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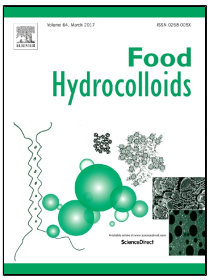

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Highlights

- Fundamentals of low frequency high power ultrasound are outlined.
- Functional modification of proteins from ultrasonic processing is described.
- The factors involved in ultrasonic emulsification are critically discussed.

Applications of ultrasound for the functional modification of proteins and nanoemulsion formation: A review

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Abstract:

This review surveys the most recent developments in low frequency, high power ultrasound for the functional modification of proteins derived from a number of food sources (*e.g.* dairy, animal, cereal, legume, tuber and fruit), and subsequently for the fabrication of nano-sized emulsion droplets. Aside from an overview of the fundamentals of ultrasound, including a cursory outline of ultrasonic cavitation, heat generation and acoustic energy determination via calorimetry, examples of ultrasound treatment for improvements in the dissolution, hydration, hydrophobicity, emulsifying and rheological performance of proteins are described. Ultrasound possesses the industrial capability to improve the functional properties of proteins, and this review emphasises the improvement to the surface active properties of proteins, which is attributed to decreases in protein aggregate size and increases in hydrophobicity, demonstrating increased molecular mobility. Finally, the utilisation of ultrasound for the fabrication of nanoemulsions is assessed with a particular focus on the intrinsic relationship between process configuration (*i.e.* batch or continuous), processing parameters (*i.e.* acoustic power and residence time) and emulsion formulation (*i.e.* emulsifier type and concentration). A better understanding of the effect of industrially relevant high molecular weight biopolymers (*i.e.* proteins) within ultrasonic emulsification processes would increase the utilisation of ultrasound as a fabrication technique for nano-sized emulsion droplets.

Keywords: Ultrasonic processing, Proteins, Functional properties, Emulsifying performance, Nanoemulsion fabrication, Sonoreactor design

1. Introduction

Low frequency, high power ultrasound, commonly referred to as power ultrasound, has gained significant interest over the past decade as it possesses a wide range of uses within a myriad of sectors making it a versatile processing technology, for the alteration, generation and modification of microstructures. As a consequence, due to ultrasonic cavitation, it is capable of mechanically altering the structure of proteins in solution without the use of additives (chemical or biological) or excess heat, and increasing specific surface area in emulsion systems for the generation nano-sized emulsion droplets (McClements, 1995; O'Brien, 2007).

Proteins are ingredients utilised within a wide range of formulations due to both their nutritional value and functionality (O'Sullivan & O'Mahony, 2016). The term 'functionality' as applied to food ingredients describes any property other than nutritional attributes that contribute to an ingredient's beneficial aspects within a formulation (Damodaran, 1997). Proteins are highly functional molecules within food systems capable of the stabilisation of oil droplets and air bubbles, formations of gel structures and the enhancement of viscosity (O'Connell & Flynn, 2007; Walstra & van Vliet, 2003). This functionality is due to the complex chemical makeup of these molecules owing to their unique amino acid sequences (Beverung *et al.*, 1999). Improvement to the functional properties of proteins is of great interest so as to increase their commercial value, and improve utilisation of these high value ingredients, which is conventionally achieved through either molecular weight modification (*i.e.* proteolysis or aggregation), or conjugation/complexation with other biopolymers (Drapala *et al.*, 2015; Grigorovich *et al.*, 2012; Kurukji *et al.*, 2015; Malaki Nik *et al.*, 2010; Mulcahy *et al.*, 2016; O'Sullivan *et al.*, 2016).

As for emulsion formation, traditionally it is achieved industrially through the implementation of homogenisers, usually two stages, operating at pressures up to 25 MPa

(McClements, 2005). Numerous technologies have shown the capacity for the fabrication of nano-sized emulsion droplets, such as microfluidics, high and ultrahigh pressure valve homogenisers, and membrane emulsification (crossflow and rotary) (Lee & Norton, 2013; Lloyd, *et al.*, 2014). However, industry is reluctant to readily adopt these technologies due to the associated capital expenditure and scalability issues.

Amongst the forthcoming technologies for the functional modification of proteins and generation of nano-sized emulsion droplets, power ultrasound, also commonly referred to as high intensity ultrasound, has garnered particular interest due in part to the mechanical nature of this process (*i.e.* ultrasonic cavitations). Traditionally, the functionality of proteins is altered by aggregation (*i.e.* increasing molecular weight), proteolysis (*i.e.* reducing molecular weight) or conjugation with other entities (*e.g.* Maillard reaction with reducing sugars). Power ultrasound offers the possibility of altering protein structures without the use of additives or excessive thermal treatments, simplifying the processing of these ingredients and generating a 'cleaner' packaging label for consumers. With adequate sonoreactor design (*i.e.* chamber volume and volumetric flow rate selection), and high throughput, cost effective generation of nano-sized emulsion droplets is readily achievable (Gogate & Kabadi, 2009; Gogate, *et al.*, 2011).

The aim of this review is to outline the fundamentals of ultrasound and critically assesses applications of ultrasound treatment for the functional modification of proteins in aqueous solution (*e.g.* solubility, hydrophobicity, rheological behaviour, emulsifying performance, etc.) and the generation of nano-sized emulsion droplets. A particular focus has been placed on the industrial relevance of ultrasonic processing within the food industry, as a cost effective, mechanical method for the generation, alteration and modification of food microstructures (*e.g.* emulsifications, lipid crystallisation, structural modification of biopolymers, etc.).

2. Fundamentals of ultrasound

Ultrasound is an acoustic wave above the threshold of human auditory perception (> 16 kHz). Acoustic waves are the propagation of mechanical waves of pressure and displacement through a medium, as longitudinal waves, exhibiting compressions (high pressure regions) and rarefactions (low pressure regions). Longitudinal waves are waves whereby the displacement of the medium is in the same direction as the wave (Mansfield & O'Sullivan, 1998).

Ultrasound can be further classified in two distinct categories based on the frequency range, high frequency (100 kHz – 1 MHz), low intensity (< 1 W cm^{-2}) ultrasound, utilised most commonly for the analytical evaluation of the physicochemical properties of food (Chemat *et al.*, 2011; Demirdöven & Baysal, 2008), and low frequency (20 – 100 kHz), high intensity (10 – 1000 W cm^{-2}) ultrasound recently employed for the alteration, generation and modification of foods, either physically or chemically (McClements, 1995). The acoustic power intensity (I_a; W cm^{-2}) is defined as the acoustic power (P_a; W) per unit area of ultrasound emitting surface (S_A; cm^{-2}). This review will focus solely upon low frequency, high power ultrasound, and hereafter will refer to it as simply power ultrasound.

The effects of power ultrasound on food structures are attributed to ultrasonic cavitation, the rapid formation and collapse of gas bubbles, generated by localised pressure differentials occurring over short periods of times (a few microseconds). These ultrasonic cavitations cause localised regions of intense hydrodynamic shear forces and a rise in temperature at the site of bubble collapse (up to 5000^{o}C), contributing to the observed effects of power ultrasound (Güzey *et al.*, 2006; O'Brien, 2007; O'Donnell *et al.*, 2010).

Acoustic waves are generated from the conversion of electrical energy into mechanical energy. A transducer, a device which converts energy from one form to another, is employed to produce acoustic waves. In acoustics, transducers are commonly referred to as tips. More

specifically, the tip, a part of the sonotrode, is the point from which the acoustic waves emanate. The piezoelectric material (*e.g.* quartz or lithium sulphate zirconate titanates) within the transducer oscillates in response to electrical energy, leading to mechanical vibrations in the tip. When the tip is submerged in liquids, the mechanical energy at the tip is delivered to the medium as the tip vibrates generating acoustic waves (Martini, 2013; Soria & Villamiel, 2010; Trujillo & Knoerzer, 2011a).

Ultrasonic emanation from the tip of the sonotrode is referred to as acoustic streaming (Nyborg, 1953; Tjøtta, 1999).There are two main acoustic streaming theories which describe this phenomena mathematically, those developed by Rayleigh (Rayleigh, 1896), Nyborg (Nyborg, 1953) and Westervelt (Westervelt, 1953), referred to as the RNW theory, and that proposed by Lighthill, the Stuart streaming theory (Lighthill, 1978). The RNW theory is only applicable to laminar systems, whereas the Stuart streaming theory is applicable to systems demonstrating acoustic jets (*i.e.* turbulent), resulting from high power acoustic beams from transducers, a computationally developed example of which is shown in Fig. 1 (Lighthill, 1978; Stuart, 1963). Ultrasonic processing utilised within the food industry for the development of microstructures and functional modification of food ingredients is usually power ultrasound processing which is most adequately modelled and explained by the Stuart streaming theory (McClements, 1995; Trujillo & Knoerzer, 2011a).

2.1. Ultrasonic cavitations

High power ultrasonic waves generate several different types of cavitation bubbles due to pressure changes during wave propagation (Servant *et al.*, 2001). Cavitation bubbles are formed at acoustic intensities greater than that of the cavitation threshold. The cavitation threshold pressure required to initiate cavitations is a strong function of stream width and acoustic power, and once triggered bubble generation increases with increasing acoustic power

(Leighton, 1995; Neppiras, 1980). Fig. 2 shows the formation and collapse of ultrasonic cavitations over a 56 μs timescale. It can be seen that over a 16 μs timeframe, cavitations are formed, and their subsequent implosion occurs, highlighting that this phenomena occurs over very short periods of time, < 20 μs in the majority of instances (Trujillo & Knoerzer, 2011). As time progresses, and more acoustic energy is provided to the system, the number of ultrasonic cavitations increases, as can be seen from 32 μs onward.

Cavitation bubbles disperse (*i.e.* reflect or scatter) and attenuate (*i.e.* gradual reduction of ultrasonic intensity) ultrasonic waves due to the acoustic impedance differential between the liquid and gaseous phases. When an acoustic wave moves from one medium to another (*i.e.* from liquid to gaseous bubbles) differences in the speed of sound and compressibility between the two phases induces an impedance mismatch (McClements, 1995; O'Brien, 2007). As a consequence, the acoustic wave is either partially or completely scattered by the bubble. The cavitation locus is situated in an area close to the tip of the sonotrode, whereby this region yields the highest levels of acoustic intensity, and thus an area of increased formation of cavitations. Therefore, the attenuation in this region is quite high and dominated by acoustic scattering (Martini, 2013), decaying exponentially with respect to distance from sonotrode tip, almost completely dissipated at distances as low as 2 cm (Chivate & Pandit, 1995; Kumar *et al.*, 2006; Kumaresan *et al.*, 2006), highlighting the importance of adequate sonotrode positioning for effective processing of liquid medium (Gogate *et al.*, 2011; Gogate *et al.*, 2003).

2.2. Heat generation

Ultrasonic processing of fluid systems yields heat generation due to a number of factors which occur as a consequence of the transmission of an acoustic wave through the medium, including molecular absorption, dissipation of turbulence, dispersion of acoustic waves by

gaseous bubbles and viscous losses. The acoustic energy transmitted to the medium manifests as both kinetic energy (*i.e.* bulk motion) and thermal energy (*i.e.* heat). The kinetic energy transmitted to the medium is dissipated as heat due to viscous losses (Tjøtta, 1999; Zisu *et al.*, 2010).

In ultrasonic processes where the attenuation coefficient, β, is high (*i.e.* a high number of ultrasonic cavitations) it can be assumed that the acoustic energy is rapidly converted to thermal energy in the locus of the sonotrode tip, from which the acoustic waves emanate (Lighthill, 1978). The validity of this assumption is true for systems exhibiting high attenuation coefficients where dissipation of acoustic energy occurs at the transducer, and additionally where the kinetic energy disperses at the sonotrode tip. Chivate & Pandit, (1995) confirmed that acoustic energy dissipates completely within close proximity of the sonotrode tip, approximately 2 cm, and it was found that the majority of kinetic energy (> 80 %) is dissipated in the form of thermal energy in a small volume (< 2 % of a 2 L batch volume) in the locus of the transducer (Kumar *et al.*, 2006; Kumaresan *et al.*, 2006).

Trujillo & Knoerzer, (2011a) employed a computational approach to investigate the distribution of temperature in a batch ultrasonic process, as shown in Fig. 3. Fig. 3 highlights, that there is a higher temperature in the immediate proximity of the sonotrode tip, owing to the aforementioned cavitation mediated ultrasonic attenuation, which to a large extent, limits transmission of energy from the sonotrode tip.

2.3. Acoustic energy determination

The determination of the acoustic energy input into a volume of liquid is a topic under investigation, however a satisfactory description of the solution has thus far to be elucidated, even though the fields of sonochemistry and ultrasonic cavitation have been under investigation for several decades. The electrical consumption of the ultrasonic process and the acoustic

power under non-cavitational conditions are attainable, however acoustic power measurements within the cavitational regime are lacking (Margulis & Margulis, 2003).

As acoustic energy is transmitted to a liquid medium via the sonotrode tip, this acoustic energy is dissipated as absorbed acoustic energy, manifesting as thermal energy, and unadsorbed energy. The absorbed acoustic energy is the active component of total acoustic energy involved in the processing. Acoustic power intensity, I_a, can be estimated from the following:

$$I_a = \frac{kf^2U}{\rho c} \quad (1)$$

Where f is the frequency of sound (Hz), U is the voltage of the transducer (V), k is a conversion of coefficient dependent on the transducer type, ρ is the density of the liquid medium (kg m^{-3}) and c is the speed of the acoustic wave in a given medium (m s^{-1}). The product of density and speed of sound (*i.e.* ρc) is known as the acoustic resistance (Margulis & Margulis, 2003). Under non-cavitational conditions the acoustic energy can be estimated accurately using *Eq.* 1, whilst in the cavitational regime the acoustic resistance is significantly reduced. The reduction of both the speed of sound and bulk density of the medium by the presence of cavitation bubbles within the medium depresses the accuracy of the acoustic intensity determination from *Eq.* 1. The underlying principles involved in the formation of and interactions between cavitation bubbles are not fully understood, hence the reliability of the acoustic resistance term and consequently *Eq.* 1 as an effective method for the estimation of the acoustic intensity within the cavitational regime is dubious (Leighton, 1995; Margulis & Margulis, 2003; O'Brien, 2007).

The drawbacks associated with *Eq.* 1 are mitigated against by the usage of a calorimetric method for the determination of absorbed energy (*cf. Eq.* 2), whereby the acoustic

196 resistance term is neglected. The main assumption for the determination of acoustic energy via
197 calorimetry is that all absorbed acoustic energy is converted to thermal energy.

198 $$I_a = \frac{P_a}{S_A} = \frac{m\, c_p\left(\frac{dT}{dt}\right)}{S_A} \quad (2)$$

199 Where P_a is the absorbed acoustic power (W), S_A is the surface area of the tip of the
200 transducer (cm^2; *i.e.* ultrasound emitting surface), m is the mass of ultrasound treated medium
201 (g), c_p is the specific heat capacity of the medium (J/gK) and dT/dt is the rate of change of
202 temperature with respect to time, starting at $t = 0$ ($^oC\ s^{-1}$). As energy emitted from the sonotrode
203 tip, it is absorbed within close proximity to the tip due to cavitational attenuation, the energy
204 is dissipated as heat, allowing for estimation of the acoustic energy absorbed without the
205 necessity to account for cavitation bubbles (*i.e.* the acoustic resistance term) (Jambrak *et al.*,
206 2008; Margulis & Margulis, 2003).

207 **3. Physicochemical alteration of food proteins via ultrasonic processing**

208 From the literature, the application of ultrasonic treatment has been related to proteins
209 derived from dairy, animal, cereal, legume, tuber and fruit sources, see Table 1.

210 *3.1. Dissolution effects of ultrasonic processing*

211 Dissolution of powder ingredients is essential for functional utilisation within a given
212 formulation system, and depending upon the specific powder, its rehydration can be
213 challenging. Broadly, high protein systems are difficult to reconstitute, with certain protein
214 fractions exacerbating this, for example, casein-dominant high-protein content powders
215 (Crowley *et al.*, 2015; O'Sullivan *et al.*, 2017). Upon addition of a powder to water, there are
216 5 stages in its complete dissolution, schematically represented in Fig. 4 for a high-protein dairy
217 powder: (1) Wetting, (2) Swelling, (3) Sinking, (4) Dispersion and (5) Dissolution (Crowley *et*
218 *al.*, 2016). The key stages where power ultrasound could affect the rehydration process is that

of dispersion, the fragmentation of wetted powder particles, and dissolution, the complete breakdown of granular structure and release of constituent molecules (Vos *et al.*, 2016).

Ultrasound treatment offers improved rates of dissolution and solubilisation of poorly soluble dairy protein powders in comparison to conventional dissolution methodologies (*i.e.* low/high shear mixing or high pressure homogenisation) (Chandrapala *et al.*, 2014; McCarthy *et al.*, 2014; O'Sullivan *et al.*, 2016). McCarthy *et al.*, (2014) demonstrated that the high levels of hydrodynamic shear associated with ultrasonic cavitations disrupt agglomerates of powder imparting greatly increased rates of solubilisation in comparison to conventional overhead mixer dispersion methodologies employed for dairy powders possessing a high degree of micellar casein (MC), whilst Chandrapala *et al.*, (2014) observed that the most effective methodology for the dissolution of dairy powders possessing a high MC content (≥ 80 wt. %) was high pressure homogenisation (single stage at either 80 or 200 bar), with ultrasonic processing being an intermediate methodology for dissolution, followed by low/high shear mixing. Enhancement of dissolution of MPC in this case may be achieved by operating at an increased ultrasonic amplitude (50% was employed by Chandrapala *et al.*, (2014), whilst 100% was utilised by McCarthy *et al.*, (2014)) and/or optimal positioning of the ultrasonic horn so as to achieve the maximum effect of the ultrasonic sound beam (*i.e.* minimisation of dead-zones) (Gogate *et al.*, 2011).

The available literature is limited to studies on the effect of ultrasonic processing for dairy powders for dissolution purposes. Be that as it may, there is a growing interest within the food industry for the use of plant derived protein ingredients rather than animal sourced systems, for a variety of reasons, such as nutritional profile, functional properties and commercial rationale (Gonzalez-Perez & Arellano, 2009). Ultrasound processing of plant protein systems could offer potential benefits for dissolution of powders, as ultrasound has

been shown to be capable to reduce aggregate size of plant proteins in aqueous solution, as discussed in the following section.

3.2. Size effects of ultrasonic processing

Ultrasound treatment reduced the size of aggregated caseins in aqueous solution (phosphocasein, calcium caseinate, milk protein concentrate from retentate and milk protein concentrate reconstituted from powder), from micron-sized entities (5 - 30 μm) to nano-sized species (~200 nm) (Madadlou, *et al.*, 2009; McCarthy, *et al.*, 2014; Shanmugam, *et al.*, 2012; Yanjun, *et al.*, 2014; Zisu, *et al.*, 2010), the expected size for casein micelles (O'Connell & Flynn, 2007). This size reduction is attributed to the high shear forces associated with ultrasonic cavitations in liquid mediums (Trujillo & Knoerzer, 2011). Be that as it may, prolonged ultrasound treatment led to growth in aggregate size toward the micron-scale, related to whey-whey or casein-whey protein interactions as a consequence of both protein denaturation and deceased solubility attributed to elevated temperatures from ultrasound treatment (McCarthy, *et al.*, 2014; Shanmugam, *et al.*, 2012). Sonication of whey protein (suspensions, concentrates, isolates, and from retentate) similarly reduced the size of protein aggregates due to disruption of non-covalent interactions, to sizes ~100 nm (*i.e.* hydrogen bonding, hydrophobic and electrostatic interactions) (Arzeni, *et al.*, 2012; Chandrapala, *et al.*, 2011; Jambrak, *et al.*, 2014; Martini, *et al.*, 2010; Zisu, *et al.*, 2010), yet similarly displayed growth of particle size attributed to increases in temperature, resulting in protein denaturation and aggregation (Gülseren, *et al.*, 2007).

Furthermore, the ultrasound treatment of proteins derived from legume sources (pea protein, soy protein, black bean protein and mung bean protein) and wheat protein displayed a significant reduction in aggregate size (> 20 μm) to entities which were submicron (~200 nm), thus enhancing the solubility of traditionally poorly soluble plant protein solutions (Charoensuk, *et al.*, 2014; Jiang, *et al.*, 2014; O'Sullivan, Beevers, *et al.*, 2015; O'Sullivan,

Murray, *et al*., 2016; O'Sullivan, Park, *et al.*, 2016b; Zhang, *et al.*, 2011). However, ultrasound treatment of egg white proteins (Arzeni, *et al.*, 2012; Krise, 2011) exhibited growth in aggregate size, from submicron (~500 nm) to micron sized entities (~100 μm), attributed to thermal denaturation of protein due to increases in temperature from prolonged ultrasonic treatment. Be that as it may, size reduction of egg white protein aggregates is achievable if the temperature is maintained well below denaturation temperatures (~40 ^{o}C) (O'Sullivan, Murray, *et al.*, 2016). Sonication of rice protein isolate, lupin protein concentrate and zein demonstrated no significant differences in size, associated with insufficient provided acoustic energy to disrupt disulphide bonding maintaining the denatured aggregate structure (O'Sullivan, Murray, *et al.*, 2016; O'Sullivan, Park, *et al.*, 2016a; Ren, *et al.*, 2015). Size reduction of protein aggregates in aqueous solution from ultrasound treatment is associated with the disruption of associative non-covalent interactions which maintain protein aggregate structure in aqueous solutions.

3.3. Molecular structure effects of ultrasonic processing

Even though ultrasound treatment has been shown to possess the capability of reducing the size of proteins in aqueous solution and enhance dissolution, it does not appear to cause scission of the primary structure for a large number of proteins, including milk protein concentrate (Yanjun *et al.*, 2014), whey protein suspensions (Martini, *et al.*, 2010), soy protein isolate (Hu, *et al.*, 2013), pea protein isolate (O'Sullivan, Murray, *et al.*, 2016), wheat gluten (Zhang *et al.*, 2011), black bean protein isolate (Jiang *et al.*, 2014), potato protein isolate (O'Sullivan, Park, *et al.*, 2016a), gelatin (O'Sullivan, Murray, *et al.*, 2016) and egg white protein (Krise, 2011), as ultrasound treatment provides insufficient energy to cause scission of the primary acid sequence (*i.e.*, peptide bond). Krise, (2011) observed a minor shift in the molecular weight distribution of egg white protein and attributed this to scission of disulphide bonds between cysteine residues present in egg white protein (Mine, 2002). The bond energy

associated with the disulphide bond is less than that of the peptide bond maintaining the primary structure of proteins (*cf.* Table 2), nevertheless, the majority of ultrasonic energy is utilised in the disruption of the associative non-covalent interactions maintaining the protein associate structure, rather than disruption of covalent linkages. However, a significant reduction in the molecular weight of α-lactalbumin (Jambrak, *et al.*, 2010) and whey protein concentrate/isolate (Jambrak, *et al.*, 2014), generating peptide species possessing molecular weights within a range of 4.5 to 8 kDa, was observed from pixel intensity plots generated from SDS-PAGE gels. Based on the acoustic intensity provided in both of these trials, the maximum and minimum of which were 1 W cm^{-2} and 48 W cm^{-2}, respectively, insufficient energy is provided to disrupt the peptide bonds, especially at the high concentrations of protein tested (up to 10 wt. %), and further testing should be conducted to further elucidate these results, such as high performance liquid chromatography (HPLC), circular dichroism (CD) or nuclear magnetic resonance (NMR) spectroscopy. The acoustic energy employed provided sufficient energy to disrupt hydrogen bonding, reducing aggregate size (as observed in these studies), with insufficient energy provided to achieve scission of covalent linkages.

3.4. Viscosity effects of ultrasonic processing

Sonication of protein solutions has been shown to either reduce the bulk viscosity, in the cases of calcium caseinate (Zisu, *et al.*, 2010), milk protein concentrate (Yanjun, *et al.*, 2014; Zisu, *et al.*, 2010), whey protein from retentate (Zisu, *et al.*, 2010), soy protein isolate (Hu, *et al.*, 2013) and egg white protein (Arzeni *et al.*, 2012), or to yield no difference in bulk viscosity, as for skimmed milk powder (Shanmugam, *et al.*, 2012) and α-lactalbumin (Jambrak, *et al.*, 2010). For the case of soy protein, a reduction from 1 to 0.2 Pa.s at a shear rate of 100 s^{-1} and concentration of 12.5 wt. % was observed (Hu, *et al.*, 2013), and for whey protein (from retentate) a reduction from 0.065 to 0.055 Pa.s at 100 s^{-1} for a 33 wt. % solution was demonstrated. The reduction in bulk viscosity is attributed to the reduction in aggregate size as

a consequence of ultrasonic cavitations. The spatial distance between adjacent protein aggregates is increased upon size reduction via ultrasound treatment, increasing the critical overlap concentration, c^*, for a given protein solution, and thus, decreasing the bulk viscosity with respect to increasing protein concentration (Lefebvre, 1982; Morris *et al.*, 1981).

3.5. Emulsifying effects of ultrasonic processing

Proteins which have been treated with power ultrasound have shown improvements in both emulsion formation and stability, for milk protein concentrates (O'Sullivan, Arellano, *et al.*, 2014; Yanjun, et al., 2014), egg white protein (O'Sullivan, Murray, *et al.*, 2016), bovine gelatin (O'Sullivan, Murray, *et al.,* 2016), soy protein isolate (Chen, *et al.*, 2012), pea protein isolate (O'Sullivan, Murray, *et al.*, 2016), potato protein isolate (O'Sullivan, Park, *et al.*, 2016a), wheat protein (O'Sullivan, Park, *et al.*, 2016b; Zhang, *et al.*, 2011) and walnut protein (Jincai, *et al.*, 2013). Yanjun *et al.*, (2014) reported a significant increase in both EAI (*i.e.*, emulsion activity index) and ESI (*i.e.*, emulsion stability index) for emulsions prepared with MPC, from 3.5 to 6 m^2 g^{-1}, and from 50 to 80 min, respectively. In addition, O'Sullivan, Murray, *et al.*, (2016) observed significant enhancements in both emulsion formation and stability for emulsions prepared with bovine gelatin. At a protein concentration of 0.1 wt. % emulsions prepared with untreated and ultrasound treated bovine gelatin yielded emulsion droplet sizes of 1.75 μm and 1 μm, respectively, and moreover emulsions prepared with ultrasound treated bovine gelatin were stable throughout a 28 day stability study, whereas their untreated counterparts were unstable at concentrations < 1 wt. %, leading to growth in emulsion droplet size.

These improvements in emulsion formation and stability for ultrasound treated proteins were associated with increases in hydrophobicity, which occurred as hydrophobic protein residues within the interior of the untreated aggregate became revealed upon treatment with ultrasound, and improved interfacial packing at the emulsion droplet interface. O'Sullivan,

Park, *et al.*, (2016a) observed a significant reduction in the hydrodynamic volume of potato protein isolate which is associated to an increase in the hydrophobicity of proteins (Khan, *et al.*, 2012), accounting for the observed enhancements in emulsion formation and stability in this instance. In addition, ultrasound treatment of whey protein (Arzeni, *et al.*, 2012; Gülseren, *et al.*, 2007), soy protein (Arzeni, *et al.*, 2012; Hu, *et al.*, 2013), black bean protein (Jiang, *et al.*, 2014) and egg white protein (Arzeni, *et al.*, 2012) increased the hydrophobicity, and the rate of protein adsorption to and interfacial packing at the oil-water interface, as measured by interfacial tension. These differences were measured for the cases of milk protein isolate (O'Sullivan, *et al.*, 2014), bovine gelatin (O'Sullivan, Murray, *et al.*, 2016), pea protein isolate (O'Sullivan, Murray, *et al.*, 2016) and soy protein isolate (Chen, *et al.*, 2012), further accounting for improvements in emulsion formation and stability. O'Sullivan, Murray, *et al.*, (2016) reported reductions in the equilibrium value of interfacial tension (*i.e.*, rapeseed oil and water) for both bovine gelatin and soy protein isolate, from 5 to 2.5 mN m^{-1}, and from 6 to 3.5 mN m^{-1}, respectively. Furthermore, O'Sullivan, Murray, *et al.*, (2016) visualised the improved interfacial packing using cryo-SEM for ultrasound treated bovine gelatin in comparison to untreated bovine gelatin. Ultrasound treatment of bovine gelatin reduced the size of the untreated fibres (*cf.* Fig. 5a) to smaller fibrils (*cf.* Fig. 5b), whereby this reduction in fibre size of bovine gelatin after sonication allowed for improved packing at the oil-water interface (*cf.* Fig. 5d), in comparison to emulsions prepared with untreated bovine gelatin (*cf.* Fig. 5c) (O'Sullivan, Murray, *et al.*, 2016).

Ultrasound treatment of a range of dairy proteins (whey protein concentrate, milk protein from retentate and calcium caseinate) utilising large scale sonoreactors demonstrated the capacity for ultrasound to modify the rheological behaviour (*i.e.*, reduction in bulk viscosity) of these proteins at pilot scale and was attributed to a reduction in protein aggregate size (Zisu *et al.*, 2010). This work highlights the potential applicability of ultrasound for the

functional modification of proteins at larger scales, whilst more work is required to fully implement this technology industrially (Gogate & Kabadi, 2009; Gogate, *et al.*, 2011).

4. Nanoemulsion fabrication from ultrasound and the associated parameters

Power ultrasound is a well-established technique for the formation of emulsions from either coarse pre-emulsions (*i.e.* $d_{3,2}$ > 50 μm) or discrete continuous and dispersed phases (Bondy & Söllner, 1935), consistently yielding nano-sized emulsion droplets (Leong, *et al.*, 2009). The resultant microstructure of emulsions is dependent upon formulation and the emulsification processing conditions. Processing configuration (*i.e.* batch or continuous processing methodologies) and associated parameters (*i.e.* acoustic power, residence time, etc.) have been extensively investigated, yet the fundamental influence of emulsion formulation with industrial relevant emulsifiers (*i.e.* high molecular weight biopolymers), geometric configuration to optimise contact time and the intrinsic interactions between processing and formulations have yet to be fully explored.

Increasing the contact time of a coarse pre-emulsion within the acoustic field can decrease the emulsion droplet size to a minimum size, provided the residence time of the emulsion within the acoustic field is sufficient and there is sufficient emulsifier present for droplet coverage (Maa & Hsu, 1999). For batch processing methodologies increasing the processing time decreases the emulsion droplet size (Abismail, *et al.*, 1999; Cucheval & Chow, 2008; Delmas, *et al.*, 2011; Jafari, *et al.*, 2007; Jena & Das, 2006; Kaltsa, *et al.*, 2013; Kentish, *et al.*, 2008; Kiani & Mousavi, 2013; Leong, *et al.*, 2009; O'Sullivan, Murray, *et al.*, 2015; O'Sullivan & Norton, 2016; Ouzineb, *et al.*, 2006; Ramisetty & Shyamsunder, 2011; Shanmugam, *et al.*, 2012; Tang, *et al.*, 2013). Similarly increasing the residence time of emulsions for continuous processing, by decreasing the flow rate, decreases emulsion droplet size (Behrend, *et al.*, 2000; Behrend & Schubert, 2001; Freitas, *et al.*, 2006; Kentish, *et al.*, 2008; O'Sullivan, Murray, *et al.*, 2015; O'Sullivan & Norton, 2016; Tang, *et al.*, 2013). For

both configurations, nano-sized emulsion droplets (~200 nm) were achieved. Nevertheless, prolonged residence time within the acoustic field can lead to growth in droplet size due to re-coalescence of emulsion droplets (*i.e.* over processing) in systems possessing insufficient emulsifier (Jafari, *et al.*, 2008; O'Sullivan, Murray, *et al.*, 2015; O'Sullivan & Norton, 2016).

Despite the size reduction of emulsion droplets as a function of increasing residence time, the same trend is not observed when considering droplet size distribution (DSD). Typically, the DSD initially increases as a function of ultrasonic processing time, followed by a decrease (Abismaïl *et al.*, 1999; Leong *et al.*, 2009). This behaviour is more pronounced for batch processing in comparison to continuous configurations, whereby there is a larger propensity for stagnant zones. Other emulsification technologies exhibit more uniform size distributions, often with minimal change in distribution width as a function of processing time, as demonstrated for valve-homogenisation and microfluidization approaches, in comparison to ultrasonic emulsification (Heffernan *et al.*, 2011; Lee & Norton, 2013).

The acoustic energy transmitted from the tip of the sonotrode to the medium is highly localised (as low as 1 cm from the sonotrode; Chivate & Pandit, 1995) due to attenuation (*i.e.* dispersion of acoustic waves from cavitation bubbles). Ultrasonic cavitation bubbles are highly unstable entities yielding implosions creating highly localised regions of hydrodynamic shear within close proximity of the tip (Kumar, *et al.*, 2006; Kumaresan, *et al.*, 2006). These ultrasonically induced implosions from cavitations result in the disruption of micron-sized oil droplets (> 50 μm) and facilitate the formation of nano-sized emulsion droplets (~200 nm). Batch processing of emulsions utilising ultrasound is often inefficient due to the nature of the emulsification process, whereby less than 2 % of the medium of a given volume experiences acoustic energy due to acoustic attenuation (Kumar, *et al.*, 2006; Kumaresan, *et al.*, 2006), and the turbulent forces generated by the acoustic streaming transfer the coarse emulsion from the bulk to within the vicinity of the tip, whereby emulsification occurs. Depending on the volume

of coarse emulsion being processed and the surface area of the tip via batch configuration this can be a time consuming process, in comparison to continuous processing methodologies, which typically demonstrate smaller chamber volumes relative to tip surface area, examples of which are shown in Fig. 6 and 7. Fig. 6b and 7a show configurations where the path of fluid flow through the system may potentially bypass the ultrasound, owing to the geometrical configuration of the chamber. Conversely, Fig. 6a, 7b, 7c and 7d depict setups where fluid flow is focused to a specific location, where there is a high probability of ultrasonic cavitations, thus, maximising the efficiency of the process.

Continuous processing configurations operate at lower residence times in comparison to batch processing (< 1 s), yet are capable of achieving comparable droplet sizes due to minimisation of chamber volume to maximise the volume of coarse emulsion within the acoustic field (*cf.* Fig. 6). By optimisation of the geometry, whereby the course of emulsion is pumped directly into the tip of the sonotrode, maximum droplet breakup can be achieved (*cf.* Fig. 6). The residence time for continuous processing is dictated by the flow rate of emulsion, whereby reduction of flow rate increases the contact time, allowing for a greater reduction in the droplet size (Freitas, *et al.*, 2006; Kentish, *et al.*, 2008; O'Sullivan, Murray, *et al.*, 2015; O'Sullivan & Norton, 2016; Tang, *et al.*, 2013).

The rate of droplet breakup can be improved by increasing the acoustic power transmitted to the coarse emulsion for both batch processing (Abismail, *et al.*, 1999; Cucheval & Chow, 2008; Delmas, *et al.*, 2011; Higgins & Skauen, 1972; Kaltsa, *et al.*, 2013; O'Sullivan, Murray, *et al.*, 2015; O'Sullivan & Norton, 2016) and continuous processing configurations (Freitas, *et al.*, 2006; O'Sullivan, Murray, *et al.*, 2015; O'Sullivan & Norton, 2016). However the minimum achievable droplet is dictated by the formulation of the emulsion (Maa & Hsu, 1999). For example, when comparing droplet sizes of emulsions prepared with 0.1 and 0.75 wt. % Tween 80, the achieved droplet sizes were 1 μm and 150 nm, respectively, highlighting

that sufficient emulsifier is necessary to achieve nano-sized emulsion droplets (O'Sullivan, Murray, *et al.*, 2015). Thus, increasing the acoustic power minimises the processing time required to achieve the minimum droplet size, dictated by emulsion formulation.

The resultant droplet size of emulsions fabricated via ultrasonic processes is dictated by the formulation of the emulsion (*i.e.* emulsifier type and concentration, dispersed phase type and volume fraction, presence of stabilisers, etc.), whilst the processing parameters determine the rate at which the resultant droplet is formed (Jafari, *et al.*, 2007). The majority of studies conducted utilise model emulsifier systems (*i.e.* low molecular weight surfactants), whereby a high degree of purity can be guaranteed. These surfactants include Tween 40 (Kentish, *et al.*, 2008), Tween 60 (Abismaïl, *et al.*, 1999), Tween 80 (O'Sullivan, Murray, *et al.*, 2015) and Span 80 (Leong, *et al.*, 2009). Increasing the emulsifier concentration decreases the droplet size to a minimum size given optimal processing conditions to achieve the minimal droplet size. Few studies have been conducted whereby industrial applicable ingredients are utilised, such as multi-component protein sources as the emulsifying agent. Kaltsa *et al.*, (2013), Heffernan *et al.*, (2011), O'Sullivan, Murray, *et al.*, (2015) and O'Sullivan & Norton, (2016) employed whey protein concentrate, sodium caseinate, milk protein isolate and pea protein isolate, respectively, as the emulsifying agent in oil-in-water emulsions. Submicron emulsion droplets have been prepared from these dairy proteins, whereby Kaltsa, *et al.*, (2013) and Heffernan, *et al.*, (2011) solely utilised batch processing, achieving ~600 and ~200 nm sized emulsion droplets, respectively. O'Sullivan, Murray, *et al.*, (2015) and O'Sullivan & Norton, (2016) comparatively assessed both batch and continuous configurations, highlighting the efficiency of continuous processing, as acoustic energy is utilised more efficiently in lower processing volumes associated with the chamber of the continuous configuration. In both cases, submicron emulsion droplets, ~200 nm, were achieved with sufficient emulsifier and adequate processing.

Power ultrasound has demonstrated a capacity for alteration of the functionality of proteins, and the efficient fabrication of emulsions, both acting through ultrasonic cavitations. However, to the author's knowledge, only one study is available comparing the effects of ultrasonic processing upon protein functionality as an emulsifier for pre- (*i.e.*, unadsorbed) and post-emulsification (*i.e.*, interfacial) (O'Sullivan, Beevers, *et al.*, 2015). Milk protein isolate and pea protein isolate were employed as the emulsifying agents in this study, and emulsions were prepared via microfluidiser (100 MPa for 1 pass). This study highlighted that emulsions prepared with ultrasound treated milk protein isolate post-emulsification yielded smaller emulsion droplets (12 μm) in comparison to emulsions prepared with either untreated or ultrasound treated pre-emulsification milk protein isolate (27.5 μm and 20 μm, respectively) at a concentration of 0.1 wt. % (O'Sullivan, Beevers, *et al.*, 2015). Emulsions prepared with ultrasound treated pea protein isolate yielded smaller droplets in comparison to their untreated counterparts, yet no significant differences were observed between ultrasound treated pea protein pre- and post-emulsification, attributed to the highly aggregated nature of pea protein in comparison to that of milk protein isolate (O'Sullivan, Beevers, *et al.*, 2015). The aggregated nature of pea protein, which is also typically observed in other plant derived protein ingredients upon solubilisation, is associated with a combination of isolation of the proteins components from the initial raw material and subsequent dehydration to produce a powder, yielding systems with hydrophobic exteriors and hydrophilic interiors (Boye, *et al.*, 2010; O'Sullivan, Murray, *et al.*, 2016).

From an industrial perspective, the most practical method for the implementation of ultrasound within a production environment is the continuous processing configuration, primarily due to the higher throughputs. Irrespective of configuration, the implementation of ultrasound within the food industry has been limited for a number of reasons: including pitting of the sonotrode tip (*i.e.* the gradual erosion of the tip material due to mechanical vibrations),

deposition of tip debris within the processed medium and poor performance of current ultrasound geometric configurations (*i.e.* dead zones). Freitas, *et al.*, (2006) developed a configuration for continuous processing of emulsions, whereby the ultrasonic probe was welded to the steel jacket (*cf.* Fig. 7c, d). Additionally the space in between the jacket and the glass tube, through which the medium passed, contained pressurised water which behaved as an acoustic conductor. This methodology prevents direct contact of the sonotrode with the medium being processed, hence removing the potential for contamination from ultrasonic pitting. Nevertheless, a fundamental understanding of energy transfer through the acoustic medium needs to elucidated. O'Sullivan, Murray, *et al.*, (2015) compared the effect of continuous processing at both lab and pilot scale, demonstrating that the pilot scale continuous configuration is dependent upon the ultrasonic amplitude (*i.e.* acoustic power), unlike the lab scale, due to bypassing of elements of pre-emulsion from the acoustic field at lower ultrasonic amplitudes, highlighting the necessity for optimisation of processing conditions at larger scales to efficiently achieve nanoemulsions.

The design of conventional continuous configurations is under investigation and continual development (Gogate *et al.*, 2011, 2003). The primary design criteria for the development of continuous ultrasonic processes are the operating conditions (*i.e.* acoustic power and processing time) and geometric parameters (sonotrode location, chamber volume, tip location within the chamber, etc.). Be that as it may, several other factors must be taken into consideration during the development and design of continuous ultrasonic systems: such as the hydrodynamic conditions within the acoustic field, variance due to the presence of discrete entities within the liquid medium (*i.e.* gaseous bubbles, immiscible liquid droplets, solid particles or high molecular weight biopolymers), the degree of acoustic attenuation chiefly due to the non-homogenous nature of food systems, and ratio of frequency irradiation to power dissipation within the locus of the tip of the sonotrode (Gogate *et al.*, 2011, 2003).

5. Conclusions and future trends

Even though low frequency, high power ultrasonic processing is a well-established technology within the food industry, numerous advances have been achieved in understanding the fundamental mechanisms for the functional modification of the physicochemical properties of proteins for specific applications and the factors associated with the efficient generation of nano-sized emulsion droplets in recent years. Ultrasound offers the potential for the functional modification of proteins through mechanical means, without the use of chemical or biological (*i.e.* enzymes) additives.

Ultrasonic treatment of proteins is related to physicochemical changes in structure, manifesting as: modifications to the functional attributes of proteins, reduction of bulk viscosity, increases of hydrophobicity and improvements in emulsion formation and stability. Ultrasound treatment of proteins in solutions affects the associative behaviour of proteins, disrupting the non-covalent forces which maintain protein aggregate structure, and reducing aggregate size.

Power ultrasound has shown to be an effective emulsification methodology, either utilising batch or continuous configurations, for the formation of nano-sized droplets. The development of nano-sized droplets is related to a combination of process parameters (*i.e.* acoustic power and contact time), geometric considerations (*i.e.* sonotrode location within the chamber, chamber geometry, etc.) and emulsion formulation (*i.e.* emulsifier type and concentrations, dispersed phase volume fraction, etc.). Emulsion formation within the acoustic field is attributed to the high levels of hydrodynamic shear generated by ultrasonic cavitations within close proximity to the tip of the sonotrode. Increasing the residence time which the coarse pre-emulsion has within the acoustic field decreases the emulsion droplet size, to a minimum droplet size as determined by the emulsion formulation. In addition, increasing the

acoustic power increases the rate by which this minimal droplet size is achieved. Nevertheless, further investigations of emulsification implementing ultrasound are required to develop optimised geometries for maximum droplet breakup, utilisation of industrial relevant ingredients (*i.e.* high molecular weight biopolymers) and the intrinsic interactions between emulsion formulation and operating conditions (*i.e.* microstructural engineering).

Lastly, it is worth mentioning that although numerous advances have been made in understanding the effects of power ultrasound upon proteins in aqueous solution and for the fabrication of nanoemulsions, this understanding is predominately at lab scale. Although studies are being conducted for both the ultrasound treatment of proteins and emulsion generation at pilot scale, further work is required to fully understand the specific design criteria to allow the effective utilisation of this versatile technology within the food industry.

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956 **Figure legends**

957 **Fig. 1.** Velocity distribution for acoustic streaming as predicted by Stuart Streaming, with chart
958 bar indicating the magnitude of velocity (m s^{-1}), adapted from Trujillo & Knoerzer, (2011a),
959 rights of use acquired from Elsevier.

960 **Fig. 2.** Images of oscillating cavitation bubbles, formation of cavity can be seen at t = 8 μs,
961 and cavity collapse at t = 16 μs. Frame rate: 125,000 fps. Image taken from Wagterveld, *et al.*,
962 (2011), rights of use acquired from Elsevier.

963 **Fig. 3.** Temperature profile distribution of an ultrasonic probe, after a 10 minute timescale,
964 with chart bar indicating temperature range (K). Image taken from Trujillo & Knoerzer,
965 (2011a), rights of use acquired from Elsevier.

966 **Fig. 4.** Schematic representation of rehydration of agglomerated high-protein dairy powder,
967 showing the 5 stages of powder rehydration. Image taken from Crowley *et al.*, (2016), rights
968 of use acquired from Springer.

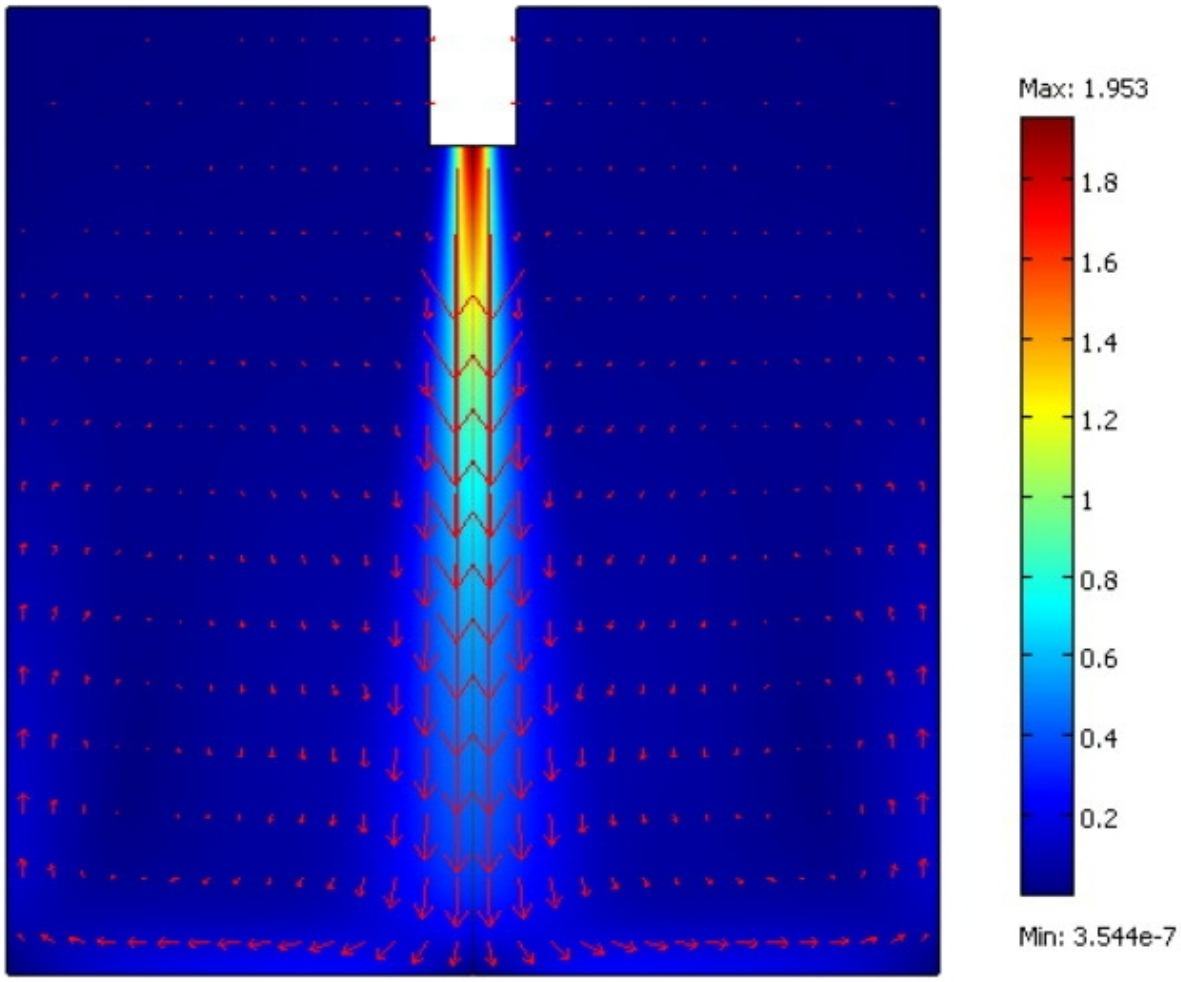
969 **Fig. 5.** Cryo-SEM micrographs of (a) 1% untreated bovine gelatin solution, (b) 1% ultrasound
970 treated bovine gelatin solution, (c) 1% untreated bovine gelatin stabilised emulsion and (d) 1%
971 ultrasound treated bovine gelatin stabilised emulsion. Scale bars are 2 μm and 10 μm for
972 solutions and emulsions, respectively. Image adapted from O'Sullivan, Murray, *et al.*, (2016).

973 **Fig. 6.** Schematic of continuous emulsification configurations for (a) lab scale and (b) pilot
974 scale processing. Image adapted from O'Sullivan, Murray, *et al.*, (2015).

975 **Fig. 7.** Examples of continuous ultrasonic configurations. Images taken from Gogate, *et al.*,
976 (2011) and Freitas, *et al.*, (2006), rights of use acquired from Elsevier.

977 **Figures**

978 Fig. 1.



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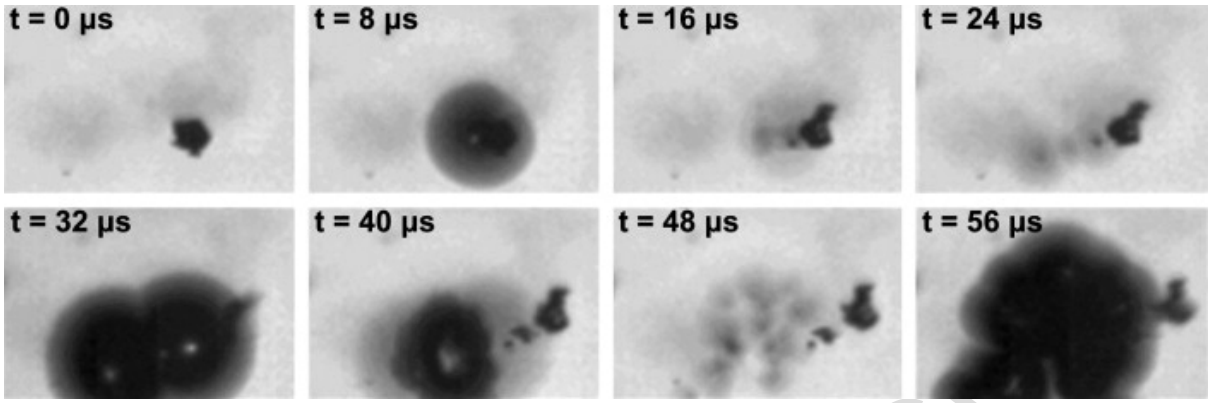
981 Fig. 2.



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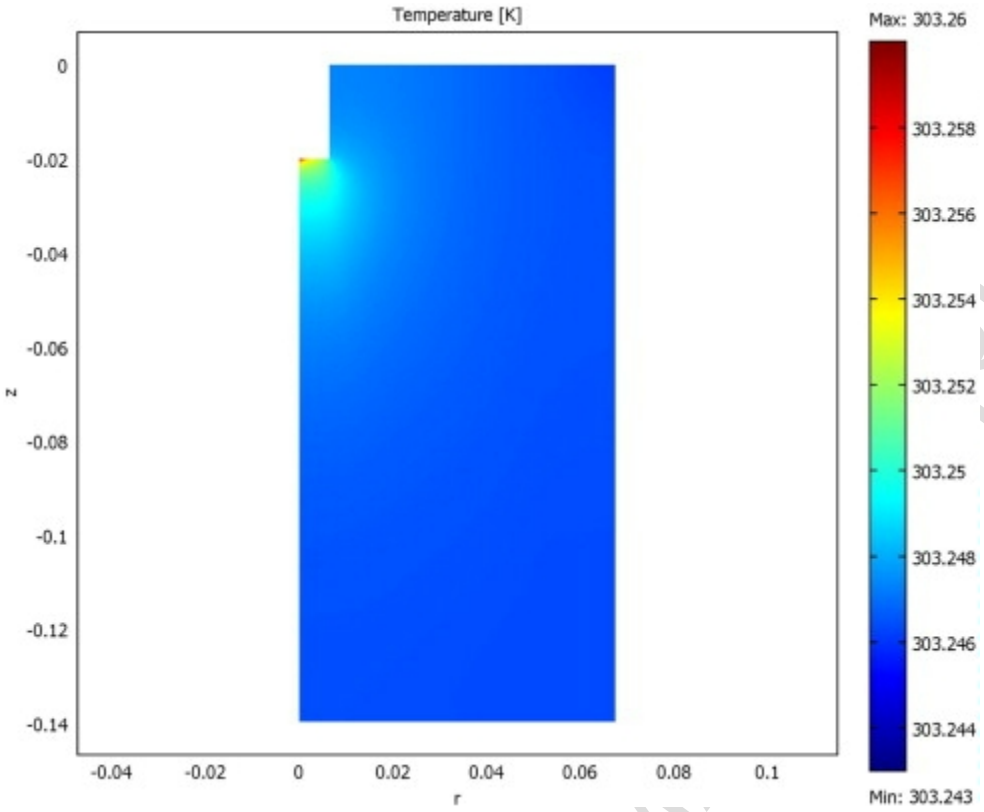
984 Fig. 3.



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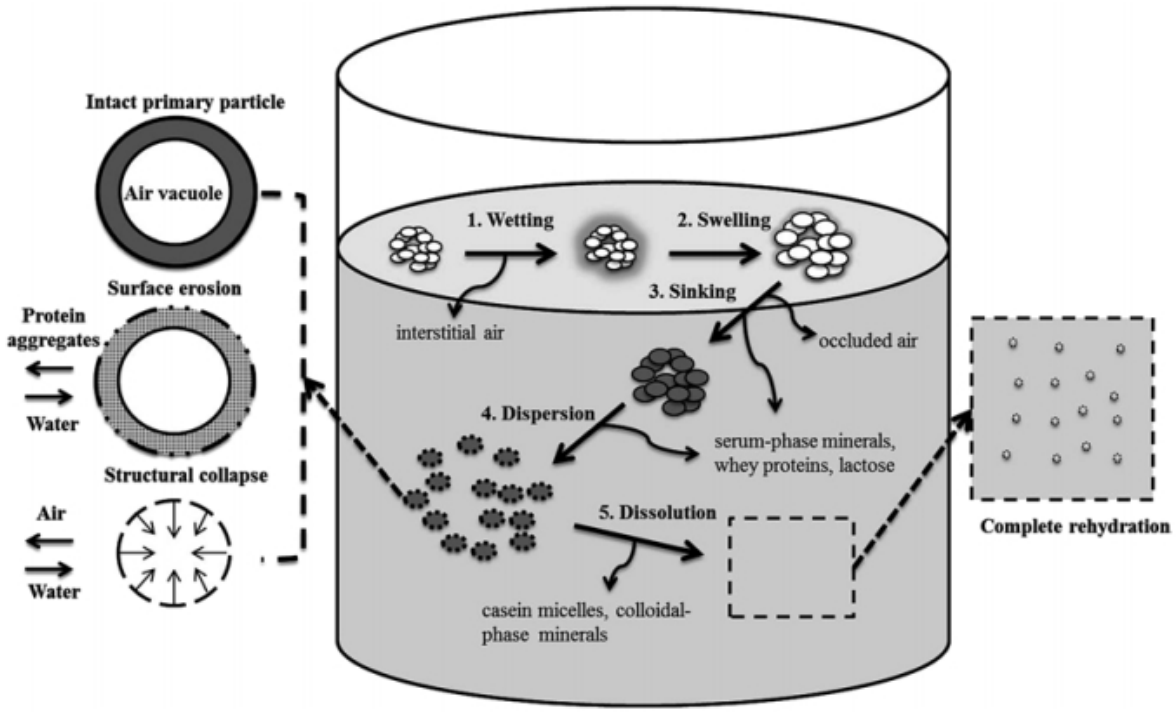
987 Fig. 4.



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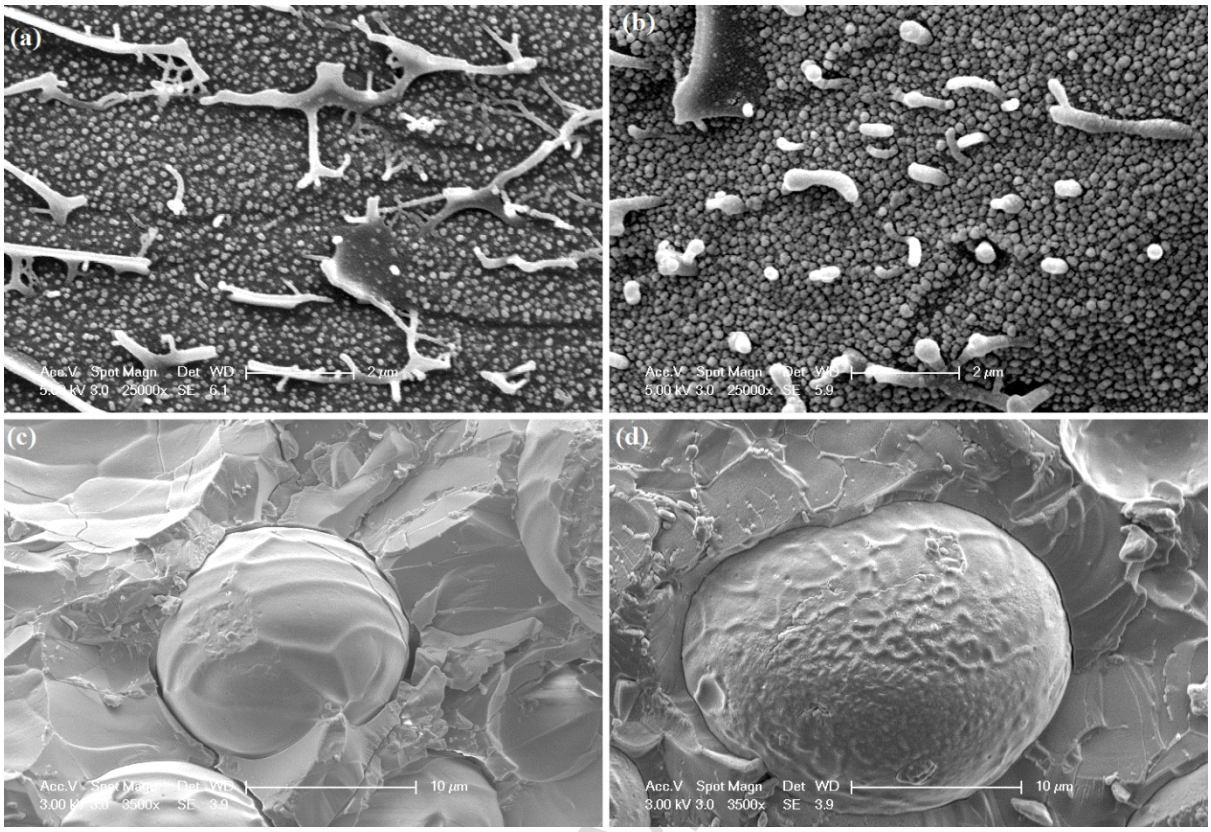
990 Fig. 5.



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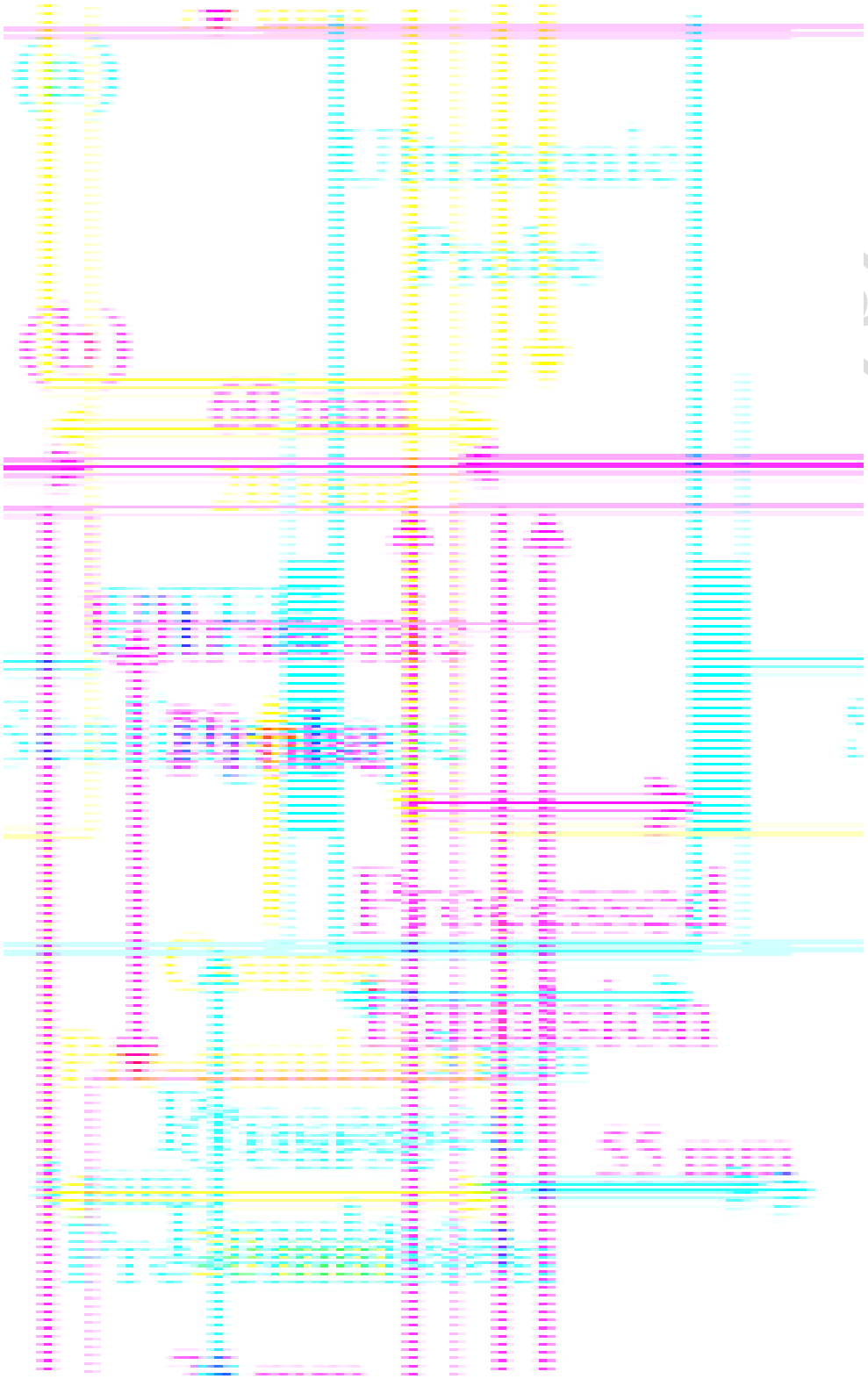
993 Fig. 6.



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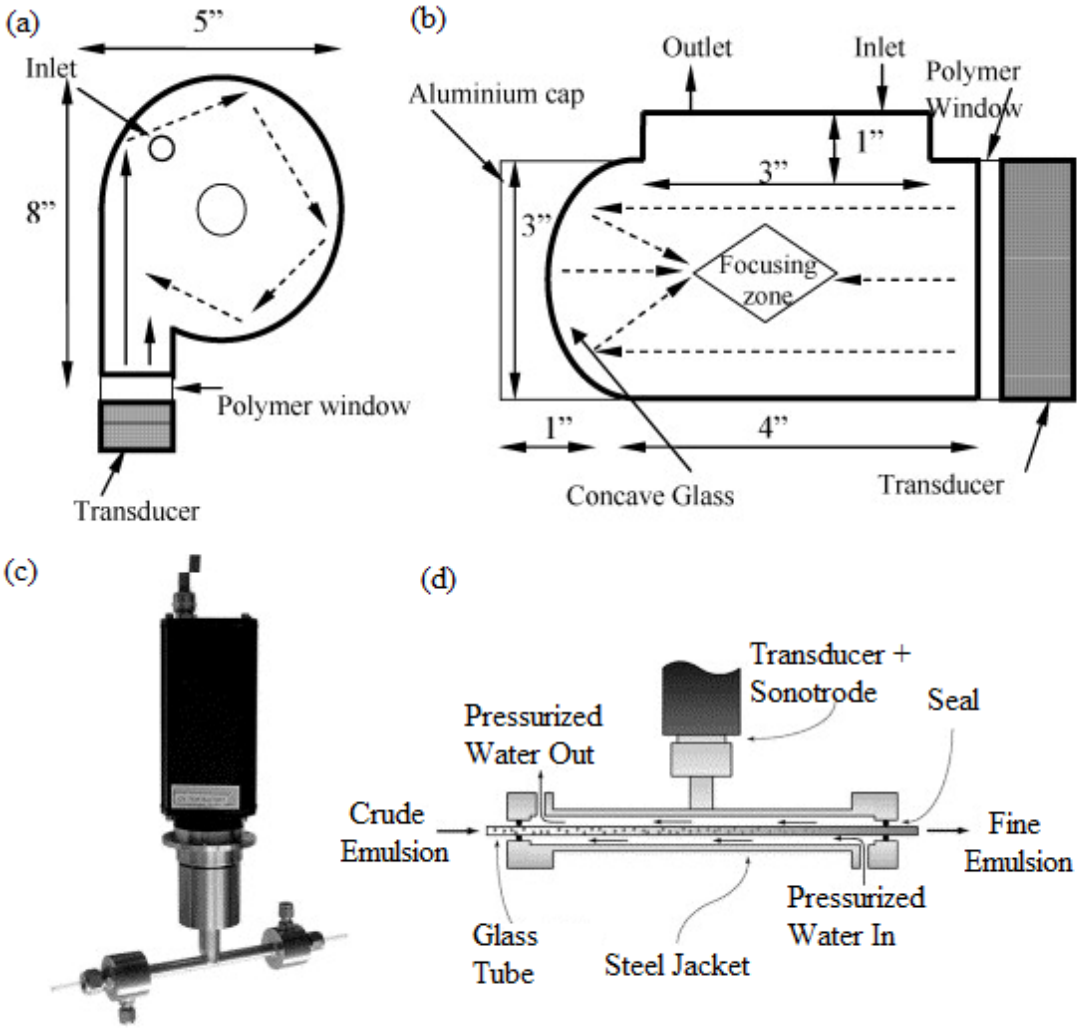
996 Fig. 7.



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999 **Tables**

1000 **Table 1.**

1001 Examples of studies examining the effect of ultrasonic treatment related to dairy, animal,
1002 cereal, legume, tuber and fruit protein sources.

Protein source		Reference
Dairy	Micellar casein	Madadlou, *et al.*, (2009)
	Sodium caseinate	O'Sullivan, Arellano, *et al.*, (2014); O'Sullivan, *et al.*, (2014), de Figueiredo Furtado *et al.*, (2017); de Figueiredo Furtado *et al.*, (2016)
	Calcium caseinate	Zisu, *et al.*, (2010)
	Milk protein concentrates/ isolates (including retentates and skim powders)	Chandrapala, *et al.*, (2014); McCarthy, *et al.*, (2014); O'Sullivan, Arellano, *et al.*, (2014); O'Sullivan, Beevers, *et al.*, (2015); Shanmugam, *et al.*, (2012); Uluko, *et al.*, (2013); Yanjun, *et al.*, (2014); Zisu, *et al.*, (2010)
	Whey protein concentrates/ isolates (including retentates, BSA and α-lactalbumin)	Arzeni, *et al.*, (2012), Barukčić, *et al.*, (2014), Chandrapala, *et al.*, (2011), Gülseren, *et al.*, (2007), Güzey, *et al.*, (2006), Guzey & Weiss, (2001), Jambrak, *et al.*, (2008), Jambrak, *et al.*, (2010), Jambrak, *et al.*, (2014), Martini, *et al.*, (2010), O'Sullivan, Arellano, *et al.*, (2014), Zisu *et al.*, (2010), Shen *et al.*, (2016), Abadía-García *et al.*, (2016)
Animal	Egg white proteins	Arzeni, *et al.*, 2012; Arzeni, Pérez, *et al.*, (2012); Krise, (2011); O'Sullivan, *et al.*, (2016); Zhou, *et al.*, (2015), Xiong *et al.*, (2016)
	Gelatin (bovine and piscine)	O'Sullivan, Murray, *et al.*, (2016)
Cereal	Rice	Li, *et al.*, (2015, 2016); O'Sullivan, *et al.*, (2016)
	Wheat	O'Sullivan, *et al.*, (2016b); Zhang, *et al.*, (2011)
	Corn	Ren, *et al.*, (2015), Zhou *et al.*, (2016)
	Millet	Nazari *et al.*, (2016)
Legume	Soy protein concentrates/ isolates (including flakes)	Arzeni, *et al.*, (2012); Chen, *et al.*, 2012; Hu, *et al.*, (2013); Jambrak, *et al.*, (2009); Karki, *et al.*, 2010; O'Sullivan, Murray, *et al.*, (2016); O'Sullivan, Park, *et al.*, (2016b), Wang *et al.*, (2017), Liu *et al,*, (2016), Zhou *et al.*, (2016)
	Pea protein isolate	O'Sullivan, Beevers, *et al.*, (2015); O'Sullivan, Murray, *et al.*, (2016), McCarthy *et al.*, (2016)
	Black bean protein isolate	Jiang *et al.*, (2014)
	Mung bean protein isolate	Charoensuk *et al.*, (2014)
	Lupin protein concentrate	O'Sullivan, Park, *et al.*, (2016a)

Tuber	Potato protein isolate	O'Sullivan, Park, *et al.*, (2016a)
Fruit	Walnut protein	Jincai *et al.*, (2013)
	Peanut protein	Chen *et al.*, (2016), Huang *et al.*, (2016)

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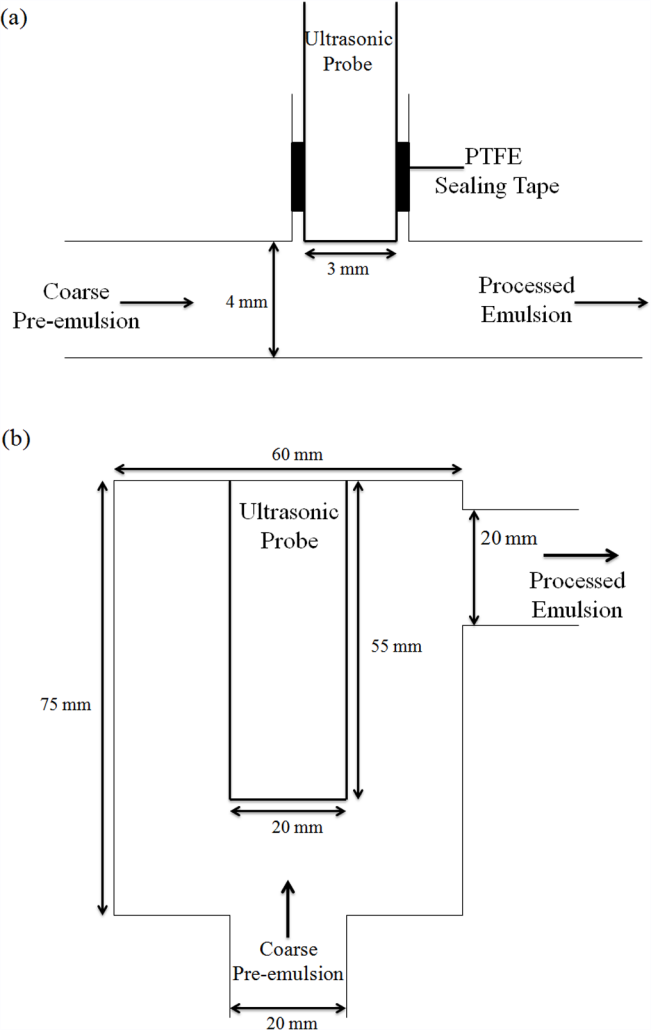
1004

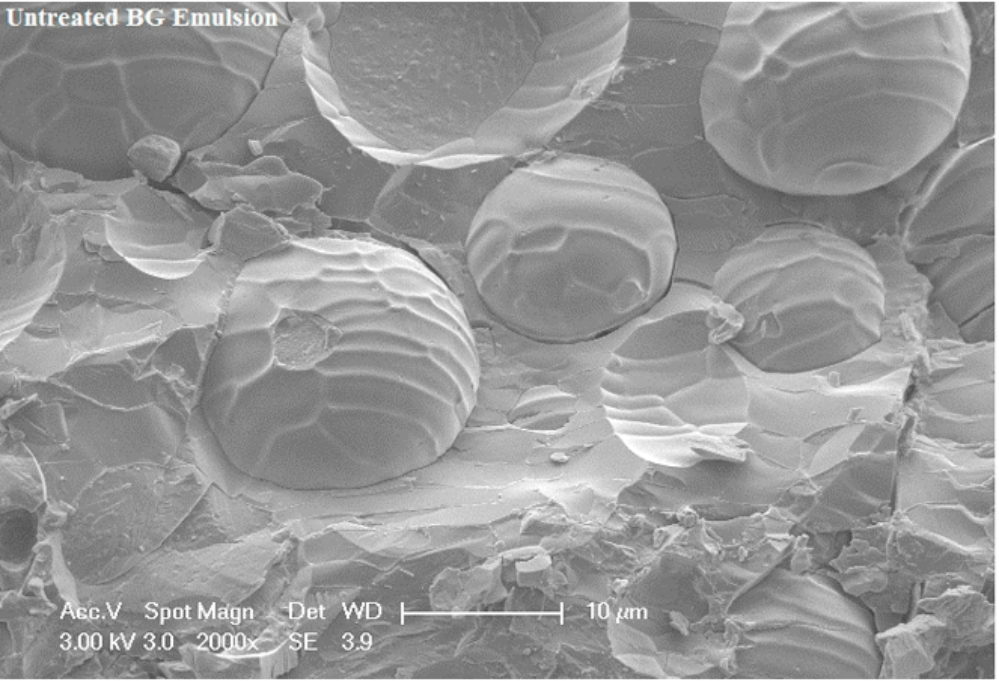
1005 **Table 2.**

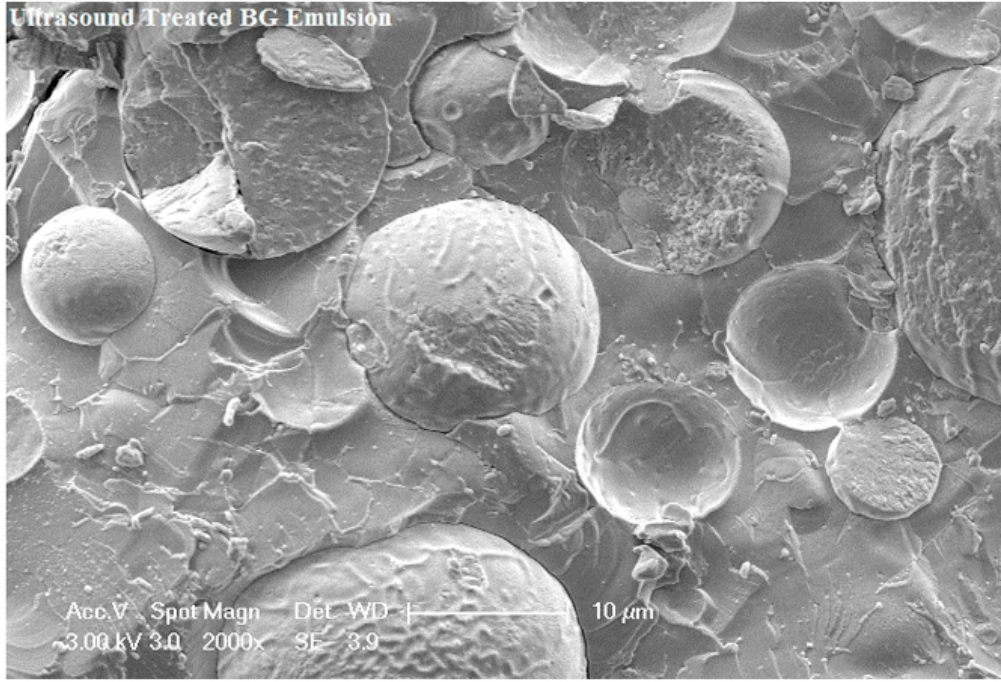
1006 Bond energy (kJ mol^{-1}) associated with intra- and intermolecular bonds present in proteins
1007 (McMurry, 2011).

	Typical bonds present in proteins	Bond energy (kJ mol^{-1})
Intramolecular bonds present within peptide chains	C-N (peptide bond)	285
	C=N	615
	C-C	348
	N-H	391
	C-H	413
	C=O	799
Intermolecular bonds occurring between amino acids	Hydrogen bonding	4 – 13
	S-S	226

1008


(a)
Ultrasonic Probe
PTFE Sealing Tape
3 mm
Coarse Pre-emulsion
4 mm
Processed Emulsion
(b)
60 mm
Ultrasonic Probe
20 mm
Processed Emulsion
55 mm
75 mm
20 mm
Coarse Pre-emulsion
20 mm


Untreated BG Emulsion
Acc.V Spot Magn Det WD 10 μm
3.00 kV 3.0 2000x SE 3.9


Ultrasound Treated BG Emulsion
Acc.V Spot Magn Det WD 10 μm
3.00 kV 3.0 2000x SE 3.9