An overview of nanoemulsion characterization via atomic force microscopy

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ABSTRACT
Nanoemulsion-based systems are widely applied in food industries for protecting active ingredients against oxidation and degradation and controlling the release rate of active core ingredients under particular conditions. Visualizing the interface morphology and measuring the interfacial interaction forces of nanoemulsion droplets is essential to tailor and design intelligent nanoemulsion-based systems. Atomic force microscopy (AFM) is being established as an important technique for interface characterization, due to its unique advantages over traditional imaging and surface force-determining approaches. However, there is a gap in knowledge about the applicability of AFM in characterizing the droplet interface properties of nanoemulsions. This review aims to describe the fundamentals of the AFM technique and nanoemulsions, mainly focusing on the recent use of AFM to investigate nanoemulsion properties. In addition, by reviewing interfacial studies on emulsions in general, perspectives for the further development of AFM to study nanoemulsions are also discussed.

KEYWORDS
Nanoemulsion; interfacial characterization; atomic force microscopy; surface force

Introduction
Nanoemulsions are colloidal dispersions composed of two immiscible liquids, typically oil and water, that contain nanometer-scaled droplets. The upper limit of droplet size in nanoemulsions has been reported differently; the variation can be seen across these publications: 100 nm (Goindi et al. 2016; Bazylińska and Saczko 2016), 200 nm (Naseema et al. 2021), or 500 nm (McClements, 2012; Pucek et al. 2020). Due to the unique properties of nanoemulsions, such as very small droplet size, very high stability, and extremely large surface area, they have become one of several emerging technologies in the design of delivery systems for encapsulating, protecting, controlling release rate, and enhancing the bioavailability of many lipophilic bioactive compounds in the pharmaceutical, cosmetics, biotechnology, and food industries (Jin et al. 2016). The development of innovative delivery systems in food industries is limited by the low solubility, stability, and bioavailability of these active compounds. Some of them can be easily volatilized and have low bioavailability, due to rapid metabolism, while others are highly susceptible to degradation or deterioration during processing and storage. However, advances in nanoemulsion technology have addressed these challenges. Food scientists and industries have successfully employed nanoemulsions to encapsulate many active compounds for the development of functional food products, as well as for the modification of food structure (McClements, Decker, and Weiss 2007; Dasgupta and Ranjan 2018; Salem and Ezzat 2018). In addition, nanoemulsions formulated with active ingredients (e.g. cinnamaldehyde and essential oils) have been incorporated into edible coatings and packaging films to enhance the quality and shelf life of many food products (Aswathanarayan and Vittal 2019).

During the preparation of nanoemulsions, either a suitable emulsifier or a combination of two or more emulsifiers is added to achieve long-term stability. Emulsifiers are surface-active molecules and are amphiphilic in nature, allowing them to diffuse, adsorb, and rearrange easily on the interfacial regions to reduce the interfacial tension by which the formation of small, stable, dispersed droplets is facilitated (Aswathanarayan and Vittal 2019). The rearrangement and interaction among the molecules in dispersed and continuous phases and the emulsifiers, which occur in the interface region, are decisive factors for the formation, stability, rheology, and ultimate applications of nanoemulsions. Therefore, to produce nanoemulsion-based functional food products with improved quality, characterization of the interfacial region in terms of interfacial compositions, structure, electrical properties, energy, rheology, and responsiveness to changes in environmental conditions is crucial. This can provide insight on how the interfacial properties affect the overall properties of nanoemulsions and how these interfacial properties are dependent on the type, concentration, and properties of the surface-active components present in the nanoemulsion system, or environmental conditions such as pH, ionic strength, and temperature (McClements 2015). Among these interfacial properties, determination of droplet...
topography and surface interaction forces between droplets or droplet and emulsifier is of great aid in designing and tailoring nanoemulsion-based delivery systems of bioactive compounds. However, such measurements are rarely found in reported studies about food nanoemulsions.

Typically, advanced microscopic techniques such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are widely used to characterize the droplet morphology and size of nanoemulsions (Jin et al. 2016). However, the samples for these techniques are required to be pretreated with staining and/or vacuum, which may result in blurred images and even the destruction of the native state of the droplets (Yang et al. 2007). Meanwhile, the surface force apparatus (SFA), which is known as a leading instrument for determining the interactions between solid surfaces in various liquids, can hardly be used to investigate the droplet interfacial interactions of nanoemulsions, because it is limited to measuring the interactions between flat surfaces to simulate the geometry of the interactions between a sphere and a flat surface (Gunning et al. 2004). With the advances and innovations of atomic force microscopy (AFM) developed since it was invented by Binnig, Quate, and Gerber (1986) more than three decades ago, AFM has become one of the most widespread technologies for investigating the interfacial properties of many organic and biological materials from micron to molecular scale (Marrese, Guarino, and Ambrosio 2017). By employing nano-structured probes to scan material surfaces at subnanometric and atomic resolution, AFM can provide information on the surface interaction forces at the nanometer scale and the imaging of micro/nano-structured surface topography under various environmental conditions (air – "dry mode", aqueous – "wet mode", and vacuum). Thus, it is emerging as a fundamental tool to investigate the behavior of molecules at the droplet interfaces of nanoemulsions. Nevertheless, in previous studies about food-grade nanoemulsions (details in section 4), the use of AFM for interfacial characterization has been limited to the imaging of surface topography under dry conditions, suggesting that the droplets after being deposited on flat substrate surfaces were dehydrated and that the AFM was operated in the air mode. Although such measurements have been successful in droplet imaging, the results may have been affected by measurement conditions that did not represent true nanoemulsion systems in liquid media. In addition, the deformable soft surfaces of food-grade nanoemulsion droplets are limiting factors for applying AFM to determine interfacial interaction forces.

The main focus in this manuscript is on reviewing and proposing perspectives for the applicability of AFM in characterizing the droplet interface properties of nanoemulsions. The current and potential applications of AFM for determining important properties of nanoemulsions, which are determined by chemical composition and physicochemical
properties of disperse and continuous phases are summarized in Figure 1. Also, the fundamental aspects of nanoemulsions and AFM are briefly described.

**Fundamentals of nanoemulsions**

Emulsions are colloidal dispersions of two immiscible liquids, typically oil and water. Depending on the size of the dispersed droplets in the emulsions, which determines their stabilization mechanisms, physical properties, and thermodynamic stability, they can be categorized into macroemulsion, nanoemulsion, and microemulsion (Jin et al. 2016). The main characteristics of nanoemulsions, as compared to other types of emulsions are summarized in Table 1.

Nanoemulsions can be produced using several methods. Based on the form of the forces used to break the dispersed phase, emulsification methods can be divided into two large categories (Figure 2): high-energy emulsifications, in which the droplets are broken by mechanically generated shear and turbulent forces, and low-energy emulsifications, in which the physicochemical energy from the diffusion and phase behavior of the emulsion system is harnessed to break the droplets (Komaiko and McClements 2016). For edible nanoemulsion systems, nanometer-scale droplet sizes are typically fabricated, using extreme mechanical shear, whereas low-energy methods are not appropriate, since they require high concentration of surfactants, which adversely affect food formulation taste and safety (Kumar et al. 2019).

This kinetic stabilization of nanoemulsions is largely governed by processes occurring at the interface and the properties imparted, during both the emulsification process and the long-term storage stability (McClements 2015). It is, therefore, necessary to describe and analyze a nanoemulsion’s interface to explain the various phenomena observed in a nanoemulsion system. The important role of a nanoemulsion’s interface begins at the emulsification process. During the course of emulsification, two processes are competing: disruption of the dispersed phase to produce smaller droplets and recoalescence of the larger droplets formed (McClements 2015). To produce nanoemulsions with small, fine droplets, the recoalescence process should be hindered, and this is a role for the interfacial layer and the emulsifiers. The presence of suitable emulsifiers lowers the interfacial tension between the continuous and dispersed phases, decreasing the energy required to form a new interface. Additionally, the emulsifiers adsorb to the newly formed interface, providing a barrier against coalescence. The various types of emulsifiers yield different results, given the differences in their adsorption behavior on the interface. This is due to their differences in molecular weight affecting interfacial adsorption rate (e.g. small-molecule surfactants versus proteins) (Stang, Karbstein, and Schubert 1994), and in molecular structure determining conformational freedom (e.g. β-lactoglobulin versus β-casein) (Dickinson 1992; Wilde 2000; Poon, Clarke, and Schultz 2001).

The stability of a nanoemulsion is also largely influenced by its interface, since many of the different mechanisms through which nanoemulsions are destabilized are governed by the properties of the interfacial layer. This is most apparent in droplet aggregation, flocculation, and coalescence, all of which involve direct contact between droplets. While it is possible to prevent effective contact by slowing the droplets’ movement (by increasing the continuous phase’s viscosity or forming a network), the interfacial layer can also be manipulated to achieve the same goal. An ionic emulsifier could introduce electric charges on the surface of the droplet, imparting electrostatic repulsion, thus reducing the chance of a collision (McClements 2015). A polymer emulsifier can either lead to enhanced stabilization by the introduction of steric hindrance or, conversely, impart polymer bridging, which leads to droplet aggregation (Dickinson 2003). Additionally, Ostwald ripening can also be slowed down by having a thicker interfacial layer, even though it does not involve droplet-droplet contact (Mun and McClements 2006). To characterize the properties of nanoemulsions, many analytical techniques for bulk measurements have been developed and well documented (Table 2). Meanwhile, those for interface properties, e.g. interface topography and interface interaction forces for understanding the behavior of emulsifiers in the creation and stabilization of nanoemulsion droplets in such a state remain a new frontier for researchers.

Nanoemulsions present interesting opportunities for various applications, especially in the medicine and food sectors. The wide potential of applications can be attributed mainly to the encapsulation of materials in the dispersed droplets, opening up opportunities to protect the enclosed materials from environmental damage and to release the contents in a controlled manner (Lovelyn and Attama 2011; McClements 2013; Tayeb and Sainsbury 2018). A wide range of

| Characteristics | Macroemulsion | Nanoemulsion | Microemulsion |
|-----------------|---------------|--------------|---------------|
| Droplet size (diameter) | 0.1–100 μm | 20–500 nm | 10–100 nm |
| Shape | Spherical | Spherical | Spherical |
| Stability | Thermodynamically unstable, low kinetic stability | Thermodynamically unstable, kinetically stable | Thermodynamically stable |
| Appearance | Turbid/opaque | Clear/translucent | Clear |
| Surface-to-mass ratio (m²/g) | 0.07–70 | 0–300 | 30–1300 |
| Surfactant load | Fairly low | Medium | Fairly high |
| Common preparation methods | Classic homogenization | Low-energy (spontaneous emulsification) and high-energy (extreme mechanical shear) | Low-energy (spontaneous emulsification) |
| Polydispersity | > 40% | 10–20% | < 10% |
Figure 2. (a) High-energy methods for producing nanoemulsions [Adapted with permission from McClements (2012)]; (b) Low-energy methods for producing nanoemulsions. PIC – phase inversion composition, PIT – phase inversion temperature, EIP – emulsion inversion point, O/W – oil-in-water emulsion, W/O – water-in-oil emulsion [Adapted with permission from McClements (2012), Saifullah, Ahsan, and Shishir (2016), Kumar et al. (2019)].

Table 2. Techniques for characterizing nanoemulsions (Jin et al. 2016, Dasgupta and Ranjan 2018).

| Techniques                                      | Measured parameters                                      |
|-------------------------------------------------|----------------------------------------------------------|
| Dynamic light scattering (Zeta-sizer)           | Size and size distribution of droplets                    |
| Zeta potential (Zeta-sizer)                     | Droplet charge - stability of nanoemulsion                |
| UV-Vis Spectrophotometer and Turbidity measurement | Optical density                                           |
| X-ray diffraction and Small-angle X-ray scattering | Optical properties                                       |
| Differential scanning calorimetry               | Crystallinity or fat crystallization                      |
| FTIR spectroscopy                               | Structure, shape and size of droplets                     |
| Nuclear magnetic resonance                      | Phase transitions, crystallization and melting of fat     |
| Rheology                                        | Solid fat or ice crystals in nanoemulsion                 |
| Transmission electron microscopy and Scanning electron microscopy | Molecular fingerprint of nanoemulsion |
|                                                 | Identification of components and their amount in nanoemulsion|
|                                                 | Quality and consistency of nanoemulsion                   |
|                                                 | Fat crystallization                                       |
|                                                 | Mobility, arrangement and environment of the oil molecules|
|                                                 | Structural information regarding molecular compounds      |
|                                                 | Flow behavior and deformation                             |
|                                                 | Imaging (morphology, structure and topography)            |
|                                                 | Size of droplets                                          |

UV-Vis = ultraviolet-visible light, FTIR = Fourier-transform infrared.
pharmaceutical compounds are lipophilic, presenting a challenge to administer them. Such compounds can be administered by nanoemulsions, taking the advantages of not only dispersing them in an aqueous medium, but also having the means of protecting them from degradation before reaching the appropriate receptors, as well as controlling their release.

Figure 3. A sketch of typical atomic force microscope (AFM) components (a) and classification of AFM tips according to their shape, materials, and coating materials (b).
(McClements 2013; Tayeb and Sainsbury 2018). Drugs contained in nanoemulsions can be administered through various routes, including topical, transdermal, intranasal spray, gastrointestinal, injection (Tayeb and Sainsbury 2018), and even by self-emulsifying drug delivery system (Tang et al. 2016; Jin et al. 2016; Ranjan et al. 2016; Saifullah, Ahsan, and Shishir 2016). In the food sector, many compounds can be encapsulated effectively and then added to food to achieve a multitude of goals, including introduction of flavor components (Rao and McClements 2011), fortification of nutrients (Oztürk 2017), formulation of functional foods (McClements 2013, Salvia-Trujillo et al. 2017), and even preservation of food against microbial spoilage (Donsi and Ferrari 2016). The droplets of nanoemulsions are small enough to minimize light scattering, suggesting that they can be added to liquid food without changing the original appearance of the food (Rao and McClements 2011; Donsi and Ferrari 2016). For detailed description, we recommend previous reports on the properties, production, characterization, and applications of nanoemulsions in various fields (Borthakur et al. 2016; Jin et al. 2016; Ranjan et al. 2016; Saifullah, Ahsan, and Shishir 2016).

**Fundamentals of atomic force microscopy and force measurement**

**Fundamentals of atomic force microscopy**

The emergence of the AFM technique for characterization of nanoemulsion interfacial properties originated not only from its advantages over conventional high-resolution interface-characterizing techniques (such as SEM and TEM), which allowed us to investigate the droplet interface under conditions resembling real nanoemulsion systems, but also from the simplicity of the instrument. In comparison to SEM and TEM, the configuration of AFM equipment is quite simple. The main components of a typical AFM system are illustrated in Figure 3a (Jalili and Laxminarayana 2004). It consists of a sharp tip (also known as a probe) attached to the end of a flexible cantilever that acts as a spring. The configurations of the AFM tips determine the resolution and quality of the images taken. The AFM tips can be classified according to their shape, materials, and coating materials; some of them are illustrated in Figure 3b. Further details about the construction and properties of the various AFM tips can be found in AFM distributor websites, such as NanoAndMore USA Corp (2021) and Bruker (2021). A photodiode detects the deflections of the cantilever during scanning over the sample surface via a laser beam focused on and reflected from the rear of the cantilever. A computer system acquires the electrical signals from the photodiode to generate feedback signals (via a feedback loop) to a piezoelectric scanner and the cantilever to maintain the tip at either a constant force or constant height above the sample and to display surface topographic images as well as the interaction forces between the atoms on the tip and sample surface (Jandt 2001; Jalili and Laxminarayana 2004; Eaton and West 2010). The main functions of these AFM components are summarized in Table 3 (Jandt 2001).

| AFM components | Main characteristics |
|----------------|----------------------|
| Cantilever     | - Designed with a very low spring constant so that it is very sensitive to any forces. |
| Tip (probe)    | - Is the part contacting the sample surface to determine its properties, causing the cantilever to deflect. |
| Feedback loop  | - Controls the force between the sample and tip, and z-sample position. |
| Laser source   | - Produces laser beam incident to cantilever. |
| Piezoelectric scanner | Converts electrical signals from the computer controlling system into mechanical scanning motion to control x, y and z-sample position with Å accuracy. |
| Computer       | - Controls the AFM system. |

**Atomic force microscope operating modes and imaging for topography**

The primary purpose of the AFM is to image the sample surface structure at the atomic level. In AFM, the sample surface topography is constructed from the deflection of the cantilever, which is determined by the sample surface features, as the cantilever tip scans over the region of interest on the sample surface. AFM can be operated under vacuum, under water, and in air, depending on the operation modes and applications. The operation of AFM can be classified into three different modes, namely (1) contact, (2) noncontact, and (3) tapping modes, depending on the manner in which the AFM tip interacts with the sample surface. In the contact mode, the AFM tip directly contacts the sample surface during scanning, and the sample surface profiles are generated by operating with either constant height (e.g. the AFM tips scan the sample surface laterally without moving...
in the z-direction) or constant force (e.g. the force between the AFM tip and the sample surface is kept unchanged). Due to frictional forces of the AFM tip applying to the sample surface, the contact AEM can damage the sample surface and possibly distort the features of the images generated and consequently is not suitable for characterization of soft materials under aqueous conditions (Siedlecki and Marchant 1998; Jalili and Laxminarayana 2004). The characteristics of these noncontact AFM operating modes could be found elsewhere (Jandt 2001, Giessibl 2003; Marchant 1998; Jalili and Laxminarayana 2004; Butt, Cappella, and Kappl 2005; Eaton and West 2010).

For the noncontact mode, the AFM cantilever tip moves about 50–150 Å above the sample surface and oscillates near or at its natural resonance frequency. As the AFM tip approaches the sample surface, the attractive van der Waals forces acting between the tip and the sample cause shifting of the resonance frequency and subsequently deflection of the cantilever. Unlike the contact mode, the noncontact AFM allows imaging of the soft materials with no contact between the tip and the sample. However, it was very difficult to obtain high-resolution images from the noncontact AFM, because the true distance between the sample surface and the AFM tip, which is an important parameter for the enhancement of topographical images, can not be determined. In addition, the noncontact AFM mode was unable to image the true sample surface, because the sample surface was typically contaminated with a fluid layer that led to large damping effects on the cantilever resonance. Therefore, the noncontact AFM is not suitable for the study of biological materials under aqueous conditions (Siedlecki and Marchant 1998; Jalili and Laxminarayana 2004).

The tapping mode is a combination of contact and noncontact modes in which the AFM tip oscillates near or at its natural resonance frequency over the sample surface and is allowed to slightly ‘tap on’ the sample surface in a minimal amount of time. Therefore, the shear forces applied on the sample surface are negligible, which enabled the tapping AFM to become the most widely used technique for high-resolution imaging of soft samples under aqueous conditions (Jalili and Laxminarayana 2004). The characteristics of these AFM operating modes are illustrated in Table 4. Further details about AFM principles, equipment, and operation modes could be found elsewhere (Jandt 2001, Giessibl 2003; Jalili and Laxminarayana 2004; Butt, Cappella, and Kappl 2005; Eaton and West 2010).


table 4. Atomic force microscopy (AFM) operating modes (Jandt 2001, Marrese, Guarino, and Ambrosio 2017).

| Operating modes | Principle | Advantages | Disadvantages |
|-----------------|-----------|------------|---------------|
| Contact (static mode) | - Operated by scanning the tip across the sample surface while maintaining the constant deflection of the cantilever - Physical contact between the tip and the surface as the tip drags over sample surface - Strong interaction force (repulsive) with constant cantilever deflection | - High scan speeds - Only mode which can provide "atomic resolution" images - Suitable for rough samples with extreme changes in vertical topography | - Damage to soft samples - Possibilities of image artifacts due to lateral forces - In air, high forces normal to the AFM tip-sample interaction due to capillary forces from the adsorbed fluid layer on the sample surface |
| Non-contact (dynamic mode) | - No contact between the tip and the sample (the cantilever oscillates close to sample surface without touching at a frequency slightly above the cantilever resonance frequency with a typical amplitude less than 10 nm) - Weak interaction force (attractive) with vibrating probe | - Low lateral resolution which is limited by the AFM tip-sample separation - No damage to sample surface due to no forces exerting on the sample surface - Possibility of atomic resolution in a ultra-high vacuum environment | - Usually only suitable for extremely hydrophobic samples, as the adsorbed fluid layer is at its minimum. Otherwise, the tip becomes trapped in the adsorbed fluid layer resulting in unstable feedback and scrapping of the samples - Slower scan speed than contact and tapping modes - Slower scan speed than contact mode |
| Tapping (dynamic mode) | - Light contact between the sample and the tip (the cantilever oscillates at or slightly below its resonance frequency with an amplitude of 20-100 nm and the tip makes repulsive contact with the sample surface at the lowest point of the oscillation) - Strong interaction force (repulsive) with vibrating probe | - Minimal damage to samples due to lower lateral forces acting on the sample surface - High lateral resolution on most samples | |

**Force measurement**

In addition to imaging of the sample surface topography, the determination of the force-distance curve, which can be employed to investigate many surface material properties including surface forces, is another important function of the AFM technique (Cappella and Dieterl 1999). To perform the force measurement in most commercial AFMs, the cantilever deflection signals from the photodiode are monitored as the piezoelectric scanner moves the sample surface up and down (z-direction), by which the cantilever tip approaches, touches on, and retracts from the sample surface to complete a circular movement. A plot of the tip sample interaction forces versus the tip sample distance, known as a force-distance curve, provides quantitative information about the interfacial forces acting between the tip and the sample surface. A typical such curve is shown in Figure 4a. The deflection of the cantilever from its original position is dependent on the distance between the tip and the sample surface. Initially, as the piezoelectric scanner begins to extend and the tip is still far from the sample surface, there
Figure 4. (a) A typical force-distance curve [Adapted with permission from Siedlecki and Marchant (1998)]. The AFM tip is far from the sample surface, with no interaction or cantilever deflection (A). Moving to the left as the tip approaches the sample surface, attractive van der Waals forces increase and induce the tip to jump to contact (B). As the tip presses into the surface, the cantilever bends upward (C point). When the tip is pulled away from the sample surface, the cantilever relaxes downward until the tip forces are in equilibrium with the surface forces (D point). Due to the adhesive forces between them, they remain in contact as piezo-retraction continues (E point). When the cantilever deflection is large enough to overcome the adhesive force, it is completely pulled away from the sample surface (F point) and returns to its starting deflection point (G point). The arrows in the small images (A–E) indicate the movement direction of the piezoelectric scanner. (b) Configurations of the force-distance curves of samples with different surface properties.
is no force interaction or cantilever deflection detected (A point). When the tip approaches the sample surface at a distance larger than 10 nm, the long-range forces such as electrostatic and hydrophobic interactions are dominant, and the cantilever deflects according to the forces that it experiences. If the forces between the tip and the sample surface are repulsive, the cantilever reflects away from the sample surface. In the case of attractive forces, the cantilever bends toward the sample surface. When the separation between the tip and the sample surface is less than 10 nm, the attractive van der Waals force begins to increase and gradually becomes larger than the spring constants of the cantilever; the tip jumps to contact the sample surface (B point) and presses on the sample surface, which makes the cantilever bend upwards (C point). When the piezoelectric scanner retracts and the tip is pulled apart from the sample surface, the cantilever relaxes downward until the tip forces are in equilibrium with the surface forces (D point). When the piezoelectric scanner retracts beyond the distance of the initial jump contact, due to the adhesive forces between the tip and the sample surface, they remain in contact (E point), and this leads to a hysteresis between the approaching and retracting curves. Since the forces induced by the cantilever deflection are larger than the adhesive forces, the tip separates from the sample surface (F point) and returns to its starting deflection point (G point) (Siedlecki and Marchant 1998; Jandt 2001; Krasowska, Prestidge, and Beattie 2014).

From the force-distance curve, the force interactions between the tip and sample can be determined when the spring constants of the cantilever are known. By functionalization and modification of the tip with substrates having different origins and natures, and by attaching colloidal particles/droplets to the tip, the applicability of the AFM for surface force measurement has been enormously expanded from two rigid solid surfaces to two deformable soft droplets, and even between two surfaces that are premodified with the functional groups desired (e.g. hydrophobic and hydrophilic groups) (Butt, Cappella, and Kappl 2005; Maver et al. 2011). In addition to surface forces, the surface elasticity of the materials analyzed can be extracted from the force-distance curve. As can be seen in Figure 4a, from point (B) to point (E) where the tip compresses on the sample surface, the cantilever deflection extent is dependent on the movement of the tip on the sample surface, which in turn is influenced by the hardness of the sample surface. If the sample surface is soft, the cantilever will reflect less, due to further compression of the tip on the sample surface as it is ascended by the piezoelectric scanner. In contrast, if the sample surface is hard, ascending it results in a larger amount of cantilever deflection. The extent of cantilever deflection measured for a certain downward movement distance of the tip indicates the elasticity of the sample surface (Morris 2013). The force-distance curve varies, depending not only on the AFM operating mode and the properties of the sample surface, but more importantly on the tip properties (especially the 'true value' for the spring constants of the cantilever) and the imaging conditions determining the contribution of additional forces to the force-distance curve, e.g. the capillary force as imaging in air and the electrostatic force, osmotic pressure, hydration force, solvation force, and adhesion force as imaging in fluids (Lal and John 1994; Jandt 2001). Figure 4(b) illustrates various configurations of the force-distance curves of samples with different surface properties (Veeco Instruments Inc. 2004).

**Characterization of nanoemulsions using atomic force microscopy**

To the best of our knowledge, presently the use of AFM to characterize nanoemulsions, especially in food science and nutrition sectors, has only been limited to the determination of the morphology (shape and structure) and the size of droplets. A summary of such reported studies is shown in Table 5. In these studies, nanoemulsions of functional compounds such as essential oils: eugenol, thymol, fish oil, lemon oil, lemongrass essential oil and basil oil; β-carotene; or curcumin were investigated using food-grade emulsifiers (e.g. whey protein isolate, sodium caseinate, maltodextrin, κ-carrageenan and lecithin), demonstrating a highly potential applicability of AFM to study nanoemulsions in food sciences. To successfully image the droplet topography, the droplets or particles of nanoemulsions must be firmly attached in their native and intact state to very smooth solid surfaces to resist the lateral forces caused by the AFM scanning tip (El Kirat et al. 2005). In addition, the droplets must be well dispersed on the solid surface, which is determined by several factors, including the exposure time and the dilution ratio of the nanoemulsions, the interfacial free energy and electrostatic energy associated with the droplets, the hydrophilic/hydrophobic forces interacting between droplets, surface, and solution, and the additives and surfactants present in the nanoemulsions. Surfaces commonly used to fix the droplets include mica, glass, and silicon oxide. Among them, mica (a nonconducting layered mineral composed of multiple 1-nm-thick layers) is the most preferred, due to its unique properties such as being atomically flat, clean after cleavage, easy to cut to desired size, relatively inexpensive, and negatively charged surface that can be modified to make the surface positive (Starostina and West 2006).

In the studies reported in Table 5, the sample preparation followed the same procedure. Most nanoemulsions must be diluted into distilled water from 100 to 1000 times to avoid agglomeration and coalescence of the droplets. The diluted solution is then deposited on the freshly cleaved mica substrate. In some cases, the deposited droplets are washed with distilled water before dehydration by either leaving it overnight in a dust-protected environment at room temperature or using a furnace/heater to accelerate the drying process (Salvia-Trujillo et al. 2013). The binding (adhesion) of droplets on the substrate surface is usually accomplished via electrostatic attraction (e.g. adsorption) between the charges on the sample and those on the mica surface. To facilitate chemical bonding between droplet and substrate, surface-coating chemicals such as poly-L-lysine, poly-D-lysine, polyethyleneimine or aminopropyltriethoxy-silane are normally added to the samples before the droplets are deposited on...
Table 5. A summary of reported studies in which atomic force microscopy (AFM) has been used to characterize the nanoemulsions with potential application in food science and nutrition.

| Encapsulated components | Components in dispersed and continuous phase, or surfactants | Preparation | AFM operating mode and results | References |
|-------------------------|-----------------------------------------------------------|-------------|--------------------------------|------------|
| - Eugenol               | - Lauric alginote  
- Lecithin | High-speed homogenizer | - Tapping mode  
- Morphology: mostly spherical particles  
- Droplet size: 90 nm (eugenol)  
- 100 nm (thymol) | Ma et al. (2016) |
| - Thymol                | - Tween 80  
- Span 80 | High-speed and high-pressure homogenizer | - Tapping mode  
- Morphology: undefined shapes | Galvão et al. (2018) |
| Pepper extract          | - Tween 80  
- Span 80 | High-speed and high-pressure homogenizer | - Tapping mode  
- Morphology: spherical particles  
- Droplet size: 29 nm (with PG)  
- 117 nm (without PG) | Xue (2015) |
| Thymol oil              | - Whey protein isolate and maltodextrin conjugates  
- Propylene glycol (PG) | High-speed homogenizer | - Tapping mode  
- Morphology: spherical particles  
- Droplet size: 134.5 nm (NaCas)  
- 147.3 nm (lecithin), and  
- 76.9 nm (NaCas and lecithin) | Xue and Zhong (2014) |
| - Sodium caseinate      | - Lecithin  
- Propylene glycol | High-speed homogenizer | - Tapping mode  
- Morphology: Mostly spherical particles  
- Droplet size: 76.8 nm | Pan et al. (2014) |
| - Casein hydrolysates   | - Sucrose stearate (SS)  
- Propylene glycol | Phase inversion temperature | - Tapping mode  
- Morphology: Discrete and mostly spherical particles  
- Droplet size: 36.1-50.4 nm depending on SS content | Su and Zhong (2016b) |
| Sodium caseinate        | High-speed homogenizer | - Tapping mode  
- Morphology: Mostly spherical particles  
- Droplet size: 76.8 nm | Pan et al. (2014) |
| Fish oil                | - Whey protein isolate  
- Tween 80  
- Span 80 | High-speed and high-power ultrasonic homogenizer | - Tapping mode  
- Droplet size: < 100 nm | Nejadmansouri et al. (2016b) |
| - β-carotene            | - WPI  
- Lecithin  
- Soybean oil | High-speed homogenizer | - Tapping mode  
- Differentiated morphology and dimensions of droplets of nanoemulsions prepared from different components | Guan et al. (2016) |
| Lemon oil               | - Sodium caseinate (NaCas)  
- Tween 20 | High-speed homogenizer and phase inversion temperature | - Tapping mode  
- Differentiated morphology and dimensions of droplets of nanoemulsions prepared from NaCas and from NaCas and Tween 20 | Su and Zhong (2016a) |
| Neem oil                | - Tween 20 | High-energy sonicator | - AFM operating mode: not mentioned  
- Morphology: Spherical shape  
- Droplet size: 48.2 nm | Anjali et al. (2012) |
| Lemongrass essential oil| - Sodium alginate  
- Tween 80 | High-speed and high-pressure homogenizer | - Tapping mode  
- Morphology: Spherical shape  
- Droplet size: 50-150 nm | Salvia-Trujillo et al. (2013) |
| Basil oil               | - Tween 80 | High-energy sonicator | - AFM operating mode: not mentioned  
- Morphology: Spherical shape  
- Morphology: Spherical shape with smooth surface  
- Droplet size: 20.1 nm | Ghosh et al. (2013) |
| β-carotene              | - Tween 20  
- Whey protein isolate | High-speed and high-pressure homogenizer | - Tapping mode  
- Morphology: Approximately spherical particle with sags and crests  
- Droplet size: 100-200 nm | Mao et al. (2009) |

(continued)
| Encapsulated components | Components in dispersed and continuous phase, or surfactants | Preparation | AFM operating mode and results | References |
|-------------------------|-------------------------------------------------------------|-------------|--------------------------------|------------|
| Eucalyptus oil          | - Tween 80                                                  | High-energy sonicator | - AFM operating mode: not mentioned  
- Morphology: Approximately spherical particle  
- Droplet size: 20.1 nm | Sugumar et al. (2013) |
| Vitamin E acetate       | - Tween 80  
- Mustard oil  
- Phase inversion component | Phase inversion component | - FM operating mode: not mentioned  
- Morphology: Approximately spherical particle | Dasgupta et al. (2016) |
| Curcumin                | - Medium chain triglycerides  
- Octenyl-succinic-anhydride-modified starch  
- Chitosan  
- Carboxymethyl cellulose | High-speed homogenizer and high-energy sonicator | - AFM operating mode: not mentioned  
- Morphology: elongated or disc-shaped droplets for primary nanoemulsion; more compact and near spherical for CMC-/chitosan-coated nanoemulsions | Abbas et al. (2015) |
| Capsicum                | - Tween 80  
- Glycerol  
- Phase inversion component | High-speed, high-pressure, and high power ultrasonic homogenizer | - Tapping mode  
- Droplet size: $< 70$ nm | Akbas et al. (2018) |
| Canola oil              | - $\alpha$-tocopherol/  
- Cinnamaldehyde  
- $\alpha$-tocopherol/garlic oil  
- $\alpha$-tocopherol/  
- Cinnamaldehyde and garlic oil  
- Span 60  
- Tween 20 | High-speed and high-pressure homogenizer | - Tapping mode  
- Droplet size: $96$ nm $- 1.07 \mu$m (lecithin) and $20$ nm $- 1 \mu$m (SMP)  
- Contact mode  
- Morphology: Uniformly sized spherical particles  
- Droplet size: $110$–$150$ nm | Pérez-Córdoba et al. (2018) |
| Lemongrass essential oil| - Chitosan olate  
- Oleic acid  
- Spontaneous emulsification | Spontaneous emulsification | - Non-contact mode  
- Spherical morphology and homogeneous dimensions | Bonferoni et al. (2017) |
| Medium chain triglycerides (Miglyol 12TM) | - Purity Gum 2000 (primary nanoemulsion)  
- Chitosan and $\beta$-carrageenan (3 and 5-layer nanocapsules) | High-speed and high-pressure homogenizer | 1). Topography and droplet size: tapping mode  
- All capsules: spherical shape, smooth surface  
- Droplet size: 50–500 nm  
2). Force-volume measurement: contact mode  
Allowed to distinguish between nanoemulsions and nanocapsules via stiffness of the wall | Preetz et al. (2010) |
| Seaweed oil             | - $\kappa$-carrageenan  
- Tween 80  
- Phase inversion component | High-speed homogenizer and ultrasonic homogenizer | - Tapping mode  
- Droplet size: $47.6$ nm (day 0) and $59.2$ nm (day 30) | Saravana et al. (2019) |
| Essential oil           | - Tween 80  
- Phase inversion component | Ultrasonic homogenizer | - AFM operating mode: not mentioned  
- Droplet morphology: spherical shape  
- Droplet size: $82.64$ nm | Javanshir et al. (2020) |
| Benznidazole            | - Medium chain triglyceride (Miglyol®812)  
- Sodium oleate  
- Soy phosphatidylcholine | Phase transition | - Non-contact mode  
- Droplet size: 79.5–241.6 nm | Streck et al. (2019) |
| $\beta$-Carotene        | - Scallop gonad protein isolates  
- Medium-chain triglycerides and corn oil | High-speed and high-pressure homogenizer | - Tapping mode  
- Droplet morphology: spherical and uniform shape  
- Droplet size: 250–300 nm | Han et al. (2020) |
| Cinnamaldehyde          | - Tween 80  
- Phase inversion component | Magnetic stirred for 24 h | | Ji et al. (2021) |
### Table 5. Continued.
| Encapsulated components | Components in dispersed and continuous phase, or surfactants | Preparation | AFM operating mode and results | References |
|-------------------------|------------------------------------------------------------|-------------|--------------------------------|-------------|
| Thyme oil               | - Sodium caseinate                                         | Self-emulsification by utilizing pH-dependent solubility of thyme oil | - AFM operating mode: not mentioned  
- Droplet size: > 97.45 nm  
- Tapping mode  
- Droplet size: 52.8–57.3 nm  
- Intermittent contact mode  
- Droplet size: 150–300 nm | Zhang and Zhong (2020) |
| Cholesterol             | - Castor oil  
- Phosphatidylcholine  
- Sodium oleate  
- Tween 80 | Sonicator | - AFM operating mode: not mentioned  
- Droplet size: < 200 nm  
- Tapping mode  
- Droplet size: 218 nm | Keykhasalar, Homayouni Tabrizi, and Ardalan (2020)  
Romes et al. (2020) |
| Seed essential oil      | - Tween 80                                           | Ultrasonic homogenizer | - AFM operating mode: not mentioned  
- Droplet morphology: spherical shape  
- Droplet size: 100–150 nm | Copetti et al. (2020) |
| Elaeis guineensis leaves extract | - Olive oil and sunflower seed oil  
- Brij L23 and Span 80  
- Span 80  
- Tween 80 | High-speed and ultrasonic homogenizer | - Non-contact mode  
- Droplet morphology: spherical shape  
- Droplet size: 150 nm | Pomputtapitak et al. (2019) |
| Astrocaryum aculeatum extract | - Span 80  
- Medium-chain triglycerides  
- Tween 80 | Spontaneous emulsification | - AFM operating mode: not mentioned  
- Provided insight of phase inversion from O/W to W/O nanoemulsion during moisture reduction | Lin et al. (2020) |
| γ-Oryzanol              | - Cetyl palmitate and caprylic/capric triglycerides  
- Span 40  
- Tween 80 | High-speed and high-pressure homogenizer | - Non-contact mode  
- Droplet morphology: regular spheres  
- Droplet size: < 100 nm | Nikolic et al. (2020) |
| Turmeric oil            | - Tween 80  
- Span 80  
- Propylene glycol | Phase transition | - AFM operating mode: not mentioned  
- Droplet morphology: spherical shape  
- Droplet size: 112.6 nm | Karami et al. (2019) |
| Curcumin                | - Medium-chain triglycerides  
- Eucalyptol and pinene  
- Polysorbate 80  
- Soybean lecithin | Spontaneous emulsification | - Non-contact mode  
- Droplet morphology: regular spheres  
- Droplet size: largely  
-< 100 nm | Medina-Alarcón et al. (2020)  
Romes et al. (2020)  
Javanshir et al. (2020) |
| Indinavir (protease inhibitor) | - Oleic acid  
- α-tocopherol  
- Span 8  
- Tween 80  
- Glycerol | High-speed homogenizer | - AFM operating mode: not mentioned  
- Droplet morphology: spherical shape  
- Droplet size: 112.6 nm | Medina-Alarcón et al. (2020)  
Romes et al. (2020)  
Javanshir et al. (2020) |

The substrate surface (Starostina and West 2006; Singh et al. 2015). The most common AFM operating mode used to characterize the droplets in nanoemulsions was the tapping or noncontact mode to avoid damaging on the droplet surface, and the imaging was performed under dry conditions. Taken images were then utilized for droplet morphology and size determination.

For the characterization of nanoemulsions, AFM is currently used as a complementary approach to other analytical methods to confirm the properties of nanoemulsions. Nevertheless, the results are not always comparable, indicating challenge in the use of AFM to study nanoemulsions. The highly compatible results in the determination of droplet size and the morphology of nanoemulsions between AFM and other analytical techniques, such as dynamic light scattering, confocal laser-scanning microscopy, and TEM were reported for thymol nanoemulsions with sodium caseinate (NaCas), lecithin, and propylene glycol (Xue and Zhong 2014); fish-oil nanoemulsions with whey protein isolate (WPI) alone or in combination with Tween 80 and Span 80 (Nejadmansouri et al. 2016b); basil oil nanoemulsions with Tween 80 (Ghosh, Mukherjee, and Chandrasekaran 2013); curcumin nanoemulsions with medium-chain triglycerides and octenyl succinic anhydride-modified starch (Abbas et al. 2015); canola oil, α-tocopherol and garlic oil, α-tocopherol and cinnamaldehyde, and α-tocopherol, cinnamaldehyde and garlic oil nanoemulsions with Tween 20 and Span 60 (Pérez-Córdoba et al. 2018); and β-carotene nanoemulsions with Tween 20 or WPI (Mao et al. 2009). Similarity in droplet size measured by AFM and dynamic light scattering was also reported for nanoemulsion of many functional components with different emulsifiers such as essential oils with Tween 80 (Javanshir et al. 2020); benznidazole with medium-chain triglyceride, sodium oleate and soy phosphatidylcholine (Streek et al. 2019); β-carotene with scallop gonad protein isolates, medium-chain triglycerides and corn oil (Han et al. 2020); cholesterol with castor oil, phosphatidylcholine and sodium oleate (Medina-Alarcón et al. 2020); elaeis guineensis leaves extract with Brij L23 and Span 80 (Romes et al. 2020); astrocaryum aculeatum extract with Span 80, medium-chain triglycerides and Tween 80 (Copetti et al. 2020); γ-oryzanol
with cetyl palmitate, caprylic/capric triglycerides, Span 40 and Tween 80 (Pornputtapitak et al. 2019); indinavir (protease inhibitor) with oleic acid, α-tocopherol, Span 8, Tween 80 and glycerol (Karami et al. 2019). An example of such a study is shown in Figure 5a, in which the size and morphology of curcumin nanoemulsion droplets determined by AFM and TEM were similar.

However, in many studies the AFM results were quite different from those obtained from other analytical techniques, making the use of AFM to characterize nanoemulsions doubtful in its accuracy and applicability. Ma, Davidson, and Zhong (2016) reported that the dehydration process during sample preparation, which can result in the flattening of droplets, made the droplet diameter of eugenol and thymol nanoemulsions with lauric alginate and lecithin, as determined by AFM, 30 nm larger than the hydrodynamic diameter (measured by particle-size analyzer). With similar reason, AFM measurement gave a larger droplet size of cinnamaldehyde nanoemulsion with Tween 80 than laser dynamic light scattering (Ji et al. 2021). Similarly, Akbas, Soyler, and Oztop (2018) and Akbas, Soyler, and Oztop (2019) found that the droplet size of capsicum nanoemulsions with Tween 80 and glycerol, and sucrose monopalmitate (or lecithin) and glycerol, respectively, measured by AFM, was much larger than that observed under TEM. Together with sample preparation, the weak adsorption of the smallest dispersed droplets to the mica surface and the increase in size of nanoparticles caused by the broadening effect of the tip being in contact with soft or sticky biomaterials, were the reasons for the larger droplet size of lemon-grass essential oil nanoemulsions with sodium alginate and Tween 80 observed in AFM images, compared with the value determined by TEM or dynamic light scattering (Salvia-Trujillo et al. 2013).

However, Guan, Wu, and Zhong (2016) reported a contrasting trend in which the particle size of β-carotene nanoemulsions with lecithin, soybean oil, eugenol and WPI measured by AFM was much smaller than the hydrodynamic diameter. A smaller droplet size determined by AFM than that measured by dynamic light scattering was also reported by Saravana et al. (2019) for seaweed oil nanoemulsions with Tween 80 and κ-carrageenan; Zhang and Zhong (2020) for thyme oil nanoemulsion with sodium caseinate; Keykhasalar, Homayouni Tabrizi, and Ardalan (2020) for seed essential oil nanoemulsion with Tween 80. Meanwhile, Bonferoni et al. (2017) stated that the reason for the much smaller droplet size of lemon-grass essential oil nanoemulsion with chitosan oleate and oleic acid, as
measured by AFM in comparison to photon correlation spectroscopy, was the presence of some aggregates that could not be completely avoided in photon correlation spectroscopy measurement, regardless of the dilution in sample preparation. Likewise, Su and Zhong (2016b) reported that the differences in the operating principles between AFM and dynamic light scattering could have been the reasons for the dissimilarities in droplet size of the thymol nanoemulsions determined by these two methods. Regarding morphology, Galvão et al. (2018) also found that unlike micrographs of AFM, which showed the droplets of pepper nanoemulsions with undefined shape, cryo-SEM indicated the presence of clear spherical droplets with sufficient uniformity and droplets of the oil phase surrounded by the aqueous phase.

In addition, AFM was also reported for its ability to differentiate the nanoemulsion properties prepared from different surfactant types. Xue (2015) found that under AFM images, thymol nanoemulsions prepared with propylene glycol had much smaller droplet size and more uniform particles than those prepared without propylene glycol, and these results were in agreement with the transparent and turbid appearance of the two samples. Likewise, Su and Zhong (2016b) found differences by AFM in the droplet size of thymol nanoemulsions with casein hydrolysate and different amounts of sucrose stearate. Adding a small amount of sucrose stearate (0.25%, w/v) led to a significant reduction in droplet size, due to the enhancement of packing of casein hydrolysate and sucrose stearate molecules caused by the hydrophobic attractions of thymol, while at a higher concentration of sucrose stearate (1%, w/v), the droplet size became larger, due to the excess amount of sucrose stearate. Lemon oil nanoemulsions prepared with NaCas and Tween 20 had relatively smaller particles than those prepared with Tween 20 alone (Su and Zhong 2016a). Importantly, AFM also allowed us to differentiate between the nanoemulsions stabilized by small- and large-molecule emulsifiers (Figure 5b). Under AFM, some aggregated droplets in the β-carotene nanoemulsions stabilized with Tween 20 could be observed, indicating the instability of the nanoemulsions, while the droplets in the WPI-stabilized nanoemulsion were better distributed and thus of higher stability (Mao et al. 2009). Results in these studies suggest that AFM can be expanded to study the adsorption behavior of surfactant(s) on the dispersed droplet surface of nanoemulsions.

In addition, there were several granted patents in which AFM was employed to determine droplet diameter of O/W nanoemulsion cosmetic moisture cream, e.g. 43–186 nm (Kim et al. 2006, Kim, Park, et al. 2010, 2012), and morphology of droplets in nanoemulsion of plant essential oils (Kim, Kim et al. 2010). In these patents, the sample preparation and operating conditions for AFM analyses are similar to those in the studies reported in Table 5. In conclusion, the reported studies and patents found in literature so far have confirmed the current application of AFM in characterizing nanoemulsion is limited to determination of shape, appearance and size of dispersed droplets which were previously dried on the flat surface, e.g. dry mode AFM. Changes in droplet characteristics induced during sample dehydration for AFM are the primarily reasons for incompatibilities in analyzed results between AFM and other complementary methods. This causes current utilization of AFM to study nanoemulsions to be doubted for their accuracy and reliability. For dry mode AFM of nanoemulsions, care must be taken, and conditions for sample preparation need to be optimized to preserve intact and native features of dispersed droplets. Also, techniques for such AFM analyses in “liquid mode” should be developed, allowing to characterize nanoemulsion droplets in “as such” state. In the following section, the further potential applicability of AFM to determine other important interfacial properties of nanoemulsions in both “dry and liquid mode” in food and nutrition fields is proposed.

**Future perspectives for atomic force microscopy development in the study of nanoemulsions**

Unlike imaging for surface topography and droplet size determination, the use of AFM to investigate the characteristics of the droplet interface of nanoemulsions such as mechanical properties (e.g. elasticity, hardness, adhesion, surface-charge densities), distribution and adsorption behaviors of surfactants, and surface interaction forces between droplets, or between droplets and surfactants; all of which are important to design nanoemulsion-based delivery systems for functional compounds in various areas including food industries; has rarely been reported, even in “dry mode AFM”. So far, only one study dedicated to determining the surface force of nanoemulsion droplets, using AFM, could be found. Preetz et al. (2010) reported the AFM procedure for force measurement to determine the mechanical properties of the interfacial layers of nanoemulsions and nanocapsules prepared from medium-chain triglycerides, octenyl succinic anhydride-modified starch, chitosan, and λ-carrageenan. In this approach, the contact AFM mode was used to enhance the force-curve analysis, and the measurement was carried out under aqueous conditions to eliminate the capillary forces that can lead to hysteresis in the force-distance curve. By analyzing the interaction forces between the AFM tip and sample surface, the authors successfully distinguished nanocapsules with a 3–5-layer shell from nanoemulsions with a one-layer shell via the stiffness of interfacial shells. Systems with a higher number of layers were of greater stiffness and, thus, of greater stability and improved capability of prolonging core release. The lack of such studies may have resulted from their extremely complex sample preparation and optimization of AFM operating conditions, due to the deformability and nanometric size of the dispersed droplets. For “dry mode” AFM, as discussed in the previous section, dehydration during sample preparation could lead to changes in droplet properties. Once this problem is solved, determination of the interfacial properties of nanoemulsion droplets can be extended beyond imaging with the currently advanced AFM techniques. For “liquid mode” AFM, there is still lack of a general technique to effectively fix nanoemulsion droplets on a flat surface. However, building on established AFM theories, we have applied the results of
studies on microdroplet emulsions to the field of nanoemulsions, and this could lead to very interesting further investigations, given the undeniable importance of nanoemulsions in many practical applications. In the remaining part of this section, we present previous works on macroemulsions that can serve as a starting point for similar approaches to nanoemulsions.

Ideas of employing AFM for the direct measurement of surface interaction forces between colloidal particles under aqueous conditions have been reported in recent decades. This method was known as a 'hard probe technique' in which a silica glass or polystyrene sphere with a diameter of several micrometers and a rigid surface was attached to the cantilever tip. Initially, this approach was developed to investigate the interaction forces between a rigid colloidal particle and a rigid, flat, smooth surface (Ducker, Senden, and Pashley 1991, 1992; Rabinovich and Yoon 1994) and between two rigid colloidal particles (Li et al. 1993; Larson et al. 1995). The AFM tip attached with the rigid sphere was then employed to probe the surface forces and surface deformation of soft surfaces, such as air bubbles (Butt 1994; Ducker, Xu, and Israelachvili 1994) and oil droplets (Mulvaney et al. 1996; Snyder, Aston, and Berg 1997; Hartley et al. 1999; Aston and Berg 2001, 2002) in aqueous environments. Gradually, as a result of the great advances in AFM technique, scientists have been able to attach an oil droplet to the AFM cantilever tip, by which direct measurement of the surface forces between a pair of deformable oil droplets under different aqueous conditions was investigated (Aveyard et al. 1996; Dagastine et al. 2004; Gunning et al. 2004; Dagastine et al. 2006; Gromer et al. 2010; Tabor et al. 2011). Preparing and attaching a single oil droplet to the cantilever tip is quite difficult, but it can be accomplished in several ways. In the method reported by Gunning et al. (2004), and Gromer et al. (2010), the oil droplets were initially sprayed onto a clean glass surface from a certain distance (e.g. ~10 cm), using an ‘ad hoc sprayer’ such that the glass surface was covered by a uniform layer of separated oil droplets. The water droplets were then carefully added to the glass surface without dislodging the oil droplets. Due to the hydrophilic nature of the glass surface, most of the oil droplets were displaced by the added water droplets.

**Figure 6.** (a) A sketch illustrating the surface deformation: (1) in the constant-compliance region for compressing two rigid surfaces (cantilever tip and rigid flat surface), (2) the slope declined as a deformable surface was pressed on the rigid surface (deformable oil droplet attached to the cantilever tip and rigid flat surface), (3) the slope declined further in compressing two deformable surfaces (deformable oil droplets attached to the cantilever tip and fixed rigid flat surface) [Adapted with permission from Morris (2013)]. (b) Photos showing hexadecane droplets stabilized by negatively charged sodium dodecyl sulfate (SDS) and nonionic Triton X-100 (TX) surfactants on a hydrophilic and hydrophobic glass surface. The photos are taken after 30 min of contacting between emulsion and glass surface at stagnant state (the first and third columns), and after flushed with continuous phases (the second column). The spreading of droplets was observed on the hydrophobic surface for SDS, and on both hydrophilic and hydrophobic surfaces for TX [Adapted with permission from Dickhout et al. (2018)].
However, some oil droplets still remained attached to the glass surface. The AFM head was then lowered down to the glass surface to sandwich the water between the liquid scanner and the glass surface. Later, the cantilever tip was directly positioned above the center of an oil droplet on the glass surface and gradually driven down into the target droplet until their engulfment was achieved. After ‘snapping on’ the oil droplet, the cantilever tip was retracted to carry the captured oil droplet way from the glass surface. Once the droplets were attached to the cantilever, the proteins and/or surfactants required were added to the AFM liquid cell to stabilize the attached oil droplet. Another method for attaching an oil droplet to the cantilever tip was also reported (Aveyard et al. 1996; Dagastine et al. 2004; Dagastine et al. 2006; Tabor et al. 2011). In this method, the oil droplet was formed under water in a special fluid cell, using a controlled syringe to inject nanoliter volumes of oil through a glass capillary. The fluid cell was mounted in the AFM, and the cantilever tip was programmed to pick up the oil droplet. These methods were successful in attaching oil droplets with diameters of 10–70 μm to the cantilever tip. The oil droplet attached to the cantilever tip was brought over another oil droplet to perform the interaction force measurement. Measuring droplet-droplet interaction forces enables to understand the destabilization mechanisms of nanoemulsions during storage.

In the reports by Gunning et al. (2004) and Morris (2013), it was found that in a hard system, as a ridge particle fabricated on the cantilever tip was brought to a hard surface, the slope of the force-distance curve (e.g. the cantilever deflection gradient) begins to change at the contact point between two surfaces. On the force-distance curve, this part is known as the region of constant compliance (solid line in Figure 6a). The point at which the slope changes is defined as the zero point, and it allowed us to determine the absolute distance of the approach or retraction of two surfaces, an important parameter for interaction force calculation from theoretical models. In a typical AFM instrument, there is no system that allows direct determination of the true separation distance between two interacting interfaces. The usual way to calculate the separation distance is by adding the cantilever deflection (which is a ratio of the photodiode voltage to the slope of the constant-compliance region) to the displacement of the piezoelectric scanner as the zero point of the separation distance is set at the constant-compliance region (Ishida and Craig 2019). However, in deformable systems (e.g. between a rigid surface and a deformable oil droplet or between two deformable oil droplets), the contact point is very difficult to define, and the slope of the force-distance curve changes, depending on the deformability of the contacting droplets/surfaces. In addition to interfacial deformability, the adsorption of any molecules on the interfaces, which may be caused by the measuring environment, and the roughness of the interfaces make the definition of the true separation distance more complicated. As indicated in Figure 6a, the slope change that occurs when pressing between a rigid surface and a deformable surface (dotted line) is much less profound than those that occur when compressing a deformable surface to another deformable surface (dashed line). In addition, the deformation of the oil droplets can mask the weak interaction forces. Instead of only inducing the AFM cantilever deflection, the weak interaction forces may lead to a small deformation of oil droplets with no measurable deflection of the cantilever. This can clearly be seen in Figure 6a, in that the turning point for the cantilever deflection in the hard system (the solid line) is much sharper than that in the deformable systems (the dotted and dashed lines). Therefore, the measurement of the surface interaction forces between the deformable droplets presents significant experimental and theoretical challenges. However, as reported by Tabor et al. (2011), the in situ use of confocal fluorescence microscopy with the AFM experiment could address the limitation of previous studies in the lack of independent measurement of the absolute separation between two oil droplet surfaces. This approach allowed us to directly visualize and determine the position and absolute separation of the contacting oil droplets within 50 nm and also to quantify the deformation in the interfacial region during the interactions. In addition, through arbitrary estimation of the absolute separation between two oil droplet surfaces, based on theoretical models, the quantitative interpretation of the dynamic forces between micrometer-sized deformable oil droplets was reported (Aveyard et al. 1996; Dagastine et al. 2004; Gunning et al. 2004; Dagastine et al. 2006; Gromer et al. 2010; Tabor et al. 2011; Jamieson et al. 2019).

The deformability of oil droplets is dependent on the structure and compositions of the droplet interface. Therefore, determining the alteration of the force-distance curve slopes allowed us not only to quantify the deformability of the droplets, but also to monitor the formation and manipulation of the interfacial structure. The force-distance profile between two oil droplets could be used to probe the surface deformability of an oil droplet stabilized by proteins (e.g. β-lactoglobulin) and/or surfactants (e.g. Tween 20), their competition for adsorption on the interface, and their interactions (Gunning et al. 2004; Woodward et al. 2010). The adsorption of β-lactoglobulins on the interface of the oil droplet resulted in the increase of the force-distance curve slope, which was attributed to the elasticity of the protein network formed on the surface of the droplet. However, once Tween 20 was added, competitive displacement of the protein by Tween 20 led to the decline in magnitude of the slope of the equilibrium value, where Tween 20 dominated on the interface. A similar study of the role of sugar beet pectin (at various concentrations) as an emulsifier in the stabilization of oil droplets under different aqueous conditions was also reported (Gromer et al. 2010). In addition to the force-distance profile, another approach based on imaging of the interface also allowed us to investigate the adsorption behavior of emulsifiers on the interface, where the Langmuire-Blodgett method was employed to prepare the film with components similar to those of the interfacial layer in actual emulsions (Gromer et al. 2010; Berton-Carabin et al. 2013).
We applied AFM techniques as well as sample preparation approaches to successfully image the droplet interface topography and to determine the surface forces of macroemulsions and nanoemulsions. However, due to the deformability of the dispersed droplets and the dynamic nature of nanoemulsion systems, several aspects must be considered during technical development. They include how to attach the nanometric dispersed droplets to the cantilever tip and to the substrate surface without affecting their native state and properties and how to determine the true contact point and absolute separation between two investigated droplets. Moreover, with advances in nanotechnology, AFM tips functioned with different chemical groups and/or coated with different surface charge, hydrophobic or hydrophilic materials are commercially available (NanoAndMore USA Corp 2021; Bruker 2021). This allows investigation of possible interactions between nanoemulsion droplets with various surfaces to provide insight of adsorption behavior of nanoemulsion-based delivery systems in different digestion systems. For both dry and liquid mode AFM, an important factor which need to be carefully considered during fixation of dispersed droplets on the flat surface is the wettability of dispersed droplets on the flat surface, which can be evaluated via contact angle measurement. The dispersed droplets should be attached on the flat surface without coalescing and/or spreading on it. An example of the attachment of hexadecane emulsion droplets (with diameter of 3–4 μm) stabilized by negatively charged sodium dodecyl sulfate (SDS) and nonionic Triton X-100 (TX) surfactants, on hydrophobic and hydrophilic glass surface with hexadecane contact angle of 155 and 60°, respectively is shown in Figure 6b. For SDS, the images in the first column indicates the adhesion of dispersed droplets after 30 min of contacting between emulsion and glass surface at stagnant state. After a fraction of attached droplets was washed out via flushing with continuous phase (the second column), it was observed that some droplets were spread out on the hydrophobic surface while the droplets on hydrophilic surface maintained their native state. For TX, the droplets were spread out on both hydrophilic and hydrophobic surfaces (Dickhout et al. 2018). Therefore, choosing a flat surface with the desired wettability is very important to preserve intact and native state of dispersed droplet during sample preparation for AFM. Depending on the physical state of dispersed droplets (e.g. oil or water), the nature of emulsifiers, and the properties of continuous phase, wettability of flat surface can be modified via numerous techniques including plasma treatment, UV irradiation, chemical vapor deposition, silanization, hydrosilylation, bulk modification with nanomaterials, sol-gel chemistry, dynamic coating with surfactants, and graft polymer coating (Wang and Zhang 2018; Dickhout et al. 2018).

Despite these experimental difficulties, such studies will be of interest to many researchers and may rapidly increase in number, due to their importance in both academic and industrial applications, given the current rapid progress in nanotechnology. These studies can provide new insights and findings about the behavior of surface-active molecules at droplet interfaces as a function of the properties of the bulk aqueous phase and interfacial compositions, while including the effects of droplet surface deformation and interdroplet interactions, which most resemble the situation in real nanoemulsion systems.

**Conclusion**

The functional properties and stability of dispersed systems such as nanoemulsions are highly dependent on the surface properties of dispersed droplets and interaction forces between them. An understanding of these properties allowed us to manipulate and effectively design nanometric delivery systems for many bioactive compounds, nutraceuticals, polyunsaturated fatty acids, prebiotics, probiotics, and pharmaceuticals in a wide range of fields, such as the chemical, pharmaceutical, food and nutrition, and cosmetic industries. For these purposes, AFM technology could emerge as one of the most meaningful techniques for imaging the surface topography and determining the surface forces of droplets in nanoemulsions. However, the AFM technique was recently employed as a complementary analytical technique for determination of the droplet morphology and size of nanoemulsions, while investigation of the mechanical properties and interaction forces on the droplet surface remains in the initial stage of research and development. The unavoidable deformation of both droplet surfaces as they come into contact, together with the extremely small size of the droplet (e.g. nanometer scale) makes both measurement and interpretation of the surface forces for nanoemulsion droplets very difficult. However, some studies have successfully characterized macromolecules, and they may be used as good starting points for scientists to extend the investigation into nanoemulsions to provide the new insights expected to occur in real nanometric dispersed systems.

**Disclosure statement**

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