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Oil-in-water emulsions stabilized by sodium phosphorylated chitosan

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ABSTRACT

Oil-in-water (O/W) emulsions with sodium phosphorylated chitosan (PCTS) were obtained via simple emulsification. PCTS in aqueous solution was amphiphilic with a hydrophilic–lipophilic balance (HLB) of 19 and a critical aggregation concentration (CAC) of 0.13% w/v. The emulsifying efficiency and emulsion stability of PCTS over oil droplets were evaluated in terms of the droplet size, droplet size distribution and microscopic observation using confocal laser scanning microscopy. PCTS preferred to cover oil droplets to produce an O/W emulsion and formed long term stable particles (90 days storage at room temperature) when using PCTS concentrations from above the CAC to 3% w/v. However, emulsions formed from PCTS concentrations below the CAC or over 3% w/v were unstable with particle agglomeration by flocculation after only 7 days storage, although they reverted to individual droplets that retained their integrity in acidic conditions. Overall, PCTS forms effective stable O/W encapsulated particles with potential applications in lipophilic drug encapsulation via a simple emulsion system.

1. Introduction

Encapsulation is a vesicular system in which the active substances (payload) are loaded into an inner core cavity surrounded by a polymeric membrane shell. The shell of the encapsulated particles can serve as an active substance or just as a payload carrier. Several methods had been developed for the production of encapsulated particles (Mora-Huertas, Fessi, & Elaissari, 2010) including polymer-coating, layer by layer, precipitation methods and so on. Among these various methods, the formation of emulsions, a heterogeneous system in which two typically immiscible liquids of different polarities are dispersed one into the other in the form of droplets, is a simple and interesting system for encapsulation since the emulsion provides either lipophilic or hydrophilic cavities where lipophilic or hydrophilic components, respectively, can be encapsulated. However, the emulsion possesses a low stability because it is thermodynamically unstable owing to the unfavorable contact between the lipophilic and hydrophilic phases (such as oil and water) that typically results in phase separation (Dickinson, 1992a, 1992b). Therefore, emulsifiers or surfactants are added into the immiscible mixture and these are important in the formation and stabilization of emulsions (Dickinson, 1992a, 1992b). Small molecular emulsifiers, so-called surfactants, function to decrease the surface tension between the immiscible phases. However, surfactants often cause irritation to the human skin (Gajjar & Benford, 1990) and respiratory pathway (Sukhapan & Brimblecombe, 2002), making them somewhat unsuitable for use in medical and cosmetic applications. Macromolecular emulsifiers not only decrease the surface tension, generate and stabilize emulsions, but they also function as an encapsulator or protective barrier for the dispersed droplets and any substances inside them, because of their alignment at the interface between the oil and water phases.

Owing to these reasons, polymers are currently widely used as emulsifiers in diverse emulsion systems, such as polyethyleneglycol stearates (Li et al., 2008), acrylate and its hydrophobically modified derivatives (Buraczewska, Berne, Linberg, Törmä, & Lodén, 2007). However, attention has also shifted to the utilization of natural polymers or biopolymers as emulsifiers instead of synthetic polymers because of their greater biological compatibility (Garti, 1999). Some natural and water soluble emulsifiers have been used to emulsify and stabilize food emulsions, including proteins

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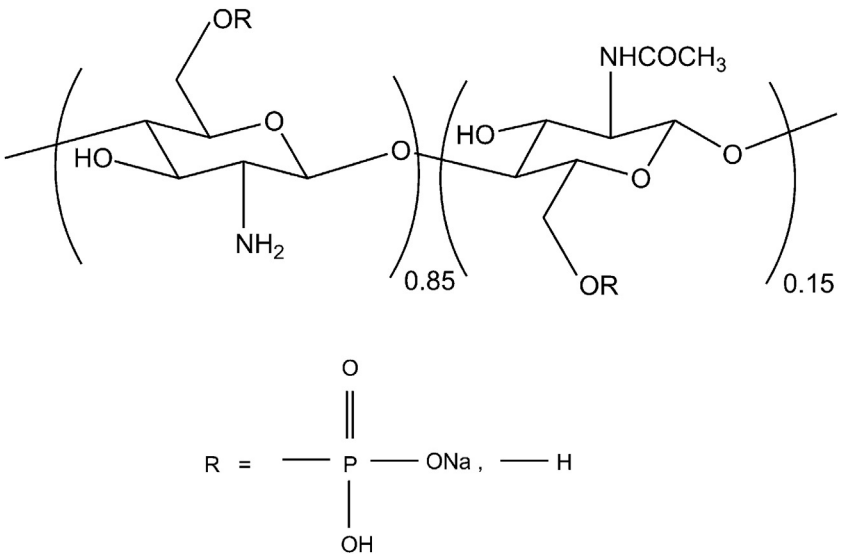

Scheme 1. Schematic structure of PCTS. The proportion of R sites that are phosphate or hydrogen moieties depends upon the degree of substitution (DS) of phosphate. The DS of the PCTS used in this study was 0.04.

(Demetriades & McClements, 1998) and glycoproteins (proteins conjugated polysaccharides) (Fecher, Knoth, Scherze, & Muschiolik, 2007). However, common abundant biocompatible polysaccharides, such as cellulose and starch, are highly hydrophilic and so require hydrophobic modification to improve their amphiphilic properties. Chitosan is one type of natural, readily available, and sustainably renewable polysaccharide that has been used to produce an emulsion (Rodríguez, Albertengo, & Agulló, 2002). Nevertheless, chitosan does not dissolve in water, making it unsuitable for emulsion based encapsulation in many cosmeceutical, pharmaceutical and medical applications.

Therefore, in order to avoid organic solvents, we are interested in developing new bio-based water soluble emulsifier suitable for emulsification so as to be able to synthesize emulsion-based encapsulation of active agents (payloads). Sodium phosphorylated chitosan (PCTS), a water soluble phosphate derivative of chitosan, has been synthesized in our laboratory (Tachaboonyakiat, Netswasdi, Srakaew, & Opaprakasit, 2010), and the possible structure of PCTS is shown in Scheme 1. PCTS has been reported to be suitable for clinical applications, including pharmaceutical drug delivery, because of its cytocompatibility (Srakaew, Ruangsri, Suthin, Thunyakitpisal, & Tachaboonyakiat, 2011) and bioabsorbability (Zhu, Wang, Cui, Feng, & Groot, 2003). PCTS would, therefore, be expected to be a good emulsifier to form a thick adsorbed layer and so play a role as a protective layer. PCTS also has the advantage of that it, and the emulsions formed from it, are surfactant-free and so potentially suitable as an active encapsulating agent in pharmaceutical and personal care products.

In this research so as to evaluate the suitability of PCTS as a water soluble emulsifier, PCTS formed emulsion-based particles were synthesized and characterized. Since this is an emulsion system, the PCTS properties in aqueous solution were characterized in terms of the hydrophilic–lipophilic balance (HLB) and critical aggregation concentration (CAC). The emulsifying efficiency and emulsion stability were investigated in terms of the droplet size, droplet size distribution, zeta potential and microscopic appearance using zetasizer and confocal laser scanning microscopy (CLSM). Furthermore, the physical mechanism underlying the instability of the emulsions was also investigated to elucidate further details on the main reasons for emulsion instability. The PCTS emulsion was then evaluated for its drug encapsulation efficiency and drug release profile using naproxen as the model lipophilic payload drug.

2. Materials and methods

2.1. Materials

PCTS with a degree of substitution (DS) of phosphate of 0.04 and a molecular weight (M_W) of 58 kDa was synthesized as previously reported (Tachaboonyakiat et al., 2010). Briefly, chitosan was reacted with phosphorous pentoxide at 0.1 equivalent mole to chitosan residue in methanesulfonic acid at 0–5 °C for 3 h. The mixture was then precipitated in acetone and dried. In order to improve the water solubility of the phosphorylated chitosan, it was then dissolved in distilled water and dialyzed sequentially against distilled water and 0.1 M sodium hydroxide (NaOH), respectively. The dialyzed product was then neutralized with 0.1 M hydrochloric acid (HCl), and redialyzed against distilled water. The water soluble PCTS was then obtained after lyophilization.

Chitosan with a M_W of 250 kDa, as determined by high performance liquid chromatography (HPLC) (Shimadzu, LC-10ATVP, Japan), and a degree of deacetylation (DD) of 85, as determined by proton nuclear magnetic resonance (^{1}H NMR) (Varian, Unity Inova, US), was purchased from Seafresh Chitosan (Lab) Co., Ltd. (Bangkok, Thailand). Mineral oil (density 0.8 g/cm^3) was purchased from Witco (Houston, USA). Isopropyl myristate (IPM) was obtained from Merck (Darmstadt, Germany). Fluorescein isothiocyanate (FITC) was supplied from Fluka (Buchs, Switzerland). Dialysis tubing, CelluSep T2 (MWCO 6000–8000) was purchased from Membrane Filtration Products, Inc. (TX, USA). Naproxen was received from Aldrich (Steinheim, Germany). All chemicals and solvents were used as received.

2.2. Determination of the HLB of PCTS

The HLB value was determined according to the method of Becher (1960) by measuring the diameter of a toluene drop laid over known HLB emulsifier solutions. The experiment was performed in triplicate. The average diameter of the toluene drops in each of various HLB solutions were plotted to form the calibration curve (Supplementary information Fig. S1). In order to determine the HLB value of PCTS, the average diameter of toluene drops on the surface of a 1% w/v PCTS aqueous solution was measured at room temperature and the HLB value was calculated from the above calibration curve.

2.3. Determination of the CAC of PCTS

In order to investigate the lowest concentration for PCTS that can form a micelle, the CAC was determined by measuring the surface tension of PCTS solutions at various concentrations. Surface tension has reported as one of the most popular means for determining the critical micelle concentration (CMC) or CAC of an emulsifier (Jönsson, Lindman, Holmberg, & Kronberg, 1998). The Du Noüy ring method was used to measure the surface tension of each PCTS solution at 25 °C using a Dataphysics DCAT 21 dynamic tensiometer (Filderstadt, Germany). The concentration of PCTS solutions were varied in the range of 0.00–0.53% w/v. From the plot of the surface tension against the PCTS concentration, the inflection point corresponds to the CAC of PCTS.

2.4. Emulsion preparation and encapsulation

The PCTS aqueous solution (1% w/v except where specified otherwise) and mineral oil were used as the aqueous and oil phases, respectively, for the preparation of the emulsion-based particle (emulsion droplet) at a 4:1 v/v ratio of the aqueous PCTS solution (pH 5.5): mineral oil. The mixture was homogenized with a high pressure homogenizer (EmulsiFlex C3, Avestin Inc., Canada) at 150 MPa for six cycles to obtain the emulsion.

For evaluation of drug encapsulation, the 1% w/v PCTS aqueous solution and IPM were used as the aqueous and oil phases, respectively. Naproxen (6 mg/mL), as the model hydrophobic payload drug, was dissolved in IPM before emulsification. The emulsion was then prepared similarly to the above mentioned method.

2.5. Indirect determination of the lipophilic and hydrophilic cavity

The drop test of the emulsion into distilled water or mineral oil was used to preliminary evaluate which phase (water or oil) was the continuous phase, and so if the emulsion was an oil-in-water (O/W) or water-in-oil (W/O) emulsion. The O/W or W/O emulsion was indirectly implied to lipophilic or hydrophilic cavity, respectively.

2.6. Determination of the emulsifying efficiency and emulsion stability

The emulsifying efficiency of PCTS over the dispersed oil droplets was determined in terms of the emulsion droplet size and droplet size distribution with respect to the PCTS concentrations.

The droplet size and droplet size distribution was measured at 25 °C by zetasizer (NanoZS, Malvern Instrument Ltd., Worcestershire, UK). A 10 μL aliquot of the fresh emulsion (within 24 h after preparation) was diluted in distilled water to a final concentration of 1×10^{-4}% v/v and shaken by vortex to prevent aggregation of the individual droplets and avoid multiple scattering effects. The droplet size is reported as the surface mean diameter (d_{32}), as defined in Eq. (1):

$$d_{32} = \frac{\sum_i n_i d_i^3}{\sum_i n_i d_i^2} \quad (1)$$

where n_i is the number of droplets with diameter d_i.

In order to visualize the emulsion at different PCTS concentrations, PCTS was labeled with FITC and visualized under CLSM (FluoView 1000, Olympus, Japan) with an Olympus IX81 microscope at 40× magnification. The imaging software incorporated with the CLSM was Olympus FV10-ASW 1.7 Viewer software.

The emulsion stability of PCTS over dispersed droplets was then investigated in terms of the change in the mean droplet size (d_{32}) over short and long term storage (7 and 90 days, respectively) of the emulsion at room temperature (30 °C).

2.7. Investigation of the physical mechanism underlying the emulsion instability

2.7.1. Visualization of the PCTS distribution on the emulsion droplet

In order to visualize the PCTS distribution on the emulsion droplets after storage of the emulsion at room temperature for 7 days, the FITC-conjugated PCTS was used in the emulsification allowing the subsequent PCTS-containing droplets to be observed under CLSM.

2.7.2. Determination of the physical droplet size alteration and zeta potential of the unstable emulsion under pH variation

A 10 μL aliquot of the PCTS emulsion after 7 days of storage at room temperature was diluted in water to a final concentration of 1×10^{-4}% v/v, set to various pH values (pH 1–13) by the addition of 0.1 M HCl or 0.1 M NaOH, as appropriate, during the dilution. The droplet size and zeta potential were evaluated by zetasizer (NanoZS, Malvern Instrument Ltd., Worcestershire, UK) at 25 °C.

2.8. Determination of the drug (naproxen) encapsulation efficiency within the PCTS emulsion

The encapsulation efficiency within the emulsion was evaluated indirectly by measuring the amount of unencapsulated naproxen. The naproxen loaded emulsion was centrifuged at 6000 rpm at 5 °C for 10 min, whereupon the emulsion separated into a cream phase and subnatant. The concentration of naproxen in the subnatant was then determined as follows: a 10 μL aliquot of the subnatant was diluted in 4.99 mL ethanol, mixed by vortexing, and then the absorbance at 229 nm was measured with a UV-vis spectrophotometer (Specord S 100, AnalytikJena, Jena, Germany). The subnatant of the emulsion without naproxen loading was used as the reference. The drug encapsulation efficiency was then indirectly evaluated as in Eq. (2):

$$\text{Encapsulation efficiency } (\%) = \frac{C_e - C_s}{C_e} \times 100 \quad (2)$$

where C_e is the total concentration of naproxen in the emulsion and C_s is the concentration of drug present in the subnatant.

2.9. In vitro release study

The in vitro release of naproxen was determined by dialysis of the encapsulated emulsion (Avgoustakis et al., 2002). The emulsion was diluted in 0.1 N HCl (pH 1.2) or phosphate buffer saline (PBS, pH 7.4) to 0.02 v/v and then 5 mL poured into snake-skin dialysis tubing (MWCO 3500, Pierce, Rockford, IL, USA) and dialyzed against the same solution (50 mL) with shaking at 37 °C using an oscillator. At the indicated time intervals, 1 mL of the external medium was removed for analysis and replaced with 1 mL of the same type of release medium. The concentration of naproxen was determined by UV-vis spectrophotometer at 229 nm.

2.10. Kinetics of the release profile

To study the release kinetics of naproxen-loaded PCTS containing oil emulsions, the release data obtained at pH 1.2 and 7.4 were fitted to the Korsmeyer–Peppas exponential equation (Eq. (3)), which is often used to predict the drug release behavior from polymeric systems when the mechanism is not well known or more

than one type of release phenomenon is involved (Costa & Lobo, 2001; Korsmeyer, Gurny, Docler, Buri, & Pepas, 1983).

$$\frac{M_t}{M_\infty} = kt^n \quad (3)$$

where M_t/M_∞ is the fraction of naproxen release at time t, k is the release rate constant and n is the release exponent. To evaluate the n exponent, only the portion of release profile where $M_t/M_\infty \leq 0.6$ is employed. To clarify the release exponent, the log value of percentage naproxen released was plotted against log time, according to Eq. (4):

$$\log\left[\frac{M_t}{M_\infty}\right] = \log k + n \log t \quad (4)$$

In this model, the value of n was used to characterize the release mechanism, where $n = 0.5$ corresponded to Fickian diffusion (diffusion-controlled release), $0.5 < n < 1$ corresponded to anomalous (non-Fickian) diffusion, $n = 1$ corresponded to case II transport (relaxation-controlled delivery), and $n > 1$ corresponded to a super case II transport (Korsmeyer et al., 1983).

3. Results and discussion

3.1. HLB of PCTS

The numerical value of the HLB indicates the relative affinity of the emulsifier for the oil and aqueous phases. A molecule with a high HLB value has a high ratio of hydrophilic to lipophilic groups, and vice versa. Thus, the HLB value of an emulsifier is a useful indicator of its solubility in the oil or the water phase and can be used to predict the type of emulsion that will be formed by the emulsifier. Emulsifiers with a low HLB number (3–6) are preferably dissolved in the oil phase and so form a stabilized W/O emulsion, whilst those with a high HLB value (10–18) are preferably dissolved in water and form a stabilized O/W emulsion (McClements, 2005).

The average diameter of toluene drops laid on the 1% w/v PCTS solution was 6.2 ± 0.1 cm, giving an HLB value for PCTS of 19.0 ± 0.9 from the calibration curve (Supplementary information, Fig. S1). Therefore, PCTS is likely to preferentially stabilize O/W emulsions, which reflects the structure of PCTS with its hydrophobic backbone and the acetamide groups that can interact with the oil surface, and its hydrophilic amino, hydroxyl and phosphate groups that can interact with the water. The chitosan structure was also contributed to have hydrophobic character along its backbone, acetamide groups and hydrophilic components of the amino and hydroxyl groups (Elsabee, Morsi, & Al-sabagh, 2009).

3.2. CAC of PCTS

The CAC is the lowest concentration of polymeric emulsifier that can promote micelle formation. Furthermore, the CAC value is important for systems in which the effect of emulsifiers is the primary consideration. For example, only low concentrations of emulsifier are desirable in personal care products but micelle formation is still required. The CAC of PCTS in aqueous solution was determined by measuring the surface tension of solutions of different PCTS concentrations.

The equilibrium values of the surface tension of PCTS at 25 °C are shown in Fig. 1. The CAC of PCTS is determined as the concentration at the inflection point of the surface tension curve, which in this case was at 0.13% w/v or 1.3 mg/mL.

As such, for PCTS to act as an emulsifier it will be required at a lower concentration than other commonly used small molecular emulsifiers, such as sodium dodecylsulphate (CMC of 8.1 mM or 2.33 mg/mL) and dodecyl-trimethylammonium bromide (CMC of 15.3 mM or 4.66 mg/mL) (Atkin, Craig, Wanless, & Biggs, 2003)

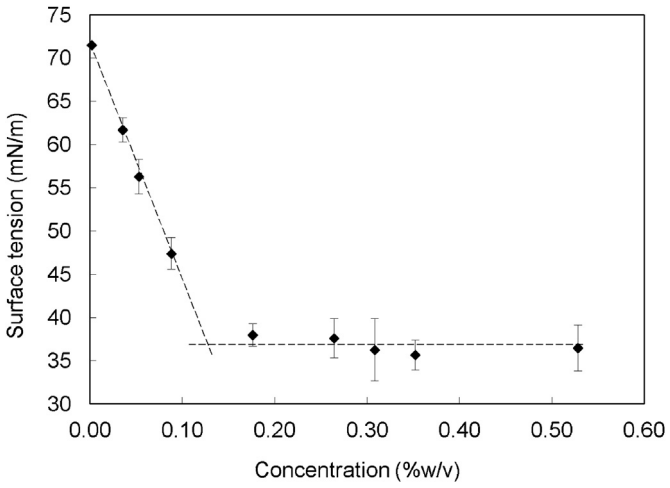

Fig. 1. Surface tension of different concentrations of PCTS in an aqueous solution at 25 °C. The inflection point (0.13% w/v) corresponds to the CAC of PCTS. Data points are the mean ± SD, derived from three independent replications.

to form micelles. Therefore, this water soluble PCTS has a high potential to form micelles due to its low CAC. Indeed, polymeric emulsifiers are generally effective at a low total concentration (Elsabee et al., 2009). Interestingly, hydrophobically modified water soluble chitosan derivatives would increase the nonpolar region leading to a decrease in their CAC. For example, (2-hydroxyl-3-butoxyl)-propylcarboxymethyl chitosan (hydrophobic substitution of 0.24) (Weiping, Changqing, Yanjing, Zhiguo, & Xiangzheng, 2006) and *N*-octyl-*N*-dimethyl chitosan (hydrophobic substitution of 0.08) (Zhang, Ding, Ping, & Yu, 2005) possess CAC values of 0.5 mg/mL and 0.43 mg/mL, respectively, compared to that of chitosan in acetic acid that exhibits a higher CAC value of around 1 mg/mL (Amiji, 1995).

3.3. Indirect determination of lipophilic or hydrophilic cavity

Whether PCTS mediated the formation of a lipophilic or hydrophilic cavity in the emulsion particles was preliminary evaluated in terms of if an O/W or W/O emulsion was formed. Whilst O/W emulsions have a lipophilic cavity and so are suitable for encapsulation of lipophilic payloads, W/O emulsions have hydrophilic cavities and so are suitable for the encapsulation of hydrophilic payloads.

The drop test of the emulsion into distilled water or mineral oil was used to preliminary investigate the continuous phase. The emulsion formed with 1% w/v PCTS dispersed spontaneously in distilled water, but aggregated in mineral oil (data not shown). This then supports that the continuous phase was the aqueous phase and that PCTS was suitable for O/W emulsion formation. Moreover, these observations of PCTS forming O/W emulsions are in agreement with the HLB value of PCTS derived in this study (~19), and a previous report (McClements, 2005), which would predict that PCTS should form an O/W emulsion and provided a lipophilic cavity.

3.4. Determination of emulsifying efficiency and emulsion stability

3.4.1. Emulsifying efficiency

The mean sizes of the newly formed O/W droplets formed with different concentrations of PCTS (0.06–5% w/v) are shown in Fig. 2. As described above, the CAC was found to be 0.13% w/v. In accord, the droplet sizes of emulsions formed with a PCTS concentration of 0.06% w/v, which is below the CAC (0.13% w/v), were large at around 350 nm, but decreased to 170 nm as the PCTS concentration was

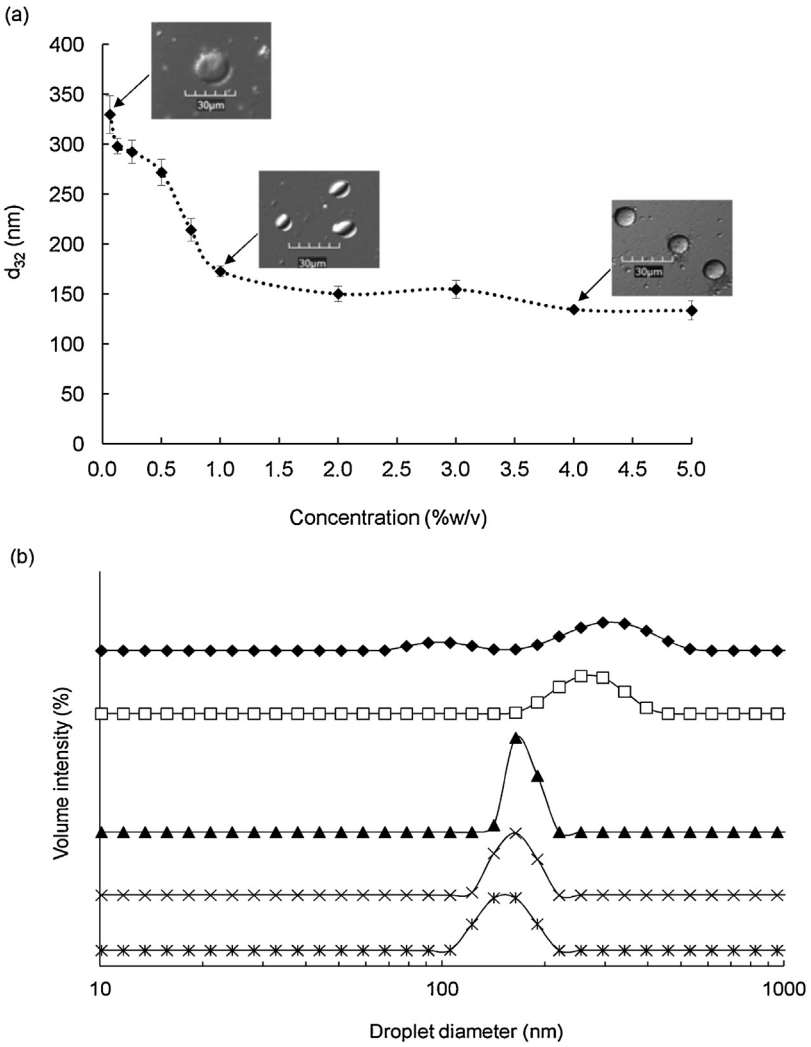

Fig. 2. (a) Zetasizer-derived surface mean diameter (d_{32}) of emulsion droplets formed at various concentrations of PCTS. Insert picture: CLSM micrograph (Nomarski image) of emulsion droplets at a magnification of 40×. (b) Droplet size distribution of the newly formed emulsion with PCTS concentrations of 0.06% w/v (♦), 0.13% w/v (□), 1.0% w/v (▲), 3.0% w/v (×) and 5.0% w/v (*). Data are shown as (a) the mean ± SD, derived from three independent replications and inserts are representative of at least three such fields of view per sample and from three independent replications, or (b) representative results of one of three independent replications.

increased to 1% w/v. With these increasing PCTS concentrations up to 1% w/v the electrostatic repulsion between charged groups can be shielded by counter ions, e.g. amino or phosphate groups, resulting in a decrease in the polymer dimension. These macromolecule particles with lower dimensions would be better adsorbed on the oil droplet and as a consequence increase the adsorption density and decrease the droplet size. When PCTS concentrations were further increased above 1–4% w/v, the emulsion droplet size obtained was almost constant at around 140 nm, suggesting that 1% w/v PCTS provided sufficient emulsifier to form a strong polymer layer at the oil–water interface and stabilize the emulsion. If a smaller droplet size is still required, this might be achieved by increasing the input energy of the homogenizer since simply increasing concentration caused no further significant decrease in the droplet particle size below 140 nm diameter (Fig. 2).

The appearance of the emulsion droplets could be visualized under CLSM by visualization of FITC-conjugated PCTS, where the PCTS was interfacially laid between the oil and water phase (Fig. 2a (insert) and supplementary Fig. S2).

The CLSM micrographs revealed that emulsion droplets could form even in the presence of only 0.06% w/v PCTS, a concentration below the CAC of PCTS (0.13% w/v). The small proportion of PCTS (relative to oil) did not completely cover all around the oil droplet. In contrast, emulsions formed using concentrations of PCTS above its CAC revealed that the PCTS was rearranged in a high density covering around the oil droplets. The CLSM micrograph revealed that PCTS aligned at the interface between the oil and water phases, and so it could function as an encapsulator or protective barrier for lipophilic active ingredients inside the oil droplets. However, it should be noted that the estimated droplet size derived from CLSM was larger than that from zetasizer because particles smaller than 5 μm were too small to observe under CLSM (Lamprecht, Schäfer, & Lehr, 2000). Accordingly, in this research only the large drops were observed under CLSM under the assumption that they were representative of all the emulsion droplets in terms of that they are assumed to exhibit similar characteristics to the small emulsion droplets.

The adsorption of PCTS onto the oil–water interface was likely due to the arrangement of the hydrophobic units of the backbone and acetamide groups, which can interact with the oil, and the hydrophilic units of the amino, hydroxyl and phosphate group which interact with the water.

The zetasizer-derived droplet size distribution of the emulsions formed from different PCTS concentrations is displayed in Fig. 2b. The emulsion formed with a PCTS concentration of 0.06% w/v (below its CAC) displayed a bimodal size distribution at ~100 nm

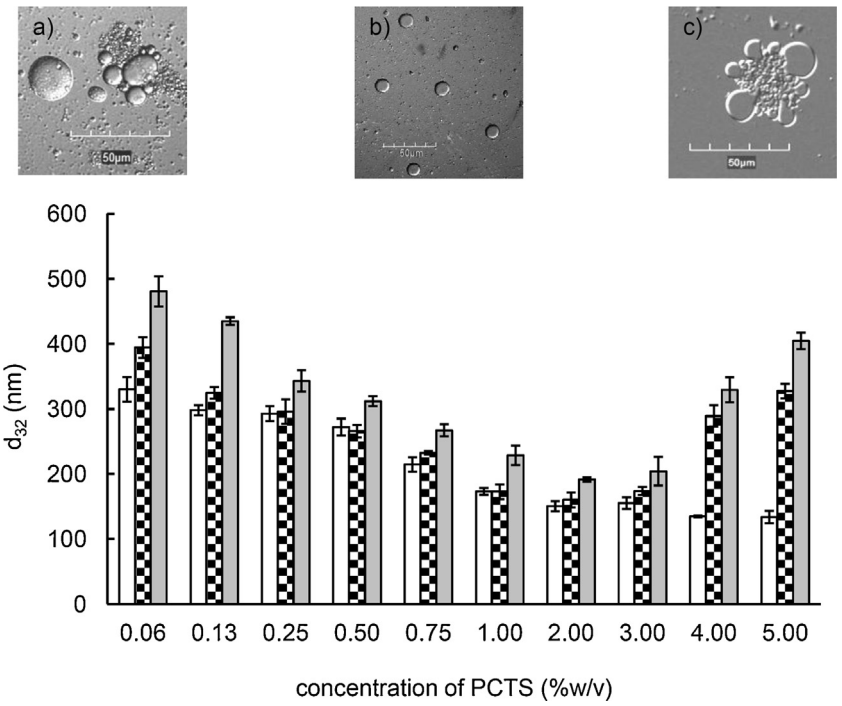

Fig. 3. Zetasizer-derived average droplet size, as the surface mean diameter (d_{32}), of the emulsions formed with different PCTS concentrations after storage for 0 (□), 7 (▩) or 90 (■) days. Insert pictures: CLSM micrographs (Nomarski image) of emulsion droplets formed with FITC-conjugated PCTS at (a) 0.06% w/v, (b) 1.0% w/v and (c) 4.0% w/v after storage for 7 days. Data are shown as the mean ± SD, derived from three independent replications.

and 200–550 nm. When the concentration of PCTS was increased to that of its CAC (0.13% w/v) the droplet size distribution became broad but unimodal at 170–500 nm. Thereafter, as the concentration of PCTS increased above its CAC value in the range of 1–5% w/v the droplet size distributions became narrow and unimodal at 100–210 nm. This indicated that PCTS stabilized the emulsion droplets by covering a higher interface surface area as a consequence of decreasing the droplet size.

From the above results, a low PCTS concentration of 1% w/v is required to cover the surface of the oil droplets and activate the interface, indicating a potentially high emulsifying efficiency.

3.4.2. Emulsion stability

In order to study the stability of the formed emulsion, the alteration in the droplet size after storage at room temperature for a short and long term (7 and 90 days, respectively) was evaluated.

Both in the short and the long term storage, the emulsions showed a significant change in their droplet size depending on the PCTS concentration that they had been formed from (Fig. 3). For short term storage, the emulsions formed from a PCTS concentration of 0.06% w/v, which is below the CAC value, or those formed at much higher PCTS concentrations of 4% w/v or 5% w/v were found to be unstable and significantly increased in size. Thus, at PCTS concentrations of 0.06% w/v, the mean diameter of the particles (d_{32}) increased 1.23-fold (by around 75 nm). Likewise, at very high PCTS concentrations of 4% w/v and 5% w/v the droplet size increased some two-fold by around 300 nm. Emulsions formed with 0.13–3% w/v PCTS were, however, relatively stable with only a slight numerical and statistically insignificant increase in the droplet size.

With respect to the long term storage (90 days), this revealed a broadly similar trend in the increase in the droplet size to that seen with short term storage, except the magnitude of the change was greater. At a PCTS concentration below or at the CAC of PCTS (0.06% w/v and 0.13% w/v, respectively), the d_{32} increased significantly (ca. 1.5-fold) with an increase in the droplet size of around 150 nm. At PCTS concentrations from above its CAC up to 3% w/v, the droplet sizes increased only slightly (1.1–1.3-fold; by ~50 nm) after storage, suggesting the droplets possessed a near optimum thickness of the PCTS interfacial layer to stabilize the individual emulsion droplets. At higher PCTS concentrations of 4% w/v and 5% w/v, the droplet size of the emulsion significantly increased (2.5–3.0-fold, respectively) by around 170 nm and 270 nm, respectively. In both cases, PCTS emulsion tended to form agglomeration and increase in droplet size.

The physical mechanism for the proposed emulsion instability by droplet agglomeration was next investigated further.

3.5. Physical mechanism that underlies the agglomeration of emulsion droplets

3.5.1. Visualization of the PCTS distribution on the emulsion droplet

From Fig. 3, the emulsions formed in the presence of a PCTS concentration below its CAC (that is at 0.06% w/v) or much higher than it (4% w/v and 5% w/v) tended to increase in droplet size on storage. This was hypothesized to be due to the agglomeration of the droplets. The physical mechanism behind an unstable emulsion can be classified into the three main categories of flocculation, coalescence and Ostwald ripening. Flocculation is generally an agglomeration of droplets, but since the agglomerated droplets can revert back to individual droplets under an applied shear stress then they must retain their individual integrity in the agglomeration. In contrast, coalescence results when two or more droplets are no longer separated by the continuous phase and the interfacial membrane of the emulsifier surrounding the droplet is ruptured leading to the fusion of the droplets to from a single larger unit. Finally, Ostwald ripening is a process of molecular diffusion transfer

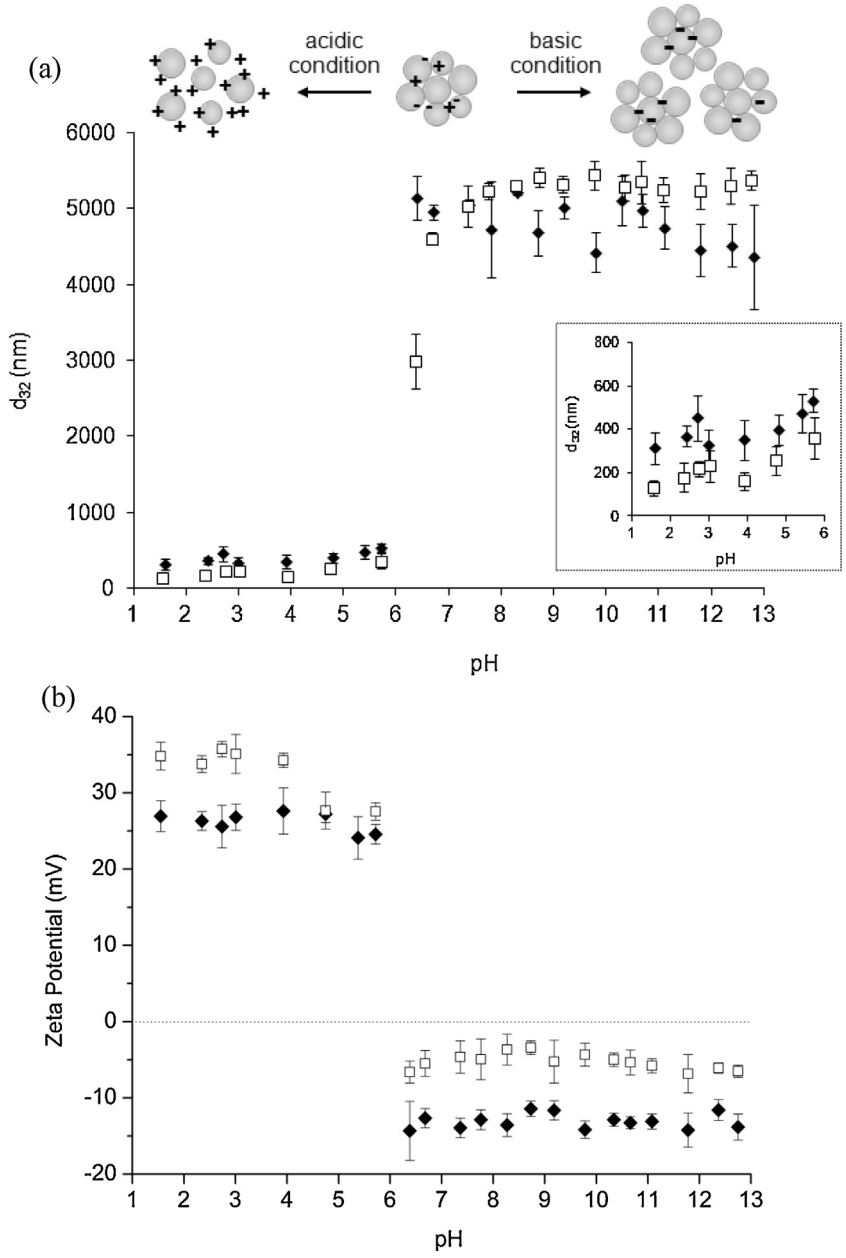

Fig. 4. (a) Zetasizer-derived surface mean diameter (d_{32}) and (b) zeta potential of the emulsion droplets formed with PCTS as a function of the pH. Emulsions were prepared with PCTS concentrations of 0.06% w/v (♦) or 4% w/v (□). Data are shown as the mean ± SD, derived from three independent replications. Insert; schematic drawing of emulsion droplets representing the charge state and proximity of individual droplets or flocculates in different environmental pH values.

of the disperse phase from the smaller droplet to the larger droplet and is driven by chemical potential differences, which are the differences in not only the droplet curvatures but also the compositions of the disperse phase.

In this research, the physical mechanism accounting for the unstable emulsions prepared with PCTS concentrations of 0.06% w/v and 4% w/v was preliminary investigated by visualization of the FITC-conjugated PCTS distribution on the emulsion droplet under CLSM.

The CLSM micrographs of the emulsion droplets formed at PCTS concentrations of 0.06% w/v and 4% w/v, after storage for 7 days (Fig. 3; inserts (a) and (c)) show individual small droplets clumped together. This contrasts to those droplets formed with a PCTS concentration of 1% w/v (Fig. 3; insert (b)), and supports that the mechanism accounting for the droplet agglomeration as a consequence emulsion instability is flocculation.

3.5.2. Droplet size and zeta potential changes in the unstable emulsion at different environmental pH values

Several different methods have been utilized to verify the flocculation of an emulsion, for example (i) the evaluation of the viscosity of the emulsion and (ii) visualization of droplet flocculation by direct observation. For the former method, the viscosity is evaluated in terms of the shear stress under various applied shear rates (Demetriades, Coupland, & McClements, 1997; Floury, Desrumauz, & Lardières, 2000). Here, low droplet-droplet interactions in emulsions without flocculation are supposed to be responsible for the Newtonian behaviors of the fluids and so the shear-thinning behavior of the emulsion is attributed to the formation of clusters or flocculated droplets. In the latter case of the direct observation of flocculation, this is performed by direct observation using optical microscopy (Chantrapornchai, Clydesdale, & McClements, 2001), where the optical image shows droplets clumped together.

Table 1
Kinetic parameters of naproxen release from oil emulsions prepared with 1% w/v PCTS in acidic (0.1 M HCl, pH 1.2) or physiological (PBS, pH 7.4) media with Korsmeyer–Peppas model fitting ($n = 3$).

Release condition	Korsmeyer–Peppas model		
	Correlation coefficient (R^2)	Diffusional exponent (n)	Type of release
pH 1.2	0.9976	0.51	Fickian diffusion
pH 7.4	0.9920	0.61	Anomalous (non-Fickian) diffusion

We hypothesize that, owing to PCTS being amphoteric, the emulsion droplets surrounded by PCTS will change their droplet size with changes to the pH of the surrounding environment if they are subject to flocculation.

The zetasizer-derived mean particle diameter (d_{32}) and zeta potential of the oil emulsion droplets formed with PCTS at 0.06% w/v or 4% w/v after storage for 7 days was plotted against the pH (Fig. 4(a) and (b), respectively), and revealed that the d_{32} of droplets from both emulsions decreased as the pH of the environment was reduced from pH 6 to pH 2. Surface charge of droplets was also changed from +25 mV to +28 mV and +28 mV to +34 mV for PCTS at 0.06% w/v or 4%w/v, respectively, when the pH of the environment was reduced from pH 6 to pH 2 (Fig. 4b). This could be due to the amino groups on the PCTS (DD 85) being protonated to charged NH_3^+ groups (pKa = ~6.5) in the acidic media and so inducing electrostatic repulsion of the positive charges between the droplets, concomitant with separating into individual particles, as a result of smaller surface mean diameter. In a basic environment (pH > 7), the phosphate groups on the PCTS structure are deprotonated to be negatively charged HPO_3^- (pKa = ~7) or PO_3^{2-} (pKa = ~12), resulting in the slight increase in negative surface charge from −12 mV to −14 mV and from −6 mV to −7 mV seen here for the emulsion droplets with PCTS at 0.06% w/v or 4% w/v, respectively. However, the PCTS used in this research has a low DS of phosphate groups (DS = 0.04), and so this may not have provided a sufficient repulsive electrostatic force to prevent agglomeration with the resulting observed large mean particle diameter size.

This indicated that the clumps of the emulsion droplets derived from PCTS at 0.06% w/v and 4% w/v could then be separated into individual droplets in acidic conditions, so the physical mechanism should be flocculation and not coalescence. As such this then has the advantage of maintaining the integrity of the individual droplets.

3.6. Encapsulation efficiency and in vitro release of emulsion

The encapsulation efficiency of naproxen (6 mg/mL of oil) within the emulsion with 1% w/v PCTS emulsion was 91.2 ± 4.7%. Therefore, the PCTS O/W emulsion had a good ability to encapsulate the model hydrophobic drug (naproxen).

To evaluate the release profile of the encapsulated naproxen from the PCTS emulsion, the in vitro release profile was performed in 0.1 N HCl (pH 1.2) and PBS (pH 7.4) whilst being shaken at 37 °C using an oscillator. The naproxen showed a slower release rate and to a lower level in the acidic condition (pH 1.2) than in PBS at pH 7.4 (Fig. 5). Thus, at pH 1.2 only 20% of the naproxen was released after 5 h compared to ~80% and 90% released naproxen within 3 h and from 4 h onwards at pH 7.4. The naproxen release from the PCTS emulsion is therefore influenced by the pH of the release media. Since naproxen is a lipophilic molecule with acidic group with is less soluble in acidic media, so it is preferentially released in a neutral environment (Duarte, Costa, Simplício, Cardosa, & Duarte, 2006). Accordingly, the solubility of drug under various pH conditions appears to have a significant effect on the rate of drug release.

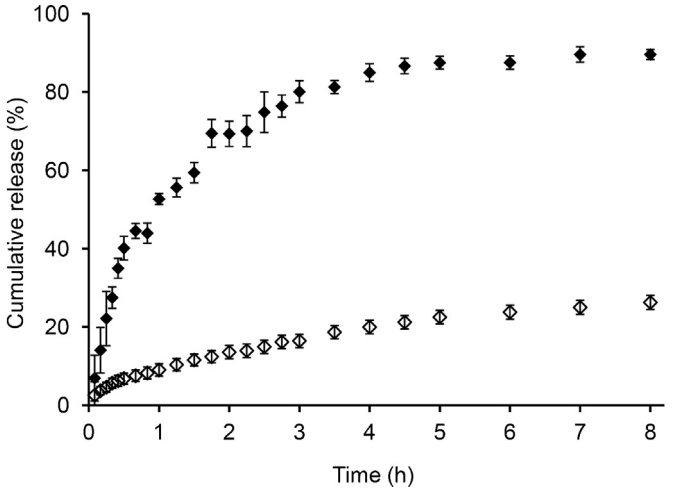

Fig. 5. Naproxen release profile from emulsions prepared with 1% w/v PCTS emulsion in 0.1 M HCl pH 1.2 (◊) and PBS pH 7.4 (♦) at 37 °C. Data are shown as the mean ± SD, derived from three independent replications.

3.7. Kinetics of the naproxen release profile

To clarify the drug release mechanism of the system, the Korsmeyer–Peppas model was applied and the derived release kinetics of naproxen-loaded PCTS emulsion in pH 1.2 and pH 7.4 are presented in Table 1. The naproxen release from the emulsion showed a good fit with the Korsmeyer–Peppas model (correlation coefficient (R^2) greater than 0.99) in both the acidic (pH 1.2) and physiological (pH 7.4) media. The release exponent (n) was used to indicate the likely release mechanism. In the pH 1.2 medium the release exponent for the naproxen-loaded PCTS emulsion ($n = 0.51$) was consistent with a Fickian diffusion controlled release, suggesting that the likely mechanism of naproxen release was by diffusion from the polymeric particles. However, in PBS at pH 7.4 the release exponent ($n = 0.61$) indicated an anomalous (non-Fickian) diffusion controlled release, where more than one mechanism may be involved, i.e. a combination of diffusion of the drug and swelling of the drug-encapsulated polymer.

4. Conclusions

PCTS with a low DS of phosphate (0.04) was evaluated as a potential emulsifier system for the encapsulation of a reactive lipophilic agent that can then be applied for cosmeceutical, pharmaceutical and medical applications. PCTS was found to potentially be suitable, yielding lipophilic encapsulation via an O/W emulsion system with a HLB of 19.0 ± 0.9 and a CAC of 0.13% w/v. Emulsions prepared in the presence of PCTS at concentrations above its CAC up to 3% w/v showed only a slight increase in droplet size after long term (90 days at room temperature) storage. However, emulsions formed from PCTS at levels either below the CAC (that is at 0.06% w/v) or considerably above it (4% w/v or 5% w/v) were unstable and flocculated upon short and long term storage. The potential to improve the stability by using PCTS with a higher DS of phosphate remains to be evaluated. The physical mechanism of

flocculation was established by evaluation of the particles under different environmental pH values. The PCTS encapsulated oil particles retained their individual integrity and so could be of use for controlled drug loading. Furthermore, naproxen, as a model lipophilic drug, was successfully encapsulated at over 90% encapsulation efficiency in the PCTS emulsion, while the in vitro release profiles at pH 1.2 and 7.4 revealed that the rate and net level of release of naproxen was significantly higher in the pH 7.4 medium, which was likely related to the increased naproxen solubility. The drug release profiles at both pH 1.2 and pH 7.4 showed a good fit into Korsmeyer–Peppas model. Indeed, PCTS based emulsions appear potentially suited as a novel particle to deliver reactive substances, owing to its amphiphilic and amphoteric properties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.03.065.

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