Manipulating and studying triglyceride droplets in microfluidic devices

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Abstract

Triglyceride is the main lipid class in nature, found as droplets in both living systems and man-made products (such as manufactured foods and drugs). Characterizing triglyceride droplets in situ in these systems is complex due to many environmental interactions. To answer basic research questions about droplet formation, structuration, stability, or degradation, microfluidic strategies were developed, allowing well-controlled droplets to be formed, manipulated, and studied. In this review, these strategies are described, starting with the presentation of droplet production devices, with applications essentially related to microencapsulation and delivery, then detailing methods to monitor droplet degradation in situ and in real time, finishing with microfluidic platforms allowing the investigation of many aspects of biological lipid droplets simultaneously.

1. Introduction

Triglyceride (also called triacylglycerol, consisting in three fatty acids esterified to glycerol) is the main lipid class found in animals, plants, and fungi. Triglycerides are present as lipid droplets in most types of cell, although at a high concentration only in some specialized cells where they are stored as an energy reservoir. Within an organism, or from one organism to the other, triglycerides undergo many hydrolysis/re-esterification processes catalyzed by various lipases, yielding free fatty acids (with or

without one monoglyceride molecule depending on lipase specificity). Most of these processes takes place for transport purposes, an especially important one being the hydrolysis of triglyceride droplets as the first step of energy production.

As a full example, upon fat ingestion in the human, hydrolysis of each triglyceride molecule within the gastrointestinal tract yields two fatty acid and one monoglyceride molecules. These lipids are then transported inside the enterocytes where they are re-esterified to triglycerides directly in chylomicrons (a lipoprotein), or transiently as lipid droplets stored for later chylomicron production. Chylomicrons are secreted into the lymph where they circulate to be distributed in the body, for subsequent energy production in most tissues after hydrolysis, and for storage as lipid droplets in adipose tissues after a hydrolysis/re-esterification process [1].

Hence, triglyceride droplets are ubiquitous in living systems, undergoing many hydrolysis/re-esterification processes for physiological purposes. However, their formation, structuration, stability, interactions, and degradation are still the subject of unresolved questions [2, 3, 4]. Moreover, studying triglyceride droplets is challenging due to various interactions occurring between them and with other molecules such as proteins or phospholipids, required to stabilize droplet interface. Hence, there is no unique structure and composition for triglyceride droplets, as this depends on their environment and their use (both for living and man-made systems). Simplified systems are thus often studied, such as diluted emulsions (where interactions still take place) or individual droplets.

With the advent of droplet-based microfluidics about 20 years ago, individual droplets can now be produced, manipulated, and studied dynamically and kinetically [5]. However, most of the investigations to date focused on aqueous droplets, the manipulation of oil droplets being more demanding due to viscosity and wetting effects, alkanes being often used as the oil phase to minimize these effects (note that oil is used here as a generic word referring to a phase that is not miscible with water, that can be alkane, mineral oil, triglyceride, edible oil…). The use of lipids is more recent, coming from the need to understand and control the behavior of biological droplets in various environments,

typically triglyceride droplets in drug or food, in the gastrointestinal tract, and in various cells after lipid absorption.

This review shows what microfluidics brings to the physicochemical study of triglyceride droplets, starting with various strategies to create droplets with well-controlled size and structure, that can be used for bioactive microencapsulation and release, then describing lab-on-chip analyses dedicated to the kinetics of triglyceride droplet hydrolysis, finishing with promising microfluidic platforms allowing in situ investigations of triglycerides in the cell.

2. Triglyceride emulsification

Common methods for triglyceride emulsification are batch methods such as high-pressure homogenization, ultrasonication, membrane and jetting methods. Despite a high yield of production, these methods do not allow a precise control of the size, polydispersity, shape, and structuration of the droplets. Moreover, a high energy input in these methods may lead to thermal alterations of heat-sensitive compounds.

On the contrary, direct droplet formation in microfluidic devices yields monodisperse droplets of controlled structure with a low energy input. They divide into two categories, based on the droplet formation mechanism.

The first one is spontaneous or interfacial tension-driven, the resulting droplet size being only influenced by the flow rate of the dispersed phase and by the geometry of the device [6]. In this method, droplets form spontaneously by interfacial tension forces, generating many droplets simultaneously (figure 1A), each being 10 µm in diameter when produced in EDGE microchips (using sunflower oil and a milk protein solution).

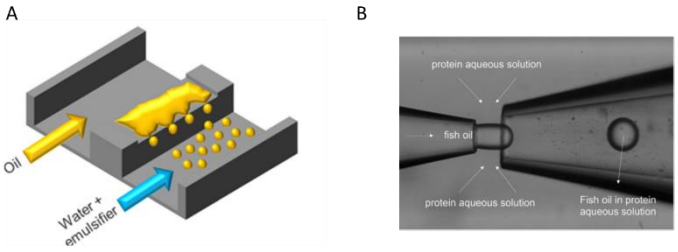


Figure 1: Droplet microfluidic devices. (A) Spontaneous formation of droplets by an interfacial tension-driven mechanism in EDGE microchips. Reprinted from [6], under the terms of the Creative Commons CC BY license. (B) Single droplet formation by a shear-driven mechanism inside a co-flowing glass microfluidic device. Reprinted from [11], with permission from Elsevier.

The second one is shear-driven (microfluidic T-, Y- or cross-junction devices; co-flowing devices), the resulting droplet size being influenced by the flow rate of both phases and by the geometry of the device (figure 1B). Here, droplets break continuously at the location of the maximum shear stress, where the continuous phase pinches the dispersed phase. Contrary to the first method, this one offers a better control of the size, shape, structure, and composition of the droplet, typically ranging between 50 and 500 µm, with possibilities to reach 10 and 1000 µm.

As an example of a cross-junction device, Kim and Vanapalli could control the shape of 500 µm palm oil particles by a temperature gradient making them liquid, semi-solid or solid [7]. The interplay between the cooling temperature and the flow rate of the continuous phase was shown to control the transition from spherical to elongated droplets. A relationship between the geometric confinement and elasticity of the particle was established to explain these results. With a similar approach but using a co-flowing microfluidic device, Capretto et al. were also able to generate solid lipid microparticles of various sizes and shapes using tripalmitin [8].

When using liquid triglyceride, coalescence has to be controlled in the microfluidic device in order to control the droplet size. This was the subject of a recent study about Pickering droplets, i.e. stabilized by solid particles [9]. Monodisperse sunflower oil droplets were generated using a microfluidic device (T-junction). Colloidal lipid particles (CLPs) made of tripalmitin particles stabilized by sodium caseinate were used as the Pickering particles. A collision channel was used to enable and monitor coalescence events between the droplets. Those were quantified by comparing images of droplets taken at the inlet and outlet of the collision chamber (figure 2A). These images were captured using a high-speed camera connected to a microscope. Coalescence was found to depend on the droplet surface coverage

by CLPs. A low coverage of CLPs induced coalescence due to droplet-droplet bridging, whereas a high coverage of CLPs totally suppressed the coalescence.

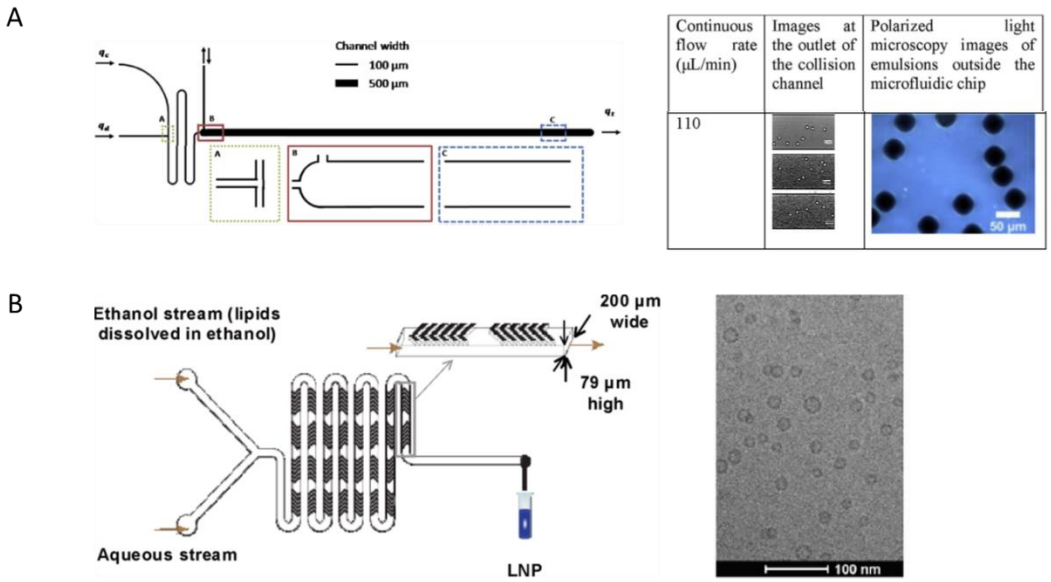


Figure 2: Microfluidic control of the droplet size. (A) Control of the Pickering droplet size by coalescence in a microfluidic device. The device was composed of three regions. A: T-junction, B: inlet collision channel, C: outlet collision channel. Reprinted from [9], with permission from Elsevier. (B) Lipid nanoparticles generation with a Staggered Herringbone microfluidic Mixing device. Production of monodisperse lipid particles in the range of 20 to 70 nm. Reprinted with permission from [10], American Chemical Society.

Biomimetic lipid droplets can also be created using microfluidics. For example, a Staggered Herringbone microfluidic Mixing device (SHM) was designed to generate various lipid nanoparticles, with either oil core (droplet made of triolein and a phospholipid), or with aqueous core (vesicle made of phospholipid alone) [10]. An ethanol phase containing the lipids was rapidly mixed with an aqueous stream in a chaotic mixer driven by asymmetric grooves on the floor of the channel. The high flow rate ratio applied increased quickly the polarity of the mixing solution and spontaneous assembly occurred, leading to the synthesis of limit size lipid nanoparticles (figure 2B). Contrary to the other microfluidic methods detailed above, this enables the generation of very small monodisperse lipid particles in the range of 20 to 70 nm. This size presents a great interest for the study of biological lipid droplets, and for the design of long-circulating drug delivery system.

3. Droplet microencapsulation

Apart from the fundamental understanding of droplet generation, triglyceride droplet microfluidics finds an important application in the design of microencapsulation systems for lipophilic compounds delivery. First, compared to common microencapsulation techniques (complex coacervation, spray drying and spray chilling), the efficiency of encapsulation using droplet-based microfluidics is closed to 100 %. Also, many lipids and lipophilic compounds are unstable in contact with light, oxygen, and heat. By microencapsulation in structured particles, various bioactive compounds can be protected in detrimental environments, and released in favorable environments.

For example, a recent work demonstrated a better protection against oxidation when fish oil was encapsulated as droplets covered by various proteins (gelatin or casein or whey protein, or soy protein) with characteristics controlled by microfluidics. Stable droplets could be obtained using all proteins except soy protein, and the best formulation against oxidation was a combination of gelatin and casein [11]. To ensure an even better protection, oil droplets are often covered by a wall material composed of a gel-forming polymer, with layer characteristics perfectly controlled by microfluidics (figure 3A). Alginate, a natural polymer, is a good candidate to form such protective layer. This polysaccharide was used to protect echium oil droplets against oxidation using the same microfluidic approach as above. This protection highly reduced the generation of MDA (malondialdehyde, an oxidation product) compared to free echium oil [12].

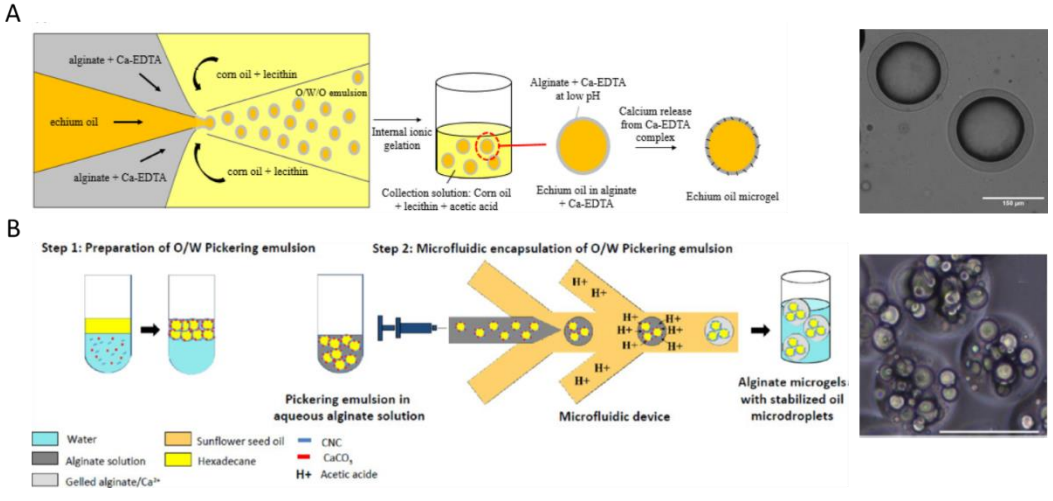
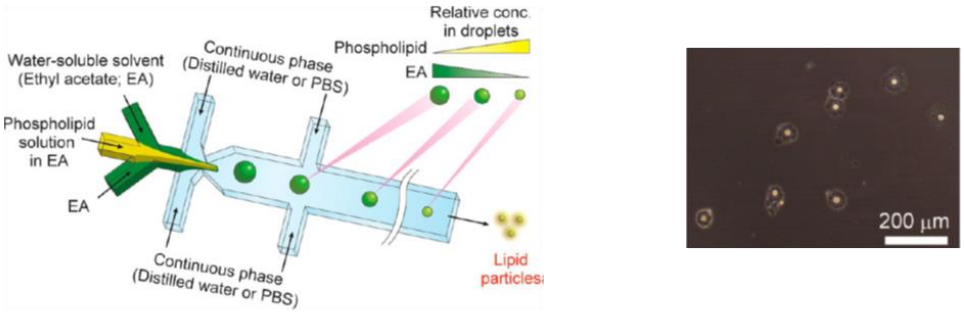


Figure 3: Microfluidic protection of droplets by a gelled shell. (A) Encapsulation process by combination of microfluidics and ionic gelation for the formation of capsules with an alginate shell and an echium oil core. Reprinted from [12], with permission from Elsevier. (B) A two-step process to generate alginate microgels containing several Pickering oil droplets (scale bar of 100 µm). Reprinted with permission from [13], American Chemical Society.

To go further, two protective barriers can be associated to encapsulate oil into alginate microgels, following a two-step approach [13]. A highly stable oil-in-water Pickering emulsion was first prepared using nanoparticles of cotton cellulose nanocrystals (CNC), forming a dense solid shell. Calcium carbonate ($CaCO_3$) particles were added during the Pickering emulsion step, adsorbing onto the cellulose layer, forming CNC/$CaCO_3$ hybrid particles. This hybrid system enabled a fine control of alginate gelation during the subsequent microfluidic microgel generation step (figure 3B). Nile red as a lipophilic compound remained encapulated within the oil droplets for days, even when the droplets were released from the microgels by degradation of the alginate gel.

Another way to create encapsulation systems in microfluidic devices is to use the self-assembly properties of lipid molecules, similarly to what was done by Zhigaltsev et al. [10], described in the previous section. A microfluidic strategy was also built to produce monodisperse multilamellar oil droplets in a three-step process [14]. After the formation of monodisperse droplets containing triglycerides and a phospholipid in a water-soluble organic solvent, a shrinkage phenomenon occurred by solvent diffusion of the organic solvent into the continuous aqueous phase, leading to the concentration of lipid molecules in a multilamellar core-shell structure (figure 4A). By modulating the ionic strength in the continuous phase, the size of the shell could be controlled. Various lipophilic compounds could be loaded and were stable in the controlled lipid structures.

A



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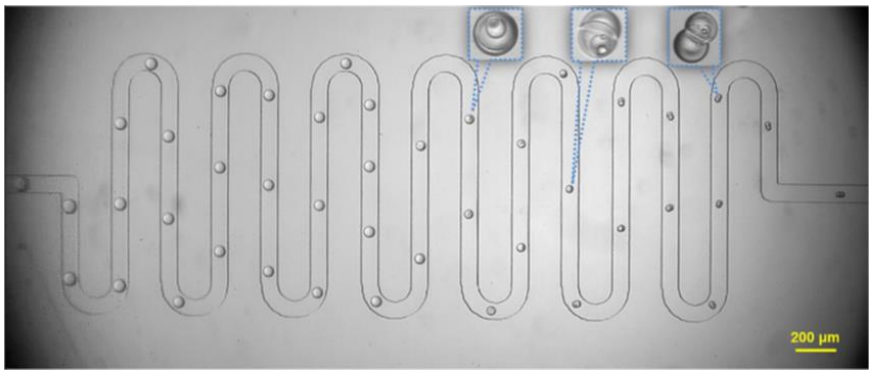

Figure 4: Microfluidic self-assembly of lipid molecules. (A) Process of lipid microparticles formation after shrinkage of droplets by solvent diffusion of the organic solvent into the continuous aqueous phase. A multilamellar core-shell structure is obtained. Reprinted with permission from [14], American Chemical Society. (B) Solvent diffusion applied to triglyceride/polymer droplets generation in winding channels to produce monodisperse Janus microparticles. Reprinted from [15], with permission from Elsevier.

Recently, this solvent diffusion approach was modified to produce monodisperse Janus microparticles (i.e. two-part particles, see figure 4B) made of triglycerides and a synthetic polymer [15]. Other morphologies could be obtained depending on the concentration of the surfactants used. A model lipophilic compound (paclitaxel) was loaded into Janus microparticles, and its release was found to exhibit two kinetics, a fast one from the triglyceride droplet part, and a slow one from the polymer droplet part.

4. Kinetics of droplet hydrolysis

As stated in the introduction, triglyceride hydrolysis is an important biological process occurring in many organs. In the context of gastrointestinal digestion, droplet microfluidic devices were used as miniaturized in vitro digestion systems with on-chip hydrolysis analysis.

In the first work on this topic, triglyceride droplets of 135-145 µm were generated in one microfluidic chip, individually immobilized in traps in another microfluidic chip, and subjected to gastrointestinal conditions in the latter chip [16]. For the gastric phase, the digestive fluid was composed of a fungal lipase (mimicking mammalian gastric lipase) at acid pH. For the intestinal phase, the digestive fluid was composed of a pancreatic lipase and a bile salt at neutral pH. The temperature inside the digestion chamber was maintained to 37 °C. During intestinal digestion, hydrolytic products solubilized in the bile salt micelles present in the continuous aqueous phase, resulting in a shrinking of the droplet. Triglyceride droplet kinetics was monitored in situ using optical microscopy. Image analysis was used to measure the droplet size as a function of digestion time, allowing a quantitative kinetic analysis. This approach provided equivalent results compared to the conventional emulsion-based method. Advantages of using droplet microfluidics included reducing the material volumes, avoiding the issue of lipid-bile stoichiometry (as digestive fluid was continuously renewed during the digestion), and enabling a real-time kinetic quantification. Droplets with a similar initial diameter but made of different edible oils were studied. The type of triglyceride (chain length and degree of unsaturation) was found to be the main parameter controlling the hydrolysis kinetics.

This initial work was recently expanded by adding a lipophilic micronutrient (beta-carotene or retinyl palmitate) in the triglyceride droplet [17, 18]. Droplets of 100 µm containing one lipophilic micronutrient were generated, individually immobilized in traps, and subjected to gastrointestinal conditions, all steps within a single microfluidic chip (figure 5). During lipid digestion, lipophilic micronutrients were co-digested with lipids and co-incorporated with hydrolytic products in bile salt micelles, resulting in a shrinking of the droplet as well as an increased micronutrient concentration inside the droplet. In this situation, the kinetics of triglyceride digestion and of micronutrient release were simultaneously monitored in situ using confocal fluorescence microscopy. The image analysis of

the autofluorescence of the micronutrient contained in the triglyceride droplet and of the transmitted light of the droplet enabled the kinetic measurements of the micronutrient release and of the hydrolytic products release during intestinal digestion. This work revealed a non-linear relation between these release kinetics. Nevertheless, the kinetics of micronutrient release was found to be controlled by the kinetics of triglyceride digestion, thus depending mainly on the triglyceride type again.

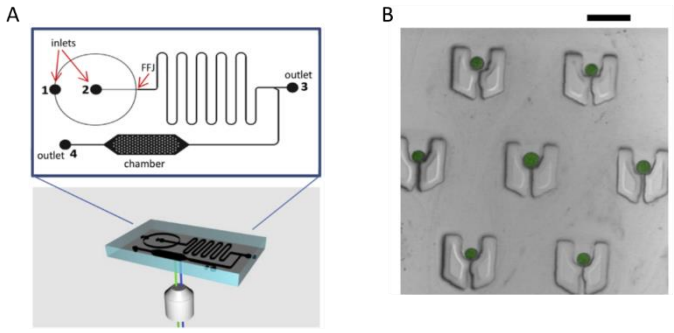

Figure 5: Microfluidic triglyceride droplet hydrolysis. (A) Single microfluidic chip allowing triglyceride droplet generation in the flow-focusing part, droplet trapping and hydrolysis in the chamber part. (B) Monitoring of trapped droplets during hydrolysis by fluorescence microscopy, green level representing the autofluorescence intensity of beta-carotene inside the droplets (scale bar of 200 µm). Reprinted from [17], with permission from Elsevier.

One other approach was used to study lipid digestion within a microfluidic device, focusing on the effect of coalescence of triglyceride droplets in gastrointestinal conditions [19]. Triglyceride droplets of 35-40 µm were injected into a digestion chamber containing traps larger than the droplet size, allowing single as well as multiple droplets trapping. For quantitative analysis, triglyceride droplets were stained with Nile red to image their fluorescence. Compared to a single droplet, coalescence of multiple droplets slowed gastric hydrolysis (catalyzed by a recombinant dog gastric lipase), and also slowed the subsequent intestinal hydrolysis (catalyzed by pancreatin in the absence of bile salt), although there was no coalescence events during most of the intestinal phase. Near the end of the intestinal digestion, massive coalescence occurred and then hydrolysis rate dramatically increased.

This final increase was not explained but was also observed for a single droplet. The last size measured being around 15 µm, this increase could simply be an artefact due to the resolution of the imaging method or to droplets escaping the trap.

5. Cellular triglyceride droplets

Lipid droplets are produced in various cells, and many studies were published concerning their genesis and interaction in the cell. In this context, microfluidic devices were only developed in two studies. This lack of microfluidic studies originates from the fact that biological microfluidics is still a recent field, requiring specific high-tech labs and multidisciplinary research teams.

The first investigation focused on the differentiation of human mesenchymal stem cells into adipocytes, as stimulated by insulin [20]. A microfluidic platform was built in which stem cells could be cultured, differentiated, and the number of lipid droplets measured in situ under an inverted microscope (figure 6A). The stem cells could also be stimulated mechanically using a membrane to apply shear stresses. The results revealed an optimal insulin concentration where a maximal number of lipid droplets are produced, and a decrease of the number of lipid droplets with an increasing shear stress.

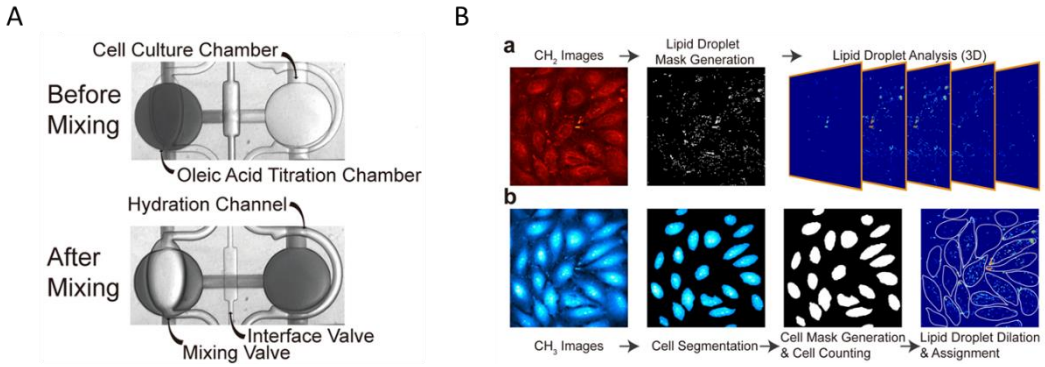

Figure 6: Cellular lipid droplets in a microfluidic device. (A) Microfluidic cell culture chamber and oleic acid chamber to study the formation of lipid droplets in cells. (B) On-chip cell (b) and lipid droplet (a) imaging, and scheme of image analysis (right). Reprinted with permission from [21], American Chemical Society.

The second investigation focused on lipid droplets formed in different cell types under oleic acid stimulation, cultured in an integrated microfluidic platform (figure 6B), and monitored in situ by stimulated Raman scattering microscopy [21]. This technique enabled a label-free quantification of the droplet size, lipid concentration in the droplet, and number of lipid droplets, in different physiological conditions and various cell lines, with simultaneous characterization of their morphology. These analyses could also be performed at the single cell level, enabling the cell-to-cell variability to be assessed. The lipid droplet growth dynamics was found to be mainly controlled by the number of lipid droplets.

6. Conclusion

To sum up, droplet microfluidics brings several advantages over conventional bulk methods to control and study triglyceride droplets. First, it allows the generation of monodisperse droplets that can be manipulated individually. Moreover, complex lipid objects can be obtained using low amounts of material, allowing novel and inexpensive microencapsulation strategies. Biological droplets can also be produced, and their ability to deliver bioactive compounds can be studied directly in the microfluidic chip in real time using appropriate microscopy techniques (optical, fluorescence, Raman). Finally, microfluidic platforms can be designed to study lipid droplet dynamics in the cell, integrating all steps such as cell culture, differentiation, stimulation, and droplet characterization. This approach holds great promises, and could be integrated to organs on chip platforms to investigate organ molecular functions and interactions. These methods could also be used to gain more understanding of the various degradation processes of triglyceride droplets and of their interaction with other (cellular) components.

Author contributions

All authors reviewed the literature. HTN wrote section 4. MM wrote sections 2 and 3, and designed the figures. SM wrote the other sections and edited the manuscript.

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