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Pickering emulsion stabilized by protein nanogel particles for delivery of curcumin: Effects of pH and ionic strength on curcumin retention

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Abstract

This study aimed to design whey protein nanogel particles (WPN)-stabilized Pickering emulsion as a delivery vehicle for curcumin (CUR). Firstly, the effectiveness of WPN to stabilize medium chain triglyceride (MCT) oil was assessed using droplet sizing, microscopy across scales, surface coverage calculations and interfacial viscosity measurements. Then, the ability of this delivery vehicle to encapsulate CUR and the effects of pH and ionic strengths on the retention of CUR were investigated in an in vitro release model at 37 °C. Results demonstrate that 1.0 wt% WPN was sufficient to create a monolayer of particles at the droplet surface resulting in ultra-stable droplets that were resistant to coalescence over a year. Addition of 500 μg/ mL of CUR did not result in any change in the droplet size of the Pickering emulsion droplets. The CUR was fully retained within the Pickering emulsions, which might be attributed to the nanometric size of the gaps (≅30 nm) at the interface that did not allow CUR to diffuse out into the release media. The partitioning of CUR to the dispersed phase was influenced by pH of the media. Increased binding affinities between CUR and WPN at the interface (binding affinity constant, $K_a=1 \times 10^4$ M^{-1}) existed at pH 3.0 as compared to that at pH 7.0 ($K_a=6.67 \times 10^1$ M^{-1}) owing to the electrostatic interactions between CUR and interfacial WPN in the former. Such binding affinities between CUR and interfacial WPN at pH 7.0 was further influenced by presence of ions.

Keywords

Curcumin; Pickering emulsion; nanogel; retention; whey protein; electrostatic interaction

Abbreviations:

WPN: whey protein nanogel particles; CUR: curcumin; E_{WPN}: Pickering emulsions stabilized by WPN, $CURE_{WPN}$: curcumin-loaded Pickering emulsions stabilized by WPN.

1. Introduction

Over more than a century ago, in 1904, Walter Ramsden first mentioned that “solid matter has the power of forming persistent emulsions” (Ramsden, 1904). After three years, Pickering published an extensive experimental study on particle-stabilized emulsions for plant spray applications, from which the term "Pickering emulsions" was coined (Pickering, 1907). In an oil-in-water Pickering emulsion, particles are irreversibly adsorbed at the oil-in-water interface due to their high energy of adsorption (thousands of kT/particle). Irreversible adsorption of micro- or nano particles to the oil-water interface provides ultra-stability against coalescence and Ostwald ripening (Dickinson, 2012).

In recent years, there has been an upsurge of research efforts in designing Pickering emulsions to overcome stability issues in conventional emulsions (Araiza-Calahorra et al., 2018; Gupta & Rousseau, 2012; McClements., 2012). Moreover, in the research domain of delivery of bioactive lipophilic molecules, such as curcumin, Pickering emulsion stabilized by food-grade laboratory-synthesized particles have been recently recognized as promising templates (Araiza-Calahorra et al., 2018).

Curcumin (CUR), the main curcuminoid present in the Curcuma longa plant, has been used in traditional medicine for many centuries in Asian countries (Goel, Kunnumakkara, & Aggarwal, 2008). More recently, its potent free-radical scavenging activity has been reported to play an important role on its multiple health-promoting properties such as anti-inflammatory, anticarcinogenic, antidiabetic, anti-aging and antioxidant activities (Wilken, Veena, Wang, & Srivatsan, 2011). The free-radical quenching properties of CUR is attributed to its chemical structure. The structure consists of two aromatic rings connected by seven carbons that hold two α,β-unsaturated carbonyl groups (Payton, Sandusky, & Alworth, 2007). However, the main drawbacks for the industrial applications of CUR as a nutraceutical or pharmaceutical

ingredient are its low water-solubility, alkaline degradation and chemical instability, which reduces its bioavailability when orally administrated (Araiza-Calahorra et al., 2018).

Recently, Pickering emulsions have been used to encapsulate CUR as the formation of a mechanical barrier by the particles can protect the encapsulated CUR against pro-oxidants and thus potentially enhance the physical and chemical stability of these bioactive compounds (Wang et al., 2015). For example, Pickering stabilizers that have been used in literature specifically for encapsulating CUR include silica (Tikekar, Pan, & Nitin, 2013), chitosan-tripolyphosphate nanoparticles (CS/TPP NPs) (Shah et al., 2016a,b), zein-tannic acid complex colloidal particles (Zou, Guo, Yin, Wang, & Yang, 2015), starch granules (Marefati, Bertrand, Sjöö, Dejmek, & Rayner, 2017), karifin (Xiao, Li, & Huang, 2015a) and gliadin particles (Zhou et al., 2018). Particularly, CUR-loaded Pickering emulsions stabilized by biocompatible sub-micron sized CS/TPP NPs, ranging from 322 to 814 nm size have been recently demonstrated to be stable for 30 days (25 ^{o}C) with a CUR half-life degradation (50 wt%) of 120 hours (22 ^{o}C, in the dark) (Shah et al., 2016b). In another example of Pickering stabilizers used for encapsulating CUR, hydrophobically modified starch granule-stabilized Pickering emulsions were developed to assess the stability and loss of encapsulated CUR (Marefati et al., 2017). When samples were not subjected to a heat treatment, release of encapsulated CUR was higher during storage as compared to that of heat-treated emulsions. Authors suggested that the large particles size of starch granules (d_{43} of granules ~ 1.8 µm) allowed rather faster release of the CUR through the gaps at the oil-water interface between these micron-sized starch granules. On the contrary, heat-treated emulsions presented a rather fused barrier of partially gelatinized starch granules at the interface, most likely closing those interfacial gaps, which retained the encapsulated CUR more efficiently. An alternative approach to reduce the size of the interfacial gaps might be to reduce the size of the Pickering stabilizers to a nanometric size. Up until now,

use of Pickering emulsions for delivering CUR is a relatively new field, and literature on the influence of interfacial properties on CUR retention is relatively scarce till date.

Besides polysaccharide-based particles, protein-based particles as Pickering stabilizers have attracted significant research attention as a potential formulation approach to develop functional food materials (Dickinson, 2012, 2017; Sağlam, Venema, van der Linden, & de Vries, 2014; Sarkar, Zhang, Holmes, & Ettelaie, 2019). Particularly, in case of protein, two classes of particles have been used by previous researchers, namely 'nanoparticles' and 'gel particles'. The protein-based nanoparticles are prepared by delicately balancing the attractive and repulsive forces of proteins. During such nanoparticle formation, change in pH, ions or solvent addition cause unfolding of dilute solutions of protein and exposure of functional groups (Liu, Ou, & Tang, 2017; Peinado, Lesmes, Andrés, & McClements, 2010; Xiao et al., 2015b). Subsequent thermal or chemical crosslinking leads to the formation of cross-linked nanoparticles. The most common preparation methods for protein nanoparticles are coacervation, solvent extraction, electrospray etc. (Jain, Singh, Arya, Kundu, & Kapoor, 2018; Verma, Gulati, Kaul, Mukherjee, & Nagaich, 2018).

On the other hand, protein-based gel particles are new entrants to the food-based particle library, these can be either microgels (micron-sized) or nanogels or (nanometric-sized) (Matsumiya & Murray, 2016; Sarkar et al., 2018a; Sarkar et al., 2016). These protein microgels or nanogels are soft colloidal particles that are produced by using a top-down technique of forming a physically cross-linked heat-set hydrogel prepared by using highly concentrated protein solutions, followed by breaking them down to gel particles under high shear forces. A combination of steric and electrostatic repulsions confer good colloidal stability to these particles in aqueous dispersions (Dickinson, 2017). Although protein microgels has been previously reported in the literature as Pickering stabilizers, controlled shearing to create protein nanogels and making Pickering emulsions with the latter has been rare until recently

(Sarkar et al., 2018a). In particular, to our knowledge, this is the first study that reports the encapsulation and stability of bioactive compounds in emulsions stabilized by whey protein nanogel particles.

Therefore, in the present work, we aimed to design oil-in-water Pickering emulsions stabilized by nanometric-sized gel protein particle as a new encapsulation system for CUR. We have created protein-based nanogel particles, hereafter named as whey protein nanogel particles (WPN) with a mean hydrodynamic diameter of < 100 nm size. It was hypothesized that due to the formation of a closely packed mechanical barrier and reduced interstitial gap size, WPN-stabilized Pickering emulsion can serve as an effective template for allowing better retention of CUR within the emulsion system. The ability of the Pickering emulsions on preserving CUR was evaluated at different pH and ionic strengths. To our knowledge, this is the first study that has employed WPN-stabilized Pickering emulsion to encapsulate CUR and investigated the mechanisms behind pH/ ion-induced changes in CUR retention and advances the current state-of-the art on Pickering emulsion delivery vehicles for CUR. Although CUR is used in this study as a model lipophilic compound, the knowledge from this fundamental study can be used for rational designing of nanogel-stabilized oil-in-water Pickering emulsions for encapsulation of any lipophilic bioactive compound.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) with ≥ 90% protein content was gifted from Fonterra Co-operative Group Limited (Auckland, New Zealand). Curcumin, CUR (≥ 65% purity), methanol, sodium chloride, sodium hydroxide, sodium phosphate monobasic monohydrate, sodium phosphate dibasic anhydrous and hydrogen chloride were purchased from Thermo Fisher Scientific, Loughborough, UK. Heptane, acetic acid, sodium acetate, and calcium chloride were

147 purchased from Sigma-Aldrich, Dorset, UK. The lipid phase consisted of medium-chain
148 triglyceride (MCT-oil) Miglyol® 812 with a density of 945 kg m^3 at 20 °C (Cremer Oleo GmbH
149 & Co, Germany). Dialysis membranes of molecular weight cut off 3,500 Da were purchased
150 from Thermo Scientific, Paisley, UK. All reagents were of analytical grade and used without
151 further purification unless otherwise reported. All solutions were prepared with Milli-Q water
152 with a resistivity of 18.2 MΩ cm at 25 °C (Milli-Q apparatus, Millipore, Bedford, UK). Sodium
153 azide (0.02 wt %) was added as a preservative.
154

155 2.2. Preparation of whey protein nanogel particles

156 The nanogel particles were created based on modification of a previous top-down technique
157 (Sarkar et al., 2018a; Sarkar et al., 2016). The WPI powder was dissolved in 10 mM phosphate
158 buffer at pH 7.0 for 2 hours to prepare whey protein solution (10 wt%). The WPI solution was
159 heated in a temperature-controlled water bath at 90 °C for 30 min to form a heat-set gel
160 (quiescent), followed by cooling down for 15 min and storage at 4 °C overnight to form heat-
161 set hydrogels. Obtained WPI gels were pre-homogenized with buffer (5 wt%) using a hand
162 blender (HB724, Kenwood) for 1 minute. The resulting 5 wt% whey protein gel was passed
163 two times through a high-pressure two-chamber homogenizer Jet homogenizer (University of
164 Leeds, UK) at 300 bars. The resulting whey protein nanogel particles (WPN) were diluted with
165 buffer and used as the continuous phase for the emulsion preparation. Emulsions were prepared
166 in triplicate.
167

168 2.3. Preparation of whey protein nanogel-stabilized emulsions (E_{WPN}), CUR-loaded
169 emulsions ($CURE_{WPN}$) and whey protein isolate-stabilized emulsions (E_{WPI})

170 Pickering emulsions (E_{WPN}) were prepared using fixed MCT oil concentration (20 wt%) and
171 WPN of varying concentrations (0.1 – 3.0 wt %). The emulsifier concentration was changed
172 by diluting the aqueous dispersion of WPN (5 wt% protein) with phosphate buffer (pH 7.0) to

get the desired protein content in the final emulsion. Briefly, coarse WPN-stabilized emulsions (20:80 w/w) were prepared using Ultra Turrax T25 homogenizer (IKA-Werke GmbH & Co., Staufen Germany) at 13, 500 rpm for 1 min. Following this, the coarse emulsions were homogenized using the Leeds Jet homogenizer at 300 bars using two passes to prepare fine E_{WPN} droplets. In case of CUR-loaded emulsions ($CURE_{WPN}$), CUR was added to the MCT-oil phase at 500 µg/ mL and stirred at 200 rpm for 30 min at 60 °C to ensure maximum solubility before the coarse emulsion formation step. The choice of MCT-oil as the lipidic phase was to ensure maximum CUR solubility in the dispersed phase (Supplementary Fig. S1).

Emulsions stabilized by whey protein isolate solution (E_{WPI}) were prepared as controls to compare the difference in the microstructure between E_{WPN} and E_{WPI} emulsions. The E_{WPI} (20 wt% MCT oil, 1 wt% WPI) were prepared following the same protocol as described above for E_{WPN}. All emulsions samples were prepared in triplicates**.**

2.4 Transmission electron microscopy

Transmission electron microscopy (TEM) was employed to observe the microstructure of WPN, E_{WPI} and E_{WPN} samples using a previously reported method (Sarkar, Li, Cray, & Boxall, 2018b; Sarkar, Zhang, Murray, Russell, & Boxal, 2017). Briefly, 10 µL of samples were fixed with 2.5% (v/v) glutaraldehyde and post-fixed in 0.1% (w/v) osmium tetroxide. The samples were then carefully exposed to serial dehydration in ethanol (20–100%) before being embedded in araldite. Ultra-thin sections (80 – 100 nm) were deposited on 3.05 mm grids and stained with 8% (v/v) uranyl acetate and lead citrate. The sections were cut on an ‘‘Ultra-cut’’ microtome. Images were recorded using a CM10 TEM microscope (Philips, Surrey, UK).

2.5 Cryogenic- Scanning Electron Microscopy

Cryogenic scanning electron microscopy (cryo-SEM) of the WPN, E_{WPI}, E_{WPN} and $CURE_{WPN}$ were conducted. Particularly, for cryo-SEM of emulsion samples i.e. E_{WPI}, E_{WPN} and $CURE_{WPN}$, heptane was used as the dispersed rather than MCT oil, to avoid interference by crystallization of oil during the freezing step as used in a previous study involving Pickering emulsions stabilized by microgels (Destribats et al., 2014). Both the systems (heptane or MCT-oil emulsions) presented the same overall microstructural behavior and therefore, the cryo-SEM images observed using heptane emulsions can be extrapolated to MCT-oil emulsions. The WPN, E_{WPI}, E_{WPN} or $CURE_{WPN}$ were mounted on rivets attached to the sample stub. The samples were plunge-frozen in liquid nitrogen "slush" at −180 °C, then transferred to the cryo-preparation chamber on the SEM. The frozen protein nanogels or emulsion droplets were cleaved and then etched at −95 °C for 4 minutes. Next, the samples were coated with 5 nm of platinum (Pt). Finally, the Pt-coated samples were transferred to the SEM for imaging at −135 °C. The heptane emulsion sample was imaged in a FEI Quanta 200F ESEM with a Quorum Polar Prep 2000 cryo system.

2.6. Confocal scanning laser microscopy (CLSM)

The microstructures of the emulsions (20 wt% MCT oil) i.e. E_{WPI}, E_{WPN} and $CURE_{WPN}$ were characterized using a Zeiss LSM 880 inverted confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). Also, the $CURE_{WPN}$ was characterized after the CUR retention experiments. A stock solution of Nile Red (1 mg/ mL in dimethyl sulfoxide, Sigma-Aldrich) was used to stain MCT-oil to a final concentration of 0.02 mg mL^{-1} and a stock solution of Fast Green (1 mg mL^{-1} in Milli-Q water) was used to stain the protein to a final concentration of 0.1 mg m^{-1}. The fluorescently labelled emulsion samples were placed on a concave confocal microscope slide, secured with a glass coverslip and finally imaged using an oil immersion 63× lens and the pinhole diameter maintained at 1 Airy Unit to filter out the majority of light scatter. Nile Red was excited at a wavelength of 488 nm and Fast Green at 633 nm. The

emission filters were set at 555 - 620 nm for Nile Red and 660 - 710 nm for Fast Green (Ong, Dagastine, Kentish, & Gras, 2011).

In general, CUR is known to bind to certain hydrophobic domain of proteins. Hence, CUR binding to WPN was imaged by placing the $CURE_{WPN}$ emulsions directly in the slide and covered them with a glass coverslip using CUR auto-fluorescence. For imaging of CUR, the auto-fluorescence of CUR was recorded using the filters set for Nile Red dye, since CUR exhibits an excitation of 455 nm and an emission at 540 nm (Minear et al., 2011).

2.7. Determination of adsorption efficiency by WPN

To determine the amount of WPN at the interface of the emulsion droplets, E_{WPN} samples were centrifuged for 15 min at 1,770 g at 25 °C (Eppendorf 5702, Hamburg, Germany). Subnatants were carefully removed using a syringe and filtered through 0.45 µm filters (Perkin Elmer, Waltham, MA, USA). The process was repeated twice, and the absorbance of the filtrates was detected using a DC protein assay kit (Bio-Rad Laboratories, Watford, UK) and a UV-Vis Spectrophotometer. The protein concentration of the filtrates was determined with the Lowry method using BSA as the standard. The adsorption efficiency was calculated as the difference between the total amount of protein used for initial emulsion preparation and the amount of protein in the continuous phase as a percentage of total protein concentration.

2.8 Interfacial shear viscosity

The interfacial shear viscosity was measured using a two-dimensional Couette-type viscometer in presence or WPI or WPN. Details have been previously described (Murray & Dickinson, 1996; Sarkar et al., 2017). Briefly, a stainless steel biconical disc (radius 14.5 mm) was suspended from a thin torsion wire with its edge in the plane of the oil-water interface of the solution contained within a cylindrical glass dish (radius 72.5 mm). The deflection of the disk

was measured by reflection of a laser off a mirror on the spindle of the disc onto a scale at a fixed distance from the axis of the spindle. The interfacial viscometer was operated in a constant shear-rate mode, as described in a recent study (Zembyla, Murray, & Sarkar, 2018). For the measurements, a layer of pure n-tetradecane was layered over an aqueous solution of whey protein isolate (WPI) or whey protein nanogel particles (WPN). A concentration of 0.5 wt% was used as the aqueous phase at pH 7.0. The constant shear rate apparent interfacial viscosity, η_i , is given by the following equation:

$$\eta_i = \frac{g_f}{\omega} K(\theta - \theta_0) \quad (1)$$

where, K is the torsion constant of the wire, θ is the equilibrium deflection of the disc in the presence of the film, θ_o is the equilibrium deflection in the absence of the film, i.e. due to the drag force of the sub-phase on the disc, g_f is the geometric factor, and ω is the angular velocity of the dish. A fixed value of $\omega = 1.27 \times 10{-}3$ rad s^{-1} was used.

2.9. Droplet and particle size distribution

Droplet size distributions of the emulsion samples (E_{WPI}, E_{WPN} and $CURE_{WPN}$) were determined using static light scattering at 25 ^{o}C using a Malvern MasterSizer 3000 (Malvern Instruments Ltd, Malvern, Worcestershire, UK). The refractive index of the MCT-oil (Miglyol® 812 oil) and the dispersion medium were set at 1.445 and 1.33, respectively. The absorbance value of the emulsion droplets was 0.001. The mean droplet size distribution of the emulsions was reported as volume mean diameter d_{43} (De Brouckere mean diameter) and surface mean d_{32} (Sauter mean diameter). The d_{43} refers to the mean diameter of a sphere with the same volume, whereas the d_{32} is the diameter of a sphere that has the same volume/surface area ratio as the sphere of interest. Both are generally used to characterize an emulsion droplet. Particle size of the WPN was determined using dynamic light scattering (DLS) at 25 ^{o}C using a Zetasizer Nano-ZS (Malvern Instruments, Malvern UK) in a PMMA standard disposable

cuvette. Particle size was measured after diluting the samples in phosphate buffer (pH 7.0). Each sample was analyzed three times and the average value was reported in the result section.

2.10. ζ-potential

The ζ-potential of the WPN, $E_{WPN,}$ and $CURE_{WPN}$ was determined using a particle electrophoresis instrument (Zetasizer, Nano ZS series, Malvern Instruments, Worcestershire, UK). Samples were diluted in Milli-Q water (0.1 wt% particle or 0.002 wt% droplet concentration) and added to a folded capillary cell (Model DTS 1070, Malvern Instruments Ltd., Worcestershire, UK). Mean and standard deviation of the ζ-potential value of each sample was calculated from three individual measurements on triplicate samples.

2.11. Measurement of CUR retention in Pickering emulsions

To recover the encapsulated CUR from Pickering emulsion, the emulsions samples (200 µL), were disrupted with methanol (1 mL). Sample-solvent mixtures were centrifuged at 1, 770 g at ambient temperature for 10 min to precipitate the WPN (Marefati et al., 2017). Noteworthy, during extraction with methanol, a distinct orange-red color was observed at neutral pH (pH 7.0) (Supplementary Fig. S2a), which can be attributed to pH-induced changes in the CUR structure. As a diarylheptanoid, CUR contains two aromatic rings joined by a seven carbons chain (heptane) with a α,β-unsaturated-β-diketone structure (Araiza-Calahorra et al., 2018). Depending on the solvent characteristics, electron delocalization and deprotonation when in neutral-alkaline environment alters the β-diketone structure undergoing keto–enol tautomerism (Khopde, Indira Priyadarsini, Palit, & Mukherjee, 2000; Nardo et al., 2008). Alteration of the tautomerism of the structure causes the optical properties of CUR to change, causing a deviation of the spectral band position in the absorption or emission spectrum of the molecule to a longer wavelength (bathochromic shift) i.e. changing CUR's color from yellow to red

297 (Tønnesen & Karlsen, 1985). Visual appearance of CUR dilution (1:1 (v/v) methanol/ buffer,
298 25 ^{o}C) as a function of different pH (2.0 – 7.0) can be observed in Supplementary Fig. S2b.
299 Hence, the supernatant of the centrifuged $CURE_{WPN}$ samples at the two biologically
300 relevant pH conditions (pH 3.0 and pH 7.0) were first diluted to appropriate concentrations for
301 quantification of encapsulated CUR (Supplementary Fig. S3). The wavelength used was 425
302 nm and its was chosen based on a scan performed on methanol containing CUR ranging from
303 300 to 500 nm. Diluted samples were placed in a cuvette to measure the absorbance in a UV-
304 VIS spectrophotometer (6715 UV/VIS Spectrophotometer, Jenway, UK). A standard curve of
305 known concentrations of CUR in methanol was prepared to convert the absorbance
306 measurements to CUR concentration.
307
308 2.12. CUR retention in Pickering emulsions
309 The capacity of the Pickering emulsions to retain CUR during short-term storage was measured
310 based on the CUR concentration recovered from the emulsions after they were subjected to pH
311 3.0 or pH 7.0 in absence and presence of ions (50 mM NaCl or 10 mM $CaCl_2$). In brief,
312 $CURE_{WPN}$ was mixed with the appropriate buffer in a 1:0.5 w/w ratio and pH was adjusted to
313 the desired value (pH 3.0 and 7.0) in absence or presence of 50 mM NaCl or 10 mM $CaCl_2$ and
314 the mixture was placed in pre-soaked dialysis membrane (100 kDa molecular weight cut-off
315 membrane, Spectrum Laboratories, USA). Subsequently, the membranes was suspended in
316 buffers at corresponding pH and ionic strengths at 37 ^{o}C with agitation (90 rpm) for 30 minutes.
317 The aqueous buffers used were sodium acetate buffer for pH 3.0, and phosphate buffer for pH
318 7.0. Since CUR is known to be hydrophobic with limited solubility in water, ethanol was added
319 into the aqueous buffer solutions at a final concentration of 15 % (v/v) based on a previous
320 study (Shah et al., 2016b). After 30 minutes, $CURE_{WPN}$ samples within the dialysis membranes

were taken out and CUR concentration in the emulsion sample and CUR released to the aqueous buffers was measured using the method described earlier.

2.13 Fluorescence measurements

Previously, ability of CUR to form complexes with numerous proteins, such as soy protein isolate or β-lactoglobulin has been reported (Chen, Li, & Tang, 2015; Sneharani, Karakkat, Singh, & Rao, 2010). Thus, binding studies of CUR with WPN was conducted at pH 3.0 and pH 7.0 in presence or absence of ions and such interactions were measured using an adapted fluorescence emission spectroscopy method described by Sahu, Kasoju, and Bora (2008). Steady-state fluorescence measurements were carried out in a CLARIOstar microplate spectrofluorimeter reader (BMG Labtech). The fluorescence of CUR was measured by keeping its concentration constant (10 μM) and by varying the WPN concentration (0 – 40 μM) in either sodium acetate buffer (pH 3.0), and phosphate buffer (pH 7.0) in absence or presence of 50 mM NaCl or 10 mM $CaCl_2$. The emission spectra were recorded from 450 to 650 nm with an excitation wavelength of 420 nm. Solutions without WPN were used as controls for the fluorescence measurements. The binding constant was determined by the following equation (Sahu et al., 2008):

$$\frac{1}{\Delta FI} = \frac{1}{\Delta FI_{max}} + \frac{1}{K_a \Delta FI_{max}[WPN]} \quad (2)$$

where ΔFI is the change in the CUR fluorescence intensity in the presence and absence of WPN, ΔFI_{max} is the maximal change in the CUR fluorescence intensity, K_a is the binding constant, and [WPN] is the concentration of WPN. The intensity data were then used to plot the double-reciprocal plot 1/[CM] versus 1/ΔFI. The intercept of the double-reciprocal plot on

345 the $1/\Delta FI$ axis is $1/\Delta FI_{max}$, which was used to calculate the binding constant from the value of
346 the slope in the plot.
347
348 2.14. Statistical analysis
349 The statistical software Minitab 16 (Minitab Inc. Stage College Pennsylvania) was used. The
350 analysis was carried out with the three individual measurements on three individual emulsion
351 samples (i.e. 9 measurements) and analyzed with two-way analysis of variance (ANOVA) and
352 Student's t-test; significance was accepted at $p < 0.05$.
353
354 **2.6 Results and discussion**
355 3.1 Characteristics of aqueous dispersions of WPN
356 The hydrodynamic diameter of WPN dispersion was determined by DLS and morphology was
357 probed using cryo-SEM and TEM across scales (Fig. 1). The particle size distribution was
358 monomodal with a polydispersity index of 0.24, and a mean hydrodynamic radius of 83.05 nm
359 (Fig. 1a). As can be observed from the cryo-SEM image (Fig. 1b), the size of WPN was in
360 close agreement with DLS and WPN showed a tendency to aggregate in the observation grid
361 (Fig. 1b). It is difficult to comment with certainty on the sphericity of the particles because of
362 the possible effects of preparation for cryo-SEM on particle morphology, as have been
363 observed previously (Sarkar, Kanti, Gulotta, Murray, & Zhang, 2017). Looking at lower length
364 scale, the TEM image (Fig. 1c) showed that WPN formed a hierarchical structure of aggregates
365 of protein of different characteristic sizes as postulated by Schmitt et al. (2010) using small
366 angle X-ray scattering experiments.
367 Previous researchers have shown the formation of microgel particles of spherical shape
368 of about 200 – 500 nm (Destribats, Rouvet, Gehin-Delval, Schmitt, & Binks, 2014; Sarkar et
369 al., 2016). Differences in size and aggregate morphology of WPN used in this study as

370 compared to the previous reports can be attributed to the variation in the processing route, such
371 as using the high-pressure homogeniser (Leeds Jet Homogeniser, University of Leeds, UK),
372 which uses turbulent flow and extremely high localized pressures as compared to conventional
373 homogenizers, as well as the initial protein concentration used to form the hydrogel (Nicolai,
374 Britten, & Schmitt, 2011; Sarkar, Kanti, et al., 2017; Schmitt, Bovay, Vuilliomenet, Rouvet, &
375 Bovetto, 2011; Torres, Murray, & Sarkar, 2017). The WPN exhibited an average ζ-potential
376 value of -30.46 mV, which suggests that the electrostatic repulsion between the particles was
377 high enough to ensure dispersion stability at pH 7.0 (Fig. 1a). The negative charge was expected
378 as WPN was above the isoelectric point (pI) and the value was within the range found in the
379 literature (Destribats et al., 2014; Sarkar et al., 2016).
380
381 3.2 Characteristics of Pickering emulsions (E_{WPN})
382 Firstly, we conducted interfacial shear rheology experiments and microstructural evaluation
383 across scales to investigate whether the WPN were forming Pickering emulsions as compared
384 to a conventional emulsion stabilized by WPI. Applying shear rheology deformation to the
385 interfacial layers in E_{WPI} or E_{WPN} will give us information on the formation and structuring of
386 absorbed protein or particle layers, respectively. This method is particularly sensitive to
387 differentiating proteins versus particles based on their interfacial flow behavior, and
388 consequently can give quantitative insight into difference between WPN and WPI (Murray,
389 Durga, Yusoff, & Stoyanov, 2011; Sarkar, Zhang, et al., 2017). Surface shear viscosity (η_i)
390 values for WPN were compared to those of WPI solution, both dispersed in phosphate buffer
391 at pH 7.0. We present the measurements of η_i values at 'short' (2 and 3 h) and 'long' (24 h)
392 adsorption time scales in Table 1. As expected, the value of η_i for WPI decreased from ~ 453
393 mN s m^{-1} at 2 h to its quarter after 24 h, which in agreement with previous works with protein
394 monolayers (Chen & Dickinson, 1995; Dickinson, Rolfe, & Dalgleish, 1990).

However, the value of η_i for WPN at the oil-water interface was twice as that of WPI in 2 h time scale. Of more interest is that the η_i became almost an order of magnitude higher than that of WPI in 24 h time scale (Table 1). The high values obtained for WPN is indicative of strengthening of the interfacial films by the presence of adsorbed particles. These quantitative results perfectly corroborate with the qualitative observation of nanogel particles at the interface of the WPN-stabilized emulsions in the CLSM, cryo-SEM and TEM images (Fig. 2).

Table 1. Interfacial shear viscosities (η_i / mN s m^{-1}) O/W interface in presence of whey protein isolate (WPI) and whey protein nanogel particles (WPN) at pH 7.0. Values represent mean ±SD of at least three independent experiments (n ≥ 3).

Adsorption time / h	0.5 wt% WPN	0.5 wt% WPI
2	916.13 ± 100.83	453.22 ± 112.46
3	969.62 ± 75.78	334.19 ± 55.87
24	1006.13 ± 278.36	127.50 ± 27.75

In particular, the sizing of the droplets (Fig. 2) highlight that E_{WPI} droplets (d_{43} = 0.89 ± 0.08 μm) were much smaller in size as compared to that of E_{WPN} droplets (d_{43} = 10.29 ± 2.31 μm), which is expected owing to the larger size of WPN particles (~80 nm, Fig. 1a) stabilizing the droplets in the latter as compared to protein molecule counterpart in the former (~ 2 nm). Also looking at the cryo-SEM and TEM images at different magnification (Fig. 2), the interface of the E_{WPI} droplets did not present any visible protein molecules that is expected owing to the size of the protein molecules being smaller as compared to the microscopic resolution in agreement with previous studies (Sarkar, Zhang, et al., 2017). However, the particles are clearly evident at the interface of E_{WPN} droplets that confirms the Pickering stabilization by these particles providing ultra-stability to these droplets against coalescence over a year storage period (data not shown).

The droplet size distribution, mean droplet size and charge of the Pickering emulsions (0.1 – 3 wt% WPN) are shown in Figs. 3a, and b, respectively. In Fig. 3a, it can be observed

416 that the droplet size distribution of E_{WPN} was mostly bimodal with two populations of droplets
417 clearly identified except for emulsions stabilized by 0.1 – 0.5 wt% WPN. The signature peak
418 in the area of 0.1 – 1 µm most likely corresponds to the unabsorbed WPN, which has been
419 reported previously (Sarkar et al., 2018a; Sarkar et al., 2016), while the more prominent peak
420 in the area of 5 to 50 µm represents the Pickering emulsion droplets. In case of 0.1 – 0.5 wt%
421 WPN, a third peak was observed in 50 – 500 µm range highlighting either coalesced or
422 flocculated droplets in these emulsions. When the concentrations of WPN was above 0.5 wt%,
423 the third peak almost disappeared with increase of second peak suggesting more adsorption of
424 particles to the droplet surface until 1.0 wt% (Fig. 3a). However, above 1.0 wt% WPN, the
425 percentage of the relative area of the first peak increased at the expense of the second peak,
426 suggesting a gradual increase of unadsorbed WPN. No significant change in the mean oil
427 droplet diameter occurred when varying the concentration of WPN, except for 0.1 – 0.25 wt%
428 samples ($p < 0.01$) (Fig. 3b).

429 The absolute magnitude of ζ-potential of all emulsions was higher as compared to that
430 of the WPN present in the aqueous phase (Fig. 3b) ($p < 0.05$). This increase in negative surface
431 charge might be attributed to the concentration of WPN at the droplet surface as compared to
432 being in the aqueous phase. Such magnitude ≥ ±30 mV is generally indicative of strong
433 electrostatic stabilization of droplets (McClements, 2004) in addition to the mechanical
434 stabilization provided by the particles.

435 The maximum adsorption efficiency for these systems was calculated to be 100% (0.1
436 wt%) (Fig. 3c). The absorption efficiency in emulsions prepared with higher concentrations of
437 WPN gradually decreased to 58.34% for 3 wt% (Fig. 3c) further supporting the unabsorbed
438 particles seen in light scattering data (Fig. 3b). Besides adsorption efficiency, surface coverage
439 (Cs) was calculated to provide a useful indication of the density of the particles anchored at the
440 oil-in-water interface for emulsions undergoing limited coalescence (Gautier et al., 2007). In

441 principle, the percentage of interfacial area covered by the particles can be calculated using
442 equation 3, as reported previously by Binks and Olusanya (2017). The simplest version of this
443 equation assumes all the particles are adsorbed at the droplet surface and the non-adsorbed
444 particles are neglected. Under these assumptions, equation 3 is defined as:
445
446 $$\frac{1}{d_{32}} = \frac{m_p}{4CsV_d\rho_p d_p} \quad (3)$$
447
448 where, m_p is the mass of the particle, V_d the volume of the oil phase, ρ_d the density of
449 the particle, d_d the particle radius and d_{32} is the mean droplet diameter. Assuming all particles
450 were monodisperse and were adsorbed at the oil-water interface in a hexagonal close packing
451 arrangement, the surface coverage should be equal to 0.907.
452 Below the critical concentration of the 1.0 wt%, the surface coverage obtained was
453 significantly below 0.907 (Table 2). In this concentration range, it can be suggested that the
454 droplet size was dictated by the particle concentration (Binks, Philip, & Rodrigues, 2005). This
455 behavior is typical of Pickering emulsions undergoing limited coalescence and has been
456 reported for particles of a similar size range, such as colored organic pigment particles, silica,
457 and poly(glycerol monomethacrylate)–poly(2-hydroxypropyl methacrylate) (PGMA–
458 PHPMA) diblock copolymer particles (Binks & Olusanya, 2017; Gautier et al., 2007;
459 Thompson et al., 2014).
460 Table 2. Surface coverage of emulsion droplets by various concentrations of WPN.

WPN (wt%)	**Cs**
0.1	0.14
0.25	0.32
0.35	0.46
0.5	0.68
1	1.35
1.5	1.95
2	2.59

3	3.45

In the case of higher WPN concentrations (1 – 3 wt%), surface coverage was greater than 0.907, suggesting either formation of a multilayer or aggregates of WPN at the interface (Binks & Olusanya, 2017) or an excess of particles that were not adsorbed. In order to characterise the morphology of particles at the droplet surface, cryo-SEM imaging of heptane droplets covered by 1 wt% nanogel particles at pH 7.0 is shown in Fig. 3b1. As can be clearly observed, the interface was covered by a network of particles where WPN adopted configurations of either individual particles or a network of particle aggregates. These observations correspond to the high surface coverage as calculated and reported in Table 2. Such visual clarity of nanometric-sized aggregates versus nanometric sized-single particle at the droplet surface has been also previously observed in cryo-SEM images of Pickering droplets by previous authors (Destribats, Rouvet, Gehin-Delval, Schmitt, & Binks, 2014).

In summary, emulsions ≥ 0.5 wt% experienced limited coalescence in the particle-poor regime, whereas ≥1 wt% it transitioned towards a particle-rich regime. It is clear that within the explored concentration range, the addition of 1 wt% demonstrated to create stable droplets with complete coverage (~1.14 monolayers theoretically). Hence, this concentration was selected hereafter to create Pickering emulsion for encapsulation of CUR ($CURE_{WPN}$), and in vitro retention of CUR.

3.3 Characteristics of CUR-loaded Pickering emulsions

The CUR content in the $CURE_{WPN}$ was 474 ± 29.4 µg/mL, which was close to the amount of 500 µg/mL added to the oil. This suggests that CUR was not degraded or lost during the emulsification process. The size distribution of $CURE_{WPN}$ was identical to the distribution of samples without the addition of CUR (E_{WPN}) (Fig. 4a) with diameters in the former ranging

between 5 and 50 µm ($p > 0.05$). This suggests that the addition of CUR did not negatively affect the droplet size of the $CURE_{WPN}$ (Araiza-Calahorra et al., 2018).

Furthermore, the typical dimensions of empty spaces (Sarkar et al. 2016) between the WPN arranged on the triangular lattice $(\sqrt{3}-1)d_{WPN}/_2$ was calculated to be $\cong 30\ nm$ for the current study with a diameter (d_{WPN}) of the nanogel particle at 80 nm. Overall, Pickering emulsion stabilized by nanometric-sized WPN allowed loading high concentrations of CUR without any effect on the size distribution of the emulsions or CUR diffusing out of the empty spaces at the interfaces. The cryo-SEM analysis allowed to observe the morphology of CUREWPN droplets (Fig. 4b2) which was in agreement with the samples without CUR (Fig. 3b) in terms of droplet size. However, it appears that the surface was not showing the same degree of WPN aggregates at the interface as observed in the samples without CUR (Fig. 4b1). This suggests that there might have been some interactions between CUR in the dispersed phase and WPN in the adsorbed phase, which is discussed in details in the next sections. However, one must be cautious with interpreting this cryo-SEM data as there might be interaction between CUR and heptane in the dispersed phase, causing some microstructural changes in these images that might be specific to thee images.

3.4 CUR retention in the $CURE_{WPN}$

After characterizing the stability and surface coverage of the Pickering emulsions, the next aim was to assess the retention of CUR within the $CURE_{WPN}$ droplets. The retention ability was assessed as a function of pH (pH 3.0 and 7.0) and ions i.e. 50 mM NaCl, and 10 mM $CaCl_2$. The choice of pH and ions were based on physiological relevance i.e. pH and ions that are commonly encountered in the gastric and duodenal regimes in human physiology. The retention of CUR in the Pickering emulsions was measured using a dialysis approach as described in the materials and methods section, based on the protocol previously used by Shah et al. (2016b).

511 Ethanol was added to the aqueous buffer media in order to solubilize the CUR and create a
512 'force-release' environment to the aqueous buffer media because CUR is poorly soluble in
513 aqueous phase.

514 The CLSM imaging was performed to characterize the microstructural changes (if any)
515 in the Pickering emulsion droplets before and after the retention experiments (Fig. 5a1-a3). As
516 can be observed, the presence of a bright ring around the droplets indicates an adsorbed layer
517 of nano-meter sized WPN at the interface at both acidic (gastric environment) and basic pH
518 (duodenal environment). This indicates that the emulsions were stable with no pH-induced
519 hydrolysis of the WPN at the interface. Noteworthy, a bright ring around the emulsion droplets
520 were evident in the non-stained samples i.e. the samples where only auto-fluorescence of CUR
521 could be observed (Fig. 5b1-b3). It is well documented in literature that CUR binds to the
522 hydrophobic domain of numerous proteins such as bovine casein micelles, bovine serum
523 albumin (BSA), human serum albumin (HAS), soy protein isolate and beta-lactoglobulin (β-
524 lg) through hydrophobic interactions (Chen et al., 2015; Sahoo, Ghosh, & Dasgupta, 2009;
525 Sahu et al., 2008; Sneharani et al., 2010; Zsila, Bikádi, & Simonyi, 2003). However, the
526 observed intensities obviously need interpreting with caution since it is well documented in
527 literature that CUR phosphorescence intensity strongly depends on the energy of the exciting
528 photons applied (Chignell et al., 1994). Nonetheless, this observation of a bright auto-fluorescing
529 ring at the particle-laden interface surrounding the $CURE_{WPN}$ emulsion droplets with increased
530 intensity and the intensity within the oil droplets might suggest that CUR was mainly retained
531 within the emulsion. This CUR might be retained in two ways: either being bound to the
532 interfacial WPN, or were dispersed within the oil droplets. Indeed, CUR release into the
533 aqueous buffer media was verified (less than 1% of CUR was the loss), which confirmed that
534 in all cases CUR remained entrapped within the emulsion systems, either bound or solubilised
535 within the oil phase. As WPN at the droplet surface might form a complex with CUR, the

partitioning of CUR in the dispersed phase versus interface might be affected. Hence, CUR retention in $CURE_{WPN}$ and further characterization of the interaction between CUR and WPN were performed using spectroscopic techniques, which is discussed in the following sections.

In the absence of ions, about 60.53% of CUR was found to be dispersed in the oil phase at acidic conditions (pH 3.0), which means that 39.47% of CUR was bound to the WPN. Fig. 6a shows both, the amount of CUR retained witin the Pickering emulsion, and the amount of CUR bound to the WPN as a function of pH. On the contrary, CUR retention within the oil droplets was statistically higher ($p < 0.05$) in neutral pH (76.85%). The CUR retention in $CURE_{WPN}$ when subjected to different salt concentration are also plotted in Fig. 6a. At pH 3.0, changes in retention parameters were not statistically significant ($p > 0.05$) on addition of ions. However, at pH 7.0, the CUR retention values were statistically significant ($p < 0.05$) in absence or presence of the divalent cations. These results points out the relevance of pH in CUR retention and binding, which was not obvious in the CLSM images (Fig. 5).

To understand this better, we measured changes in fluorescence intensity to quantify binding constants (K_a) (Fig. 6b) between CUR and WPN (corresponding fluorescence spectra reported in Supplementary Fig. S4).

3.5 Binding of CUR and WPN

Fig. 6b shows the double-reciprocal plot 1/[WPN] versus 1/ΔFI that has been used to calculate the binding constant from the value of the slope in the plot. The binding constants were estimated to be in the range of $6.67 \times 10^1 – 1.33 \times 10^4\ M^{-1}$ (Fig. 6b). Binding constants in the order of 10^4 show that there existed a strong affinity between WPN and CUR at pH 3.0. The K_a results obtained are in agreement with previous studies that have reported similar values for β-lg and CUR ($10^5\ M^{-1}$), and other dietary polyphenols, such as resveratrol ($10^4–10^6\ M^{-1}$), epigallocatechin-3-gallate ($10^4–10^5\ M^{-1}$), and catechin and epicatechin ($10^3\ M^{-1}$) (Kanakis et

al., 2011; Liang, Tajmir-Riahi, & Subirade, 2008; Shpigelman, Israeli, & Livney, 2010; Sneharani et al., 2010). Nevertheless, this is the first study that provides binding constant values between CUR and WPN. Interestingly, the K_a at neutral pH was found to be 6.67×10^1 M^{-1}, which is three orders of magnitude lower than that at acidic pH 3.0 (1.00×10^4 M^{-1}). Findings of our study are in close agreement with previous studies that have reported that at neutral pH, the K_a for CUR with denatured β-lg was $7.0 \pm 0.2 \times 10^2$ M^{-1} (Sneharani et al., 2010), which might explain the increased partitioning of CUR to the oil phase as shown in Fig. 6a.

The influence of ions on the K_a is also shown in Fig. 6b. Interestingly, the K_a for the CUR/ WPN mixture at pH 3.0 was not significantly affected by addition of 50 mM NaCl and 10 mM $CaCl_2$ (1.33×10^4 M^{-1}). These results suggest that addition of ions did not alter the physical stability and solubility of CUR at pH 3.0. On the contrary, at pH 7.0, the presence of both, monovalent and multivalent ions enhanced the binding affinity between CUR and WPN (6.67×10^3 and 8.00×10^3 M^{-1}, for 50 mM NaCl and 10 mM $CaCl_2$, respectively), although these values were still one order of magnitude lower than the ones calculated at pH 3.0. These results can be explained in terms of pH-induced changes in CUR conformation and/or WPN charge distribution.

At acidic pH, CUR primarily exists in the open enol tautomeric form (Nardo et al., 2008). In the open conformation, the valence electrons of the carbonyl and enolic oxygen act as H-bond acceptors and the enolic proton as H-bond donor, with charges of -0.73, -0.70, and 0.50, respectively in water (Balasubramanian, 2006). The H-bond accepting and donating capabilities of the molecule expands the number of possible interaction sites that account for CUR's increased binding behaviour. A possible mechanism of the interaction between CUR and WPN, focusing on amino acid residues in βA sheet domain of β-lg, at pH 3.0 is schematically shown in Fig. 7a. The βA sheet domain of β-lg was used since studies have indicated that, upon partial denaturation, Tyr20, which is located close to the base of the that

586 is generally considered to be the binding pocket, is highly accessible, and that Trp19 is critical
587 for the interaction of β-lg and CUR (Brownlow et al., 1997; Mohammadi, Mahmudian, Moeeni,
588 & Hassani, 2016). Here, the enol tautomeric form of CUR (Litwinienko & Ingold, 2004) allows
589 hydrophobic interactions with aromatic residues of WPN, such as tyrosine (Tyr) and tryptophan
590 (Trp). Also, worth noting that CUR possess a weak net negative charge (Fig. 7a), whereas WPN
591 undergoes protonation at pH 3.0 (pI ~ 5.2) and is strongly positively charged. Hence, at pH 3.0,
592 both electrostatic and hydrophobic interactions play a role in CUR-WPN binding (Fig. 7a) that
593 support their high binding affinities (Fig. 6b).

594 Conformation of the β-diketone group in CUR is highly dependent on the chemical
595 environment that successively dictates its intermolecular bonding behaviour (Heger, van
596 Golen, Broekgaarden, & Michel, 2014). Upon changing the environment to pH 7.0 (Fig. 7b),
597 CUR adopts the diketo tautomeric conformation characterized by a visible red band or shoulder
598 present in steady-state absorption spectra measurements (Khopde et al., 2000; Nardo,
599 Andreoni, Bondani, Másson, & Tønnesen, 2009; Nardo et al., 2008). Underscored by the fact
600 that a red shift in the absorption spectrum of CUR was observed at neutral pH (Supplementary
601 information S2), it is suggested that changes in pH to neutral pH reduced CUR binding
602 behaviour limiting its migration to the interfacial layer of WPN and increasing the CUR
603 concentration in the dispersed phase (Fig. 6a). It is also worth noting that WPN and CUR both
604 possessed a net negative charge contributing to higher degree of repulsive interactions, further
605 contributing to limited binding affinity as observed in Fig. 6b. Hence at pH 7.0, the binding
606 between CUR and WPN might be attributed only to the hydrophobic interactions (Fig. 7b) as
607 well as higher solubility of CUR in the dispersed phase, all of which contributing to higher
608 partitioning to the oil (Fig. 6a).

609 Retention of CUR in $CURE_{WPN}$ was compared with similar measurements reported in
610 the literature using other particle-laden interfaces. In Pickering emulsions stabilized by sub-

micron sized chitosan-tripolyphosphate nanoparticles (CS/TPP NPs) ranging in size from 322 – 814 nm, 44% and 63% of the encapsulated CUR was retained after 24 hours at pH 2.0 and 7.4, respectively (Shah et al., 2016b), which is lower than the ranges observed in the current study. Overall, these results suggests that WPN-laden interface can be used to increase the retention of CUR, and such retention is largely associated with the mechanical barrier provided by the WPN at the droplet surface that are present either as particle or as network of aggregated particle reducing the gap size. In addition, the partitioning of the CUR retained within these emulsions might be dictated by electrostatic and/or hydrophobic interactions between CUR and interfacial WPN as well as solubility of the CUR in the oil phase, largely affected by the pH and ionic conditions of the medium.

4. Conclusions

In this study, whey protein nanogel particles were used to stabilize oil-in-water Pickering emulsions for encapsulation and controlled delivery of curcumin. We have investigated the influence of particle concentration on the structure and stability of emulsions containing 20 wt% MCT-oil stabilized by these nanogel particles. Comparing microstructure at multiple length scales, droplet size and interfacial rheology of emulsions stabilized by protein molecules and protein nanogel particles, we confirmed that the emulsions were Pickering stabilized ones in the latter case. Pickering emulsions presented a monolayer of particles at the droplet surface at a critical concentration of 1.0 wt% whey protein nanogel particles. Structural visualization (TEM and cryo-SEM) of emuliosn stabilized by 1.0 wt% particle concentration revealed that whey protein nanogels adsorbed in two different conformations, as a closely packed layer of individual particles, and as network of aggregated particles.

Furthermore, whey protein nanogel-stabilized emulsions were used to encapsulate curcumin. The droplet size and stability of the curcumin-loaded Pickering emulsions were maintained after curcumin incorporation. It was demonstrated that curcumin retention in these Pickering emulsions were associated with the mechanical barrier provided by the whey protein nanogels at the interface and reduced interfacial gap size, latter associated with the nanometric size of these nanogel particles. Furthermore, the partitioning of curcumin in the dispersed phase varied as a function of pH in an in vitro release model with lower partitioning at pH 3.0 as compared to that at pH 7.0. This was attributed to the electrostatic and hydrophobic interactions that allowed more binding of curcumin to whey protein nanogel particles at the interface at pH 3.0 as compared to that at pH 7.0. The binding of curcumin to whey protein nanogel particles at the interface at pH 7.0 was also affected by the presence of mono- and divalent cations. Overall, our study demonstrates the design principles for developing Pickering emulsions for controlled delivery of curcumin, with mechanisms unraveled behind curcumin binding to the interfacial whey protein nanogel particles as a function of pH and ionic strengths.

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