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Influence of Soy Lecithin and Sodium Caseinate on The Stability and *In-Vitro* Bioaccessibility of Lycopene Nanodispersion

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SUMMARY

Research background. Various approaches have been used to present functional lipids including lycopene in a palatable food form to consumers. However, being highly hydrophobic, lycopene is insoluble in aqueous systems and has a restricted absorption level in the body. Producing lycopene nanodispersion is expected to improve the properties of lycopene, but its stability and bioaccessibility are also affected by emulsifier type and environmental conditions such as pH, ionic strength, and temperature.

Experimental approach. The influence of soy lecithin (LC), sodium caseinate (SC), and soy lecithin: sodium caseinate (LC:SC) at one to one ratio on the physicochemical properties and stability of lycopene nanodispersion prepared using the emulsification-evaporation methods before and after

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being subjected to different pH, ionic strength and temperature treatment were investigated. The *in-vitro* bioaccessibility of the nanodispersions was also studied.

Results and conclusion. Under neutral pH conditions, LC-stabilized nanodispersion showed the highest physical stability by exhibiting the smallest particle size (78 nm), lowest PDI value (0.180), and highest zeta potential (-64 mV) but lowest lycopene concentration (1.826 mg/100mL). SC-stabilized nanodispersion conversely had the lowest physical stability. Combining the LC with SC at 1:1 ratio resulted in a physically stable lycopene nanodispersion with the highest lycopene concentration (2.656 mg/100mL). The lycopene nanodispersion produced by LC also exhibited high physical stability under different pH range (pH=2-8) where the particle size, PDI, and zeta potential remained fairly consistent. The SC-containing nanodispersion was unstable with respect to droplet aggregation when the pH was reduced close to the isoelectric point of SC (pH=4-5). The particle size and PDI value of LC:SC stabilized nanodispersion increased sharply when the NaCl concentration increased above 100 mM while the LC and SC counterparts were more stable. All of the nanodispersions showed good stability with respect to temperature changes (30-100 °C) except for the one stabilized by SC, which exhibited an increased particle size when heated to a temperature above 60 °C. The combination of LC and SC was found to increase the bioaccessibility of the lycopene nanodispersion. The physicochemical properties, stability, and extent of the lycopene nanodispersion digestion were highly dependent on the emulsifier types.

Novelty and scientific contribution. Producing a nanodispersion is considered one of the best ways to overcome the poor water solubility stability, and bioavailability issues of lycopene. Currently, studies related to lycopene-fortified delivery systems, particularly in the form of nanodispersion are still limited. The information obtained on the physicochemical properties, stability, and bioaccessibility of lycopene nanodispersion is useful for the development of an effective delivery system for various functional lipids.

Keywords: lycopene; nanodispersion; bioaccessibility; sodium caseinate; lecithin; emulsifier

INTRODUCTION

An emulsifier has a major role in the food industry, especially in the production of dairy products, baked goods, and beverages. In recent years, in-depth studies of the use of various emulsifiers in the production of nanoemulsions or nanodispersions as a delivery system for poorly water-soluble compounds (such as carotenoids, antioxidants and flavor compounds) for food application has been extensively conducted. Many studies have shown that nanoemulsions can improve the solubility of hydrophobic compounds, increase the absorption rates, enhance the

bioavailability and improve physical stability against destabilization processes (*i.e.*, flocculation, creaming, coalescence, and Ostwald ripening) and environmental stresses (*1,2,3*). However, the characteristics and stability of the resulting nanoemulsion are also dependent on the type of emulsifier used.

SC and LC are examples of food-grade emulsifying agents that are being widely used in the food industry. The SC molecules are able to absorb, unfold and spread at the droplet surface to form a thick coating layer that generates strong steric stabilization. LC is a phospholipid containing phosphatidylcholine (PC), phosphatidylthenolamine (PE), and phosphatidylinositol (PI) up to 29-46 %, 21-34 % and 13-21 %, respectively (*4*). It also contains minor amounts of phosphatidic acid (PA), phosphatidylserine (PS), and non-phospholipids components such as triglycerides, free fatty acids, and carbohydrates. LC contributes good stability to the emulsion by absorbing rapidly onto the droplet surface, efficiently reducing the interfacial tension between the oil and water phases, and imparting a high negative net charge to the emulsion, which results in high repulsive force interactions against the destabilization processes. One of the most common approaches to produce an emulsion with the desired characteristics is by combining different types of emulsifier to stabilize the emulsion such as chitosan (*5*); starch (*6*), protein (*7*), sodium caseinate, and soy lecithin (*8*).

The emulsifying properties of food emulsifier as the sole stabilizer or in combination with other types of emulsifier is strongly related to the properties of the surrounding environment such as pH, ionic strength, and temperature. The use of various processing methods and conditions as well as numerous ingredients in food processing may expose foods to various environmental conditions such as pH, ionic strengths, and temperatures which might affect the emulsion properties.

Bioaccessibility should also be taken into consideration when designing food products as it is affected by the structure and particle size of the compounds, the nature of the food matrix, and its interaction with other food constituents (*9*). An evaluation of bioaccessibility is sufficient to estimate the bioavailability of carotenoids such as *in-vitro* digestion methods as it is considered rapid, inexpensive, reproducible, and reliable (*10*). Many different *in-vitro* models have been developed based on the food or carotenoids being studied, but the general steps are largely similar, *i.e.*, simulation of the digestion conditions in the gastrointestinal (GI) tract, *i.e.*, in the mouth, stomach, small intestine and colon, followed by quantification of the carotenoid fraction released from the food matrix and incorporated into the bile salt micelles via high-performance liquid chromatography (HPLC) (*11,12*).

Lycopene is a natural carotenoid pigment consisting of a straight hydrocarbon chain with 11 conjugated double bonds and 2 non-conjugated double bonds that are susceptible to degradation,

oxidation, and/or *trans-cis* isomerization under light, oxygen, heat, and acidic or alkaline conditions (*13*). The presence of unsaturated double bonds in the lycopene demonstrated several biological activities such as antioxidant (*14*), immunoregulation (*15*), and anticancer (*16*) as preventive effects against nerval degenerative diseases (*17*), breast cancer (*18*), and cardiovascular diseases (*19*).

In this study, lycopene nanodispersions were prepared by using different emulsifier types namely (i) lecithin (LC), (ii) sodium caseinate (SC), and (iii) a combination of lecithin and sodium caseinate (LC:SC) at 1:1 ratio, using emulsification and evaporation method. The effects of emulsifier types and environmental conditions (pH, ionic strength, and temperature) on the particle size, polydispersity index, zeta potential, and lycopene content of the nanodispersions were determined. The in-vitro bioaccessibility of the lycopene nanodispersions was also investigated using a 2-phase static in vitro gastrointestinal digestion model.

MATERIALS AND METHODS

Materials

Lycopene (95 %) was purchased from Shaanxi Jinjiankang Biological Technology Co., Ltd. (Xi'an, China). Sodium caseinate, soy lecithin, sodium chloride, and sodium hydroxide were purchased from Fisher Scientific (Leicestershire, UK). Ascorbic acid solution, pancreatin from porcine pancreas, bile extract, pyrogallol, DL-α tocopherol, potassium chloride, calcium chloride dihydrate, potassium phosphate monobasic, magnesium chloride hexahydrate, and sodium bicarbonate were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The deionized water for nanodispersions preparation was produced using a Sartorius Stedim Biotech Arium 611DI system (Goettingen, Germany).

Preparing a lycopene nanodispersion

Different types of emulsifiers namely (i) lecithin (LC), (ii) sodium caseinate (SC), and (iii) a combination of lecithin and sodium caseinate (LC:SC) in the proportion of 1:1 were used to prepare lycopene nanodispersion using the emulsification-evaporation method. All samples were prepared at neutral pH. For the LC:SC mixture, both emulsifiers were mixed before the emulsification process. The 1:1 ratio of LS:SC was chosen as it showed the best synergistic effects on the lycopene nanodispersion with the smallest particle size and the highest zeta potential during our preliminary study.

Pre-emulsification step

Lycopene powder extract was dissolved in dichloromethane using a magnetic stirrer (MR Hei-Tec; Heidolph, Schwabach, Germany) at room temperature (25 °C) to form an organic phase. The

aqueous phase consisting of an emulsifier dissolved in deionized water was also prepared using magnetic stirring. Coarse oil in water emulsion was obtained by mixing the organic phase with the aqueous phase at a ratio of 1:9 by volume in a rotor-stator homogenizer (Silverson L4R, Buckinghamshire, UK) at 5000 rpm for 5 min.

Emulsification in a high-pressure homogenizer

The resulting coarse pre-emulsion was passed through a high-pressure homogenizer (Panda Plus 2000; GEA Niro Soavi, Parma, Italy) for 2 homogenization stages at 300 bar for 2 cycles. The dichloromethane in the fine emulsion was removed by using a rotary evaporator (Eyela NE-1001, Tokya Rikakikai Co. Ltd, Tokyo, Japan) under a reduced pressure of 25 kPa for 20 min at 40 °C and 100 rpm.

Pre-treatment

pH

The effects of pH (pH=2-8) on the characteristics of lycopene nanodispersions were performed using a Zetasizer Nano-ZS instrument (Malvern Instruments Ltd., Worcestershire, UK) coupled with an automatic titrator unit (Autotitrator MPT-2). The titrations were done automatically in a plastic sample container, which was connected through a capillary system and a peristaltic pump with a folded capillary zeta potential cell (DTS 1060, Malvern Instruments Ltd., Worcestershire, UK).

Ionic strength

The effects of ionic strength on the characteristics of lycopene nanodispersion were performed by adding appropriate amounts of 1 M NaCl solutions into the freshly prepared samples to obtain final NaCl concentrations of 0, 100, 200, 300, 400, and 500 mM, respectively. The diluted samples were stored in glass amber bottles at ambient temperature for 24 h prior to analysis. The experiments were carried out under low light conditions.

Temperature

The effects of temperature on the stability of lycopene nanodispersions were studied by incubating the freshly prepared samples stored in amber bottles in a water bath (Waterbath WBU 45, Memmert) set at 30, 40, 60, 80, and 100 °C for 1 h. Then, the samples were kept in a dark place at ambient temperature for 24 h prior to analysis. The experiments were carried out under low light conditions.

In-vitro bioaccessibility

The bioaccessibility of the lycopene nanodispersion stabilized by different emulsifier types was measured by the 2 phase static *in-vitro* model simulating the digestion process in the stomach (gastric phase) and upper part of the small intestine (duodenum) as described by Ha *et al*. (*20*) with minor modifications. The oral phase was not included in the model because it does not have a significant impact on the bioaccessibility of the lycopene nanodispersion, as the main digestive process that occurs in the mouth is starch digestion by amylase, and the lycopene nanodispersions do not contain starch. Furthermore, lycopene is categorized as a lipid compound, and lipid digestion occurs in the stomach and small intestine (*21*). Digestion in the large intestine was not studied because most chemical digestion and nutrient absorption take place in the small intestine (*22*). The bioaccessibility of the prepared lycopene nanodispersion was evaluated by measuring the lycopene concentration released from the food matrix (lycopene in the digesta supernatant) and the lycopene concentration incorporated into micelles.

To simulate the first half of the stomach phase, 5 mL of NaCl/ascorbic acid solution [0.9 % (*m/V*) NaCl and 0.1 % (*m/V*) ascorbic acid] and 5 mL of electrolyte solution (3 g NaCl/L, 1.062 g KCl/L 1.468 g $CaCl_2.2H_2O$/L, 0.470 g KH_2PO_4/L and 0.740 g $MaCl_2.6H_2O$/L) were added to the sample solution (10 mL). The pH was adjusted to pH=4 by adding 1 M HCl. Then, 5 mL of pepsin solution was added to the mixture to prepare the simulated stomach conditions. The second half of the preparation of the simulated stomach conditions included reducing the pH to pH=2 by adding 1 M HCl. The mixtures were incubated in a water bath shaken at 250 rpm for 30 min at 37 °C.

To simulate the small intestinal phase, the pH of the digesta from the previous simulated stomach phase was increased to 6.9 by adding $NaHCO_3$. Then, 3 mL of pancreatin/bile extract solution [0.4 % (*m/V*) pancreatin from porcine pancreas, 2.5 % (*m/V*) bile extract, 0.5 % (*m/V*) pyrogallol, and 1 % (*m/V*) DL-α tocopherol] was added, and the mixture was incubated in a water bath shaken at 250 rpm for 2 h at 37 °C.

To measure the lycopene content released from the food matrix, the *in-vitro* digesta solution was centrifuged (Model 4000 Centrifuge, Kubota Corporation, Japan) at 5000 x g for 15 min. The supernatant was vacuum-filtered, and the lycopene concentration in the supernatant was measured using HPLC. The concentration of lycopene incorporated in the bile salt micelles was measured by extracting the lycopene from the solution of the *in-vitro* digesta as follows: 2 mL of the solution of the *in-vitro* digesta was added to a 4 mL hexane: acetone: ethanol (2:1:1) mixture containing 0.1 % butylated hydroxytoluene (BHT). Hexane (non-polar) was used to dissolve the lycopene, whereas acetone and ethanol (polar solvents) were used to remove water-soluble compounds in the samples and to obtain phase separation. The mixture of hexane, acetone, and ethanol has previously been

used to optimize lycopene extraction, particularly from tomato products (*9*). BHT was added as an antioxidant. NaCl solution (1 mL of 25 % *m/m*) was added, and the mixture was vortexed for 10 min. The added NaCl was responsible for increasing the polarity of the polar phase and improving the separation between the polar and non-polar phases. The mixture was centrifuged at 5000 x g for 10 min, and the organic phase in the upper layer was collected. The lycopene concentration in the organic phase was measured by using HPLC.

The *in-vitro* bioaccessibility was calculated using the following formula:

$$\text{Bioaccessibility (\%)} = [(c_r + c_m) / c_i] \times 100 \quad /1$$

where c_r = concentration of lycopene released from the food matrix (mg/mL), c_m = concentration of lycopene in micelles (mg/mL), and c_i = initial lycopene concentration (mg/mL).

Characterizing the lycopene nanodispersion

Particle size and polydispersity index (PDI)

The particle size and PDI of lycopene nanodispersions were determined by using a dynamic light scattering device (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK). The particle size and PDI were measured at 25 °C under low light conditions. The mean particle diameters are reported as “Z-average” diameters (the scattering intensity-weighted mean diameter) in nm.

Zeta potential

The stability of the nanodispersions was analyzed by determining the net electrical charge using Zetasizer Nano-ZS. One milliliter of freshly prepared lycopene nanodispersion was injected into disposable folded capillary cells (DTS 1060) and placed in the cell holder. The sample was then equilibrated at 25 °C for 120 s before measurement. The analysis was performed under low light conditions.

Determining the lycopene concentration

An aliquot of lycopene nanodispersion (1 mL) was mixed with 2 mL of hexane: acetone (2:1) and vortexed at 1000 rpm for 5 min. Then, the sample was centrifuged at 800 g for 10 min. The hexane upper layer containing lycopene was collected, and the extraction method was repeated once again. The lycopene in the hexane layer was collected and subjected to HPLC analysis.

An aliquot of extracted lycopene nanodispersion (1 mL) was then filtered through a 0.45 µm-pore membrane filter, and 20 µL of the filtrate was injected into an HPLC system [Waters e2695 HPLC separation modules equipped with a Waters 2489 UV-Vis detector (Waters, Milford, MA, USA)]. HPLC analysis was performed with a Waters liquid chromatography system equipped with a Diode Array

Detector and a Nova-Pak C18 (3.9 x 300 mm) Waters HPLC column with an isocratic mobile phase consisting of 15 % (*V/V)* tetrahydrofuran, 30 % (*V/V)* acetonitrile, and 55 % (*V/V)* methanol at 1 mL/min and temperature of 30 °C. Detection was performed at 472 nm. All steps were performed under subdued light.

Statistical analysis

All measurements taken from freshly prepared samples were expressed as mean ± standard deviation (SD) of triplicate determination (n=3). The data were analyzed with SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA) (*23*). Duncan's multiple range test was used to compare significant differences between sample means at a 5 % significance level ($p<0.05$).

RESULTS AND DISCUSSION

Influence of emulsifier type

In this study, the physical and chemical stability of lycopene nanodispersions stabilized by LC, SC, and LC:SC was investigated by evaluating the particle size, PDI, zeta potential, and lycopene concentration of the freshly prepared sample. Based on the results listed in Table 1, all three emulsifiers were capable of producing lycopene nanodispersion at a nano-size range whereby the smallest mean particle size was exhibited by LC (78 nm), followed by LC:SC (91 nm) and SC (159 nm). The LC-stabilized nanodispersion showed the lowest PDI value, indicating the narrowest size distribution. The SC-stabilized nanodispersion on the other hand exhibited the highest PDI value, demonstrating the broadest and less homogenous size distribution. The small particle size and low PDI value in an LC-stabilized nanodispersion were attributed to its small molecular weight and ability to adsorb rapidly onto the droplet surface. LC is a naturally occurring amphiphilic surfactant that is able to absorb at the oil-water interface and reduce the interfacial tension between oil and water. Its hydrophilic head group comprising of PA, PC, PE, PI, or PS attached to the phosphate groups is attracted to water while its hydrophobic tail group consisting of two fatty acids (*24,25*) is attracted to the oil phase. During the homogenization process, LC is absorbed at the oil-water interface rapidly and formed a protective layer that prevents droplets aggregation and coalescence. The hydrophilic head group of lecithin which can be anionic (phosphatidylinositol) or zwitterionic (phosphatidylcholine and phosphatidylethanolamine) also helps to prevent droplets coalescence by providing a negative surface charge to the dispersed droplets keeping the droplets remained small in size.

SC is a high-molecular weight protein emulsifier with good surface activity and is able to unfold, absorb at the oil-water interface, and reduce the interfacial tension between the phases. The emulsifying ability of proteins is influenced by the presence of lipophilic amino acids such as

phenylalanine, leucine, and isoleucine (*26*). During the emulsification process, the lipophilic groups modify their confirmation and penetrate the oil phase, whereas the hydrophilic groups protrude into the aqueous phase. Protein such as SC produces a loop structure that causes steric hindrance to the oil droplets, preventing droplets' flocculation and coalescence (*27*). The presence of ionic functional groups in protein molecules also provides electrostatic stabilization keeping the nanodispersion physically stable. In comparison to LC, SC reduces the interfacial tension between the oil and water phase less efficiently because it needs to undergo a conformational change at the interface while LC, being a small molecular weight emulsifier with a clear partitioning of the hydrophobic and hydrophilic parts would absorb rapidly at the interface (*28*). Therefore, during the emulsification process, the lycopene droplets may have enough time to merge before being fully coated by the SC molecules, resulting in the formation of a single larger entity. Another contributing factor was bridging flocculation, which usually occurs when the SC chains adsorb to multiple droplets via their extended long loops and tails (*29*).

The LC:SC-based nanodispersion showed an intermediate particle size and PDI value, suggesting that both emulsifiers adsorbed at the droplet surface. In the presence of proteins, small molecule surfactants such as phospholipids may interact or compete with the proteins to absorb at the droplet surface (30). During the emulsification process, both emulsifiers were expected to absorb at the surface of the newly created droplets and reduce the interfacial tension between the oil and water phases preventing them from coalescing. The absorption of the LC molecules at the droplet surface would affect the absorption mode of the SC molecules. With a more limited surface area available on the droplet surface, the absorbed SC molecules would protrude into the aqueous phase rather than elastically spreading to cover a maximum area (*31*). This consequently has resulted in steric stabilization, which may prevent the lycopene droplets from aggregating. The LC molecules will also compete with SC to adsorb at the oil-water interface (30) and interact with adsorbed SC to form a strong and densely-packed protective layer around the droplet. The absorption of both emulsifiers on the droplet surface resulted in a combination of electrostatic and steric stabilization, which in turn provided good stability to the emulsion. However, not all the adsorbed protein is displaced by LC (*31*), suggesting that the surface of the droplets was occupied by LC and SC. Small molecule surfactants, such as LC, could also influence the nanodispersion properties by displacing the absorbed biopolymers from the interface. The ability of the LC to displace proteins at the interface was attributable to its small molecular weight, maneuverability, and strong affinity towards the interface (*32*). It has been reported that, during emulsification with protein as the main emulsifier, the presence of even a small quantity of rapidly adsorbing surfactant can facilitate a substantial reduction in the mean droplet size (*33*). This result shows that natural emulsifier such as LC and a combination

of LC and SC can be used to produce lipid nanodispersion with particle size less than 100 nm, comparable to synthetic surfactants such as Tween 80 and sodium dodecyl sulfate (SDS) (*34*). Fig. 1 shows the schematic illustration of the lycopene nanodispersions stabilized by LC, SC, and LC:SC.

The influence of emulsifier types on the stability of lycopene nanodispersion against droplet aggregation was determined by measuring the droplet surface charge (zeta potential). Stable lycopene nanodispersion can be produced by using LC and SC separately or in combination (LC:SC), as all samples showed a zeta potential of more than -30 mV (Table 1). A zeta potential value exceeding ±30 mV indicates good stability of a colloidal system. Stabilizing lycopene nanodispersion with LC resulted in a stable system with a high negative charge (-64 mV). This was due to the presence of negatively-charged phospholipid components, *i.e.*, PI, PA, and lysophosphatides. Moreover, the small particle size of the LC-stabilized nanodispersion resulted in Brownian effects dominating the gravitational force and thus keeping it stable against aggregation. Although the zeta potential value of the SC-stabilized nanodispersion was considerably high, it was the lowest among all samples, presumably due to its tendency to form a network structure. The combination of LC:SC resulted in a significantly lower droplet charge (*i.e*., -48 mV) in comparison with LC alone. The reduced zeta potential was probably due to the depletion flocculation by non-absorbing SC and LC in the continuous phase. In comparison to SC alone (*i.e.* -45 mV), the LC:SC-stabilized nanodispersion had a higher zeta potential value. Fang and Dalgleish (*31*) found that LC can increase the stability of emulsions made from protein.

The lycopene concentration in the nanodispersions was quantified using HPLC analysis. Fig. 2 shows the HPLC chromatograms of lycopene in the lycopene nanodispersions prepared in this study. Quantification of the lycopene concentration using HPLC showed that LC:SC-stabilized nanodispersion had the highest lycopene concentration followed by SC and finally LC (Table 1). It can be postulated that the combination of LC:SC at neutral pH provides better protection to the lycopene against degradation in comparison with the LC and SC alone. The enhanced stability of the LC:SC sample was ascribed to the interaction between LC and SC at the interface, as well as the formation of a strong and thick interfacial layer around the droplets (30) that prevented lycopene from chemical degradation. The interaction between phospholipids such as LC and protein would affect the surface activity, protein structure, and net charge of the emulsion (*35*). The SC-stabilized nanodispersion had a relatively high lycopene concentration. The SC coating layer and extended protein chains from the adsorbed SC at the interface generated a strong steric hindrance that protects the droplets against oxidative degradation (*36*). The high stability of lycopene in the SC and LC:SC-stabilized nanodispersions was also due to the phosphorylated casein peptides that can chelate metal

ions (*37*). The SC has also been reported to exhibit antioxidative properties thus minimizing the lycopene loss in the nanodispersion sample (*34*).

Effect of pH

The effects of pH on the particle size, PDI, and zeta potential of all lycopene nanodispersions are shown in Fig. 3. From Fig. 3a and Fig. 3b, the LC-stabilized nanodispersion was highly stable over a wide pH range (pH=2-8), indicating that the LC layer on the droplet surface was resistant to pH changes and was able to protect the lycopene droplets from aggregating. This is presumably due to the high negative charge carried by PA, PI, and anionic components in LC that makes the droplets repulse each other. Other contributing factors are stabilization via electrostatic and hydration forces by PA and PS, as well as the high content of surface-active impurities other than phospholipids in LC (*38*).

The particle size of SC-stabilized nanodispersion decreased with decreasing pH value from pH=8 to pH=6. However, at pH=5, a sharp increase in the particle size was observed, indicating the occurrence of extensive droplet aggregation. Reducing the pH value close to the isoelectric point of SC (between pH=4-5) resulted in a loss of net charge close to zero. The loss in the surface charge caused the droplets to be attracted to each other because the repulsive force between them was insufficient to overcome the attractive force. When the pH of the nanodispersion was reduced further until pH=2, a significant decrease in the particle size was observed. The reduction in the pH value towards a highly acidic condition resulted in the increased surface charge of SC molecules, thus increasing the repulsive force among the coated droplets, preventing droplets aggregation.

Based on the graph in Fig. 3a and Fig. 3b, the particle size and PDI of LC:SC-stabilized nanodispersion remained small when the pH was reduced from pH=8 to pH=6. At high pH values (pH=6-8), the droplets were highly negatively charged due to the absorption of both SC and LC molecules on the droplet surface. Consequently, the droplets tended to repel each other. In addition, the LC and SC molecules can also interact with each other via attraction interaction between positive patches in SC with negatively-charged LC components and vice versa, thus forming a strong LC-SC complex around the droplet surface that prevents the droplets from aggregating. However, under more acidic conditions (≤ pH=5), the nanodispersion became highly unstable, especially at pH=4 and pH=5, which were near the isoelectric point of SC. When the pH was reduced further to pH=3 and pH=2, a significant size reduction and reduced PDI value were observed. The SC molecules became increasingly positive with increasing acidity. Thus, they were able to interact with negatively-charged LC which led to an increased repulsion force between droplets, thus preventing droplets from aggregating.

The effects of pH on the zeta potential of all nanodispersions are shown in Fig. 3c. Generally, the LC-stabilized nanodispersion was stable over a wide pH range by exhibiting a high negative net charge exceeding -30 mV at every pH value except at pH=2 (-29 mV). The zeta potential value decreased gradually from pH=8 to pH=2. The reduced negative net charge of the nanodispersion was related to the protonation of the phosphate group of the LC molecules (*39*).

Nanodispersion made from SC and LC:SC showed significant changes in droplet charge (*i.e.*, from highly negative to highly positive when the pH was reduced from pH=8 to pH=2) with a similar trend. This result suggests that SC molecules have also been absorbed on the droplet surface and that not all the absorbed casein had been displaced by LC molecules. At pH below the isoelectric point of SC, the zeta potential value of LC:SC was slightly lower than that of the SC-stabilized nanodispersion, indicating lower stability. The presence of non-absorbing SC in the LC:SC-stabilized nanodispersion (which resulted from the competitive absorption of LC molecules and displacement by LC molecules at the droplet surface) may have resulted in depletion flocculation, hence promoting droplets aggregation (40).

Effect of ionic strength

The particle size and PDI of LC and SC nanodispersions were less severely affected by ionic strength in comparison with their combination (LC:SC) (Fig. 4). The particle size of the LC-stabilized nanodispersion was small (82 nm) at low NaCl concentrations (≤100 mM), but increased to 108 nm with further increase in the NaCl concentration. An increase in the NaCl concentration beyond 100 mM resulted in the reduction of droplet surface charge due to the electrostatic screening effect (*41*). The increase in the NaCl concentration caused the ionic strength to increase, resulting in reduced droplet charges, hence causing the droplets to enlarge. The reduced curvature of the phospholipid membranes by salt may also promote droplet aggregation (*42*).

The particle size of SC-stabilized nanodispersion decreased appreciably from 148 nm (without NaCl) to 71 nm (at 100 mM), and it decreased slightly to 66 nm with a further increase in NaCl concentration up to 500 mM. Srinivasan *et al*. (*43*) found that absorption of α-casein at the droplet surface was enhanced by the addition of NaCl solution up to 40 mM; when 200 mM of NaCl was added, reduced droplet flocculation and improved creaming stability were observed. Dalgleish (*44*) reported that the change in the ionic strength would change casein conformation at the interface, which would in turn affect the steric stabilization.

For LC:SC-stabilized nanodispersion, the particle size increased drastically from 91 nm (at 100 mM) to 265 nm (at 200 mM), and it continued to increase up to almost 4 μm (at 500 mM) (Fig. 4a). The same trend was observed in the PDI result, where the value increased drastically up to 1

when the NaCl concentration exceeded 100 mM, indicating a broad and inhomogeneous size distribution. At low NaCl concentration (≤100 mM), the electrostatic repulsive force was strong enough to overcome the attractive forces between droplets, thus keeping them away from one another. However, beyond 100 mM, the loss in the surface charge of the droplets weakened their repulsive force, thus causing them to be attracted to each other.

The increase in the NaCl concentration resulted in decreased zeta potential (Fig. 4c). Starting from 100 mM to 500 mM NaCl, all samples showed zeta potential values of less than -30 mV, indicating poor physical stability. Among all samples, nanodispersion stabilized by LC showed better stability at different NaCl concentrations. This may be due to its smaller particle size and narrower size distribution, thus decreasing the tendency to aggregate.

Effect of temperature

The particle size and PDI of LC and LC:SC-stabilized nanodispersions were remarkably stable over a wide temperature range (Fig. 5). Ozturk *et al*. (*45*) reported that good thermal stability of LC resulted from high electrostatic and steric repulsion. In other previous research, LC was added to milk and combined with other emulsifier types such as whey protein isolate in the emulsion to enhance their thermal stability (*30*). The displacement of SC by LC at the interface, the formation of LC-SC complex, and the increase in the surface charge of casein micelles (*46*) may have contributed to the high thermal stability in LC:SC-stabilized nanodispersion. Additionally, it has been reported that the emulsification activity of a soybean protein-lecithin complex increased after heat treatment (*47*). As for the SC-stabilized nanodispersion, the particle size was stable when heated at 30 °C and 40 °C, but it started to increase gradually with a further increase in temperature. The same trend was observed in the PDI result. The high stability of the SC-stabilized nanodispersion at a moderate temperature under neutral pH was related to the crosslinking between adsorbed SC molecules on the same droplet (*48*).

All samples showed good stability at various heating temperatures (30-100 °C) by exhibiting zeta potential values exceeding -30 mV. Comparing the SC and LC:SC-stabilized nanodispersions, the combination of LC and SC (LC:SC) showed better heat stability than the one stabilized by SC alone. The presence of LC in the emulsion was associated with improved heat stability (*30*).

The stability of the lycopene nanodispersions against thermal degradation was also investigated by observing the color intensity of the nanodispersions (Fig. S1) as chemical degradation of carotenoids can be indicated by color fading (*49*). In Fig. S1, the color of the LC-stabilized nanodispersion started to fade at a lower temperature in comparison with the SC-containing nanodispersion. The color fading became more significant at a higher temperature, indicating that the

LC-coated lycopene droplets were susceptible to degradation, particularly at high temperatures. The degradation of the lycopene in the nanodispersion was also accelerated by its small particle size. The SC-lycopene nanodispersion, showed higher color intensity than the LC-stabilized nanodispersion at all the temperature points, indicating better lycopene stability against degradation. This was attributed to the thick stabilizing layer of SC at the droplet surface, which hindered the lycopene from degradation. The LC:SC-stabilized nanodispersion was the most stable among all samples. The absorption of both LC and SC molecules at the same droplet surface resulted in a combination of electrostatic and steric stabilization, thus providing good protection to the lycopene droplets from the chemical degradation.

In-vitro bioaccessibility of lycopene nanodispersion

The influence of particle size and emulsifier type on the bioaccessibility of the lycopene nanodispersion was investigated. The particle size of the lycopene nanodispersion was examined just before the samples were subjected to *in-vitro* bioaccessibility analysis. Based on the results of particle size in Table 2, the particle size of the lycopene nanodispersions made from different emulsifier types increased in the following order: LC < LC:SC < SC.

For bioaccessibility, the LC-stabilized nanodispersion was less bioaccessible than the LC:SC and SC counterparts although its initial particle size was smaller (77 nm). Theoretically, smaller particles are more bioaccessible due to the increased surface area that is available for enzyme adsorption. However, an apparent aggregation was observed in the digesta containing the LC-stabilized nanodispersion when the pH was increased to pH=4 during the first half of gastric digestion. The aggregation decreased significantly when the pH of the digesta was reduced to pH=2 in the second half of the gastric digestion and then increased when pH=6.9 in the small intestinal digestion. The changes in the particle size, and structure of the nanodispersion might have reduced its bioaccessibility. The changes in the pH of the digesta will alter the electrical charge of the emulsifier used to coat the lipid and consequently will cause the structure, interaction, and susceptibility to aggregation to change (*50,51*). Adjusting the pH digesta to pH=4 might have reduced the zeta potential of the LC-stabilized nanodispersion, thus promoting droplet flocculation by reducing the repulsion force between droplets. Aggregation may have prevented the lycopene droplets from being easily accessed by the enzymes, leaving the lycopene droplets undigested or minimally digested.

Based on visual observation, no significant aggregation was observed in the digesta containing the LC:SC- or SC-stabilized nanodispersion. Therefore, the initial particle size value of these samples was considered when justifying the bioaccessibility. The lycopene nanodispersion made from LC:SC showed the greatest bioaccessibility. This result was ascribed to its smaller particle

size. As previously noted, a lycopene nanodispersion with a small particle size provides more surface area for enzyme adsorption, thus enhancing enzyme access to the lycopene droplet or accelerating any chemical reactions occurring at the oil-water interface (*50*). Smaller particle size has been associated with higher solubility, higher micellization rates (increased transfer rate into the bile salt micelles) (*52*), and higher diffusivity rates (enhanced release from the food matrix). Kipp (*53*) claimed that a significant improvement in the bioavailability of a nanoparticle can be expected when the size of the particle is below 100 nm. Additionally, the bioaccessibility of the lycopene nanodispersion was also influenced by the LC and SC molecules at the droplet surface. In the gastric digestion phase, the SC molecules might have been extensively digested by pepsin, while the LC molecules left at the lycopene droplet surface may protect lycopene from chemical degradation and aggregation (via electrostatic repulsion). In the subsequent small intestinal phase, the lycopene nanodispersion was continually digested by pancreatin in the presence of bile salts. The SC molecules were preferentially digested by proteases, and LC by phospholipase. This consequently facilitated the release of lycopene into the digesta and increased the amount of lycopene incorporated into the bile salt micelles.

The lower bioaccessibility of the SC-stabilized nanodispersion compared with the LC:SC-stabilized nanodispersion was particularly related to its larger particle size. Although the SC molecules at the droplet surface might have been degraded extensively by pepsin and proteases, the digestion process might have been less effective due to the smaller surface area available for adsorption.

CONCLUSIONS

This study showed that lycopene nanodispersion with nano-sized particles ($d<200$ nm), good distribution (PDI<1), and physically stable (zeta potential ≥30 mV) can be produced from LC and SC, either as the sole stabilizer or in combination (LC:SC) in the proportion of 1:1, by using the emulsification-evaporation method. Under neutral pH conditions, the nanodispersion stabilized by LC was the most physically stable with the smallest particle size, the lowest PDI, and the highest zeta potential value, but it was the least stable against lycopene degradation. To overcome the lycopene degradation issue in the LC-based nanodispersion, combining LC with SC at the proportion of 1:1 was not only able to produce lycopene nanodispersion with small particle size but also enhanced the lycopene stability against degradation. Under different environmental conditions, the LC-stabilized nanodispersion was not appreciably affected by the changes in pH, ionic strength, and temperature enhancing its potential to be used in various food systems at wide pH, ionic strength, and temperature ranges. Although combining the LC with SC was able to produce stable lycopene nanodispersion, its application in the food systems with high ionic strength may cause physical destabilization leading to

droplet coalescence. The particle size of SC-stabilized nanodispersion on the other hand reduced with increasing ionic strength but changing the pH of the nanodispersion near the isoelectric point of SC (pH=4-5) and heating above 60 °C caused the particle size to increase. The presence of SC seemed to contribute to the physical instability of LC:SC-stabilized nanodispersion at pH=4-5 but enhanced the in vitro bioaccessibility of the lycopene nanodispersion. The lycopene nanodispersion bioaccessibility was not only affected by the particle size but also the emulsifier types used to prepare the samples. The in vitro bioaccessibility of lycopene nanodispersion increased in the following order: LC > SC > LC:SC. The information obtained from this study not only provides a better understanding of lycopene nanodispersion physicochemical properties, stability, and bioaccessibility in response to various factors but also the potential emulsifiers for fabricating a delivery system for poor water soluble functional lipids.

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CONFLICT OF INTEREST

Declarations of interests: none

SUPPLEMENTARY MATERIALS

All supplementary materials are available at: www.ftb.com.hr.

AUTHOR'S CONTRIBUTION

Nor Shariffa Yussof designed the study, performed experiments, collected data, drafted and edited the manuscript. Tan Chin Ping, Tan Tai Boon, Uthumporn Utra, and Muhammad Ezzudin contributed to the critical revisions and improvement of the final manuscript.

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Table 1. Particle size, PDI, zeta potential and lycopene concentration of lycopene nanodispersion

Sample	*d*(particle)/nm	PDI	*ζ*/mV	*γ*(lycopene)/(mg/100 mL)
LC	(78.0±0.9)^{a}	(0.180±0.008)^{a}	(-64.0±0.2)^{a}	(1.826±0.096)^{c}
SC	(159.0±1.2)^{b}	(0.411±0.010)^{c}	(-45.0±0.1)^{c}	(2.510±0.023)^{b}
LC:SC	(91.0±0.5)^{c}	(0.315±0.034)^{b}	(-48.0±0.2)^{b}	(2.656±0.051)^{a}

The results are expressed as mean value ± S.D. (N=3). $^{a-c}$ Different letters show statistically significant differences between acceptable result quality of responses ($p<0.05$) in each column

Table 2. Particle size and *in vitro* bioaccessibility of lycopene nanodispersion

Sample	*d*(particle)/nm	*γ*(lycopene)/(mg/100 mL)			Bioaccessibility/%
		Initial sample	In food matrix	In micelles	
LC	(77.0±1.2)^{a}	(4.56±0.26)^{c}	(0.28±0.06)^{a}	(0.72±0.01)^{c}	(21.92±0.29)^{c}
SC	(156.1±2.4)^{b}	(8.17±0.18)^{a}	(0.25±0.01)^{a}	(1.77±0.01)^{b}	(24.73±0.30)^{b}
LC:SC	(95.0±0.9)^{c}	(6.96±0.38)^{b}	(0.26±0.09)^{a}	(1.93±0.02)^{a}	(31.47±0.14)^{a}

The results are expressed as mean value ± S.D. (N=3). $^{a-c}$ Different letters show statistically significant differences between acceptable result quality of responses ($p<0.05$) in each column

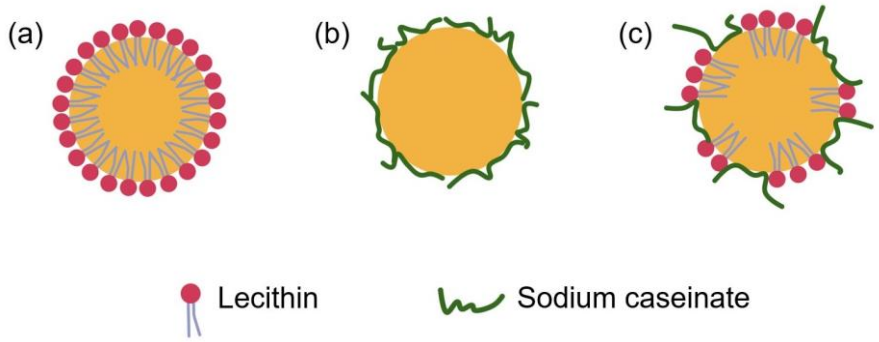

Fig. 1. The schematic illustration of lycopene nanodispersion stabilized by (a) soy lecithin (LC), (b) sodium caseinate (SC), and (c) soy lecithin and sodium caseinate (LC:SC)

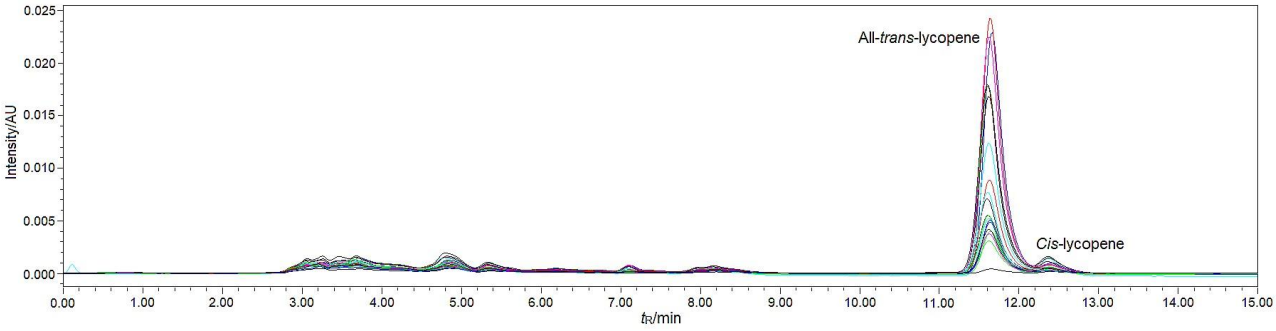

Fig. 2. The HPLC chromatogram of lycopene in lycopene nanodispersion produced using emulsification-evaporation technique

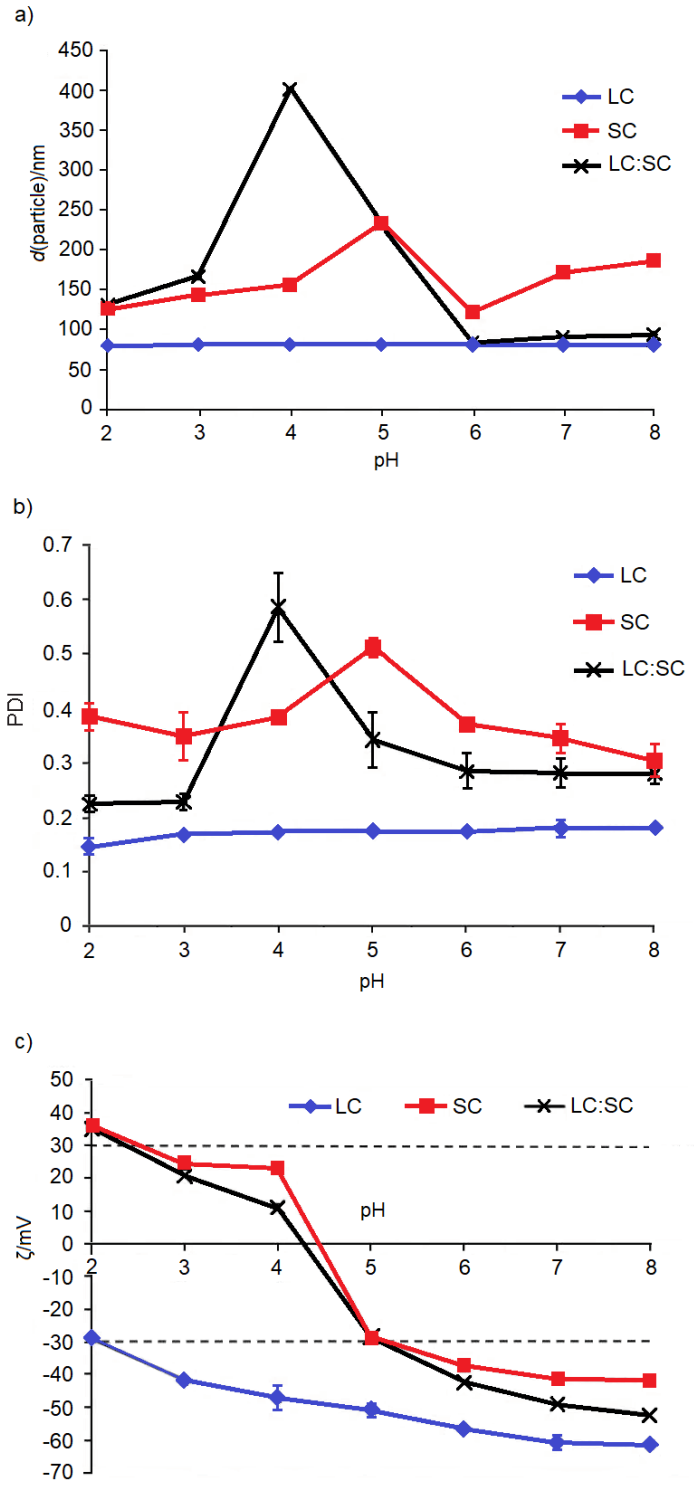

Fig. 3. The effects of pH on the (a) particle size, (b) polydispersity index (PDI) and (c) zeta potential of lycopene nanodispersion stabilized by soy lecithin (LC), sodium caseinate (SC), and soy lecithin and sodium caseinate (LC:SC)

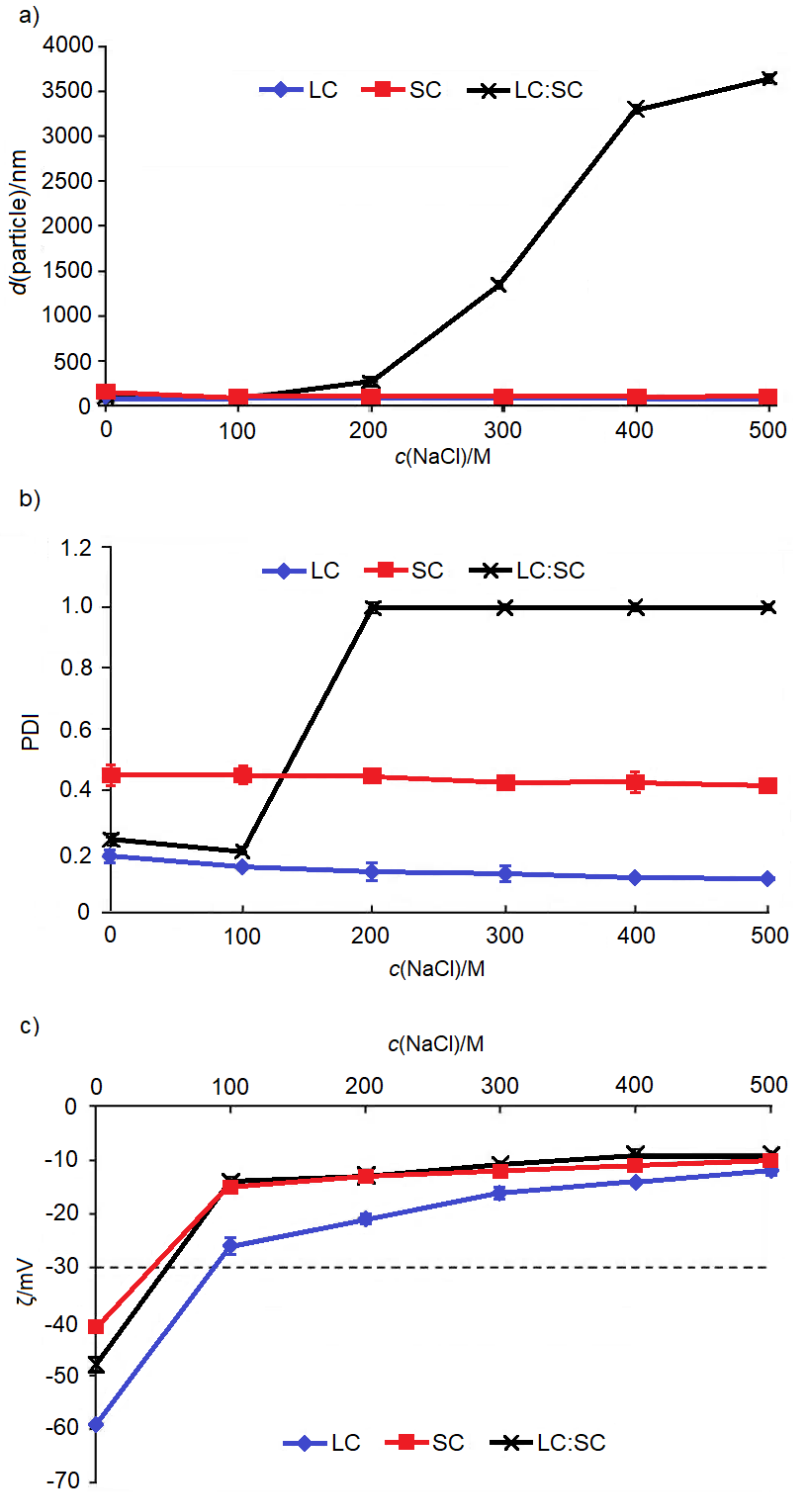

Fig. 4. The effect of ionic strength on the (a) particle size, (b) polydispersity index (PDI) and (c) zeta potential of lycopene nanodispersion stabilized by soy lecithin (LC), sodium caseinate (SC), and soy lecithin and sodium caseinate (LC:SC)

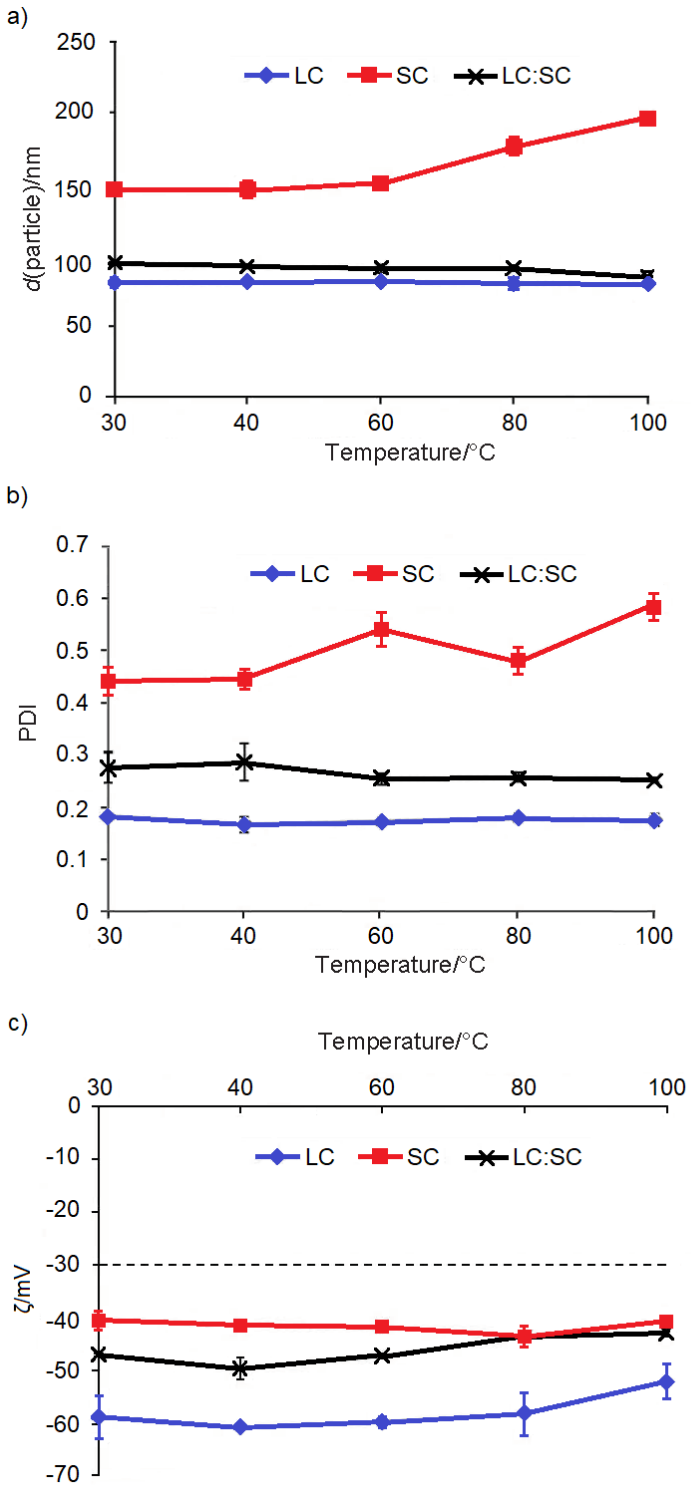

Fig. 5. The effect of temperature on the (a) particle size, (b) polydispersity index (PDI) and (c) zeta potential of lycopene nanodispersion stabilized by soy lecithin (LC) sodium caseinate (SC), and lecithin and sodium caseinate (LC:SC)

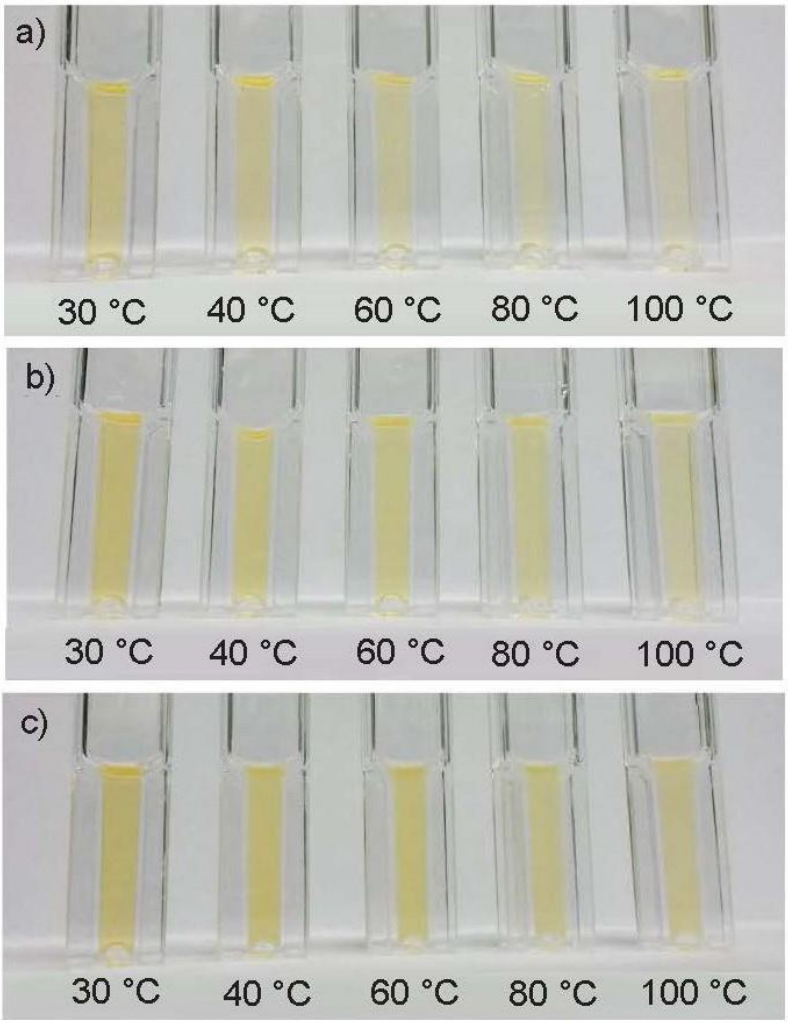

Fig. S1. Stability of lycopene nanodispersion stabilized by (a) soy lecithin (LC), (b) sodium caseinate (SC), and (c) soy lecithin and sodium caseinate (LC:SC), incubated at different temperatures for 1 h

GRAPHICAL ABSTRACT