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Review

Advances in edible nanoemulsions: Digestion, bioavailability, and potential toxicity

David Julian McClements [a,b,*]

[a] Department of Food Science, University of Massachusetts, Amherst, MA 01003, USA
[b] Department of Food Science & Bioengineering, Zhejiang Gongshang University, 18 Xuezheng Street, Hangzhou, Zhejiang 310018, China

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ABSTRACT

The design, fabrication, and application of edible nanoemulsions for the encapsulation and delivery of bioactive agents has been a highly active research field over the past decade or so. In particular, they have been widely used for the encapsulation and delivery of hydrophobic bioactive substances, such as hydrophobic drugs, lipids, vitamins, and phytochemicals. A great deal of progress has been made in creating stable edible nanoemulsions that can increase the stability and efficacy of these bioactive agents. This article highlights some of the most important recent advances within this area, including increasing the water-dispersibility of bioactives, protecting bioactives from chemical degradation during storage, increasing the bioavailability of bioactives after ingestion, and targeting the release of bioactives within the gastrointestinal tract. Moreover, it highlights progress that is being made in creating plant-based edible nanoemulsions. Finally, the potential toxicity of edible nanoemulsions is considered.

1. Introduction

Nanoemulsions are a type of colloidal dispersion that consists of small droplets of one fluid dispersed in another immiscible fluid [1,2]. Typically, the two fluids are oil and water, but other immiscible liquids can also be emulsified. There are two common types of nanoemulsions that can be distinguished from each other by their structural organization: oil-in-water (O/W) and water-in-oil (W/O) types (Fig. 1). O/W nanoemulsions consist of small oil droplets dispersed within water, whereas W/O nanoemulsions consists of small water droplets dispersed in oil. Nanoemulsions with more complicated structures are also possible, such as W/O/W or O/W/O (Fig. 1), but these are less common. Currently, O/W nanoemulsions are the most widely used for encapsulation and delivery purposes and so they will be the main focus of this article.

A frequent source of confusion in this area is the difference between emulsions, nanoemulsions, and microemulsions [3]. Indeed, many of the papers published in this area employ incorrect terminology when referring to the colloidal systems they are working with. It is therefore important to clearly distinguish between these systems. Nanoemulsions and emulsions are both thermodynamically unstable because the free energy of the separate oil and water phases is lower than that of the emulsified system. This is because of the positive free energy (ΔG) required to increase the surface area (ΔA) between the oil and water phases: $\Delta G = \gamma \Delta A$, where γ is the interfacial tension [3]. From a thermodynamic perspective, nanoemulsions are therefore less stable than emulsions because they have a higher surface area, *i.e.,* there is a greater driving force for them to separate. However, from a kinetic perspective, nanoemulsions are usually much more stable than emulsions because their smaller droplet size reduces the tendency for gravitational separation (creaming/sedimentation) or droplet aggregation (coalescence/flocculation) to occur, *i.e.,* there is a higher kinetic energy barrier between the emulsified and non-emulsified states (Fig. 2). The only difference between nanoemulsions and emulsions is the droplet size. Typically, nanoemulsions are considered to have mean droplet diameters below 200 nm, whereas emulsions have them above this value [4]. Even so, there is currently no single definition that is widely used. Researchers often use different mean diameters as the demarcation point between nanoemulsions and emulsions, such as 100, 200, 500, or even 1000 nm. Moreover, it is not clear what mean droplet diameter to use, *e.g.,* the number- (d_{10}), surface- (d_{32}), volume- (d_{43}), or intensity- (d_Z) weighted average [1]. It should be noted that there are no dramatic changes in the properties of these colloidal systems when the particle size moves from above to below these critical cut-off points, but it would

* Corresponding author at: Department of Food Science, University of Massachusetts Amherst, Amherst, MA 01003, USA.
E-mail address: mcclements@foodsci.umass.edu.

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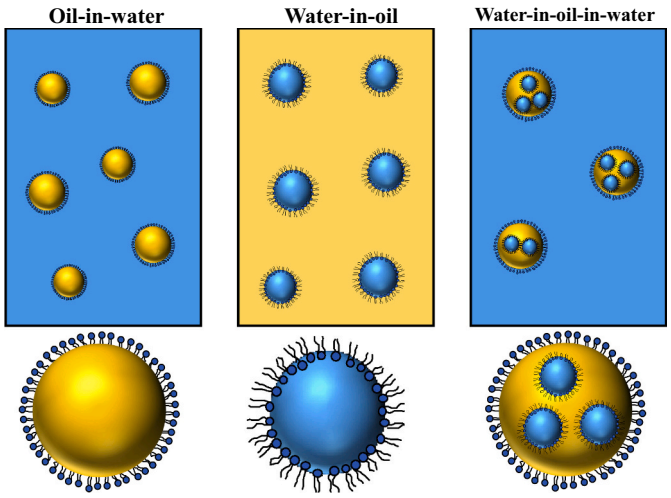

Fig. 1. Nanoemulsions may be of the oil-in-water or water-in-oil type, but more complex types are also possible, such as the water-in-oil-in-water type.

be useful to have a more consistent definition. To be consistent with government regulations on nanoparticles, such as those of the European Union, nanoemulsions should have number-weighted mean diameters below 100 nm. In this article, a looser definition will be employed so as to include systems with mean diameters below about 500 nm, since a large majority of the published studies on nanoemulsions fall into this size range.

Nanoemulsions are often confused with microemulsions in the literature, but they are distinct types of colloidal dispersion, despite having some similarities. Unlike nanoemulsions, microemulsions are thermodynamically stable systems, *i.e.,* the separated components (usually oil, water and surfactant) have a higher free energy than the colloidal dispersion [3]. Consequently, microemulsions should form spontaneously when their constituents are brought together. In practice, some external energy (such as mixing and/or warming) is required to overcome kinetic energy barriers between the separated and microemulsified states. Once formed, microemulsions should remain stable indefinitely, provided their composition and the environmental conditions are not altered, and there is no chemical degradation or microbial contamination. The reason that microemulsions are thermodynamically stable, despite having a large specific surface area, is because the interfacial tension is very low when the surfactant monolayer adopts it's optimum curvature [3]. As a result, entropy of mixing dominates the overall free energy difference between the separated and microemulsified states. Like nanoemulsions, microemulsions are dispersions of small emulsifier-coated oil droplets in water, which can be used to encapsulate hydrophobic bioactives. Microemulsions are often optically transparent and resistant to gravitational separation because of their very small particle size, which is similar to nanoemulsions that contain very small droplets.

This review article focuses on the most recent advances in the application of nanoemulsions as delivery systems for bioactive components. In particular, it focuses on the formulation of nanoemulsions from plant-based ingredients, since this is a major thrust in many industries at present, as well as recent advances in understanding the gastrointestinal fate of nanoemulsions, particularly their digestibility and bioavailability, using both *in vitro* and *in vivo* methods. In addition, recent applications of nanoemulsions to encapsulate and deliver a variety of hydrophobic bioactive substances are highlighted, including omega-3 fatty acids, antimicrobials, vitamins, nutraceuticals, and cannabinoids. Finally, approaches for extending the functionality of nanoemulsions are highlighted, including interfacial engineering, droplet clustering, lipid phase solidification, and microgel encapsulation technologies.

2. Advantages of nanoemulsions

Nanoemulsions have a number of potential advantages over emulsions and microemulsions for the encapsulation and delivery of hydrophobic bioactives.

2.1. Nanoemulsions versus emulsions

The main advantages of nanoemulsions over emulsions are related to the smaller size of the oil droplets in the nanoemulsions [1,3]. When the mean droplet diameter is below about 50 nm, it is possible to create nanoemulsions that are optically transparent, which is beneficial for some applications, such as fortified waters, fruit juices, or soft drinks. It should be noted, however, that it is often challenging to create nanoemulsions containing droplets that are this small. Typically, high

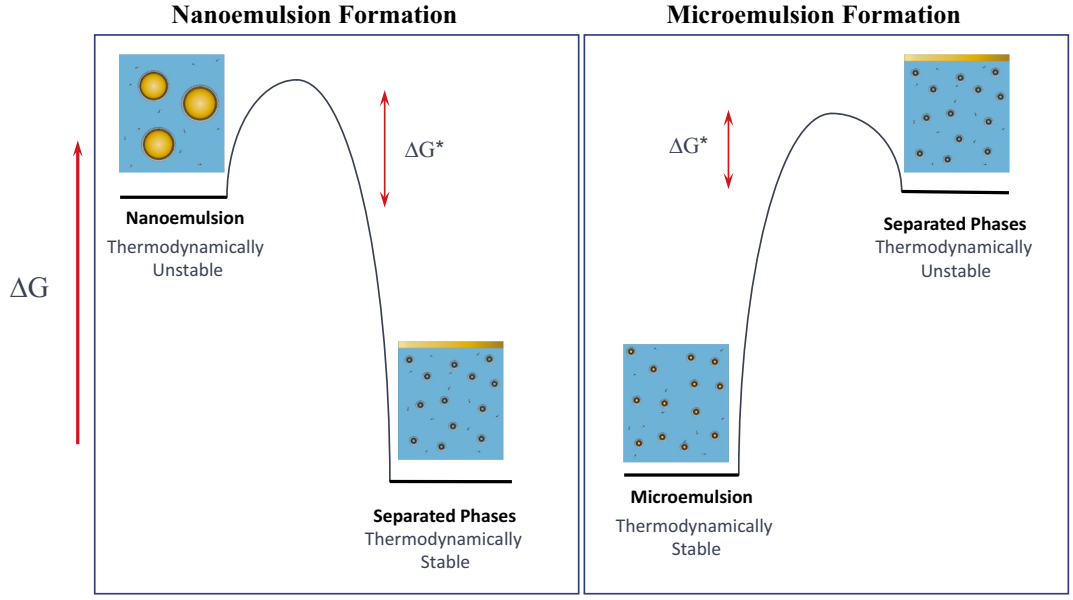

Fig. 2. Schematic diagram of the free energy of microemulsion and nanoemulsion systems compared to the phase separated state. Microemulsions have a lower free energy than the phase separated state, whereas nanoemulsions have a higher free energy. The two states are separated by an activation energy ΔG*.

concentrations of synthetic surfactants are required. When the droplet diameter is below about 200 nm, nanoemulsions are highly resistant to gravitational separation (usually creaming), which is useful for developing fluid formulations that are expected to have an extended shelf-life. Finally, the smaller size of the oil droplets in nanoemulsions means that they are digested more rapidly and fully in the gastrointestinal tract, which can increase the bioavailability of encapsulated hydrophobic bioactives.

2.2. Nanoemulsions versus microemulsions

A major advantage of nanoemulsions over microemulsions is that they can be formulated from a much wider range of ingredients, such as emulsifiers and oils [1,3]. In particular, nanoemulsions can be created from natural emulsifiers, including proteins, polysaccharides, phospholipids, biosurfactants, and saponins [5–8]. This is becoming increasingly important as more and more consumers are adopting commercial products that have cleaner labels, which means that industry is trying to replace synthetic ingredients with natural ones. In contrast, high concentrations of synthetic surfactants are usually required to prepare microemulsions, which is undesirable for cost, taste, toxicity, and labeling reasons. Another important advantage is that nanoemulsions typically have a higher loading capacity than microemulsions, *i.e.,* they can incorporate more hydrophobic bioactives per unit mass of delivery system [9]. This is mainly because microemulsions only have a relatively small hydrophobic domain inside them that can accommodate bioactives.

3. Formulation and fabrication of nanoemulsions

In general, there are two main approaches for producing nanoemulsions, which have been described in detail elsewhere [10–12]: low-energy and high-energy fabrication methods. Typically, low-energy methods are based on the spontaneous formation of tiny oil droplets when the composition or temperature of a system containing oil, water, and surfactant is changed in a specific way. This category of methods includes spontaneous emulsification, phase inversion temperature, and emulsion inversion methods. In contrast, high-energy methods are based on applying intense mechanical forces to a mixture of oil, water, and surfactant, which causes the oil phase to breakup into tiny surfactant-coated oil droplets that are dispersed within water. This category of methods includes microfluidization, sonication, and high-pressure homogenization. A considerable number of recent research efforts have focused on identifying the optimum compositions and operating conditions required to produce nanoemulsions using these methods.

One of the main areas of focus in recent research has been on creating nanoemulsions entirely from natural food ingredients, rather than synthetic ones, due to increasing consumer demands for clean label products [5,8]. In particular, there has been great interest in using plant-based (rather than animal-based) natural ingredients, such as oils, phospholipids, proteins, polysaccharides, biosurfactants, or saponins isolated from plant materials [5,8]. It has proved very difficult to formulate nanoemulsions using low-energy methods from natural emulsifiers because they do not have the required solubility characteristics and phase behavior [13,14]. High-energy methods are much more suitable for this purpose since they can create nanoemulsions from a wide range of different kinds of emulsifiers and oils [5,8]. Typically, plant-based emulsifiers have to be highly water-soluble, surface active, have rapid adsorption kinetics, and be present at a high enough concentration to cover all the oil droplet surfaces formed during homogenization. A number of plant-based emulsifiers have these characteristics, including many phospholipids, proteins, polysaccharides, biosurfactants, and saponins.

A few examples of plant-based nanoemulsions prepared using high-energy methods are given here. Sonication has been used to prepare nanoemulsions from a number of different plant-based emulsifiers, including soy proteins, rice proteins, peanut proteins [15], soy protein/phospholipid mixtures [16], pea proteins [17,18], soy lecithin, tea saponins [19], and quillaja saponins [19]. Similarly, microfluidization has been used to prepare nanoemulsions from various kinds of plant-based emulsifiers, including soy proteins [20], soy protein-tea polyphenol complexes [21], soy protein-dextrin conjugates [22], sesame proteins [23], lentil proteins [24,25], pea proteins [26,27], pea protein-tannic acid complexes [28], gum arabic [29], soy lecithin [30,31], sunflower lecithin [32], quillaja saponin [33], and tea saponin [34]. The stability and functionality of these nanoemulsions is highly dependent on the nature of the plant-based emulsifier used to formulate them. In some cases, the functional performance of protein-coated oil droplets in nanoemulsions can often be improved by adding charged polysaccharides that adsorb to the droplet surfaces and form a protective coating [25]. Taken together, these studies indicate that nanoemulsions can be successfully formulated from plant-based ingredients, at least using high-energy homogenization methods, which may be important for many applications in the modern food industry.

4. Factors affecting bioavailability

One of the most important applications of nanoemulsions within the food industry has been to increase the bioavailability of beneficial bioactive substances, such as oil-soluble vitamins and phytochemicals [12,35]. For this reason, it is important to understand the major factors that impact the overall bioavailability (BA) of bioactive substances after they have been ingested [36]. The bioavailability can be taken to be the proportion on an ingested substance that reaches the site of action within the body in a biologically active form, which can be simplistically expressed by the following Eq. [37]:

$$BA(t) = B^*(t) \times A^*(t) \times D^*(t) \times M^*(t) \times E^*(t) \quad (1)$$

The bioavailability depends on numerous events that occur within the human GIT and body [38], which are shown schematically in Fig. 3 [4]. In this expression, B*(t), A*(t), D*(t), M*(t), and E*(t) represent the bioaccessibility, absorption, distribution, metabolism, and excretion of the bioactive substance over time (*t*) [36]. The *bioaccessibility* is the fraction of the substance inside the GIT fluids that is in a form that can be absorbed. For hydrophobic substances, this is usually the fraction that is solubilized within the mixed micelle phase, which is comprised of micelles and vesicles. The *absorption* is the fraction of the bioaccessible substance in the gut that passes through the epithelium cells and enters the body. Absorption may occur through a variety of passive or active transport mechanisms. Typically, more hydrophilic substances pass through the epithelium layer and enter the portal vein, where they then move to the liver before entering the systemic circulation. In contrast, more hydrophobic substances are packaged into lipoproteins within the epithelium cells, which are then travel through the lymphatic system, before reaching the systemic circulation, thereby avoiding first pass metabolism. The *distribution* reflects the fraction of the bioactive substance located at the intended site of action, which takes into account the fact that the absorbed substance is distributed around the human body to various tissues and organs. In practice, the concentration of the substance in the systemic circulation (bloodstream), rather than in specific tissues, is often used to represent the effective concentration. The *metabolism* is the fraction of a substance remaining within a biologically active form at the site of action, taking into account any chemical or metabolic changes that have occurred inside the gut and body. Finally, the *excretion* is the fraction of substance remaining at the site of action after any of the normal excretion processes operating in the body, such as expulsion through the urine, feces, breath, or sweat, have occurred. In practice, the overall bioavailability of a substance may be mainly determined by one or more of these processes. The concentration of a bioactive substance at a particular site in the body varies over time after ingestion of a food because all of the processes mentioned are time

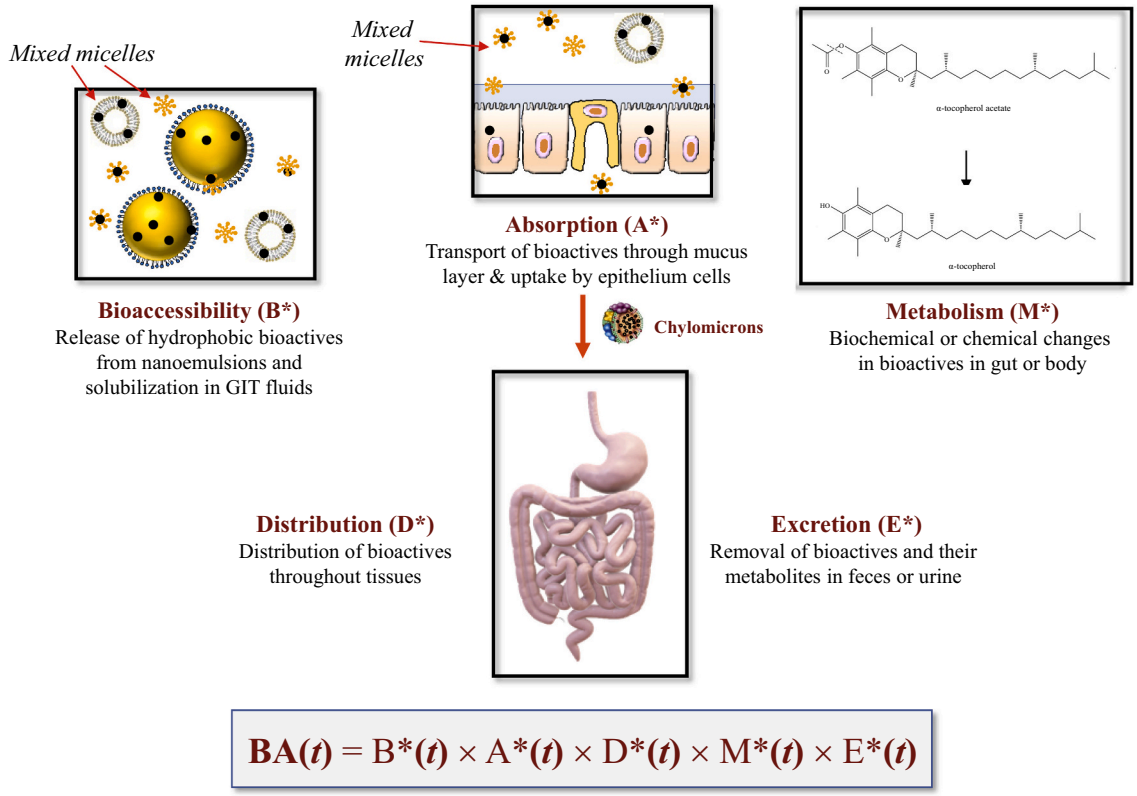

Fig. 3. The overall bioavailability of hydrophobic bioactives encapsulated inside nanoemulsions depends on numerous factors, including their bioaccessibility, absorption, distribution, metabolism and excretion.

dependent. The resulting pharmacokinetic profile of an ingested component determines its bioactivity (Fig. 4).

A great deal of research on nanoemulsion-based delivery and excipient systems has focused on formulating them to increase the bioavailability of strongly hydrophobic substances, like oil-soluble vitamins (A, D, E, and K), nutraceuticals, and long-chain triglycerides [9]. The rate limiting step for many of these hydrophobic substances is their bioaccessibility, which is the fraction taken up by the mixed micelles in the small intestine [36]:

$$B^{*} = 100 \times \frac{c}{c_T} \quad (2)$$

here, c_M and c_T are the concentrations of the bioactive substance present in the mixed micelle phase and in the total digest, respectively. The mixed micelle phase contains a mixture of colloidal particles (such as micelles and vesicles) that are comprised of bile acids and phospholipids (secreted by the gut), as well as free fatty acids and monoacylglycerols (generated during digestion of the ingested lipid). Hydrophobic bioactives are solubilized within the hydrophobic interiors of the mixed micelles and then carried to the epithelium cells where they can be absorbed [39]. One of the advantages of using nanoemulsions to increase the bioavailability of hydrophobic substances is that they are rapidly and completely digested in the small intestine, thereby releasing these substances. Moreover, they rapidly form mixed micelles that can solubilize the released hydrophobic substances.

Studies have been carried out to elucidate the link between the composition and structure of nanoemulsions, the properties of the mixed micelles formed in the small intestine, and the bioaccessibility of bioactive substances [9]. These studies indicate that the hydrophobic domains inside the mixed micelles (micelles and vesicles) have to be sufficiently large to accommodate the bioactive substances [40,41]. Otherwise, they will remain in the aqueous phase and may crystallize or form a separate oily phase, which reduces their bioaccessibility. In general, the size, composition, and physical state of the lipid droplets in nanoemulsions has to be optimized to increase their bioaccessibility [9].

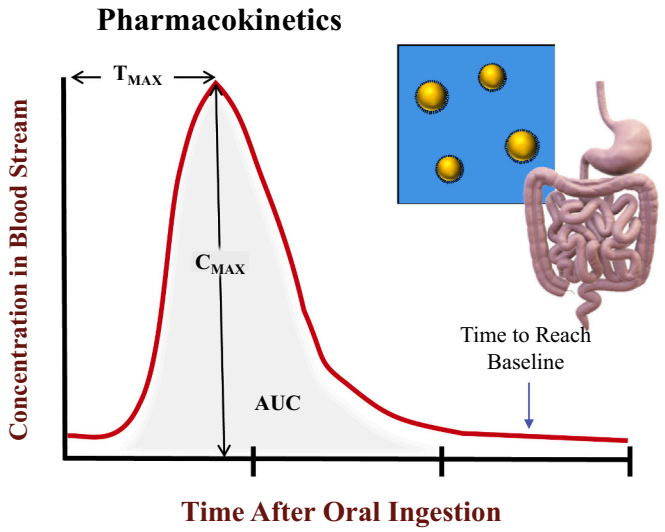

Fig. 4. The pharmacokinetics of bioactive components are governed by the digestion and absorption of nanoemulsions and can be described by the change in concentration in the bloodstream over time. Here, T_{MAX} is the maximum concentration achieved, T_{MAX} is the time to reach the maximum, and AUC is the area under the curve.

5. Advances in assessing gastrointestinal fate of nanoemulsions

Knowledge about the potential gastrointestinal fate of nanoemulsions has increased greatly over the past few years as a result of the

development and application of both *in vitro* and *in vivo* testing methods [42,43].

5.1. In vitro gastrointestinal studies

Many researchers are now using simulated gastrointestinal models to understand how different nanoemulsion formulations might behave inside the human gut [24,44,45]. A number of simulated GIT models have been developed for this purpose, which vary in their sophistication, accuracy, and ease of use, which often makes comparison of results from different studies challenging [46,47]. For this reason, standardized models have recently been developed for this purpose. The most widely used of these models is the static *in vitro* digestion method developed by the INFOGEST international consortium [46,48]. This consortium includes an international network of scientists whose aim is to improve the healthiness of foods by understanding how they behave within the human gastrointestinal tract (www.cost-infogest.eu). As part of this work, a standardized *in vitro* GIT model was established to better simulate the events occurring inside the human gut, as well as to harmonize the results obtained from different research laboratories. The INFOGEST model is designed to simulate the mouth, stomach, and small intestine regions of the human GIT. It provides detailed instructions on the conditions that should be used in each region of the GIT, including incubation times, mechanical forces, temperatures, pH values, mineral compositions, enzyme activities, mucin levels, and bile salts concentrations. In addition, it gives instructions on how the data obtained should be collected, analyzed, and reported. The INFOGEST model is a "static" approach because the conditions used in each region of the GIT are fixed. The results of the INFOGEST model have been compared to those obtained in animal and human feeding studies, which has shown that there is a good qualitative *in vitro – in vivo* correlation in many cases [49,50]. As a result, the INFOGEST method is now widely used to rapidly test new formulations intended for oral ingestion, which is reducing the need for ethically challenging and costly animal and human studies. It should be noted, however, that a simple static digestion model cannot accurately reflect the complexity and diversity of the human gut. This problem can partly be overcome by using more sophisticated "dynamic" *in vitro* digestion models, where the conditions in each gastrointestinal region are varied over time, *e.g.*, by continuously pumping in acids, enzymes, or bile salts based on feedback loops [49,51,52]. For instance, dynamic *in vitro* digestion models have been used to study the bioavailability and bioactivity of curcumin encapsulated within nanoemulsions [53], as well as the bioaccessibility of tocopherols in nanoemulsions [54]. Although these dynamic models are better at simulating real GIT conditions, they are more laborious, costly, and time-consuming to carry out. Moreover, the results obtained from dynamic models are often more difficult to interpret. These factors have limited their more widespread application.

In vitro digestion models are particularly useful for providing insights into the changes in the structure and composition of nanoemulsions as they pass through the GIT, the rate and extent of macronutrient digestion, and the bioaccessibility of encapsulated bioactive substances. They may also be used to provide information about the chemical or biochemical transformation of encapsulated components within the GIT (such as oxidation or hydrolysis). Occasionally, they have been extended to provide insights into the potential absorption of bioactives through the epithelium cells lining the small intestine. For instance, some researchers have used dialysis bags to model the epithelium cells. The dialysis bags are placed in the small intestinal fluids to measure the concentration of the bioactive component that is released from the test sample and then transported through the pores [55]. A more sophisticated approach is to take the contents of the digest produced after the small intestine phase, dilute it with an appropriate buffer, and then place it on a cell culture model of the epithelium cells, such as Caco 2 cells [56,57]. The amount of the bioactive substances transported into or through the model epithelium cells can then be determined, as well as their packaging into lipoproteins, such as chylomicrons [58]. In humans, hydrophobic bioactive substances are usually packaged into chylomicrons within the epithelium cells, which are small triacylglycerol-rich particles coated by phospholipids and proteins, and then carried through the lymphatic system to the bloodstream. It should be noted that many researchers simply place their formulated nanoemulsions on cell culture models, without carrying out a digestion step first, which may lead to unrealistic or misleading results.

5.2. In vivo gastrointestinal studies

A more accurate understanding of how nanoemulsions behave inside the human gut can often be obtained using animal feeding studies [59,60]. A nanoemulsion formulation is usually prepared and then orally administered to animals (usually rats or mice). Typically, this is done by oral gavage, which involves placing the nanoemulsion in a syringe and then injecting it directly into the animal's stomach. The advantage of this method is that the amount of nanoemulsion administered to the animal can be carefully controlled. However, it is also possible to have the animal drink a liquid form of the nanoemulsion or to eat a powdered form, which may be produced by freeze or spray drying. The advantage of this approach is that it causes less discomfort to the animal, and the nanoemulsion passes through the mouth and esophagus, which could alter its properties. The gastrointestinal fate of the nanoemulsion can then be monitored by sacrificing the animal and measuring the concentration, size, and properties of the oil droplets in different regions of the GIT. In addition, the concentration of the administered bioactive components can be measured in the bloodstream and specific organs (such as the liver, kidney, heart, adipose tissue, muscles, intestinal tissue, brain, *etc.*) after a fixed time [59]. Ideally, measurements should be made over a range of times using different animals to obtain information about how the bioactives are absorbed by the body, and then distributed, metabolized, and excreted. In many studies, the concentration of the administered bioactive agent and/or its metabolites is simply measured in the bloodstream of the animal over time by periodically taking blood samples [59]. Thus, the impact of nanoemulsion formulation (such as particle size, oil type, and emulsifier type) on the pharmacokinetics of the bioactive component can be established. Pharmacokinetic experiments can also be carried out using human feeding studies, provided the formulation is known to be safe and appropriate approval has been obtained [61]. However, few human studies have been reported in the literature on the gastrointestinal fate of ingested nanoemulsions. In one study, it was shown that nanoemulsions improved the oral bioavailability of ω-3 fatty acids in humans after they were incorporated into yogurts, when compared to a bulk oil [61]. In another human feeding study, it was shown that emulsified cod liver oil had a significantly higher bioavailability than bulk cod liver oil [62].

5.3. Selecting appropriate reference systems

Bioaccessibility, bioavailability or pharmacokinetic studies of bioactives usually involve comparing the results obtained using different formulations (Fig. 5). It is important to select an appropriate reference sample to compare to the test sample, otherwise the results will be misleading. The gastrointestinal behavior of a hydrophobic bioactive encapsulated within a test nanoemulsion may be compared to various kinds of reference sample:

- *Bulk form*: In this case, a solid bioactive may be used in a powdered bulk form [63] or a liquid bioactive may be used in a fluid bulk form [61,64]. The bulk sample is then simply mixed with simulated GIT fluids or administered to an animal. This format often has a relatively low bioaccessibility and bioavailability due to the large surface area and poor solubility in gastrointestinal fluids;

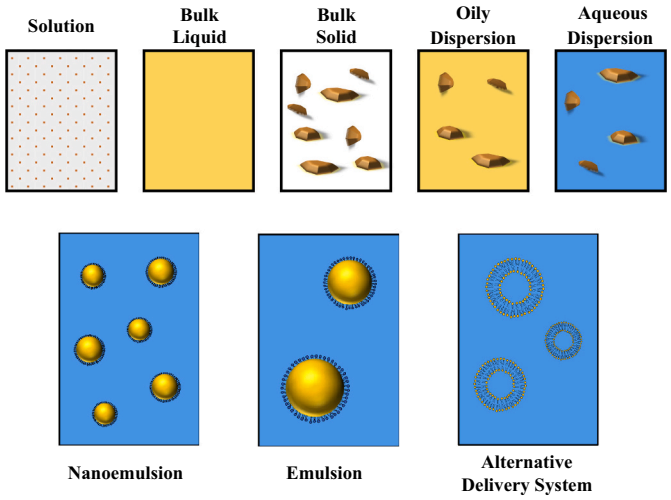

Fig. 5. The efficacy of a bioactive component encapsulated in a test nanoemulsion can be compared to various kinds of reference system, which will effect the nature of the results obtained.

- *Non-encapsulated dispersed form:* In this case, a crystalline bioactive is suspended in an aqueous or oily dispersion in the form of small crystals [65]. Typically, this format also has a relatively low bioaccessibility and bioavailability for the same reasons;
- *Dissolved form:* In this case, a crystalline bioactive is dissolved in a suitable organic solvent (like DMSO) [66,67]. This format may not give an accurate comparison, since these organic solvents cannot be used in food applications;
- *Emulsion form*: In this case, the bioactive is encapsulated within a conventional emulsion ($d > 200$ nm), but the size of the oil droplets used may vary considerably [68,69]. For this reason, it is often informative to use emulsions with different mean droplet diameters in the comparison (*e.g.*, ≈500, 1000, 2000, 5000 and 10,000 nm). Typically, it is important that the composition of the emulsion is similar to that of the test nanoemulsion (*e.g.*, oil and emulsifier type) to make reliable comparisons.
- *Nanoemulsion form:* In this case, the bioactive is encapsulated in another nanoemulsion ($d < 200$ nm) with a different composition or structure. For instance, nanoemulsions with different mean droplet diameters, emulsifier types, or oil phase compositions could be compared to the test nanoemulsion [69–72].
- *Alternative delivery system form:* The purpose of some studies is to compare the efficacy of a test nanoemulsion formulation with one or more other colloidal delivery systems, such as biopolymer nanoparticles, nanoliposomes, nanocrystals, or microgels [73–75].

It is always important to select an appropriate reference formulation when establishing the efficacy of a test nanoemulsion-based delivery system for a specific bioactive component. It is also important to ensure that the concentration of the bioactive is similar in the reference sample as in test nanoemulsion.

6. Applications

6.1. Healthy lipids: Omega-3 fatty acids and conjugated linoleic acids

Nanoemulsions have been used to encapsulate flaxseed oil, which is rich in alpha-linoleic acids, an important source of plant-based omega-3 fatty acids in the diet [76]. The researchers showed that stable nanoemulsions could be formed using a combination of whey protein and sodium alginate to stabilize them. The whey protein acted as an emulsifier that adsorbed to lipid droplet surfaces, while the sodium alginate formed a protective polysaccharide coating around the protein-stabilized lipid droplets. In an *in vivo* feeding study using broiler chickens, the authors showed that the level of omega-3 fatty acids in the flesh of the chickens was appreciably higher when emulsified flaxseed oil was used rather than bulk flaxseed oil. This result can be attributed to the faster and more extensive digestion of the small oil droplets within the gastrointestinal tracts of the birds. In this example, nanoemulsions may be used as effective delivery systems to increase the healthy lipid profile of meat products.

An *in vivo* animal study has been used to study the efficacy of nanoemulsions at delivering a bioactive oil rich in tocopherols: kenaf (*Hibiscus cannabinus* L.) seed oil [77]. These systems were stabilized using complexes of β-cyclodextrin, sodium caseinate and Tween 20. The researchers compared the pharmacokinetic profiles of nanoemulsions, emulsions, and bulk oil after oral administration to adult female Sprague–Dawley rats. In general, the level of tocopherols detected in the animal's bloodstream increased more rapidly in the following order: nanoemulsions > emulsions > bulk oil. After 180 min, the bioavailability of the tocopherols in the blood was 1.4- and 1.7-fold higher in the nanoemulsions than in the emulsions and bulk oil, respectively. This result can again be attributed to the faster and more extensive digestion of the lipid phase as its surface area increases (particle size decreases). These results were consistent with an earlier *in vitro* study by the same authors that showed the nanoemulsions were digested more rapidly and had a higher bioaccessibility than bulk oil [54]. Hence, their appeared to be a good *in vitro-in vivo* correlation, at least qualitatively, for this system.

In another *in vivo* animal study using hypercholesterolemic Sprague-Dawley rats, the same authors examined the impact of delivery format (nanoemulsion, emulsion, or bulk oil) on the ability of kenaf seed oil to reduce cholesterol levels [78]. The nanoemulsion format was shown to exhibit the strongest cholesterol-lowering effects, as well as the strongest weight control and reduced liver fat levels, which was mainly attributed to the higher bioavailability of the kenaf seed oil when it was encapsulated within small lipid droplets.

In a human feeding study, it was shown that administering an ω-3-rich algal oil in a nanoemulsion form improved its bioavailability compared to a bulk oil form [61]. The authors incorporated the different forms of algal oil into a model yogurt product and used a randomized crossover design (11 participants) to study the pharmacokinetics. Their results showed that the blood levels of ω-3 fatty acids were significantly higher for the nanoemulsion than for the bulk oil, which indicated that nanoemulsions could be used to enhance the bioavailability of these healthy lipids. In another human feeding study, a randomized crossover design (47 participants) showed that ω-3-rich cod liver oil had a significantly higher oral bioavailability when administered in an emulsified form than in a bulk form [62].

6.2. Antimicrobials

There has been great interest in the utilization of oil-in-water nanoemulsions to encapsulate natural antimicrobials, particularly essential oils, over the past few years [79–81]. Essential oils are hydrophobic liquids that can be isolated from many kinds of plants, which often contain a variety of different antimicrobial constituents [82]. This is probably because these substances are secreted by plants to protect them from a variety of predators, such as microbes, insects, or herbivores. The key molecular mechanisms responsible for the antimicrobial action of essential oils are believed to be disruption of the microbial cell walls and interference with key biochemical pathways [82]. Due to the increasing interest from consumers in clean-label foods, the food industry has been trying to identify effective alternatives to synthetic antimicrobial agents. Essential oils are hydrophobic liquids that are largely insoluble in water. Consequently, for many applications, they need to be converted into colloidal dispersions containing essential oil-rich colloidal particles dispersed in water. Oil-in-water nanoemulsions are particularly suitable for this purpose since they can easily be

formulated using food grade ingredients using existing production technologies. Typically, essential oil nanoemulsions are fabricated from an essential oil, a ripening inhibitor, an emulsifier, and water, but other components may sometimes be incorporated. For instance, other kinds of antimicrobial phytochemicals can also be included in the oil droplets. In this section, a brief overview of recent research on the development of antimicrobial nanoemulsions is given.

Many of studies have shown that a ripening inhibitor is essential for formulating antimicrobial nanoemulsions from essential oils [83,84]. A ripening inhibitor is a strongly hydrophobic oil that has a very low water-solubility, such as a medium- or long-chain triglyceride oil (such as MCT, corn, or sunflower oils). It is typically mixed with the oil phase prior to homogenization. The presence of the ripening inhibitor slows down droplet growth due to Ostwald ripening through an entropy of mixing effect. A major trend in this area has been the formulation of all-natural antimicrobial nanoemulsions [5]. In this case, natural emulsifiers (such as saponins, proteins, or phospholipids) are used to replace the synthetic ones that are typically used (such as Tweens and Spans). As an example, antimicrobial thymol-nanoemulsions have been formulated using a mixture of lecithin and gelatin as emulsifiers to stabilize them [85]. Similarly, antimicrobial thyme oil-nanoemulsions have been formulated using sodium caseinate as an emulsifier [86]. Other studies have shown that antimicrobial essential oil nanoemulsions can be stabilized by soy lecithin [87,88], quillaja saponins [88–90], and bovine serum albumin [88] These studies indicate that it is possible to create clean-label antimicrobial nanoemulsions that are efficacious and should be better for the environment and human health.

Another area where there have been considerable advances in recent years is the application of antimicrobial nanoemulsions to real food products. For instance, research has shown that hexanal-nanoemulsions were effective at inactivating both spoilage and pathogenic organisms in apple juice, without altering their desirable organoleptic properties [91]. Similarly, cinnamon oil nanoemulsions have been shown to be effective at inhibiting spoilage and pathogenic organisms (*Listeria monocytogenes* and Salmonella spp.) on melons [92]. Research has shown that carvacrol nanoemulsions can be used to inhibit microbial growth (*Salmonella enterica* Enteritidis and *Escherichia coli* O157:H7) in contaminated mung bean and alfalfa seeds [93,94], broccoli and radish seeds [94,95]. Geraniol and carvacrol loaded nanoemulsions have been used as antimicrobial treatments for meat products, where they were shown to increase the shelf life of fresh goat meat [96]. In these examples, it is important that the antimicrobial nanoemulsions do not adversely affect the desirable organoleptic properties of the foods, such as their appearance, mouthfeel, or flavor profile. Thus, it is important to select an essential oil that is compatible with the food that the nanoemulsion is applied to.

Antimicrobial nanoemulsions have also been incorporated into edible coatings and packaging materials to increase the shelf life of foods [97,98]. In this case, the nanoemulsions are usually mixed with natural polymers prior to casting the coatings or films. The essential oils then slowly leach out of the coatings or films over time, which helps to inactivate or inhibit the growth of microbes, yeasts, or molds. The essential oils may diffuse through the films as individual molecules or inside oil droplets, depending on the pore size and interactions within the polymer network. As an example, thymol-, cinnamaldehyde-, and eugenol-based nanoemulsions have been incorporated into biodegradable films formed from pullulan and their release properties have been studied [99]. Thymol oil-nanoemulsions have been loaded into gelatin films and demonstrated to exhibit good antimicrobial activity [85]. The thymol was gradually released from the gelatin films and was effective at inhibiting both Gram-positive and Gram-negative bacteria. Essential nanoemulsions containing cinnamaldehyde and garlic oil have been loaded into composite biopolymer films prepared using gelatin and chitosan, where they were shown to exhibit good antimicrobial properties against *Pseudomonas aeruginosa* [100]. Finally, Thymus daenensis oil-based nanoemulsions have been incorporated into hydroxypropyl methyl cellulose edible films and shown to exhibit good antimicrobial properties [101].

6.3. Vitamins

Nanoemulsions have been used to encapsulate a number of different kinds of oil-soluble vitamins to improve their dispersibility, stability, bioavailability, and bioactivity. As an example, vitamin A (retinol) has been encapsulated in nanoemulsions formulated from different kinds of oils and emulsifiers [102]. The authors reported that the degradation of vitamin A during storage could be inhibited by selection of an appropriate combination of emulsifier and oil.

The impact of encapsulating vitamin D_3 (cholecalciferol) in nanoemulsions on its *in vitro* and *in vivo* bioavailability have been assessed [103]. The *in vitro* experiments, which were carried out using a simulated GIT model, showed that the vitamin D_3 had a higher bioaccessibility when encapsulated in nanoemulsions than in emulsions. The *in vivo* experiments, which involved oral administration of the samples to mice, showed that the serum 25(OH)D-3 levels increased by about 36% and 73% compared to the control for the emulsions and nanoemulsions respectively, again highlighting the ability of the nanoemulsions to improve the oral bioavailability. These effects were attributed to the faster and more extensive digestion of the nanoemulsions due to their higher surface areas.

The efficiency of nanoemulsions at increasing the bioavailability of vitamin D_2 (ergocalciferol) has also been examined using a combination of *in vitro* and *in vivo* studies [68]. The *in vitro* studies, which were performed using a simulated GIT model, indicated that the vitamin bioaccessibility increased with decreasing droplet size, which was linked to a faster rate of lipid digestion and micelle solubilization. Interestingly, the *in vivo* studies, which involved oral administration of the samples to rats indicated that vitamin absorption was higher for emulsions than nanoemulsions. The authors attributed this poor *in vitro-in vivo* correlation to a number of factors: a simulated GIT cannot closely mimic the complex nature of a real GIT; the level of vitamin in the bloodstream was only measured at a single time point after ingestion in the *in vivo* experiments (rather than measuring the full pharmacokinetic profile).

In an *in vitro* bioactivity study, it was shown that Vitamin D-fortified nanoemulsions exhibited cytotoxicity to model human colorectal cancer cell lines HCT116 and HT29 [104]. The nanoemulsions in this study consisted of vitamin D-loaded oil droplets that were coated by a layer of pea protein, with pectin added as an extra stabilizer. The authors suggested that the vitamin D was released from the oil droplets and interfered with key biochemical pathways, leading to cell growth arrest and apoptosis.

In another bioactivity study, the potential of nanoemulsions to increase the anticancer effects of a vitamin E analog (α-tocopherol succinate) was examined [105]. The authors showed that encapsulating the vitamin E analog in nanoemulsions increased its anticancer activity against a model human breast cancer cell line (MCF-7) and a human oral epithelial cancer cell line (KB), relative to the bulk form. In addition, an *in vivo* study where the samples were orally administered to rats showed that the nanoemulsion form of the vitamin analog led to a higher bioavailability than the bulk form. Moreover, pharmacokinetic studies showed that there was a faster and higher rise in the blood serum levels for the nanoemulsions than the bulk form.

6.4. Nutraceuticals

Nanoemulsions have been widely used as a means of encapsulating, protecting, and delivering hydrophobic nutraceuticals [9,11,42]. Typically, the nutraceuticals are dissolved in the oil phase prior to homogenizing the nanoemulsions. The loading capacity of the nanoemulsions is then determined by the maximum solubility of the nutraceutical in the oil phase under the conditions used (particularly storage temperature),

as well as the amount of oil droplets present in the final product. In some situations, the nutraceuticals can be added to a nanoemulsion after it has been formed. For instance, curcumin can be incorporated into nanoemulsions by adding it as a powder and then heating [106], dissolving it in an organic solvent and then mixing, or dissolving it in an alkaline solution and then mixing [107]. There have been a huge number of studies on the utilization of nanoemulsions for this purpose, and so only a few examples are given here, with a focus on *in vivo* studies of their bioavailability and/or bioactivity.

Many researchers have used *in vitro* digestion models to study the impact of nanoemulsion composition and structure on the bioaccessibility of encapsulated hydrophobic nutraceuticals, which has been reviewed in detail recently [42]. In general, these studies show that nutraceutical bioaccessibility depends on many factors, including oil droplet content, oil droplet size, oil digestibility, and emulsifier type [9,42]. Typically, bioaccessibility increases with decreasing droplet size, since this leads to faster and more complete lipid digestion. The dimensions of the hydrophobic domains within the mixed micelles (micelles and vesicles) formed after lipid digestion are also important. To be solubilized within the intestinal fluids, nutraceuticals must be small enough to fit inside the hydrophobic domains inside the mixed micelles. It is for this reason that long hydrophobic molecules, like β-carotene, have a high bioaccessibility when delivered in nanoemulsions formulated from long-chain triglycerides but not in those formulated from medium-chain triglycerides [108]. *In vitro* digestion studies have shown that nanoemulsions can increase the bioaccessibility of many different nutraceuticals, including β-carotene [109,110], lycopene [111–113], lutein [114,115], astaxanthin [116,117], curcumin [106,107,118], pterostilbene [119] and quercetin [120,121]. It should be noted, however, that reducing the lipid droplet size does not always improve the bioavailability of nutraceuticals. For instance, it has recently been shown that the chemical degradation of curcumin is worse in nanoemulsions than conventional emulsions because of the larger surface area of the oil droplets in the former [122]. Consequently, it may be important to optimize nanoemulsion-based delivery systems to obtain good bioaccessibility and chemical stability.

Essential oil (*Carum Carvi*) nanoemulsions have been shown to exhibit anticancer (apoptotic and cytotoxic effects) on model colon cancer cells (HT-29) using an MTT assay [123]. The same study showed that the cytotoxicity of the anticancer nanoemulsions was much lower on model normal cells (Huvec) than on the model cancer cells, with the IC_{50} values being 50 and 12.5 μ g/ml, respectively. Another study showed that nanoemulsions loaded with a botanical extract (goldenberry) exhibited greater cytotoxicity against cancer cells than non-cancer cells [124]. These nanoemulsions were formulated using a mixture of non-ionic surfactants (Tween 80 and Span 80, using medium chain triglycerides (MCT) as a carrier oil.

An *in vitro* and *in vivo* study has been carried out to establish the impact of tea polyphenols on the storage stability and gastrointestinal fate of ingested nanoemulsions containing β-carotene [123]. The incorporation of tea polyphenols in the water phase of the Tween 80-stabilized nanoemulsions increased the chemical stability of the carotenoids during storage, which was attributed to the antioxidant activity of the polyphenols. The nanoemulsions were then orally administered to Sprague-Dawley (SD) rats. Later, the rats were euthanized and the liver, feces, and contents of their GIT tracts (small and large intestine) were collected and analyzed. The authors also measured the levels of vitamin A in the liver, since β-carotene is converted into this oil-soluble vitamin there. The study showed that the addition of tea polyphenols to the nanoemulsions increased the amount of vitamin A detected, which was attributed to the ability of the tea polyphenols to inhibit the degradation of the carotenoids prior to absorption.

The ability of nanoemulsions to increase the bioavailability of curcumin has been assessed using a combination of *in vitro* and *in vivo* methods [118]. These nanoemulsions were stabilized using a thiol-modified chitosan. The curcumin-loaded nanoemulsions did not exhibit any cytotoxicity on model normal cells (mouse fibroblasts) but they did exhibit cytotoxicity against model colon cancer cells (HT29). A pharmacokinetic study showed that the nanoemulsions led to a faster and higher level of curcumin in the bloodstream after oral administration than for the non-encapsulated systems. Moreover, they showed that the presence of piperine increased the concentration of curcumin in the blood, which was attributed the ability to block efflux mechanisms in the epithelium cells. The *in vivo* rat feeding study also showed that the anti-inflammatory properties of the curcumin were enhanced after it was loaded into nanoemulsions, especially in the presence of piperine, which was mainly attributed to the increase in overall bioavailability. Another recent *in vivo* study showed that nanoemulsions were more effective than emulsions at increasing the bioavailability of curcumin when orally administered to Wistar rats [59]. The authors also showed that using conjugated linoleic acid (CLA) to formulate the nanoemulsions led to a higher curcumin bioavailability than using fish oil. These studies highlight the importance of optimizing the formulation of nanoemulsions to enhance their biological activity.

Nanoemulsions have also been used to orally deliver quercetin, which is a nutraceutical that is claimed to have anti-obesity effects [125]. *In vitro* cell culture studies (Caco-2 cell) showed that quercetin loaded in nanoemulsions had a 3.4-fold higher absorption than free quercetin. Moreover, an *in vivo* study that involved oral administration of the samples to mice showed that the nanoemulsion form of quercetin had about a 34-fold higher oral bioavailability than the free quercetin. Finally, there was a greater reduction in weight gain (around 24%) by rats fed a high fat diet for the nanoemulsions. Similarly, an *in vivo* study showed that nanoemulsions increased the oral bioavailability of Coenzyme Q10 (compared to a bulk oily formulation) when orally administered to male Wistar rats [126].

6.5. Cannabinoids

The utilization of nanoemulsion-based delivery systems for cannabinoids, such as THC or CBD, has been a growing area over the past few years, with numerous examples of commercial companies using this technology to create cannabis-fortified edibles [35]. Nevertheless, there have only been a few academic publications in this area due to the fact that cannabinoids are not legal in many states and countries. Even so, there has been considerable progress made by industrial researchers who have created a range of cannabis edible products using nanoemulsions. The nanoemulsions are used to encapsulate the cannabinoids, protect them from degradation during storage, and then produce a specific pharmacokinetic profile after ingestion. Typically, a rapid release of cannabinoids into the bloodstream is desirable so as to quickly get the desired effects. In one academic study, reported in a conference abstract, nanoemulsions were used to incorporate cannabis into beverages [127]. In another study, a combination of fermentation and sonication were used to prepare emulsified cannabis formulations suitable for incorporation into beverage products [127]. Researchers have also prepared oil-in-water nanoemulsions from hemp oil [128,129], and examined the factors that impact their physical and oxidative stability during storage [130]. This area appears to be one that would certainly benefit from more academic research in the future, as there have been many advances made in nanoemulsion technology that could lead to more reliable and efficacious cannabinoid formulations.

6.6. Excipient foods

Recently, "excipient" nanoemulsions have been developed to increase the bioavailability of hydrophobic substances (such as vitamins and nutraceuticals) in co-ingested foods [40,131]. Excipient nanoemulsions are typically oil-in-water systems whose composition and structure are carefully controlled to create an environment within the human gut that boosts the bioaccessibility, stability, and/or absorption of co-ingested bioactive substances [132]. Excipient nanoemulsions

could be used as a basis to form a variety of products intended for use in the food or supplement industries: (i) cooking sauces that are consumed with cooked vegetables; (ii) salad dressings that are poured onto salads; (iii) rich creams that are poured onto fruit; or (iv) creamy beverages that are drunk with nutritional supplements. Nanoemulsions are especially suitable for this purpose because they can be designed to contain small oil droplets that are quickly digested within the human gut, thereby rapidly producing mixed micelles that can solubilize and transport the hydrophobic substances liberated from the foods or supplements consumed with them [133–135]. Previously, it has been shown that excipient nanoemulsions can enhance carotenoid bioaccessibility in spinach [135,136], tomatoes [137], carrots [138], and dietary supplements (capsules and pills) [139]. It should be noted, excipient nanoemulsions also have the potential to enhance the bioavailability of potentially harmful hydrophobic substances in foods, *e.g.,* the pesticides used to treat agricultural produce [140–142]. Nevertheless, these studies suggest that only highly hydrophobic pesticides are impacted by the presence of excipient nanoemulsions. The nature of the lipid phase used to formulate excipient nanoemulsions is important because it determines the solubilization capacity of the mixed micelles formed within the small intestine, thereby influencing the bioaccessibility of hydrophobic substances in foods. Nevertheless, other components can also be added to excipient nanoemulsions to increase the bioavailability of hydrophobic substances, such as substances to inhibit their chemical degradation (such as antioxidants and chelating agents) or substances to increase their absorption (such as permeation enhancers or efflux inhibitors).

7. Advanced nanoemulsion-based technologies

Nanoemulsions have some advantages due to their small particle sizes but they also have some disadvantages for the same reason. For instance, bioactive release rates tend to be very rapid due to the small particle dimensions and bioactive degradation rates tend to be rapid due to the high droplet surface area. The functional performance of nanoemulsions can be improved using a variety of strategies, a few of which are shown schematically in Fig. 6. A brief overview of these strategies is given here, along with some of their potential applications.

7.1. Lipid phase solidification

The lipid core of nanoemulsion droplets can be fully or partially crystallized by careful selection of the type of lipids used, as well as the thermal history of the system (Fig. 6) [143–147]. This is typically achieved using long-chain saturated triacylglycerols (digestible) or edible waxes (indigestible). When the lipid phase is fully crystallized, the particles are usually referred to as solid lipid nanoparticles (SLNs) but when it is only partially crystallized, they are referred to as nanostructured lipid carriers (NLCs). Crystalline lipid nanoparticles can be used to protect encapsulated bioactive components from release or from chemical degradation during storage, since the diffusion coefficient of the bioactives or other components within the particle matrix is reduced [148]. Moreover, they can be used to reduce lipid digestion or prolong bioactive release, since solid nanoparticles are digested more slowly than equivalent lipid nanoparticles [149–152]. Nevertheless, the SLNs or NLCs have to be carefully formulated to ensure they are physically stable and have the required functional performance.

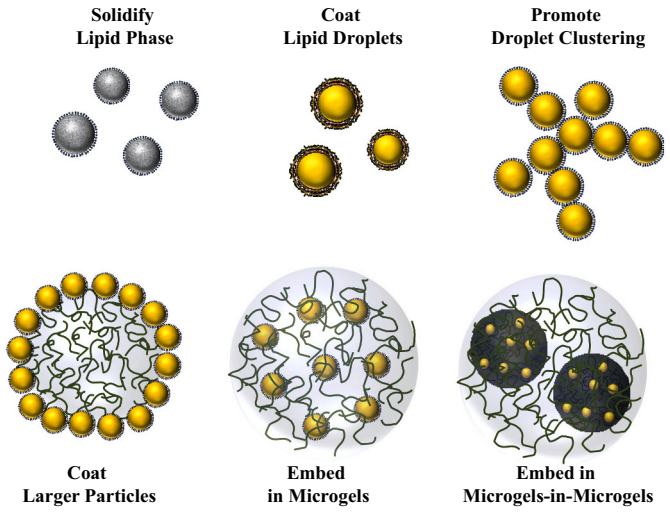

Fig. 6. Examples of approaches that can be used to improve the functional performance of nanoemulsions.

7.2. Interfacial engineering

The lipid droplets in nanoemulsions can be coated with layers of edible biopolymers to enhance their stability and functionality (Fig. 6) [153–155]. Typically, the electrostatic layer-by-layer (LbL) method is used to form laminated biopolymer layers around emulsifier-coated lipid droplets [156]. First, a nanoemulsion is created using an electrically charged emulsifier, such as an ionic surfactant, phospholipid, or protein (1-layer system). Second, this nanoemulsion is mixed with a solution of biopolymers with an opposite charge to the emulsifier-coated lipid droplets, which causes a layer of biopolymers to form around the lipid droplets (2-layer system). Third, the nanoemulsion is then mixed with another biopolymer solution that has an opposite charge to the first biopolymer, which causes a second layer of biopolymer to form around the lipid droplets (3-layer system). This process can be repeated numerous times to create multiple layers around the lipid droplets. The functional performance of a nanoemulsion can be modulated by controlling the composition, thickness, and external charge of the nanolaminated coatings [157]. This can be achieved by using different types, numbers, and sequences of biopolymers to carry out the coating. Numerous studies have shown that this approach can be used to increase the resistance of nanoemulsions to environmental stresses (such as changes in pH, ionic strength, heating, freezing, and dehydration), to protect encapsulated substances from chemical degradation during storage, and to control the gastrointestinal fate of bioactives [153,158].

7.3. Lipid droplet clustering

In many applications it is desirable for the droplets in nanoemulsions to be isolated from each other, since this improves the overall stability of the systems. In some cases, however, it may be useful to promote controlled clustering of the lipid droplets in nanoemulsions (Fig. 6) [159]. For instance, clustering can lead to an increase in the viscosity or gel strength of a nanoemulsion, which may be useful for the creating of highly viscous or semi-solid materials, such as creams or pastes [160,161]. This approach has been used to enhance the mouthfeel of nanoemulsions by increasing their perceived creaminess and thickness [162]. Moreover, clustering of oil droplets inside the gastrointestinal tract can reduce the rate of lipid digestion, which may prolong the release of encapsulated bioactive components [163–165]. The clustering of emulsifier-coated oil droplets can be controlled by manipulating the attractive and repulsive interactions acting between them, such as the electrostatic, hydrophobic, depletion, and/or bridging forces [166].

7.4. Particle coating

The small lipid droplets in nanoemulsions can be used to coat larger particles, such as microgels, to alter their functional attributes [167] (Fig. 6). Typically, this is achieved by inducing an attractive interaction between the surfaces of the emulsifier-coated lipid droplets and the surfaces of the larger particles. Electrostatic attraction is typically used

for this purpose, *e.g.,* cationic lipid droplets can be made to form a coating around anionic microgels. This approach may be useful for creating reduced calorie food products – a microgel coated with lipid droplets may exhibit similar characteristics as a larger fat droplet, thereby reducing the overall fat content of the system.

7.5. Microgel encapsulation

Finally, lipid droplets can be embedded inside larger particles to alter their functional properties [168–170]. For instance, nanoemulsion droplets can be trapped inside biopolymer microgels (Fig. 6). These filled microgels can be formed by mixing a nanoemulsion with a gelling biopolymer (such as alginate) and then injecting the mixture into a gelling solution (such as calcium chloride). This process leads to the formation of microgel particles with small lipid droplets trapped within them. The composition, external dimensions, shape, pore size, and charge characteristics of the microgels can be controlled by using different ingredients and processing methods to formulate them. In addition, lipid droplets can be encapsulated within microgel-in-microgel systems [171] (Fig. 6), where each microgel phase may be made of a similar or different type of biopolymer. As a result, delivery systems with a broad range of functional attributes can be created. For instance, encapsulation of bioactive-loaded lipid droplets inside microgels can be used to protect the bioactive agents from chemical degradation during storage [172–174]. Alternatively, encapsulation can be used to prolong the release of a bioactive component, such as a hydrophobic flavor molecule during cooking [175,176]. Biopolymer microgels have also been used to control the gastrointestinal fate of nanoemulsions. For instance, encapsulation of the lipid droplets can slow down the rate of lipid digestion under simulated GIT conditions, as well as prolong the release of bioactive components [171,177].

There are numerous other strategies that can be employed to improve nanoemulsion performance, but these few examples highlight the potential of using these approaches. As a result, the functional properties of nanoemulsion-based systems can be extended and tailored for specific applications.

8. Potential toxicity of edible nanoemulsions

There has been some concern about the potential toxicity of nanoparticles in foods [178–180]. However, the nature of the nanoparticles concerned have a major influence on their potential toxicity, *e.g.,* their size, shape, charge, and digestibility. Nanoemulsions contain lipid nanoparticles that may exhibit toxicity through various mechanisms as a result of their small dimensions and the ingredients used to formulate them. A number of the most important ones are briefly highlighted here:

- *Greater penetration through biological barriers*: In principle, nanoparticles can penetrate through biological barriers, such as the mucus layer and epithelium cells, more easily than larger particles [181]. In practice, most food-grade nanoemulsions are rapidly digested by the enzymes within the upper gastrointestinal tract (such as lipases and proteases) and are therefore broken down before reaching these biological barriers. Nevertheless, there are some potential exceptions. First, lipid droplets that are not partially digested in the stomach do not produce free fatty acids there, which the body normally uses as a signal to regulate gastric emptying [181]. As a result, a high concentration of lipid droplets may enter the small intestine and not be fully digested before they reach the epithelium cells. Second, the droplets in nanoemulsions formulated from indigestible oils (such as mineral oils) are resistant to digestion in the upper GIT [182]. As a result, they may be able to penetrate through the mucus layer and reach the epithelium cells (provided they do not coalesce or flocculate first). Third, nanoemulsions may be encapsulated within indigestible food matrices (such as dietary fiber coatings or microgels) prior to ingestion [171]. In this case, they may be released in the small intestine and then travel through the mucus layer before being fully digested. Fourth, some of the components used to formulate nanoemulsions (such as surfactants and alcohols) could increase the permeability of the epithelium cells by disrupting the lipid bilayers, thereby allowing the lipid droplets to penetrate through the cells more easily [181]. Finally, studies have found that there is often an optimum nanoparticle size for absorption, around 50 to 100 nm [181].
- *Increased bioavailability into a toxic level*: Many studies have shown that nanoemulsions can increase the bioavailability of hydrophobic bioactive substances [9]. Usually, this enhancement is desirable because it increases the bioactivity but there may be circumstances where it is not. For instance, some bioactive agents have been reported to promote adverse health effects when consumed at high levels, *e.g.,* increasing the levels of β-carotene consumed has been reported to increase the risk of lung cancer in smokers [183]. Consequently, if nanoemulsions greatly increase the bioavailability of these substances in foods they could lead to health problems in certain populations. Similarly, if nanoemulsions increase the bioavailability of any undesirable (toxic) substances in foods (such as pesticides) they may have adverse effects on health [141].
- *Dysregulation of metabolism or hormonal system*: Bulk oils or conventional emulsions are typically digested relatively slowly because of their large lipid domains (small surface areas). As a result, free fatty acids and monoglycerides are released and absorbed relatively slowly leading to a moderate increase in blood lipids (Fig. 2). Conversely, nanoemulsions are digested rapidly, which could lead to a spike in blood lipids. Over time, this could lead to dysregulation of the metabolic and hormonal systems, *e.g.,* feelings of hunger and satiety. To the authors knowledge, no research has previously been carried out in this area, but this could be a fruitful area for future studies.
- *Enhanced toxicity due to ingredients used*: Another potential source of toxicity is related to the type and amount of ingredients used to formulate nanoemulsions. Nanoemulsions are typically formulated using the same ingredients as conventional emulsions, but higher emulsifier levels are required to stabilize their higher surface areas. Some studies have shown that certain types of food-grade emulsifiers potentially have adverse effects on human health, *e.g.,* by altering the gut microbiome or permeability [181,184–187]. In some cases, nanoemulsions with small droplet sizes can only be created using small molecule synthetic surfactants, which may be more toxic than natural emulsifiers (such as proteins or phospholipids).

A number of researchers have carried out *in vitro* and *in vivo* studies to assess the potential toxicity of various nanoemulsion formulations [188]. The most common method of assessing the potential toxicity of nanoemulsions is to use cell culture models, typically model normal cells, such as fibroblasts. As an example, the potential cytotoxicity of unloaded and curcumin-loaded nanoemulsions was tested using mouse fibroblasts (3T3) [118]. These studies showed that neither type of nanoemulsion exhibited appreciable cytotoxicity against the model normal cells, but the curcumin-loaded nanoemulsions did exhibit strong cytotoxicity against model colon cancer cells (HT29). A similar finding was reported for essential oil nanoemulsions, which were reported to exhibit strong cytotoxicity against HT-29 cells ($IC_{50} = 12.5$ μg/ml), but much less against model normal cells (Huvec) ($IC_{50} = 50$ μg/ml) [123]. A study using nanoemulsions loaded with a botanical extract (goldenberry) reported that they exhibited much stronger cytotoxicity against cancer cells than non-cancer cells [124]. Nanoemulsions formulated from tocopheryl polyethylene glycol succinate (TPGS), lemon oil, Tween-80, and water have also been shown to be non-toxic when tested on Hep G2 cells [189]. In contrast, bergamot oil nanoemulsions have been shown to exhibit some cytotoxic activity against Caco 2 cells when applied at sufficiently high levels, although these authors did not expose the nanoemulsions to a simulated GIT prior to applying them to the cells

[190].

In a comprehensive *in vitro* (cell culture) study of the potential toxicity of nanoemulsions it was shown that loading bioactive agents within them (β-carotene), actually increased their cytotoxicity [181]. This effect was mainly attributed to the formation of reactive oxidative species inside the cells due to oxidation and metabolism of β-carotene. These authors passed the nanoemulsions through a simulated upper GIT and then applied them to a cell culture model. The translocation of the ingredients was then observed by confocal fluorescence microscopy, whereas the cytotoxicity was determined using cell viability methods (Caco 2 cells). Interestingly, they found for Tween 80-stabilized nanoemulsions the cytotoxicity actually increased with increasing droplet diameter (45 to 200 nm), although these effects were only observed at relatively high doses. The increased toxicity with increasing droplet size was attributed to the higher concentration of non-absorbed surfactants (Tween 80) and alcohol (ethanol) in the nanoemulsions, which were able to disrupt the cell membranes. Overall, the authors suggested that the toxicity of the nanoemulsions was not due to their small size but due to the presence of specific ingredients (such as surfactants, alcohols, and carotenoids).

Recently, an *in vivo* study was carried out using male Wistar rats to assess the potential toxicity of nanoemulsions [188]. The nanoemulsions were orally administered to the rats for 21 days at lipid concentrations of 200, 400 or 800 mg/kg body weight. The authors then sacrificed the rats and measured "biochemical, hematological, oxidative stress, and genotoxicity parameters". Oral ingestion of the nanoemulsions did not alter the organ weights or biochemical parameters of the rats compared to the controls. The authors concluded that "the results from this study suggest that [nanoemulsions] can be considered safe for oral administration.

Taken together, these results suggest that nanoemulsions do not typically exhibit strong cytotoxic effects, provided they are formulated using food-grade ingredients. The small droplet size means that they are rapidly converted into monoglycerides and free fatty acids in the small intestine, which would not be expected to have toxic effects because these are normal digestion products.

9. Conclusions and future directions

There have been major advances in the formulation, characterization, and utilization of edible nanoemulsions over the past decade or so. Studies have shown that stable nanoemulsions can be formulated from either synthetic or natural ingredients. Recently, however, there has been an emphasis on formulating nanoemulsions from botanical ingredients, such as plant-based oils and emulsifiers, due to consumer desires for more label friendly commercial products. There is accumulating evidence from *in vitro* and *in vivo* studies showing that nanoemulsions are highly effective at increasing the bioavailability and bioactivity of orally administered hydrophobic bioactives, such as nutraceuticals, vitamins, healthy lipids, and pharmaceuticals. In the future, it will be important to establish the efficacy of these formulations using human feeding studies, provided they are shown to be safe for consumption first. Moreover, more *in vivo* research needs to be carried on the next generation of nanoemulsion-based systems, such as filled microgels, clusters, or multilayer systems.

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