**Article:**
Zhang, S, Holmes, M, Ettelaie, R et al. (1 more author) (2020) Pea protein microgel particles as Pickering stabilisers of oil-in-water emulsions: Responsiveness to pH and ionic strength. Food Hydrocolloids, 102. ISSN 0268-005X

https://doi.org/10.1016/j.foodhyd.2019.105583

# Pea protein microgel particles as Pickering stabilisers of oil-in-water emulsions: Responsiveness to pH and ionic strength

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## Abstract

The aim of this study was to design plant protein-based microgel particles to create Pickering emulsions (20 wt% sunflower oil, 0.05-1.0 wt% protein) and investigate the role of electrostatic interactions on colloidal behaviour of such emulsions. Pea protein microgels (PPM) were designed using a facile top-down approach of heat-set protein gel formation followed by controlled shearing. The aqueous dispersion of PPM had hydrodynamic diameters ranging from 200 to 400 nm at pH 7.0 to pH 9.0 with high negative charge (-30 to -35 mV) and pI was pH 5.0. With increasing ionic concentration from 1 to 250 mM NaCl, the ζ-potential of PPM changed to -8 mV due to charge screening effects, in line with theoretical calculations of the electrostatic potential. The Pickering emulsions with smallest droplet sizes ($d_{43}$) ~25 µm exhibited excellent coalescence stability and high adsorption efficiency of PPM at the oil-water interface (>98%) at pH 7.0, with the latter being supported by confocal microscopy showing effective adsorption of the PPMs at the droplet surface. Adjusting the pH of the emulsions to pI demonstrated aggregation of adsorbed PPM at the particle-laden interface providing a higher degree of adsorption as well as enhancing inter-droplet flocculation and the shear-thinning character as compared to those at pH 7.0 or pH 3.0. Charge screening effects in presence of 100 mM NaCl resulted in PPM-PPM aggregation and enhanced viscosity of the emulsions. Findings from this study on pea protein microgels would open avenues for rational designing of sustainable Pickering emulsions in the future.



**Keywords:** Pickering emulsion; pea protein; microgel; interaction potential; plant protein; particle-stabilized interface

## 1. Introduction

In recent times, gradual dietary changes towards sustainable plant-based ingredients has increased and, consequently designing food emulsions using plant proteins have been the preferred direction for emulsifiers to reduce food-production associated environmental footprints (Rayner, et al., 2014). Furthermore, plant proteins are appreciated by consumers as they are "green", "vegan-friendly" and are considered to be less allergenic and are less expensive as compared to the most commonly used dairy proteins. A range of plant proteins, such as soybean, pea, chickpea, faba bean, lentil, cowpea, French bean, sweet lupin, tomato seed and zein protein (Ben-Harb, et al., 2018; Jayasena, et al., 2010; Karaca, et al., 2011; Kimura, et al., 2008; Pan, et al., 2015; Sarkar, et al., 2016a) have been investigated for stabilizing oil-in-water (O/W) emulsions. However, many if not most, of these plant proteins have limited aqueous solubility (Makri, et al., 2006; Nesterenko, et al., 2013; Sarkar, et al., 2016a) and are less digestible as compared to the dairy proteins (de Folter, et al., 2012; Laguna, et al., 2017). This restricts easy replacement of dairy proteins in food products by plant proteins.

In comparison to the conventional protein-stabilized emulsions, the investigation of plant protein-based particles to create Pickering emulsions can be particularly interesting as these emulsions involve particle-stabilization of the droplets and therefore do not need perfect solubilisation of these proteins in the aqueous phase. In other words, these emulsions need the particles to be partially wettable by the oil and aqueous phases. In addition, Pickering emulsions have gained remarkable research interest in the food colloids community in recent years due to their distinctive stability against coalescence and Ostwald ripening as well as their ability to alter lipid digestion kinetics of emulsions post consumption (Aveyard, et al., 2003; Binks, 2002; Dickinson, 2012; Dickinson, 2013; Sarkar, et al., 2019). Hence, there is a strong

demand in food industries to find cheaper plant protein-based Pickering stabilizer alternatives that can effectively stabilize emulsion droplets for longer term against coalescence.

As compared to the extensive studies on particles derived from animal-based proteins being used as Pickering stabilizers, *e.g.* whey protein microgels (Destribats, et al., 2014; Sarkar, et al., 2016b) and lactoferrin nanoparticles or nanogels (David-Birman, et al., 2013; Gal, et al., 2013; Meshulam, et al., 2014; Sarkar, et al., 2018), those involving particles obtained from plant proteins are fairly recent and are attracting significant research attention. For example, Liu and co-authors (Liu, et al., 2017; Liu, et al., 2013; Liu, et al., 2014; Liu, et al., 2016; Peng, et al., 2020; Peng, et al., 2018; Zhu, et al., 2017) investigated the ability of soy protein nanoparticles aggregates (SPN) (~ 100 nm, created via heat-treatment at 95 ºC for 15 min) to act as Pickering stabilizers. On the other hand, Chen, et al. (2014) prepared heat denatured soy protein nanogel particles at various pH values (pH 2.0-7.0) and added ions (0-200 mM NaCl). In another recent study, Zhu, et al. (2018) suggested the importance of the electrostatic screening by ions (100-200 mM NaCl) to improve freeze-thaw stability of Pickering emulsions stabilized by soy protein-based nanoparticles. Besides the commonly used soy protein-based particles, peanut protein microgel particles have been recently investigated, where these microgel particles were prepared *via* enzyme treatment and had hydrodynamic diameters ranging from 200 to 300 nm and were used to stabilize high-internal-phase Pickering emulsions with 87 % oil volume fractions (Jiao, et al., 2018). Water-insoluble zein-based colloidal particles and kafirin nanoparticles have also been reported as possible Pickering stabilizers. For example, de Folter, et al. (2012) fabricated zein-based colloidal particles with an average diameter of ~ 70 nm. Xiao, et al. (2016) used an anti-solvent precipitation method to produce kafirin nanoparticles with a mean diameter of 206.5 nm. Gliadin colloidal particles (GCPs)

94 with average diameter of ~120 nm at acidic pH that were prepared by an anti-solvent procedure
95 (Hu, et al., 2016) were suggested as Pickering stabilisers.
96
97 Due to the significant academic and industrial interests resulting from their low cost, Liang, et
98 al. (2014) created pea protein nanoparticles for the first time. In such a process, an aqueous
99 dispersion of pea protein isolate (PPI) was adjusted at pH 3.0 to produce pea protein-based
100 particles with hydrodynamic diameter of 100-200 nm. In addition, such particles generated oil-
101 in-water (O/W) Pickering emulsion with 20 wt% oil volume faction. In another study, Shao, et
102 al. (2015) found that such pea protein particle-stabilized Pickering emulsion enabled controlled
103 release of lipophilic bioactive compounds during *in vitro* gastrointestinal digestion. Compared
104 to the emulsion stabilized with untreated PPI, the Pickering-stabilised emulsion had a sustained
105 release behaviour due to their gel-like inter-droplet network formation. Another recent study
106 by Cochereau, et al. (2019) designed pea protein microgel particles with protein concentration
107 of 1-4 wt% at pH 6-8 via slow and modest heat treatment (*i.e.* 20- 40 °C).
108
109 It is thus clear from the literature that stabilizing Pickering emulsions using plant
110 protein-based particles is a relatively recent endeavour. In particular, considering the
111 strong research interests by food industries and academic community in pea protein, it
112 is surprising that relatively little attention has been devoted to designing pea protein-
113 based particles for the purpose of stabilizing Pickering emulsion droplets. Although the
114 gelation properties of pea proteins (Bora, et al., 1994; Mession, et al., 2015; Shao, et al.,
115 2015), pea protein-based aggregates, such as heat-treated fibrillar aggregates (Munialo
116 et al., 2014), mixed pea globulin aggregates (Mession, et al., 2017), as well as thermal
117 aggregates from mixed pea globulin and β-lactoglobulin (Chihi, et al., 2016; Chihi, et
118 al., 2018), have been widely studied, there has only been two studies that have used pea

protein particles to prepare Pickering emulsions (Liang, et al., 2014; Shao, et al., 2015). Furthermore, these two studies have been performed using pea protein gelation at just one particular pH (pH 3.0), thus restricting the use of such emulsions over a wider range of pH and ionic strengths. To our knowledge, there has been no study that has systematically characterized the colloidal properties of thermally-crosslinked pea protein microgel in the aqueous phase, as well as when present at the droplet surface, and in particular also investigated the role of electrostatics on the colloidal behaviour of these types of emulsions. Considering the recent demand of plant-based protein particles, it is necessary to characterise the ability of such Pickering emulsions at a wide range of pH and ionic strengths to understand their responsiveness to environmental conditions during their processing and indeed after consumption.

Hence, in this study, pea protein has been used to design pea protein microgels (PPM) using a top down approach for creating a heat-set hydrogel, followed by controlled shearing to investigate their potential to stabilize O/W Pickering emulsions, which has not been reported in literature to date. We hypothesized that pea protein microgel already adsorbed to the interface would aggregate at the droplet surface by suitably adjusting the pH to acidic pH, forming a densely packed layer of particles further protecting the oil droplets against coalescence. Colloidal stability of pea protein microgel particles (PPM) in aqueous phase and PPM-stabilized emulsions were systematically characterized as a function of pH (pH 2.0-9.0) and ionic strength (1-250 mM NaCl) using particle sizing (dynamic light scattering), droplet sizing (static light scattering), optical microscopy, confocal laser scanning microscopy (two dimensional (2D) as well as three dimensional (3D) images), apparent viscosity, adsorption efficiency assessment and ζ-potential measurements. The composition of PPM at the

interface was assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In addition, we calculated the interaction potentials of the particles as a function of pH and ionic strengths at close separation distances to identify the electrostatic contribution of these particles at the droplet surface during any droplet aggregation phenomena.

## 2. Materials and Methods

### 2.1 Materials

Commercial pea protein concentrate (Nutralys S85XF) (PPC) with 85% protein content was kindly gifted by Roquette (Lestrem, France). Sunflower oil was purchased from local supermarket and used without any further purification. Mini-PROTEAN TGX Gels, ProtoBlue Safe Colloidal Coomassie G-250 stain and all sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) chemicals were purchased from Bio-Rad Laboratories, UK. Sodium azide, Nile Red and Nile Blue were purchased from Sigma Aldrich (Dorset, UK). All other chemicals were of analytical grade and purchased from Sigma-Aldrich Dorset, UK) unless otherwise specified. All solutions were prepared with Milli-Q water (water purified by a Milli-Q apparatus, Millipore Corp., Bedford, MA, USA) with a resistivity of 18.2 MΩ cm at 25 ℃.

### 2.2 Methods

#### 2.2.1 Preparation of pea protein microgel (PPM)

Pea protein-based Pickering particles were prepared using slight modification of the top-down method developed by Sarkar, et al. (2016b). Briefly, the PPC powder at 15 wt% powder *i.e.* 12.75 wt% protein was dispersed in 20 mM phosphate buffer at pH 7.0. The aqueous dispersion

168 of PPC was mixed for 2 hours using magnetic stirring at 300 rpm, and then stored at 4 ºC
169 overnight. The PPC dispersion was heated at 90 ºC for 1 hour to allow formation of heat-set
170 gel (**Figure 1**). During heat treatment, the globular pea proteins were denatured and unfolded
171 (Laguna, et al., 2017). And then the denatured proteins aggregated *via* the disulphide
172 crosslinking forming a macroscopic protein-based hydrogel. After cooling to room temperature
173 using flowing water at 25 ºC, the pea protein hydrogel was stored at 4 ºC overnight. These
174 hydrogels were mixed with 20 mM phosphate buffer (1: 1 v/v) at pH 7.0 and were broken to
175 macrogel particles using a blender (HB711M, Kenwood, UK) at speed 3 for 5 minutes. After
176 removing the air bubbles generated during blending using vacuum (25-30 bar) for 15 min, the
177 macroscopic gel particle dispersion was homogenized using two passes through a two-stage
178 valve homogenizer (Panda Plus 2000, GEA Niro Soavi Homogeneizador Parma, Italy)
179 operating at first/ second stage pressures of 250/ 50 bars, respectively. The resultant particles,
180 termed as pea protein microgels (PPM) contained 6.375 wt% protein.

181

182 **2.2.2 Preparation of PPM-stabilized Pickering emulsions (PPM-E)**

183 Sunflower oil was mixed with appropriate quantities of PPM at 20:80 oil: protein (w/w) ratio
184 using rotor-stator (L5M-A, Silverson machines, UK) mixing at 8,000 rpm for 5 minutes. The
185 PPM dispersion was diluted using phosphate buffer to have 0.05, 0.10, 0.25, 0.50 and 1.0 wt%
186 protein content in the final emulsions, henceforth, such emulsions are referred as E0.05, E0.1,
187 E0.25, E0.5 and E1.0, respectively. The pre-homogenized oil-PPM mixture was further
188 homogenized by a two-stage valve homogenizer (Panda Plus 2000, GEA Niro Soavi
189 Homogeneizador Parma, Italy) operating at two stages, of 250 and 50 bar pressures (**Figure 1**)
190 resulting in Pickering emulsions (PPM-E, E0.05-E1.0). Sodium azide (0.02 wt%) was added
191 as an antimicrobial agent.

192 **2.2.3 Coalescence stability of PPM-E during storage**

193 The PPM-Es (E0.05, E0.10, E0.25, E0.50 and E1.0) were stored at 4 ºC for a period of 28 days
194 and were monitored using droplet sizing, ζ-potential measurement and visual observation to
195 select the most stable emulsions for pH and ion treatment.

196

197 **2.2.4 pH and ion treatment**

198 To investigate the colloidal stability of PPM and PPM-E (E1.0, chosen based on the
199 coalescence stability study) under environmental conditions, the samples were subjected to
200 different pH and ionic strengths. The pH adjustment (pH 2.0-9.0) was done by both "low to
201 high" and "high to low" methods by adding 1 M HCl or 1 M NaOH, without any salt addition
202 (Adal, et al., 2017). For "high to low" pH adjustment, the PPM at pH 9.0 was rapidly adjusted
203 to a target pH while mixing. For "low to high", the PPM at pH 2.0 was adjusted to a target pH
204 quickly while mixing. In case of E1.0, the pH responsiveness was checked at pH 3.0, 5.0 and
205 7.0. For ionic strengths, the pH value of PPM was kept constant at pH 7.0 and ionic strength
206 was adjusted from 1-250 mM NaCl. For E1.0, the physicochemical stability was assessed by
207 subjecting the emulsions to 1 mM, 10 mM and 100 mM NaCl, respectively.

208

209 **2.2.5 Size and ζ-potential measurements.**

210 The hydrodynamic diameters of PPM dispersion at various pH and ionic strengths were
211 measured at 25 ºC using a Zetasizer Nano-ZS (Malvern Instruments Ltd, Malvern,
212 Worcestershire, UK). The PPM sample was diluted to 0.004 wt% particle concentration with
213 Milli-Q water before measurement. Assuming that there is no particle–particle interaction in
214 the diluted sample, the hydrodynamic diameter ($D_h$) of the droplets was calculated using the
215 Stokes–Einstein equation (Equation 1):

$$D_h = \frac{k_B T}{3\pi\eta D_t} \quad (1)$$

where $D_t$ is the translational diffusion coefficient, $k_B$ is Boltzmann's constant, $T$ is thermodynamic temperature, and $\eta$ is dynamic viscosity. The refractive index of PPM was set at 1.52. The absorbance of the protein was assumed to be 0.001.

Droplet size distribution of PPM-Es (E0.05, E0.10, E0.25, E0.50 and E1.0) as a function of storage and E1.0 as a function of different pH or with various ionic strengths were determined using static light scattering (SLS) techniques using a Malvern MasterSizer 3000 (Malvern Instruments Ltd, Malvern, Worcestershire, UK) at 25 ℃. Samples were added dropwise in distilled water until the instrument gave an obscuration rate between 4 and 6%. The average droplet size of the emulsion was reported as the volume mean diameter ($d_{43}= \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$) and surface mean ($d_{32}= \frac{\sum n_i d_i^3}{\sum n_i d_i^2}$)

where, $n_i$ is the number of droplets with a diameter, $d_i$. The refractive index of sunflower oil and the dispersion medium were set at 1.46 and 1.33, respectively. The absorbance value of the emulsion droplets was set at 0.001.

Zetasizer Nano ZS was used to measure the ζ-potential of PPM dispersion, PPM-Es (E0.05, E0.10, E0.25, E0.50 and E1.0) as a function of storage and E1.0 as a function of pH and ionic strength. Before measurement, PPM dispersion at different pH values was diluted to 0.004 wt% particle concentration and E1.0 with different pH values was diluted to 0.005 wt% droplet concentration using Milli-Q water adjusted to relevant pH (2.0-9.0). Similarly, PPM or E1.0 containing different concentrations of NaCl was diluted Milli-Q water adjusted to relevant NaCl concentrations (1-250 mM). The diluted sample was then added into a folded capillary cell (DTS1070 cell, Malvern Instruments Ltd., Worcestershire, UK), which had two electrodes.

After 120 seconds of equilibration in the Zetasizer at 25 °C, the PPM particles or E1.0 droplets moved towards oppositely charged electrodes. The magnitude of ζ-potential was determined from the terminal speed of the particle motion using Henry's equation, with Smoluchowski approximation appropriate here since the thickness of the diffused double layer is expected to be much smaller than the size of the particles.

### 2.2.6 Adsorption efficiency of PPM at the oil-water interface

To determine the adsorption efficiency of PPM at the oil-water interface after pH-treatment (adjusting the pH of PPM-E to pH 3.0, 5.0 and 7.0) or salt-treatment (100 mM NaCl), the quantity of PPM in the emulsion phase was determined (Araiza-Calahorra, et al., 2019; Sarkar, et al., 2016b). Briefly, PPM-E (E1.0) at different pHs (pH 3.0, 5.0 and 7.0) and ionic strength (100 mM NaCl) were diluted (1:4 w/w) with phosphate buffer (pH 7.0) or Milli-Q water (adjusted to pH 5.0 or pH 3.0) or phosphate buffer at H 7.0 containing 100 mM NaCl. All diluted emulsions were centrifuged for 40 mins at 10,000 rpm, 20 °C (Fresco 21 centrifuge, Thermo Fisher Scientific, Germany). The subnatants were carefully collected using a syringe and then measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) on UV-Vis Spectrophotometer with an adsorption wavelength of 750 nm. The adsorption efficiency of PPM at the interface was calculated by subtracting the amount of PPM in the subnatant from the total amount of PPM used initially to prepare the emulsions as a percentage of total protein concentration in the emulsion.

### 2.2.7 Apparent viscosity measurement

The apparent viscosity of PPM-E (E1.0) as a function of pH and ionic strength were determined at 25 °C using a Kinexus ultra rheometer (Malvern Instruments Ltd, Malvern, UK). The

apparent viscosities ($\eta_a$, Pa s) were recorded as a function of shear rates ranging from 0.1 to 1000 $s^{-1}$. All measurements were done in triplicates and were reported as the mean and standard deviation. In order to determine the flow type the emulsions as a function of pH and ionic strength, the flow curves were fitted using power-law model (Ostwald-de Waele model) (see Equation (2)):

$$\eta_a(\dot{\gamma}) = K(\dot{\gamma})^{n-1} \quad (2)$$

where, $K$ is consistency coefficient (Pa $s^n$), $n$ is power-law index and $\dot{\gamma}$ is shear rate ($s^{-1}$).

### 2.2.8 Microscopy

Optical microscopy (Nikon, SMZ-2T, Japan) was used to observe the microstructure of PPM and E1.0 as a function of pH and ionic strengths. The samples undergoing optical microscopy needed to be diluted 10 times in respective buffer. Zeiss confocal microscope (Model LSM 700, Carl Zeiss MicroImaging GmbH, Jena, Germany) was used for microstructural characterization of PPM at the interface of E1.0 droplets. The oil droplets in E1.0 were stained with 100 $\mu L$ of Nile Red (2% w/v in in dimethyl sulfoxide) and the protein stabilizing the oil droplets was stained with 500 $\mu L$ of Nile Blue (10% w/v in Milli-Q water). Nile Red was excited by at 488 nm whereas Nile Blue was excited at 635 nm. The stained samples were mixed with an appropriate amount of xanthan gum (1 wt %) to reduce the Brownian motion of oil droplets. The prepared samples were placed onto a microscope slide, covered with a cover slip and observed at 63 × (oil) magnifications.

### 2.2.9 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions was used to determine the composition of protein in the initial pea protein

solution, PPM dispersions and the adsorbed phase of PPM-E (E1.0). The samples included PPM dispersions that are pH-treated (adjusting the pH of PPM to pH 3.0, 5.0 and 7.0) and salt-treated (100 mM NaCl) PPM dispersions as well as PPM-E (E1.0) at different pHs (pH 3.0, 5.0 and 7.0) and ionic strength (100 mM NaCl). To determine the protein compositions of absorbed phase *i.e.* the particles at the interace, all the PPM-E samples after pH and ionic treatments were centrifuged at 14,500 *g* for 45 min, and then the cream phases were carefully collected, dispersed in Milli-Q water and centrifuged again for 45 min at 14,500 *g*. Approximately, 65 µL of pea protein dispersion, PPM samples and cream layer of PPM-E (E1.0) samples were mixed with 25 µL pf SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue) and 10 µL of diothiothreitol (DTT, of a final concentration of 50 mM), heated at 95 ℃ for 5 min.The SDS-PAGE was carried out by loading 5 µL of protein marker and 10 µL of these samples-SDS buffer mixtures in the Mini-PROTEAN 8-10% TGX Gels in a Mini-PROTEAN II electrophoretic unit Bio-Rad Laboratories, Richmond, CA, USA). The resolving gel contained 16% acrylamide and the stacking gel was made up of 4% acrylamide.

The running process had two stages; one at 100 V for 10 min at first stage and then 200 V of about 20 min for the second stage. After the run, the gel was stained for 2 hours using Coomassie Blue solution, which consisted of 90% ProtoBlue Safe Colloidal Coomassie G-250 stain and 10% ethanol. The gel was then destained using Milli-Q water overnight and scanned using the ChemiDoc™ XRS+ with image Lab™ Software (Bio- Rad Laboratories, Inc, USA). Each band within the lanes was selected automatically by the software to cover the whole band. Background intensity was subtracted after scanning an empty lane. The SDS-PAGE experiments were carried out in triplicates and band intensities was reported as an average and standard deviation of three reported readings.

### 2.2.10 Statistical analysis

All experimental results were reported as mean and standard deviations of five measurements on triplicate samples (n = 5 × 3). The statistical analyses were conducted using one-way ANOVA and multiple comparison test using SPSS software (IBM, SPSS statistics, version 24) and the significant difference between samples were considered when $p < 0.05$.

## 3. Results and Discussion

### 3.1. Characteristics of aqueous dispersion of PPM

The aqueous dispersion of PPM in phosphate buffer at pH 7.0 had a narrow size distribution with single peak in the size range of 100 to 1000 nm (**Figure 2**). The PPM had a hydrodynamic diameter ($D_h$) of about 232 nm (polydispersity index (PDI) < 0.2) (**Figure 3a**). Although an aqueous dispersion of PPC showed multimodal size distribution with high PDI of nearly 1.0 (data not shown), preparing PPM via the top-down approach of heat-set gel formation and controlled shearing appears as a feasible approach to create plant protein-based particles with high colloidal stability. For instance, the PPM were highly negatively charged (-40 mV) (**Figure 3b**) showing no particle aggregation or macroscopic sedimentation (**Figure 3c**) at pH 7.0, respectively over a year storage.

### 3.2 Colloidal stability of aqueous dispersions of PPM

As shown in **Figure 3a**, $D_h$ of PPM ranged from 200 to 400 nm at neutral to alkaline pH (pH 7.0 to pH 9.0) ($p > 0.05$). However, at pH 6.0, PPM showed a marked increase in $D_h$ supported by correspondingly steep increase in PDI to 0.8 as compared to that at neutral pH ($p < 0.05$). The $D_h$ of PPM dispersions increased to the highest values of > 8,000 nm at pH 5.0 followed

335 by a decrease to < 2,000 nm at 3.0 < pH < 5.0 (**Figure 3a**). High degree of particle aggregation
336 and sedimentation observed using optical microscopy and macroscopic images, respectively,
337 indicate pH 5.0 to be the isoelectric point (pI) (**Figure 3c**). Caution should be exercised while
338 interpreting these $D_h$ results with values above 1000 nm, when using dynamic light scattering
339 (**Supplementary Figure S1**). Thus, focusing on the trend of increasing values of $D_h$, the
340 colloidal stability of aqueous dispersions of PPM was limited at pH 6.0 with high PDI (**Figure**
341 **3a**) and excessive particle aggregation (**Figure 3c**).

342

343 The colloidal behaviour of PPM as a function of pH was investigated using ζ-potential
344 measurements of PPM (**Figure 3b**). The ζ-potential of PPM increased from -40 mV to +30 mV
345 with pH change, from pH 9.0 to pH 2.0 ($p < 0.05$). Noticeably, the net surface charge was close
346 to zero at pH 5.0 (**Figure 3b**) validating this to be the pI, corroborating with the largest
347 hydrodynamic diameter and PDI (almost close to 1.0) data (**Figure 3a**). At this pH, the
348 positively-charged amino-groups in PPM were balancing the negatively-charged carboxyl
349 groups. Interestingly, the pI of PPM (**Figure 3b**) shifted slightly from that of the PPC, where
350 latter is reported to be around 4.0 (Adal, et al., 2017). Such discrepancy in pI values between
351 protein concentrates and protein microgel particles are possible owing to the unfolding process
352 during thermal treatment of the latter and consequently exposure of some charged groups. At
353 or below pH 4.0, PPM showed net positive charge ranging from +20 to +30 mV. An interesting
354 study by Destribats, et al. (2014) demonstrated that in whey protein microgel particles (WPM),
355 some larger particle were present even when the pH value was far from the pI and the particles
356 possessed electrostatic charge. The larger particles were postulated to be associated with the
357 swelling of WPM *i.e.* the solvation of the exposed protein groups enabling WPM to swell.
358 Nevertheless, in the present study even if some degree of swelling of PPM might have occurred,

359 due to the solvation of the protein groups that was promoted at acidic pH, such effects have
360 been overshadowed by the dominant particle aggregation as observed in **Figure 3c**.

361

362 It is worth noting that the PPM in presence of electrolytes showed no significant difference in
363 $D_h$ and PDI (**Figure 4a**) as a function of ionic strengths. This was in close agreement with no
364 aggregation or sedimentation observed in optical microscopy or macroscopic images (**Figure**
365 **4c**). However, a progressive decrease of ζ-potential magnitude from -31 to -7.5 mV was
366 observed (**Figure 4b**) as the ionic strength increased from 1 to 250 mM NaCl. Similar salt-
367 induced reduction in ζ-potential in plant protein-based particles has been observed in the case
368 of soy protein-based nanoparticles, where Liu, et al. (2013) demonstrated that the increasing
369 NaCl concentration (0-500 mM) led to the decrease of absolute magnitude of ζ-potential of soy
370 protein nanoparticles. This is due to a decreased Debye length which for constant charged
371 surfaces at any rate will lead to a lower ζ-potential.

372

373 Colloidal particles, such as PPM in this study, dispersed in aqueous solutions will experience
374 Derjaguin-Landau-Verwey-Overbeek (DLVO) forces, other types of short-ranged, attractive
375 non-DLVO forces (Hogg, et al., 1966) as well as possible steric repulsion. To understand the
376 role of interaction in the colloidal stability of PPM, we used DLVO theory to calculate the
377 inter-particle electrostatic-mediated activation energy barrier between sub-micron sized
378 PPMs, as two such particles approach each other at varying pHs and ionic strengths (**Figure**
379 **5**). This is done using equation (3) (Cosgrove, 2010):

380 $$U_R = 2\pi\varepsilon\psi^2 a \ln[1+\exp(-kh)] \quad (3)$$

381 for the electrostatic component of particle-particle interaction. For equation (3), $\varepsilon$ is the
382 permittivity of the system, *i.e.* $\varepsilon_0\varepsilon_r$ ($\varepsilon_0$ is the permittivity of vacuum, and $\varepsilon_r$ is relative

permittivity of water), $\psi$ is the surface potential approximately equal to the ζ-potential values measured at different pHs or ionic strengths, $a$ is the radius of PPM particle (*i.e.* 118 nm), $h$ is the particle-particle surface separation distance ranging from 0.1 to 5 nm and $\kappa^{-1}$ is the Debye length, which was calculated from equation (4):

$$\kappa = \left[\frac{N_A e^2}{\varepsilon k T} \sum_i z_i^2 c_i^{\infty}\right]^{\frac{1}{2}} \quad (4)$$

Here, $z_i$ is the valency of $i^{th}$ type of ion, $c_i^{\infty}$ is the number density of that ion, $N_A$ is the Avogadro's number, and $T$ is the temperature (298 K).

The total interaction potential ($U$) between PPMs is the summation of electrostatic repulsion ($U_R$) and van der Waals attraction ($U_{VW}$). In this calculation, the pH independent van der Waals attractive energy ($U_{VW}$) between two equal sized PPM was calculated using equation (5):

$$U_{VW} = -\frac{aA_H}{12h} \quad (5)$$

where, $A_H$ is the Hamaker constant which has been assumed to be 1 $k_BT$, similar to other reported protein particles (Tuinier, et al., 2002).

The total interaction potential was higher than 100 $k_BT$ at pHs < 3.0 and pHs > 6.0 (**Figure 5a**), suggesting an electrostatically-induced energy barrier being sufficient to slow down any aggregation to negligible rates (see **Supplementary Figure S2a** for electrostatic repulsion). However, the van der Waal's attractive forces particularly for PPM in the acidic pH *i.e.* pH 2.0-4.5 played a dominant role when at close PPM-PPM separation distance (< 0.15 nm). This caused the total energy maximum to fall below ~10 $k_BT$ (**Figure 5a**), in line with high $D_h$, high PDI (**Figure 3a**) and extensive particle aggregation (**Figure 3c**). The ζ-potential of PPM reflected minimal surface charge (almost close to zero, **Figure 3b**) at pH 5.0 and the correspondingly lack of sufficient electrostatic repulsion barrier, to provide a large energy in

405 the presence of strong van der Waal's attraction (**Figure 5a**). A low energy barrier accelerated
406 the aggregation of the particles (**Figures 3a** and **3c**).

407

408 The $U_R$ and $U_{VW}$ between PPMs with varying ionic strengths were also calculated using
409 equations (3-5), as shown in **Figure 5b** (see **Supplementary Figure S2b** for $U_R$ and $U_{VW}$)**.**
410 Interestingly, at 1-10 mM NaCl, the interaction was mainly dominated by electrostatic
411 repulsive forces with PPM-PPM interaction potential of > 100 $k_BT$ overshadowing effects of
412 van der Waal's attractive forces (**Figure 5b**) over most separation distances. As expected, the
413 electrostatic repulsion between particles was screened, down to nearly zero, with the increase
414 in the NaCl concentration (**Figure 5b**). Once the salt concentration was above 50 mM, the
415 dominating van der Waal's attractive forces between PPMs should result in particle
416 aggregation, leading to an unstable dispersion. However, this was not the case experimentally.
417 In fact, larger particle aggregates were not observed (**Figure 4c**), and the size of PPM was
418 stable at a range of 230 to 240 nm, with lower PDI values (**Figures 4a**). The possible
419 explanation for the discrepancy between the theoretically predicted aggregation and an
420 experientially observed stable dispersion might be attributed to the steric repulsion effects
421 associated with the hairy PPM particles, which is not strongly influenced by electrolyte
422 concentration. This requires experimental investigation in the future. This behaviour is unlike
423 that of WPM, soy protein nanoparticles, zein colloid particles and kafirin nanoparticles (de
424 Folter, et al., 2012; Destribats, et al., 2014; Liu, et al., 2013; Xiao, et al., 2016), where NaCl
425 addition is known to cause aggregation of particles in the aqueous dispersions.

426

427 **3.3 Characteristics of Pickering O/W emulsions stabilized by PPM (PPM-E)**

428 **3.3.1 Stability of PPM-E prepared using various concentration of PPM**

To determine the optimum concentration of PPM to stabilize PPM-E, coalescence stability of PPM-Es stored under refrigerated conditions for a period of 28 days were characterized using size, charge and visual imaging of any oiling off. **Table 1** shows the emulsion droplet size and ζ-potential, and **Figure 7** presents the corresponding visual images of the freshly prepared PPM-Es (E0.50-1.0) after 7, 14 and 28 days storage, respectively, at 4 °C. The cream layer was evident in all the freshly prepared emulsions (E0.05-0.5), except in E1.0 (**Figure 7**), however, all the emulsions reverted back to a homogenous dispersion after gentle hand shaking without any visual evidence for coalescence. After a week of storage, although no phase separation was discernible in E0.05 and E0.1 macroscopically (**Figure 7**), larger oil droplets were visible after diluting the emulsions with buffer (**Supplementary Figure S3**) with clear signs of coalescence and hence the size and ζ-potential could not be measured (**Table 1)** in these emulsions. This is expected as the insufficient quantities of PPM in E0.05 and E0.1 prevented sufficient particle coverage to enable stabilization of the large surface area of such high fraction of oil droplets (20 wt%). The network formed by PPM in continuous phase appears to encapsulate the oil droplets, and this prevented the macroscopic oil phase separation (**Figure 7**).

The remaining three emulsions (E0.25, E0.5 and E1.0) did not show any coalescence upon dilution within the first week of storage (**Supplementary Figure S3**) and the ζ-potential values ranging from -38 to -41 mV (**Table 1)** indicated that the droplets had high net negative surface charge providing sufficient electrostatic stability to the droplets. After two weeks of storage, emulsions E0.25 and E0.5 showed an increase in droplet size ($p < 0.05$), however, no change in droplet size was observed in the case of E1.0 ($p > 0.05$) (**Table 1)**. This was associated with decreasing values of ζ-potential for E0.25 and E0.5 ($p < 0.05$) and no significant change in ζ-potential value in the case of E1.0 ($p > 0.05$) over time (**Table 1)**. More importantly, after storage over a month, oil droplets were detected in the diluted E0.25 and E0.5 emulsions

(**Supplementary Figure S3**). There was no significant difference in droplet size and ζ-potential of emulsion E1.0 over this 28 day storage period ($p > 0.05$), which is also in line with results of other Pickering emulsions, where 1 wt% of particles were needed to provide sufficient surface coverage of the droplets (Araiza-Calahorra, et al., 2019; Du Le, et al., 2020). In summary, 1.0 wt% PPM was sufficient to create small-sized ($d_{43}$ ~25 μm) stable droplets that showed excellent resilience against coalescence (E1.0). Hence, hereafter, 1 wt% PPM was chosen as the optimum concentration to create Pickering emulsions (E1.0) and test their response to pH and ionic strengths.

### 3.3.2 Composition of proteins in the adsorbed phase of E1.0

Compositions of PPC, PPM dispersions and the adsorbed phase of E1.0 were analysed by reduced SDS-PAGE to understand if there was any difference in composition of the protein **subunits** in bulk phase and those that were adsorbed at the interface in case of E1.0. The protein mixture in PPC or the laboratory-synthesized PPM had more than ten polypeptides (**Figure 6**) and the bands at range of 36-42 kDa had the highest proportion. The polypeptide composition of PPC or PPM was similar to those reported by Mession et al. (2015) and Peng, et al. (2016) containing the three main proteins, legumin, vicilin and convicilin. Convicilin was the band at 66 kDa. Legumin is a globular protein with an acidic subunit (Lα) at about 42 kDa and basic subunit (Lβ) at 18- 24 kDa. Vicilin proteins consisted of three subunits (Vi1-3), which are respectively observed in fractions of around 50 kDa, 36-30 kDa and 20 kDa. Overall, SDS-PAGE results indicate that the formation of PPM from PPC involved all protein subunits, which is in accordance with results obtained previously that heat treatment did not affect the protein composition (Laguna, et al., 2017). In addition, the adsorbed phase of E1.0 showed similar molecular weight profiles to that of PPM that were not influenced by pH-treatment or exposure to ions. This suggests that adsorption or environmental stresses (pH or ions) applied to PPM

had limited effect on the protein composition of the particles. In addition, a significant proportion of PPM did not enter the resolving gel and were retained in the stacking gel suggest that they were oligomers above 250 kDa. This suggests that DTT was not sufficient to break covalent disulphide bridges in the PPM effectively.

### 3.3.3 Influence of pH on behaviour of E1.0 droplets

**Figure 8a** shows the mean droplet size distribution of E1.0 (20 wt% sunflower oil) as a function of pH (pH 3.0, pH 5.0 and pH 7.0) with corresponding Sauter mean diameter ($d_{32}$), De Brouckere mean diameter ($d_{43}$) and ζ-potential shown in **Table 2**. The initial E1.0 (pH 7.0) has a significantly larger proportion of droplets in the peak area of 10-100 μm. The smaller peak in the range of 0.1-1 μm as observed in **Figure 8a**, overlaps neatly with the size distribution of PPM estimated using dynamic light scattering (**Figure 2**), suggesting that these small particles in **Figure 8a** might be the free microgel particles that were not adsorbed to the droplet surface during the homogenization process (Sarkar, et al., 2016b). Comparing the mean diameter of the PPM particles of ~230 nm (**Figure 3a**) and the mean size of the oil droplets ($d_{43}$) of ~ 25 μm (**Table 2**), the ratio of oil droplet size to PPM size ranges from 100:1 to 1000:1, which is a signature of a classical Pickering emulsion (Ettelaie, et al., 2015; Sarkar, et al., 2016b). The ζ-potential of E1.0 is about -41 mV (**Table 2**), similar to that of PPM (**Figure 3b**) at pH 7.0. This suggests that perhaps a monolayer of PPM might be present at the surface of droplets.

Interestingly, after adjusting the pH of E1.0 to pH 5.0 or pH 3.0, the $d_{43}$ values were comparable to that of E1.0 at neutral pH ($p > 0.05$). However, $d_{32}$ values of E1.0 at different pHs showed a significant difference ($p < 0.05$) (**Table 2**). This might be attributed to the fact that $d_{32}$ value was more affected by the changes in free (unadsorbed) microgel peak as a function of pH as

503 compared to $d_{43}$ value, which is line with the behaviour of PPM in aqueous phase as observed
504 in **Figure 3a**. The width of the peak at the range 10-100 μm in the droplet size distribution was
505 narrower with a higher peak height at a lower pH (pH 3.0) than that seen for E1.0 at pH 7.0
506 (**Figure 8a**). Also, one might not expect such narrow droplet size distribution in E1.0,
507 particularly at pH 5.0 considering it is the isoelectric point (pI) of PPM, where E1.0 droplets
508 will also possess negligible surface charge (**Table 2**). This is a unique behaviour, unlike that
509 observed in PPM in the aqueous phase (**Figures 3a and 3c**) as well as previous studies, where
510 pH adjustment of emulsions stabilized by pea protein isolate to lower pH increased the
511 emulsion droplet size and reduced the emulsion stability (Adebiyi, et al., 2011).

512

513 To understand the aggregation behaviour, the apparent viscosities of the emulsions (E1.0) at
514 pH 7.0, pH 5.0 and pH 3.0 were determined using shear rate ranging from 0.1 to 1000 $s^{-1}$
515 (**Figure 8b**). The Ostwald de Waele model was applied to fit the flow curves and the
516 corresponding fit parameters (consistency coefficient ($K$), flow index ($n$), regression coefficient
517 ($R^2$) were summarized in **Table 3**. The $R^2$ of all samples was ≥ 0.98, confirming a good fit to
518 the model. For E1.0 at different pH, $n$ varied from 0.63 to 0.80, suggesting that E1.0 was a
519 pseudoplastic fluid showing shear-thinning behaviour at all the tested pH values. Emulsions at
520 pH 5.0 showed the highest $K$ and the lowest $n$ ($p < 0.05$) (**Table 3**). This is expected owing to
521 strong inter-droplet flocculation in E1.0 at pI (pH 5.0) due to a reduction in electrostatic
522 repulsive forces. Nevertheless, such aggregates were not strong and therefore possibly broken
523 down by the shearing process during the static light scattering experiments and thus were not
524 evident as a second peak in **Figure 8a**. Interestingly, at pH 3.0, the viscosity appeared to be
525 higher than at pH 7.0 (**Figure 8b**), nevertheless, there was no significant difference ($p > 0.05$)
526 in either $K$ or $n$ as compared to those at pH 7.0 ($p > 0.05$) (**Table 3**) suggesting that the droplet
527 flocs that were broken down in the direction of shear were similar at pH 7.0 and pH 3.0.

528 Visual images of E1.0 after 3 months of storage showed no distinct oil layers again confirming
529 the ability of PPM to to act as effective Pickering stabilizer (**Figure 9a**). Confocal laser
530 scanning two-dimensional (2D) (**Figure 9a**) and three-dimensional (3D) (**Figure 9b**)
531 micrographs revealed a thick layer of PPM (Nile Blue staining the protein microgels, displayed
532 in green) adsorbed at oil-water interface (Nile Red staining the oil droplets, displayed in red),
533 acting as a barrier to coalescence as observed visually**.** The confocal micrographs showed
534 evidence of bridging flocculation as pH was reduced to pI (pH 5.0) with visual signs of
535 creaming. This is in agreement with the higher viscosities and consequently higher consistency
536 coefficients of the emulsions at pH 5.0 as compared to those at pH 7.0 and pH 3.0 ($p < 0.05$)
537 (**Figure 8b, Table 3**). When the pH was adjusted to pH 3.0 (**Figure 9b**), the aggregation of
538 adsorbed PPM at interface as well as bridging flocculation between the droplets were still
539 evident. To understand whether the reduction of pH had an effect on PPM that were present at
540 the interface, **Table 2** shows the adsorption efficiency of the PPM as a function of pH. The
541 PPM had very high degree of adsorption (> 98%) to the droplet surface at all pH (**Table 2**),
542 with slightly yet significantly higher adsorption at pH 5.0 as compared to those in pH 7.0 or
543 pH 3.0 ($p < 0.05$). This is also evident visually from the images of the subnantant
544 **(Supplementary Figure S4a)** after dilution and centrifugation of the emulsions suggesting
545 that the majority of the PPM particles were either adsorbed at the droplet surface or somehow
546 associated with interconnecting the neighbouring droplets in a PPM-PPM network. Overall,
547 E1.0 maintained high stability to coalescence when the pH was adjusted from pH 7.0 down to
548 pH 5.0 or pH 3.0, where the adsorbed PPM on the droplet surface increased aggregation as
549 well as the PPM attached to neighbouring droplets.

550

551 **3.3.4 Influence of background electrolyte concentration on behaviour of E1.0 droplets**

With the increase in ionic strength from 1 mM to 10 mM, the droplet size distribution (**Figure 10a**) and corresponding mean droplet diameters ($d_{43}$, $d_{32}$) (**Table 2**), showed no statistically significant differences ($p > 0.05$). Although the net surface potential of charged droplets became less negative (from - 40 mV to -28 mV), the electrostatic repulsion was still sufficient to inhibit extensive flocculation in E1.0. The emulsions showed shear thinning behaviour irrespective of ionic strengths (**Figure 10b)**. There was no significant difference between viscosities and the *n* values of these emulsions in the presence of 1 mM and 10 mM NaCl, especially in the region of 0.1-10 $s^{-1}$ shear rate ($p > 0.05$) (**Table 3**). Interestingly, the viscosity of emulsions in presence of 100 mM NaCl was higher than the other emulsions with consequently lower *n* value and higher *K* value ($p < 0.05$) (**Table 3**). The unaltered adsorption efficiency (**Table 2**, **Supplementary Figure S4b)** upon ion treatment (100 mM NaCl) and enhanced viscosity suggested inter-droplet flocculation in E1.0 with 100 mM NaCl, in line with lower net surface charge at the droplet surface (-12 mV) (**Table 2**). No oiling off or phase separation were observed after rheology measurements, and even after an extended period of storage (**Figure 11**). Looking closely at confocal micrographs (**Figure 11**), droplet flocculation can be observed after addition of 100 mM NaCl corroborating with the bulk rheological measurements (**Figure 10b**). In a previous study, Pickering emulsions stabilized by kafirin nanoparticles showed a reduction in average droplet size on increasing ionic strength from 10 mM to 50 mM, which was mainly attributed to enhanced nanoparticle interaction *via* electrostatic screening effects. On the other hand, de Folter, et al. (2012) suggested that Pickering emulsions stabilized by both positively- and negatively-charged zein particles at very high ionic strength (1 M) aggregated and exhibited an emulsion–gel phase. Comparing our results with these afore-mentioned plant protein-based Pickering emulsions, we hypothesize that a weak gel-like emulsion structure might have been formed which is apparent from the droplet aggregation observed in the confocal images (**Figure 11**). However, the structure of the O/W

emulsion might not be as strong as that of a 'true gel', but may exhibit a small yield stress, which requires future rheological characterization.

## 4. Conclusions

Results from our research demonstrate the ability of a new class of plant protein particles *i.e.* pea protein microgels created using a facile top down approach to stabilize O/W Pickering emulsions with ultrastability against coalescence. To understand the characteristics of these Pickering O/W emulsion droplets as a function of microgel concentration, pH- or salt-treatment, the colloidal behaviour of pea protein microgel dispersions in aqueous phase was first investigated at various pH values (pH 2.0 to pH 9.0) or salt concentrations (1 to 250 mM NaCl) was investigated. Aqueous dispersions of pea protein microgels showed highest degree of particle aggregation at pH 5.0 as the activation energy barrier in particle-particle interaction potential was calculated to be extremely low at this pH. Meanwhile, high salt concentrations resulted in charge screening effects in PPM dispersion but the resulting reduction in electrostatic potential did not affect the hydrodynamic diameter of microgels, suggesting that other, possibly steric effects might also be playing a role in the colloidal stability of these particles. Interestingly, when the pea protein microgels were present at the oil-water interface, ultra-stable emulsion droplets were obtained only at microgels with 1.0 wt% protein concentration with all protein subunits *i.e.* legumin, vicillins and convicillins, being simultaneously present on the microgel-laden interface. The packed layer of PPM particles stabilizing the oil droplets allowed the emulsions to be stable over few months against coalescence. Upon pH reduction to pH 5.0, both intra-droplet and inter-droplet aggregation of PPM occurred resulting in higher adsorption efficiency and higher viscosity, respectively. The emulsions also showed responsiveness to ions, especially at 100 mM NaCl with enhancement

in viscosity and shear-thinning character. To our knowledge, this is the first comprehensive study that has systematically demonstrated the role of electrostatics in the colloidal stability of the plant-based microgel particles in bulk phase versus particles adsorbed at the surface of the droplets. Findings from this comprehensive study might open door for applicability of these pea protein-based microgels in a range of food products and allied soft-matter applications, where alternative plant-based sustainable Pickering stabilizers are increasingly necessary. Ongoing research is focussing on tuning the surface and bulk properties of pH and ion-responsive pea protein microgel-stabilized emulsions, which can help to tailor the *in vitro* gastrointestinal digestion kinetics of these microgel-stabilized emulsified lipids and allow their usage for controlled release applications in the future.

## Acknowledgements

The authors would like to gratefully acknowledge the contributions of Dr Sally Boxal for her technical support in confocal microscopy at the Bio-imaging Facility within the Faculty of Biological Sciences of University of Leeds.

## References

Adal, Sadeghpour, Connell, Rappolt, Ibanoglu, & Sarkar. (2017). Heteroprotein Complex Formation of Bovine Lactoferrin and Pea Protein Isolate: A Multiscale Structural Analysis. *Biomacromolecules, 18*(2), 625-635.

Adebiyi, & Aluko. (2011). Functional properties of protein fractions obtained from commercial yellow field pea (Pisum sativum L.) seed protein isolate. *Food Chemistry, 128*(4), 902-908.

Araiza-Calahorra, & Sarkar. (2019). Pickering emulsion stabilized by protein nanogel particles for delivery of curcumin: Effects of pH and ionic strength on curcumin retention. *Food Structure, 21*, 100113.

Aveyard, Binks, & Clint. (2003). Emulsions stabilised solely by colloidal particles. *Advances in Colloid and Interface Science, 100*, 503-546.

Ben-Harb, Panouillé, Huc-Mathis, Moulin, Saint-Eve, Irlinger, Bonnarme, Michon, & Souchon. (2018). The rheological and microstructural properties of pea, milk,

mixed pea/milk gels and gelled emulsions designed by thermal, acid, and enzyme treatments. *Food Hydrocolloids, 77*, 75-84.

Binks. (2002). Particles as surfactants—similarities and differences. *Current opinion in colloid & interface science, 7*(1-2), 21-41.

Bora, Brekke, & Powers. (1994). Heat Induced Gelation of Pea (Pisum sativum) Mixed Globulins, Vicilin and Legumin. *Journal of Food Science, 59*(3), 594-596.

Chen, Lin, Sun, & Zhao. (2014). Stable and pH-Sensitive Protein Nanogels Made by Self-Assembly of Heat Denatured Soy Protein. *Journal of agricultural and food chemistry, 62*(39), 9553-9561.

Chihi, Mession, Sok, & Saurel. (2016). Heat-Induced Soluble Protein Aggregates from Mixed Pea Globulins and β-Lactoglobulin. *Journal of agricultural and food chemistry, 64*(13), 2780-2791.

Chihi, Nicolas, & Saurel. (2018). *Acid gelation of mixed thermal aggregates of pea globulins and β-lactoglobulin* (Vol. 85).

Cochereau, Nicolai, Chassenieux, & Silva. (2019). Mechanism of the spontaneous formation of plant protein microcapsules in aqueous solution. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 562*, 213-219.

Cosgrove. (2010). *Colloid science: principles, methods and applications* (2nd ed.). Chichester, U.K: Wiley.

David-Birman, Mackie, & Lesmes. (2013). Impact of dietary fibers on the properties and proteolytic digestibility of lactoferrin nano-particles. *Food Hydrocolloids, 31*(1), 33-41.

de Folter, van Ruijven, & Velikov. (2012). Oil-in-water Pickering emulsions stabilized by colloidal particles from the water-insoluble protein zein. *Soft Matter, 8*(25), 6807-6815.

Destribats, Rouvet, Gehin-Delval, Schmitt, & Binks. (2014). Emulsions stabilised by whey protein microgel particles: towards food-grade Pickering emulsions. *Soft Matter, 10*(36), 6941-6954.

Dickinson. (2012). Use of nanoparticles and microparticles in the formation and stabilization of food emulsions. *Trends in Food Science & Technology, 24*(1), 4-12.

Dickinson. (2013). Stabilising emulsion-based colloidal structures with mixed food ingredients. *Journal of the Science of Food and Agriculture, 93*(4), 710-721.

Du Le, Loveday, Singh, & Sarkar. (2020). Pickering emulsions stabilised by hydrophobically modified cellulose nanocrystals: Responsiveness to pH and ionic strength. *Food Hydrocolloids, 99*, 105344.

Ettelaie, & Lishchuk. (2015). Detachment force of particles from fluid droplets. *Soft Matter, 11*(21), 4251-4265.

Gal, Shani Levi, Tal, & Lesmes. (2013). *Emulsions stabilization by lactoferrin nano-particles under in vitro digestion conditions* (Vol. 33).

Hogg, Healy, & Fuerstenau. (1966). Mutual coagulation of colloidal dispersions. *Transactions of the Faraday Society, 62*, 1638-1651.

673 Hu, Yin, Zhu, Qi, Guo, Wu, Tang, & Yang. (2016). Fabrication and characterization
674 of novel Pickering emulsions and Pickering high internal emulsions stabilized
675 by gliadin colloidal particles. *Food Hydrocolloids, 61*, 300-310.

676 Jayasena, Chih, & Nasar-Abbas. (2010). Functional properties of sweet lupin protein
677 isolated and tested at various pH levels. *Research Journal of Agriculture and*
678 *Biological Sciences, 6*(2), 130-137.

679 Jiao, Shi, Wang, & Binks. (2018). High-Internal-Phase Pickering Emulsions
680 Stabilized Solely by Peanut-Protein-Isolate Microgel Particles with Multiple
681 Potential Applications. *Angewandte Chemie International Edition, 57*(30),
682 9274-9278.

683 Karaca, Low, & Nickerson. (2011). Emulsifying properties of chickpea, faba bean,
684 lentil and pea proteins produced by isoelectric precipitation and salt
685 extraction. *Food Research International, 44*(9), 2742-2750.

686 Kimura, Fukuda, Zhang, Motoyama, Maruyama, & Utsumi. (2008). Comparison of
687 Physicochemical Properties of 7S and 11S Globulins from Pea, Fava Bean,
688 Cowpea, and French Bean with Those of Soybean · French Bean 7S
689 Globulin Exhibits Excellent Properties. *Journal of agricultural and food*
690 *chemistry, 56*(21), 10273-10279.

691 Laguna, Picouet, Guàrdia, Renard, & Sarkar. (2017). In vitro gastrointestinal
692 digestion of pea protein isolate as a function of pH, food matrices,
693 autoclaving, high-pressure and re-heat treatments. *LWT, 84*, 511-519.

694 Liang, & Tang. (2014). *Pea protein exhibits a novel Pickering stabilization for oil-in-*
695 *water emulsions at pH 3.0* (Vol. 58).

696 Liu, Ou, & Tang. (2017). Ca 2+ -induced soy protein nanoparticles as pickering
697 stabilizers: Fabrication and characterization. *Food Hydrocolloids, 65*, 175-186.

698 Liu, & Tang. (2013). Soy protein nanoparticle aggregates as Pickering stabilizers for
699 oil-in-water emulsions. *Journal of agricultural and food chemistry, 61*(37),
700 8888-8898.

701 Liu, & Tang. (2014). Emulsifying properties of soy protein nanoparticles: influence of
702 the protein concentration and/or emulsification process. *J Agric Food Chem,*
703 *62*(12), 2644-2654.

704 Liu, & Tang. (2016). Soy glycinin as food-grade Pickering stabilizers: Part. III.
705 Fabrication of gel-like emulsions and their potential as sustained-release
706 delivery systems for β-carotene. *Food Hydrocolloids, 56*, 434-444.

707 Makri, & Doxastakis. (2006). Study of emulsions stabilized with Phaseolus vulgaris
708 or Phaseolus coccineus with the addition of Arabic gum, locust bean gum and
709 xanthan gum. *Food Hydrocolloids, 20*(8), 1141-1152.

710 Meshulam, & Lesmes. (2014). Responsiveness of emulsions stabilized by lactoferrin
711 nano-particles to simulated intestinal conditions. *Food & Function, 5*(1), 65-73.

712 Mession, Chihi, Sok, & Saurel. (2015). Effect of globular pea proteins fractionation
713 on their heat-induced aggregation and acid cold-set gelation. *46*, 233-243.

714 Mession, Roustel, & Saurel. (2017). Interactions in casein micelle – Pea protein
715 system (part I): Heat-induced denaturation and aggregation. *Food*
716 *Hydrocolloids, 67*, 229-242.

717 Nesterenko, Alric, Silvestre, & Durrieu. (2013). Vegetable proteins in
718 microencapsulation: A review of recent interventions and their effectiveness.
719 *Industrial Crops and Products, 42*, 469-479.

720 Pan, Tikekar, Wang, Avena-Bustillos, & Nitin. (2015). Effect of barrier properties of
721 zein colloidal particles and oil-in-water emulsions on oxidative stability of
722 encapsulated bioactive compounds. *Food Hydrocolloids, 43*, 82-90.

723 Peng, Kong, Chen, Zhang, Yang, & Hua. (2016). Effects of heat treatment on the
724 emulsifying properties of pea proteins. *Food Hydrocolloids, 52*, 301-310.

725 Peng, Xu, Li, & Tang. (2020). Improving the emulsification of soy β-conglycinin by
726 alcohol-induced aggregation. *Food Hydrocolloids, 98*, 105307.

727 Peng, Xu, Liu, & Tang. (2018). Molecular Mechanism for Improving Emulsification
728 Efficiency of Soy Glycinin by Glycation with Soy Soluble Polysaccharide. *J*
729 *Agric Food Chem, 66*(46), 12316-12326.

730 Rayner, Marku, Eriksson, Sjöö, Dejmek, & Wahlgren. (2014). Biomass-based
731 particles for the formulation of Pickering type emulsions in food and topical
732 applications. *Colloids and Surfaces A: Physicochemical and Engineering*
733 *Aspects, 458*, 48-62.

734 Sarkar, Ademuyiwa, Stubley, Esa, Goycoolea, Qin, Gonzalez, & Olvera. (2018).
735 Pickering emulsions co-stabilized by composite protein/ polysaccharide
736 particle-particle interfaces: Impact on in vitro gastric stability. *Food*
737 *Hydrocolloids, 84*, 282-291.

738 Sarkar, Kamaruddin, Bentley, & Wang. (2016a). Emulsion stabilization by tomato
739 seed protein isolate: Influence of pH, ionic strength and thermal treatment.
740 *Food Hydrocolloids, 57*, 160-168.

741 Sarkar, Murray, Holmes, Ettelaie, Abdalla, & Yang. (2016b). In vitro digestion of
742 Pickering emulsions stabilized by soft whey protein microgel particles:
743 influence of thermal treatment. *Soft Matter, 12*(15), 3558-3569.

744 Sarkar, Zhang, Holmes, & Ettelaie. (2019). Colloidal aspects of digestion of Pickering
745 emulsions: Experiments and theoretical models of lipid digestion kinetics.
746 *Advances in Colloid and Interface Science, 263*, 195-211.

747 Shao, & Tang. (2015). *Gel-like pea protein Pickering emulsions at pH3.0 as a*
748 *potential intestine-targeted and sustained-release delivery system for β-*
749 *carotene* (Vol. 79).

750 Tuinier, & De Kruif. (2002). Stability of casein micelles in milk. *The Journal of*
751 *chemical physics, 117*(3), 1290-1295.

752 Xiao, Wang, Gonzalez, & Huang. (2016). Kafirin nanoparticles-stabilized Pickering
753 emulsions: Microstructure and rheological behavior. *Food Hydrocolloids, 54*,
754 30-39.

755 Zhu, Zhang, Lin, & Tang. (2017). Freeze-thaw stability of pickering emulsions
756 stabilized by soy and whey protein particles. *Food Hydrocolloids, 69*, 173-184.

757 Zhu, Zheng, Liu, Qiu, Lin, & Tang. (2018). Freeze-thaw stability of Pickering
758 emulsions stabilized by soy protein nanoparticles. Influence of ionic strength
759 before or after emulsification. *Food Hydrocolloids, 74*, 37-45.

760