Impact of Chitosan-Capric Acid Nanogels Incorporating Thyme Essential Oil on Stability of Pomegranate Seed Oil-in-Water Pickering Emulsion

Shahbazi, Nayer; Rajaei, Ahmad*[+]

Department of Food Science, School of Agricultural Engineering, Shahrood University of Technology, Shahrood, I.R. IRAN

Tabatabaei, Meisam*[+•]

Microbial Biotechnology Department, Agricultural Biotechnology Institute of Iran (ABRII), Agricultural Research, Education, and Extension Organization (AREEO), 31535-1897 Karaj, I.R. IRAN

Mohsenifar, Afshin[••]

Nanosystems Research Team (NRTeam), Karaj, I.R. IRAN

Bodaghi, Hojatollah

Department of Horticulture and Plant Protection, School of Agricultural Engineering, Shahrood University of Technology, Shahrood, I.R. IRAN

ABSTRACT: *The aim of this study was to obtain a stable Pomegranate Seed Oil (PSO)-in-water Pickering emulsion stabilized by chitosan (CS)-capric acid (CA) nanogels incorporating Thyme Essential Oil (TEO). Firstly, CS-CA nanogels were synthesized at different ratios of CA to CS (0.25:1, 0.5:1, and 0.75:1). Scanning electron microscopy images showed that by increasing the CA to CS ratio, the uniformity of particles was increased. In the following, CS-CA nanogels were used to stabilize PSO-in-water emulsions. The findings revealed that the most stable emulsion was obtained at pH 8, CA-to-CS ratio of 0.5:1, and oil-to-nanogel ratio of 10:1. In addition, the interfacial structure of emulsion droplets indicated that the CS-CA nanogels contributed to the stability of emulsion through both the formation of an interface layer and a network on the surface of dispersed droplets. Finally, the oxidative stability and microstructure of the emulsions stabilized by CS-CA nanogels incorporating TEO (0.1%) were evaluated. The results showed that TEO increased the oxidative stability of the emulsion and reduced the emulsion droplet size.*

KEYWORDS: *Chitosan-capric acid nanogels; Pomegranate seed oil; Pickering emulsion; Thyme essential oil; Oxidative stability.*

***** *To whom correspondence should be addressed.*
+ *E-mail*: *ahmadrajaei@gmail.com & meisam_tab@yahoo.com ; meisam_tabatabaei@abrii.ac.ir*
● *Other Address: Nanosystems Research Team (NRTeam), Karaj, I.R. IRAN*
●● *Other Address: Research & Development Department of Nanozino, Tehran, I.R. IRAN*
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INTRODUCTION

Nowadays there is a dramatic increase in the use of bioactive compounds in the food, pharmaceutical, and cosmetics industries [1,2]. Pomegranate Seed Oil (PSO) is an attractive source of bioactive compounds such as punicic acid and ortho-diphenols. In various studies, punicic acid has shown many outstanding functions associated with human health, such as anti-carcinogenic, anti-diabetes, anti-hyperlipidemia, anti-inflammatory, anti-cancer, and anti-atherosclerotic properties [3,4]. The use of PSO as a whole in the formulations of food, pharmaceutical, and cosmetic products possesses some limitations due to the lipophilic nature of the oil impeding its simple and direct dispersion into a polar phase. In addition to that, the chemically unstable nature of the oil makes it very susceptible to oxidative degradation. Encapsulation has great potential for overcoming the challenges associated with using PSO [5].

One of the common methods for encapsulation and delivery of bioactive compounds like Poly Unsaturated Fatty Acids (PUFAs) in the food and pharmaceutical industries is the use of emulsions. Emulsions, either Oil-in-Water (O/W) or Water-in-Oil (W/O), can be produced by different approaches including adding surfactants to decrease interfacial tension, hydrocolloids to form steric interfacial films, and surface-active colloidal particles to create physical barriers [6]. Pickering Emulsions (PEs), which are stabilized by colloidal particles, are recognized as a kind of emulsion with long-term stability [7].

Chitosan (CS), which is the deacetylated form of chitin, is a linear polysaccharide including D-glucosamine and N-acetyl-D-glucosamine. In previous research, CS has been used as an edible coating [8], for encapsulating bioactive compounds [9,10], a drug delivery [11,12], in nanocomposite [13–15], for removing reactive blue dye [16], and in stabilizing Pikering emulsions [7,17,18]. The use of CS in PEs can be a new possibility for the development of bio-based Pickering emulsifiers. CS particles have been used in stabilizing emulsions, but CS is not a good surfactant for preparing the emulsions due to its high hydrophilic properties, therefore it requires surface modifications to improve its surfactant property [19]. Previous research has shown that modifying the structure of CS can increase the emulsifying property of CS [7,17,20,21].

Most studies about essential oils have been performed on their antioxidant, antimicrobial and anti-cancer properties [22-25]. However, previous research has shown that essential oils can interact with other compounds and alter their functional properties [26-29]. For example, cinnamaldehyde, the known volatile compound in some essential oils, in combination with CS, was able to increase the emulsifying property of CS [26,28]. The essential oil of thyme with the scientific name of *Thymis vulgaris* is one of the well-known natural antioxidants [30].

Therefore, the present study was first aimed at generating CS nanogels through interaction between the existing amino groups of CS, and the carboxyl group of Capric Acid (CA) as a new modifier, to improve the emulsifier property of CS. In the following, the impacts of different parameters, i.e., pH, CA-to-CS ratio, and oil-to-nanogel ratio on the stability of the PSO-in-water Pickering emulsion were studied. Finally, given the benefits of adding natural, plant-based antioxidants to lipids to prevent lipid oxidation [31], the microstructure and oxidative stability of encapsulated PSO *via* either shell or core antioxidant (Thyme essential oil) were compared.

EXPERIMENTAL SECTION

Materials

CS (90% deacetylated, 50-190 KDa), Nile red and fluorescein isothiocyanate (FITC) from Sigma (Germany), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) from Fluka (USA), methanol, acetic acid, NaOH, CA, ethanol, 1,1,3,3-tetraethoxypropane, ammonium thiocyanate and Iron (II) from Merck (Germany) were obtained. Furthermore, Thyme essential oil (TEO), which its main components were thymol (46.3%), p-cymene (18.4%), and γ-terpinene (16.9), was obtained from Barij Essence Co. (Iran). Moreover, PSO with 1.3 (meq/kg) peroxide value was purchased from Nayeri Co. (Iran). Ultra-pure water was also used throughout the study.

Preparation and characterization of CS-CA nanogels

CS-CA nanogels were synthesized by creating amide bonds between CA and CS *via* an EDC-mediated reaction [7]. Initially, 250, 500, and 750 mg CA dissolved in a certain amount of ethanol and mixed with EDC at a constant ratio. Then, the different mixtures were added to CS solution prepared by dissolving 1 g of CS in 100 mL of acetic acid (1% v/v). After that, the mixtures were stirred for 24 hours in a dark environment. In the following, pH of solutions was adjusted to 8.5–9 using sodium hydroxide (1 M) and sonicated for 5 min to prepare the nanogels. The mixtures

were then centrifuged to separate the nanogels and washed several times with ethanol and distilled water to remove unreacted materials and EDC. The nanogel solutions were finally filtered by a filter with a 0.2 μm mesh.

Subsequently, to study the chemical structure of CS-CA nanogels, Fourier Transformation InfraRed (FT-IR) spectrums of pure CS and CA as well as different CS-CA nanogels, were obtained using an FTIR-430 (Jascow, Japan) at the range of 500-4000 cm^{-1}. Before the analysis, the dried nanogels, CS and CA were mixed with 100 mg potassium bromide.

In addition, to study the size and morphology of CS-CA nanogels, a scanning electron microscope (SEM) (Philips: XL30) was used.

Preparation *of PEs*

Different PEs were prepared by mixing nanogels and PSO based on one factor at a time technique through which different parameters, i.e., pH, CA-to-CS ratio, and oil-to-nanogel ratio were taken into account. Upon preparation, mixtures were homogenized at 6000 rpm (IKA T10 basic, IKA Werke GmbH and Co., Germany) for 2 min at room temperature in a glass vial to prepare the PEs.

Initially to study the influence of pH, PEs were made at different pH (i.e., 2, 5, 8, and 10), at a fixed oil-to-nanogel ratio of 10:1 (5% O/W emulsion), and CA-to-CS ratio of 0.5:1. In the following, PEs were prepared at different CA-to-CS ratios (i.e., 0:1, 0.25:1, 0.5:1, and 0.75:1), at a fixed oil-to-nanogel ratio of 10:1, and at the best pH in terms of both stability and droplet size as determined in the prior step. Finally, PEs were made at different oil-to-nanogel ratios (i.e., 5:1, 10:1, 20:1, and 40:1), at the best pH, and the best CA-to-CS ratio. Furthermore, the drop test was used to verify the O/W emulsion [32].

Creaming index and microstructure of PEs

After forming PEs, the height of the cream layer (Hc), and the total height of the formulation (Ht) (after 3 h and 7 d) were recorded. The creaming index was reported as (Hc/Ht) × 100 [33].

To study the microstructure of PEs, a GX optical microscope (Australia) equipped with a CCD camera (CM TCAM3) was used. For a precise analysis, a drop of different emulsions was gently deposited at a the water-air interface on a microscope slide. Following that, image J1.46 software was applied to measure the oil droplet size.

Study of interfacial structure

To investigate the interfacial structure of oil droplets in PEs, a Confocal Laser Scanning Microscope (CLSM) (Leica TCS SPE, Leica Microsystems Inc., Heidelberg, Germany) was applied. Before preparing the emulsion, FITC-labeled CS-CA (FITC-CS-CA) was first produced [34]. Then, the PE was produced by using FITC-CS-CA and Nile red-stained PSO. Finally, the dyed PE was deposited on a concave confocal microscope slide and gently covered with a coverslip. The argon lasers at 488 nm and at 532 nm were used to excite the fluorescent dyes of FITC and Nile Red, respectively.

Oxidation stability

Regards the results of previous steps, the best PE in terms of both the creaming index and the droplet size was applied to evaluate the oxidative stability of BSO along with TEO in both core and shell mode. The following PEs were prepared:

i. PSO-in-water emulsion with 0.1% (w/w) TEO added in the core of encapsulated PSO; before adding PSO to the emulsion, the TEO added to the oil.

ii. PSO-in-water emulsion with 0.1% (w/w) TEO added in the shell of encapsulated PSO. TEO was encapsulated in CS-CA nanogel [35] before preparing the emulsion.

Subsequently, 10 mL from the different emulsions was poured into the sealed screw-cap glass tubes and reserved in an oven for 22 d at 35 °C. Following that, an appropriate amount of emulsions was periodically taken out and the hydroperoxides produced in the samples were measured as described in the literature [36]. In brief, first, the samples were added to 9.8 mL chloroform: methanol (7:3, v/v), and mixed for 5 s on a vortex mixer. Then, ammonium thiocyanate solution (50μL) was added to the mixtures. In the following, 50μL iron (II) solution was added. Finally, the absorbance of the samples was measured at 500 nm against a blank by a spectrophotometer (Unico, UV2150, USA) after 5 min storage. It is necessary to mention that the number of lipid hydroperoxides was calculated by using the standard curve of cumene hydroperoxide.

Statistical analysis

Microsoft Excel (2013) software was used to measure the means and Standard Deviations (SD). Moreover, to compare the differences among the means, Duncan's multiple range tests were used at the level of 0.05 by using the SPSS 16.0 software. All the tests also were carried out in triplicate unless otherwise is stated.

RESULTS AND DISCUSSION

Characteristics of CS–CA nanogels

In this study, CS-CA nanogels were prepared by creating amide bonds, which was verified by FT-IR, between CS and CA (CA-to-CS ratios of 0:1, 0.25:1, 0.5:1, and 0.75:1) through EDC. In Fig. 1, FT-IR spectrums of CA, CS, and CS-CA nanogels are shown. According to CA spectrum shown in Fig. 1, the C-H stretching vibrations produce the peaks at 2918 and 2849 cm^{-1} region. In addition, the peaks at 936, 1261, 1467, and 1710 cm^{-1} regions were due to O-H bending vibrations, C-O stretching vibrations, C-H bending vibrations, and C=O stretching vibrations, respectively. Moreover, the peak was recorded at 687 cm^{-1} is that of C-H bending vibrations. These peaks verified the structure of CA and the existence of carboxylic acid groups.

In Fig. 1, (CS) presents the spectrum of the CS (CA-to-CS ratio of 0:1), the peak observed at 3440 cm^{-1} is related to the partial overlap of –OH, and N–H stretching vibrations. In addition, C=O stretching vibrations in amide linkages, i.e., acetylated amine produced a peak at 1653 cm^{-1} region. Besides, the observed peak at 2873 cm^{-1} is related to the stretching vibrations of methyl and methylene groups. Moreover, the peaks at 1159 cm^{-1} and 1071 cm^{-1} relevant to anti-symmetric stretching of C-O-C, and stretching vibration of C-O respectively, are integral parts of CS.

According to the spectrums of CS-CA nanogels shown in Fig. 1, there is a peak at 1630 cm^{-1} in all of the spectrums that can be associated to both amide groups belong to acetylated amines, and new amide connections due to the reaction between carboxylic acid groups of CA and amine groups of CS. Moreover, a peak is seen at 1528 cm^{-1} in all of the spectrums relevant to NH vibration in the amide II group, which can be due to the reaction between CS and CA. In addition, by comparing the nanogel spectrums with

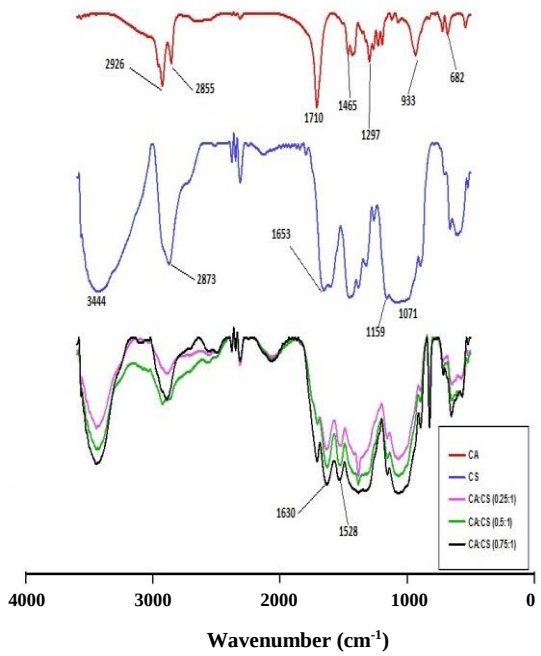

Fig. 1: Fourier Transform InfraRed (FT-IR) spectroscopy analysis obtained for CA, CS and nanogel with different CA-to-CS ratio.

each other, it can be seen that increasing the ratio of CA to CS from 0.25 to 0.75 led to increasing intensity of peak at the 1630 cm^{-1} and 1528 cm^{-1}, which can be due to an increase in amide bonds on CS. These results indicate that the amide connections between CS and CA were well-formed. In addition, there is a peak at 1706 cm^{-1}, which is observed also in the spectrum of CA (1710 cm^{-1}) associated with the C=O stretching vibrations, in the spectrums of nanogels. The observed peak at 1706 cm^{-1} could be due to the electrostatic interactions between carboxylic acid groups of CA and amine groups of CS. In a previous study, similar results were obtained from the spectrums of CS-stearic acid nanogels and CS-myristic acid nanogels [7,17].

In Fig. 2, the SEM images of CS-CA nanogels are shown. Accordingly, it can be stated that with increasing the ratio of CA-to-CS, the nanogels formed better. As presented in Fig. 2D, CS-CA nanogels at ratio of 0.75:1 were better in terms of particle homogeneity compared to the other ratios. This result can be interpreted as higher ratios of CA

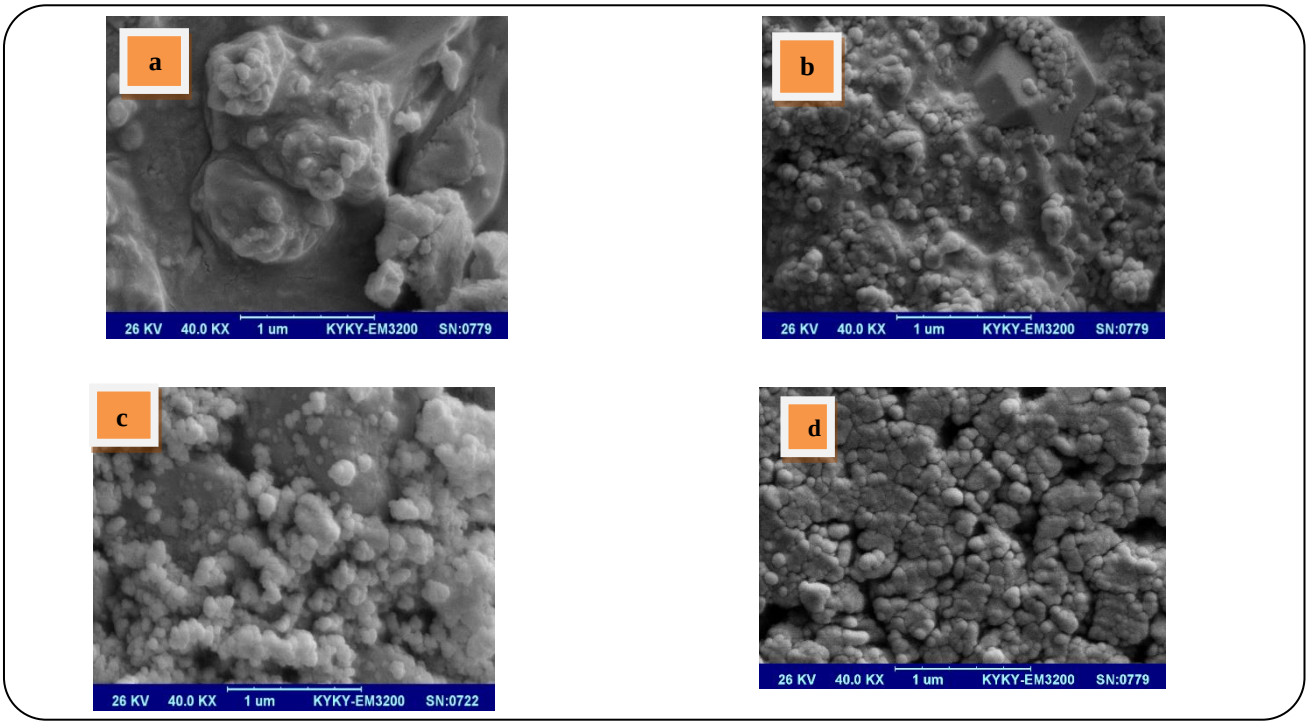

Fig. 2: Scanning electron microscopy (SEM) micrograph of CS (a) and CS–CA nanogels with different CA-to-CS ratio of 0.25:1 (b), 0.5:1 (c), and 0.75:1(d).

cause more hydrophobicity, which results in better accumulation and better particle formation. These results were consistent with the results of former works on the modifying of CS structure with stearic acid and myristic acid [7,17].

Stability of CS-CA nanogels-stabilized Pickering emulsions

Fig. 3 shows the creaming index (after 7 d of storage) of the PEs with different pH (2-10), CA-to-CS ratios (0-0.75), and oil-to-nanogel ratios (5-40). According to Fig. 3a, the most instability was observed in PEs prepared at pH values of 10 and 2, while the least instability was found in PEs prepared at pH values 5 and 8. This result revealed that very acidic and very alkaline pH values could lead to instability of PSO-in-water emulsions. Based on the data presented (Fig. 3b), the most instability was observed in the PE stabilized by CA-to-CS nanogels at the ratio of 0:1. This outcome confirmed previous findings [7,20], proving that CS is a weak emulsifier due to its high hydrophilic properties. As can be understood from Fig. 3c, by increasing the proportion of oil to the nanogel, the creaming index increased.

Influence of pH, CA-to-CS ratio, and oil-to-naogel ratio on microstructures of PEs

Fig. 4 provides the results of the emulsion droplet size at different pHs. Looking at Fig. 4, the largest oil droplets were obtained in PE at pH 2 after 3 h of storage. After 3 h of storage, there was no significant difference ($p>0.05$) among PEs at pH 5, 8, and 10 in terms of droplet size. On the 7th day, PE at pH 10 had the largest oil droplets. Moreover, on the 7th day, the smallest oil droplets were observed in the emulsion at pH 8. These results suggest that CS-CA nanogels in a slightly alkaline environment can have a better effect on the stability of PSO-in-water emulsion. This result can be interpreted that CS is a cationic polysaccharide; therefore the positive charges of CS being neutralized in the neutral and alkaline environment, so that CS chains can better cover the surface of the oil droplets [37]. In addition, the results of the present study showed that with decreasing pH, the particle size and creaming index increased, which is probably due to the higher solubility of CS and consequently thinner CS coating around oil droplets. These results are in line with the findings of previous studies [7,37-39].

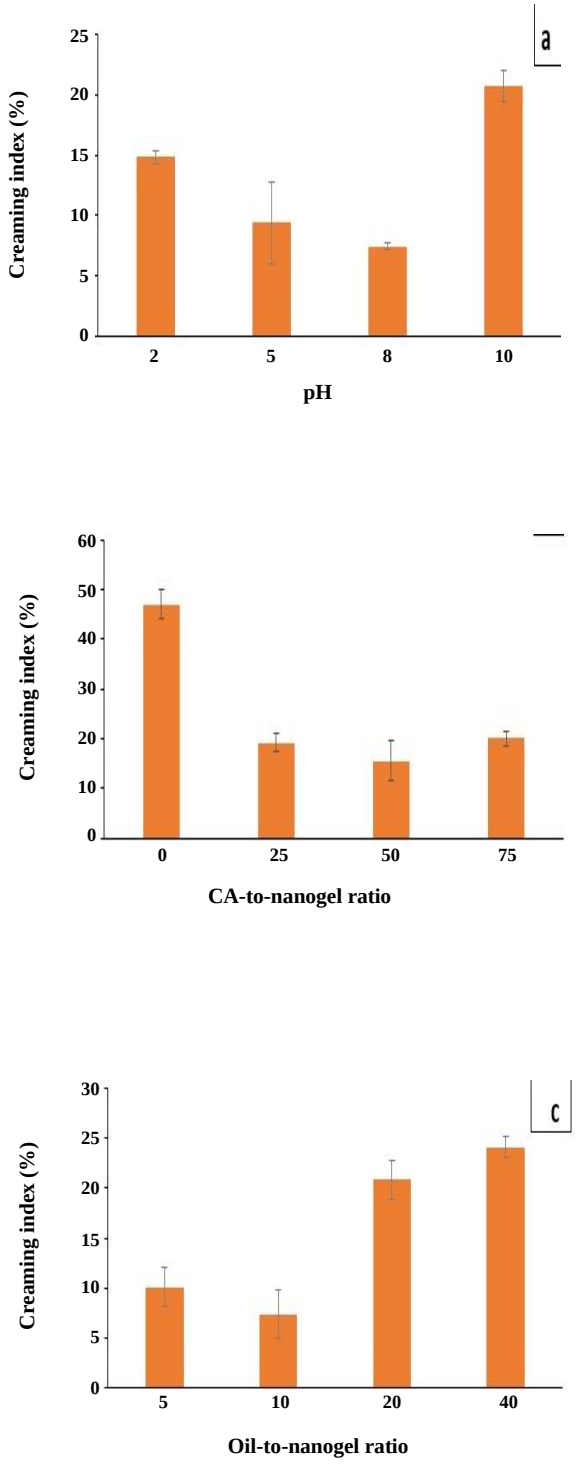

Fig. 3: Creaming index of the Pickering emulsions stabilized by CS-CA nanogels at different pH values (a), CA-to-CS ratios (b), and oil-to-nanogel ratios (c) after 3 h and 7 d of storage.

The results of PE droplet size at different CA ratios are shown in Fig. 5. According to this data, after 3 h, PE stabilized with nanogels at CA ratio of 0.5 had the lowest and PE stabilized with nanogels at the ratio of 0 had the largest droplet size. Moreover, after 7 days of storage, PE stabilized with nanogels at CA ratio of 0.75 had the smallest droplet size. These results showed that at higher ratios of CA, more stable emulsions were attained. Such a result can be associated to the decrease of positive charges and the increased hydrophobicity of CS because of the increase of the amide linkages in response to the increasing CA content. The results of previous studies [7,20,21] also confirm the results of the present study.

The results of droplet size related to PEs at different PSO-to-nanogel ratios are shown in Fig. 6. After 3 h, PE with a PSO-to-nanogel ratio of 10:1 had the lowest average droplet size, although no significant difference ($p>0.05$) was observed between the average droplet size of PEs at PSO-to-nanogel ratios of 10:1 and 5:1. In addition, PE prepared at PSO-to-nanogel ratio of 40:1 had the highest average droplet size. On the seventh day, PE with PSO-to-nanogel ratio of 10:1 had the lowest average droplet size, although there was no significant difference ($p>0.05$) between ratios of 10:1 and 5:1 that this finding was similar to that of the 3th h. These results show that by increasing PSO fraction in PEs, CS-CA nanogel was not adequate to coat the oil droplet surfaces which was also reported by the other works [7,40,41].

Interfacial structure

Since interfacial properties such as composition, thickness, and integrality can partly determine physical stability, CLSM was used to examine the interfacial structure of PE (pH 8, CA-to-CS ratio of 0.5:1, and oil-to-nanogel ratio of 10:1) that the images obtained from CLSM are shown in Fig. 7.

The green (Fig. 7A) and red (Fig. 7B) fluorescence fields represented nanogels stained with FITC and oil phase stained with Nile red, respectively. In addition, Fig. 7C shows overlay fluorescence images of 7A and 7B. From Fig. 7C, the oil phase was in the inside of the droplets, whereas the nanogels made a packed layer at the boundary of droplets. Moreover, Figs. 7A1, 7B1, and 7C1 show the higher magnification CLSM photos of a single droplet. According to the CLSM images, it can be seen that the CS-CA nanogels formed an interface layer on the

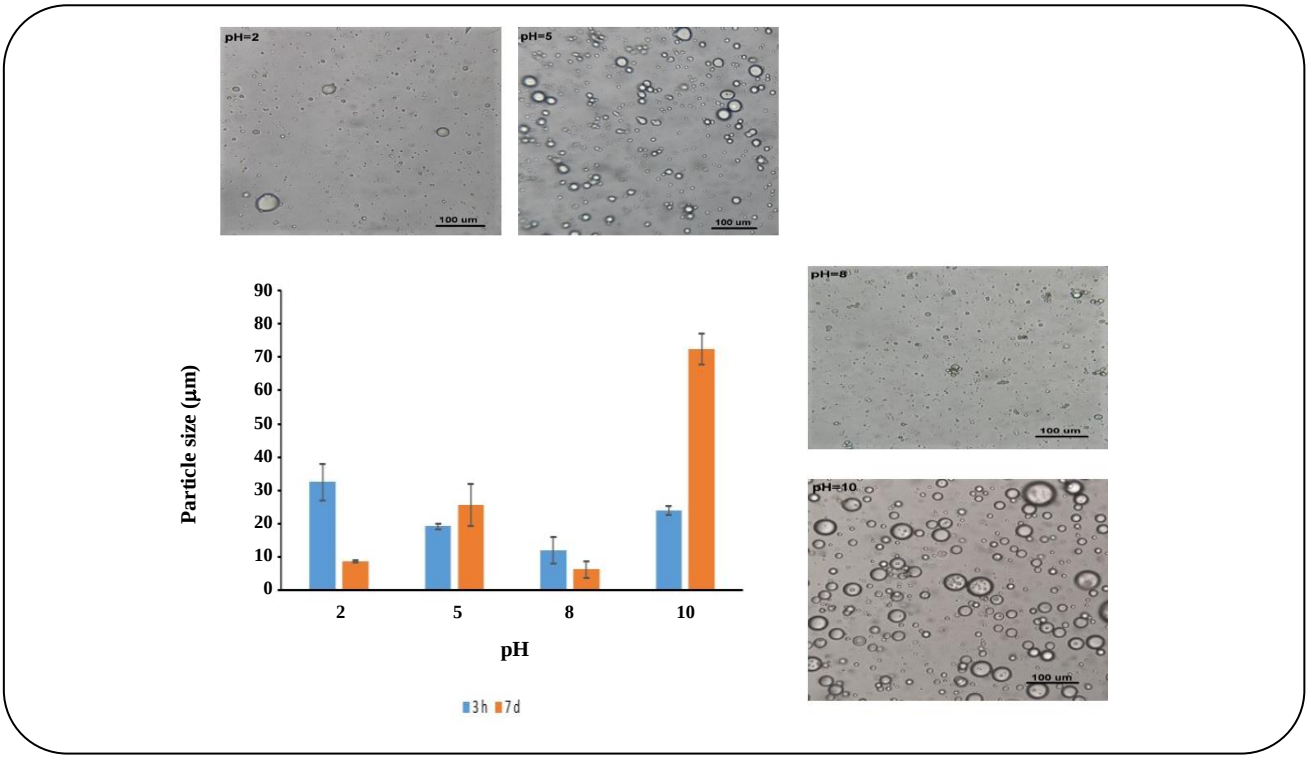

Fig. 4: Emulsion droplet size (after 3h and 7d) and optical microscopic images (after 7d) of the Pickering emulsions stabilized by CS-CA nanogels with different pH (2-10) at the fixed oil-to-nanogel ratio of 10:1 and CA-to-CS ratio of 0.5.

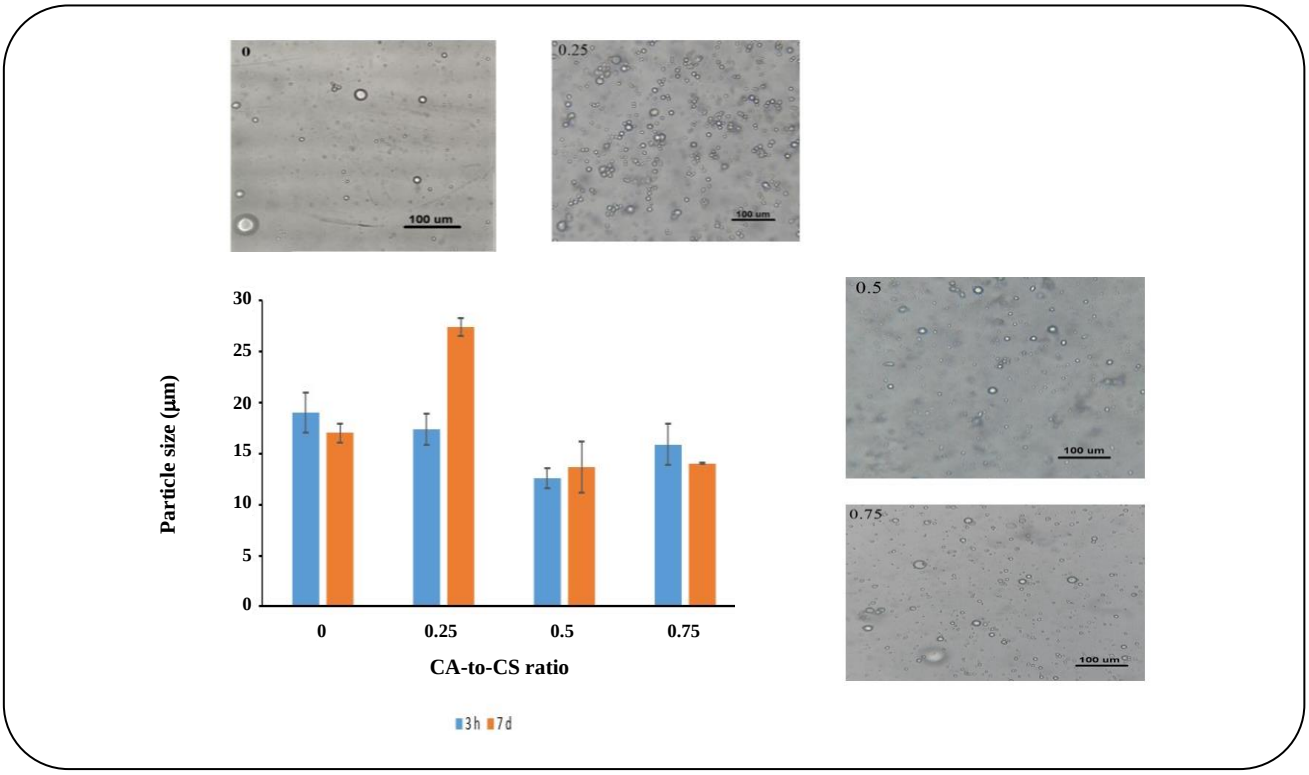

Fig. 5: Emulsion droplet size (after 3h and 7d) and optical microscopic images (after 7d) of emulsions stabilized by CS nanogels at different ratio of CA-to-CS at the fixed oil-to-nanogel ratio of 10:1 and pH 8.

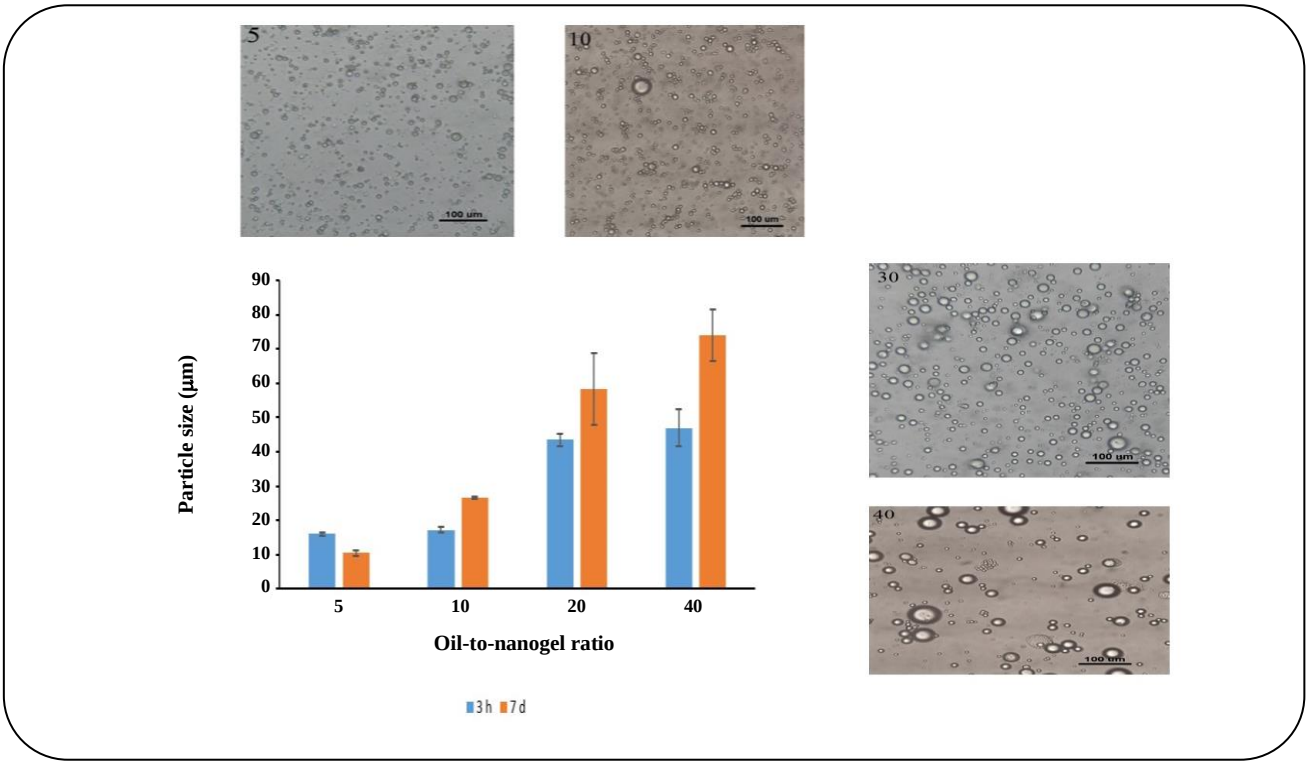

Fig. 6: Emulsion droplet size (after 3h and 7d) and optical microscopic images (after 7d) of Pickering emulsions stabilized by CS-CA nanogels with different oil fraction at the fixed pH of 8 and CA-to-CS ratio of 0.5.

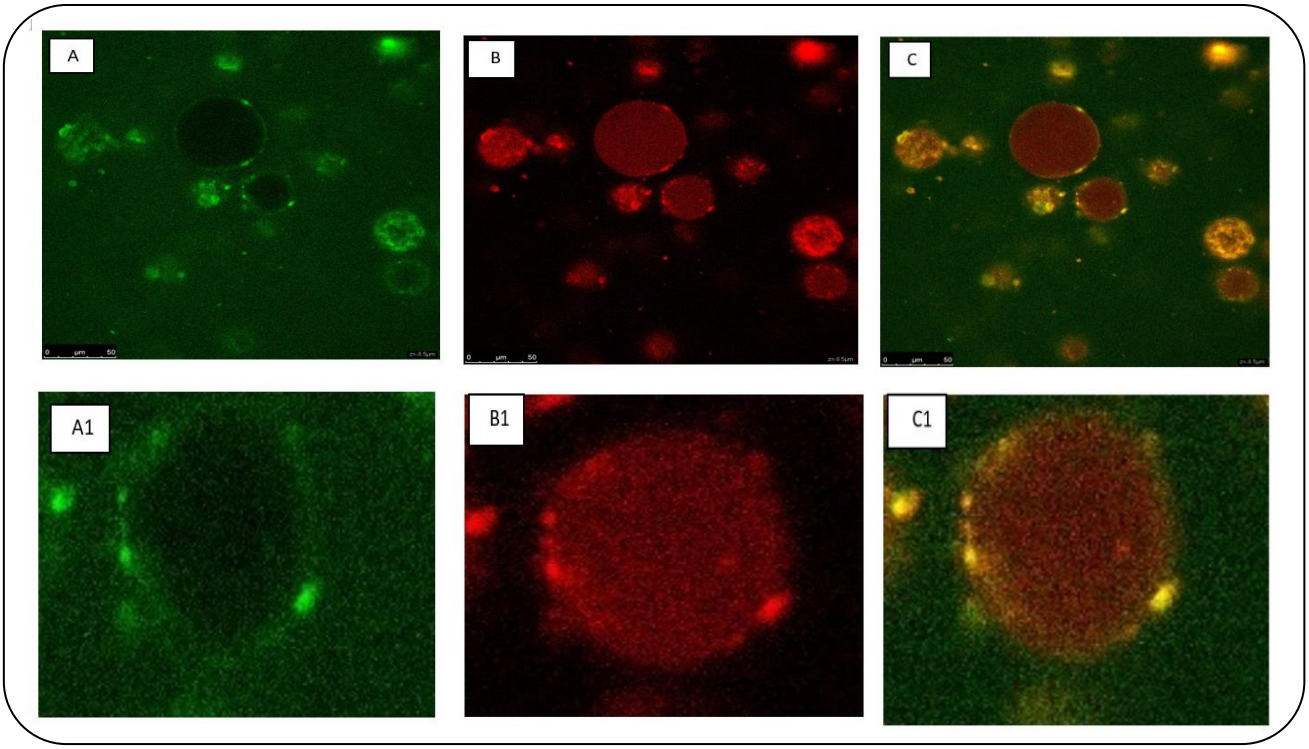

Fig. 7: CLSM images of CS-SA nanogels-stabilized Pickering emulsions at 5% oil fraction: CS-CA nanogels was stained by FITC (green) and was excited at 488 nm (A, A1); PSO was stained with Nile Red (red) and was excited at 488 nm (B, B1); C is the combined image of A and B while C1 is the combined image of A1 and B1.

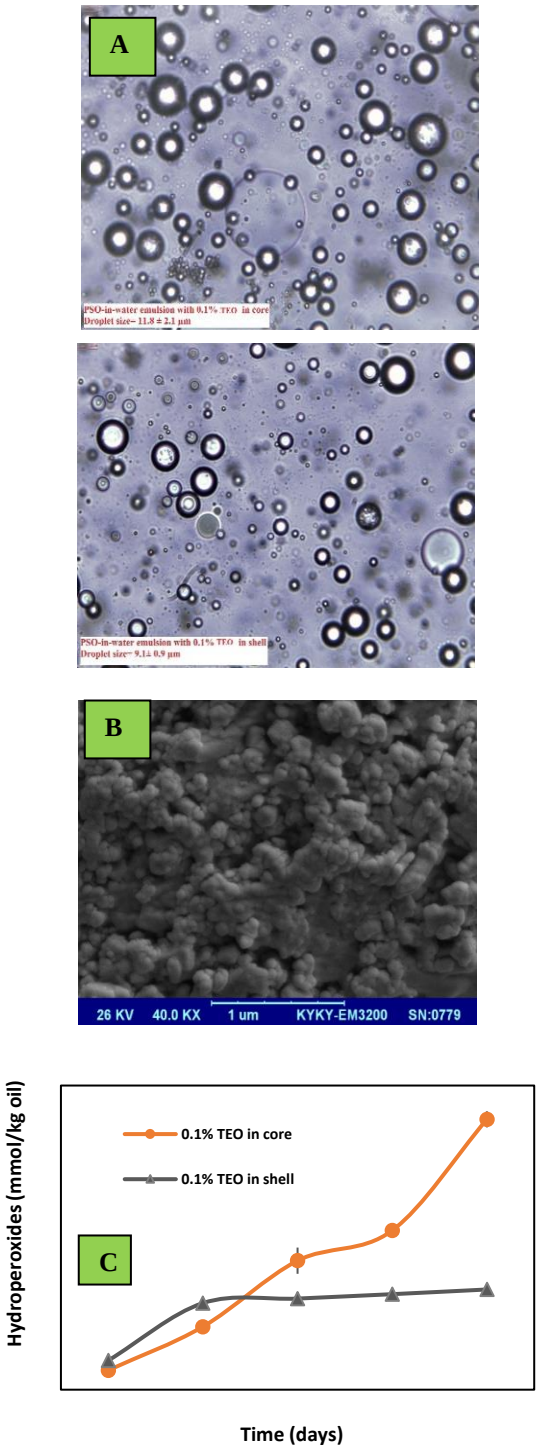

Fig. 8. Emulsion droplet and optical microscopic images (after 3h) (A), scanning electron microscopy (SEM) micrograph of CS–CA nanogel with CA-to-CS ratio of 0.5:1 incorporating 0.1% TEO (B) and formation of lipid hydroperoxides (mean ± SD, n = 3) of PSO-in-water emulsion with 0.1% TEO in core and PSO-in-water emulsion with 0.1% TEO in shell during 22 days storage at 35 °C (C).

surface of oil droplets, which can prevent the oil droplets from coalescence with the steric barrier. Also, these results showed that CS-CA nanogels can make a network on the surface of oil droplets. It has been proposed that the particle-based barrier in PEs is not a simple bilayer or monolayer, but a network of particles, adsorbed at the oil−water interface. The observations made in this work were in line with those reported by the former researchers [7, 42, 43].

Oxidative stability and microstructure of PE incorporating TEO

The effect of both TEO and the location of TEO (inside the core or inside the shell of the encapsulated PSO) on the lipid oxidation and the microstructure of PSO-in-water emulsion was examined. The emulsion droplet size and optical microscopic images (after 3h) of the PSO-in water emulsion along with shell and core TEO are shown in Fig. 8A. From Fig. 8A, the largest and smallest droplet sizes were related to PSO-in water emulsion with 0.1% core TEO and PSO-in water emulsion with 0.1% shell TEO, respectively. This finding revealed that the addition of TEO in the shell of oil droplets along with the CS-CA nanogel was efficient in decreasing the droplet size of the emulsion, probably by increasing nanogel hydrophobicity due to the hydrophobic nature of the essential oils. In addition, the SEM image of CS-SA nanogel incorporating 0.1% TEO is shown in Figure 8B. By comparing Figs. 8B and 2c (CS-CA nanogel without TEO), it could be realized that the formation of nanoparticles and their uniformity in CS-CA nanogels incorporating 0.1% TEO was better compared with the CS-CA nanogel without TEO, telling that TEO could change the shape of the nanogels. This change in structure was probably due to the change in the hydrophilicity and hydrophobicity of CS-SA nanogel. This finding showed that by adding hydrophobic compounds like TEO, the emulsifier properties of CS-CA nanogel could be improved. The results of some other studies in line with the results of the present work have shown that the addition of essential oils to the CS structure can increase the emulsifying property of CS [26, 28].

The formation of lipid hydroperoxides of PSO-in-water emulsion with 0.1% TEO in core and PSO-in-water emulsion with 0.1% TEO in shell during 22 d storage at 35 °C, is shown in Figure 8C. From Figure 8C, on the final day, the highest hydroperoxides were observed in PSO-in- water

emulsion with 0.1% TEO in the core. Also on the last day, the lowest hydroperoxides were measured in PSO-in-water emulsion with 0.1% TEO in the shell. In general, by comparing the formation of hydroperoxides on different days, it can be concluded that encapsulating TEO in the shell of oil droplet had more efficient than admixing TEO in the core of the oil droplet in reducing lipid oxidation. These results can be interpreted by considering the fact that the oxidation of emulsions happens at the interface region so that the presence of antioxidants at the interface region is more efficient than when the antioxidants are within the oil droplets [42]. *Wang et al.* [42] reported that the encapsulation of curcumin as an antioxidant in zein/chitosan complex particles had a more oxidative stability effect than when the curcumin was added directly to the corn oil. Furthermore, lower formation of hydroperoxides in emulsion with 0.1% shell TEO compared to the emulsion with 0.1% core TEO can be related to the smaller droplet size of emulsion with 0.1% shell TEO (Figure 8A). *Nakaya et al.* [44] reported that reducing the droplet size of the emulsion can reduce the formation of hydroperoxides.

CONCLUSIONS

Findings revealed the high proportion of CA to CS stimulated to favor the formation of nanoparticles. The present results also implied that the higher stability of PSO-in-water emulsion could be attained by using CS-CA nanogels with a high CA ratio and at the relative alkaline pH values. The obtained results stated that TEO into CS-CA nanogels applied for covering of PSO led to the highly oxidative stability with less droplet size in comparison with that of TEO in the core of the oil droplets. In addition, the results revealed that TEO could improve the emulsifying property of CS-CA nanogels. The findings of the present work could offer a procedure to improve the emulsifying property of CS by the connection between carboxylic acid groups of CA and amine groups of CS as well as by the use of non-polar compounds such as essential oils incorporating CS.

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