bioactive molecules are gaining increased popularity. Due to their proven health benefits, they have been extensively explored in pharmaceutical, food and cosmetic fields. In addition to their key roles in disease prevention and treatment, cosmetic care and food supplementation, these versatile molecules play a significant role in the development of innovative packaging materials. However, the application of natural antioxidants is still limited due to their inherent sensitivity to environmental conditions. The use of nanoemulsions as suitable carriers for natural antioxidants presents a promising approach to overcoming their stability issues and improving their biological performance. This chapter aims to comprehensively cover recent and relevant findings regarding the formulation design of nanoemulsions (selection of oils and stabilisers, and their ratio) and their preparation methods (high- and low-energy processes). In addition, it provides useful methodological information on the characterisation procedures for the antioxidant-loaded nanoemulsions (antioxidant activity assessment, *in vitro* biological safety and efficacy evaluation). Aiming to guide researchers towards successful formulation development and reliable assessment protocols for these promising colloidal systems, this chapter combines established theoretical concepts with recent experimental results, alongside the most relevant literature sources.

4.1 Introduction

Antioxidants derived from natural sources are gaining increased popularity in the past few decades as an alternative to synthetic molecules, which could exhibit side effects or low bioactivity, while imposing ecological burden and negative perception by the consumers (Lou *et al*., 2017; Raut and Karuppayil, 2014; Aburjai and Natseh, 2003). Phytotherapy and cosmetic use of botanical raw materials dates back to ancient times (Weber *et al*., 2009), but the renewal of interest in botanical actives is markedly accelerated due to recent advancements in analytical methods for their characterisation and bioactivity assessment, as well as the use of advanced carrier systems to optimise their performance (Majeed *et al*., 2015).

It is well-known that human body gets exposed to various external factors that generate free radicals, mostly reactive oxygen species (ROS) for example superoxide anion (O_2^-), peroxyl radical (RO_2), hydroxyl radical (OH), nitric oxide radical (NO), hydrogen peroxide (H_20_2). There are endogenous factors that serve to neutralise these ROS, but sometimes, due to increased and chronic exposure to reactive molecules, the skin antioxidant capacity becomes overwhelmed and it experiences oxidative stress. This condition could lead to the ROS-induced damage of lipids, proteins and DNA, causing

(photo)aging, reduction of immune response or cancer (Naidoo and Birch-Machin, 2017; Reis Mansur *et al*., 2016; Chen *et al*., 2012;). Therefore, a well-planned intake of antioxidants through balanced diet, topical application and supplementation, in line with healthy lifestyle, should boost body's ability to prevent and fight many pathological conditions (Lobo *et al.*, 2010; Masaki, 2010).

In addition to the health aspects, encapsulation of natural antioxidants and their application in the material and packaging industry is constantly increasing, aiming to develop novel "green" materials and packaging solutions that will improve safety, quality and shelf-life of the final product by minimising the amount of externally added additives (Han *et al.*, 2018, Prakash *et al*., 2018).

An important class of natural lipophilic substances with high bioactivity are plant/fruit seed oils containing essential polyunsaturated fatty acids and antioxidant molecules such as carotenoids, tocopherols, tocotrienols and polyphenols (*e.g.* olive, grape seed oil, sunflower, passion fruit, pomegranate, wheat germ, blackberry and red raspberry seed oils) (Michalak and Dadasiewicz, 2018; Pereira *et al*., 2016; Bushmann *et al.*, 2004). Some of these oils, such as olive oil, have been used for centuries in human nutrition and skincare preparations (Aburjai and Natseh 2003). Another important group of lipophilic bioactives are plant essential oils, complex mixtures of up to 70 various volatile and aromatic compounds, which are known for their antimicrobial, antioxidant and anticarcinogenic activity. Each essential oil is characterised by two or three principal compounds (usually phenolic constituents, flavonoids and terpenoids) which can act alone or synergistically, ensuring the important biological effects (Majeed *et al*., 2015). Essential oils prepared from clove, oregano, thyme, sage, rosemary and many other herbs and spices are known as potent antioxidants (Raut and Karuppayil 2014; Viuda-Martos *et al*., 2010; Dorman *et al*., 2000). Therefore, it is no wonder that there is an intensive focus on plant bioactives among pharmaceutical, cosmetic, agricultural and food industries. It is worth mentioning that the exact mechanism of action and safety profiles of many natural antioxidants are still unknown, because of the large number of naturally-derived plant extracts, their intrinsic composition variations and the fact that they are sometimes not precisely characterised (Gledovic *et al*., 2020). Isolated compounds from essential oils (*e.g.* thymol, carvacrol, eucalyptol, eugenol) and other extracted compounds from plant sources (*e.g.* curcumin, resveratrol, lycopene, β-carotene) with defined composition are more convenient for formulation development compared to the

multicomponent extracts, because they are standardised and more concentrated (Weber *et al.*, 2009).

Although many of these natural oils and isolated molecules are generally recognised as safe (GRAS) for humans, they are prone to heat degradation, oxidation, polymerisation and hydrolysis in aqueous formulations, which is a limiting factor for their application. Therefore, adequate formulations are necessary to preserve their bioactivity and safety profile. In response to that need, many researches have been carried outshowing the feasibility of nanoemulsion carriers for natural antioxidants (Ahmadi and Jafarizadeh-Malmiri 2020; Lou *et al.*, 2017; Pereira *et al.*, 2016).

Nanoemulsions present innovative colloidal delivery systems with very small droplet sizes (usually up to 300 nm) consisting of the organic phase (natural and/or synthetic oils and lipophilic actives), water and surfactants (natural and/or synthetic), and optionally some hydrophilic and/or rheological additives, prepared *via* appropriate low-energy or high-energy methods. Depending on the obtained droplet sizes, nanoemulsions are transparent, translucent or milky white fluids, with typical blue-shining appearance. Small droplet size of nanoemulsions increase their stability towards gravity-induced phenomena (creaming and sedimentation), leading to improved shelf-life compared to standard macroemulsions. In addition, an increased surface area between oil and water phases leads to increased solubility and bioavailability of the lipophilic actives that are entrapped into the internal phase of oil-in-water nanoemulsions (O/W nanoemulsions), while at the same time the surfactant layer protects bioactives form deterioration. Theoretically, controlled release and/or targeted delivery are possible when lipophilic antioxidants are incorporated into O/W nanoemulsions. Most importantly, improved stability can be achieved for the natural antioxidants, which are usually sensitive to heat, air and light exposure (Ahmadi and Jafarizadeh-Malmiri 2020; Pavoni *et al.*, 2020; Pereira *et al*., 2016). It is worth mentioning that hydroglycolic plant extracts can also be added to O/W nanoemulsions, which can sometimes lead to synergistic antioxidant effects with the lipophilic and hydrophilic molecules in the nanoemulsion carrier and improved stability of such multicomponent products (Gledovic *et al*., 2020). Taken all together, natural antioxidants can be powerful adjuvants in combination with other active ingredients, which makes them very promising materials for future research and application.

With all of the above in mind, the aim of this chapter is to present a comprehensive review of the literature related to the formation, properties and formulation optimisation

of nanoemulsions with various lipophilic and hydrophilic plant extracts and isolated compounds with proven antioxidant activity. Special focus will be placed on the multidisciplinary role of natural antioxidants in nanoemulsion carriers. This will be presented with respect to the structural specificities of nanoemulsions and the interactions between natural antioxidants and their nanoemulsion carrier, encompassing *in vitro* antioxidant and cytotoxicity assays.

4.2 Encapsulation of antioxidants in nanoemulsions: methods and basic principles

Based on the energy-efficiency and underlying mechanisms responsible for nanoemulsion formation, nanoemulsions can be produced *via* different methods, generally classified as low-energy and high-energy emulsification methods (Fig. 4.1). In the following subsections, the main advantages and disadvantages of each method for preparation of nanoemulsions containing natural antioxidants will be briefly described. Original research papers reflecting the complexity of formulation development and production optimisation of nanoemulsion carriers with natural antioxidants will be discussed as a guide for the researchers in this field (Table 4.1).

4.2.1 High-energy emulsification methods

High-energy methods represent a top-down approach to nanoemulsion formation. These methods are based on the usage of mechanical devices, for instance high-pressure homogeniser (HPH), ultrasonic homogeniser (USH), microfluidiser (MF) and high-speed mixer (HSM) to produce intense disruptive forces such as collision, compression and cavitation, which can break the micrometre emulsion droplets into the smaller nanosized dimensions. The input energy density in these high-energy processes is very high (the order of 10^8–10^{10} W kg^{-1}), but only a small amount (around 0.1%) of this energy is used for emulsification and a large amount of energy is dissipated as friction due to the presence of high shear rates, which make them cost-inefficient (Azmi *et al*., 2019; Gupta *et al.*, 2016). This dissipated energy is converted into heat, which raises the nanoemulsion temperature. Therefore, a cooling system is necessary to prevent heat damage of the sensitive ingredients such as vitamins, proteins and enzymes (Espitia *et al.*, 2018; Chong *et al*., 2018). However, the main advantages of the high-energy methods in nanoemulsions preparation are the possibility to use lower amounts of surfactants and smaller surfactant-to-oil ratios (SOR), which is especially important in food or certain pharmaceutical applications where high surfactant concentration is a limiting factor

(Gothani and Prasert, 2014; Solans and Sole, 2012). The two most widely used high-energy emulsification methods on the laboratory scale are HPH and USH, while for the industrial application HPH and MF are more suitable (Azmi *et al*., 2019; Gupta *et al.*, 2016).

High-pressure homogeniser (HPH)

HPH is a device originally used in the food and beverage industry. It is the most straightforward approach for nanoemulsification due to its versatility and suitability in laboratory settings and easy scale-up (Espitia *et al*., 2018). Three main process parameters influence HPH emulsification: pressure, number of cycles and temperature. Conventional HPHs employ pressures between 50 and 100 MPa, however, operating pressures of about 350 to 400 MPa can also be used. HPH is based on the forced passage of pre-emulsion/pre-mix (prepared *via* HSM) through a specially designed narrow valve, causing a sudden pressure drop across HPH to reach a few thousand bars. Multiple passes are employed until formation of uniform nanodroplets of desired sizes as a result of the combination of intensive disruptive forces such as shear stress, cavitation and turbulent flow conditions (Salem and Ezzat, 2018). In general, droplet sizes decrease with an increased number of passes, until a minimum value is reached. When the droplet size plateau is reached, further homogenisation may lead to coalescence and increase in droplet dimension due to the over-processing phenomenon (Azmi *et al*., 2019). Viscosities of the oil and water phases are important formulation parameters, as well as sufficient amount of surfactant necessary to quickly and effectively cover the surfaces of the newly formed droplets (Gupta *et al.*, 2016).

Ultrasonic homogeniser (USH)

An USH involves inserting the sonicator probe into a sample. The probe creates sound waves of high frequency (> 20 kHz), causing shock waves which result in turbulence due to cavitation in the surrounding liquid. The mechanical vibrations lead to the formation of liquid jets at high speed, while the collapse of the micro-bubbles generates intense disruptive forces that lead to droplet disruption and the formation of nanodroplets. It is recommended to prepare a pre-emulsion before USH (Rinaldi *et al*., 2017). The droplet sizes significantly decrease when the intensity of ultrasonic waves, sonication time, power level and surfactant concentration increase, while over-processing can cause droplet coalescence and instability (Azmi *et al*., 2019). The amount of

surfactant and the viscosity of the oil and aqueous phases are among important parameters in nanoemulsion formation, therefore should be precisely optimised. The USH method has several advantages, such as simple manipulation using the lower-cost equipment, easy operation, cleaning and servicing (Rebolleda *et al*., 2015). The main disadvantage of USH is the production of small batches on a lab scale. Moreover, USH can lead to protein denaturation, polysaccharide depolymerisation and lipid oxidation during homogenisation, which could limit its application in industrial settings (Salem and Ezzat, 2018).

Microfluidiser (MF)

In MF device, the liquid mixture is passed through the interaction chamber comprising microchannels under a high pressure, resulting in the breakage of micrometre-sized droplets into nanodroplets. Similar to other high-energy methods, the pre-emulsion is prepared first and then it is passed through the interaction chamber consisting of two separate flow channels. Due to the specific design of the channels, the two streams of the pre-emulsion collide at high velocity, generating a very high shearing action, which results in droplet breakage and formation of nanoemulsion. The desired droplet size and distribution are achieved through multiple passages with sufficient amount of surfactant. MF is operating based on similar principles as HPH. Therefore, these two homogenisation methods are usually interchangeable, and both are applicable at a laboratory and industrial scale (Salem and Ezzat, 2018; Salvia-Trujillo, 2013).

High-speed mixer (HSM) such as rotor-stator devices as a single technique is not suitable to generate nanoemulsions with small droplet sizes and narrow polydispersity. However, it is widely used to form pre-emulsions as a primary step, before applying other high-energy emulsification techniques (Salem and Ezzat, 2018). Alternatively, they can be combined with low-energy emulsification methods, as a final homogenisation step, once the nanoemulsion is formed (Teo *et al*., 2010).

4.2.2 Low-energy emulsification methods

Low-energy methods represent a bottom-up approach, which is based on the tendency of certain surfactants to self-assemble and produce nanodroplets due to the release of chemical energy when selected ingredients are mixed in a specific way. The requirements for the formation of nanoemulsion through low-energy methods are

dominantly related to the surfactant-oil-water (SOW) composition, which is limited to only certain types of surfactants, oils and additives (Solans and Sole, 2012). In other words, the low-energy emulsification methods are based on the control of interfacial phenomena at the boundary between organic and aqueous phases and depend upon the intrinsic properties (*e.g.* solubility and molecular geometry) of any surface-active molecules present (*e.g.* surfactants and co-solvents such as polyols) (Nikolic *et al*., 2018; Chang and McClements, 2014). Since these methods require significantly lower energy density input (10^3–10^5 W kg^{-1}), nanoemulsification can be performed applying simple equipment (magnetic stirrer or vortex mixer) to mix the components and release the chemical energy responsible for the nanoemulsion formation (Espitia *et al*., 2018; Gothani and Prasert, 2014). Many of these low-energy methods can be performed at room temperature, which is a significant benefit for the thermosensitive ingredients.

Classification of low-energy emulsification methods is sometimes unclear, giving the fact that many mechanisms of low-energy nanoemulsion formation are rarely fully understood. The most straightforward distinction could be done if any phase inversion from the spontaneous surfactant curvature is produced during the nanoemulsion formation or not (Solans and Sole, 2012). In spontaneous emulsification (SE) method (sometimes marked as self-emulsification), the organic phase (surfactants and oil) is being added to the water phase stepwise under continuous mixing, and ultrafine nanoemulsion droplets are formed as a result of rapid diffusion of surfactant to the continuous aqueous phase, without any change in surfactant curvature (Nikolic *et al*., 2108; Chang and McClements, 2014). However, when the aqueous phase is added to the organic phase, the process is classified as emulsion phase inversion (EPI) method (sometimes also referred to as the phase inversion composition or phase inversion concentration – PIC method). When the change of spontaneous surfactant curvature is caused by the change in temperature, the approach is named as phase inversion temperature (PIT). Many possible transient phases can occur at the phase transition point, such as liquid crystalline phase (cubic or lamellar), microemulsion (ME), oil-in-water-in-oil (O/W/O) multiple emulsions. They are all characterised by low interfacial tension, thus enabling the formation of nanodroplets (Azmi *et al.*, 2019; Gupta *et al*., 2016).

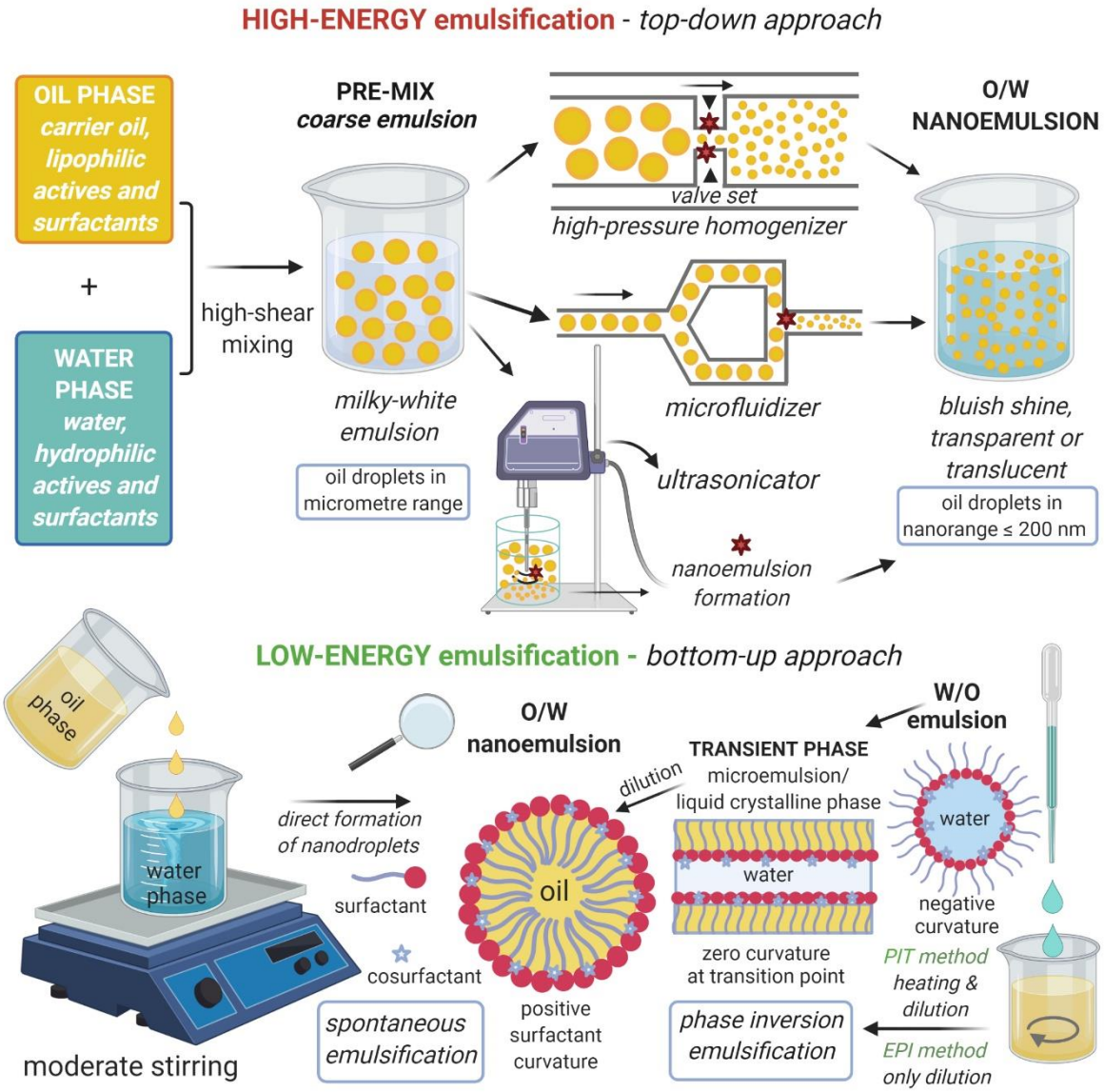

Fig. 4.1 Schematic representation of O/W nanoemulsion formation through high-energy and low-energy emulsification methods

In the past decade the low-energy methods gained higher popularity over conventional high-energy techniques due to several reasons: low-energy consumption, inexpensive equipment, simple manipulation and fast preparation. Most importantly, they are particularly suitable for the production of nanoemulsions with shear- and thermo-sensitive natural ingredients, such as essential or plant seed oils and fruit extracts (Chang and Mc Clements, 2014; Solans and Sole 2012). The main disadvantages of these methods are using relatively high concentration of surfactants (usually 10 to 20 wt%) and/or the addition of co-solvents (polyols) which is necessary to form small nanodroplets (< 100 nm). Also, there is a lack of published data regarding the scale-up and the industrial application, which is unfortunate since these methods seem to hold great promise for

future applications (Azmi *et al*., 2019; Salem and Ezzat, 2018; Gupta *et al*., 2016). Therefore, the key requirement for the widespread use of low-energy emulsification methods is the fundamental understanding of how to control the transient phases responsible for nanoemulsion formation, so that the nanoemulsions can be readily reproduced with the desired characteristics in the industrial settings.

4.3 Formulation optimisation of nanoemulsions with natural antioxidants

Natural antioxidants are challenging ingredients because of their complex composition and instability to environmental stress (heath, light, air, changes of pH values or ionic strength). Therefore, it can be a very difficult task to create nanoemulsions with ultra-fine droplets and long-term physicochemical stability (Zhong *et al.*, 2017; Bajerski *et al.*, 2016). The properties and the ratio of the oil phase, water phase and surfactants, as well as the production method, contribute significantly to the final characteristics and performance of nanoemulsions (Azmi *et al.*, 2019; Gupta *et al*., 2016; Gothani and Prasert, 2014). Therefore, it is necessary to meticulously choose all ingredients and carefully adjust the production procedure to ensure the stability of these sensitive bioactive molecules in a particular nanoemulsion carrier.

An overview of recently published papers is presented in Table 4.1. Current trends in formulation development and some important unresolved issues regarding the encapsulation of natural antioxidants in nanoemulsion-based carriers are mentioned in this table.

4.3.1 Stability of antioxidant-loaded nanoemulsions

There are several reasons for the lack of physical and/or chemical stability in nanoemulsion systems. The primary reason is the choice of surfactants. The most widely employed surfactants, regardless of the nanoemulsion production method, are hydrophilic low molecular weight non-ionic surfactants: Tween 80 (polyoxyethylene 20 sorbitan monooleate) and Tween 20 (polyoxyethylene 20 sorbitan monolaurate). The Tweens are known for their suitability in food, pharmaceutical and cosmetic industry and their versatility and excellent compatibility with various natural and synthetic oils and other ingredients, as well as having a good safety profile. However, these surfactants exhibit a decrease of hydrophilic-lipophilic balance (HLB) value at elevated temperatures that changes their affinity for water and oil phases, consequently leading to physical instability

of nanoemulsions (*e.g.* increase in droplet sizes and polydispersity index (PDI) values) (Chuesiang *et al*., 2018). The monolayer made of single surfactant may not be sufficient to prevent the influence of pro-oxidants, air and light on the nanoemulsion lipid core, leading to chemical instability of the incorporated bioactives. Thus, it is always advisable to employ a blend of surfactants (Nikolic *et al*., 2018; Mao *et al*., 2009). Lecithin is one of the recommended surfactants compatible with Tween 80, whose positioning at the oil-water interface increases Z-potential and provides electrostatic stabilisation of nanoemulsion. Additionally, lecithin has the potential to block the permeation of peroxyl radicals across the oil/water interface and to decrease the rate of oxidation of bioactive encapsulates (Pan *et al.*, 2013). An increase in surfactant concentration leads to a tighter packing of surfactant molecules at the oil-water boundary, providing an efficient physical barrier to oxidative species. Alternatively, some findings support the opinion that the excess of surfactant forms micelles in the aqueous phase which can encapsulate pro-oxidative species (McClements and Decker, 2000). However, when using higher amounts of surfactants, the formed micelles can sometimes facilitate the migration of smaller oil droplets to the larger ones (Ostwald ripening), or an excess of surfactant may alter the properties of the interfacial layer, thereby enhancing coalescence (Chuesiang *et al*., 2018). Lipophilic non-ionic surfactants Span 80 (Sorbitan monooleate) and Span 20 (Sorbitan monolaurate) are also commonly used to decrease the HLB value of the Tween-Span mixture and to match it with the HLB of natural oils, leading to a decrease in droplet size and an increase in physical and/or chemical stability (Chong *et al.*, 2018; Zhong *et al.*, 2017; Rocha-Filho *et al.*, 2014). However, looking at the available data presented in Table 4.1, it can be concluded that lecithin and Spans, without additional stabilisers, were not particularly effective at inhibiting chemical instability at elevated temperatures.

To conclude, impaired physical and/or chemical stability at elevated temperatures is apparent in most nanoemulsion systems with delicate compounds such as carotenoids (β-carotene and astaxanthin), tocopherols (α-tocopherol/α-TOC and tocopheryl acetate/VE acetate), curcumin, ɣ-oryzanol and plant seed oils (Pereira *et al*., 2016; Zhong *et al*. 2017; Hategekimana *et al*., 2015). In order to preserve the initial optimal nanoemulsion properties (*e.g.* small droplet sizes and high entrapment efficiency of the isolated compounds), the general recommendation is to store the antioxidant-loaded nanoemulsions at temperatures lower than 25˚C, protected from light and air. However, in a real-life scenario, it is not always possible to retain such ideal storage conditions. Thus, researchers are putting more effort in improving the stability of antioxidant-loaded

nanoemulsions under more challenging conditions, such as elevated temperature, UV-irradiation or oxygen exposure (Kaur *et al.*, 2017; Kim *et al.*, 2011; Bernardi *et al.*, 2011). There have been several promising solutions reported to improve the nanoemulsion stability at elevated temperatures, which are presented as follows.

- **Introduction of high molecular weight surfactants**

In a study, the influence of different surfactants and production parameters was investigated on the physicochemical properties of β-carotene nanoemulsions developed *via* HPH. The resulting nanoemulsions stabilised with Tween 20 and decaglycerol monolaurate (DML) had significantly smaller droplet sizes, but they were less stable compared with the ones stabilised with octenyl succinate anhydride modified starch (OSA-MS) and whey protein isolate (WPI). It was found that WPI was the only emulsifier able to protect β-carotene effectively from degradation, ensuring that 72% of β-carotene remained after 12 days of storage at 55 °C. It was pointed out that the low molecular weight surfactants (Tween 20 and DML) generally do not provide highly cohesive or viscous surface layers around the nanodroplets, and generate low Z-potential values (~-5 mV), which can explain the obtained poor stability. However, the large molecule emulsifiers (OSA-MS and WPI) can form mechanically strong interfacial layers and cause steric hindrance to prevent droplet coalescence. Droplets in WPI emulsions showed more negative zeta potential values (~-17 mV), while OSA-MS solution showed a relatively high viscosity, which might improve their stabilising properties. It has also been discussed that WPI can act as an antioxidant since the main constituents of WPI (β-lactoglobulin and α-lactalbumin) both contain cysteyl residues, disulphide bonds and thiol functional groups, which can inhibit lipid oxidation by scavenging free radicals. Interestingly, the combination of Tween 20 and WPI did not result in a synergistic protective effect. Instead, the β-carotene degradation rates were just between those in the nanoemulsions stabilised by WPI and Tween 20 alone. Droplet sizes of β-carotene nanoemulsions could be further reduced by increasing the homogenisation pressure to 140 MPa. However, the processing conditions of 80 MPa and 5 cycles were found to be optimal to avoid possible formation of free radicals during the HPH process and temperature rise, which would lead to thermal and oxidative degradation of β-carotene (Mao *et al*., 2009).

- **Introduction of lipophilic and/or hydrophilic antioxidants**

In another study, the physical and chemical stability of β-carotene enriched nanoemulsions were tested using different types of surfactants (Tween 20 or β-lactoglobulin) and different types of water-soluble (EDTA or ascorbic acid) and oil-soluble antioxidants (coenzyme Q10 or VE acetate). Nanoemulsions were prepared with 10 wt% oil phase (0.5 wt% β-carotene in corn oil) and 90 wt% aqueous phase (2% β-lactoglobulin in buffer solution), *via* MF method (3 passes, at 9000 psi) with temperature control, to avoid degradation of β-carotene and other antioxidants. It was found that the addition of antioxidants to the nanoemulsions did not have a major influence on the droplet size (around 90 nm) and long-term physical stability. A possible explanation for this phenomenon is that the antioxidants were primarily present in the oil phase; they were not particularly surface-active and did not compete with the globular protein at the oil-water interface. However, β-carotene degradation at 55˚C and consequent colour fading of the product could be effectively supressed by adding water-soluble or oil-soluble antioxidants to the nanoemulsions, with the following order of effectiveness: EDTA> ascorbic acid> coenzyme Q10 > VE acetate. The effectiveness of EDTA in inhibiting colour loss was attributed to its ability to strongly chelate and inactivate transition metals (such as iron) that normally promote carotenoid oxidation. Among the oil-soluble antioxidants, coenzyme Q10 was shown to provide better protection against colour fading than α-TOC, which might be due to its ability to regenerate other antioxidants present in the system (such as tocopherols in the corn oil used as a carrier oil). Similar to the previously described study (Mao *et al.*, 2009), protein-stabilised nanoemulsions were found to exhibit better β-carotene stability and less colour fading than non-ionic surfactant-stabilised nanoemulsions, which was attributed to the antioxidant effects of globular proteins Interestingly, the synergistic effect between the hydrophilic (EDTA) and lipophilic antioxidant (VE acetate) was not observed in this study (Qian *et al.*, 2012).

In another interesting study, astaxanthin-loaded nanoemulsions were prepared *via* the HPH using a mixture of natural surfactants (glyceryl/citrate/lactate/linoleate/oleate) or hydrogenated lecithins with different coantioxidants. The glyceryl ester stabilised-nanoemulsions had uniform droplet sizes after three passes at 1000 bar, which was then became independent of the number of additional cycles. However, the hydrogenated lecithin nanoemulsions had much larger droplet sizes, which were not constant with the increasing the number of cycles. Therefore, the glyceryl ester was superior to the lecithin in terms of forming semi-transparent nanoemulsions with uniform droplet distribution,

even at the high concentration of astaxanthin (5.5%). Among tested coantioxidants, only α-TOC or hydroxy dimethoxy benzyl malonate (HDBM) were suitable to keep the monomodal distribution and to partially inhibit the colour change after UV-irradiation of the tested nanoemulsions. The astaxanthin content remained stable (>90%) after exposure to potentially destabilising physical stresses (- 5, 5, 25 and 45 ˚C, 12h on each temperature, for 4 weeks), indicating the robustness of the astaxanthin-loaded nanoemulsions. The improved stability of glycerol-stabilised nanoemulsions was confirmed by SEM micrographs, in which a tight multilayer structure with a lamellar form was observed, whereby astaxanthin was completely incorporated in the hydrophobic core of nanodroplets (Kim *et al*., 2011).

Several other studies concluded that VE acetate can act as an additional surfactant due to its ability to position at the oil-water interface. It was observed that it can decrease droplet size of the nanoemulsions and improve their physical stability (Gledovic *et al*. 2020, Rocha-Filho *et al.,* 2014; Teo *et al*., 2010). For example, in the latter study, VE acetate and Pluronic F-68 were found to co-stabilize the formulations and nanoemulsions with droplet size ~ 94 nm, physically stable for 4 weeks at > 45˚C were obtained. The optimal formulation contained 24 wt% Tween 80, 2.4 wt% Pluronic F-68 10 wt%, palm oil esters (POEs), 10 wt% α-TOC and 53.6 wt% deionised water (Teo *et al.,* 2010).

4.3.2 The multifunctional role of natural (essential and seed) oils in nanoemulsions

The choice of oil phase is another crucial factor in nanoemulsion formation and stability. The incorporation of natural oils instead/in addition to the traditionally used carrier oil medium-chain triglycerides (MCT) is currently the focus of research (Zhong *et al.,* 2017; Hategekimana *et al.,* 2015; Bernardi *et al.,* 2011). The incorporation of essential oils in O/W nanoemulsions converts them into an aqueous-based product suitable for oral and topical usage since they cannot be used undiluted. Given the fact that essential oils are liquids composed of small aromatic and volatile molecules that can act as an additional surfactants (*e.g.* alcohols and esters), they play an important role in nanoemulsion formation (Pavoni *et al.,* 2020; Rocha-Filho *et al.,* 2014). The formulation development of nanoemulsions loaded with essential oils is usually done empirically when a part of the carrier oil is replaced by essential oil. At certain oil phase composition, the droplet size tends to decrease and stable nanoemulsions can be formed. Since essential oils have a complex composition, a detailed characterisation is needed for the standard

molecules isolated from them, in order to discover which components are involved in co-stabilising action and which ones remain entrapped in the nanoemulsion droplet core.

The low-energy methods that can be performed at room temperature (EPI and SE) are the preferred choice for the production of essential oil-loaded nanoemulsions. In the study involving the formation of orange oil nanoemulsions by SE, it was found that the surfactant type and concentration and oil phase composition (orange oil/MCT ratio) had notable effects on nanoemulsion formation and stability. Transparent nanoemulsions could be formed under certain conditions: 20 wt% surfactant (Tween 40, 60, or 80) and 10 wt% oil phase (4−6 wt% orange oil and 6−4 wt% MCT). Surfactant type and oil phase composition also affected thermal stability of the nanoemulsions. The system that retained ultrafine nanodroplets of ≈ 25 nm even after thermal cycling (from 20 to 90°C and back to 20°C) was the nanoemulsion prepared with 20 wt% Tween 80, 5 wt% orange oil and 5 wt% MCT, which is a clear indication of the role of the oil phase composition on the nanoemulsion stability (Chang and Mc Clements 2014). In a similar study, citrus essential oil was incorporated in Tween 80-stabilised nanoemulsions prepared *via* SE method. It was also observed that the smallest droplets (≈ 73 nm) were obtained using relatively high amount of Tween 80 (20 wt%) at the optimal ratio of essential oil to MCT of 1:1 (Lou *et al.*, 2017).

PIT method was also found suitable for the production of nanoemulsions with lavender essential oil, employing a carrier oil with natural origin of passion fruit oil (PFO). The minimum surfactant concentration necessary for the formation of nanoemulsions was 5.0 wt%. The addition of lavender essential oil (LO) to the system consisting of PFO and mixed surfactants with HLB = 10 (PEG-30 castor oil and sorbitan monooleate) reduced the droplet size compared to nanoemulsions without LO, due to its courfactant properties. LO-loaded nanoemulsions could be formed at different PFO:LO ratios *e.g.* 5:1, 5:2 and 5:5 (giving droplet sizes of ≈ 105 nm, 54 and 38 nm, respectively), but the optimal ratio was selected as 5:5, a similar finding aforementioned studies (Chang and McClements, 2014 and Lou et al., 2017). The chromatographic analysis of LO-loaded nanoemulsions indicated no change to the LO main constituents at the temperatures up to 25˚C, but the degradation of linalyl acetate and increased concentration of linalool after thermal cycling indicated some chemical instability at higher temperatures (Rocha-Filho *et al*., 2014).

Sometimes, the essential oil nanoemulsions are prepared without using any carrier oil, which in this case, it seems that the high-energy methods are more appropriate, as in

low-energy approaches the amount of surfactant to form nanoemulsions is insufficient. For example, lemongrass oil (LEO)-loaded nanoemulsions were prepared by MF method with LEO: Tween 80 ratio of 1:1, and sodium-alginate 1 vol% was used as a costabiliser. The mechanism of action of food hydrocolloids such as sodium alginate is related to their ability to adsorb to the interfacial layer, causing possible interactions and competition with the main surfactant. Also, they can modify the viscosity in the aqueous continuous phase which can decrease the rate of creaming and coalescence Mao *et al*., 2009). The nanoemulsion with very small average droplet sizes (≈7.35 nm, PDI≈ 0.34) were prepared after three passes through a MF device working at 150 MPa and the temperature in the reaction chamber was kept to $< 20^{\circ}C$, to prevent the evaporation of LEO (Salvia-Trujillo *et al.,* 2013). Basil oil nanoemulsions were prepared by HSM (up to 17000 rpm) with temperature control, containing basil oil 7.5 wt%, Tween 80 2wt% and Span 80 2 wt% as surfactants, and water. These nanoemulsions showed good physicochemical stability for after 90 days of storage in refrigerator (4˚C), retaining 87 % of estragole as the basil oil main constituent in the nanoemulsion (Da Silva Gundel *et al.,* 2018).

As it can be seen from the literature review (Table 4.1), the usage of natural plant seed oils (*e.g*. sunflower, corn, olive, wheat bran and berry fruit seed oils) as carriers and actives is a growing trend in formulation development (Pereira *et al.,* 2016; Rebolleda *et al*., 2015; Bernardi *et a*l., 2011). These oils are a rich source of fatty acid triglycerides, thus they can substitute the commonly used MCT or long-chain triglycerides (LCT). However, the minor constituents of these oils (*i.e.* tocopherols, tocotrienols, carotenoids, phenolic compounds, phytosterols) which play a big role in their bioactivity, could vary depending on the oil production procedure, climate, soil and plant material (Gledovic *et al*., 2020; Chong *et al.,* 2018). Some of these molecules such as tocopherols and phenolics are also potential surface-active ingredients. Therefore, they can significantly impact the nanoemulsion formation and stability.

It is well known that there are different grades of the same natural raw materials available on the market, but not much is known about the impact of such fine differences on the formation of nanoemulsions *via* low-energy methods, given the fact that these methods are very sensitive to any change in composition (Chang and McClements, 2014; Solans and Sole, 2012). Aiming to elaborate on this topic, Gledovic *et al*. investigated the impact of different red raspberry seed oils (ROs) on nanoemulsion formation *via* the EPI method at room temperature. The oils used were representatives of several groups, *i.e.* cold-pressed oils: non-organic, refined (RO1) *vs.* organic, unrefined (RO2) and CO_2-

extracted unrefined oils: non-organic (RO3) *vs.* organic (RO4). A very important finding was that all ROs can form nanoemulsion in a simple ternary system composed of Tween 80/RO/water by moderate mixing. The minimal surfactant to emulsion ratio (SER) was 10, and the minimal SOR value was 1.0 (50:50 ratio). However, the obtained nanoemulsions had significantly different droplet sizes and preliminary stability, which can be ascribed to the fine differences in their composition (saturated *vs.* unsaturated fatty acid profiles and the content of tocopherols and carotenoids). Raman spectroscopy confirmed these chemical differences among various ROs as well as their respective nanoemulsions, and it also approved the interactions among nanoemulsion components. It detected interaction among RO2, Tween 80 and glycerol in an aqueous environment which resulted in the formation of nanoemulsions with the smallest droplets (size: ≈125 nm, PDI<0.1), compared to the nanoemulsions prepared with other oils (≈ 144 to 157 nm, PDI<0.14) and poorer stability. Therefore, one RO could not be directly exchanged for the other (*e.g.* the cold-pressed with CO_2-extracted oil) without a significant impact on nanoemulsion properties. In addition, the textural analysis confirmed that cubic liquid crystalline gel phase was a necessary step in the preparation of RO-loaded nanoemulsions *via* the EPI method, and that all ingredients that disturb this step are unfavourable (*e.g.* polyol concentration above 15 wt% or fruit hydro-glycolic antioxidant extracts above 10 wt%, relative to the water phase). Similarly to the other previously mentioned studies (Kim *et al.*, 2011, Qian *et al.*, 2012), this study also revealed that lipophilic (VE acetate) and/or hydrophilic antioxidants (in this case hydro-glycolic fruit extracts from red raspberry fruit - RE or French oak fruit - FE) improve the stability of the nanoemulsions. Although they were physically stable at all tested temperatures (5, 25 or 40˚C, for 45 days), the only formulation which could inhibit the oil oxidation and rancid odour was the nanoemulsion containing FE extract in the water phase (4 wt%) (Gledovic *et al.*, 2020).

4.3.3 Conclusions and future guidelines for formulation development

Based on the presented findings, it is clear that formulation optimisation of nanoemulsions with natural antioxidants is a complex task and many formulation parameters and production variables have to be taken into account to obtain a stable product. Central Composite Design (CCD) is one of the statistical analysis techniques of Response Surface Methodology (RSM), which is sometimes used as a tool in the

formulation development (Chong *et al*., 2018; Alzorqi *et al.*, 2016). This methodology was found helpful in assessing the interactive effect of the independent variables (*e.g.* surfactant concentration, SOR, water content, costabiliser content) and production variables (homogenisation pressure, number of passes, ultrasonication, irradiation time and power) on the dependent variables related to nanoemulsion physical characteristics (*e.g.* droplet size and PDI viscosity).

Future research should be focused on the multicomponent systems (*e.g*. systems with mixed surfactants, coantioxidants and rheological additives, Fig. 4.2) to mimic the more realistic scenario of a product ready for the market. The group of natural emulsifiers are markedly underexplored, especially in combination with the low-energy methods; however, recent reports on the usage of WPI, OSA-MS, sodium stearoyl lactate (SSL) and glycerol esters (glyceryl citrate/lactate/linoleate/oleate) in combination with HPH and USH methods are promising (Kim *et al*., 2011, Qian *et al*. 2012; Mao *et al.*, 2009). It should be noted that the literature data regarding the long-term physicochemical stability of nanoemulsions (*e.g.* 6 - 12 months), even at the room temperature, are practically non-existent. *In vitro* data regarding cytotoxicity and *in vivo* data on human volunteers are also insufficient, having in mind the number of natural antioxidants available in the market. The advancements in these research areas should lead to more reproducible scaling-up procedures and industrial applications as a step towards the creation of stable, safe and efficient nanoemulsions.

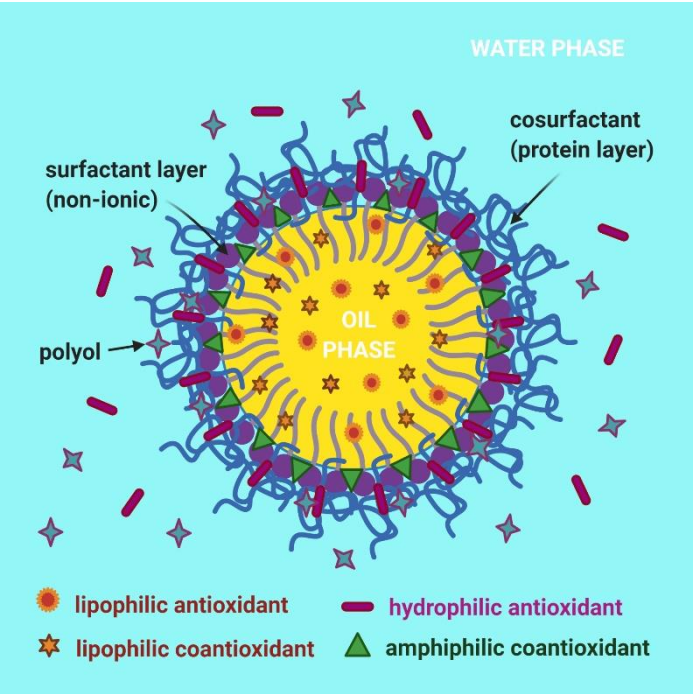

Fig. 4.2. Prototype of a stable O/W nanoemulsion with natural antioxidants

Table 4.1 Nanoemulsions with natural antioxidants – formulation optimisation *via* high-energy and low-energy methods

Antioxidant/ Carrier oil	**Surfactants**	**Water phase**	**Production method**	**Main findings (droplet size distribution, stability)**	**Application/ Reference**
Astaxanthin/ Liquid paraffin, Cholesterol, Ceramide, Ethanol, Coantioxidant	Glyceryl citrate/ lactate/ linoleate/ oleate or hydrogenated lecithin	Water Glycerin Tween 60	**HPH** 1. Pre-mix prepared at 70-75˚C, 3000-4000 rpm, 10 min. 2. Cooling to 50 ˚C, then homogenised with astaxanthin, at 3000-4000 rpm, 5 min, then cooling to RT 3. HPH at 1000 bar.	Nanoemulsionss prepared with mixed glycerol esters (3 wt%) and up to 5.5 wt% astaxanthin had smaller droplets and PDI, and they were more stable then lecithin-based nanoemulsions. The obtained droplet size was: 160 to 190 nm, narrow size distribution. Astaxanthin nanomulsions were stabilised by a multilamellar surfactant layer, as observed by FF-SEM. Coantioxidants (α-TOC or HDBM) protected astaxanthin from heat and light degradation.	Cosmetics/ Pharmaceutics Kim *et al.*, 2011.
β-carotene suspension (30 wt% of β-carotene in sunflower oil)/ MCT	Small molecular weight: Tween 20 DML High molecular weight: OSA-MS WPI	50 mM Aqueous phosphate buffer solution of pH 7 Preservative: Sodium azide 0.01%	**HPH** 1. β-carotene suspension dissolved in MCT (0.03% β-carotene), at 140˚C for a few seconds. 2. Pre-mix: surfactants (1 wt%) dissolved in buffer solution added to the oil phase under continuous mixing at 5000 rpm. 3. HPH at 20, 80 or 140 MPa, for 3 cycles, then cooling to room temperature.	Optimal pressure was 80 MPa, although at 140 MPa nanoemulsions had smaller droplets. Size: Tween 20 - 132.0± 2.5 nm; DML -132.5±2.9 nm; WPI - 183.3± 3.6 nm; WPI+ Tween 20 - 140± 2.3 nm; OSS - 212.2± 2.3; PDI for all nanoemulsions was <0.22 (at 80 MPa). Nanoemulsions stabilised with Tween-20 and DML had smaller droplet sizes, but poorer stability, compared with the OSA-MS and WPI-stabilised nanoemulsions. WPI was able to protect β-carotene from degradation, whereas OSA-MS was not when nanoemulsions were stored at 55 °C for 12 days. The mix of Tween-20 + WPI improved nanoemulsions physical stability, but it did not improve the stability of β-carotene in the nanoemulsions.	Food/ Pharmaceutics/ Cosmetics Mao *et al.*, 2009
β-carotene/ corn oil	β-lactoglobulin or Tween 20	10 mM Aqueous phosphate buffer solution of pH 7, Preservative: Sodium azide 0.01%	**MF** 1. β-carotene 0.5 wt% dispersed in corn oil with mild heating (<5 min, 50-60˚C), then stirring at RT ~ 1 h, to fully dissolve. 2. β-lactoglobulin 2 wt% or Tween 20 1.5% dissolved in the aqueous phase. 3. Pre-mix: 10 wt% oil phase was mixed with 90 wt% (w/w) aqueous phase at RT, with a high-speed blender, 2 min. 4. MF: 3 cycles at 9000 psi.	Size: initially around 90 nm for all β-lactoglobulin nanoemulsions, after 15 days storage at 55˚C, there was little change (<7%) in droplet size, but the distribution became bimodal, probably due to flocculation. Therefore, antioxidants did not influence the nanoemulsion physical stability, *i.e.* droplet size and distribution. The rate of β-carotene degradation decreased upon antioxidant addition with the following order of efficacy: EDTA> ascorbic acid> Q10> VE acetate. No synergism was observed between ETDA and α-TOC. Nanoemulsions stabilised by β-lactoglobulin were more stable to colour fading than those stabilised by Tween 20.	Food/ Pharmaceutics/ Cosmetics Qian *et al.*, 2012
β-D-glucan (from *Ganoderma Lucidum)*/ Palm olein, refined	Polyoxyl 40 Hydrogenated Castor Oil (hydrophilic surfactant)/ Oleoyl macrogol-6 glycerides (lipophilic surfactant)	Water, deionised	**USH** 1. Water phase: hydrophilic surfactant and β-glucan dispersed in water at 40˚C. 2. Lipophilic surfactant dissolved with stirring in the oil phase at 40 ˚C. 3. Pre-mix: oil phase was added to the water phase dropwise, at 40˚C, and then mixed with at 12000 rpm, 20 min at RT. 4. Ultrasonication at 30˚C, max 1000 W,	Nanoemulsions were optimised with experimental design: CCD+ RSM The optimal composition of formulated nanoemulsions (water: 85%, oil/surfactant ratio: 3), as well as the ultrasonic emulsification conditions, (power: 700 W, irradiation time: 300 s) enhanced the nanoemulsions' physical properties by producing lower droplet diameter (263 nm), narrower PDI (0.244) and lower viscosity (1.85 cP). β-D-glucan nanoemulsions exhibited higher stability at lower concentration (1%) compared to the highest concentration (85%) and showed higher antioxidant activity than the free β-glucan (FRAP and DPPH assays).	Cosmetics/ Pharmaceutics Alzorqi *et al.*, 2016

	HLB of the mix: 10		at 20 kHz, with cooling.		
Curcumin/ VE Coantioxidant: Benzyl isothiocyanate-BITC	Tween 80/ SSL	Water, deionised Ethanol	**USH** 1. Pre-mix: the organic phase was added to the water phase (containing SSL) in a drop-wise manner with continuous stirring. 2. Ultrasonication for 10 min and then ethanol was added and probe-sonicated for an additional 3 min (20% amplitude, 60 W).	The optimal ratio of water: surfactant: oil was (94:4.5:1.5) with (SSL/Tween-80) = 0.023, and Ethanol (2 wt%), to yield transparent nanoemulsions. Size: pure nanoemulsion: 38± 3nm, curcumin nanoemulsion: 49± 3nm, curcumin + BITC nanoemulsion: 53± 2 nm. All nanoemulsions exhibited good storage stability up to 90 days at 4, 25 and 37 ˚C. Nanoemulsions protected curcumin from UV light i.e. only 7% curcumin degraded in nanoemulsion compared to 78% in water/ethanol system after 120 min. The IC_{50} value for pure nanoemulsion, curcumin nanoemulsion, BITC nanoemulsion and curcumi + BICT nanoemulsion was: 85.46 µM, 104.24 µM, 58.73 µM and 75.35 µM, respectively (DPPH assay). Curcumin + BITC nanoemulsion shows a synergistic antioxidant effect.	Pharmaceutics/ Cosmetics/ Food Kaur *et al.,* 2017
Curcumin/ MCT	Tween 80/ Lecithin (soybean) HLB at 9:1 ratio ~14	Water, ultrapure	**SE** 1. Lecithin was dissolved in the MCT, Tween 80 was added, and mixing for 30 min. 2. Curcumin was dissolved in the organic phase 3. The oil and surfactant blend was added dropwise to water under constant stirring at 1000 rpm, with a magnetic stirrer.	The optimal formulation components: Tween 80: Lecithin (9:1, 10 wt%), MCT 10% and ultrapure water 80 wt% + curcumin (1, 2 and 3 mg/mL). Physicochemical stability was demonstrated during 3 months at RT (mean droplet size: 111.3–146.8 nm; PDI < 0.2; pH: 4.73–5.73). Potent antioxidant activity of curcumin in nanoemulsions was confirmed *via* DPPH (IC_{50}=0.1187 mg/mL) and FRAP (1.19 ± 0.02 mmol/g), with no alterations after incorporation in the formulation.	Pharmaceutics/ Cosmetics Nikolic *et al.,* 2018
VE acetate/POEs	Tween 80/ Poloxamer 188 (Pluronic 68) Optimal ratio: 40:1	Water, deionised	**EPI** 1. The surfactant mixture was first dissolved into a mix of POEs and VE acetate, stirring at 150 rpm. 2. Deionised water was added dropwise while stirring at 150 rpm, after that it was homogenised at 250 to 350 rpm for 4 hours. 3. High-shear homogenisation at 10000 rpm, 5min.	The optimal formulation contained: 10 wt% POEs, 10 wt% VE acetate, 24 wt% Tween 80, 2.4% Pluronic F-68 and 53.6% deionised water. This formulation is considered to be the best as a nanocosmeceutical product due to the small droplet size (94.21 nm), low occurrence of Ostwald ripening and stable at different storing temperatures (5, 25 and 45°C) for four weeks. In conclusion: TA and Pluronic improved nanoemulsion stability and elevated temperatures, and TA decreased droplet sizes< 100 nm (at 8 or 10 wt%).	Cosmetics/ Pharmaceutics Teo *et al.,* 2010
α-TOC / Olive oil (as a source of LCT) MCT Short-chain triglyceride - SCT	Tween 80	Water, Citric buffer of pH 3.0	**EPI** 1. The organic phase containing the carrier oil, Tween 80 and α-TOC was stirred for 30 min at 500 rpm with a magnetic stirrer. 2. The water phase (citric buffer, pH 3.0) was titrated into the oil phase at a flow rate of 20 drops per 10 s, with constant stirring at 500 rpm.	The optimal formulation contained: 8wt% α -TOC, and 2wt% carrier oil, SER 1, SOR 1. Droplet sizes of nanoemulsions prepared with different carrier oils: 82.6 ±0.7, 113.4±1.4 and 87.7±2.1 nm) using SCT, MCT and LCT as carrier oils, respectively. α-TOC -loaded nanoemulsions with SCT, MCT and LCT showed physical stability to heat shock (30–90°C, 30 min), ionic strength (0–500 mM), pH (2.0–8.5) and long term storage (60 days, under light and darkness, 4, 25, 40 °C), but there was significant α-TOC degradation in heat processed and long-term storage samples.	Pharmaceutics/ Cosmetics Hategekimana *et al.,* 2015
ɣ- Oryzanol/ MCT Fish oil (as a source of LCT)	Tween 80/ Span 20 Optimal ratio 3:1 HLB mix: 13.4	Water, deionised Citric acid 1% Sodium benzoate 0.1%	**SE** 1. γ-oryzanol (1% wt) was dissolved in the oils phase, then the surfactant mix was added and stirred for a minimum of 20 min at RT to obtain the organic phase.	Optimal formulation contained: 1 wt% γ-oryzanol, 10 wt % oil phase (MCT to fish oil =7:3) and 10 wt% surfactant mix. Nanoemulsion droplet size: ~ 157 nm, PDI< 0.14.	Food/ Pharmaceutics/ Cosmetics

			2. The organic phase was added to the water phase under stirring (850 rpm), with a titrating speed of 60 drops per minute. 3. Homogenisation: additional 5 min, at 850 rpm.	γ-oryzanol nanoemulsions were physically stable at a broad pH range (2–7), high salt levels (≤0.8 mol L−1), high sugar concentrations (≤16%), and heating temperatures below 50 °C. The oxidative stability of the nanoemulsions compromised at temperatures above 37˚C, as confirmed by elevated peroxide and p-anisidine values, therefore storage at temperatures <23˚C are recommended.	Zhong *et al.*, 2017
Cinnamon essential oil/ MCT	Tween 80	Water, deionised	**PIT** 1. Pre-mix: Cinnamon oil and MCT were mixed for 3 min, and then Tween 80 and deionised water were added and mixed for 30 min. 2. Pre-mix is heated to temperature 15 ˚C above the PIT. 3. A two-step cooling process: cooling to the PIT to form the microemulsion phase. Secondly, rapid cooling by adding cold deionised water (4˚C) to the system with stirring 3 min.	The cinnamon oil-to-carrier oil ratio in the lipid phase impacted the PIT temperature, initial mean droplet diameter, droplet size distribution, and stability of the cinnamon oil nanoemulsions. Optimal Cinnamon oil: MCT ratio was found to be 4: 6 (10 wt% oil phase). Cinnamon oil nanoemulsions prepared using a PIT with cooling-dilution method had droplet diameters ~ 100 nm (at 10 wt% Tween 80) and 20 nm (at 20 wt% Tween80), and they were stable during storage at low temperature (4˚C) or ambient temperature (25 ˚C) for at least 31 days.	Food/ Pharmaceutics Chuesiang *et al.*, 2018
Orange peel essential oil/ MCT	Tween 20, Tween 40, Tween 60, Tween 80, Tween 85 Span 20	5 mM Aqueous citrate buffer of pH 3.5	**SE** Nanoemulsions were prepared by titration of a mixture of orange oil, carrier oil -MCT, surfactant (Tween) into an aqueous solution (5 mM citrate buffer at pH 3.5) with continuous stirring. The oil/emulsion ratio content was kept constant (10 wt %), while the SER varied (2.5−20 wt %).	Transparent nanoemulsions could be formed under certain conditions: 20 wt % surfactant (Tween 40, 60, or 80) and 10 wt% oil phase (4−6 wt% orange oil + 6−4 wt% MCT). Surfactant type and oil-phase composition also affected the thermal stability of the nanoemulsions. Most of the nanoemulsions broke down after thermal cycling (from 20 to 90 °C and back to 20 °C). Only one system remained transparent (droplet size ~ 25nm) after thermal cycling, the formulation with: 20 wt% Tween 80, 5 wt% orange oil, and 5 wt% MCT.	Food/ Pharmaceutics Chang and McClements, 2014
Basil essential oil/ MCT	Tween 80 Span 80	Water, deionised	**HSM** 1. The organic phase (basil oil + lipophilic surfactant Span 80 and the water phase (water + hydrophilic surfactant Tween 80) were mixed separately with a magnetic stirrer. 2. High shear homogenisation: the organic phase was added to water phase at 10000 rpm, then speed was increased to 17000 rpm and mixed for 30 minutes, with cooling.	The optimal formulation contained: basil oil 7.5 wt%, Span 80 2 wt%, Tween 80 2wt%, water 88.5 wt%, with a droplet size of ~119 nm, PDI~0.16. The obtained nanoemulsions were physically stable at 4, 25 up to 45 days, while at 45˚C the pH value decreased due to hydrolysis of fatty esters in the aqueous surrounding. Therefore, storage at 4˚C is recommended, in which case, 87% of estragole remained after 90 days. Moreover, basil-loaded nanoemulsions maintained antioxidant activity in NE carrier (DPPH test) with reduced cytotoxicity.	Food/ Pharmaceutics Da Silva Gundel *et al.*, 2018
Citrus medica essential oil/ MCT	Tween 80	5 mM Aqueous citrate buffer of pH 6.0	**SE** 1. The organic phase (essential oil, surfactant and MCT were mixed, at 500 rpm with a magnetic stirrer. 2. The water phase (aqueous citrate buffer) was previously prepared. 3. The organic phase was titrated into the water phase at 2 ml/min, under continuous mixing at 500 rpm.	Optimal formulation: Citrus essential oil 5 wt%, MCT 5%, Tween 80 20wt% and water phase 70 wt%. Obtained citrus oil nanoemulsions had droplet size: ~73 nm, and were stable 30 days, at RT. The nanoemulsification significantly increased the antioxidant, antibacterial and antibiofilm activity of essential oil. DPPH assay - citrus oil: 44.3% *vs.* citrus nanoemulsion: 72.4%; Hydroxyl radical scavenging - citrus oil: 26.1% *vs.* citrus nanoemulsion: 58.7%	Food/ Pharmaceutics/ Cosmetics Lou *et al.*, 2017

				Iron reducing power - citrus oil: -0.106 *vs.* citrus nanoemulsion: 0.218, at 0.48 mg/ml.	
LEO	Tween 80	Water, deionised Sodium alginate	**MF** 1. Water phase: sodium alginate (1 wt%) was dissolved in hot water at 70 ˚C and continuous stirring. 2. Pre-mix emulsion was made by mixing the water phase and LEO (1 vol%) as a lipid phase plus Tween 80 (1 vol% (as a surfactant, with an HSM, at 3400 rpm for 2 min**.** 3. MF: at 50, 100 or 150 MPa, for 1, 2, 3, 4, 5 and 10 cycles, with temperature constantly cooled to <20˚C	The average droplet size, viscosity and whiteness index of nanoemulsions decreased by increasing the processing pressure and the cycles through the interaction chamber of the microfluidizer device. After homogenization with MF, the interfacial electrical charge of droplets ranged between -36.66 and -51.95 mV, irrespectively of the pressure applied and the number of cycles. Therefore, LEO-alginate nanoemulsions obtained with MF are more stable than pre-mix. The optimal formulation was obtained after 3 cycles at 150 MPa, with very small droplet sizes: ~7 nm, PDI~ 0.34.	Food/ Pharmaceutics/ Cosmetics Salvia-Trujilo *et al.* 2013
LO/PFO	Tween 80 Span 80 PEG-30, PEG-40, PEG-60 Castor oil	Water, deionised	**PIT** 1. The water phase and oily phase + surfactants blend were heated separately at 75 ± 3ºC, 2. The water phase was added to the oily phase (PFO with or without LO) under 600 rpm 3. Cooling 25 ± 3ºC under stirring.	The minimum surfactant concentration necessary for the formation of nanoemulsions was 5.0 wt%. LO caused the reduction in droplet sizes in mixed LO+PFO oil phases due to its co-stabilizing properties. LO-loaded nanoemulsions could be formed at several PFO: LO ratios: 5:1, 5:2 and 5:5 (droplet sizes ~ 105 nm, 54, and 38 nm, respectively). There were no observed changes in the LO main constituents in LO-loaded nanoemulsions at temperature up to 25˚C, but the degradation of linalyl acetate was observed after thermal stress.	Cosmetics/ Pharmaceutics Rocha-Filho *et al.*, 2014
RO/Isostearyl isostearate (ISIS) Coantioxidant: VE acetate	Tween 80	Water, deionised Glycerol or Hydro-glycolic antioxidant fruit extracts: RE or FE	**EPI** 1. The organic phase (RO and Tween 80, with or without ISIS and/or VE acetate) and the water phase (water, glycerol/hydro-glycolic extracts) were mixed separately at 1300 rpm. 2. The water phase was added gradually, to the organic phase, with hand mixing with glass laboratory sticks until the gel phase is crossed, and then vortex mixing at 1300 rpm continued until the nanoemulsion was formed and the sample was homogenised 2 min at 1300 rpm.	It was found the oil type had a major impact on nanoemulsion formation and stability. The organic, cold-pressed, unrefined oil RO2 gave the optimal nanoemulsions with the smallest droplets and PDI and overall stability, regarding all oil phase (VE acetate, ISIS) and water phase variations (size: 125 to 135 nm; PDI < 0.1). The synergistic free radical scavenging effect was pronounced in nanoemulsions with combined lipophilic (in RO2) and hydrophilic antioxidants (in FE) with very high DPPH and ABTS results (>90% inhibition), and good stability at 40˚C. All raw materials and low-energy nanoemulsions showed satisfactory safety profiles in the MTT test on MRC-5 cells, while the anti-proliferative effect was more pronounced on HeLa cells when using nanoemulsionsthan neat ingredients.	Cosmetics/ Pharmaceutics Gledovic *et al.*, 2020
Red palm oil (RPO)	Tween 80/ Span 80	Water, deionised Glycerol Preservative: Citric acid 0.08%	**HPH** 1. The organic phase (RPO and Span 80) was added to the water phase (water, glycerol, Tween 80, citric acid) mixed using high shear at 6000 rpm for 10 minutes. 2. Pre-mix then were passed through high-pressure homogeniser (up to 7 cycles, at 500 – 900 bar).	The formulation was optimised using CCD coupled to RSM and it contained: 6.09 wt% mixed surfactant (Tween 80/ Span 80 (63:37, wt)), 20 wt% glycerol as a cosolvent *via* homogenisation pressure (500 bar). The optimised RPO-based nanoemulsion had droplet size and PDI were 119.49 nm and 0.286, respectively, which was in agreement with the RSM predicted values, and nanoemulsions were preliminary stable for 35 days at RT.	Cosmetics/ Pharmaceutics Chong *at al.*, 2018
Rice bran oil (RBO) Coantioxidant:	Span 80 PEG-30 Castor oil	Water, deionised Preservative:	**PIT** Water and organic phases were heated separately at 75°C, the water phase was added into the organic phase (RBO and surfactants)	The optimal nanoemulsion was composed of 10% RBO, 10% mix, 0.05% antioxidant and 0.50% preservatives in water (size: 69±17 nm). Nanoemulsions were physically stable at 4, 25 and 40˚C for 90 days.	Cosmetics/ Pharmaceutics Bernardi *et al.*,

butyl hydroxytoluene	Surfactant mix HLB: 8.0	0.5%	with continuous stirring at 600 rpm. After nanoemulsion formation, the mixture was cooled to 25°C while stirring.	*In vivo* studies showed that nanoemulsions have hydrating properties on healthy volunteers and psoriasis patients and maintained normal skin pH. *In vitro* HET-CAM test revealed the non-irritant nature of RBO-loaded nanoemulsions as opposed to the slight irritant nature of the neat surfactant.	2011
Wheat bran oil (WBO)	Tween 80 Tween 20 Span 80 Diacetyl tartaric acid ester of mono- and diglycerides (DATEM)	Water, deionised	**USH** 1. Pre-mix preparation: WBO and surfactants were mixed separately before water was added and homogenised with a high-speed blender at 29000 pm. 2. High-intensity ultrasonication: at 500 W, 20 kHz, at 20% amplitude and in pulses of 5 s (5 s ultrasound and 5 s pause) to avoid heating of the sample.	The optimal nanoemulsion was obtained *via* CCD and RSM when 1 wt% of WBO and 7.3 wt% of a surfactant mixture of Span 80: Tween 80 (37.4:62.6) were emulsified in water by high-intensity ultrasonication for 50 s after pre-emulsification with a high-speed blender for 5 min (~39 nm, PDI~0.25). Nanoemulsions showed good stability when stored at 4˚C during 60 days. Antioxidant activity of WBO nanoemulsions and pure oil were confirmed *in vitro* with several tests (DPPH, FRAP and ABTS) and formulation also inhibited mushroom tyrosinase activity (skin whitening action).	Cosmetics/ Pharmaceutics Rebolleda *et al.*, 2015

4.4 Screening the antioxidant activity – an overview of the well-established methods and future perspectives

An important feature of hydrophilic nanoemulsions in pharmaceutical, food and cosmetic industry is their ability to effectively solubilise/encapsulate lipophilic active and/or functional components such as vitamins and nutraceuticals, flavours, colouring agents, antioxidants, preservatives. Due to their properties, nanoemulsions provide improved stability and handling, facilitated incorporation of the specific component within a product, increased (bio)availability and efficacy, with good visual appearance of the final product (McClements and Rao, 2011).

The concept of antioxidant capacity first originated from chemistry and it was later adapted to biology, medicine, epidemiology and nutrition, describing the ability of redox molecules to scavenge free radicals (Floegel *et al*., 2011). For the determination of antioxidant ability of different antioxidant molecules *per se*, as well as the ability of antioxidant-loaded nanoemulsions, different methods have been introduced.

A standardised method for antioxidant activity should, ideally, meet the following requirements (Prior *et al*., 2005):

- It utilises a biologically relevant radical source;
- It is simple;
- It has defined endpoint and chemical mechanism;
- Required chemicals and instrumentation are readily available;
- It has good within-run and between-day reproducibility;
- It should be adaptable for assay of both hydrophilic and lipophilic antioxidants and radicals from different sources;
- It should be as close as possible to the real application;
- It is adaptable to “high-throughput” analysis for routine quality control analyses.

In line with the assessment of other nanoemulsion properties, the antioxidant activity assessment should not be based on a single antioxidant test. It is always advised to perform several *in vitro* antioxidant evaluation procedures. Moreover, due to significant differences underlying each method, the obtained results cannot be easily compared (Alam *et al.*, 2013). Therefore, the results should always be critically analysed before reaching any conclusion.

In general, antioxidant can deactivate free radicals by hydrogen atom transfer (HAT) or single electron transfer (SET). Consequently, methods developed for antioxidant activity assessment can be categorised as HAT-based or SET-based methods. HAT-based methods measure the ability of an antioxidant to quench free radicals by hydrogen donation, whereas SET-based methods detect the ability of a potential antioxidant to transfer one electron and reduce any compound. The two mechanisms may occur in parallel, but the dominating one will be determined based on the antioxidant structure and properties (Prior *et al.*, 2005). In addition, it is noteworthy that nanomaterials may possess inherent antioxidant ability (regardless of the presence of an antioxidant molecule) due to their possibility to trap or adsorb free radicals, thus preventing them from continuing oxidative reactions. Therefore, such property should also be appropriately checked (Valgimigli *et al*., 2018).

The most popular assays of radical trapping share a simple principle: the change in absorbance or fluorescence of an indicator solution (free radical/oxidising agent) is measured upon addition of an antioxidant (Fig. 4.3). The measurement is generally performed after a certain time in order to allow equilibrium to be established (Li *et al.*, 2015). Even though translation of the results obtained after colourimetric antioxidant assays to physiological context may be difficult, they are fast and easy to perform, presenting good screening tools. In this section, several commonly applied methods for antioxidant determination will be described underlining their advantages and disadvantages with respect to antioxidant-loaded nanoemulsion testing. The most important findings are summarised in Table 4.2.

4.4.1 Spectrophotometric methods

4.4.1.1 DPPH radical scavenging assay

This method is widely used for the determination of free radical scavenging activity. DPPH• (2,2-diphenyl-1-picrylhydrazyl) represents a stable free radical. It is soluble in organic solvents (*e.g.* ethanol, methanol), rendering intensively purple-coloured solutions (λ_{max} ~ 515-520 nm). An antioxidant with proton-donating ability can react with DPPH•, forming DPPH-H, which fades out the purple colour of DPPH• solution. The degree of discolouration depends on the potency and concentration of the antioxidant, but also on the reaction time (duration of the experiment). During the experiment, prepared mixtures of the radical with different concentrations of the

antioxidant should be protected from light and continuously shaken. The inhibition percentage (%) of radical is calculated applying the following formula (Eq. 4.1):

$$\%\ of\ inhibition = \frac{A_{blank\ sample} - A_{test\ sample}}{A_{blank\ sample}} * 100 \quad (4.1)$$

After plotting the inhibition percentage versus antioxidant concentration, results are usually expressed as IC_{50} (inhibition concentration of 50 %), defined as the concentration of the potential antioxidant able to decrease 50% of the initial absorbance of the radical (Nile *et al*., 2012). However, taken alone, this parameter does not provide comparable results with those obtained applying the same assay, but for different reaction time. It can be used only to compare the activity of different antioxidants evaluated in the identical experimental setting (Amorati and Valgimigli, 2015). In addition, there are some disadvantages when lipid-based antioxidant carriers (*e.g.* nanoemulsions) are tested through this methodology, since it is performed in an organic solvent. By dissolving the lipid-based components of the carrier by in the organic solvent, the antioxidant would be released from the formulation immediately upon the contact with the solvent. Consequently, it cannot be claimed that measured effect comes from the loaded formulation, but from the antioxidant released upon disruption of the carrier's structure. However, even with that disadvantage, this method can be used as a stability assessment tool for antioxidants incorporated in a carrier (Nikolic *et al.*, 2018).

4.4.1.2 ABTS radical scavenging assay

ABTS scavenging assay is also a spectrophotometric test. In this assay, ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)) is oxidised to its radical cation, $ABTS^{\bullet+}$, which has intensive blue-green colour (Gledovic *et al*., 2020). Originally, this assay used metmyoglobin and H_2O_2 to generate ferrylmyoglobin, which then reacted with ABTS, forming $ABTS^{\bullet+}$ (Miller *et al.,*. 1993). In the following years, various oxidising agents have been used to generate the radical (potassium persulfate – $K_2S_2O_8$ (Re *et al*., 1999), manganese dioxide – MnO_2 (Miller *et al.*, 1996) or oxidising enzymes (Cano *et al.*, 2013). The $ABTS^{+}$ has strong absorption at 734 nm. Upon reaction with an antioxidant, there is a decrease in the colour intensity, which correlates with the antioxidant ability of the tested compound. The antioxidant activity is defined as the amount of $ABTS^{\bullet+}$ quenched after a fixed time, usually 6 to 30 minutes, which has to be optimised (Amorati and Valgimigli, 2015). Since this test is usually performed in an aqueous environment (such as phosphate-buffered saline, pH 7.4), when the hydrophilic nanoemulsions loaded

with antioxidants are tested, their structure can be preserved (Gledovic *et al.*, 2020, Rebolleda *et al.*, 2015). In the comparative study of the antioxidant activity of a variety of fresh fruits, vegetables and beverages consumed as a source of antioxidants, it was observed that the antioxidant capacity detected by ABTS assay was significantly higher compared to that by DPPH assay. It was concluded that the high-pigmented and hydrophilic antioxidants were better reflected by ABTS assay (in PBS buffer - aqueous environment) than DPPH assay (in methanol) which suggests that ABTS assay may be more useful than DPPH assay for detecting antioxidant capacity in a variety of foods (Floegel *et al*., 2011). Another advantage of the ABTS test is the fact that it can also be performed in other solvents *e.g.* ethanol, methanol and dimethyl sulfoxide (DMSO) (Rinaldi *et al*., 2017). Therefore, the choice of solvent is one of the crucial parameters in ABTS test and it can be adjusted to the solubility of the tested antioxidants.

4.4.1.3 FRAP assay (Ferric reducing antioxidant power)

This is a frequently used assay, based on the reduction of Fe^{3+} ion. Prior to the analysis, FRAP reagent, consisting of acetate buffer (pH 3.6), TPTZ (2,4,6-tripyridyl-s-triazine) solution in HCl and $FeCl_3x6H_2O$ solution in purified water, should be formed (Nile *et al.*, 2012). The antioxidant should react with the reagent for 30 minutes under controlled temperature conditions (37°C). During the reaction, Fe^{2+} ion is generated, rendering blue solution and absorbance can be detected at 595 nm. The results are expressed as FRAP value - mmol of Fe^{2+} per gram of dry matter. The test is useful for the assessment of hydrophilic and lipophilic antioxidants, as well as antioxidant-loaded carriers, such as hydrophilic nanoemulsions (Nikolic *et al.*, 2018). However, as the mechanism involved is SET-based, the assay may not detect the activity of antioxidants, acting only *via* HAT (Pisoschi *et al.*, 2016).

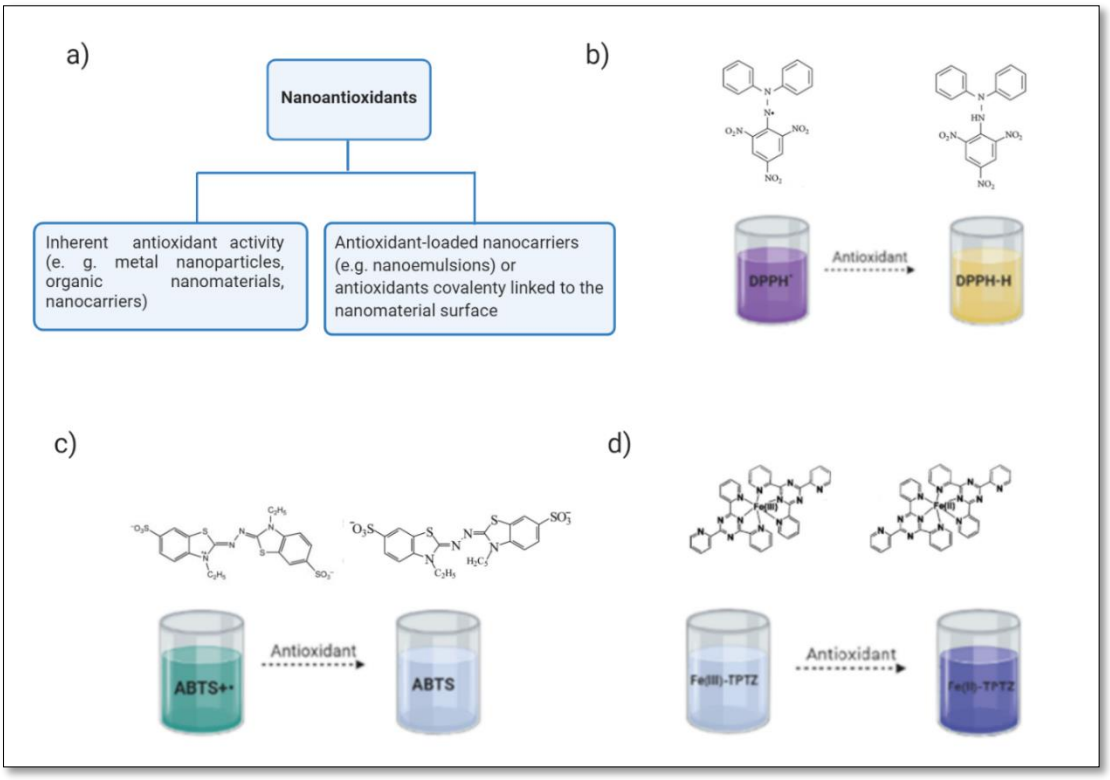

Fig. 4.3 a) Nanoantioxidants: structural properties determine the antioxidant activity; b) Schematic representation of the DPPH radical scavenging assay; c) Schematic representation of the ABTS radical scavenging assay; d) Schematic representation of the FRAP assay

4.4.2 Non-spectrophotometric methods

4.4.2.1 Electron paramagnetic resonance spectroscopy

Antioxidant activity evaluation through electron paramagnetic resonance (EPR) spectroscopy represents a valuable method and an alternative to spectrophotometric approaches, avoiding some interferences that may occur due to the overlap of the absorption bands of the probe or of the reaction products with that of the nanomaterial (Valgimigli *et al.*, 2018). The method itself involves the absorption of microwave energy by paramagnetic species (molecules with unpaired electron – such as free radicals) produced during transition of spin states in the presence of an external magnetic field. Therefore, it can be used in the antioxidant activity assessment (Nawab *et al.*, 2017). Unlike other usually applied methods, EPR spectroscopy enables detection of the inherent antioxidant activity of a nanocarrier (*e.g.* nanoemulsions). EPR antioxidant activity tests can be performed in aqueous environment, enabling experiments with hydrophilic lipid-based systems, such as O/W nanoemulsions. One of the widely used free radicals in this

kind of experiments is Tempol (4-hydroxy-2,2,6,6 - tetramethyl-l-piperidinyloxy) - a stable nitroxide radical, characterised by a well-defined EPR spectrum consisting of three peaks. In the presence of compounds with antioxidant activity, the EPR spectrum of Tempol is reduced after the scavenging reaction with the antioxidant. The % of inhibition of the EPR spectrum is calculated from the following equation (Eq. 4.2):

$$\%\ of\ inhibition = \frac{A_0 - A_S}{A_0} * 100 \quad (4.2)$$

where A_0 represents the integral intensity of the EPR spectrum of a control sample (blank), and A_s is the integral intensity of the EPR spectrum in the presence of the test sample.

Many research groups have successfully applied EPR to perform antioxidant activity evaluation of various types of antioxidants or antioxidant-loaded nano-dispersed systems (Mitsou *et al.*, 2019; Sanna *et al*., 2019; Aboudzadeh *et al.*, 2018; Chatzidaki *et al.*, 2015). However, the main disadvantage is associated to the costs of the equipment.

4.4.2.2 Electrochemical method based on cyclic voltammetry (CV)

Another promising non-spectrophotometric method which can be used to assess the antioxidant activity of natural antioxidants before and after nanoemulsification is electrochemical method based on CV. Unlike the standardly used DPPH and ABTS radicals, which do not occur in nature, the oxygen-derived free radicals are readily produced in biological systems, causing damage to lipids, proteins and DNA, as a main cause of many diseases. Therefore, it can be of great interest to directly test the radical scavenging activity of a potential antioxidant with a more biologically compatible ROS. Prior to electrochemical measurements, the investigated antioxidant compounds are usually dissolved in DMSO, but it has been reported that O/W nanoemulsions, or standard antioxidants diluted in sunflower oil, can be analysed without the use of organic solvents, *e.g.* in their original state, which is a very favourable experimental setting (Benedetti *et al.*, 2012).

In the electrochemical approach, CV is used to electrocatalytically reduce oxygen to O_2•-, which further reacts with a radical scavenger. The information regarding a radical scavenging activity can be obtained analysing the evolution of the electrochemical response upon successive addition of a radical scavenger. The antioxidant activity ranking is established by evaluating the decrease of charge under anodic wave upon addition of antioxidant (Q), relative to the charge observed in O_2-saturated solution without

antioxidants (Q_0), where O_2•− scavenging activity is quantified as an absolute value of a slope of a Q/Q_0 vs. concentration of the sample (Janosevic Lezaic *et al*., 2014; Dimitric Markovic *et al*., 2012). Two possible mechanisms for the reaction of polyphenols with O_2•− (proton-transfer and H-transfer i.e. radical-transfer), can be investigated based on the analysis of their cyclic voltammograms. For instance, in the presence of polyphenols (which are main antioxidant actives in many natural extracts and oils), there is no increase of cathodic peak current upon sample addition into O_2-saturated solution, and the appearance of cathodic pre-peaks (or pre-waves) is indicative of a prevailing H-transfer (radical-transfer) mechanism. Although electrochemical analysis is a convenient method that can be performed in an aqueous environment (Benedetti *et al*., 2012), it should be noted that a direct comparison of radical scavenging activities of two different compounds or systems can be made only if the electrochemical assay is performed under identical conditions (Janosevic Lezaic *et al*. 2014; Dimitric Markovic *et al*., 2012).

Table 4.2 Selection of commonly applied tests for antioxidant activity, accompanied with useful references providing methodological entries

Antioxidant assay	Principle/Detection method	Advantages	Disadvantages	Useful references [2]
DPPH radical scavenging assay	• Mixed HAT- and SET-based method • UV-Vis Spectrophotometric determination	• Affordable, simple, fast and reproducible method • Applicable to both hydrophilic and lipophilic antioxidants • Apart from spectroscopy measurements, DPPH radical can be determined applying EPR-spectroscopy, as well.	• DPPH radical may react with reductants having no antioxidant activity. For instance, DPPH• can be completely reduced by H_2O_2, which cannot be considered an antioxidant. • Results highly depend on the reaction time, so obtained data can be compared only in case of identical experimental setting. • It cannot determine the reaction kinetics. • The reaction milieu (organic solvents) breaks the structure of lipid-based carriers (*e.g.* nanoemulsions).	• Gledovic *et al.*, 2020 (nanoemulsions prepared with RO and/or hydrophilic antioxidant fruit extracts) • Nikolic *et al*., 2018 (curcumin and curcumin-loaded nanoemulsions) • Rinaldi *et al.*, 2017 (nanoemulsions prepared with neem oil) • Zugic *et al*., 2015 (different Usnea barbata extracts)
ABTS radical scavenging assay	• SET-based mechanism • UV-Vis Spectrophotometric determination	• It is usually performed in aqueous environment (PBS buffer), so that hydrophilic nanoemulsions containing antioxidants can retain their structure. • As, various solvents can be used (ethanol, methanol, DMSO), this method is adaptable to hydrophilic and lipophilic antioxidants.	• ABTS radical has to be generated at the beginning of the reaction • Time consuming • Results are dependent on the reaction time	• Gledovic *et al.*, 2020 (nanoemulsions prepared with RO and/or hydrophilic antioxidant fruit extracts) • Rinaldi *et al.*, 2017 (nanoemulsions prepared with neem oil) • Rebolleda *et al.*, 2015 (WBO-loaded nanoemulsions)
FRAP assay	• SET-based mechanism • UV-Vis spectrophotometric determination	• It is performed in aqueous environment, adaptable to hydrophilic and lipophilic antioxidants, as well as their carriers (such as hydrophilic nanoemulsions)	• Time consuming – the FRAP reagent should be prepared prior to the reaction • Temperature control is required • Detects antioxidant activity only for the antioxidants acting via SET mechanism.	• Nikolic *et al*., 2018 (curcumin and curcumin-loaded nanoemulsions) • Rebolleda *et al.*, 2015 (WBO-loaded nanoemulsions)
EPR spectroscopy	• Determination of the EPR spectra of a free radical	• It can be performed both in aqueous environment and in organic solvents • Interference due to the overlap of the absorption bands of the probe or of the reaction products with the nanomaterial that may hinder spectroscopic measurements ca be avoided. • It is possible to evaluate the kinetics of the process • It is possible to detect inherent antioxidant activity of the nanocarrier	• Experiments are costly due to sophisticated equipment (EPR spectrometer)	• Nikolic *et al.*, 2020 (curcumin and curcumin-loaded nanoemulsions) • Mitsou *et al.*, 2019 (Hydroxytyrosol-loaded microemulsions) • Sanna *et al.*, 2019 (myrtle hydroalcoholic extracts) • Aboudzadeh *et al.*, 2018 (VE-loaded microemulsions) • Chatzidaki *et al*., 2015 (microemulaina loaded with various phenolic antioxidants) • Polovka *et al*., 2003 (green, black and mixed fruit tea samples)
Electrochemical method (CV)	• Analyses changes in the CV recorded upon successive addition of sample containing antioxidant molecules that originate from its chemical reaction with $O2^{\bullet -}$.	• Gives insight into free radical scavenging activity of a tested compound in reaction with a biologically compatible ROS, such as superoxide anion ($O2^{\bullet -}$). • It can be performed both in aqueous environment and in organic solvents. • It is inexpensive and easy to reproduce.	• The results of different studies can be compared only if the test is performed in an identical way.	• Benedetti *et al.*, 2012 (nanoemulsions loaded with olive oil, or caffeic acid in sunflower oil). • Janosevic Lezaic *et al.*, 2014 (polyaniline tannate solid microspheres).

[2] Tested antioxidants or antioxidant-loaded carriers are indicated in the parentheses.

4.4.3 Conclusion

Due to the growing interest in the substances of natural sources with antioxidant effects, the area of their application has been significantly enlarged. A great diversity of antioxidant test methods is described in the literature, but the three presented methods (DPPH, ABTS and FRAP) remain well-established since they are applicable to various isolated compounds and antioxidant-loaded emulsions. However, the comparison of the results obtained in different studies remains a difficult task, due to large variations in experimental setting, solvent type and test concentration range. In order to generate valid conclusions, the selection of appropriate methods and their concomitant use is an imperative for a reliable assessment of antioxidant activity of the molecule *per se*, but also of the antioxidant-loaded carrier. Non-spectrophotometric methods are a valuable addition to the standard protocols, since they can be performed with nanoemulsions in their original state (in the aqueous environment). They should be used more often in future work.

4.5 *In vitro* safety and efficacy screening of nanomaterials using cell cultures: focus on nanoemulsions

Despite evident increase in the use of nanomaterials in medicine and consumer products, there is a general lack of standardised protocols for their characterisation, especially in terms of safety aspects and biological interactions (Rischitor *et al.*, 2016). Even though *in vitro* test methods with cell cultures represent substantial tools for both mechanistic toxicity studies in fundamental research and for toxicity screening purposes, the existing established protocols usually apply to pure chemicals/test compounds, whereas they are not completely applicable to complex nanoformulations, such as nanoemulsions (Gioria *et al.*, 2018).

Due to the observed cell-specific effects, the selection of optimal cell line for toxicity screening should be done based on the intended route of administration, target tissue and estimated exposure, preferably applying more than one test (Aslantürk, 2018; Joris *et al.*, 2016). However, many studies indicate that, after systemic exposure, most of the nanoparticulate systems are eliminated from the body through liver and kidneys, making these organs suitable for toxicity tests. Therefore, there are some available protocols for cytotoxicity assessment applying porcine kidney (LLC-PK1) and human cancerous liver cells (Hep G2) (Potter and Stern, 2010). As common methodologies, 3-

(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) release are performed. To complement the findings, and to reveal the exact mechanism of the obtained effects, genotoxicity assessment is also advised applying, for example, COMET assay (Krug and Wick, 2011).

Being a highly interdisciplinary field, working with nanosystems in a biomedical context requires a strong understanding of colloidal behaviour and familiarity with specific characterisation methods, biology, and bio-nano interactions. When reporting results, some seemingly simple and trivial experimental details may be overlooked, giving misleading interpretations (Moore *et al*, 2019). Reliable experimental protocols are essential for proper processing of the results. Much work has been done in order to investigate the effect of particle physicochemical properties on particle-cell interactions, targeting, cellular uptake and toxicity (Kinnear, 2017; Zhao *et al.*, 2011). However, issues of reproducibility still persist, which are, at least partly, caused by lack of reporting, non-standardised characterisation of nanomaterials and variations in biological assays (Faria *et al.*, 2018).

Due to their diversity, and sometimes inherent complexity, there are numerous obstacles that may be involved in the *in vitro* screening of nanomaterials. A thorough characterisation of the tested nanomaterial and its behaviour both before and during toxicity assessment is a prerequisite to properly address these issues (Bouwmeester *et al.*, 2012). With this in mind, in the following section, we present the methods used for *in vitro* nanomaterial safety and efficacy assessment, underlining some useful considerations aiming to prevent commonly occurring pitfalls. Some specific aspects related to the nanoemulsions are also addressed.

4.5.1 Commonly used *in vitro* cell viability assays

As previously mentioned, MTT and LDH represent routinely performed *in vitro* assays for cytotoxicity assessment. Despite some limitations, according to ISO 10993–5:2009 and ASTM E2526–08, they are considered as standard methods for biological evaluation of nanomaterials.

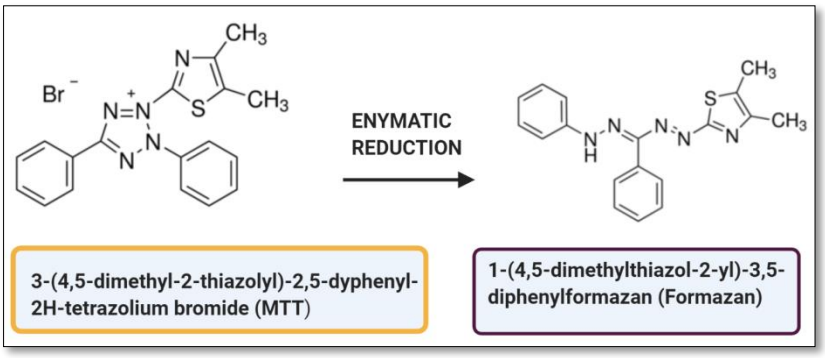

Fig. 4.4 Principle of the MTT assay: reduction of the MTT reagent and formazan formation, which can be spectrophotometrically assessed

MTT assay represents the most commonly used colourimetric test, providing insights into the proliferation and viability of cultured cells. The test itself is based on the reduction activity of metabolically active cells (Aslantürk, 2018). Namely, MTT is a water-soluble yellow die, which can, following reduction by dehydrogenases present in metabolically active cells, be turned into a water-insoluble violet-blue formazan product (Fig. 4.4). Formazan deposits are extracted and calorimetrically assessed (Stockert *et al.*, 2018). The intensity of violet-blue colour indicates the extent of metabolic activity, which is related to the cell viability. Even though there are findings suggesting that MTT reduction may occur elsewhere, it mainly takes place in mitochondria. Therefore, this test is considered as an efficient assay for mitochondrial function (Potter and Stern, 2010).

On the other hand, LDH represents a cytosolic enzyme which can be released upon cell damage. The extent of the LDH release can be correlated with cell membrane integrity (Fig. 4.5).

Therefore, MTT and LDH assay are complementary methods for *in vitro* cytotoxicity assessment. The principle of this assay lays in the ability of LDH to oxidise lactate to pyruvate. Pyruvate can then react with a tetrazolium salt, forming water-soluble formazan, which can also be detected spectrophotometrically (Kaja *et al.*, 2018).

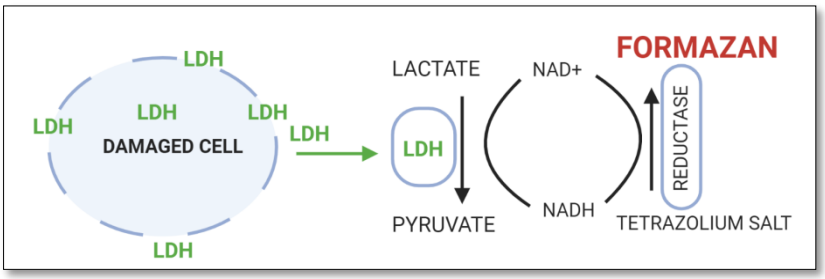

Fig. 4.5 Scheme of the LDH assay: two-step reaction and formation of coloured formazan

Even though these methods are commonly applied, there are some identified pitfalls due to the colourimetric detection and the possibility of nanomaterial interferences with the test reagents (Gioria *et al*, 2018). In addition to the obtained results, to avoid any misleading interpretation, careful visual examination of the treated cells should be an important step in any assessment (Guadagnini *et al*, 2013).

In general, standardised protocols should be followed, but there are some specific considerations that ought to be addressed when testing nanoparticulated material. Hence, they are discussed in more details.

4.5.2 Adsorption of proteins: formation of a protein corona

Apart from their apparent difference from small molecules, some properties of the nanomaterials may change significantly during the assay, which can further complicate the data interpretation. For instance, a well-known phenomenon is the formation of a protein corona (an adsorbed layer of various proteins) around the nanomaterial upon its contact with the serum or cell culture medium (Fig. 4.6). Such occurrence may affect the nanomaterial-cell interaction by either facilitating or making the internalisation harder (Monopoli *et al.*; 2012; Walkey *et al.*, 2012; Lynch *et al.*, 2009). It has been shown that components of the biological surrounding (proteins and lipids) can affect recognition and processing of the nanomaterials by the cells. In other words, biological outcomes, at least partially, are dependent on the identity of the protein corona (its structure and protein orientation) and its residence time on the nanodroplet surface (Albanese *et al.*, 2014).

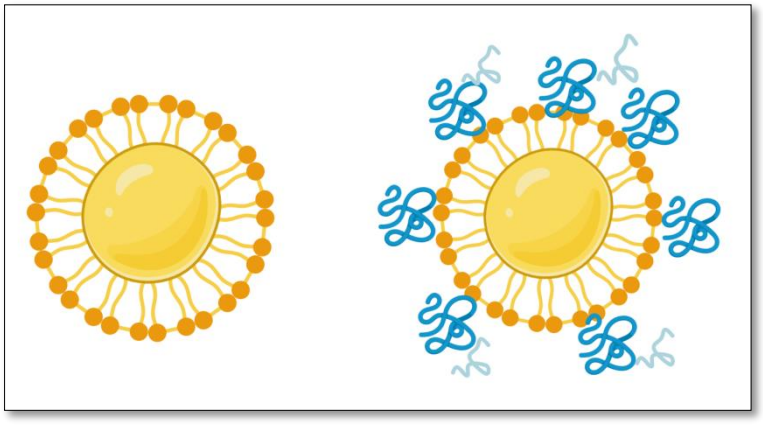

Fig. 4.6 Schematic representation of a nanoemulsion droplet *per se* (left), and in physiological environment/cell culture medium (right): adsorption of proteins at the interface (formation of protein corona) is visible

Any nanomaterial exposed to a physiological environment interacts with proteins. However, the level of these interactions, as well as the properties of the formed protein

corona, depend on the so called “synthetic“ identity of the nanomaterial, meaning that size, composition, topography and curvature of the nanomaterial surface are the most important parameters governing interactions with proteins, which further determines the extent of cellular uptake (Walkey *et al.*, 2012). Despite considerable research effort, the exact relationship between nanomaterial properties and protein adsorption has not yet been completely elucidated. What can be generalised is that nanomaterials with hydrophobic or charged surfaces tend to bind more proteins, whereas highly curved nanomaterial surfaces tend to decrease interactions with proteins (Aggarwal *et al.*, 2013).

Literature provides an overview of techniques that are appropriate for the estimation of some properties of the protein corona. They can be broadly divided into *in situ* (the ones that allow direct measurements of the nanomaterial in the biological medium) and *ex situ* (the ones that require nanomaterial isolation). Both approaches have their advantages and disadvantages. Even though the *in situ* techniques are more relevant, they are limited in terms of the amount of information they could provide. On the other hand, nanomaterial isolation for *ex situ* techniques inevitably causes some structural changes in the protein corona and the loss of the loosely attached proteins (Weber *et al.*, 2019). Nevertheless, the structural assessment of the protein corona encompasses its thickness, density, protein identity and affinity towards the cells (Walkey *et al.*, 2012).

One of the commonly applied techniques for the size and size distribution assessment of nanomaterials is dynamic light scattering, which also finds its place in the estimation of the protein corona thickness. Protein corona evaluation may be easily performed by comparing the size in water (the usual dispersant) to the size obtained using cell culture medium or full serum as a dispersing medium. Applying this technique, Walczyk *et al*. (2010) have obtained reproducible results with surface-carboxylated polystyrene particles, showing that protein corona in full serum is formed in a relatively stable manner after 1 h. Obtained results were consistent with those extracted through TEM analysis, as an example of *ex situ* technique. Other corona parameters and selection of appropriate techniques for its assessment are summarised in Table 4.3. As isolation techniques for *ex situ* analysis, differential centrifugation and size exclusion chromatography are typically applied (Walkey *et al.*, 2012).

Table 4.3 Selected methods for protein corona assessment

Corona parameters	***In situ* techniques**	***Ex situ* techniques**
Thickness	• Dynamic light scattering • Fluorescence correlation spectrometry	• Differential centrifugal sedimentation • Size exclusion chromatography • Transmission electron microscopy

Density of the adsorbed proteins		• Colourimetric protein assays
Identity and quantity of the adsorbed proteins		• Poly(acrylamide) gel electrophoresis • Liquid chromatography tandem mass spectrometry
Protein conformation		• Circular dichroism • Fluorescence quenching
Affinity		• Size exclusion chromatography • Surface plasmon resonance • Isothermal titration calorimetry

In addition to this, *in silico* simulations of protein–nanomaterial interactions have been attracting attention recently. So far, such approach has not been sufficiently powerful to take into consideration all the peculiarities and to predict the complexity of interactions in physiological environment. However, it is expected that the improvement of the computational methodologies will enable them to reach the appropriate level of accuracy (Di Felice and Corni, 2011).

In spite of all difficulties, the characterisation and analysis of protein adsorption to the nanomaterial represents a step towards understanding the true nature of its biological effects (Weber *et al*, 2019). It provides important insights into the cellular uptake mechanism, cytotoxicity, inflammation potential and other biological effects caused by the nanomaterials (Saptarshi *et al*, 2013).

4.5.3 Relation between nanomaterial physicochemical properties and dosimetry

Because of the small size and specific stabilisers, nanomaterials are practically not affected by the gravitational force, representing stable dispersions. Such property presents a potential difficulty for *in vitro* assays, where cells adhering to the bottom of the well may not be exposed to the sufficient amount of the tested nanomaterial (Lison *et al.*, 2008).

In order to avoid any misleading results from *in vitro* tests, determination of the dose of that can effectively get in contact with the cells during the specific assay is also an important aspect to be considered (Rischitor *et al.*, 2016). Usually, when chemicals are being tested in *in vitro* studies, the initial concentration added to the culture medium would be considered as the effective concentration (due to diffusion and expected homogeneity). However, such approach should not be taken with nanomaterials. In contrast to chemical compounds, nanomaterials interact with culture medium, and depending on their properties (size and size distribution, shape, mass density and

solubility) utilise different transport mechanisms to reach the cell monolayer (Cohen *et al.*, 2014; Cho *et al.*, 2011; Teeguarden *et al.*, 2007). Furthermore, apart from aforementioned protein corona, other dynamic modifications of the nanomaterial properties may occur in the cell culture medium, leading to potential agglomeration and aggregation. Consequently, the particle size of the test nanocarrier could be changed, directly influencing its transport towards the cell monolayer and the cellular uptake (internalisation) of the tested material (Cohen *et al.*, 2014). Having in mind the importance of this specific aspect, much research has been done so far dealing with dosimetry (Rischitor *et al.*, 2016).

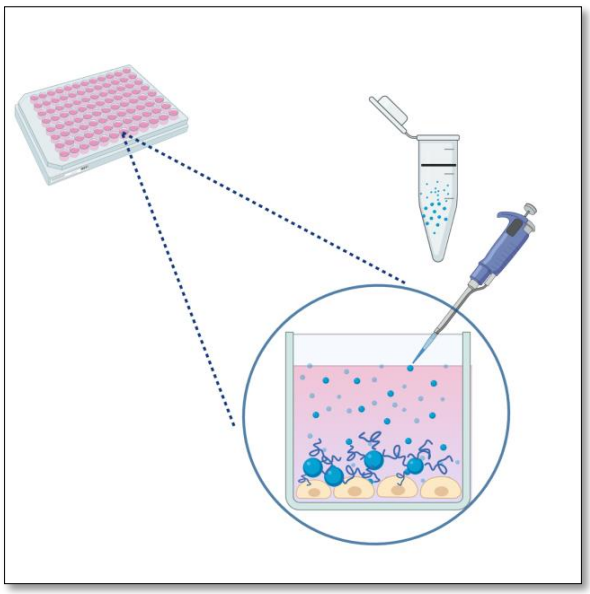

Fig. 4.7 Deposition of the nanomaterial onto the cell monolayer

Studies of cellular uptake are closely related to the transport of the nanomaterial to the cell monolayer (Fig. 4.7). *Cellular dose* – the number of nanounits that reach the cell membrane and that can eventually be uptaken, depends on the transport of the nanomaterial and its affinity to the cells. Rischitor *et al*. (2016) presented an interesting work, calculating the amount of deposited and/or uptaken gold nanoparticles by measuring the UV-Vis spectra of the cell culture medium supernatant from the wells at several time points and comparing it to the spectra obtained for the supernatant from the well without cells. The authors found that the fraction of nanoparticles deposited on the cellular layer is dependent on their size and density, as these parameters govern their transport towards the cell monolayer. Also, the duration of exposure is an important experimental condition that should be controlled.

It is worth noting that initial nanomaterial concentration used in the *in vitro* assay should be carefully selected because an excess of nanostructured material can, in some cases, be misinterpreted as toxicity, even though a decrease in cell viability is actually caused by mechanical obstruction due to the overload (Wittmaack *et al.*, 2011).

Interestingly, Moore and *et al.* (2019) demonstrated that even the manner of administration of the nanoparticles to the well could significantly influence the observed interactions. They evaluated concentrated (bolus) administration, concentrated administration followed by pipette mixing, and premixing to the desired concentration with culture medium prior to the addition. Observed differences in cellular response were attributed to the different ability of nanoparticles to deposit onto cell monolayer.

4.5.4 Specific observations related to the nanoemulsions

Nanoemulsions, as soft matter nanosystems, are usually applied as carries for medicinal compounds, nutrients, food additives and cosmetic ingredients (McClements and Jafari, 2018; Nikolic *et al.*, 2018). It is well known that certain components, such as nanoemulsion stabilisers, may induce toxicity to cultured cells due their solubilising effects (Vater *et al.*, 2019), even though they are declared as biocompatible. Therefore, attention should be paid to the selected concentration range for toxicity testing. Whenever possible, for any tested nanomaterial, cell exposure to the test sample should realistically reflect intended application (Krug and Wick, 2011).

It has been reported that some antioxidants possess additional properties, such as anticancer and/or antigenotoxic activities, which should be assessed applying cell cultures (Gledovic *et al.*, 2020; Nikolic *et al.*, 2020; Nikolic *et al*., 2018). In order to estimate their efficacy when applied in the form of nanoemulsion, it is crucial to eliminate any potential effect of the empty carrier. It addition, it is sometimes possible to discover a beneficial contribution of this specific delivery system, comparing the outcomes of the "free" antioxidant to the one solubilised in the formulation. Therefore, it is advised that the placebo naoemulsion formulation is tested first, in order to obtain a specific concentration range with no observed effects. Further on, based on the findings related to the "free" compound, the loaded formulation can be diluted to the desired concentration for efficacy assessment (Nikolic *et al.*, 2020; Theochari *et al*., 2017). Moreover, since nanoemulsions are designed as carriers for the active ingredient (*e.g*. antioxidants), in some cases, there is no need for the uptake of the whole nanoemulsion droplet. It is expected that nanoemulsions should provide stability for the antioxidant, enabling its efficacy at the

moment of application by facilitating internalisation of the compound into the cell (Pavone *et al.*, 2020). Therefore, appropriate release studies should be conducted with a view to estimate the amount of available active ingredient.

4.5.5 Novel approaches for *in vitro* testing: Microfluidic technology

In order to overcome some of the mentioned limitations of the classical *in vitro* biological assessment, considerable attention is currently being devoted to the cell-based microfluidic model systems, an emerging biomimetic screening tools. In such setting, cells are not static, but exposed to the constant flow of the culture medium, which is an environment with physiological relevance (Jie and Li, 2018; Perestrelo *et al.*, 2015). Moreover, microfluidic cell platforms enable culturing and screening of a range of miniaturised 3D organ and tissue models, representing elegant and reliable tissue/organ-on-a-chip platforms (Trietsch *et al.*, 2013). Described technology could bridge the existing gap between the *in vitro* experimental setting and *in vivo* application, while reducing experimental costs (smaller amounts of samples and reagents are needed), with perspectives that are beyond the proof-of-concept stage (Esch *et al.*, 2015).

4.5.6 Conclusion

For meaningful evaluation of nanomaterials it is necessary to perform their complete physicochemical assessment (including size, morphology, surface charge determination, loading and release profile assessment) in addition to the characterisation of interactions between the nanomaterial and the culture medium, considering all potential experimental issues. Such comprehensive approach represents an important step towards the next stage, a more relevant *in vitro* screening. There is a need for further standardisation and development of robust methods for nanomaterial characterisation, which should lead to a reliable and physiologically closer assessment of their safety and efficacy aspects.

4.6 Closing remarks

Based on the findings presented in this chapter, it is clear that the preparation of nanoemulsions as carriers for natural antioxidants is a complex task. Numerous factors should be considered in order to achieve the formulation with good chemical stability, desired release profile and high antioxidant efficacy. Those factors include right selection

of oils and stabilisers, appropriate preparation method and processing conditions, often followed by the adjustment of characterisation protocols. The theoretical concepts and experimental results presented in this chapter, alongside relevant literature sources, should provide a useful guide to formulating innovative solutions for tailor-made nanoemulsions with natural antioxidants

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