Comparative assessment of the effect of ultrasound treatment on protein functionality pre- and post-emulsification

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HIGHLIGHTS

- Ultrasonic (US) effect on protein structure pre- and post-emulsification assessed.
- US of MPI post-emulsification yielded smaller droplets than other counterparts.
- US of PPI yielded no differences in droplet, in comparison to untreated PPI.
- US PPI exhibited emulsion stability in comparison to untreated PPI, at ≤0.5 wt.%.

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GRAPHICAL ABSTRACT

ABSTRACT

The effect of ultrasound treatment (∼34 W cm⁻² for 2 min) on unadsorbed and adsorbed milk protein isolate (MPI) and pea protein isolate (PPI) was investigated in terms of the ability to form and stabilise emulsion droplets. Submicron emulsions were prepared using a microfluidiser with a single pass at 100 MPa with untreated proteins (control), proteins ultrasound treated prior to pre-emulsification (unadsorbed) or proteins sonicated post pre-emulsification (adsorbed). Emulsions were also prepared with a low molecular weight surfactant, Tween 80, for comparative purposes.

Ultrasound treatment reduced the size of both MPI and PPI to the nanoscale (∼150 nm) from micrometre sized aggregates (∼20 μm). Emulsions prepared with ultrasound treated post pre-emulsification MPI yielded significantly smaller emulsion droplet sizes than those prepared with untreated or unadsorbed MPI. This behaviour is ascribed to rearrangement of interfacial protein allowing for the formation of smaller emulsion droplets. In contrast, emulsions produced with PPI yielded no significant differences, regardless of treatment, in emulsion droplet size, this was attributed to the more highly structured nature of PPI in contrast to MPI. Nevertheless, emulsions prepared with ultrasound treated PPI, both unadsorbed and adsorbed, yielded significantly more stable emulsion droplets than untreated PPI. This behaviour is associated with an enhanced interfacial layer and greater electrostatic repulsive forces as observed by an increased zeta-potential.

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1. Introduction

Proteins are highly functional ingredients widely utilised within a myriad of industries and in numerous applications, such as foaming, gelation and emulsification. Proteins are of particular interest...
due to their emulsifying capacity, owing to their ability to adsorb at oil–water interfaces and the development of viscoelastic layers [1,2]. The surface active nature of proteins is due to their amphiphilic character, owing to the presence of both hydrophilic and hydrophobic amino residues in their primary amino acid sequences [3]. Upon adsorption of proteins at oil–water interfaces protein–protein interfacial interactions occur, leading to the formation of strong viscoelastic films that are more resistant to coalescence and provide either steric or electrostatic stabilisation offering enhanced emulsion stability [4,5]. Therefore, it is of great importance for the food industry to investigate and understand methodologies that possess the capacity for enhancing the emulsifying performance of proteins.

Low frequency (≤100 kHz), high power (10–100 W cm⁻²) ultrasound has garnered particular interest for utilisation within the food industry over the past decade for the enhancement of ingredient functionality [6]. The effect of ultrasound upon the physicochemical structure of molecules is ascribed to ultrasonic cavitations (i.e. rapid formation and collapse of gas bubbles), which are generated by highly localised pressure differentials (up to 50 MPa) and heat (up to 5000 °C), occurring over very short periods of time (on the scale of milliseconds) [7]. Additionally, high shear forces and turbulence resulting from these cavitations contribute to the observed effects of ultrasound [8].

The application of ultrasound to proteins in aqueous solution has been related to a range of dairy (sodium caseinate, whey protein isolate and milk protein isolate), animal (gelatin and egg white protein) and vegetable (pea protein isolate, soy protein isolate and rice protein isolate) proteins [9–17]. Ultrasound treatment of proteins has been shown to typically reduce the size of protein associates, ascribed to disruption of non-covalent and electrostatic interactions maintaining the structure of these aggregates [9,18]. This reduction in associate size allows for increased molecular mobility through the bulk to the oil–water interface and improved interfacial packing as measured from interfacial tension data. Decreased aggregate size and increased surface activity of ultrasound treated proteins allow for the fabrication of smaller and more stable emulsion droplets in comparison to those prepared with their untreated counterparts [6]. Furthermore, proteins have been employed as the emulsifying agent in studies where ultrasound was utilised as the emulsification methodology post pre-emulsion formation, such as for sodium caseinate [19], whey protein [20], milk protein isolate [21] and pea protein isolate [22].

The work of O'Sullivan et al. [21] and O'Sullivan and Norton [22] on milk protein isolate (MPI) and pea protein isolate (PPI), respectively, highlight the possibility that ultrasound treatment affects protein structure differently as to whether the protein is free in solution (i.e. unadsorbed) or adsorbed at the oil–water interface. Emulsions prepared in these cases were with interfacial proteins, whilst the same proteins were utilised in the works of O'Sullivan et al. [9,10,23] were sonicated prior to pre-emulsification, yielding different emulsion droplet sizes at emulsifier concentrations <1 wt.%. Comparison of these studies suggest that ultrasound treatment of proteins prior to pre-emulsification yields an improvement in the emulsifying performance in comparison to those treated post pre-emulsification. However, different emulsification methodologies were employed in both instances for the fabrication of submicron emulsions, high pressure valve homogenisation versus sonication, which may account for the observed differences in emulsion droplet size. Therefore, it is necessary to comparatively assess the effect of ultrasound upon unadsorbed and adsorbed proteins for the formation and stability of emulsions utilising the same emulsification technique to further understand its effects upon protein structure in a range of systems.

Milk protein isolate (MPI) is a mixture of micellar casein (~80%) and whey protein (~20%) [24]. The casein in MPI has a micellar structure similar to the native form found in milk, and the whey proteins are present in the globular native form [25]. Casein is a mixture of four protein fractions: αs1-, αs2-, β- and κ-casein [26]. In solution, these caseins form spherical colloidal micelles, due to regions of high hydrophobicity and the charge distribution arising from the amino acid sequence, phosphorylation and glycosylation [27]. The internal structure of the casein micelle is constituted of the calcium sensitive fractions (αs1- and αs2-), which are maintained by associative hydrophobic interactions and calcium–phosphoserine crosslinking. The micelle is stabilised by κ-casein which is predominately situated on the micelle surface due to its highly hydrophilic carboxylic–terminus protruding into the aqueous phase. β-casein exists in a temperature dependant equilibrium between the aqueous phase and the micelle [28,29]. The main protein fractions in whey are β-lactoglobulin (β-Lg), α-lactalbumin (α-lac) and bovine serum albumin (BSA) [30].

Pea protein isolate (PPI) is an ingredient utilised within the food industry and has gained significant interest owing to its functionality [31,32], and moreover its hypoallergenic characteristics [33]. PPI, a pulse legume, is extracted from Pisum sativum, and is the predominant cultivated protein crop in Europe [34]. The protein fractions found within PPI are albumins, globulins, and other minor fractions, such as prolamins and glutelins [33,35].

In this work, milk protein isolate (MPI) and pea protein isolate (PPI) were investigated in order to assess the effect of power ultrasound on the emulsifying performance of pre- and post-emulsification. The objective of this research was to probe the effect of ultrasonic processing upon the structure of proteins free in solution and adsorbed at emulsion interfaces discerned in terms of differences in emulsion droplet size (d3,2), long term emulsion stability and zeta-potential; the industrial rationale being to ascertain the optimal stage within a process stream for ultrasonic processing to occur. Oil-in-water submicron emulsions were prepared with untreated, ultrasound treated prior to pre-emulsification and ultrasound treated post pre-emulsification MPI and PPI at different concentrations, and compared between them and to a low molecular weight surfactant, Tween 80.

### 2. Materials and methods

#### 2.1. Materials

Milk protein isolate (MPI) and pea protein isolate (PPI) were both kindly provided by Kerry Ingredients and Flavours (Listowel, Ireland). The composition of these proteins is provided in Table 1, acquired from the material specification forms from suppliers. Tween 80 and sodium azide were purchased from Sigma–Aldrich (UK). The oil used was commercially available rapeseed oil. The water used in all experiments was passed through a double distillation unit (A4000D, Aquatron, UK). All materials were used with no further purification or modification of their properties.

**Table 1**

| Composition and pH of milk protein isolate (MPI) and pea protein isolate (PPI). |
|-----------------|-----------------|-----------------|
| **MPI**         | **PPI**         |
| Protein (wt.%)  | 86              | 86              |
| Moisture (wt.%) | 4               | 7.2             |
| Fat (wt.%)      | 1.5             | 0               |
| Carbohydrate (-) | 1              | pos.            |
| Ash (wt.%)      | 6               | 4.85            |
| pH at a concentration 1 wt.% (-) | 6.74 | 7.45 |
2.2. Methods

2.2.1. Preparation of emulsifier solutions

MPI, PPI, and Tween 80 were dispersed in water at 40 °C for 6 h to obtain solutions within a protein concentration range of 0.1–3 wt.%. MPI, and Tween 80 were soluble at the investigated range of concentrations, whilst PPI exhibited a sedimenting component irrespective of hydration time. Sodium azide (0.02 wt.%) was added to the solution to mitigate against microbial activity.

2.2.2. Ultrasound treatment of protein solutions and protein stabilised emulsions

An ultrasonic processor (Viber Cell 750, Sonics, USA) with a 12 mm diameter stainless steel probe was used to ultrasound treat 50 ml aliquots of protein solutions or protein stabilised emulsions (cf. Section 2.2.4) in 100 ml plastic beakers, whereby the sonotrode was immersed with a depth of 3 mm in all cases, which were placed in an ice bath to reduce heat gain. The protein solutions and protein stabilised emulsions were sonicated with a frequency of 20 kHz and amplitude of 95% (wave amplitude of 108 µm at 100% amplitude) for up to 2 min, whereby these processing conditions were selected based on the works of O’Sullivan et al. [9,10]. This yielded an ultrasonic power intensity of ~34 W cm⁻², determined calorimetrically by measuring the heat gain of the sample as a function of treatment time, under adiabatic conditions. The acoustic power intensity, \( I_a \) (W cm⁻²), was calculated as follows [36]:

\[
I_a = \frac{P_a}{S_A} \quad \text{where} \quad P_a = m \times c_p \left( \frac{dT}{dt} \right)
\]

(1)

where \( P_a \) (W) is the acoustic power, \( S_A \) is the surface area of the ultrasound emitting surface (1.13 cm²), \( m \) is the mass of ultrasound treated solution (g), \( c_p \) is the specific heat of the medium (4.18 kl/gK) and \( \frac{dT}{dt} \) is the rate of temperature change with respect to time, starting at \( T = 0 \) (°C/s).

The temperature of protein solutions and protein stabilised pre-emulsions was measured before and after sonication by means of a digital thermometer (TGST3, Sensor-Tech Ltd., Ireland), with an accuracy of ±0.1 °C. Prior to ultrasound treatment, the temperature of protein solutions and protein stabilised pre-emulsions was within the range of 5–10 °C. After ultrasonic irradiation, the temperature was raised to approximately ~45 °C, for both protein solutions and protein stabilised emulsions.

2.2.3. Characterisation of untreated and ultrasound treated protein solutions

2.2.3.1. Microstructure characterisation. The size of untreated and ultrasound treated MPI and PPI was measured by static light scattering (SLS) using the Mastersizer 2000 (Malvern Instruments, UK). Protein size is reported as a size distribution data. The protein size distributions are reported as the average and standard deviation of three repeat measurements.

2.2.3.2. Microstructure visualisation. Cryogenic scanning electron microscopy (Cryo-SEM; Philips XL30 FEG ESEM) was utilised to visualise the microstructure of untreated and ultrasound treated MPI and PPI. One drop of protein solution was frozen to approximately –180 °C in liquid nitrogen slush. Samples were then fractured and etched for 3 min at a temperature of ~90 °C inside a preparation chamber. Subsequently, samples were sputter coated with gold and scanned at a voltage of 3 kV, during which the temperature was maintained below ~160 °C by addition of liquid nitrogen to the system.

2.2.3.3. Protein electrophoretic potential characterisation. The electrophoretic potential of protein solutions, more commonly referred to as the zeta-potential, was measured by electrophoretic mobility using the Zetasizer Nano Series (Malvern Instruments, UK). Zeta-potential measurements were conducted at a solids concentration of 0.1 wt.%, by dilution of protein solutions with distilled water, and added to a specialised disposable capillary cell for measurement. Zeta potential measurements are reported as the average and standard deviation of three repeat measurements.

2.2.3.4. Molecular structure characterisation. The molecular structure of untreated and ultrasound MPI and PPI was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using a Mini-Protein 3 Electrophoresis System (Bio-Rad, UK), where proteins were tested using the reducing method. 100 µl of protein solution at a concentration of 1 wt.% was added to 900 µl of Laemmli buffer (Bio-Rad, UK; 65.8 mM Tris–HCl, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue) and 100 µl of β-mercaptoethanol (Bio-Rad, UK) in 2 ml micro tubes and sealed. These 2 ml micro tubes were placed in a float in a water bath at a temperature of 90 °C for 30 min, to allow the reduction reaction to take place. A 10 µl aliquot was taken from each sample and loaded onto a Tris–acylamide gel (Bio-Rad, UK; 4%~20% Mini Protein TGX Gel, 10 wells). A molecular weight standard (Bio-Rad, UK; Precision Plus Protein™ All Blue Standards) was used to determine the primary protein structure molecular weight profile of the samples. Gel electrophoresis was carried out initially at 55 V (1 × 20 mA) for 10 min, then at 155 V (1 × 55 mA) for 45 min in a running buffer (10 × Tris/Glycine/SDS Buffer, Bio-Rad, UK; 4% Tris, 15% glycine, 0.5% SDS). The gels were removed from the gel cassette and stained with Coomassie Bio-safe stain (Bio-Rad, UK; 4% phosphoric acid, 0.5% methanol, 0.05% ethanol) for 1 h and de-stained with distilled water overnight.

2.2.4. Preparation of oil-in-water emulsions

10 wt.% dispersed phase (rapeseed oil) was added to the continuous aqueous phase containing either MPI or PPI, or the low molecular weight surfactant, Tween 80, at different concentrations, ranging from 0.1 to 3 wt.%. Oil-in-water pre-emulsions were prepared by emulsifying these mixtures at 8000 rpm for 2 min using a high shear mixer (SL2T, Silverson, UK), whereby coarse emulsions with a droplet size (\( d_{32} \)) of approximately 10 µm was achieved regardless of emulsifier type or concentration employed. Three different types of protein stabilised emulsions were prepared, one set was prepared with untreated proteins (i.e. control), another set with ultrasound treated proteins prior to pre-emulsification, and the final set prepared by initially forming pre-emulsions and subsequently treating the samples with ultrasound. Submicron emulsions were then prepared by further emulsifying these pre-emulsion using an air-driven microfluidiser (M110S, Microfluidics, USA), at 100 MPa for a single pass. The initial temperature of these emulsions was 5 °C to minimise the potential for protein aggregation from the high processing pressures. The final temperatures of emulsions prepared after homogenisation was ~30 °C.

2.2.5. Characterisation of oil-in-water emulsions

2.2.5.1. Droplet size measurements. The droplet size of emulsions was measured by SLS using a Mastersizer 2000 (Malvern Instruments, UK) immediately after emulsification. Emulsion droplet size values are reported as the volume–surface mean diameter (Sauter diameter; \( d_{32} \)). The stability of the emulsions was assessed by droplet size measurements over 28 days, where emulsions were stored under refrigeration conditions (4 °C) throughout the duration of the stability study. The droplet sizes and error bars are reported as the mean and standard deviation, respectively, of measured emulsions prepared in triplicate.

2.2.5.2. Interfacial tension measurements. The interfacial tension between the aqueous phase (pure water, protein solution, or sur-
factant solution) and oil phase (rapeseed oil) was measured using a
tensiometer K100 (Krüss, Germany) with the Wilhelmy plate
method. The Wilhelmy plate has a length, width and thickness of
19.9 mm, 10 mm and 0.2 mm, respectively, and is made of plat-
ium. The Wilhelmy plate was immersed in 20 g of aqueous phase
to a depth of 3 mm. Subsequently, an interface between the aque-
ous phase and oil phase was created by carefully pipetting 50 g of
the oil phase over the aqueous phase. The measurement was con-
ducted over 3600 s and the temperature was maintained at 20 °C
throughout the duration of the test. The interfacial tension values
and the error bars are reported as the mean and standard deviation,
respectively, of three repeat measurements.

2.2.5.3. Emulsion visualisation. Cryogenic scanning electron
microscopy (Cryo-SEM; Philips XL30 FESEM) was used to
visualise the interface of pre-emulsions prepared using untreated
and ultrasound treated prior to pre-emulsification MPI or PPI as
previously described for protein solutions (cf. Section 2.2.3.2).

2.2.5.4. Emulsion droplet electrophoretic potential characterisation.
The electrophoretic potential (i.e. zeta potential) of emulsion
droplets stabilised with untreated, ultrasound treated unadsorbed
and ultrasound treated adsorbed MPI and PPI at a concentration
of 0.75 wt.% were measured as previously described for protein solu-
tions (cf. Section 2.2.3.3). A protein concentration of 0.75 wt.% was
employed as the works of O’Sullivan et al. [9,10,21,22] indicate that
this concentration is such that there is sufficient emulsifier present
to stabilise the nanoemulsion droplets (~120 nm).

2.3. Statistical analysis

Student’s t-test with a 95% confidence interval was used to
assess the significance of the results obtained. t-test data with
P<0.05 were considered statistically significant.

3. Results and discussions

3.1. Effect of ultrasound on the physicochemical properties of MPI
and PPI solutions

The effect of ultrasound treatment on the size of MPI and PPI was
investigated. Protein solutions at a concentration of 1 wt.% were
sonicated for 2 min with a frequency of 20 kHz and an ultrasonic
amplitude of 95%. Protein size distributions for untreated and ultra-
sound treated MPI and PPI are shown in Fig. 1. As can be seem
from the results in Fig. 1 there is a significant reduction (P<0.05)
in the size of both MPI and PPI after ultrasound treatment. Both
untreated MPI and PPI possess a unimodal distribution, whereby
the peak of untreated MPI (cf. Fig. 1a) depicts denatured protein
aggregates, either composed of whey–whey or casein–whey inter-
actions, and the peak of untreated PPI (cf. Fig. 1b) represents the
denatured component within PPI, both situations ascribed to ther-
mal denaturation during the preparation of the isolate, allowing for
the formation of disulphide bridges (~S–S~) between amino acid
residues containing sulphur (i.e. cysteine and methionine). Similar
results (i.e. protein aggregate size reduction) were observed at the
lower (0.1–0.75 wt.%) and higher (1.5 and 3 wt.%) concentrations
utilised in this study. The disruption of the micron sized entities
is attributed to disruption of non-covalent and electrostatic in-
teractions maintaining these aggregated structures. The micron sized
peak in the case of MPI was wholly removed, whereas, the micron
sized peak for PPI was not completely eliminated, associated with
insufficient energy provided from ultrasound to achieve scission of
the covalent bonds maintaining these aggregated structures [9].
The acoustic energy (~34 W cm⁻²) transmitted to protein these
protein solutions provides only sufficient energy to achieve disrup-
tion of the associative non-covalent interactions which maintain
these aggregates (3–14 kJ mol⁻¹), whilst insufficient energy is sup-
plied to achieve scission of disulphide bridges [226 kJ mol⁻¹] and
hydrolysis of peptide bonds, as demonstrated in the works of
O’Sullivan et al. [9,10]. The peptide bond exhibits a resonant struc-
ture with the adjacent carboxylic group and the associated bond
energies of −C−N− and −C=N− are 285 kJ mol⁻¹ and 615 kJ mol⁻¹,
respectively [37].

Cryo-SEM micrographs of untreated and ultrasound treated MPI
and PPI solutions at a concentration of 1 wt.% are shown in Fig. 2.
As can be seen from Fig. 2, untreated aggregates of MPI appear to
be distributed in a densely packed network (cf. Fig. 2a), whilst soni-
cated MPI appears to be distributed uniformly as discrete entities
with a smaller overall size (cf. Fig. 2b), in comparison to untreated
MPI. The structure of untreated PPI in solution appears to be highly
aggregated with large entities (cf. Fig. 2c), whereas, ultrasound
treated PPI exhibits a distinct reduction in protein size (cf. Fig. 2d).
These findings are consistent with the previously observed reduc-
tion in aggregate size of ultrasound treated MPI and PPI (cf. Fig. 1),
and confirms our hypothesis that sonication disrupts non-covalent
interactions maintaining protein aggregates.

The molecular structure of untreated and ultrasound treated
MPI and PPI was investigated next. Protein solutions at a concen-
tration of 1 wt.% were ultrasound treated for 2 min at 20 kHz, with a
power intensity of ~34 W cm⁻². Electrophoretic profiles obtained

![Fig. 1. Protein size distributions for (a) 1% untreated MPI (solid line) and 1% ultra-
sound treated MPI (dashed line) and (b) 1% untreated PPI (solid line) and 1% ul-
trasound treated PPI (dashed line).](image-url)
by SDS-PAGE for untreated and ultrasound treated MPI and PPI, and the molecular weight standard, are shown in Fig. 3. No difference in the protein fractions was observed between untreated and sonicated MPI and PPI (cf. Fig. 3). These results are in concurrence with those reported by Krise [38] who showed no difference in the primary structure molecular weight profile between untreated and ultrasound treated egg white, with a treatment conducted at 55 kHz, 45.33 W cm \(^{-2}\) for 12 min. Moreover, the obtained protein fractions are in agreement with the literature for MPI [23,39] and PPI [9,31].

3.2. Comparison of ultrasound treatment pre- and post-emulsification of MPI and PPI stabilised emulsion

A range of oil-in-water emulsions were prepared with 10 wt.% rapeseed oil and either untreated, ultrasound treated prior to pre-emulsification or ultrasound treated post pre-emulsification MPI and PPI, or a low molecular weight surfactant, Tween 80, at different concentrations (0.1–3 wt.%). Emulsion droplet sizes were measured immediately after emulsification are shown in Fig. 4.

Significant differences \((P<0.05)\) were observed between the emulsion droplet sizes prepared with untreated, unadsorbed ultrasound treated and adsorbed ultrasound treated MPI at concentrations <1 wt.% (cf. Fig. 4a), where ultrasound treatment of adsorbed MPI yielded the smallest emulsion droplet size. The greater exhibited reduction in emulsion droplet size of ultrasound treated adsorbed MPI is attributed to structural rearrangement of protein adsorbed at the oil–water interface allowing for a potential improvement in the interfacial packing of protein molecules. Furthermore, no significant differences \((P>0.05)\) in emulsion droplet size were observed for emulsions prepared with MPI at higher concentrations (>1 wt.%), regardless of treatment, attributed to the excess of MPI present within the systems. In relation to ultrasound treatment of unadsorbed milk proteins, it has been associated with physicochemical changes in protein structure (i.e. reduction in aggregate size) manifesting as an enhancement in the emulsifying performance (i.e. smaller, more stable emulsion droplets) of ultrasound treated isolates (MPI), as shown by O’Sullivan et al. [10], and milk protein concentrates (MPC), as shown by Yanjun et al. [39], in comparison to untreated MPI, in agreement with the observed results (cf. Fig. 4a).

In the case of PPI, no significant differences \((P>0.05)\) were observed between emulsions prepared with untreated, unadsorbed ultrasound treated and adsorbed ultrasound treated PPI, at all tested concentrations. This behaviour is attributed to the highly aggregated nature of PPI in comparison to MPI, whereby dena-
tured aggregates of PPI are maintained by the presence of covalent interactions prohibiting their complete size reduction as shown by the previously discussed protein size distributions (cf. Fig. 1b) [33], which has the potential of minimising differences in molecular mobility between the tested PPI samples. Nevertheless, these results are in contradiction to those presented by O’Sullivan et al. [9] who established an improvement in the emulsifying performance, as demonstrated by a significant reduction ($P < 0.05$) in the emulsion droplet size, of ultrasound treatment of unadsorbed PPI in comparison to untreated PPI. These differences are attributed to different methods of emulsification, whereby emulsions in this study and that of O’Sullivan et al. [9] were prepared utilising an air-driven microfluidiser (100 MPa for 1 pass) and a high pressure valve homogeniser (125 MPa for 2 passes), respectively.

Emulsions prepared with Tween 80 yielded significantly smaller ($P < 0.05$) emulsion droplet sizes at all investigated concentrations when compared to both MPI and PPI regardless of treatment owing to molecular weight differences allowing for greater molecular mobility, whereby Tween 80 has a molecular weight of 1.31 kDa and both MPI and PPI have molecular weights $>20$ kDa (cf. Fig. 3). This behaviour can be interpreted by comparing the interfacial tension of the studied systems. Fig. 5 presents the interfacial tension between water and rapeseed oil, for Tween 80, untreated MPI and PPI, and unadsorbed ultrasound treated MPI and PPI all at concentrations of 0.1 wt.%. In order to evaluate the presence of surface active impurities within the dispersed phase, the interfacial tension between distilled water and rapeseed oil was measured.

The interfacial tension of all systems decreases continually as a function of time (cf. Fig. 5). In light of these results, the decrease in interfacial tension with time is predominately ascribed to the nature of the dispersed phase utilised, and to a lesser extent the type of emulsifier employed. Gaonkar [40,41] described that the time dependant nature of interfacial tension of commercially available vegetable oils against water was due to the adsorption of surface active impurities present within the oils to the oil–water interface. Additionally, Gaonkar [40,41] reported that after purification of vegetable oils the time dependency of interfacial tension was no longer observed. Purification of these vegetable oils was achieved by percolation through a bed of synthetic magnesium silicate.

The initial value of interfacial tension provides information about the rate of adsorption of surface active agents to interfaces, whilst the equilibrium value provides information about both the facilitation of droplet breakup and packing of emulsifier molecules at the oil–water interface [3]. Significant differences ($P < 0.05$) in interfacial tension were observed in the initial value of interfacial tension of Tween 80 ($\sim 6$ mN m$^{-1}$) in comparison to both investigated proteins ($\sim 14$ mN m$^{-1}$), whereby this behaviour is ascribed to the significant ($P < 0.05$) molecular weight differences between the low molecular weight surfactant and tested proteins, allowing for greater molecular mobility through the bulk to the oil–water interface.
In relation to the interfacial tension of MPI (cf. Fig. 5a) there is a marginal reduction in the initial value and significant reduction ($P<0.05$) in the equilibrium value of interfacial tension. This result is consistent with the previously discussed emulsion droplet size data (cf. Fig. 4a), and confirms that size reduction of aggregates of MPI (cf. Fig. 1a) allows for increased rates of protein adsorption and enhanced interfacial packing at the oil–water interface. With respect to the interfacial tension of PPI (cf. Fig. 5b) the initial value of ultrasound treated PPI interfacial tension is significantly lower ($P<0.05$) than untreated PPI, whilst no significant differences ($P<0.05$) were observed in the equilibrium values of interfacial tension. Additionally, the interfacial tension data for adsorbed ultrasound treated proteins was unattainable due to both the nature of sample preparation, the necessity for prior emulsification, and measurement of interfacial tension requires both discrete aqueous and dispersed phases. These hypotheses were explored by cryo-SEM of pre-emulsions, to allow for visualisation of the emulsion interface, prepared with untreated and adsorbed ultrasound treated MPI and PPI stabilised emulsions, at an emulsifier concentration of 1.5 wt.% for all investigated pre-emulsions (cf. Fig. 6).

Emulsion droplets of pre-emulsion prepared with untreated MPI (cf. Fig. 6a) exhibit a textured surface upon the droplet, whereas emulsion droplets of pre-emulsion prepared with unadsorbed ultrasound treated MPI (cf. Fig. 6b) appear to have a smoother interface, suggesting improved interfacial packing of MPI at the oil–water interface, accounting for the observed lower equilibrium value of interfacial tension (cf. Fig. 5a) and the decrease in emulsion droplet size (cf. Fig. 4a). The droplet surfaces of pre-emulsions prepared with untreated PPI (cf. Fig. 6c) appear to have a rough surface, and similarly pre-emulsions prepared with unadsorbed ultrasound treated PPI (cf. Fig. 6d) appear to have an equivalently rough emulsion droplet surface. These results are consistent with the interfacial tension data (cf. Fig. 5), where a significant reduction ($P<0.05$) in the equilibrium interfacial tension upon sonication of MPI, whilst no significant reduction ($P<0.05$) in equilibrium interfacial tension was observed for ultrasound irradiated PPI, and accounted for by visualisation of interfacial packing of protein.

The stability of oil-in-water emulsions prepared with untreated, ultrasound treated prior to pre-emulsification and ultrasound treated post pre-emulsification MPI and PPI, and Tween 80 at comparative purposes, was assessed over a 28 day period. Fig. 7 shows the evolution of droplet size ($d_{32}$) as a function of time for emulsions prepared with untreated, sonicated unadsorbed and sonicated adsorbed MPI and PPI, as well as Tween 80, at emulsifier concentrations of 0.1 wt.%; 0.75 wt.% and 3 wt.%.

Emulsions prepared with MPI (cf. Fig. 7a) irrespective of treatment, untreated, ultrasound treated prior to pre-emulsification and ultrasound treated post pre-emulsification, were stable against coalescence and flocculation over the 28 days of this study, whilst emulsions prepared with Tween 80 (cf. Fig. 7) exhibited a growth in emulsion droplet size, solely at a concentration of 0.1 wt.%; owing to insufficient emulsifier for long-term emulsion droplet stabilisation. Emulsions prepared with higher concentrations ($>0.5$ wt.%) of Tween 80 and MPI were all stable and resistant to emulsion instabilities. In the case of PPI stabilised emulsions (cf. Fig. 7b), both unadsorbed sonicated and adsorbed sonicated PPI demonstrated emulsion stability throughout the 28 day emulsion stability study, whilst emulsions prepared with untreated PPI exhibit growth in emulsion droplet size as a function of time. This behaviour is ascribed to sonication yielding an improved interfacial packing and conformation of ultrasound treated PPI in comparison to that of untreated PPI owing to the significant reduction in protein aggregate size (cf. Fig. 1 and Table 2). Thus, ultrasound treatment of PPI, irrespective of unadsorbed or adsorbed, improves the emulsion stability at lower emulsion concentrations ($<0.5$ wt.%). Additionally, emulsions prepared with higher concentrations ($>0.5$ wt.%) of PPI were stable regardless of treatment and the emulsion droplet size remained static throughout the 28 days of the study, and this behaviour is ascribed to a sufficiency of protein molecules to allow for complete interfacial coverage.

Differences in the adsorption behaviour of untreated and ultrasound treated (unadsorbed and interfacial) MPI and PPI was explored in terms of differences of zeta-potential. Table 2 shows the zeta-potential of MPI and PPI solutions and emulsions sta-

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![Fig. 6. Cryo-SEM micrographs of protein stabilised pre-emulsions: (a) 1.5% untreated MPI stabilised emulsion, (b) 1.5% unadsorbed ultrasound treated MPI stabilised emulsion, (c) 1.5% untreated PPI stabilised emulsion and (d) 1.5% unadsorbed ultrasound treated PPI stabilised emulsion. Scale bar is 10 μm in all cases.](image-url)
Fig. 7. Effect of emulsifier type on droplet size as a function of time for O/W emulsions stabilised with: (a) 0.1% MPI, (b) 0.1% PPI, (c) 0.75% MPI, (d) 0.75% PPI, (e) 3% MPI and (f) 3% PPI, all related to Tween 80. (Δ) represents untreated proteins, (○) represents ultrasound treated unadsorbed protein and (▼) represents ultrasound treated adsorbed protein, in all cases.

Table 2
Effect of sonication on the zeta-potential of untreated and ultrasound treated protein solutions (MPI and PPI) at a concentration of 0.1 wt.% and emulsions stabilised with 0.75 wt.% untreated, ultrasound treated unadsorbed and ultrasound treated adsorbed MPI and PPI, diluted to a concentration of 0.1 wt.% for zeta-potential measurement.

|                      | Zeta-potential (mV) | MPI       | PPI       |
|----------------------|---------------------|-----------|-----------|
| **Protein solutions**|                     |           |           |
| Untreated            | −25.4 ± 0.8         | −27.2 ± 0.6 |
| Ultrasound treated   | −29.7 ± 0.5         | −36.8 ± 0.5 |
| **Emulsions**        |                     |           |           |
| Untreated            | −35.1 ± 0.6         | −15.2 ± 0.4 |
| Ultrasound treated unadsorbed | −34.8 ± 1.1 | −19.8 ± 0.6 |
| Ultrasound treated adsorbed        | −35.4 ± 0.9         | −20.1 ± 0.7 |
bilised with 0.75 wt.% MPI and PPI (untreated, ultrasound treated unadsorbed and ultrasound treated adsorbed). As can be seen from Table 2 there is a significant increase (P<0.05) in the zeta potential of both MPI and PPI upon ultrasound treatment and this behaviour is ascribed to structural rearrangement of the protein resulting from ultrasonic cavitations. Moreover, the increase in zeta-potential exhibited in protein solutions is additionally observed for the aforementioned emulsion systems prepared with PPI, whereby emulsions prepared with ultrasound treated PPI possessed a significantly greater (P<0.05) zeta-potential in comparison to their untreated counterparts. This behaviour explains the observed significant improvement (P<0.05) in emulsion stability of emulsions prepared with ultrasound treated PPI, both unadsorbed and adsorbed, in comparison to those prepared with untreated PPI at emulsifier concentrations of 0.1 wt.% (cf. Fig. 7b), allowing for increased electrostatic repulsive forces between emulsion droplets. In the case of emulsion prepared with MPI, no significant differences (P<0.05) were observed in the zeta-potential, accounting for the long term stability of emulsions prepared with MPI, whether untreated or ultrasound treated.

4. Conclusions

This study showed that ultrasound treatment (20 kHz, \(-34\) W cm\(^{-2}\) for 2 min) of MPI and PPI significantly reduced (P<0.05) the size of protein aggregates, whereby complete disruption to the nanoscale was achieved for MPI, whilst a micron sized component remained for PPI, attributed to the presence of covalent bonding maintaining the structure of these denatured aggregates. Ultrasonic size reduction of protein aggregates is attributed to hydrodynamic shear forces associated with ultrasonic cavitations, and disruption of associative hydrophobic and electrostatic interactions maintaining these entities.

Emulsions prepared with ultrasound treated post-pre-emulsification MPI yielded significantly (P<0.05) smaller emulsion droplets than those prepared with either untreated or ultrasound treated prior to pre-emulsification MPI. This behaviour is associated with rearrangement of MPI at the interface during ultrasound treatment. By comparison, emulsions prepared with PPI, irrespective of treatment, yielded no significant (P<0.05) differences in emulsion droplet size. However, ultrasound treatment, both of unadsorbed and adsorbed PPI, enhanced emulsion stability, whereas emulsions prepared with untreated PPI exhibited growth in emulsion droplet size. This improvement in emulsion stability is attributed to an improvement in the interfacial layer resulting, as shown by zeta potential measurements, from ultrasonic irradiation. These results highlight that ultrasound treatment is capable of improving the emulsifying performance of proteins. Moreover, it depends as to whether ultrasound treatment occurs pre- or post-emulsification within an industrial process, and the protein source being utilised within a given formulation.

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