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**Application of Flow Cytometry as Novel Technology in Studying Lipid Oxidation in Oil-in-Water Emulsions**

Peilong Li

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APPLICATION OF FLOW CYTOMETRY AS NOVEL TECHNOLOGY IN STUDYING LIPID OXIDATION IN OIL-IN-WATER EMULSIONS

A Thesis Presented

by

PEILOGIN LI

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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Department of Food Science
APPLICATION OF FLOW CYTOMETRY AS NOVEL TECHNOLOGY IN STUDYING LIPID OXIDATION IN OIL-IN-WATER EMULSIONS

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ABSTRACT

APPLICATION OF FLOW CYTOMETRY AS NOVEL TECHNOLOGY IN STUDYING LIPID OXIDATION IN OIL-IN-WATER EMULSIONS

SEMPTEMBER 2019

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Directed by: Professor Eric A. Decker

The body of literature on the impact of emulsion particle size on oxidation rates is unclear. This could be because emulsions are typically polydisperse and the oxidation rate of individual droplets is impossible to discern. Flow cytometry is a technique for studying individual cells and their subpopulations using fluorescence technologies. It is possible that individual emulsion droplets could also be characterized by flow cytometry as a novel approach for studying lipid oxidation.

Typical emulsion droplets are too small to be visualized by flow cytometer, so emulsions were prepared to have droplets > 2 μm; weighting agent and xanthan gum were added to minimize creaming during storage. A radical-sensitive lipid-soluble fluorescence probe (BODIPYr665/676) was added to the lipid used to prepare the emulsion so that the susceptibility of individual emulsion droplets could be determined. The results showed that in a polydisperse emulsion system, small droplets were oxidized faster than large droplets. Using mixtures of emulsions with and without prooxidants, it was possible to see the transfer of prooxidants between droplets, a process that is influenced by surfactant and salt concentrations. For
example, surfactants micelles can transfer prooxidants to neighboring non-oxidized droplets and cause fluorescence loss when surfactant concentration was higher than critical micelle concentration (CMC). Transfer of prooxidants was promoted by adding NaCl and free fatty acid which could be attributed to the lower CMC. This study showed the potential for applying flow cytometry on oxidation of individual emulsion droplets.
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CHAPTER 1

INTRODUCTION

Lipid oxidation of unsaturated lipids not only can impair food quality but also may cause potential adverse effect on human health due to lipid oxidation endproducts (Frankel, 1980; Kanner, 2007; Vieira et al., 2017). In addition to the bulk form of oils, lipids in foods are often present as heterogeneous emulsion systems (McClements & Decker, 2000). Dispersion of oil in an aqueous system can dramatically enlarge surface area due to size reduction, which poses a challenge to food shelf life because lipid droplets in emulsion can be oxidized more rapidly than bulk oils (Yuji et al., 2007). The inverse relationship between droplet diameter (d) and droplet specific surface area (A_s, area per unit mass) can be expressed as A_s = 6/ρd (Decker et al., 2017), showing that reduction of particle dimension significantly enlarges surface area. Reduction of droplet size will increase interface area which allows amphiphilic molecules such as hydroperoxides to concentrate at the emulsion droplet surface resulting in interactions with transition metals in continuous phase that decompose hydroperoxides to free radicals therefore accelerating oil oxidation. Thus, it has been hypothesized that the oxidation rates in oil-in-water emulsions could be influenced by lipid droplet surface area.

Numerous researchers have investigated the effect of particle size on lipid oxidation in emulsions. Several studies reported that oxidation in smaller droplets were faster than in larger droplets. Gohtani et al. (1999) found that the peroxide value of DHA emulsion made of smaller droplets was higher than the group with larger
droplets after storage. Lethuaut et al. (2002) reported that a coarse sunflower oil-in-water emulsion showed a slower oxygen consumption than an emulsion with smaller droplets. Similar observations were also reported in corn oil emulsions homogenized with a microfluidizer (Lee et al., 2010), and flaxseed oil emulsion prepared with a high-pressure homogenizer (Kuhn & Cunha, 2012). Ma et al. (2013) also reported that the oxidation of a α-linolenate emulsion was promoted by reduction of droplet size.

Additionally, fish oil emulsion with smaller droplets were also found to exhibit accelerated oxidation due to stronger light penetration in the presence of riboflavin (Uluata et al., 2016).

However, several research publications observed opposite results. Nakaya et al. (2005) found that hexanal generation was lower in small emulsion droplets and the authors suggested the possible reason was the wedge effect, which reduced mobility of triacylglycerols in the interior of the small emulsion droplets. Similar results were also reported by others (Imai et al., 2008; Kikuchi et al., 2014; Miyagawa et al., 2017; Nguyen et al., 2013). A possible reason is that there could be less prooxidants per droplet due to a dilution effect (Decker et al., 2017). Additionally, some papers also reported that oxidation was not affected by droplet size (Dimakou et al., 2007; Kiokias et al., 2007; Osborn & Akoh, 2004).

These disparity of results indicates that the conclusion on how emulsion droplet size impact lipid oxidation is still unclear and further investigation is required. Decker et al. (2017) pointed out that the homogenization techniques applied in these papers do not produce uniform droplets but instead a wide range of particle sizes with
authors reporting the mean diameter to represent the emulsion droplet size.

Unfortunately, mean droplet size is not an accurate representation of the heterogeneity of lipid droplet size in a polydisperse system emulsion. In order to have a holistic understanding on the effect of droplet size, new techniques are needed in polydisperse emulsion, which can separate or classify oxidation in different sized individual droplets (Villeneuve et al., 2018).

If oxidation is affected by droplet size and initiates in subpopulation, can oxidation products move to the neighbors? In fact, components of lipid droplets can exchange rapidly. Conceptually, there are three possible mechanisms of mass transfer in emulsion systems: diffusion, collision exchange, and micelle-assisted transportation (Laguerre et al., 2017).

Diffusion refers to components in lipid phase that can partition out of a droplet and migrate to another one through the emulsion continuous phase. This phenomenon usually occurs in emulsion made from oil with relatively high polarity such as flavor oil (McClements et al., 2012), and hydrocarbons (Weiss et al., 1999). This phenomenon leads to Ostwald ripening, a process that molecules of small droplets can thermodynamically diffuse out and move to large droplets, which eventually causes phase separation. Collision-exchange transfer refers to the process when two droplets merge together and then separate (Rharbi & Winnik, 2002). During this process, materials can be transferred from one droplet to the other.

The third mechanism is micelle-assisted transfer between emulsion droplets. Surfactants in emulsion can adsorb on the droplet surface, and when the surface area
is saturated, the unadsorbed surfactants partition in the aqueous (or lipid phases). As the concentrations of unabsorbed surfactants increases, they will form micelles (or reverse micelles). The concentration of micellization is called critical micelle concentration (CMC). Surfactant concentrations used in foods and many experimental conditions are often higher than CMC, and thus surfactant micelles can be found in many oil-in-water emulsions (Berton - Carabin et al., 2014). The hydrophobic core of micelles can solubilize nonpolar components out of lipid droplets which contributes to mass transport between droplets and thus may affect lipid oxidation (Decker et al., 2017). According to the work conducted by Nuchi et al. (2002), the partitioning of lipid hydroperoxides into the water phase of an oil-in-water emulsions was enhanced by increasing surfactant concentrations meaning that hydroperoxides can be solubilized by micelles. Raudsepp et al. (2014a) reported that AMVN-derived radicals were moved between droplets by surfactant micelles. Hydroperoxides and peroxyl radicals are examples of prooxidants that could be transferred between emulsions droplet resulting in alterations in lipid oxidation kinetics.

Another hypothesis for prooxidant transfer is that the surface-active lipid oxidation products, e.g. hydroperoxides (LOOHs) can assemble micelles by themselves even when surfactant concentration was lower than the CMC. Brimberg (1993) observed that LOOHs formed reverse micelles in bulk oil and promoted oxidation but hydroperoxide micelles have not be reported in oil-in-water emulsions. Laguerre et al. (2017) suggested that at the beginning of oxidation, without surfactant micelles, LOOHs might not be able to partition into the continuous phase by
themselves, but later in oxidation when the emulsion droplet surface is more saturated with LOOHs, hydroperoxides could bud off from the droplets surface to form micelles. However, this hypothesis has not been confirmed and thus future studies are required.

Although the ability of surfactant micelles to transfer components between emulsion droplets has been reported, we still do not know if this process can promote oxidation from one droplet to another. A challenge of studying this type of transfer phenomenon is that it is difficult to analyze lipid oxidation components in individual droplets. Conventional methods such as peroxides determination cannot reveal oxidation of separated droplets. Confocal microscopy combining with fluorescence probes would be capable of visualizing individual droplets but cannot quantify the counts of targets efficiently. Another approach is required in studying transfer rate on the scope of individual droplets (Villeneuve et al., 2018).

Flow cytometry is a technique that has been utilized in many fields such as microbial strain classification, apoptosis progression, immunophenotyping, and chromosomes studies (Robinson, 2004). Using flow cytometry, cells can be categorized into groups based on properties such as particle size, fluorescence, granularity, and inclusion complexity. The advantage of this technology is mainly derived from its high resolution and high-throughput analysis of individual cells or other units. Once an analysis template is established, routine analysis is convenient and reproducible. Samples analyzed by flow cytometry are injected into a pressurized air line with a sheath fluid. The coaxial flow enables cells to align in
single stream by hydrodynamic focusing, with the cells then passing the optical unit equipped with argon laser. When an individual particle is illuminated by the laser beam, light scattering is generated and collected by detectors. The scatter signals can be affected by particle size and fluorescence. Different fluorescence probes can be detected with different band pass wavelength filters. Imaging flow cytometry equipped with camera is an upgrade of conventional flow cytometry, which is able to capture the images of particles in the flow. Particle size and fluorescence can be analyzed by pixels on image data.

As an emulsion droplet share similar structure as a cell and thus can scatter light, this study aims to apply flow cytometry as a novel technology in studying lipid oxidation in oil-in-water emulsions. The first objective is to investigate in a polydisperse system whether oxidation is faster in small droplets or in large droplets. The second objective is to study if oxidation products in a subgroup of lipid droplets can be transferred or not.
CHAPTER 2
LITERTURE REVIEW

2.1. Lipid oxidation

2.1.1. Mechanism

Although the consumption of unsaturated lipids, such as fish oil, is widely encouraged for the health benefits, the oxidative instability of these oils has been a challenge which limits them to be incorporated in food products (Holman & Elmer, 1947). Lipid oxidation not only decreases nutritional value, but also produces unpleasant off-flavor and oxidation endproducts having potential adverse effect on human health (Frankel, 1980; Kanner, 2007). Therefore, inhibition of lipid oxidation has received considerable attention from both the food industry and academia.

The mechanism of lipid oxidation can be categorized into three stages: initiation, propagation, and termination (Gray, 1978). Due to the presence of double bonds, hydrogen is prone to be disassociated resulting in the exposure of unpaired electron forming alkyl radicals. Oxygen in ground state, as biradical, can react with alkyl radicals to produce peroxyl radicals (Eq. i), which can then rob a hydrogen from another non-oxidized lipid molecule producing a hydroperoxide and new alkyl radical (Eq. ii). Hydroperoxides, as a primary oxidation product, is unstable, which can be further cleaved into short-chain molecules in β-scission, including ketones, aldehydes, and hydrocarbons. The off-flavor of lipid oxidation is largely attributed to these volatile compounds. In the presence of transition metals, the decomposition of peroxides into peroxyl radicals and alkoxy radical (Eq. iii & iv) promotes the chain
reaction (Eq. v), which can propagate throughout the system until radicals polymerize with each other (Eq. vi).

i. \( \text{L}^\bullet + \text{O}_2 \rightarrow \text{LOO}^\bullet \)

ii. \( \text{LH} + \text{LOO}^\bullet \rightarrow \text{L}^\bullet + \text{LOOH} \)

iii. \( \text{LOOH} + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \text{LOO}^\bullet + \text{H}^+ \)

iv. \( \text{LOOH} + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{LO}^\bullet + \text{OH}^- \)

v. \( \text{LH} + \text{LO}^\bullet \rightarrow \text{L}^\bullet + \text{LOH} \)

vi. \( \text{LOO}^\bullet + \text{LOO}^\bullet \rightarrow \text{Nonradical products} \)

2.1.2. Lipid oxidation in emulsion system

Instead of the bulk form, food lipids are usually present in heterogeneous emulsion system such as milk, mayonnaise, and salad dressing. Emulsions can be categorized as oil-in-water (o/w) emulsion and water-in-oil (w/o) emulsion. This review will focus on o/w emulsion.

Dispersion of oil in water can significantly enlarge surface area by droplet size reduction, posting a challenge to food shelf life because lipid droplets in emulsion can be oxidized more rapidly than bulk oil (Yuji et al., 2007). The droplet size of food emulsion ranges from 0.05 to 100 \( \mu \text{m} \). Lipid oxidation in emulsion is more complicated than in bulk oil considering a variety of factors having influence on reaction rate (McClements & Decker, 2000). For example, the dispersion of oil in aqueous phase provides an interface for surface-active lipid hydroperoxides to concentrate on and thus accelerates the reaction with transition metals from continuous phase, which could be the reason for the faster oxidation in the contrast to
oxidation of bulk oil. Similarly, considering the difference of surface area, change of droplet size of emulsion may also influence the oxidation rate. Another factor making emulsion oxidation research more complicated is mass transfer phenomena: materials may exchange between droplets. Mass transfer is closely associated with surfactant micelles in continuous phase when droplet surface is saturated with surfactants. This review highlights the effect of droplet size and mass transportation on lipid oxidation in emulsion system.

2.1.2.1 Droplet size

The inverse relationship between droplet diameter (d) and droplet specific surface area ($A_s$, area per unit mass) can be expressed as $A_s = 6/\rho d$ showing that the reduction of particle dimension significantly enlarges surface area (Decker et al., 2017). A variety of facilities and techniques, including microfluidizer, ultrasonication, colloidal mill, high presser homogenization and membrane homogenization, are applied in emulsion preparation, and the processing factors greatly affect emulsion droplet size.

Although numerous previous studies have been conducted to investigate the effect of droplet size on lipid oxidation in emulsions, the results were not consistent (summarized in Table 1). Gohtani et al. (1999) reported that after storage study the peroxide value of DHA emulsion with smaller droplets prepared by membrane homogenizer was higher than the larger-droplet group. Sunflower oil emulsion prepared by two-stage homogenizer had faster oxygen consumption when homogenizer pressure was increased (Lethuaut et al., 2002). Similar results were also
reported on corn oil emulsion homogenized by microfluidizer (Lee et al., 2010), and flaxseed oil emulsion prepared by high-pressure homogenizer (Kuhn & Cunha, 2012). Ma et al. (2013) also agreed that the reduction of particle size adversely affected oxidative stability and found a faster progression of autoxidation of methyl α-linolenate small-droplet emulsion. The elevation of oxidation rate can be attributed to the increase of surface area, which promoted the reaction between interfacial hydroperoxides and transition metals in continuous phase (Decker et al., 2017). Additionally, fish oil emulsion with smaller droplet size was also found with an accelerated oxidation progression due to stronger light penetration, which oxidized riboflavin to lumiflavin and thus attacked unsaturated fatty acids (Uluata et al., 2016).

On the contrary, some studies observed opposite results. Nakaya et al. (2005) found that after storage, an emulsion with larger droplets (12.8 µm) was more rancid than an emulsion with small droplets (0.831µm), and the authors indicated the possible reason was the wedge effect, which reduced mobility of interior triacylglycerols and thus inhibit the oxidation at interface. Imai et al. (2008) measured the fraction of non-oxidized lipid of a series of emulsions with different particle size and found oxidation was inhibited by the size reduction of lipid droplets. A higher oxidative stability of rice bran oil emulsion was also observed with smaller droplets (Nguyen et al., 2013). Similar results were also tested using mathematic modeling (Kikuchi et al., 2014; Miyagawa et al., 2017). The possible reason was attributed to that the increase of surface area could enable droplets to adsorb more surfactants which consequently deceased oil concentration of individual droplet and thus reduced
susceptibility of triacylglycerol to radicals (Adachi, 2015). Additionally, there could be less prooxidants per droplet with smaller diameter, and owing to the increase of surface area, the interaction between transition metal and hydroperoxides at interface might be diluted (Decker et al., 2017). Several papers also reported that the oxidation rate was not affected by the change of droplet size (Dimakou et al., 2007; Kiokias et al., 2007; Osborn & Akoh, 2004).

The disparity of the results in Table 1 indicated the effect of droplet size on oxidation is still unclear and further investigation is required. It should be noticed that the majority of these storage studies were conducted in high temperature and short storage time (such as one day at 55°C), which was too short to differentiate lag phases between groups. A reason for using high-temperature short-time storage study could be due to the challenge of physical instability of large-droplet emulsion which is subjected to creaming. Future studies need an approach to inhibit droplet coalescence process and use lower temperature to carry out longer storage study to have a better comparison. Decker et al. (2017) also pointed out that the homogenization techniques used in previous studies were not able to produce emulsions with uniform droplet size and authors only measured the mean droplet diameters of a polydisperse emulsion. This might not be representative enough for the total droplet populations. Most of these studies were conducted in conventional approaches, evaluating oxidation for the whole polydisperse system, such as hexanal, peroxides, oxygen consumption. For having a holistic understanding on the effect of droplet size on lipid oxidation, study on microscope is needed, which can separate or classify individual droplets from a
polydisperse system (Villeneuve et al., 2018).

### 2.1.2.2 Mass transfer

The onset point of lipid oxidation in emulsion is believed to be at the interface, where hydroperoxides meet transition metals and decompose into more reactive radicals. Therefore, the partitioning of prooxidants and antioxidants have been shown that can impact lipid oxidation. However, partitioning properties only reveal the static status, from which we cannot know whether components stay at the specific site permanently or not. If oxidation occurs in a subpopulation, can prooxidants generated move to other droplets? In fact, materials can exchange rapidly between phases. Conceptually, there are three possible mechanisms of mass transfer in emulsion system: diffusion, collision exchange, and micelle-assisted transportation.

Diffusion refers to that components in lipid phase can be dissolved out of droplet and migrate to another one through the water. This phenomenon usually occurs on emulsion made of oil with relatively high polarity such as flavor oil (McClements et al., 2012) and hydrocarbons (Weiss et al., 1999). This phenomenon leads to Ostwald ripening, a process that molecules of small droplets can thermodynamically diffuse out and move to large droplets, which eventually causes phase separation. However, water-path transfer is unlikely with poorly water-soluble lipids such as vegetable oil due to the molecular dimension and strong hydrophobic force (Coupland et al., 1996).

However, Raudsepp et al. (2014b) observed the transfer of AMVN-derived peroxyl radicals between droplets using an oxidizable fluorescence probe. If radicals cannot migrate through water path, how can the probes in neighboring droplets be
oxidized? There are two other possible mechanisms. The theory, collision-exchange-separation, refers to the process when two droplets merge together and then separate (Rharbi & Winnik, 2002). During this process, materials can be transferred from one droplet to the other. However, a study of our lab showed that the transfer rate of collision-based mechanism was minimal when the emulsion with lipophilic fluorescence dye was blended with another blank emulsion.

The third mechanism is micelle-assisted transfer. Surfactants in emulsion can adsorb on the droplet surface and reduce interfacial tension. When the surface area is saturated, excessive surfactants in aqueous phase can aggregate into micelles. The concentration of micellization is critical micelle concentration (CMC). In fact, the amount of surfactants used in daily foods or emulsion researches is usually much higher than CMC, which means there are considerable amount of surfactant micelles in continuous phase. As the meta-analysis reported by Berton - Carabin et al. (2014) among 80 emulsion studies only 7 of them used surfactant concentrations below CMC. Micelles with hydrophobic core can solubilize nonpolar components out of lipid droplets which can alter partitioning. A study of our lab showed that micelle-assisted transfer was much more efficient than collision-assisted mass transfer, which plays a more important role in emulsion lipid oxidation. Raudsepp et al. (2014a) reported that AMVN-derived radicals may transfer between droplets with the help from surfactant micelles. This mechanism may also fit with autooxidation-derived prooxidants, such as lipid hydroperoxides and peroxyl radicals.

It should be noticed that lipid hydroperoxides (LOOHs) are also suitable for mass
transportation. Firstly, lipid hydroperoxides are much more stable than radicals, which allows them to migrate a longer distance. Additionally, hydroperoxides, with the help of oxygens, are surface active, and the presence of hydroperoxides at interface has been reported in several manuscripts (Decker et al., 2017). According to the work conducted by Nuchi et al. (2002), the partition of hydroperoxides in water phase was enhanced by the increase of surfactants, indicating that peroxides can be solubilized by micelles. The surface activity may help peroxide escape droplets when surfactants bud off to form micelles. However, can lipid hydroperoxides still move when surfactant concentration is lower than CMC?

Another hypothesis for prooxidant transfer is that the surface-active LOOHs can assemble micelles by themselves even when surfactant concentration was lower than the critical value. Brimberg (1993) observed that LOOHs formed reverse micelles in bulk oil and promoted oxidation, though it has not been tested in o/w emulsion system. The hypothesis proposed by Laguerre et al. (2017) stated that at the beginning of oxidation, without surfactant micelles, LOOHs cannot escape out by themselves, but when the lipid droplet surface is saturated with LOOHs, peroxides can bud off from droplets (shown in Figure 1). The polar –OOH groups point outwards, which can be cleaved into radicals in the presence of transition metals, and then attack fresh droplets. The LOOHs self-assembled micelles are not necessarily made of pure hydroperoxides, which can be also incorporated with surfactants forming mixed micelles. However, this hypothesis has not been investigated yet and future studies are required.
Another possibility is that free radicals could migrate between droplets. Of the free radicals, peroxyl radicals (LOO•) are the most likely one that could be transferred because of their lower reduction potential (1000 mV) and thus their life span is longer (0.5-7 seconds; Pryor, 1986). In comparison, alkoxy radicals (LO•) have a reduction potential of 1600 mV (Buettner, 1993) and extremely short life span (10⁻⁶ s) (Pryor, 1986). The distance a radical can migrate can be calculated from the equation \( x = \sqrt{2D_{1/2}} \), where \( D \) is the diffusion coefficient and \( t_{1/2} \) is the half-life of radical (Termini, 2003). With a diffusion coefficient of \( 10^{-6} \text{ cm}^2\text{s}^{-1} \) (Sutherland et al., 2008) the maximum distance a peroxyl radicals could move though the continuous phase would be 30 μm, making it possible to transfer to the BODIPY-containing emulsion droplets. Using the same calculation, the transfer distance of alkoxy radicals is one million times shorter than peroxyl radicals. Raudsepp et al. (2016) observed the transfer of AMVN-generated peroxyl radicals between droplets but did not observe the transfer of diterbutyl-generated alkoxy radicals. Alkyl radical (L•) is the first radical in the oxidation chain reaction with the lowest reduction potential (600 mV) when a triacylglycerol loses a hydrogen. The long life span may allow alkyl radical migrate a long distance, but the hydrophobicity of an alkyl radical is almost the same with the original TAGs meaning that it is unlikely be solubilized in both the presence and absence of micelles and thus would not transfer. Peroxyl radical would likely have the same transfer limitation as hydroperoxides meaning that free fatty acid peroxyl radical would more readily transfer than TAG peroxyl radicals as discussed above.
Micelles not only can translocate prooxidants but also can affect antioxidants efficacy. The polar paradox proposed thirty years ago stated that polar antioxidants are more effective in bulk oil whereas nonpolar antioxidants are more effective in o/w emulsion (Porter et al., 1989). However, the data of later studies did not corroborate the paradox. For example, it has been reported that caffeic acid esters showed the higher antioxidant activity when 8-carbon alkyl chain was attached while an increase of hydrophobicity, such as with 20 carbons, impaired the antioxidant activity (Sørensen et al., 2014). Similar results were reported extensively (Alemán et al., 2015; Costa et al., 2015; Lee et al., 2013; Panya et al., 2012; Shahidi & Zhong, 2011). This phenomenon was attributed to the cut-off effect behind the polar paradox when the increase of antioxidant hydrophobicity reduces the antioxidant partitioning at the interface. It was reported that the addition of surfactant lead to the increase of the antioxidant activity and the partition of 20-carbon ester in water, suggesting that surfactant micelles can solubilize antioxidants in water and thus alter oxidation rate.

2.2. Flow cytometry

Flow cytometry is a technology used primarily to characterize cells (Robinson, 2004). Using flow cytometry, individual cell samples can be separated and subgroups can be classified by properties including cell size, fluorescence, granularity, and inclusion complexity, which has been applied in different areas such as immunophenotyping, apoptosis analysis, and chromosomes studies. The advantage of flow cytometry is the high throughput in characterizing individual cells which is able
to scan thousands of samples per second. Routine analysis is available if an analysis template is established.

2.2.1. Mechanism

The typical structure of a flow cytometer is illustrated as Figure 2. Cells in flow cytometer are injected into pressurized airline with sheath fluid. The coaxial flow aligns cells in single profile by hydrodynamic focusing, which then passes the optical unit equipped with argon laser. When an individual particle is illuminated by the laser beam, a forward scatter light (FSC) is projected on the detector. The intensity of signal recorded by detector can reflect cellular size. Besides forward scatter, a side scatter (SSC) can be generated at 90°. The side scatter can help to explain the cell granularity and internal structure in apoptosis study. Another common factor used to differentiate samples is fluorescence, which is either located at surface using antibody or interior of cells using probes. Different fluorescence dyes can be detected after scatters pass through corresponding band pass wavelength filters. Sorting function can also be equipped when flow cytometer has deflection plates, namely fluorescent activated cell sorters (FACS), which is able to capture cells with pre-defined fluorescent label from a complex population by electrostatic deflection (Wilkerson, 2012).

Imaging flow cytometry (IFC) is an upgrade version of conventional flow cytometry combining with fluorescence microscopy, which is capable of visualizing individual cells and recording real images. The first imaging flow cytometer, Imagestream 100, was firstly launched by Amnis Corporation in 2005. As an
emerging technology, the installation of IFC only takes up 5% of the installation of conventional flow cytometers (Zuba-Surma & Ratajczak, 2011). Imaging flow cytometers were equipped with charge-coupled device (CCD) cameras operating by time-delay intergradation (TDI), which is able to detect synchronized images of cells in motion. The excitation and emission wavelength of imaging flow cytometer (Amnis Imagestream Mark II) is listed in Table 2. Comparing with conventional flow cytometry, the major merit of IFC is the image data on morphological characteristics. Gates used in conventional FC data analysis are usually established empirically based on data dot density, which could be inaccurate in identifying the boundary between target sample and false-positive results such as cell debris (Barteneva et al., 2012). Using imaging flow cytometry, it is possible to improve the accuracy of gates by directly examining morphological information of each suspicious data dot. The data acquisition speed of IFC (300-1000 events/s) is slower than conventional flow cytometry (3000-10000 events/s), but slower flow rate is beneficial to maintain the shape of samples (Ma et al., 2015).

2.2.2. Application of flow cytometry in studying microparticles

Microparticles, such as liposomes and lipid droplets, share the similar structure with cells and can scatter lights. Therefore, it is possible to use flow cytometer to analyze microparticles. Vorauer - Uhl et al. (2000) firstly applied flow cytometry in size measurement of liposomes, and Hai et al. (2004) used the technique to evaluate drug release behavior of w/o/w emulsion and authors indicated the results of release behavior obtained from flow cytometer was in agreement with the results obtained
from conventional *in-vitro* digestion methods. Similarly, in the field of drug release, Sivakumar et al. (2009) and Petersen et al. (2010) applied flow cytometry to quantify lipid particles loaded with lipophilic drug. This technique was also used to characterize soy bean oil emulsion (Fattaccioli et al., 2009) and silicone oil particles (Ludwig et al., 2011). In addition, double emulsions and liposomes were investigated by many studies using flow cytometry due to the similar membrane structure as cells (Chan et al., 2017; Sato et al., 2006; Sunami et al., 2010; Yan et al., 2013; Zinchenko et al., 2014). Imaging flow cytometry was firstly applied to visualize liposomes by (Matsushita-Ishiodori et al., 2019). The previous literatures have shown the potential of grafting flow cytometry on microparticle researches.

### 2.2.3. Can flow cytometry be used in lipid oxidation study?

To our best knowledge, this technique has not been extended in lipid oxidation study yet. In emulsion system, a great number of lipid droplets are dispersed in water. It is unlikely that oxidation can occur simultaneously in all droplets but instead may initiate in a subgroup and then gradually propagate to the whole system. However, it is still unclear that which subpopulation is more prone to be oxidized. A hypothesis is that the oxidation rate might be affected by surface areas. In a polydisperse system, emulsion droplets are not uniform, which is distributed in a wide range of size. It is reasonable that smaller droplets with relative larger specific area could be oxidized earlier. As flow cytometry is capable of separating individual particles and characterize size, studying the effect of droplet size on lipid oxidation in polydisperse system is possible. Additionally, using lipophilic radical-sensitive fluorescence
probes, the oxidation status of individual droplets can be revealed.

If the oxidation occurs in the subpopulation it would generate prooxidants, such as radicals and peroxides, that could be transferred to neighboring droplets? How fast is the transfer? Using confocal microscopy is able to determine the total fluorescence loss but it is hard to quantify the transfer rate on the scale of individual droplets. As flow cytometry is able to examine individual droplets in high throughput, it can be used to study the transfer phenomenon of minor components in emulsion system.

2.3. Fluorescence dyes used in lipid oxidation study

In order to use flow cytometry to study lipid oxidation of emulsion, two fluorescence probes should be added. The first fluorescence dye should be oxidation-sensitive to monitor the oxidation status of individual droplets. Another dye is non-oxidizable to visualize droplets, such as Nile red, which is excited by laser at 560 nm. The emission band of Nile red ranges from 550 nm to 780 nm.

Lag phase is important to lipid oxidation study, which is usually determined by the start point of lipid oxidation, which are originated from peroxyl radicals. By quantifying peroxyl radicals, we would be able to identify the start point of lipid oxidation. According to Gomes et al. (2005) several fluorescence probes can be used to test peroxyl radicals (shown as Table 3). However, not all of them are applicable to the flow cytometry study. Criteria for choosing suitable probes are listed as below:

1) The excitation laser used should be available on flow cytometer (Table 2).
2) The radical-sensitive dye should be lipophilic and stay in oil droplet consistently.
3) The emission wavelength should avoid the band of autofluorescence of oxidation products (~500 nm).

4) Overlap on emission band of Nile red is unfavorable.

2.3.1. cis-Parinaric acid

* cis-Parinaric acid (λ<sub>excitation</sub> = 320 nm; λ<sub>emission</sub> = 432 nm) is a polyunsaturated free fatty acid with an 18-carbon backbone. Ordinary fatty acids are not fluorescent but due to the *cis* structure, the conjugated double bonds are able to absorb energy and produce fluorescence. Peroxyl radicals can oxidize the double bonds and trigger fluorescence loss. *cis*-Parinaric acid has been used to evaluate lipid peroxidation (Kuypers et al., 1987) and antioxidant activity (Naguib, 1998). As a fatty acid, *cis*-parinaric acid is suitable to be incorporated in lipid droplet, and considering the nature as a lipid, *cis*-parinaric acid shows similar reaction potential as triacylglycerides which is able to reflect the oxidation kinetic accurately. Unfortunately, the excitation wavelength of *cis*-parinaric acid is ultraviolet which is not equipped on imaging flow cytometer (Table 2).

2.3.2. DPPP

Diphenyl-1-pyrenylphosphine (DPPP) originally is not fluorescent, but it can react with peroxyl radicals and produce DPPPO (diphenyl-1-pyrenylphosphin oxide) giving fluorescence (λ<sub>excitation</sub> = 351 nm; λ<sub>emission</sub> = 380 nm). DPPP not only can react with peroxyl radicals but also hydroperoxides, which can be used as endpoint analysis as an alternative for those current peroxide value tests. DPPP is lipophilic and has been reported that it only reacted with hydroperoxides in lipid domain but not
hydrophilic peroxides in aqueous phase (Okimoto et al., 2000). The high hydrophobicity allows it to stay in lipid droplets consistently which shows the potential to monitor oxidation of individual droplets, but similarly due to the UV excitation and emission wavelength, it is difficult to be used in flow cytometry.

2.3.3. Dipyridamole

Dipyridamole is a fluorescent compound ($\lambda_{\text{excitation}} = 415 \text{ nm}; \lambda_{\text{emission}} = 480 \text{ nm}$), which is used as a drug for coronary artery disease. It was reported that dipyridamole has antioxidant property which can react with peroxyl radicals. Iuliano et al. (2000) reported that the fluorescence of dipyridamole was quenched by peroxyl radicals initiated by AAPH, a hydrophilic initiator. The excitation and emission wavelength of dipyridamole can be visualized in Channel 7 of imaging flow cytometry (Table 2) and avoid the emission band of Nile red (channel 2 to 6). Nevertheless, as the compound is water-soluble, dipyridamole can diffuse out from lipid phase quickly. The exchange of fluorescence is not favorable for lipid oxidation study. For example, in studying effect of droplet size on emulsion lipid oxidation, if the dye can be spread to the whole system freely, the effect of droplet size on oxidation rate would be hard to quantify.

2.3.4. DCFH-DA

2,7-Dichlorodihydrofluorescien diacetate (DCFH-DA) has been used in cell study to test radical generation. DCFH-DA can be hydrolyzed by intracellular enzyme forming DCFH, a non-fluorescent compound, which then can react with reactive species, producing 2,7-dichlorodihydrofluorescien ($\lambda_{\text{excitation}} = 498 \text{ nm}; \lambda_{\text{emission}} = 522$ nm).
DCFH-DA was also applied to evaluate the radical-scavenging capacity of antioxidants by TRAP method (total peroxyl radical trapping potential) (Valkonen & Kuusi, 1997). In TRAP method, AAPH is used as radical initiator and DCFH-DA is used as fluorescence indicator. Target antioxidants are added, and the lag phases of fluorescence are used to compared with the standard lag phase of Trolox. DCFH-DA is suitable for lipid oxidation study and antioxidant assessment, but it is hard to be grafted on flow cytometry due to the partition in water (Cathcart et al., 1983). The partition coefficient of DCFH is 2.62 which can diffuse out of lipid compartment.

### 2.3.5. β- Phycoerythrin

β- Phycoerythrin is a fluorescent protein ($\lambda_{\text{excitation}} = 540 \text{ nm}; \lambda_{\text{emission}} = 565 \text{ nm}$), which is used in measuring antioxidant activity, namely Oxygen Radical Absorbance Capacity (ORAC) method. ORAC was firstly established by Cao et al. (1993) using β- phycoerythrin as fluorescence indicator sensitive to reactive oxygen species. Similar with TRAP method, ORAC uses AAPH as radical initiator to produce peroxyl radicals and react with antioxidants or fluorescence probe (Xu & Chang, 2007). The result is expressed in equivalent of mmol Trolox for comparison. However, a disadvantage limits the compound to be used is the photosensitivity. Additionally, it can react with polyphenols because of non-specific protein links (Litescu et al., 2014). Since this compound is a water-soluble protein, it is unlikely to be used in flow cytometry study.

### 2.3.6. Fluorescein & 6-carboxyfluorescein

Fluorescein ($\lambda_{\text{excitation}} = 495 \text{ nm}; \lambda_{\text{emission}} = 515 \text{ nm}$) is a well-known fluorophore
which has been extensively used in assessment of antioxidant activity (Dávalos et al., 2004). The reaction with reactive oxygen species leads to fluorescence quenching. It is highly pH-dependent which is non-fluorescent at pH 4 but provides fluorescence with high quantum yield above pH 4.5 (Martin & Lindqvist, 1975). As it is more photostable and does not react with others, fluorescein was used to replace β-phycoceryth in ORAS assay (Ou et al., 2001). 6-Carboxyfluorescein is a derivative with an additional carboxyl group, which is membrane-impermeable, and thus is used to stain cells or liposomes (Makrigiorgos et al., 1997). However, due to the hydrophilicity, fluorescein is transferable between subjects which cannot be confined in lipid particle.

2.3.7. **Lipophilic derivatives of fluorescein**

In order to enhance the partition of fluorescein in lipid phase, it was modified by attaching alkyl group with different carbon number (C\(_{11}\), C\(_{16}\), C\(_{18}\)). Due to the presence of non-polar carbon chains, fluorescein is more surface-active and thus more sensible to surface-active peroxyl radicals. Maulik et al. (1998) used C\(_{11}\)-fluor, C\(_{16}\)-fluor, and C\(_{18}\)-fluor to stain cell membrane and found that although the lipophilicity has been enhanced, probes were still able to transfer between subject. The transfer rate was inversely related with the length of alkyl chains, which showed that probe transfer can be inhibited by increasing lipophilicity.

Fluoresceinated phosphoethanolamine (fluor-DHPE) is another modified fluorescein by bridging the probe with phospholipid, which shows much higher lipophilicity than C\(_{11}\)-fluor, C\(_{16}\)-fluor, and C\(_{18}\)-fluor. Maulik et al. (1998) reported that
fluor-DHPE was unable to exchange between particles which showed the excellent potential to be applied in flow cytometry. However, a study of our lab showed that certain lipid oxidation products showed green fluorescence which overlapped on the emission band fluor-DHPE. The autofluorescence of lipid oxidation products has been reported previously (Gatellier et al., 2007). In this case, the autofluorescence of oxidized droplets can cancel out the diminishing of fluor-DHPE.

2.3.8. BODIPY\textsuperscript{581/591}

\textit{4,4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid} (BODIPY\textsuperscript{581/591}) is a fluorescent compound (\(\lambda_{\text{excitation}} = 581\) nm; \(\lambda_{\text{emission}} = 591\) nm). Owing to the conjugated double bonds, BODIPY\textsuperscript{581/591} is susceptible to be oxidized by peroxyl radicals, which is thus used for testing cell membrane peroxidation (Drummen et al., 2002). The reaction rate of peroxyl radicals and BODIPY\textsuperscript{581/591} is two order of magnitude higher than it of peroxyl radicals and polyunsaturated fatty acids (Yoshida et al., 2003). After oxidized, the emission peak at 591 nm can be shifted to 520 nm. Due to the 11-carbon chain, BODIPY\textsuperscript{581/591} is hydrophobic and can be consistently retained in lipid domain which is suitable for emulsion lipid oxidation study. However, there are two limitations for BODIPY\textsuperscript{581/591} to be applied in flow cytometry. Firstly, the excitation laser (581 nm) is not typically available (Table 2). Using the laser at 488 nm is able to excite the probe, but the according to the spectrum, the intensity generated is only 10% of the intensity excited by \(\lambda_{\text{max}}\), which requires high dose of probes. The addition of probes may alter oxidation kinetics which act as antioxidants. Another limitation of this probe is that it
is difficult to be used with typical neutral dye such as Nile red due to the overlap of emission bands, which requires complicated wavelength compensation procedure.

2.3.9. **BODIPY**<sup>665/676</sup>

(E,E)-3,5-bis-(4-phenyl-1,3-butadienyl)-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY<sub>665/676</sub>) is another fluorescent probe (λ<sub>excitation</sub> = 665 nm; λ<sub>emission</sub> = 676 nm). Similar with BODIPY<sub>581/591</sub>, the conjugated polyene between phenyl ring and BODIPY core is also prone to be oxidized by peroxyl radicals (Raudsepp et al., 2014a). The evidence of prooxidant transfer was reported by (Raudsepp et al., 2014b) using BODIPY<sub>665/676</sub> and confocal microscopy. Upon oxidation, the emission wavelength of BODIPY<sub>665/676</sub> can be shifted to 605 nm. The major advantage of this probe is that the emission band is 676 nm (Channel 11 of Table 2) which shows no crosstalk with most of fluorophores and thus can avoid the interferences such as autofluorescence. Additionally, a study of our lab shows that BODIPY<sub>665/676</sub> is heat-stable and highly hydrophobic which was not able to diffuse out of lipid droplets. Due to the laser of 642 nm is close to the λ<sub>max</sub> (665 nm), BODIPY<sub>665/676</sub> is able to provide high fluorescence intensity with an extremely low probe concentration (10<sup>3</sup> times lower than detectable peroxides). So far, BODIPY<sub>665/676</sub> is the most suitable probe for using flow cytometry in studying emulsion lipid oxidation.
CHAPTER 3
APPLICATION OF FLOW CYTOMETRY AS NOVEL TECHNOLOGY IN
STUDYING THE EFFECT OF DROPLET SIZE ON LIPID OXIDATION IN
OIL-IN-WATER EMULSIONS

3.1. Abstract

Despite several published studies, the impact of emulsion droplet size on lipid oxidation rates is unclear. This could be because oil-in-water emulsions are typically polydisperse and the oxidation rate of individual droplets is difficult to discern. Flow cytometry is a technique for studying individual cells and their subpopulations using fluorescence technologies. It is possible that individual emulsion droplets could also be characterized by flow cytometry as a novel approach for studying lipid oxidation. Typical emulsion droplets are too small to be visualized by flow cytometer so emulsions were prepared to have droplets > 2 μm that were stabilized by weighting agent and xanthan gum to minimize creaming during storage. A radical-sensitive lipid-soluble fluorescence probe (BODIPY\textsuperscript{665/676}) was added to the lipid used to prepare the emulsion so that the susceptibility of individual emulsion droplets could be determined. The results showed that in a polydisperse emulsion system, small droplets were oxidized faster than large droplets. A conventional method was also carried out by blending two emulsions with different droplet sizes and oil densities, and results showed that small droplets were oxidized faster which was in agreement with the observation obtained from flow cytometry. As a new approach, flow cytometry could be utilized in oil-in-water emulsion studies to reveal
insights of lipid oxidation mechanisms in individual droplets.

**Key Words:** Lipid oxidation, droplet size, individual droplets, flow cytometry, fluorescence.

### 3.2. Introduction

Lipid oxidation of unsaturated lipids not only can impair food quality but also may cause potential adverse effect on human health due to lipid oxidation end products (Frankel, 1980; Kanner, 2007; Vieira et al., 2017). In addition to the bulk form of oils, lipids in foods are often present as heterogeneous emulsion systems (McClements & Decker, 2000). Dispersion of oil in an aqueous system can dramatically enlarge surface area due to size reduction, which poses a challenge to food shelf life because oil-in-water emulsion oxidized more rapidly than bulk oils (Yuji et al., 2007). The inverse relationship between droplet diameter \(d\) and droplet specific surface area \(A_s\) (area per unit mass) can be expressed as \(A_s = \frac{6}{\rho d}\) (Decker et al., 2017), showing that reduction of particle dimension significantly enlarges surface area. Increase of droplet interfacial area allows amphiphilic molecules such as lipid hydroperoxides to concentrate at the emulsion droplet surface resulting in interactions with transition metals in continuous phase that decompose hydroperoxides to free radicals therefore accelerating oil oxidation. Thus, it has been hypothesized that the oxidation rates in oil-in-water emulsions could be influenced by lipid droplet surface area.

Numerous researchers have investigated the effect of particle size on lipid
Several studies reported that oxidation in smaller droplets were faster than in larger droplets. Gohtani et al. (1999) found that the peroxide value of DHA emulsion made of smaller droplets was higher than the group with larger droplets. Lethuaut et al. (2002) reported that a coarse sunflower oil-in-water emulsion showed slower oxygen consumption than an emulsion with smaller droplets. Similar observations were also reported in corn oil emulsions homogenized with a microfluidizer (Lee et al., 2010), and flaxseed oil emulsion prepared with a high-pressure homogenizer (Kuhn & Cunha, 2012). Ma et al. (2013) also reported that the oxidation of a α-linolenate emulsion was promoted by reduction of droplet size. Additionally, fish oil emulsion with smaller droplets were also found to exhibit accelerated oxidation due to stronger light penetration in the presence of riboflavin (Uluata et al., 2016).

However, several research publications observed opposite results. Nakaya et al. (2005) found that hexanal generation was lower in small emulsion droplets and the authors suggested the possible reason was the wedge effect, which reduced mobility of triacylglycerols in the interior of the small emulsion droplets. Similar results were also reported by others (Imai et al., 2008; Kikuchi et al., 2014; Miyagawa et al., 2017; Nguyen et al., 2013). Additionally, some papers also reported that oxidation was not affected by droplet size (Dimakou et al., 2007; Kiokias et al., 2007; Osborn & Akoh, 2004).

These disparity of results indicates that the conclusion on how emulsion droplet size impact lipid oxidation is still unclear and further investigation is required.
Decker et al. (2017) pointed out that the homogenization techniques applied in these papers do not produce uniform droplets but instead a wide range of particle sizes with authors reporting the mean diameter to represent the emulsion droplet size. Unfortunately, mean droplet size is not an accurate representation of the heterogeneity of lipid droplet size in a polydisperse system emulsion. In order to have a holistic understanding on the effect of droplet size, new techniques are needed in polydisperse emulsion, which can separate or classify oxidation in different sized individual droplets (Villeneuve et al., 2018).

Flow cytometry is a technique that has been utilized in many fields such as microbial strain classification, apoptosis progression, immunophenotyping, and chromosomes studies (Robinson, 2004). Using flow cytometry, cells can be categorized into groups based on properties such as particle size, fluorescence, granularity, and inclusion complexity. The advantage of this technology is mainly derived from its high resolution and high-throughput analysis of individual cells or other units. Once an analysis template is established, routine analysis is convenient and reproducible. Samples analyzed by flow cytometry are injected into a pressurized air line with a sheath fluid. The coaxial flow enables cells to align in single stream by hydrodynamic focusing, with the cells then passing the optical unit equipped with argon laser. When an individual particle is illuminated by the laser beam, light scattering is generated and collected by detectors. The scatter signals can be affected by particle size and fluorescence. Different fluorescence probes can be detected with different band pass wavelength filters. An imaging flow cytometer
equipped with camera is an upgrade of conventional flow cytometry, which is able to capture the images of particles in the flow. Particle size and fluorescence can be analyzed by pixels on the image data.

As an emulsion droplet share similar structure as a cell and thus can scatter light, this study aims to apply imaging flow cytometry as a new approach to achieve separation of individual droplets of a polydisperse emulsion system and study the effect of droplet size on oxidation rates.

3.3. Materials and methods

3.3.1. Materials

Soybean oil was purchased from local store and stored at -20°C in dark until use. Brominated vegetable oil was purchased from Spectrum Corp. (New Brunswick, NJ). Xanthan gum was donated by TIC Gums (White Marsh, MD). (E,E)-3,5-bis(4-phenyl-1,3-butadienyl)-4,4-difluoro-4-bora-3a,4a-diaza-sindacene (BODIPY665/676), a lipophilic radical-sensitive probe, was purchased from Thermo Fischer (Eugene, OR). Sodium dodecyl sulfonate (SDS), cumene hydroperoxide, Nile red, barium chloride, and ferrous sulfate were obtained from Sigma-Aldrich (St. Louis, MO). Ammonium thiocyanate was purchased from Fischer Scientific (Pittsburg, PA). All other reagents were at or above high-performance liquid chromatography grade. Distilled and deionized water was used throughout the experiment.

3.3.2. Preparation of stripped soybean oil

Soybean oil was stripped to remove minor components such as antioxidants and
pro-oxidants according to the method established previously (Chen et al., 2014). A chromatographic column (3.0 cm internal diameter × 35 cm height) was prepared with three layers: the bottom layer was packed with 22.5 g of silicic acid (washed with DDI water); activated charcoal (5.6 g) was used as the second layer and another 22.5 g of silicic acid was packed as top layer. Soybean oil (30 g) was dissolved in 30 ml of hexane and loaded onto the column which was then eluted through the column using additional 270 ml of hexane. The organic solvent was removed from the oil using a vacuum rotary evaporator (Rotavapor R 110, Buchi, Flawil, Switzerland) at 25°C. Residual hexane was evaporated by flushing with nitrogen. Stripped soybean oil (SSO) was stored at -80°C until use.

3.3.3. Preparation of oil-in-water emulsion samples

Nile red was dissolved in chloroform to prepare a 10 μg/ml stock solution. BODIPY\textsuperscript{665/676} was dissolved in hexane to obtain a 1.25 μg/ml stock solution. Brominated vegetable oil (1.33 g/ml oil) was used as weighting agent at 25% of the total oil concentrations to match oil density with water density to inhibit emulsion creaming. Nile red was used as a lipophilic non-oxidizable dye to visualize the emulsion droplets in the flow cytometer. BODIPY\textsuperscript{665/676}, a lipophilic radical-sensitive fluorescence probe, was used to monitor oxidation in individual droplets. In one gram of oil sample, 1 ml of Nile red stock solution, 0.5 ml of BODIPY\textsuperscript{665/676} stock solution, and 1 ml of hexane were mixed. Organic solvents were removed by nitrogen flushing.

An SDS solution (0.5 mM) was prepared with xanthan gum (1 g/L), added to
enhance viscosity to inhibit droplet coalescence. Oil-in-water emulsions were prepared with 0.2 g of each oil treatment and 99.8 g of the SDS-xanthan gum solution, which were then homogenized by high-speed mixer (M133/1281-0, Biospec Product Inc., Bartlesville, OK) for 2 min. During homogenization, the emulsion was immersed in an ice bath to minimize oxidation. Particle size was monitored throughout storage study using a size analyzer (Malvern Mastersizer Instruments, Worchester, UK) (Coupland & McClements, 2001).

3.3.4. Evaluation of lipid oxidation

3.3.4.1. Determination of lipid hydroperoxides

Lipid hydroperoxides were measured as primary oxidation products using the method described by Matalanis et al. (2012). Emulsion samples (1 ml) were added to 7 ml of an chloroform:methanol (2:1, v/v) solvent mixture, vortexed 3 times for 10 s each and centrifuged for 5 min at 1300 g. After centrifugation, 0.5 ml of the lower organic solvent layer was mixed with 2.5 ml of methanol:butanol solution (2:1, v/v). Each sample was then mixed with 15 μl of 3.94 M ammonium thiocyanate and 15 μl of ferrous iron solution which was prepared by mixing 1 ml of 0.132 M barium chloride in 1 ml 0.144 M ferrous sulfate. The mixture was vortexed and absorbance was measured at 510 nm using a spectrophotometer (Thermo-Spectronic, Waltham, MA) after 20 min of incubation in the dark at room temperature. Hydroperoxide concentrations were calculated using a standard curve prepared with cumene hydroperoxide (0-20 μM).
3.3.4.2. Determination of headspace hexanal

Hexanal was determined as a secondary product of lipid oxidation. One milliliter of emulsion sample was transferred into a 10-ml glass vials and sealed with poly(tetrafluoroethylene) butyl septa at the beginning of each experiment. Headspace hexanal was measured using a method adapted from Cui et al. (2016) using solid-phase microextraction-head space gas chromatography with flame ionization detection (SPME-GC-FID). After the incubation of the sample vials for 10 minutes at 45 ºC, a 50/30 μm divinylbenzene / carboxen / polydimethylsiloxane (DVB/CAR/PDMS) stableflex solid phase microextraction (SPME) fiber (Supelco Analytical; Bellefonte, PA) was injected into the vials and exposed to the headspace for 2 minutes. The volatile compounds were then desorbed from the fiber in the injector port for 3 minutes at 250 ºC at a split ratio of 1:7. Volatiles were separated using an Equity-1 fused-silica capillary column (30 m × 0.32 mm i.d. × 1 μm) coated with 100% polydimethylsiloxane (Supelco Analytical; Bellefonte, PA). Helium (1.0 ml/min) was used as carrier gas. The flame ionization detector was operated at 250 ºC while the oven was at 65 ºC. The sample run time was 10 minutes and the peak for hexanal was recorded at 4.9 minutes. Peak area integration was calculated using Shimadzu Lab Solutions version 5.87. A standard curve was prepared using an emulsion spiked with hexanal.

3.3.4.3. Evaluation of oxidation of individual droplets by flow cytometry

The oxidation of individual droplets was monitored using an ImageStream Mark II imaging flow cytometer operated by INSPIRE software (Amnis/Millipore, Seattle,
WA) and calibrated by an ASSIST program (Amnis/Millipore, Seattle, WA).

Standard beads (Amnis/Millipore, Seattle, WA) were used as internal standard which were run concurrently for real-time velocity detection and autofocusing. Lasers at 488 nm (200 mW) and 642 nm (150 mW) were used for excitation of fluorescence probes. The side scatter detectors and brightfield were turned off. The fluidics system was set on low-speed/high-sensitivity mode. Images were collected at 40× magnification. Data acquisition was stopped after 2000 events were collected for each sample.

Flow cytometry data files were processed by IDEAS software (Amnis/Millipore, Seattle, WA). Mask A (Nile red, excitation 488 nm) was established on Channel 4 at an intensity range from 200 to 4095; mask B (BODIPY\(^{665/676}\), excitation 642 nm) was established on Channel 11 with intensity range from 100 to 4095. Diameter (D) of each droplet was quantified on mask A and the intensity (I) of BODIPY\(^{665/676}\) was quantified on mask B. Relative intensity (RI) of BODIPY\(^{665/676}\) was calculated by \(I/D^3\). Small droplets were selected as those having a diameter from 2 to 8 μm and large droplets were gated on diameters over 20 μm. Scatter plot of droplets was established between diameter and relative intensity. Oxidized subpopulation was gated on relative intensity from -5 to 15. Non-oxidized subpopulation was gated on relative intensity from 15.01 to 500. Percentages of oxidized and non-oxidized subpopulations were obtained from the gates.

3.3.5. Verification study

As flow cytometry is a new method, the effect of droplet size on lipid oxidation
in polydisperse system was also investigated by a conventional method. Two groups of soybean oil were mixed with weighting agent at ratios of 1:0.15 (density < 1 g/ml) and 1:0.5 (density > 1 g/ml), respectively.

The oil with lower density was used to prepare emulsion with larger droplets by homogenizing 3 g of oil with 97 g of surfactant solution (2 mM SDS + 0.1% xanthan gum) using high-speed mixer (M133/1281-0, Biospec Product Inc., Bartlesville, OK) for 2 min. Heavier oil (more weighting agent) was used to prepare emulsion with smaller droplets. Briefly, coarse emulsion (6% oil concentration + 2 mM SDS) was prepared using high-speed mixer for 2 min, which was then homogenized by two-stage homogenizer at 1000 psi for 12 passes. An ice bath was used during homogenization to minimize oxidation. The small-droplet emulsion was diluted to 3% of oil concentration with the surfactant solution (2 mM SDS + 0.2% xanthan gum). Therefore, two emulsions with different droplet size and oil densities were prepared at the same oil content (3%), SDS concentration (2 mM), and thickener concentration (0.1%). Droplet size of emulsions were examined by size analyzer (Malvern Instruments, Worchester, UK) (Coupland & McClements, 2001).

Ten milliliters of each emulsion were blended in a 100-ml foil covered bottle. Bottles were placed on a quake shaker at 37 °C to gently shake the emulsion mixture to inhibit creaming and sedimentation. To measure lipid hydroperoxides in subgroups at each sampling day, 0.1 ml of emulsion was transfer to microcentrifuge tube and diluted with 1.4 ml of water to lower viscosity allowing for easier separation. Diluted samples were centrifuged at 13,000 rpm for 10 min. The cream layer and
sediment layer were separated and then collected by 0.75-ml pipette. Lipid hydroperoxides of each layer were determined by the method described above (Matalanis et al., 2012).

3.3.6. Statistical analysis

Analyses were conducted in triplicates and results are presented as means ± standard deviations. Data were tested by one-way analysis of variance (ANOVA) followed by Duncan’s multiple comparison using SPSS (Version 19, IBM Co., USA).

3.4. Results and discussion

3.4.1. Method development

This study applied imaging flow cytometry combined with fluorescence technology as a novel method to determine if oxidation in emulsions could be successfully analyzed on individual droplets. Several articles have applied traditional flow cytometry in droplet or particle research. Vorauer-Uhl et al. (2000) applied flow cytometry to measure the size of liposomes, and Hai et al. (2004) used the technique to evaluate drug release behavior of w/o/w emulsion. The later study indicated the results of release behavior obtained from flow cytometer was in agreement with the results obtained from conventional methods. Similarly, in the field of drug release, Sivakumar et al. (2009) and Petersen et al. (2010) applied flow cytometry to quantify lipid particles loaded with lipophilic drug. Flow cytometry was also used to characterize soy bean oil emulsions (Fattaccioli et al., 2009) and silicone oil particles (Ludwig et al., 2011). In addition, double emulsions and
liposomes were investigated by many studies using flow cytometry due to the similar membrane structure as cells (Chan et al., 2017; Sato et al., 2006; Sunami et al., 2010; Yan et al., 2013; Zinchenko et al., 2014). More recently, imaging flow cytometry was applied to visualize liposomes by Matsushita-Ishiodori et al. (2019). To our best knowledge, this technique has not been extended to the study of lipid oxidation in oil-in-water emulsions.

A challenge of studying oil-in-water emulsions with flow cytometry is that the method needs droplets > 1 µm for imaging. Thus, typical high pressure homogenized emulsion droplets (smaller than 1µm) are too small to be detected. Therefore, the emulsions used in this study were prepared with relatively low homogenization and low surfactant concentration to obtain large droplet dimension. However, physical stability has been reported as a major problem in studying large-droplet emulsions. Creaming velocity can be calculated by Stokes’s Law: \( v = -\frac{2r^2\Delta \rho g}{9\eta} \), which is associated with droplet radius \( r \), density contrast \( \Delta \rho \), viscosity of aqueous phase \( \eta \), and gravity acceleration \( g \) (Chanamai & McClements, 2000). Due to the large droplet diameter in emulsions that can be imaged by flow cytometry, the emulsions were susceptible to creaming, and once creamed the droplets were packed tightly, they can coalesce resulting in further increases in droplet size. To slow down creaming, density contrast can be minimized, and viscosity of aqueous phase can be enhanced. In this study, xanthan gum was used as thickener to increase emulsion viscosity, and brominated vegetable oil was used as weighting agent to match oil and water density. The application of 25% weighting agent to improve
physicals stability has been reported previously (Chanamai & McClements, 2000). Using only weighting agent or xanthan gum resulted in creaming in two days. The preliminary studies showed that that combination of thickener and weighting agent significantly enhanced emulsion droplet physical stability and prevented creaming for 26 days (data not shown). Emulsion droplets with and without brominated vegetable oil had the same lipid oxidation rates (Figure 3), which indicated future lipid oxidation studies can use weighting agent to stabilize emulsion without altering results. It should be noticed that the concentration of xanthan gum (0.1%) was critical: a lower concentration can lead to bridging flocculation and a higher concentration can lead to depletion flocculation (McClements, 2000). Future researchers studying emulsions with large droplets may take similar approaches to stabilize physical stability.

The size distribution of emulsion droplets was measured by light scattering size analyzer and flow cytometer, shown in Figure 4. The results showed similar size distribution curve from 2 to 40 µm, which indicated that flow cytometer is reliable in studying the size of lipid droplets. The accuracy of using traditional flow cytometer in measuring size of liposomes and w/o/w emulsions has been reported previously (Chan et al., 2017; Vorauer - Uhl et al., 2000), but it was also reported that droplets of o/w emulsion were deformed at high flow speed (20 cm/s) of conventional flow cytometer (Ma et al., 2015). Our results suggested that lipid droplets in o/w emulsion can maintain the shape well in imaging flow cytometer due to the low fluidic flow rate (2.3 cm/s).
3.4.2. Effect of droplet size on lipid oxidation in polydisperse emulsion

Figure 5 shows the formation of lipid hydroperoxides and hexanal in the entire emulsion. Sharp increases in both lipid hydroperoxides and hexanal were observed after 8 days. Figure 6 shows the droplets stained by Nile red (left column) and BODIPY<sub>665/676</sub> (right column) at different stages during oxidation. Figure 6a shows that two probes in droplets can be well visualized by flow cytometry when emulsions were fresh on day 0 for both small droplets (2-8 μm) and large droplets (20-40 μm). After oxidation, BODIPY<sub>665/676</sub> can be bleached by radicals, but non-oxidizable Nile red was still present to examine droplets (Figure 6c). As the detection channels were parallel on different fluorescence emission bands, BODIPY<sub>665/676</sub> was not interfered by Nile red (Figure 6c). Figure 6b shows selected images of small droplets (2-8 μm) and large droplets (20-40 μm) after 7 days of storage, which shows that both small and large droplets can still be observed with Nile red but the BODIPY<sub>665/676</sub> fluorescent has lost in small some droplets indicating that oxidation has occurred.

Figure 7a shows the percent of all small (2-8 μm) or large (20-40 μm) unoxidized fluorescent droplets. The results showed that initially no droplets were oxidized as the percent of fluorescent droplets was 100%. However, a sharp decrease was observed after 6 days for the small droplets and after 7 days for the large droplets. The correlation between droplet size and the percent of fluorescent droplets on day 8 were shown in Figure 7b, which indicated oxidation was inversely associated of particle dimension.

To verify the observation that small droplets were oxidizing faster than large
droplets, we prepared two emulsions with different droplet sizes and oil densities, then blended the emulsions and separated the two droplet populations by centrifugation. Figure 8 shows the particle size distribution of each emulsion separately and the blended emulsion. The blended emulsion was stored at 37°C and at each sampling time the emulsion was centrifuged and lipid hydroperoxides were determined in both the large (cream layer) and small (sediment layer) droplets. Hexanal could not be used to evaluate oxidative status as much of the hexanal would be lost in the centrifugation step. The lag phases of small droplets and large droplets were 5 and 7 days respectively (Figure 9), again suggesting that in the polydisperse system smaller droplets were oxidized earlier than larger droplets, which corroborates with the results obtained from flow cytometry study.

The susceptibility of smaller droplets has been reported previously (Gohtani et al., 1999; Lethuaut et al., 2002; Ma et al., 2013; Uluata et al., 2016). For example, Lethuaut et al. (2002) reported that emulsion with smaller droplets showed faster oxygen consumption rate than that of larger droplets. They suggested that possible underlying reason could be attributed to the increase of surface area of the smaller droplets which could increase the opportunity for lipid substrate to contact with oxygen. This assumes that the oxygen would primarily reside in the aqueous phase, but oxygen solubility is actually greater in oil than water and since emulsion are typically saturated with oxygen after homogenization (Johnson et al., 2017), it would be expected that similar oxygen levels would be found in all phases of the emulsion. However, it is possible that as oxygen proceeds, the lipid phase oxygen is rapidly
depleted and then oxygen must diffuse into the droplet thus making a higher droplet surface area an important factor in lipid oxidation rates. In addition, the enlarged surface area of the small droplets may promote more reactions between the lipid hydroperoxides present at emulsion droplet interfaces (Nuchi et al., 2002) and the metal ions in aqueous phase.

Due to the nature of flow cytometer, this study only focused on emulsion droplets over 2 μm. The effect of droplet size on lipid oxidation in nanoemulsions could be different. For example, if nanoemulsions have such large surface areas that interfacial oxidation reactions are not impacted by droplet size; e.g. surface area did not limit the reaction rates, then oxidation rates could be the same in all droplets. However, many food products have emulsions droplets > 2 μm such as mayonnaise (Jacobsen et al., 2000) and pourable salad dressings (Perrechil et al., 2010) and thus this research could apply to commercial food emulsion products.

3.5. Conclusions

Flow cytometry was able to isolate, visualize, and characterize individual droplets in oil-in-water emulsions with high throughput. The combination of weighting agent and xanthan gum was found to be an applicable approach to improve physical stability in studying emulsion with large droplets. Using BODIPY$^{665/676}$ as radical sensitive fluorescence probe, smaller droplets in a polydisperse emulsion system were found to be more prone to be oxidized than larger droplets. Flow cytometry showed the potential to provide unique insights in emulsion science and oxidation of
emulsions. In addition, this research suggests that in commercial emulsions with large droplet such as mayonnaise, altering droplet size could impact shelf-life.
CHAPTER 4
APPLICATION OF FLOW CYTOMETRY AS NOVEL TECHNOLOGY IN
STUDYING LIPID OXIDATION AND MASS TRANSPORT PHENOMENA IN
OIL-IN-WATER EMULSIONS

4.1. Abstract

It has been postulated that lipid oxidation in oil-in-water emulsions begins in a subpopulation of droplets and then propagates to the whole system. However, this hypothesis is difficult to investigate as conventional methods cannot study individual droplets. Flow cytometry, a technique for studying individual cells, was therefore used to determine if lipid oxidation products could transfer between emulsion droplets. Experiments were carried out using blends of emulsions, one with medium chain triacylglycerols and the oxidizable fluorescent dye, BODIPY\textsuperscript{665/676} and another with stripped soybean oil. Results showed that when sodium dodecyl sulfate (SDS) concentration was lower than critical micelle concentration (CMC), lipid oxidation products of triacylglycerols were not able to be solubilized out of soybean oil emulsions droplets until the emulsions were extremely oxidized. In the presence of surfactant micelles, oxidation of BODIPY\textsuperscript{665/676} was much faster suggesting that prooxidants were transferring between droplets. In the presence of free fatty acids, transfer between droplets was observed even when surfactant concentration was lower than CMC. The linoleic acid oxidation product, 2,4, decadienal was also found to transfer between droplets. The effect of surfactant concentration on prooxidant transfer was also investigated using the lipid soluble radical generator, 2,2'-azobis
(2,4-dimethylvaleronitrile) (AMVN). Results showed that surfactants promoted oxidation at low concentrations and inhibited oxidation at high concentration. The CMC of SDS was decreased by both fatty acids and NaCl thus forming micelles are lower SDS concentrations which could affect the transfer of prooxidants between droplets.

Key words: Lipid oxidation, transfer, flow cytometry, surfactant micelles

4.2. Introduction

Lipid oxidation has been a major challenge for incorporation of healthy polyunsaturated fats in foods products as oxidation adversely affects food flavor, nutritional value, and human health (Frankel, 1980; Vieira et al., 2017). Food lipids are not always present in bulk form, but often as heterogenous emulsion systems such as salad dressing and mayonnaise. One gram of oil creates $2.1 \times 10^{12}$ lipid droplets with a 1 μm diameter (McClements & Decker, 2000) thus creating a huge water-oil interfacial area. Therefore, lipid oxidation rates in emulsions are accelerated compared to bulk oils which highlights the need for studying lipid oxidation in dispersion systems to decrease food waste (Yuji et al., 2007). In a heterogenous emulsion, it is unlikely that lipid oxidation occurs simultaneously in all droplets suggesting that oxidation might be initiated in a subpopulation of droplets which then gradually propagates to the whole system (Decker et al., 2017). It is unknown if oxidation products generated in one droplet can move to the neighboring droplets to further promote oxidation. Conceptually, this could occur by three possible
mechanisms of mass transfer in emulsion systems: diffusion, collision exchange, and micelle-assisted transportation (Laguerre et al., 2017).

Diffusion refers to components in lipid phase that can partition out of a droplet and migrate to another one through the emulsion continuous phase. This phenomenon usually occurs in emulsion made from oil with relatively high polarity such as flavor oil (McClements et al., 2012), and hydrocarbons (Weiss et al., 1999). Since oxidation products of triacylglycerols have lower molecular weight and higher polarity due to the addition of oxygen, it is possible that they would exchange between droplet by diffusion. Collision-exchange transfer refers to the process when two droplets merge together and then separate (Rharbi & Winnik, 2002). During this process, materials can be transferred from one droplet to the other.

The third mechanism is micelle-assisted transfer between emulsion droplets. Surfactants in emulsion can adsorb on the droplet surface, and when the surface area is saturated, the unadsorbed surfactants partition in the aqueous (or lipid phases). As the concentrations of unabsorbed surfactants increases, they will form micelles (or reverse micelles). The concentration of micellization is called critical micelle concentration (CMC). Surfactant concentrations used in foods and many experimental conditions are often higher than CMC, and thus surfactant micelles can be found in many oil-in-water emulsions (Berton - Carabin et al., 2014). The hydrophobic core of micelles can solubilize nonpolar components out of lipid droplets which contributes to mass transport between droplets and thus may affect lipid oxidation (Decker et al., 2017). According to the work conducted by Nuchi et al.
The partitioning of lipid hydroperoxides into the water phase of an oil-in-water emulsions was enhanced by increasing surfactant concentrations meaning that hydroperoxides can be solubilized by micelles. Raudsepp et al. (2014a) reported that 2,2'-azobis (2,4-dimethylvaleronitrile) (AMVN), a lipid soluble free radical generator, was transferred between droplets by surfactant micelles. Hydroperoxides and peroxyl radicals are examples of prooxidants that could be transferred between emulsions droplet resulting in alterations in lipid oxidation kinetics in other droplets.

Another hypothesis for prooxidant transfer is that the surface-active lipid oxidation products, e.g. hydroperoxides (LOOHs) can assemble micelles by themselves even when surfactant concentration was lower than the CMC. Brimberg (1993) observed that LOOHs formed reverse micelles in bulk oil and promoted oxidation but hydroperoxide micelles have not be reported in oil-in-water emulsions. Laguerre et al. (2017) suggested that at the beginning of oxidation, without surfactant micelles, LOOHs might not be able to partition into the continuous phase by themselves, but later in oxidation when the emulsion droplet surface is more saturated with LOOHs, hydroperoxides could bud off from the droplets surface to form micelles. However, this hypothesis has not been confirmed and thus future studies are required. Although the ability of surfactant micelles to transfer components between emulsion droplets has been reported, we still do not know if this process can promote oxidation from one droplet to another. A challenge of studying this type of transfer phenomenon is that it is difficult to analyze lipid oxidation components in individual droplets.
Flow cytometry is a technique that has been utilized in many fields such as microbial strain classification, apoptosis progression, and immunophenotyping (Robinson, 2004). Using flow cytometry, cells can be categorized into groups based on properties such as particle size and fluorescence. The high resolution and high throughput of flow cytometry have shown advantages in characterizing individual cells or other units. Samples analyzed by flow cytometer are injected into a pressurized air line with a sheath fluid. The coaxial flow enables cells to align in single stream by hydrodynamic focusing, which then passes the optical unit equipped with argon laser. When an individual particle is illuminated by the laser beam, light scattering is generated and collected by detectors. The scatter signals can be affected by particle size and fluorescence. Different fluorescence probes can be detected with different band pass wavelength filters. Imaging flow cytometry is an upgrade of conventional flow cytometry as it is equipped with a camera, which can capture the images of particles in the flow. Particle and fluorescence in imaging flow cytometry can be quantitated by pixels from image data (Zuba-Surma & Ratajczak, 2011).

Similar with cells, lipid droplets can also scatter light meaning that they would be detectable in flow cytometer. This study aims to use flow cytometry as a new approach to study mass transfer of lipid oxidation products in oil-in-water emulsions. The effect of free fatty acids, surfactants, and NaCl on mass transfer were investigated.
4.3. Materials and methods

4.3.1. Materials

Soybean oil was purchased from local store and stored at -20°C in dark until use. Medium-chain triacylglycerols (MCT) were obtained from Stepan Co. (Northfield, IL). Brominated vegetable oil was purchased from Spectrum Corp. (New Brunswick, NJ). Xanthan gum was donated by TIC Gums (White Marsh, MD). A lipophilic free radical initiator, 2,2'-azobis (2,4-dimethylvaleronitrile) (AMVN), was obtained from Markelin (Shanghai, China). (E,E)-3,5-bis(4-phenyl-1,3-butadienyl)-4,4-difluoro-4-bora-3a,4a-diaza-sindacene (BODIPY 665/676), a lipophilic radical-sensitive probe, was purchased from Thermo Fischer (Eugene, OR). Sodium dodecyl sulfate (SDS), benzoyleacetone (BZA), cumene hydroperoxide, linoleic acid (LA), trans,trans-2,4-decadienal, Nile red, barium chloride, and ferrous sulfate were obtained from Sigma-Aldrich (St. Louis, MO). Ammonium thiocyanate was purchased from Fischer Scientific (Pittsburg, PA). All other reagents were at or above high-performance liquid chromatography grade. Distilled and deionized water was used in all experiments.

4.3.2. Preparation of stripped soybean oil

Soybean oil was stripped to remove minor components such as antioxidants and pro-oxidants according to the method established previously (Chen et al., 2014). A chromatographic column (3.0 cm internal diameter × 35 cm height) was prepared with three layers: the bottom layer was packed with 22.5 g of silicic acid (washed with DDI water); activated charcoal (5.6 g) was used as the second layer and another 22.5
50 g of silicic acid was packed as top layer. Soybean oil (30 g) was dissolved in 30 ml of hexane and loaded onto the column which was then eluted through the column using an additional 270 ml of hexane. The organic solvent was removed from the oil using a vacuum rotary evaporator (Rotavapor R 110, Buchi, Flawil, Switzerland) at 25°C. Residual hexane was evaporated by flushing with nitrogen. Stripped soybean oil (SSO) was stored at -80°C until use.

4.3.3. Preparation of oil-in-water emulsion samples

Nile red was dissolved in chloroform to prepare a 10 μg/ml stock solution. BODIPY<sub>665/676</sub> was dissolved in hexane to obtain a 1.25 μg/ml stock solution. AMVN was dissolved in hexane immediately before use to obtain a 0.4 mg/ml working solution.

Emulsion compositions are summarized in Table 1. Brominated vegetable oil (1.33 g/ml) was used as weighting agent in every emulsion at 25% of the total oil concentrations to match oil density with water density to prevent emulsion creaming. Nile red was used as a lipophilic non-oxidizable dye to visualize the all emulsion droplets in the flow cytometer. BODIPY<sub>665/676</sub>, a lipophilic radical-sensitive fluorescence probe, was used to monitor oxidation in individual droplets. As the content of BODIPY<sub>665/676</sub> in lipid was 1.4 μmol/kg oil (1000 times lower than detectable hydroperoxides), it was assumed that its antioxidant effect would be negligible. Stripped soybean oil or linoleic acid were used as oxidizable substrates and in some experiments AMVN was used as a lipid soluble free radical generator. Organic solvents in all treatments were removed from the lipids by nitrogen flushing.
prior to making the emulsions.

An SDS solution (0.5 mM) was prepared with xanthan gum (1 g/L) to enhance viscosity to inhibit droplet coalescence. Oil-in-water emulsions were prepared with 0.8 g of each oil treatment (MCT, stripped soybean oil, and/or linoleic acid) and 99.2 g of the SDS-xanthan gum solution, which were then homogenized by high-speed mixer (M133/1281-0, Biospec Product Inc., Bartlesville, OK) for 2 min. During homogenization, the emulsions were immersed in an ice bath to minimize oxidation.

4.3.4. Transfer study of primary products

To study the transfer phenomena of oxidation products, emulsion A (MCT + BODIPY<sub>665/676</sub>) was gently blended with emulsion B (stripped soybean oil) at a ratio of 1:1. Two milliliters of this emulsion mixture was pipetted into test tubes. Additional surfactant and/or NaCl solutions were the added to the emulsions to achieve 4 mM SDS + 0 mM NaCl, 16 SDS + 0 mM NaCl, 4 mM SDS + 50 mM NaCl, and 16 SDS + 50 mM NaCl. The emulsion samples were prepared to a final volume of 8 ml, 0.2% oil, and 0.1% xanthan gum.

To study the transfer phenomena in the presence of free fatty acids, emulsion A (MCT + BODIPY<sub>665/676</sub>) was gently blended with emulsion C (MCT + linoleic acid) at a ratio of 1:1. Additional surfactant (4 and 16 mM final concentration) was added and emulsion samples were adjusted to 8 ml as describe above.

AMVN, a liposoluble radical initiator, was used to study the effect of salt on transport of prooxidants. Emulsion A (MCT + BODIPY<sub>665/676</sub>) and emulsion D (MCT + AMVN) was gently blended at a ratio of 1:1. Emulsions were then adjusted
to have an additional 0.5, 2, 4, 8, 12 and 16 mM SDS, and 0 and 50 mM NaCl as
described above.

Samples were stored in 42 °C and lipid hydroperoxides, headspace hexanal, and
the percent of non-oxidized droplets were monitored during storage as described
below.

4.3.5. Evaluation of lipid oxidation

4.3.5.1. Determination of lipid hydroperoxides

Lipid hydroperoxides were measured as primary oxidation products using a
method described by Matalanis et al. (2012). Emulsion samples (1 ml) were added
to 7 ml of an chloroform:methanol (2:1, v/v) solvent mixture, vortexed 3 times for 10
s each and centrifuged for 5 min at 1300 g. After centrifugation, 0.5 ml of the lower
organic solvent layer was mixed with 2.5 ml of methanol:butanol solution (2:1, v/v).
Each sample was then mixed with 15 μl of 3.94 M ammonium thiocyanate and 15 μl
of ferrous iron solution which was prepared by mixing 1 ml of 0.132 M barium
chloride in 1 ml 0.144 M ferrous sulfate. The mixture was vortexed and absorbance
was measured at 510 nm using a spectrophotometer (Thermo-Spectronic, Waltham,
MA) after 20 min of incubation in the dark at room temperature. Hydroperoxide
concentrations were calculated using a standard curve prepared with cumene
hydroperoxide (0-20 μm).

4.3.5.2. Determination of headspace hexanal

Hexanal was determined as a secondary product of lipid oxidation. One
milliliter of emulsion sample was transferred into 10-ml glass vials and sealed with
poly(tetrafluoroethylene) butyl septa at the beginning of the experiments. Headspace hexanal was measured using a method adapted from Cui et al. (2016) using solid-phase microextraction-head space gas chromatography with flame ionization detector (SPME-GC-FID). After the incubation of the sample vials for 10 minutes at 45 °C, a 50/30 μm divinylbenzene / carboxen / polydimethylsiloxane (DVB/CAR/PDMS) stableflex solid phase microextraction (SPME) fiber (Supelco Analytical; Bellefonte, PA) was injected into the vials and exposed in headspace for 2 minutes. The volatile compounds were then desorbed in the injector port for 3 minutes at 250 °C at a split ratio of 1:7. Volatiles were separated using an Equity-1 fused-silica capillary column (30 m × 0.32 mm i.d. × 1 μm) coated with 100% polydimethylsiloxane (Supelco Analytical; Bellefonte, PA). Helium (1.0 ml/min) was used as carrier gas. The flame ionization detector was operated at 250 °C while the oven was at 65 °C. The sample run time was 10 minutes and the peak for hexanal was recorded at 4.9 minutes. Peak area integration was calculated using Shimadzu Lab Solutions version 5.87. A standard curve was prepared using an emulsion spiked with hexanal.

4.3.5.3. Evaluation of oxidation of individual droplets by flow cytometry

The oxidation of individual droplets was monitored using an imaging flow cytometer (ImageStream Mark II operated by INSPIRE software, Amnis/Millipore, Seattle, WA) and calibrated by an ASSIST program (Amnis/Millipore, Seattle, WA). Standard beads (Amnis/Millipore, Seattle, WA) were used as internal standard which were run concurrently for real-time velocity detection and autofocusing. Lasers at
488 nm (200 mW) and 642 nm (150 mW) were used for excitation of the fluorescence probes in the emulsions. The side scatter detectors and brightfield were turned off so images could be collected. Images were collected at 40× magnification. Data acquisition was stopped after 2,000 events were collected for each sample.

Flow cytometry data files were processed by IDEAS software (Amnis/Millipore, Seattle, WA). Mask A (Nile red, excitation 488 nm) was established on Channel 4 at an intensity range from 200 to 4095; mask B (BODIPY<sup>665/676</sup>, excitation 642 nm) was established on Channel 11 with intensity range from 100 to 4095. Diameter (D) of each droplet was quantified on mask A and the intensity (I) of BODIPY<sup>665/676</sup> was quantified on mask B. Relative intensity (RI) of BODIPY<sup>665/676</sup> was calculated by I/D<sup>3</sup>. The droplet population was gated from 3 to 50 μm to minimize interference. Scatter plot of droplets was established between diameter and relative intensity. Oxidized subpopulation was gated on relative intensity from -5 to 15. Non-oxidized subpopulation was gated on relative intensity from 15.01 to 500. The percent of non-oxidized droplets was obtained from the gate.

4.3.6. Transfer study of secondary products

As an oxidizable secondary lipid oxidation product, 2,4-decadienal was used to study the ability of prooxidants to transfer between emulsion droplets. In one emulsion, oil was mixed with 2,4-decadienal and weighting agent (33.3% of the total oil concentrations) to obtain an emulsion droplet density heavier than water. The second emulsion was prepared with MCT alone which would have a density less than water. Both emulsions were prepared by homogenizing 0.2 g of each oil with 99.8 g
of 0.5 mM SDS solution (without xanthan gum) for 1 min using hand-held mixer. The two emulsions were then blended at a ratio of 1:1. This mixture was incubated for 10 minutes at 25 ºC and then centrifuged for 10 min at 10,000 rpm. The creamed emulsion droplet layer and sediment emulsion droplet layer (with weighting agent) were collected with a pipette. One milliliter of each sample was transferred to 10-ml glass vial sealed with poly(tetrafluoroethylene) butyl septa and 2,4-decadienal was quantified by SPME-GC-FID chromatography as described above.

4.3.7. Determination of critical micelle concentration

Critical micelle concentration (CMC) was measured based on the mechanism that the absorbance of the enol form of benzoylacetone (BZA) increases significantly at the presence of micelles (Domínguez et al., 1997). Briefly, 1 mg/ml of BZA stock solution was prepared in methanol and was diluted with water to obtain a 0.03 mM working solution. SDS was then added from 0 to 20 mM. Samples were measured at 312 nm using a spectrophotometer (Thermo-Spectronic, Waltham, MA) after 10 min. CMC was determined as the concentration of SDS after which absorbance rapidly increased. The effects of NaCl (50 mM) and free linoleic acid (0.1%) on CMC were also tested.

4.3.8. Statistical analysis

Analyses were conducted in triplicates and results are presented as means ± standard deviations. Oxidation lag phases were defined as the first data point statistically higher \( (p < 0.01) \) than day zero, which were tested by one-way analysis of variance (ANOVA) followed by Duncan’s multiple comparison using SPSS (Version
4.4. Results and discussion

4.4.1. Effect of surfactants on molecular transfer and lipid oxidation in soybean oil-in-water emulsions

A preliminary study (data not shown) that was conducted with the MCT emulsions containing BODIPY$^{665/676}$ at 42 ºC showed that no loss of fluorescence for 25 days showing that the BODIPY$^{665/676}$ in the MCT emulsions was stable. Figure 10 shows the images of emulsion droplets recorded by the flow cytometer, from which Nile red and BODIPY$^{665/676}$ were both observed on day 0 when emulsions were fresh (Figure 10a). After 5 days (Figure 10b), the free radical-sensitive BODIPY$^{665/676}$ probes were bleached in most of droplets due to oxidation of soybean oil, but Nile red was still available to visualize lipid droplets. A mixture of MCT emulsion containing BODIPY$^{665/676}$ and MCT alone emulsion in the presence of 4 mM SDS showed that no probe was transferred between droplets for 6 days (data not shown).

The next experiment contained two separate emulsions: a medium chain triacylglycerol (MCT) oil-in-water (o/w) emulsion containing BODIPY$^{665/676}$ which was blended with a stripped soybean oil (SSO) emulsion. This experiment was done to determine if products from the oxidation of stripped soybean oil could be transferred to MCT droplets where they would oxidize the BODIPY$^{665/676}$ in the absence and presence of surfactant micelles (Figure 11). When the concentration of
sodium dodecyl sulfonate (SDS) was 4 mM (lower than CMC = 8 mM, Figure 11a), the lipid hydroperoxides and hexanal lag phases were 4 days. The percent of non-oxidized BODIPY-containing droplets was maintained at 100% for 12 days after which BODIPY\textsuperscript{665/676} fluorescence decreased to 66.5% after 16 days. This suggests that in the absence of surfactant micelles, prooxidants such as lipid hydroperoxides and other lipid oxidation products were not able to move to the MCT droplets where they could oxidize BODIPY\textsuperscript{665/676} until well after the lag phase of hydroperoxide and hexanal formation. However, after 12 days, the decay of BODIPY\textsuperscript{665/676} was observed suggesting that some prooxidants could transfer without surfactant micelles when the oxidation level in the SSO emulsions was high.

The stripped soybean oil primarily contains triacylglycerols (TAGs). Transfer of TAGs by Ostwald ripening has been observed with TAGs containing short chain fatty acids such as tributyrin (Li et al., 2009) but not with TAGs containing long chain fatty acids such as those found in the stripped soybean oil (McClements et al., 2012). Even in the presence of surfactant micelles, solubilization of triacylglycerols with long chain fatty acids (corn oil) is thermodynamically unfavorable considering that the size of the micelle core (1.7 nm) is smaller than molecular size of triacylglycerol (3.4 nm). For example, Coupland et al. (1996) used light scattering to show that Tween-20 micelles were not able to solubilize corn oil TAGs but could solubilize n-hexadecane which has similar molecular size (1.8 nm) as a micelle core.

However, when an increased amount of SDS (16 mM) was added to the blended MCT and soybean oil emulsions to produce surfactant micelles (Figure 11b), the loss
of BODIPY\textsuperscript{665/676} fluorescence occurred at a similar time as hydroperoxides (4 day) and hexanal (6 day) formation. During this experiment, the surfactant micelles were able to transfer approximately 25\% of the BODIPY\textsuperscript{665/676} from the MCT emulsions to the soybean oil emulsions in 6 days (data not shown). This can also be seen in the increase in the number of droplets with BODIPY\textsuperscript{665/676} fluorescence in the first 4 days of incubation (Figure 11b). The BODIPY\textsuperscript{665/676} transferred to the soybean emulsions would be oxidized by lipid radicals which would help explain why fluorescence decreased after the hydroperoxide and hexanal lag phases. Although a portion of probes were transferred and bleached in soybean oil droplets, the sharp decrease (69.5\%) of the percent of fluorescent droplets after 20 days suggested that the probe degradation could also have occurred in MCT droplets meaning that oxidation products of soybean oil were transferred by surfactant micelles. The possible oxidation products responsible for the transfer could be surface-active lipid hydroperoxides, or of unsaturated lipid oxidation decomposition products.

\textbf{4.4.2. Effect of surfactants on molecular transfer and lipid oxidation in linoleic acid-in-water emulsions.}

Lipid oxidation kinetics and transfer phenomena in emulsion containing free linoleic acid were also determined (Figure 12). In the presence of 4 mM SDS (below the CMC), the lag phases of both lipid hydroperoxides and headspace hexanal were 6 days. Unlike the soybean oil emulsions, the loss of BODIPY\textsuperscript{665/676} fluorescence started at a similar time as hydroperoxide formation (day 4). This rapid loss of BODIPY\textsuperscript{665/676} fluorescence in emulsions with free fatty acids but not in
emulsions with triacylglycerols suggests that the fatty acids themselves or their oxidation products were able to transfer between lipid droplets even without surfactant micelles. This could also be due to the free fatty acid or its oxidation products moving by themselves or due to the formation of SDS-linoleic acid mixed micelles which would have a CMC lower than SDS itself therefore allowing formation of micelles and thus transfer of linoleic acid or its oxidation products.

Determination of the CMC of SDS solution containing 0.1% linoleic acid showed that the presence of free fatty acids decreased the CMC from 8 mM to 6 mM (Figure 13). This decrease in CMC indicates that SDS-linoleic acid mixed emulsions were forming. Oxidation products of linoleic acid would also be surface-active and could further decrease the CMC of SDS allowing for promoted transfer of prooxidant to the MCT emulsions where they could degrade BODIPY<sub>665/676</sub>.

When additional SDS (16 mM) was added to the linoleic acid emulsions to form micelles, lipid hydroperoxides and headspace hexanal formation were faster with lag phases of 0 and 2 days, respectively (Figure 12b). Once again, an increase in BODIPY<sub>665/676</sub> fluorescence increased in the first 4 days of storage suggesting that the micelles were transferring the BODIPY<sub>665/676</sub> from the MCT to the linoleic acid emulsion droplets. However, Figure 12b shows that the loss of BODIPY<sub>665/676</sub> in the linoleic acid emulsions decreased faster and almost to completion in the 16 days of storage with an oxidation rate of 14.9% droplets/day compared with the soybean oil group (6.8% droplets/day Figure 11b). This is likely due to the combination of BODIPY<sub>665/676</sub> transferring to the linoleic acid droplets where they could be oxidized.
by the lipid radicals and the transfer of linoleic acid and/or its oxidation products to the MCT emulsion droplet where they degraded BODIPY\textsuperscript{665/676}.

**4.4.3. Ability of the lipid oxidation product, 2,4-decadienal, to transfer between MCT emulsion droplets**

Upon the oxidation of fatty acids in TAGs or free fatty acids, more polar, lower molecular weight oxidation products are produced during the decomposition of lipid hydroperoxides. For example, the oxidative decomposition of linoleic and linolenic acids in the stripped soybean oil would produce compounds such as 2,4-decadienal and 2,4,7-decatrienal, respectively. Since these oxidation products have double bonds, if they are transferred to unoxidized emulsion droplets they could be further oxidized, generate free radicals and propagate oxidation in a new emulsion droplet.

To determine if transfer of low molecular weight oxidation products was possible an MCT emulsion was blended with an MCT emulsion containing excess weighting agent and 2,4-decadienal. The blended emulsions were centrifuged so that the two different emulsions could be physically separated (the regular emulsion would cream and the emulsion with the excess weighting agent would sediment) and the droplets from the two different emulsions were analyzed for 2,4-decadienal. Table 2 shows that 10 min after mixing, the 2,4-decadienal was found in both emulsion droplets even without SDS micelles suggesting that these lower molecular weight oxidation products could transfer by diffusion. Therefore, fatty acid decomposition products such as 2,4-decadienal might be a possible source for the observed loss of BODIPY\textsuperscript{665/676} fluorescence in the emulsions made with triacylglycerols or free fatty acids.
4.4.4. Transfer of the free radical initiator, AMVN in the absence and presence of NaCl

AMVN is a lipid-soluble compound that can thermally decompose to a free radical and in the presence of oxygen form hydroperoxides. The oxidation of soybean oil is complex and produces many prooxidative factors that could be transferred between droplets (e.g. hydroperoxides and fatty acid decomposition products). Therefore, AMVN was used in the following experiment to create a simpler model to study the transfer of prooxidants between emulsion droplets. NaCl has also been shown to be a prooxidants in oil-in-water emulsions and its mechanism has been postulated to be due to interactions with transition metals (Cui et al., 2016). However, NaCl can also impact the CMC of ionic surfactants and this interaction could impact micelle-assisted transfer of prooxidants. Thus, the aim of the following experiments was to determine if AMVN-derived prooxidants could be transferred between emulsion droplets and thereby impact oxidation kinetics and to determine if NaCl influences the ability of surfactant micelles to change oxidation mechanisms.

These experiments had an MCT droplet subpopulation containing AMVN that was blended with an MCT droplet subpopulation containing BODIPY\textsuperscript{665/676}. The percent of non-oxidized BODIPY\textsuperscript{665/676} containing droplets as a function of time is shown in Figure 14. Without NaCl, the percent of non-oxidized droplets in the presence of low added SDS concentrations (0.5 and 2 mM) decreased slowly (Figure 14a). On the contrary, emulsions with 8 mM added SDS showed a sharp drop in
fluorescence (only 5% of non-oxidized droplets left after 4 days) suggesting a fast transfer rate of prooxidants. However, when higher surfactant concentrations (12 and 16 mM added SDS) were used, the transfer rates were slower than 8 mM added SDS. The transfer kinetics in the presence of NaCl (50 mM) is shown in Figure 14b. The greatest reduction of fluorescence was found in emulsions with 2, 4, and 8 mM added SDS. To summarize this data, the percent of non-oxidized droplets on day 2 for all emulsions with and without NaCl was plotted against surfactant concentration (Figure 14c). In emulsions with and without NaCl, loss of BODIPY<sub>665/676</sub> exhibited similar patterns: accelerated BODIPY<sub>665/676</sub> degradation occurred when low concentration of added SDS was added, followed by inhibition of BODIPY<sub>665/676</sub> loss at higher SDS concentrations. The maximum decay rates in the absence of added NaCl was at 8 mM added SDS, and 4 mM in the presence of NaCl. In the presence of added NaCl, the transfer of prooxidants was promoted in the emulsions with 2 and 4 mM SDS compared to emulsions without NaCl.

The measurement of CMC of SDS in the presence and absence of NaCl was shown in Figure 15. The CMC of SDS in water was 8 mM and was reduced to 2 mM when 50 mM NaCl was added. Thus, in the presence of NaCl, micelles would be present in the emulsions with 2 and 4 mM added SDS while without NaCl no micelle would be present until added SDS concentrations were greater than 8 mM. This suggests that AMVN and BODIPY<sub>665/676</sub> would be transferred between droplets by micelles at lower SDS concentration in the presence of NaCl and thus loss of BODIPY<sub>665/676</sub> would be greater (Figure 14c).
It was unexpected that the AMVN-triggered degradation of BODIPY\textsuperscript{665/676} would be decreased by an increase of surfactant micelles at high SDS concentration (Figure 14c). A possible explanation is that the greater concentration of micelles could result in a portion of the AMVN partitioning in the micelles instead of being transferred to the BODIPY\textsuperscript{665/676} containing emulsion droplets. If this was the case, the surfactant solubilization would partition the prooxidants into the aqueous phase where its ability to promote the oxidation of BODIPY\textsuperscript{665/676} would be lower.

The effect of NaCl on BODIPY\textsuperscript{665/676} oxidation in the soybean oil emulsion was also determined (Figure 16). In the presence of 4 mM SDS and added NaCl, lipid hydroperoxide and hexanal lag phase where 0 and 2 days, respectively, (Figure 16a) compared to 4 days in the absence of added NaCl (Figure 11a). This agrees with previous research that showed that NaCl is prooxidative in oil-in-water emulsions (Cui et al., 2016). BODIPY\textsuperscript{665/676} degradation was also faster occurring almost immediately which is in contrast to non-NaCl group (Figure 11a, 4 mM SDS + 0 NaCl) where BODIPY\textsuperscript{665/676} oxidation was not observed for 12 days. These differences are likely due to the reduction of the CMC of SDS by 50 mM NaCl from 8 mM added SDS to 2 mM SDS (Figure 15). Thus, the 4 mM SDS + 50 mM NaCl would have micelles and could transfer BODIPY\textsuperscript{665/676} from the MCT to the soybean oil emulsion droplets or soybean oil oxidation product to the MCT droplets both which would cause BODIPY\textsuperscript{665/676} fluorescence degradation.

When the SDS concentration was increased to 16 mM in the presence of 50 mM of NaCl, (Figure 16b), lipid oxidation was slower than with 4 mM SDS and
BODIPY$^{665/676}$ oxidation occurred at similar time as oxidation of the soybean oil (6-10 days). The inhibition of lipid oxidation could be due to the ability of water soluble negatively-charged SDS to chelate metals and partition them away from the lipid since salt promoted oxidation is thought to occur by enhancement of metal reactivity (Cui et al., 2016).

### 4.5. Conclusions

This study used imaging flow cytometry as a novel approach in studying mass transportation of lipid oxidation products by allowing analysis of individual emulsion droplets in o/w emulsions. Results showed that triacylglycerol oxidation products were not able to migrate between individual droplets without micelles unless the system was extremely oxidized. Surfactant micelles increased oxidation rates by transferring components between droplets. Free fatty acids and their oxidation products can transfer between lipid droplets even below the CMC of SDS. This research also showed that NaCl decreases the CMC of SDS. Thus, in emulsions with anionic surfactants, the prooxidant effect of NaCl could also be due to its ability to increase surfactant micelle concentrations which in turn could increase transfer of lipid and lipid oxidation product such as hydroperoxides, free fatty acids and fatty acid decomposition products from oxidized to unoxidized emulsion droplets. Future research could use flow cytometry to study the transfer phenomena of minor components of lipids such as antioxidants with different polarity to have a more holistic understanding of partitioning of minor components in food emulsions.
CHAPTER 5

CONCLUSIONS AND FUTURE WORK

This study used weighting agent and xanthan gum to prepare physically stable oil-in-water emulsions with droplet size from 2-40 μm, and successfully applied flow cytometry as a novel technology in separating and visualizing individual droplets of the emulsions. Combining Nile red and free radical-sensitive BODIPY\textsuperscript{656/676} probes, individual droplets were characterized with high throughput. Results showed that in a polydisperse system, smaller droplets were more prone to be oxidized than larger droplets. In addition, oxidation products were able to transfer from an oxidized subpopulation to the neighboring droplets in the presence of surfactant micelles, and the possible prooxidants responsible for the transfer could be lipid hydroperoxides or decomposition products. Linoleic acid was able to escape out of lipid droplet even when surfactant concentration was lower than critical micelle concentration due to the formation of mixed micelles. The effect of surfactant concentration on transfer rate was investigated using radical generators AMVN, and results showed that the addition of surfactant promoted transfer at beginning but inhibited transfer at high concentration by partitioning prooxidants away from lipid phase. Critical micelle concentration was decreased by NaCl and thus can also affect transfer phenomena. This study has shown the potential of flow cytometry to be used in the study of emulsions and lipid oxidation. Future work may apply flow cytometry in studying how antioxidants be transferred in emulsion system.
## APPENDIX
### TABLES AND FIGURES

Table 1: Inconsistent results of the effect of droplet size on lipid oxidation in emulsions

<table>
<thead>
<tr>
<th>Faster oxidation</th>
<th>Oil</th>
<th>Surfactant</th>
<th>Homogenizer</th>
<th>Droplet size*</th>
<th>Oxidation study</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smaller droplets</td>
<td>DHA</td>
<td>Decaglycerin monostearate</td>
<td>Membrane homogenizer</td>
<td>Small: 2.5-5 μm</td>
<td>25°C</td>
<td>Gohtani et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>Sunflower oil</td>
<td>Serum albumin</td>
<td>Two-stage homogenizer</td>
<td>Small: 0.1-1μm</td>
<td>47°C</td>
<td>Lethuaut et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>Corn oil</td>
<td>Whey protein isolate</td>
<td>Microfluidizer</td>
<td>Small: 0.04-0.15 μm</td>
<td>37°C</td>
<td>Lee et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>Flaxseed oil</td>
<td>Whey protein isolate</td>
<td>Two-stage homogenizer</td>
<td>Small: 0.5-5 μm</td>
<td>25°C</td>
<td>Kuhn and Cunha (2012)</td>
</tr>
<tr>
<td></td>
<td>α-Methyl linolenate</td>
<td>Decaglycerol monolaurate</td>
<td>Membrane homogenizer</td>
<td>Average diameters: 0.45, 0.8, 5, 20, and 29 μm</td>
<td>55°C</td>
<td>Unoxidized lipid</td>
</tr>
<tr>
<td></td>
<td>Fish oil</td>
<td>Tween 80</td>
<td>Microfluidizer</td>
<td>Small: 0.025-0.12 μm</td>
<td>37°C</td>
<td>PV, Propanal</td>
</tr>
<tr>
<td>Larger Droplet</td>
<td>Soybean oil</td>
<td>Decaglycerol lauryl ester</td>
<td>Membrane homogenizer</td>
<td>Small: 0.5-1.2μm</td>
<td>40°C</td>
<td>PV, hexanal, O₂ consumption</td>
</tr>
<tr>
<td></td>
<td>Methyl linoleate</td>
<td>Polyglyceryl-10 laurate</td>
<td>High-pressure homogenizer</td>
<td>Average diameters: 0.041, 0.079, 0.92, 1.7, and 5.9 μm</td>
<td>40°C</td>
<td>Unoxidized lipid</td>
</tr>
<tr>
<td></td>
<td>Rice bran oil</td>
<td>Tween 80, Span 80</td>
<td>Sonicator</td>
<td>Small: 0.08-1 μm</td>
<td>50°C</td>
<td>PV</td>
</tr>
<tr>
<td></td>
<td>Canola oil</td>
<td>Whey protein isolate</td>
<td>High-pressure homogenizer</td>
<td>Small: 0.5-5 μm</td>
<td>50°C</td>
<td>PV, anisidine</td>
</tr>
<tr>
<td></td>
<td>Sunflower oil</td>
<td>Tween-20</td>
<td>Two-stage homogenizer</td>
<td>Average diameters: 3.23, 1.4, 0.84, and 0.67μm</td>
<td>60°C</td>
<td>PV, TBARS</td>
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### Table 2: Optical configuration of imaging flow cytometer (Imagestream Mark II)

<table>
<thead>
<tr>
<th>Channels</th>
<th>Excitation wavelength (nm)</th>
<th>Emission wavelength (nm)</th>
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<tbody>
<tr>
<td></td>
<td>405</td>
<td>488</td>
</tr>
<tr>
<td>1</td>
<td>Brightfield</td>
<td>Brightfield</td>
</tr>
<tr>
<td>2</td>
<td>N/A</td>
<td>480-560</td>
</tr>
<tr>
<td>3</td>
<td>N/A</td>
<td>560-595</td>
</tr>
<tr>
<td>4</td>
<td>N/A</td>
<td>595-642</td>
</tr>
<tr>
<td>5</td>
<td>N/A</td>
<td>642-745</td>
</tr>
<tr>
<td>6</td>
<td>N/A</td>
<td>745-780</td>
</tr>
<tr>
<td>7</td>
<td>435-505</td>
<td>N/A</td>
</tr>
<tr>
<td>8</td>
<td>505-570</td>
<td>N/A</td>
</tr>
<tr>
<td>9</td>
<td>Brightfield</td>
<td>Brightfield</td>
</tr>
<tr>
<td>10</td>
<td>595-642</td>
<td>N/A</td>
</tr>
<tr>
<td>11</td>
<td>642-745</td>
<td>N/A</td>
</tr>
<tr>
<td>12</td>
<td>745-780</td>
<td>N/A</td>
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### Table 3: Summary of fluorescence probes used to detect peroxyl radicals

<table>
<thead>
<tr>
<th>Probes</th>
<th>Intensity change</th>
<th>$\lambda_{\text{excitation}}$ (nm)</th>
<th>$\lambda_{\text{emission}}$ (nm)</th>
<th>Solubility</th>
<th>References</th>
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<tbody>
<tr>
<td>cis-Parinaric acid</td>
<td>Quenching</td>
<td>320</td>
<td>432</td>
<td>Lipophilic</td>
<td>Naguib (1998)</td>
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<tr>
<td>DPPP</td>
<td>Enhancing</td>
<td>351</td>
<td>380</td>
<td>Lipophilic</td>
<td>Okimoto et al. (2000)</td>
</tr>
<tr>
<td>Dipyridanole</td>
<td>Quenching</td>
<td>415</td>
<td>480</td>
<td>Hydrophilic</td>
<td>Iuliano et al. (2000)</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>Enhancing</td>
<td>495</td>
<td>522</td>
<td>Hydrophilic</td>
<td>Valkonen and Kuusi (1997)</td>
</tr>
<tr>
<td>β-Phycocerythrin</td>
<td>Quenching</td>
<td>563</td>
<td>574</td>
<td>Hydrophilic</td>
<td>Cao et al. (1993)</td>
</tr>
<tr>
<td>Fluorescein &amp; 6-carboxyfluorescein</td>
<td>Quenching</td>
<td>495</td>
<td>515</td>
<td>Hydrophilic</td>
<td>Ou et al. (2001)</td>
</tr>
<tr>
<td>Lipophilic derivatives of fluorescein</td>
<td>Quenching</td>
<td>495</td>
<td>515</td>
<td>Lipophilic</td>
<td>Maulik et al. (1998)</td>
</tr>
<tr>
<td>BODIPY$^{581/591}$</td>
<td>Quenching</td>
<td>581</td>
<td>591</td>
<td>Lipophilic</td>
<td>Yoshida et al. (2003)</td>
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<tr>
<td>BODIPY$^{656/676}$</td>
<td>Quenching</td>
<td>656</td>
<td>676</td>
<td>Lipophilic</td>
<td>Raudsepp et al. (2016)</td>
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</table>
Table 4: Preparation of oils and emulsions

<table>
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<tr>
<th>Emulsion</th>
<th>Oil</th>
<th>Weighting agent</th>
<th>Nile red (10 μg/ml)</th>
<th>BODIPY665/676 (1.25 μg/ml)</th>
<th>AMVN (0.4 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.75 g MCT</td>
<td>0.25 g</td>
<td>1 ml</td>
<td>0.5 ml</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.75 g SO</td>
<td>0.25 g</td>
<td>1 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.375 g MCT +0.375 LA</td>
<td>0.25 g</td>
<td>1 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.75 g MCT</td>
<td>0.25 g</td>
<td>1 ml</td>
<td></td>
<td>0.338 ml</td>
</tr>
<tr>
<td>E</td>
<td>1 g MCT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>1 g MCT + 10 mg 2,4-decadienal</td>
<td></td>
<td></td>
<td>0.5 g</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Transfer of 2,4-decadienal between emulsion droplets

<table>
<thead>
<tr>
<th>Emulsions</th>
<th>Individual emulsions before mixing</th>
<th>Mixed and separated emulsions (after 10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCT</td>
<td>0.11 ± 0.09</td>
<td>35.52 ± 2.79</td>
</tr>
<tr>
<td>MCT + 2,4-decadienal</td>
<td>81.61 ± 5.32</td>
<td>44.98 ± 3.86</td>
</tr>
</tbody>
</table>
Figure 1: Hypothesis of lipid hydroperoxides self-assembled micelles when surfactant concentration is below CMC. Adapted from Laguerre et al. (2017)

Figure 2: Schematic of instrumental structure of conventional flow cytometry. Adapted from Adan et al. (2017)
Figure 3: Formation of lipid hydroperoxides in emulsions without and with the weighting agent, brominated vegetable oil. Data points represent means (n=3) ± standard deviation. Some standard deviations lie within the data points.

Figure 4: Size distribution of a sodium dodecyl sulfate-stabilized soybean oil-in-water emulsion droplets measured with a Malvern Mastersizer light scattering size analyzer (a) and ImageStream Mark II imaging flow cytometer (b)
Figure 5: Formation of lipid hydroperoxides (PV) and hexanal in sodium dodecyl sulfate-stabilized soybean oil-in-water emulsion stored at 32°C. Data points represent means (n=3) ± standard deviation. Some standard deviations lie within the data points.

Figure 6: Images of sodium dodecyl sulfate-stabilized soybean oil-in-water emulsion droplets analyzed for either Nile Red or BODIPY 656/676 as determined by imaging flow cytometry during storage at 32°C. The left column of each image pair is Nile red and the right is BODIPY 656/676. (a) Day 0; total emulsion. (b) Day 7; small and large droplets shown separately (c). Day 10; total emulsion.
Figure 7: Percent of non-oxidized small (2-8 μm) and large (20-40 μm) sodium dodecyl sulfate-stabilized soybean oil-in-water emulsion droplets during storage at 32°C (a); Relationship between droplet size and the percent of non-oxidized droplets on day 8 (b). Data points represent means (n=3) ± standard deviation. Some standard deviations lie within the data points.
Figure 8: Size distribution of sodium dodecyl sulfate-stabilized soybean oil-in-water emulsion droplets. Small droplet alone (a), large droplets alone (b), and their polydisperse mixture (c).
Figure 9: Lipid hydroperoxides formation in the subpopulations of a small and large droplet blended sodium dodecyl sulfate-stabilized soybean oil-in-water emulsion during storage study (37°C). Data points represent means (n=3) ± standard deviation. Some standard deviations lie within the data points.
Figure 10: Images of sodium dodecyl sulfate-stabilized soybean oil-in-water emulsion droplets analyzed for either Nile red or BODIPY\textsuperscript{665/676} as determined by imaging flow cytometry during storage at 42 °C. The left column of each image pair is Nile red and the right is BODIPY\textsuperscript{665/676}. (a) Day 0; (b) Day 5.
Figure 11: Formation of lipid hydroperoxides, hexanal, and the percent of non-oxidized droplets of sodium dodecyl sulfate-stabilized emulsions stored at 42°C with stripped soybean oil as oxidizable substrate and different surfactant concentrations: 4 mM SDS (a) and 16 mM SDS (b). The critical micelle concentration of SDS was 8 mM. Data points represent means (n=3) ± standard deviation. Some standard deviations lie within the data points.
Figure 12: Formation of lipid hydroperoxides, hexanal, and the percent of non-oxidized droplets of sodium dodecyl sulfate-stabilized emulsions stored at 42°C with linoleic acid as oxidizable substrate and different surfactant concentrations: 4 mM SDS (a) and 16 mM SDS (b). The critical micelle concentration of SDS was 8 mM. Data points represent means (n=3) ± standard deviation. Some standard deviations lie within the data points.
Figure 13: Critical micelle concentration of sodium dodecyl sulfate was decreased by 0.1% linoleic acid. Data points represent means (n=3) ± standard deviation. Some standard deviations lie within the data points.
Figure 14: Transfer kinetics of AMVN-derived prooxidants in MCT oil-in-water emulsions with 0 mM NaCl and different SDS concentrations (a); Transfer kinetics of AMVN-derived prooxidants in MCT oil-in-water emulsions with 50 mM NaCl and different SDS concentrations (b); The percent of non-oxidized droplets of MCT oil-in-water emulsions with different SDS and NaCl concentrations after two days (c). Data points represent means (n=3) ± standard deviation. Some standard deviations lie within the data points.
Figure 15: Critical micelle concentration of sodium dodecyl sulfate was decreased by 50 mM NaCl. Data points represent means (n=3) ± standard deviation. Some standard deviations lie within the data points.
Figure 16: Formation of lipid hydroperoxides, hexanal, and the percent of non-oxidized droplets of sodium dodecyl sulfate-stabilized emulsions stored at 42°C in the presence of 50 mM NaCl with stripped soybean oil as oxidizable substrate and different surfactant concentrations: 4 mM SDS (a) and 16 mM SDS (b). The critical micelle concentration of SDS was 8 mM. Data points represent means (n=3) ± standard deviation. Some standard deviations lie within the data points.
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