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► **To cite this version:**

Xinyue Wang, Halina Anton, Vandamme Thierry, Nicolas Anton. Updated insight into the characterization of nano-emulsions. *Expert Opinion on Drug Delivery*, 2023, 20 (1), pp.93-114. 10.1080/17425247.2023.2154075 . hal-04304121

HAL Id: hal-04304121

<https://hal.science/hal-04304121v1>

Submitted on 24 Nov 2023

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Updated insight into the characterization of nano-emulsions

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Abstract

Introduction: In most of the studies, nano-emulsion characterization is limited to their size distribution and zeta potential. In this review, we present an updated insight of the characterization methods of nano-emulsions, including new or unconventional experimental approaches to explore in depth the nano-emulsion properties.

Area covered: We propose an overview of all the main techniques used to characterize nano-emulsions, including the most classical ones, up to *in vitro*, *ex vivo* evaluation, and *in vivo* fate. Innovative approaches are then presented in the second part of the review presents innovative, experimental techniques less known in the field of nano-emulsion such as the nanoparticle tracking analysis, small angle X-ray scattering, Raman spectroscopy, and nuclear magnetic resonance. Finally, in the last part we discuss the use of lipophilic fluorescent probes and imaging techniques as an emerging tool to understand the nano-emulsion droplet stability, surface decoration, release mechanisms, and *in vivo* fate.

Expert opinion: This review is mostly intended for a broad readership and provides key tools regarding the choice of the approach to characterize nano-emulsions. Innovative and uncommon methods will be precious to disclose the information potentially reachable behind a formulation of nano-emulsions, not always known in first intention and with conventional methods.

Keywords: Nano-emulsion; characterization; fluorescence; surface properties; innovative methods.

1. Introduction

Nano-emulsions (NEs) are defined as the mixture of two immiscible liquid phases stabilized by surfactants, in the form of nano-droplets, generally of the oil-in-water type, sizing from 20 nm to 300 nm. NEs present some similarities with macro-emulsion, such as thermodynamic instability and adaptable properties (*e.g.*, organoleptic and rheological properties); however, due to their nanometric size range NEs have some specificities such as optical clarity, high stability and high surface area. For these reasons, they have attracted, , great attention in research. In the last 5 years, there were thousands of publications focusing on nano-emulsions (from the database of Web of Science), dedicated to various applications in a large range of fields, from cancer treatments to sunscreens, from functional beverages to biodegradable pesticides.

In pharmaceutical field, the commercially available nano-emulsions are still very limited. Like most of the advanced formulation processes of nanoparticles, low reproducibility and low production yields are the key issues hindering their industrial scale-up. Nano-emulsions are lipid-based systems that can be formulated with FDA compatible components, approved for the chosen administration route. After the formulation, nanoparticle characterization is a crucial step required to fully establish the relationship between the nanoparticle properties and their interactions with the biological systems, and subsequently, to transfer their use of laboratories into clinical applications [1].

Whatever the formulation approach—low-energy or high-energy methods—the nano-emulsions present similar properties. In fact, as nano-emulsions are described with a generic structure, independent of the formulation methods. Furthermore, the process can also impact on the results if encapsulated molecules are sensitive to the high temperature or to the high-shear process (they can be altered). As well, size distribution and polydispersity can depend on the process, thus impacting on the stability or emulsion properties. However, it is to be noted that, if two nano-emulsions present similar properties, they can be considered as similar even if they were formulated with different formulation methods. Classical NE characterization techniques comprise the NEs size distribution including mean size and polydispersity, both crucial parameters to be controlled for optimizing stability and applicative properties. Beside the stability, other crucial parameters are the stability of encapsulated molecules, release mechanisms in the presence of acceptor media, their ability to target specific tissues, the control of their half-life in blood circulation and/or interaction with cells, and the efficacy of the drug delivery.

In this review, we first propose an updated overview of the basic characterization of nano-emulsion (distribution, morphology, surface properties, rheological properties, and stability). The following part focuses on *in vitro* and *ex vivo* evaluation, and *in vivo* fate depending on oral, topical and intra-nasal administration. From this general panel of widespread approaches, the second part of the current review focus on emerging techniques applied to the nano-emulsion characterization, their interest and complementarities: nanoparticle tracking analysis (NTA), small angle X-ray/neutron scattering (SAXS), Raman spectroscopy and nuclear magnetic resonance. The last part of this review discuss fluorescent nano-emulsions, and show how the use of fluorescent probes allows monitoring droplet stability, integrity, drug leakage, and surface modifications. This is obtained by using different fluorescent phenomena like aggregation caused quenching (ACQ), aggregation induced emission (AIE), Förster resonance energy transfer (FRET), fluorescence lifetime microscopy (FLIM), and fluorescence correlation microscopy (FCS). The last part of the fluorescence *in vivo* imaging concludes the review. It is important to note that we will focus the discussion on the characterization of nano-emulsions, obviously, some experimental approaches cited are also applicable to other types of nanoparticles or nano-systems.

2. Basic characterization

A complete characterization and evaluation of nanocarriers refer to the measurement of different physicochemical properties (such as particle size, surface charge and stability), *in vitro* characterization (like drug encapsulation, drug release), *ex vivo* evaluation (e.g., cytotoxicity, cellular uptake), as well as the *in vivo* fate. The characterization methods for different nanocarriers can appear similar, however, owing to the type of nanocarrier important differences can arise. In this section, we describe important physicochemical properties and characterization methods commonly used for nano-emulsions, as well as for *in vitro* and *in vivo* studies.

2.1. Composition

The choice of the nano-emulsion composition is a prime importance parameter, respective to their potential application. The final application determines the choice of the type of oil and surfactant used in the formulation. For instance, in the case of development of nano-emulsions intended for the parenteral administration route, parenteral grade and FDA-approved compounds are privileged, like the couple Kolliphor ELP[®] (from BASF) and medium chain triglycerides [2]. In a second time, the regulatory aspects define the maximum concentration allowed, also depending on the application and administration route. On the other hand, the stability of active components encapsulated in nano-emulsion droplets needs investigations and monitoring, to ensure the absence of degradation and of interactions with excipients. These controls may be performed by differential scanning calorimetry (DSC), and provide information about the interactions between different components and their change over time. A original study by Zhu *et al.* [3] showed that the volume fraction and surfactant concentration can be determined through the measurement of refractive index, using an Abbé refractometer and fitting the results with an appropriate model.

2.2. Size distribution

NEs size distribution is the most important parameters of nano-emulsions since it is related to many other properties, such as stability, applicative properties, pharmacokinetics and bioavailability of encapsulated active pharmaceutical ingredients (APIs). Thus, a good control of the granulometry through the formulation process is essential. The average particle size, size distribution and polydispersity index qualify the homogeneity of the dispersion. Methods used for size characterization include photon correlation spectroscopy (PCS) (*i.e.*, dynamic light scattering, DLS) [4], fluorescence correlation spectroscopy (FCS) [5], electron microscopy [6]. Among all of them, DLS is the most currently used technique, probably due to the wide dissemination of apparatuses, along with the simplicity of use. Particle size is monitored by recording the variation in the light scattering of an incident light passing through the sample as a function of time. These fluctuations are analyzed by an autocorrelation function (which is the Fourier transform of the power spectrum), providing the diffusion coefficient of the particles, and thus hydrodynamic radius. Various methods for size measurements are reported in Table 1, emphasizing their respective advantages and disadvantages. These methods are generally complementary and based on different physicochemical principles.

It is noteworthy that these characterization methods concern *direct nano-emulsions* (water-in-oil, W/O), as the more studied type of nano-emulsions. However, *reverse nano-emulsions* (oil-in-water, O/W) can also be produced and studied for cosmetics or pharmaceuticals formulations, as intermediate states to create double emulsions or double nano-emulsions [7,8]. In that case, the characterization approach is quite different. Since oil is the continuous phase and cannot be

evaporated, reverse NEs are not compatible with transmission electron microscopy (TEM) or atomic force microscopy (AFM). In general, size distribution is determined by DLS, under a rigorous protocol due to some specific experimental constraints, without which the size measurement is very difficult and not reproducible. Indeed, W/O nano-emulsion can be visually very different from O/W ones, as a result, the W/O NEs sample does not exhibit a bluish and translucent aspect—which allows diluting O/W NEs up to suitable conditions for DLS. As described in literature reports [9,10], W/O NEs are milky when concentrated, and fully transparent when diluted; the challenge consists of diluting the sample to obtain *milky* but sufficiently *transparent* sample aspects, in order to meet quality criteria for DLS.

Table 1: Comparison of different methods used for particle size determination of nano-emulsion. Adapted with permission from [11].

Method	Size range	Concentration range	Advantages	Disadvantages	Cost
Electron microscopy	> 5nm	0.1%-50%	Allows structure visualization	Destructive Needs sample preparation Time-consuming Expensive	Very expensive
SLS	30 nm-1000 μm	< 0.1%	Easy to operate Fast	Needs sample preparation	Expensive
DLS	1 nm - 6 μm	From < 0.01% to > 10%	Easy to operate Fast	Needs sample preparation	Expensive
FCS	1 nm - 6 μm	< 0.01%	Accurate Provides particle number	Custom-made apparatuses Needs specific expertise in performing experiment and results interpretation	Expensive
Coulter counter	0.4-1200 μm	< 0.1%	Fast Reliable and accurate	Needs sample preparation Influence of measurement conditions Needs various glass tubes	Normal
Sedimentation	1 nm and 1 mm	Depends on the method	Allows droplets' fractionation	Needs sample preparation Destructive	Inexpensive
Ultrasonic spectroscopy	0.1-1000 μm	< 50%	Fast Precise No sample preparation Non-destructive Applied to optically opaque samples <i>In situ</i> use	Knowledge of physicochemical properties Interference of air bubbles	Expensive
NMR	0.2 and 100 μm	< 80%	No sample preparation Non-destructive Applied to optically opaque samples	Expensive	Very expensive

2.3. Morphology

Determination of droplet morphology is performed by direct visualization of the droplets, by electron microscopy. Since the resolution of the conventional optical microscopy is limited to ~ 200 nm, electron microscopy based on electron diffraction, like TEM and scanning electron microscopy (SEM) are widely used for examining the NE structure. The magnification of electron microscopes can reach as much as 100,000 times, while the resolution ranges from 0.1 nm to 0.5 nm [12]. TEM gives a two-dimensional projection of the sample, which allows visualization of droplet inner structure, *i.e.*, the structure confirmation of water-in-oil-in-water (w/O/W) double emulsion [7]. The sample preparation is rather simple, the sample is deposited on the grid and dried [9]. In case of low electron-contrasting materials, staining agent can be added in the samples before evaporation. In the case of nano-emulsions, generally, there is no need to use staining agent when using a low-power electron beam (70 kV) to avoid oil vaporization during measurement, SEM permits observing the surface of the samples, but requires a metalization of the samples. In both techniques, TEM or SEM, samples are observed under vacuum. During drying, lipid droplets can be concentrated in evaporating aqueous droplets, and can flocculate as clusters—and potentially merge. A solution to override this issue is the preparation of the concentration gradient of NEs suspensions, up to dilution for which the droplets can be individualized when dried. It should be stressed that electron microscopy techniques under vacuum remain destructive for the samples, especially for nano-emulsions which are made with liquid oil droplets. Oil nano-droplets are spread on the carbon support and—depending on their rheological properties—their shape can be modified. In that case, the size observed can be overestimated, compared to their real size or from the hydrodynamic diameter.

Cryogenic transmission electron microscopy (cryo-TEM) represents a complementary approach to TEM specifically adapted for water containing samples. The samples are frozen in liquid nitrogen in the form of thin liquid films and conserve their native structure and morphology. This approach allows a direct investigation of colloids in vitrified or frozen-hydrated state, *i.e.* close to their native state [13]. Environmental scanning electron microscopy (ESEM) is also an alternative to SEM, which allows studying non-conducting samples without beforehand preparation. The chamber contains a gas that avoids vacuum constraints. AFM has been recently used to explore the surface morphology of nano-emulsion formulations. By detecting and amplifying the forces between the cantilever and the sample surface, AFM allows a resolution at the atomic and molecular level [14,15]. The summary of different microscopic methods is displayed in Table 1, and examples of images produced by different methods shown in Fig. 1.

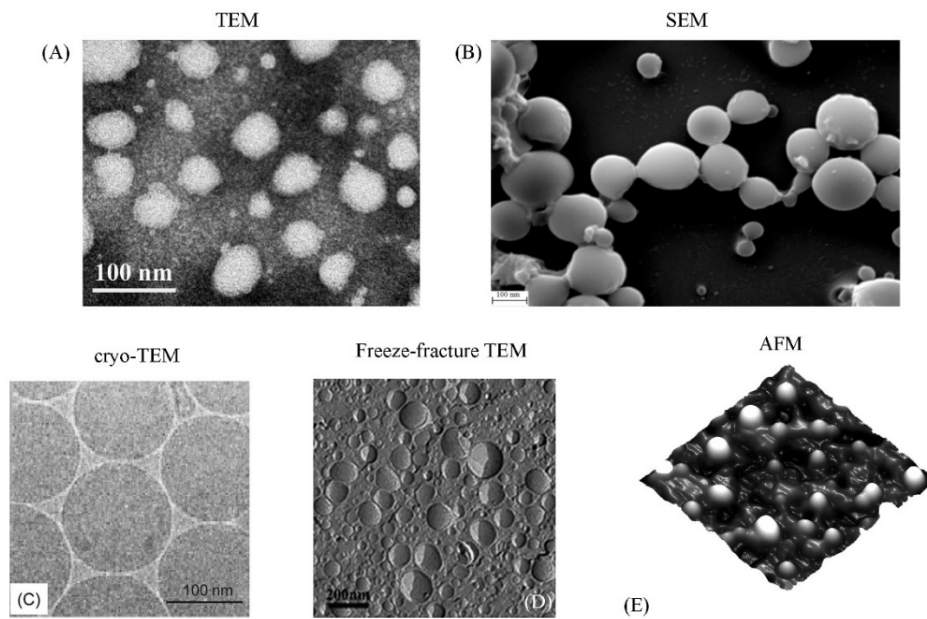


Figure 1: Examples of nano-emulsions characterized by different types of electron microscopy (transmission electron microscopy, scanning electron microscopy, combined with cryogenic techniques), and AFM. Adapted with permission from [16–19].

Table 2: Comparison of microscopic methods used for morphological characterization of nano-emulsions. Adapted with permission from [11,20].

Attribute	Type of microscope		
	Electron microscope		AFM
	TEM	SEM	
Visualization factor	Electron		Microprobe
Max. magnification	100 000×		100 000 000×
Max. resolution (nm)	0.1-0.5	1–10	0.2-horizontal 0.05-vertical
Sample preparation	Complex		Complex
Sample state	Dried or frozen ^a	Hydrated, dried or frozen ^b	Hydrated or dried
Projection type	2D	2D-3D	3D

a) Dried: negative staining, frozen: freeze-fracture TEM, cryo-TEM.

b) Hydrated: ESEM (Environmental-SEM, see details in the text), dried: SEM, frozen: freeze-fracture SEM, cryo-SEM.

2.4. Surface charge

The particle charge is an important property determining the stability and functional activity of nano-emulsions. Surface charge originates from ionized constituents present at the surfaces of NE droplets, such as ionic surfactants, phospholipids, proteins, polysaccharides, or mineral ions [21]. ζ -potential is the most widely used parameter for representing the electrical characteristics of nanoparticles. It is defined as the potential difference between the dispersion medium and the stationary layer of fluid attached to the particle [22]. Stern potential—first layer where ions are bounded to the droplet surface—presents an absolute charge, while the ζ -potential is measured at slipping plane, and can already be impacted by a charge screening from counterions. As surface charge contributes to inter-droplet repulsive interactions, values of ζ -potential are directly related to the stability of emulsions. Generally, emulsions with high ζ -potential (> 30 mV) are electrically stabilized while emulsions with low ζ -potential tend to coagulate or flocculate [23]. Importantly, in the case of nano-emulsion, the high droplet stability is not considered to be only due to the contribution of the surface charge, but also to the steric hindrance [24]. Numerous articles report the formulation of nano-emulsions made with nonionic surfactants with neutral surface charge [25], however, with conserving high stability. On the other hand, ζ -potential measurements are crucial to design surface-charged controlled nano-emulsions, for instance, used for sustained ocular delivery [26], or to design specific shell architecture by layer-by-layer technique [27]. ζ -potential is calculated using theoretical models by determining the electrophoretic mobility (μ_e). Those measurements are based on two main principles, electrophoresis and electroacoustics. In electrophoresis, under the application of electric field electrophoretic mobility of charged particles is obtained by measuring the light scattering and further deducing ζ -potential by Henry's equation :

$$\mu_e = \frac{2\varepsilon_{rs}\varepsilon_0}{3\eta} \zeta f(\kappa a)$$

where μ_e is the electrophoretic mobility, ε_0 is the dielectric constant, ε_{rs} is the relative dielectric constant of the electrolyte solution, η is the viscosity of solvents, $f(\kappa a)$ is Henry's function, which varies from 1.0 to 1.5 depending on the medium.

Electrophoresis light scattering (ELS), which is also known as laser-Doppler electrophoresis, is the most common technique used for ζ -potential determination, and it allows the particle size and ζ -potential being measured on the same equipment. For this method, an appropriate dilution of a sample is required to avoid interactions between droplets. Electroacoustic measurement of ζ -potential is based on sound wave vibration, used similarly to light-based methods, and has the advantage of being applicable in non-diluted samples [21,28,29].

2.5. Rheology

The rheological properties of nano-emulsions can be tuned from flowing fluid to a solely relaxing fluid or to a gel-like system by controlling the dispersed phase volume fraction and droplet size, or by adding additives in the bulk [30]. However, the rheological behavior allows revealing modifications in the interactions between droplets, able to impact on their 3D networking and flowing properties. The rheological characterization of nano-emulsions refers to two parameters: dynamic elastic/storage modulus (G') and dynamic viscous/loss module (G''), on which the apparent shear viscosity (η), and the sol/gel transition can be obtained. If $G' > G''$, the NE behaves as solid or gel, whereas when $G' < G''$ it is fluid liquid or viscous in nature. Oscillatory rheology has been used to characterize the type and properties of nano-emulsion-based gels, in function of droplet size and storage time [31].

Besides the emulsion rheological behavior in volume, the study of interfacial properties, surface tension and dilatational rheology is getting more and more attention in recent years. These studies are generally performed on a water/oil model interface, and allow evaluating the molecular properties when adsorbed at the interface, and the particular intermolecular interactions of molecules adsorbed on the interfaces. After adsorption, these molecules are indeed, abnormally concentrated—unlike in volume solubilization—and can adopt a particular arrangement or even form closed packed layers [32]. This organization has an impact on the interfacial stabilization, and allows understanding emulsion stability at the droplet scale. Parameters given by this approach are monolayer elasticity, interfacial concentrations, and compactness between small surfactant and macromolecules at interfaces [33].

2.6. Stability

Nano-emulsions are thermodynamically unfavorable systems and therefore have a tendency to break down over time due to Ostwald ripening. Ostwald ripening is created from the difference of the radius between droplets in suspension, which induces the difference of chemical potential within the drops. This leads to mass transfer from smaller droplets to larger ones [34]. Since Ostwald ripening is extremely slow process, the NEs can be considered as stable over months.

Beside the physical stability—followed-up by monitoring the size distribution over time—the chemical stability is also important for quality control of the drug-loaded nano-emulsions. The chemical degradation (such as oxidation, reduction or hydrolysis) of the lipid phase or of the encapsulated active molecule may modify the structure and stability of the nano-emulsion, or induce a premature drug release—if the partitioning coefficient of the drug is modified. It is worth noting that, compared with macro-scaled emulsions, the relatively high surface area of nano-emulsions increases the possibility that some fragile APIs come in contact with the surrounding environment, and are degraded. The chemical stability can be tested by spectroscopic and chromatographic methods, together with electrophoresis or mass spectrometry [35,36].

A last and very important point to emphasize is the stability of encapsulation, that is to say, the control of the release of encapsulated cargo materials when the nano-emulsion droplets are in contact with acceptor media in their continuous phase. We have shown in previous reports [37] that a classical hydrophobic dye, *i.e.* Nile Red, can exhibit a very high stability in lipid nano-emulsion droplets. However, in presence of acceptor medium or biological tissue, this dye undergoes a very rapid leakage. In this same study, we have shown that increasing the oil solubility of Nile Red by grafting lipophilic chains, strongly prevents the premature dye leakage, even in presence of acceptor media. In the same line, other studies were undertaken, emphasizing the crucial importance of studying the cargo stability in biological environment [38], of following-up destabilization of the droplets *in vivo* with a Forster's resonance energy transfer (FRET) approach [39]. The use of fluorescent probes for nano-droplet characterization is developed in a specific section below.

2.7. *In vitro* and *ex vivo* evaluation

In vitro and *ex vivo* studies represent a useful approach that provides first insight into the evaluation of the NEs in biological environment. They consist of studying the drug release, cytotoxicity, bioavailability and their relationship with the physicochemical properties, structure and composition of the NE formulations. Nano-emulsions are delivery systems for biopharmaceutical classification system (BCS) class II and class IV drugs, *i.e.* drugs with low

solubility. Thus, characterizing the drug encapsulation stability and release, when the droplets are in contact with cells, are of crucial interest. Numerous *in vitro* models have been introduced over the years, specifically adapted to various purposes of studies. As presented in the *stability* section above, in the case of lipophilic molecules encapsulated in nano-emulsions, studying the release profiles from nano-droplets also depends on the properties of the release medium, able to partially solubilize the leaking compound. To design the drug release study, the released free drugs are quantified after separation from the NE by dialysis, filtration or centrifugation. Analytical tools—*e.g.* UV visible spectrometry, HPLC—are then designed in function of the drug properties. Pitfalls can arise from the potential interferences with excipients released along with APIs, and should be considered when designing the blank and control experiments. A special care has to be taken regarding the drug separation from nano-emulsions and excipients during sample collection.

For orally delivered nano-emulsions, samples are tested by sequentially mixing with simulated oral, gastric and intestinal fluids (shown in Fig. 2 A). Digestion model, with an addition of bile salts and digestion enzymes (amylases, proteases and lipases) is employed for release study since the oily core of nano-emulsions usually consists of fatty acids [40]. For transdermal administration, the skin permeability is monitored by using Franz diffusion cells [41] clipped with animal abdominal skin, (shown in Fig. 2 B). For study of intra-nasal delivery, the human nasal cavity is simulated by a nasal cast model, filled with model nasal fluid and mucus [42].

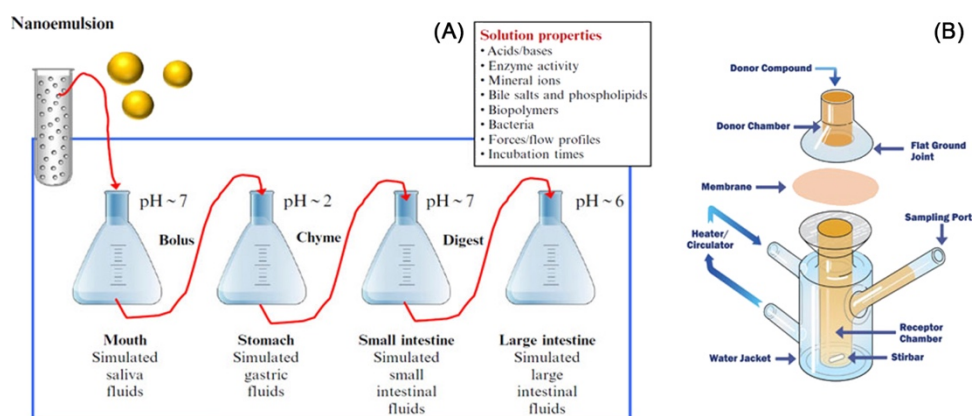


Figure 2: *In vitro* characterization methods for nano-emulsions; (A) *In vitro* simulated fluids used for evaluating oral delivered nano-emulsions, adapted with permission from; [40]. (B) Franz diffusion cells for transdermal evaluation, adapted with permission from [41].

2.8. Cytotoxicity of NE

The more straightforward way to evaluate the cellular toxicity of NEs is to incubate the formulation with cells in culture, and monitor the effect on the cell viability by classical approaches such as 1) lactate dehydrogenase (LDH) release resulting from the loss of the plasma membrane integrity 2) mitochondrial dehydrogenase activity by following the reduction of tetrazolium based compounds MTT (3-(4,5-(dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) or MTS (3-(4,5-(dimethylthiazol-2-yl)-5-(Carboxymethoxyphenyl)-2-(4-Suphophenyl)-2H-tetrazolium) to an insoluble precipitate formazan [43]. When formulated with biodegradable components, the NEs show in general low toxicity. The NEs cytotoxic effect is often related to the encapsulated active molecule and to the targeted cell types.

Selective toxicity on cancer cell lines compared to normal cells have been reported for delivery of anticancer drugs [44,45], curcumin loaded NEs [46], or for essential oil nano-emulsions [47]. In all these studies, the toxicity of “empty” NEs was significantly lower compared to loaded samples.

Specific cell lines are used to mimic contact environment related to the NEs application. Human keratinocytes or fibroblasts are used for testing potential toxicity of NE designed for dermal/topical applications [48]. Acute dermal toxicity and skin sensitization can also be evaluated *in vivo* by applying the formulation on the back of Wistar rats [49] or by using hen’s egg Chorioallantoic membrane [50]. The irritation affects the membrane vasculature and leads to membrane discoloration, hemorrhage, and increased perfusion. The hen’s egg model also serves for evaluation of ocular irritancy of NEs designed for ophthalmic applications [51].

For the edible NEs, cytotoxic effects may result from the high content of synthetic surfactants, or use of indigestible oils in the formulation. Besides the cell toxicity, the NEs may affect the gut microbiome or the permeability of the epithelium. Caco-2 cell model is commonly used to evaluate the toxicity of compounds absorbed by the small intestine [52]. Besides, changes in intestinal permeability and epithelial tight junction integrity can be evaluated by measuring transepithelial electrical resistance [52]. These perturbations are generally caused by the presence of the surfactants. Tween 20 was shown to cause more harm to Caco-2 monolayers compared to Tween 80 [45]. The study of Wooster *et al.* showed that, in addition to the surfactant composition, the toxicity of edible nano-emulsions is related to their size and the presence of ethanol in the formulation. Canola oil/Tween80 NEs loaded with β -carotene was nontoxic up to 10 mg/mL for small < 120 nm droplets and arose when droplet size increased. Ethanol in the formulation composition as well increased the cell toxicity, probably by increasing the permeability of cell membranes. The encapsulation of β -carotene decreased the IC50 (concentration causing inhibition of 50% of the tumoral activity) by a factor of 5. Authors concluded that observed toxicity increase results from oxidation and generation of reactive oxygen species induced by carotene metabolites [53].

Excised animal nasal mucosa is used to test the permeability and the toxicity of NEs formulations designed for nose-to brain applications. The nasal mucosa is excised before or after the NEs treatment and prepared for histological analysis [54]. The decrease of toxicity and irritation of nasal mucosa by NEs formulation was shown for Amiloride loaded mucoadhesive emulsion [55]. Similarly NEs encapsulated anti neuro-AIDS drug, sequinavir mesylate increased significantly drug delivery efficiency to CNS compared to intravenous administration without altering the mucosal epithelium [56]. Curcumin NEs were shown to be safe for intranasal since no nasal ciliotoxicity were observed and the formulation did not impact the SK-N-SH cell viability and proliferation up to 80 μ g/mL [57]. Complementary to these classical approaches, Elbardisy *et al.* [58] measured the nasal toxicity of Talafil nano-emulsions by biochemical analysis of Caspase-3 and tumor necrosis factor- α markers of apoptosis and inflammation, in the nasal tissues.

2.9. *In vivo* fate

The parameters typically monitored *in vivo* are pharmacokinetics, pharmacodynamics and toxicological evaluation. The experimental approach/technique employed to evaluate the fate of NE *in vivo* depends on the administration route. The biodistribution of NE allows determining the efficiency of the droplet targeting after intravenous delivery. However, the

question how drug-loaded nano-emulsions are absorbed, distributed, metabolized and eliminated *in vivo* remains unclear, and represents still a challenge for researchers.

Oral administration

Due to the lack of lipases in the stomach, the first step of lipolysis of the oily core occurs in the small intestine [59]. When the lipid is digested by gastric and pancreatic lipases, it forms free fatty acids that are incorporated along with bile acids and phospholipids into mixed micelles that can solubilize and transport active compounds to epithelium cells [60]. There is a debate over whether lipid-based nanocarriers could cross biomembranes without losing their integrity [61,62]. Schematic delineation of the *in vivo* fate of nano-emulsions is shown in Fig. 3. The left panel shows possible scenarios if lipid-based carriers are already destroyed in the lumen, being gradually degraded (Fig. 3 (I)), followed by the drug uptake in vesicles (Fig. 3 (II)) or in mixed micelles (Fig. 3 (III)), or a transfer between them (Fig. 3 (IV)). In the right panel, four possible scenarios for release mechanisms through the intestinal barrier are described: (a) the first one shows the case in which the drug leaked out of the nanocarriers before the intestinal barrier, (b) the nano-droplets can cross the barrier and release the cargo within the enterocytes, (c) the encapsulated drug remains in the droplets, which cross the barrier, (d) the lipid droplet is disrupted in the lumen, and the drugs are uptaken by vesicle or mixed micelles. However, many questions in this scheme remain, to date, not clarified. For example, questions on the possible precipitation of drugs in the gastrointestinal tract, or on the forms actually absorbed (free drugs and/or secondary carriers and/or native droplets), deserve further investigation.

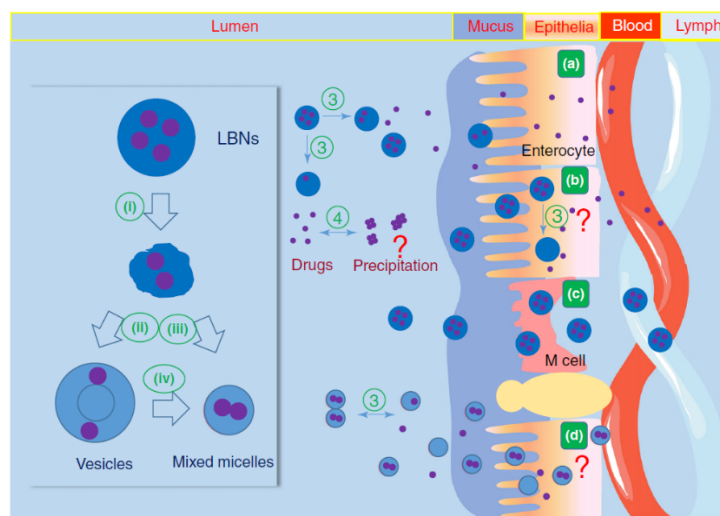


Figure 3: Schematic delineation of the *in vivo* fate and problems of NEs via oral delivery. *Left panel:* show possible scenarios if lipid-based carriers are already destroyed in the lumen (see detail in the text). *Right panel:* possible mechanisms of adsorption of NEs and/or drugs through the intestinal barrier. (a) Absorption of drugs by passive diffusion. (b) Absorption of integral lipid-based nanoparticles (LBN) through enterocytes. (c) Translocation through M cells. (d) Absorption through mixed micelles. (I) Lipolysis. (II)(III)(IV) Transformation into secondary carriers. (3) Release of the drugs; and (4) precipitation of the drugs. “?” refers to processes that await verification. Adapted with permission from [62].

Intravenous administration

When administered by intravenous injection drug travels far, extravasating with blood into various organs. Depending on their size and surface features, nano-emulsions are adsorbed by opsonins and recognized by the reticuloendothelial system (RES). Thus, they are driven into

RES reservoirs such as in spleen or liver, where immune cells (platelets, monocytes, leukocytes) usually reside [63]. Figure 4 shows an example of the biodistribution of doxorubicin-loaded NEs injected in mice by the tail vein. The NE biodistribution was monitored by fluorescence imaging. Accumulation of the nano-emulsions was detected in liver, kidneys and tumors 6 h after injection [64]. In this work, Cao *et al.* discuss the potential of lipid NEs to co-encapsulate lipid molecules having a synergetic effect together. In that case, an anticancer doxorubicin (DOX) and a P-glycoproteins (P-gp) inhibitor bromotetrandrine (W198), were encapsulated in the same NE droplets. The idea is to reduce or reverse the multidrug resistance resulting from the P-gp mediated efflux of DOX in breast cancer. Main results emphasized in the figure, showed that NEs loaded with DOX/W198 achieved an intratumoral distribution by passing the P-gp efflux.

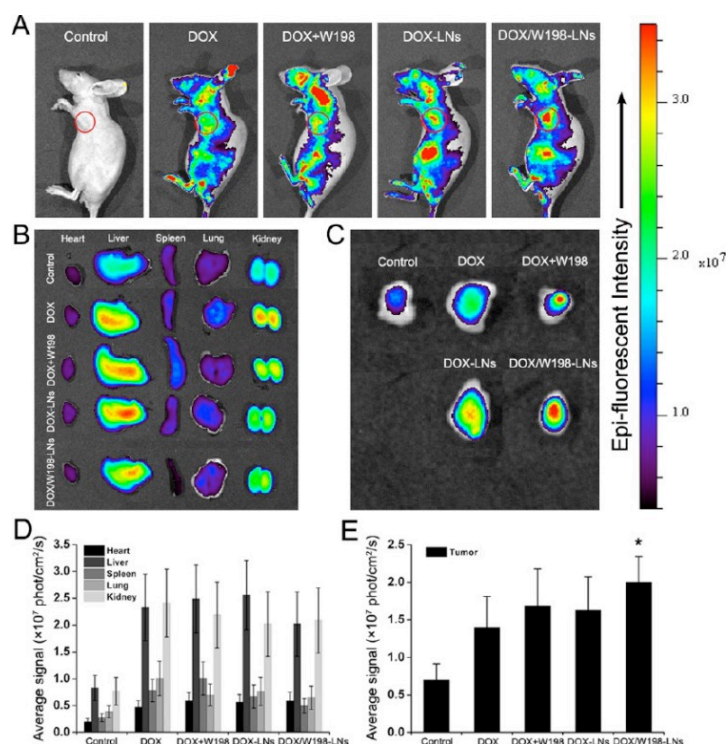


Figure 4: Biodistribution of DOX-loaded nano-emulsions in MCF-7/ADR-bearing mice, 6 h after the injection. (A) The whole body fluorescent images of MCF-7/ADR-bearing mice; (B, C) representative fluorescent images of excised organs and tumors after the termination of treatments; (D, E) semiquantitative fluorescent intensity of excised organs and tumors. Adapted with permission from [64].

Topical administration

NEs cross the epidermis via three possible routes: transcellular route, intercellular route and the route via hair follicles or sweat ducts. Even though integral NE droplets are generally considered not able to permeate the horny layers of the skin, they are still useful when applied as topical treatment of skin diseases. Compared with conventional emulsions, the reduced size of NEs promotes the skin penetration and enhanced delivery of hydrophobic drugs. As shown in Fig. 5, the lipid structure, nano-scaled size and charges can favor the penetration of nano-emulsions [65]. Fig. 5 illustrates the different mechanisms upon which NEs can penetrate the skin layers. We can cite mechanisms based on : (A) hydrophobic affinities between stratum corneum and oil composing nano-emulsions favoring the droplet penetration; (B) penetration enhanced by the size, the smaller the droplets the higher the penetration; (C) enhancement of the NEs penetration due to the surface charge; (D) direct drug delivery determined by the

solubility of the encapsulated molecules that activates its release when NEs are administrated (skin is considered as an acceptor medium); (E) using excipients to hydrate skin (absorption promoter) and favor intercellular passage; (F) using alternative ways such as hair follicles to enhance the skin penetration.

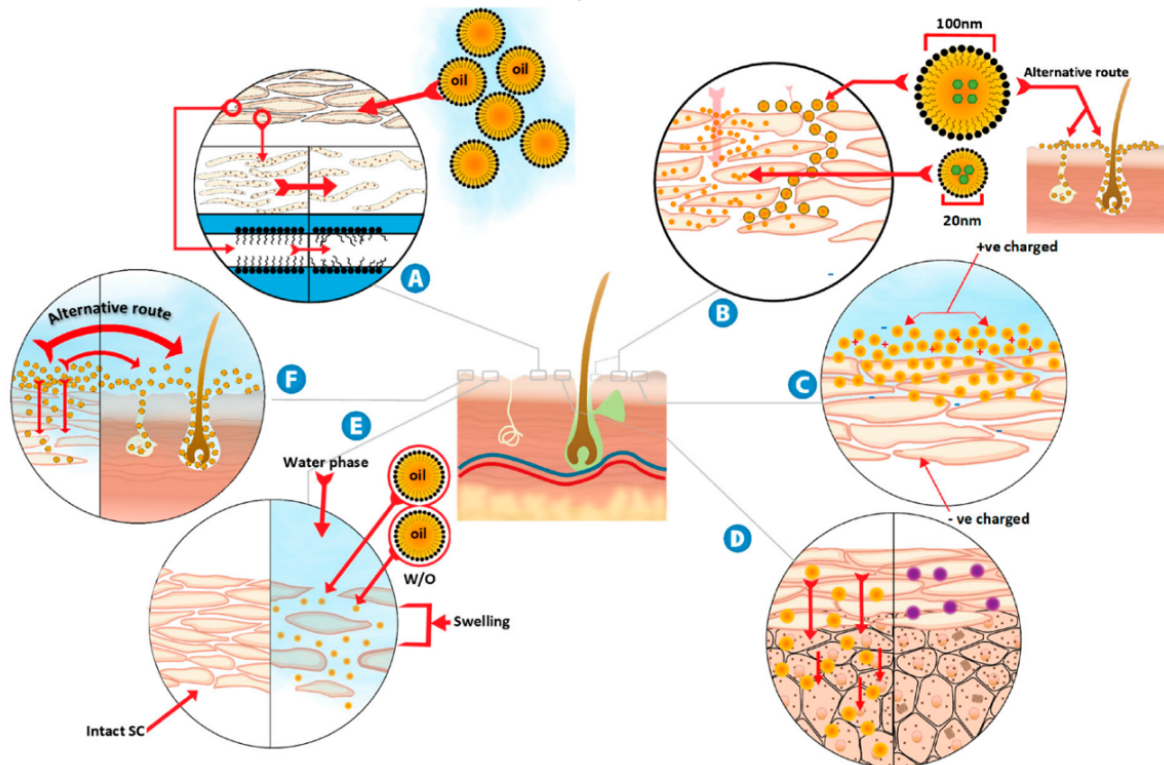


Figure 5: Mechanisms of transdermal enhancement of hydrophobic drugs from nano-emulsions: (A) Structural affinity of nano-emulsions with the stratum corneum; (B) size-dependent drug diffusion; (C) electrostatic-attraction induced penetration of positively charged nano-emulsions; (D) drug partition changes into skin layers; (E) hydrating skin and the dilation of the stratum corneum intercellular channels; (F) changing the pathway to follicular delivery. Adapted with permission from [65].

Intranasal administration

The uptake of drugs by intra-nasal administration occurs via two different pathways: systemic circulation that subsequently reaches the brain by crossing the blood brain barrier and the olfactory pathway by which drugs partly travel from the nasal cavity to cerebrospinal fluid and/or brain tissue. It was reported that, risperidone, a water-insoluble antipsychotic, has a 17-fold enhancement of brain targeting efficiency after 30 min when administered via intra-nasal delivery compared with intravenous delivery [66]. The mechanisms of drug transport from nose-to-brain are not fully understood yet, but several neuronal pathways are also supposed to be involved. Besides, toxicity issues induced by repeated administration, and/or irregular nasal absorption also need further investigations [67].

2.10. Therapeutic nano-emulsions

Beyond the basic characterization of droplet size, composition, stability, interactions with cells, and behavior after administration, an important point lies in the evaluation of the therapeutic activity of NE. In other words, answering the question of the impact of the nano-formulation of

lipophilic drugs, on a potential increase of the therapeutic efficiency. Recent literature reports show an emerging research activity on the formulations of nano-emulsion for antiviral and antimicrobial applications [68–73]. These works highlighted the potential of NEs as drug carriers that can be tailored for the encapsulation of a variety of lipophilic active pharmaceutical ingredients, able to present a therapeutic effect on very different pathogens, from Ebola, COVID-19, to resistant bacterial strains.

3. Advanced instrumental characterization methods

Important technological progress of the last decade, led to significant improvements in characterization and evaluation of nanoparticles, *i.e.*, advanced microscopes allow a better visualization of nanoparticles. At the same time, the costly equipment which used to be very limited for application becomes more accessible. In this section are introduced, new techniques that are more and more used in NE characterization.

3.1. Nanoparticle tracking and analysis (NTA)

NTA technology was firstly commercialized by a British company NanoSight in 2003. Based on a principle similar to classical dynamic light scattering method, NTA apparatus measures the fluctuations of scattered light crossing the sample. The main difference lies in the fact that the signal detection is done by a camera connected with an optical microscope, allowing tracking individual particles in two dimensions. The analysis of their trajectories provides the value of the diffusion coefficient, which gives the hydrodynamic diameter by applying the Stoke-Einstein equation (see principle in Fig. 6).

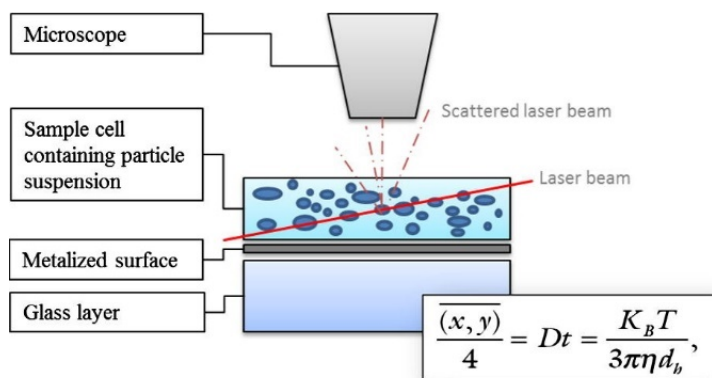


Figure 6: Scheme and principle of nanoparticle tracking analysis (NTA) and Stoked Enstein equation for free diffusion.

Filipe *et al.* has compared the accuracy of size measurement between NTA and DLS [74]. They found that, for monodispersed nanoparticles, the average size determined by NTA is close to DLS results, giving a small standard derivation and a sharp distribution. However, NTA showed a better sensitivity and higher distinction between two populations of different sizes co-existing in the same suspension. This is illustrated in Fig. 7, comparing of DLS and NTA for monodisperse and polydisperse samples. This is an important point for the correct size determination since the emulsification process may produce polydispersed size distribution. Besides, the analysis of different populations present in the samples provides information about its stability and may be used for identification of destabilization/dephasing process related to the size changes with time [75].

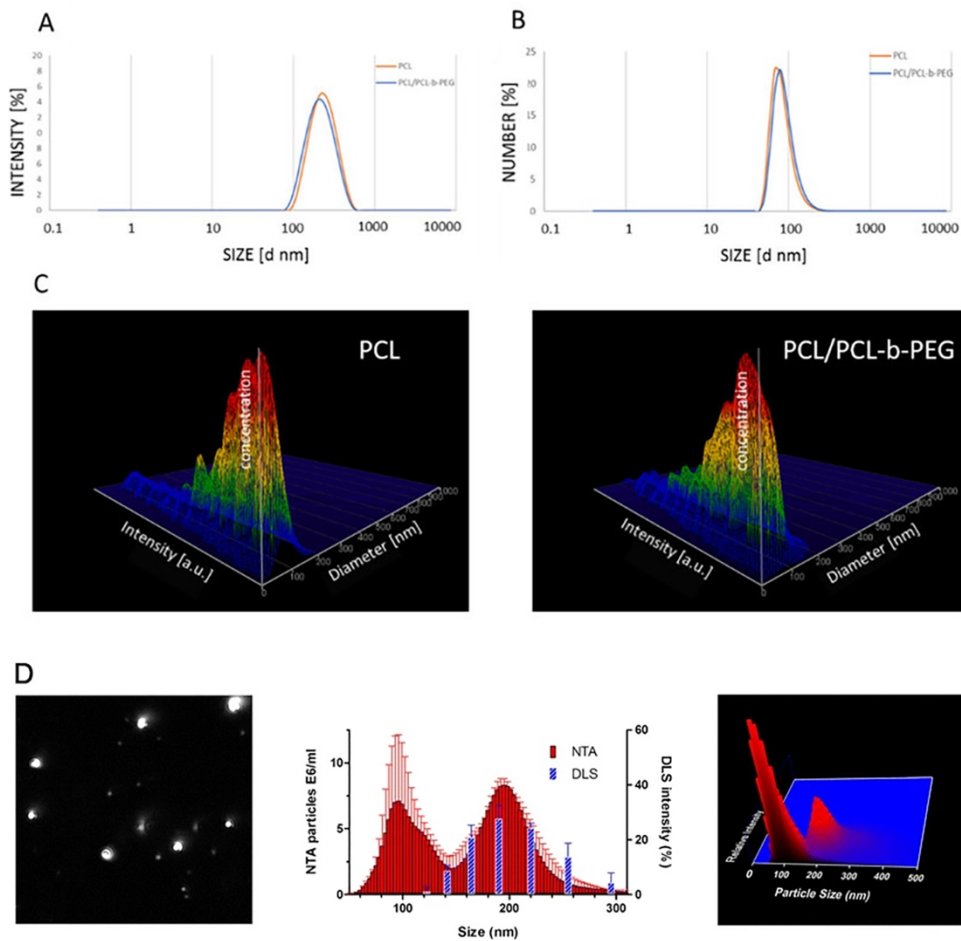


Figure 7: Comparison of DLS and NTA for characterizing size distribution of NEs and nanoparticles. Nanoemulsion stabilized by poly-epsilon-caprolactone by DLS (A, B) and NTA (C) from [76], and compared to poly-dispersed polystyrene beads (D), left panel NTA video frames, middle size distribution, and right 3D representation, from [74].

Actually, the US National Cancer Institute Nanotechnology Characterization Laboratory (NCI-NCL) and the European Nanomedicine Characterization Laboratory (EUNCL) have published a step-by-step protocol to correctly monitor the size distribution of nanoparticle suspensions [77]. The protocol is composed of three steps, prescreening, particle size determination with higher resolution, and particle size determination in plasma. Importantly, these regulatory preconizations include in all stages using NTA together with classical methods like DLS and TEM [77].

3.2. Small-angle scattering

SAXS and small-angle neutron scattering (SANS) are scattering techniques based on the diffraction (normally with an angle between 2° and 5°) of electrons or protons beam due to the difference in electron cloud density between the sample and surrounding medium. Small-angle scattering techniques are useful for studying the structure and interactions of complex fluids and colloids, allowing the determination of form factors: particle size, surface area, and particle shape in concentrated solutions [78–81]. This technique also evidence the structure factor—which allow calculating the interactions in the system.

Applied in the field of NEs, small-angle scattering provides the essential understanding of the behavior of surfactants, polymers or proteins on the droplet's water/oil interface [82,83], and NPs stabilized at the water/oil interface in the case of Pickering emulsions [84]. In addition, SAXS enables further investigations of stability-related deformation of droplets at a molecular scale [85]. For example, Lutz *et al.* [86] introduced SAXS as a tool to investigate the structure of a nano-scaled double emulsions. Compared with the microemulsion, the NEs (called in Ref. [86] *emulsified microemulsion*, EME), NEs have a narrower diffraction width, suggesting a less ordered structure in the emulsion core. Besides, a significant decrease of the diffraction intensity suggested the instability of the multi-layer structure over time. Fatouros *et al.* employed SAXS for monitoring the lipid digestion of self-emulsified NEs [87]. The multilamellar liquid-crystalline phases formed during the digestion process gave specific intensity signals in SAXS curves. Gradual changes of the SAXS signal and Bragg peak intensities with time are shown in Fig. 8, which indicates an energy loss before a fast-moving electron stops, and thus a phase change of colloid system. In the case of lipolysis, a hexagonal phase appears to co-exist with the lamellar phase; when, up to 80% of hydrolysis is complete, and at the end of the process, the hexagonal phase becomes dominant.

Compared to SAXS, SANS can provide similar information on the sample size, shape, structure, inter-droplet interactions, but with different experimental approach and constraints. SANS is performed in large facilities when SAXS is readily available in laboratories, however, SANS does not induce the radiation damages caused by SAXS. SANS was used for structure confirmation of emulsion polymerization by fitting the radius, density and aggregation number of molecules [88]. This method can also be implemented in combination with rheological approach, called rheological small-angle neutron scattering (rheo-SANS) and used to investigate the structural evolution and rheology changes during the formation of NE gels [89].

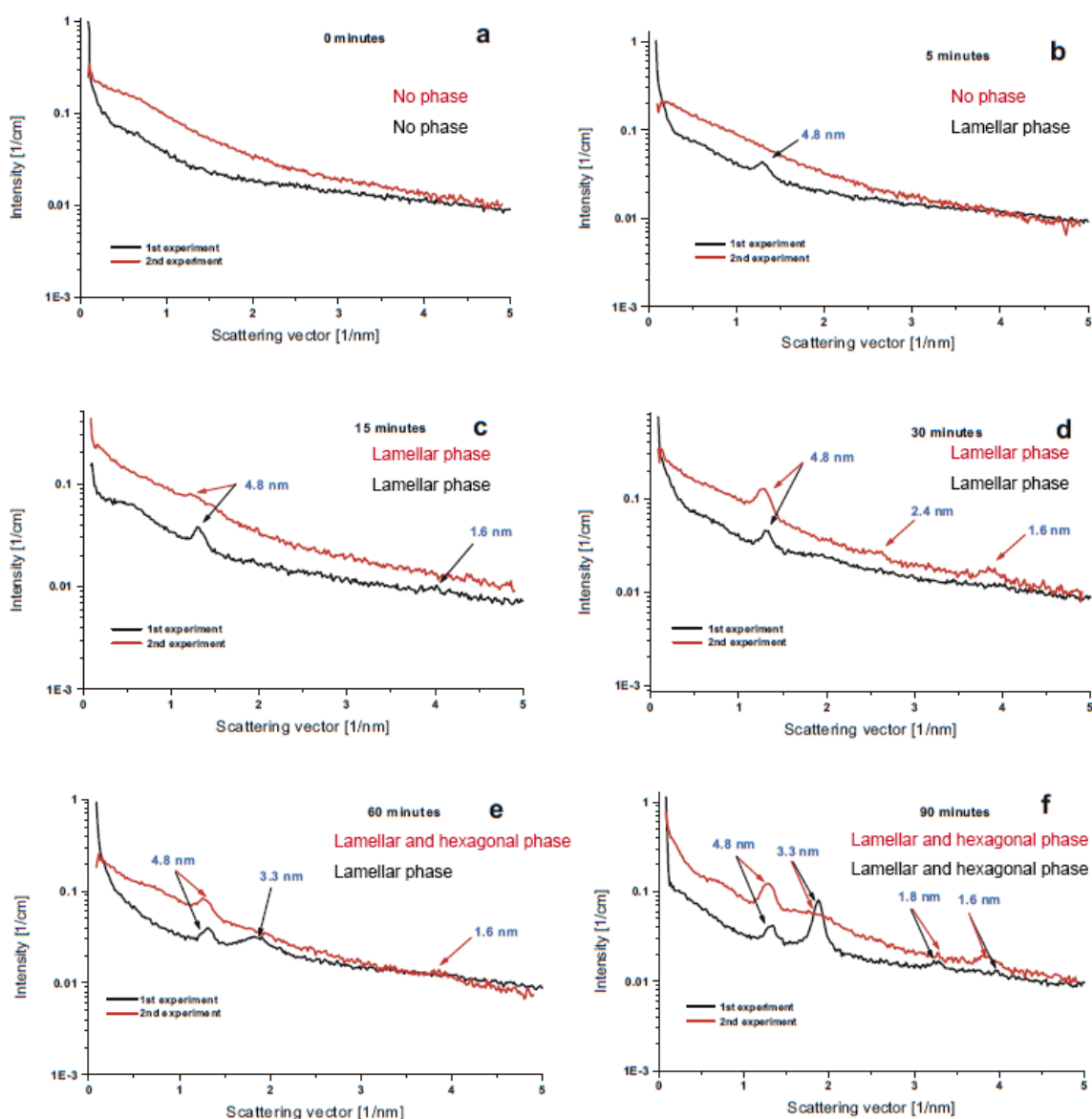


Figure 8: SAXS spectra recorded for two spontaneous nano-emulsification drug delivery systems during lipid digestion over time. Adapted with permission from [87], see details in the text.

3.3. Raman spectroscopy

Raman spectroscopy is a fast and non-destructive fingerprinting analysis technique based on inelastic light scattering, which provides detailed information about chemical structure, phase and polymorphism, crystallinity and molecular interactions. In lipid colloid systems, Raman spectroscopy can be used to characterize the chemical nature of lipids, surfactants, co-solvents, and interactions between different components, and between colloidal systems and biomembranes [90,91]. Raman spectra of lipids are sensitive to the conformation, packing, and dynamic changes involving hydrocarbon chains. Gledovic *et al.* [91] prepared nano-emulsions by low-energy emulsification method with different red raspberry seed oils (ROs) and analyzed the Raman signals of the different components, comparing them to each other in pure form of formulated in NEs. Firstly, as shown in Fig. 9 A, Raman spectra give different bands and intensities for the four oils due to different unsaturation degree, with similar bands related to the fat positioning: $\sim 1747\text{ cm}^{-1}$ (C=O), $\sim 1659\text{ cm}^{-1}$ (C=C), $\sim 1440\text{ cm}^{-1}$ (CH_2 in fatty acid chains), $\sim 1305\text{ cm}^{-1}$ (methylene twisting), $\sim 1266\text{ cm}^{-1}$ (C=C-H) and at ~ 1079 (skeletal C-C

stretching vibrations). On the other hand, after emulsification (Fig. 9 B), all the spectra presented the specific bands of P80 (the surfactant) and glycerol, but a little bit shifted compared to the pure P80 spectra. By comparing the intensity ratio between the oils characteristics peaks (1305 cm^{-1} and 1266 cm^{-1}), the authors emphasized a specific structure in the NEs formulation with RO2, attributed to conformational ordering of the oil, surfactant and glycerol, leading to the most stable NE.

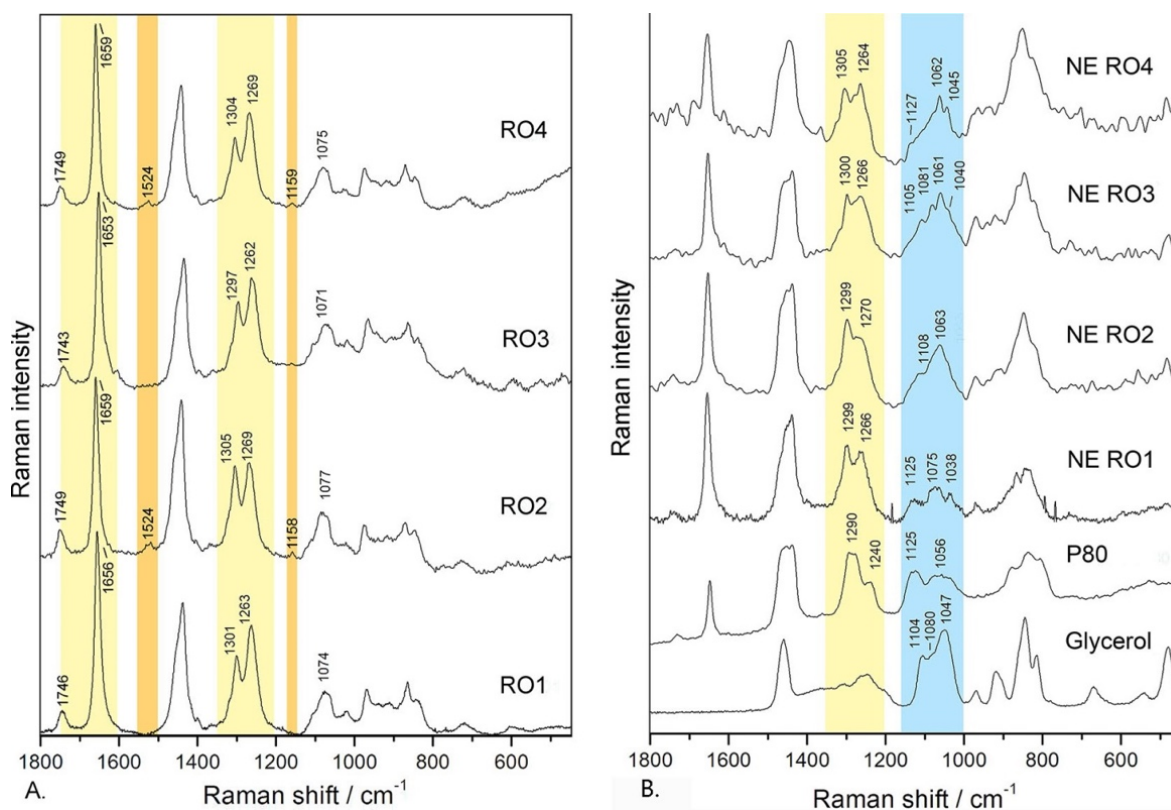


Figure 9: Raman spectra of NEs. A) Raman spectra of different red raspberry oils (ROs): RO1—cold pressed refined, non-organic oil; RO2—cold-pressed, unrefined, organic oil; RO3—CO₂ extracted unrefined, non-organic oil; RO4—CO₂ extracted, unrefined, organic oil. B) Raman spectra emulsified oils as nano-emulsions. Adapted with permission from [91], see details in the text.

In another example, Wang *et al.* [92] used Raman spectroscopy as a quantification method for α -tocopherol content in oil-in-water emulsion, and compared the obtained results with HPLC. Interestingly, Raman spectroscopy showed a comparable accuracy to HPLC. In addition this approach allowed to follow the homogeneity of the α -tocopherol distribution within the droplet, with the evolution of their spectral signature. These authors disclosed a gradual segregation of α -tocopherol in the interfacial region. Although Raman spectroscopy provides a convenient way for component analysis, in the case of complex mixtures, the analysis of the fingerprinting peaks becomes relatively complex. Moreover, low Raman scattering intensity resulting in low sensitivity of the method is the main limits for applying this experimental approach in routine or industrial scale.

3.4. Nuclear magnetic resonance (NMR)

Besides its common use for chemical characterization, in recent years, NMR was also used in NE characterization. This method reveals information on the arrangement of surfactants and

co-surfactant on the oil-water interface by the modification of their chemical shift. Xie *et al.* chose ^1H and ^{13}C NMR as the major approach to identify the functional groups on the droplet's interface, through following the variation of the peak intensity and comparing formulations with different interfacial composition [93]. Another NMR-based technology, so-called pulsed field gradient NMR (PFG-NMR), can be used to determine the particle size and size distribution for single and double emulsions [21]. This technique analyzes the reduction of the molecular diffusion, when they are confined in the discrete phase—*i.e.* emulsion droplets—in comparison to those located in the interfacial region. Analyzing when the diffusion becomes restricted provides an estimation of the droplet size. Beyond the information in size, PFG-NMR allows the fine characterization of assembled states and structures, level of sedimentation or creaming of emulsions [94]. Figure 10 illustrates the size determination of the droplet suspension by PFG-NMR, showing that this technique is less affected by light scattering and permits to track the diffusion properties of individual components in turbid and concentrated samples, by measuring their characteristic spectra (inherently chemically selective).

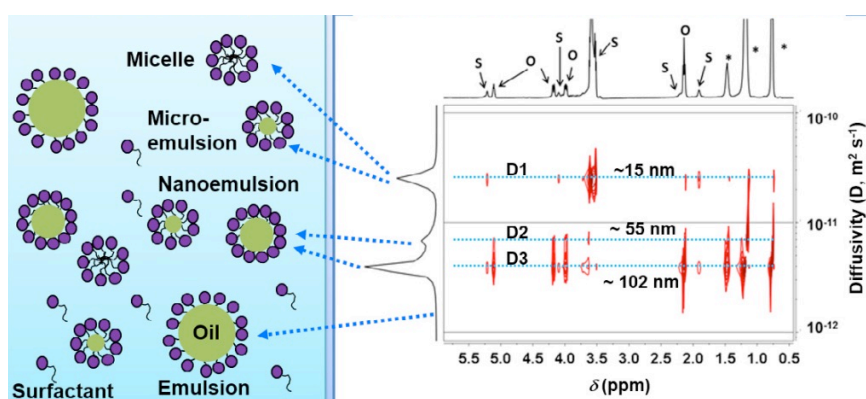


Figure 10: Diffusion ordered NMR spectroscopy (PFG-NMR) used for size characterization of NEs. The larger droplets induce a lower diffusivity of surfactants. Adapted with permission from [95].

Other complementary analytical techniques are important to be mentioned here, such as diffusing-wave spectroscopy for analyzing thick concentrated samples and multiple emulsions [96], Cryogenic electron energy-loss spectroscopy (EELS) and energy-dispersive X-ray spectroscopy (EDX) also showed unique advantages to study core-shell wet materials [17].

4. Fluorescence-based characterization

Fluorescence technology is widely used in biomedical research, and it became an essential tool for the characterization of drug delivery systems such as nanoparticles, copolymers, micelles or NEs. In recent years, several studies (described below) present the combination of lipophilic fluorescent molecules with NEs for original applications, either for NE bio-imaging or for the nano-droplet characterization. New advanced techniques were developed, based on specific photo-physical phenomena such as dye solvatochromism, aggregation-caused quenching (ACQ), aggregation-induced emission (AIE) and FRET. New experimental approaches including time-lapse, intra-vital microscopy, fluorescence lifetime microscopy (FLIM) [97] and FCS [5,37,98], have opened a new perspective for NE characterization.

4.1. Advanced fluorescence phenomena used in bioimaging

ACQ refers to fluorescence quenching of dyes via π - π stacking of the hydrophobic planar

molecules [99]. ACQ effect is common to most aromatic hydrocarbons and their derivatives since conventional luminophores are typically comprised of planar aromatic rings [100]. In most of the bioimaging applications, the nanocarrier should exhibit a high brightness, and thus ACQ may limit the maximal dye loading. This drawback can be circumvented by different strategies, by introducing chemical modification to prevent molecular aggregation (*e.g.*, by grafting alkyl chains on the dye), or by adding bulky side groups or bulky counterions that preserve dye self-aggregation, and thus, conserve fluorescence emission [38,101].

ACQ effect is also used to determine critical micellar concentration (CMC) or in tracking studies. Hydrophobic dyes with an absolute ACQ effect emit strong fluorescence when dispersed in an organic solvent, but aggregate and quench upon contact with water. These properties ensure a high contrast of the labeled nano-droplets with low background noise (possibly arising from the dye release), and thus enable to track single nano-droplets *in vivo* [102].

In 2001, a phenomenon named aggregation-induced emission (AIE) was first described by Tang's group. They observed that the fluorescence emission from propeller-like siloles was very weak in solution but became intense when the dye aggregated [103]. The AIE effect is opposed to the ACQ one, and can be used to quantitatively follow aggregation process. Illustration is reported in Fig. 11, comparing emissive and non-emissive aggregates.

AIE effect is related to the rheological change of fluorescence environment. Unlike solid nanoparticles which contain a hard core, the oily core of nano-emulsion does not seem to favor AIE triggered fluorescence emission. Consequently AIE based delivery systems described so far are nanocrystals [104], micelles [105] and nano-dots [106]. However, since NEs are widely used for various advanced formulations [24], we could expect a further investigation of AIE probes on the polymerized NEs, for example fluorescent acrylate emulsions [107] and hybrid NEs [108–110].

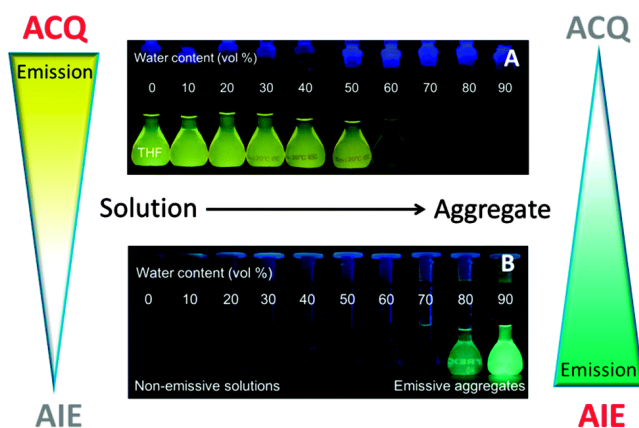


Figure 11: (A) ACQ of N,N-dicyclohexyl-1,7-dibromo-3,4,9,10-perylenetetracarboxylic diimide (DDPD) and (B) AIE of hexaphenylsilole (HPS), all in tetrahydrofuran/water mixture. Adapted with permission from [111].

Another phenomenon widely used for the characterization of NE is the FRET. It is an electrodynamic phenomenon occurring between a donor (D) fluorophore in the excited state and an acceptor (A) fluorophore in the ground state [112]. When the transfer of energy occurs, the intensity of the donor emission decreases, while the one of the acceptor increases. The prerequisites of FRET are (i) the overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, (ii) the distance between donor and acceptor molecules being within ~1–10 nm. (iii) relative orientation of the fluorophore dipoles (as shown in Fig. 12).

The efficacy of the energy transfer E indicates the percentage of the excitation photons that contribute to FRET and is defined as:

$$E = 1 - \frac{I_{DA}}{I_D}$$

where (I_{DA}) and (I_D) represent donor fluorescence intensity, in the presence and in the absence of the acceptor, respectively. E also depends on the donor-to-acceptor separation distance R with an inverse 6th order law due to the dipole-dipole coupling mechanism:

$$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}$$

with R_0 being the Förster distance between donor and acceptor at which the FRET efficiency is 50%. This dependency makes FRET extremely sensitive to small changes in the distance; thus this technique was recognized as the “molecular ruler” and has become a popular tool in biological and chemical investigations.

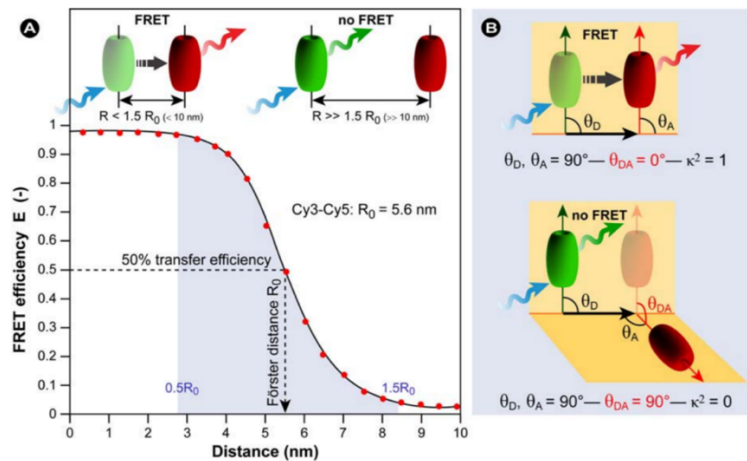


Figure 12: Principle of FRET. A) The FRET efficiency affected by donor-acceptor distance. B) The FRET efficiency affected by orientation factors. Adapted with permission from [113].

- CMC measurements

In relation to the emulsion formulation, these fluorescent techniques can serve to determine surfactant properties such as CMC, considered as a key parameter to optimize the emulsification process. CMC is typically measured physicochemical methods including surface tension measurement, DLS, or by conductivity measurements.

The fluorescent methods for CMC determination are based on the difference of fluorescence properties (intensity, emission wavelength) of the probes when (i) in solution and (ii) in micelles. Zhu *et al.* introduced a C6-unsubstituted tetrahydropyrimidine (THP-T1) probe with aggregation-induced emission enhancements and fluorescence-turn-on probe (THP-1), for the CMC determination for anionic, cationic, zwitterionic, and nonionic surfactants [109,110]. The fluorescence intensity of these probes increases with the surfactant concentration, until a concentration of micelle formation where the emission of the dye drops due to AIE. The CMC can thus be retrieved from this inflection point [114,115].

4.2.Modification and labeling of nano-emulsions

With the development of surface functionalized NEs, more and more polymer-based NE formulations are proposed. The objective of this modification is to anchor a macromolecule into the labile water/oil interface, with the possibility to graft a ligand on this anchor. The main advantage of using polymers to functionalize NEs lies to the surface anchorage which is much stronger with polymers compared to small molecules adsorbed at the droplet interface. In this context, Bou *et al.* [116] used gel electrophoresis to evaluate whether an amphiphilic polymer (labeled in red) is well attached to the oil nano-emulsion droplet (labeled in green). As illustrated in Fig. 13, without amphiphilic polymers, the neutral droplets do not migrate. However, once attached to the droplet, the polymers migrate taking the droplets with it. The migration profile shows a yellow band corresponding to NE effectively wrapped by the polymer and, for high concentrations, a red smear that reflects the polymer excess. Hence, this approach enables easy discrimination of the different nanomaterials that can be obtained during the formulation, along with their combination. Electrophoresis method is well suited for evaluating the droplet functionalization and stability of lipid nanomaterials [116].

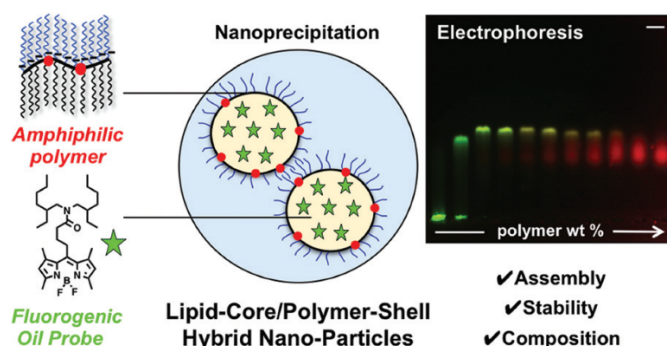


Figure 13: Dual-color labeling electrophoresis used for lipid-based nanoparticles. Adapted with permission from [116].

Our group has recently developed a new ACQ-based method for investigating the stability of NE encapsulation [117]. Fluorescent dye NR668 (lipophilic Nile red derivative) insoluble in water, was encapsulated in NEs at a concentration in which ACQ occurred. If the NE content leaks towards an acceptor medium the quantum yield increases, together with an emission shift due to the dye solvatochromism. By changing the acceptor with various proportions of non-labeled NE, it was possible to identify the key parameters responsible for the dye leakage [117]. This study disclosed that molecular release follows a similar mechanism of Ostwald or compositional ripening, in function of the partial solubility of the encapsulated dye in the continuous water phase. In addition, it emphasized that equilibrium governing the loading of acceptors, is not between droplets, but rather between the acceptor droplets and the dyes partially solubilized in the continuous phase [117].

FRET is also used as a tool to monitor the integrity of NE droplets. The sensitivity of this approach is directly related to the FRET efficiency of the dye pair used therefore an optimization experiments were performed in order to maximize the NE loading. Inside the hydrophobic core of droplets, the two dyes are in close proximity, therefore when exciting the donor, the energy transfer occurs and the acceptor emits a fluorescence signal. If the nanodroplet breaks or loses its integrity, the dyes are released and diluted, the FRET does no longer occur, and donor fluorescence is detected. This approach was used to study the integrity of NE loaded with DIL-TPB/DID-TPB (cyanine dyes with its perchlorate counterion) while passing a model of intestinal epithelium [61]. The FRET signal from the native droplets was

lost, when the NE was in contact with the Caco-2 cell layer, indicating that, while crossing the cell membrane, the droplets lose their integrity and release the loaded component [61]. The structure and photochemical properties of the dye pair used in this study are illustrated in Fig. 14. This approach has also been carried out efficiently *in vivo* in mice model bearing xenograft tumors, showing the specific droplet disintegration after reaching their target by enhanced permeation and retention effect (EPR) effect. One strategy to increase FRET efficiency in droplets is increasing the dye concentration by substituting their counterion. For example, hydrophobic tetraphenyl borate bearing lipophilic cyanine dyes as a donor/acceptor pair [61]. The bulky substitutions increase the solubility of the dyes in oily core, minimize ACQ and the dye leakage [118]

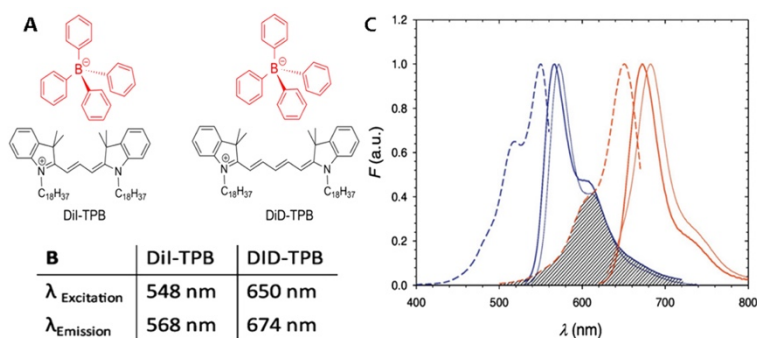


Figure 14: Counterion-based FRET pair used for investigation of nano-emulsions. A) Structure of the FRET pair. B) Excitation and emission wavelength of the FRET pair. C) Normalized spectra of the FRET pair, with dashed lines of absorption, full line of emission and thin-full line of emission in oil phase. Adapted with permission from [61].

A last and very powerful tool to obtain information on the nano-emulsion droplet integrity is FCS. Based on similar theory to dynamic light scattering, this method is more sensitive because it analyzes the fluorescence signal—instead of the light scattering for DLS—that is analyzed to calculate the autocorrelation function (as illustrated in Fig. 15). As a result FCS provides information on the number, size and brightness of fluorescent nano-droplets. Klymchenko *et al.* employed FCS for characterizing Nile red derivatives (NR668) loaded NEs [37]. In these works, FCS allows an estimation of loading efficiency through the calculation of fluorescent brightness per particle from the total brightness and the volume of each spherical droplet. This method also gives information on the dye leakage resulting in a decrease of brightness and increase of the number of emitting species [5,37].

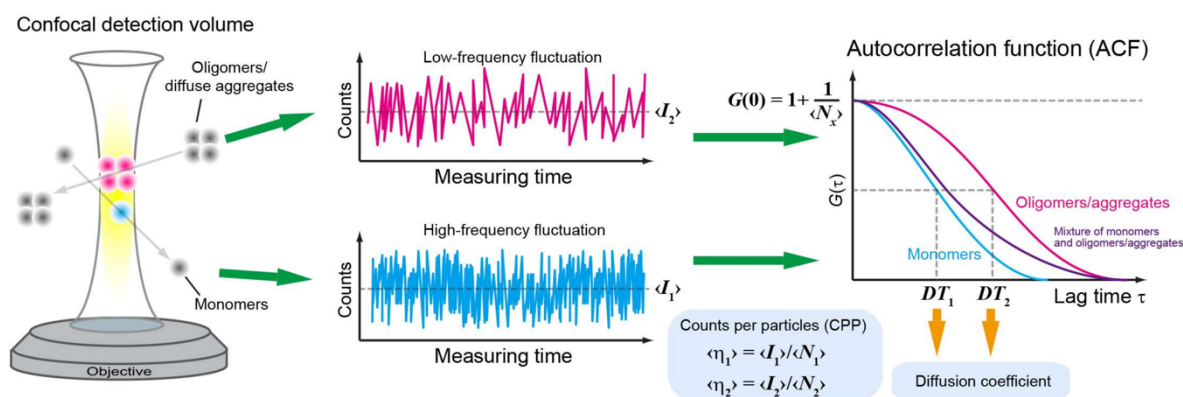


Figure 15: Principle of fluorescence correlation spectroscopy, adapted with permission from [119].

4.3. Fluorescence single particle tracking (fSPT)

Single particle tracking (SPT) is a fluorescence microscopy technique that allows following the displacements of individual molecules or particles in time and to record their trajectories. Obtained data are analyzed to retrieve the diffusion parameters and regime (free diffusion, confined movement, or active transport), traveled distances and directionality of the displacements. The quality of single particle tracks depends on the performance of the microscope, the sensitivity of the fluorescence detection and relies on the labeling efficacy of the imaged objects. In nano-emulsion-based fSPT studies, the fluorophores used are optimized to achieve a high brightness, thanks to: 1) high solubility in oily core to reach high loading and avoid leakage; 2) sufficient steric hindrance to avoid ACQ; 3) good photo-stability to permit tracking the droplets during several seconds. During the last years numerous bright fluorophores that fulfill these criteria were developed. Among them, Nile red derivative [37], BODIPY derivatives [116], rhodamine derivatives [120] are the most frequently used. An example reported in [121] shows a 40 nm NE droplet tracking in Hela cells, using a newly developed dioxaborine barbituryl styryl (DBS) dyes with high brightness and good photostability. These results revealed the fine interactions between droplets and cells, showing a significant difference of diffusion of neutral and charged droplets *in cellulo*. Individual NR668-loaded NE was also tracked *in vivo* in living zebrafish embryo, where single nano-droplets were detected in the blood flow at high acquisition rates [38].

4.4. *In vivo* fate

In vivo studies of nanoparticles and nano-emulsions are of prime importance since they pave the way towards their clinical applications. One of the biggest challenges is following and monitoring in real time the nanocarrier location and concentration. Following the encapsulated probe (*e.g.*, radioactive or fluorescent molecules) is not sufficient to conclude on the location of the nanocarrier since, the premature release, or disintegration of the carrier, is possible. To circumvent this limitation it was suggested to use specific probes like environment-sensitive probes (for which the fluorescence emission depends on the environment). These dyes change the fluorescence intensity or the wavelength when they pass from hydrophobic oily core of the NE to the aqueous environment; therefore they permit to clearly identify the *in vivo* distribution of intact NE. Nowadays, environment-sensitive fluorescent nanoparticles based on ACQ and AIE are becoming research hotspots. They were used to monitor the degradation or destruction of nanocarriers or the release of dye molecules, by measuring on-to-off (in the case of ACQ and FRET) or off-to-on (in the case of AIE) switching of the fluorescent signal.

Wu group has established a series of water-quenching methods based on a near-infrared aza-BODIPY (aza-boron-dipyrromethene BODIPY) dye for monitoring of lipid-based nanoparticles, polymeric micelles and NEs *in vivo* [122–124]. In nano-emulsions, when aza-BODIPY was encapsulated in the oily core, emulsion droplets presented strong fluorescence, while their release into the aqueous environment induced a fast fluorescence quenching. This method is sensitive and stable enough for *in vivo* kinetics and biodistribution study [124]. Illustrated in Fig. 16, the loss of signal in blood with time is clearly the result of the droplets disintegration, emitting when solubilized in the droplet core, and then quenched when leaked in the continuous phase after droplet degradation.

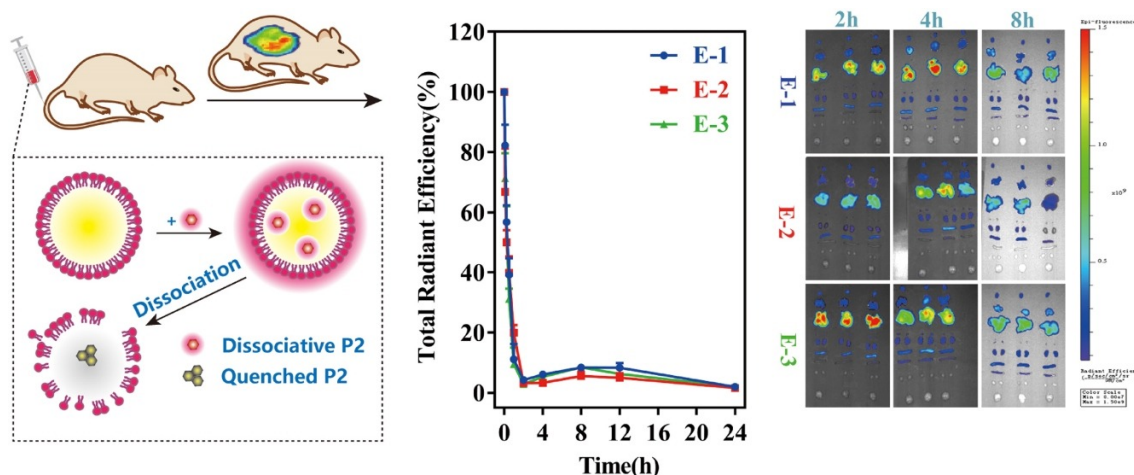


Figure 16: Schematic representation of the imaging based on ACQ (left, see details in the text); *In vivo* pharmacokinetic (middle) and biodistribution of isolated organs (right) for three different types of emulsions E-1, E-2 and E-3, adapted with permission from [124].

Similarly FRET imaging permits to monitor the integrity of NEs—composed of droplets containing the two dyes—and to measure the drug release kinetics. PEGylated NEs containing lipophilic donor acceptor dyes were synthesized and injected in the mice [125]. The FRET signal remained stable up to 24 h post-injection in PEGylated emulsion while non-PEGylated nanocarriers were rapidly cleared from blood. Bouchaala *et al.* [39] quantified the integrity of drug nanocarriers directly in blood circulation and tumor of living mice *via* near-infrared ratiometric FRET imaging. In this study, the fluorescence emission was monitored by an *in vivo* imaging system after systemic administration of NE labeled with lipophilic near-infrared cyanines FRET pairs. A strong FRET (acceptor) signal was observed in native droplets, in the tumor site located in mice’s flank. This indicates that intact lipid nano-emulsions extensively accumulated in the tumor site. After several hours, the donor signal gradually appeared, as a sign of the nano-emulsion disintegration in the tissues. These observations showed that the droplets have successfully targeted the tumor site, and have been destroyed there.

5. Expert opinion

Due to tunable properties, particle size, surface charge, rheological properties, along with high compatibility for biological applications nano-emulsions became one of the important soft materials used as carriers for drug delivery. However, the majority of the research studies focused on the formulation of NEs limit their characterization to the determination of size distribution and surface charge. The origin of this problem likely comes from the ease of use of the dynamic light scattering apparatus available on the market, compared to other experimental approaches that need a real investment in methods specifically dedicated to NE characterization. However, if DLS is a commonly recognized method, it also remains limited to acquire mono-modal and log-normal distribution of droplets suspension. It is mainly due to the fact that DLS is based on an indirect method, and can miss important features regarding the polydispersity of the droplet population, droplet morphology, or characterization of the surface chemical modifications. Herein we reviewed various classical and original methods used to describe NEs, and emphasized their added values compared to DLS. Alternative chemical analysis methods, *e.g.*, SAXS allows exploring the structure and interactions of the molecules in the droplets’ interface, Raman spectroscopy explores the inner composition of the droplets’ core and discloses fine variations, while NMR allows an accurate disclosure of the polydispersity, differentiating populations with different sizes (with, however, no information

on the droplet concentration of each population). This latter example illustrates the interesting bridge that can be done with fluorescence-based techniques. Fluorescence correlation spectroscopy provides data about droplet size and concentration in the sample (complementarity with NMR). At this point, the techniques mentioned remain indirect approaches, highlighting the strength of the direct observations given by electron microscopy/AFM, generally proposed as direct control or confirmation of the former experimental methods.

This first panel of methods of NE characterization aimed to propose a description of the droplet size, dispersity, morphology, properties and concentration. On the other hand, a second aspect of the NE characterization regards their behavior in a given environment. These techniques, based on the use of fluorescent dyes with original photophysical properties, allow monitoring the stability of encapsulated molecules, their potential leakage, and the impact of the administration route on the molecule delivery—*i.e.*, functional characterization. To this end, specific fluorescent probes are incorporated in the NE droplet, the dyes take part in the droplets' formulation. This approach provides information on the droplets stability and integrity, droplet location after interactions with a given environment –*in vitro* or *in vivo*, and also using dyes allows to finely characterize the modification of their surface such as the decoration with specific ligands, polymers, etc.

Under this sight, it is clear that these different characterization methods, developed during the last years, provide a large range of information about the NE properties and behavior, compared to classical DLS measurements. However, such techniques imply using a specific technical environment, specific to the experimental approach chosen. Interesting outlooks regarding NE characterization would be combining these novel approaches in order to improve the understanding of the droplets properties, emulsification processes and impacts on potential applications.

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