In-vitro stress stability, digestibility and bioaccessibility of curcumin-loaded polymeric nanocapsules

Shabbar Abbasa, Dawei Changb, Naveeda Riazc, Abid Aslam Maand, Muhammad Kashif Iqbal Khand, Istiaque Ahmadg, Suliman A. Alsgabyf, Ahmed El-Ghorabg, Mazhar Alih, Muhammad Imranj, Azmat Ullahj, Tahir Mehmoodk, Muhammad Zeeshan Hydera, Muhammad Sajjadh, Muhammad Umera, Asghar Shabbira and Muhammad Inam Afzala

aDepartment of Biosciences, COMSATS University Islamabad, Park Road, Tarlai Kalan, Islamabad 45550, Pakistan; bSchool of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi’an, Shaanxi 710021, P.R. China; cDepartment of Bioinformatics and Biotechnology, International Islamic University, Islamabad, 44000, Pakistan; dDepartment of Food Engineering, University of Agriculture, Faisalabad, 38000, Pakistan; eDepartment of Dairy Technology, University of Veterinary and Animal Sciences, Lahore, Pakistan; fDepartment of Medical Laboratories Sciences, College of Applied Medical Sciences, Majmaah University, Majmaah, 11932, PO Box 1712, Saudi Arabia; gCollege of Science, Chemistry Department, Jouf University, Sakaka, aljuf, 2014 king Saudi Arabia; hDepartment of Environmental Sciences, COMSATS University Islamabad, Vehari Campus, Vehari, 61100, Pakistan; iUniversity Institute of Diet and Nutritional Sciences, Faculty of Allied Health Sciences, The University of Lahore, Lahore, 54000, Pakistan; jDepartment of Food Science and Human Nutrition, University of Veterinary and Animal Sciences, Out Fall Road, Civil Lines, Lahore, 54000, Pakistan; kInstitute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences-UVAS, Lahore, 54000, Pakistan

ABSTRACT
Nanoemulsion-based delivery systems have a considerable potential for the encapsulation, protection and delivery of lipophilic bioactives. In the current study, in-vitro stability of curcumin-loaded single (NEI), double (NEII), and triple-layered nanostructures was investigated against varying environmental stress (temperature, pH, ionic strength) and storage conditions. In addition, their functional performance, as influenced by the number of polymer layers, was also assessed by comparing in-vitro lipid digestibility and curcumin bioaccessibility. Initially, NEI was prepared through ultrasonic homogenisation technique, and stabilised with modified starch. Next, sequential deposition of chitosan and carboxymethyl cellulose (CMC) on the NEI droplets resulted into the formation of NEII and nanocapsules, respectively. Nanocapsules showed good stability against aggregation over a wide range of pH (3.0–7.0), salt concentration (50–300 mM NaCl) and temperature (30–90 °C for 30 min). Digestion studies suggested that polymeric coatings interfered with lipolysis as the digestibility (% free fatty acid released) of oil droplets was considerably reduced with increasing the number of layers surrounding the oil droplets. However, curcumin bioaccessibility increased with adding the...
number of coating layers. Overall, results indicate that the developed nano-system could be helpful in improving the oral delivery of curcumin besides incorporating it into functional foods and beverages.

1. Introduction

Nanoemulsion-based delivery systems play an exceptional role for the encapsulation, protection and delivery of lipophilic bioactives [1]. Multilayered-nanocapsules, having a bioactive-enriched oil core, can be prepared using nanoemulsion as a template. Such nanocapsules, when incorporated in food, offer many potential benefits for the oral delivery of lipophilic bioactives. For food applications, these systems are prepared from the food-grade material. Very small droplet size makes nanoemulsions more stable than conventional emulsions. Besides, nanoemulsions are known to improve the physicochemical stability and bioaccessibility of the loaded compound [2]. However, emulsions are prone to lipid oxidation and environmental stress during storage, food-processing operations and in the gastrointestinal (GI) tract after ingestion [3]. To design more efficient and stable nano-system, emulsions are often used as a template. For instance, multilayered O/W nanoemulsions can be constructed using polyelectrolyte through layer-by-layer deposition technique [4–6]. In this technique, polyelectrolytes are electrostatically deposited onto the oppositely charged surface. Consequently, a multi-composite protective layer is formed, surrounding the oil droplet. Adsorption and repeated formation of such layers result in the formation of multilayer structures [4,5]. Charge at the droplet surface can be neutralised or even reversed (over-compensated) depending upon the concentration and charge density of polyelectrolyte used. Additionally, depositing polyelectrolyte molecules should have much lower charge density than that of droplet surface, as greater charge may form a polyelectrolyte monolayer instead of composite layers [7].

Biopolymer-coated multilayered emulsions (so called nanocapsules) are an excellent tool to encapsulate and deliver lipophilic food bioactives. Furthermore, such delivery systems are known to have better stability than conventional emulsions against environmental stress and food-processing conditions [8–10]. For the food-related applications, polysaccharides and proteins are most commonly used material for the preparation of multilayer structures. Comparatively, polysaccharides offer better stability to the designed nano-system than proteins against the environmental stress conditions [11,12]. Chitosan and CMC have been used recently in the fabrication of self-assembled biodegradable polymeric films/layers [13,14]. Chitosan belongs to weak polyelectrolyte, positively charged in acidic environment having an isoelectric point (pI) value around 6.3–7.0, while CMC is polyanionic with negatively charged when pH is higher than 2.0 with a pKa range of 2.0–4.0 [15].

Previously, we optimised the preparation of curcumin loaded O/W nanoemulsions stabilised with OSA (octenyl succinic anhydride) modified starch [16]. Prepared nanoemulsion was used as template for the development of nanocapsules, as described in another study [17], using optimum concentrations of chitosan and CMC. These nanocapsules were characterised for size and charge using dynamic light scattering and electrophoretic light scattering, respectively. Transmission Electron Microscopy (TEM), Confocal Laser Scan Microscopy (CLSM), and Atomic Force Microscopy (AFM) were also used [17]. Results showed that nanocapsules had almost spherical shape and well-defined structure having particle size of 159.85 ± 0.92 nm. Although, droplet/particle size of all three types of nano systems remained stable during one-month storage period, further study is needed to assess
their durability against the environmental stress conditions [18]. In addition, it is important to determine the stability of loaded curcumin. Type of biopolymer is also very critical as it determines the stability and functionality of the designed nano system [6, 19].

Several researchers have confirmed that polymeric shell modulates the lipid digestibility, bioaccessibility and release behaviour of the loaded compound [2,20,21]. A number of factors, including chemical nature of polymeric material, number and order of layers, and the preparation conditions govern these modulations [19,20,22]. Hence, it is important to understand the lipid digestion and bioaccessibility of the encapsulated bioactives. During the digestion of lipids, lipolytic enzymes (lipases in the presence of bile salts) hydrolyse the oil droplets, thus, facilitating their absorption in the human body in the form of free fatty acids [23]. The lipid digestion studies could be accomplished by using an in-vitro lipolysis model, as described in the recent studies [14]. Mixed micelles obtained after digestion, containing the fraction of lipophilic bioactive, are helpful to assess the release properties (bioaccessibility) of the designed delivery system [23].

The aim of this study was to investigate the influence of different environmental stresses (pH, ionic strength, temperature), typically encountered by food products, on the prepared nanostructures. Additionally, in-vitro digestion and bioaccessibility of the loaded curcumin, as affected by the number and type of polymeric layers, were also determined. Finally, the stability of loaded curcumin was noted over time under different incubation conditions. As physicochemical stability and functional performance of the curcumin nanocapsules was assessed, this study will have implications in the preparation and incorporation of nano-curcumin in different functional foods and nutraceutical products.

2. Experimental

2.1. Chemicals and reagents

Medium chain triglycerides (MCT) with hydrophilic-lipophilic balance of ~11.0 and curcumin (77.90% purity) were obtained from Lonza Inc. and Nanjing Zelang Medical Technology Co. Ltd. (Allendale, NJ, USA). Purity Gum Ultra (PGU) and OSA-modified starch were the products of Ingredion (USA) supplied through Rafhan Maize Products (Faisalabad, Pakistan). Chitosan (93.4% degree of deacetylation), was obtained from Qingdao Jinhu Crust Product Company Ltd., (Qingdao, China). Carboxymethyl cellulose (CMC), (97 wt.% purity and 90–150 mPa.s viscosity for 2% w/v) solution was obtained from Shandong Yulong Cellulose Technology Co., Ltd. (Linyi, China). Other chemicals and reagents were of analytical grade and supplied by Sinopharm Chemical Reagent Company (China). Double distilled water was used for the preparation of solutions and nanoemulsions.

2.2. Solutions preparation

OSA-modified starch (PGU) solution (1.5% w/v) was prepared by dispersing it into warm water at 50°C and magnetically stirring for 30 min. Acetate buffer solution (pH 4.5, 0.1 M) was used to prepare 0.075% (w/v) chitosan dispersion by stirring overnight. Na-CMC dispersion (0.1% w/v) was prepared using distilled water.

2.3. Nanoemulsion (NEI) preparation

Five mL of curcumin enriched MCT (curcumin concentration: 5.6 mg/mL MCT) was coarsely homogenised with 95 mL of PGU solution using a high speed blender (Ultra-
Turrax T25 IKA Works Inc., Wilmington, NC, USA) at 14,000 rpm for 2 min. Curcumin loaded oil phase was prepared as described in our previous research [16]. NEI was obtained through ultrasonication (JY98-IIIDN, Ningbo Scientz Biotechnology Co., Ningbo, China, frequency: 20 kHz, rated power: 1200 W, probe diameter: 20 mm, volume processing capacity: 50–1000 mL) at a power density of 1.36 W/mL for 7 min, as described in our previous study [16]. NEI droplets were coated with a single biopolymer layer conferred by modified starch.

2.4. Multilayered nanocapsules preparation

Nanoemulsion (NEI) having negative charge was used as a template to fabricate NEII and nanocapsules, as described by Abbas et al. [17]. Briefly, 20 ml of chitosan dispersion was added drop wise into 40 mL of NEI and homogenised for 5 min. Next, the colloidal mixture was sonicated for 3 min (controlled sonication; power density: 1.36 W/mL) to obtain double layered nanoemulsions or simply NEII, having positive charge. In the next phase, 13.3 mL of Na-CMC dispersion was added drop wise into 40 mL of NEII and coarsely homogenised for 5 min, followed by sonication for 25 s (controlled sonication) to obtain triple layered nanocapsule suspension, bearing net negative charge. Flow diagram of the whole preparation process of nanocapsules suspension is given in Figure 1.

2.5. Particle size and zeta potential analysis

Nanoemulsion/suspension samples were diluted with distilled water (1:200 (v/v) and analyzed for their size and charge. The size of NEI, NEII droplets and nanocapsules was determined using a DLS instrument (Zetasizer Nano ZS series, Malvern Instruments, UK). Charge (ζ-potential) on the droplets/capsules was estimated through measuring their electrophoretic mobility using a capillary electrophoresis cell. Results were triplicated for each sample and expressed in mV.

2.6. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) analysis was conducted, as described in our previous work [17], to observe the core-shell structure of prepared nanocapsules. Zeiss LSM 710 confocal microscope (Leica, Heidelberg, Germany), was used at 40X for the analysis. Nile Red (0.02%, w/v) and fluorescein isothiocyanate (FITC) (0.05%, w/v) fluorescent dyes were used to mark the oil and chitosan molecules, respectively. Nile red dye was excited at 543 nm, while FITC at 488 nm.

2.7. Environmental stresses

Developed nanostructures, when incorporated into food, may encounter extreme food process conditions. Therefore, it is relevant to investigate the influence of environmental stresses (pH, ionic strength, heating) on the size and charge of NEI, NEII droplets and nanocapsules to evaluate their integrity.

i. pH stability

Curcumin loaded nanoemulsion (NEI) was prepared with deionised distilled water while double and triple layered nanocapsules were prepared in 0.1M acetate buffer...
environment (pH:4.5–4.7). To assess the pH stability of prepared emulsions and nanosuspension, various five mM/L acetate buffer solutions, with pH values ranging from 3 to 7, were prepared. To obtain the same final oil (MCT) volume fraction, 0.1, 0.15 and 0.2 mL of NEI, NEII and suspension, respectively, were individually added to buffer solutions (4.5 mL each) and the total of all the samples were made up to 5 mL. pH was adjusted to the desired level, using either 0.1 M HCl or 0.1 M NaOH. Samples were stirred for 30 min and then stored at room temperature overnight before analysis.
ii. Ionic strength stability

Different concentrations of NaCl (0–300 mM) were added to the NEI, NEII and nanocapsule suspension to study the effect of ionic strength on their stability. NEI and NEII samples were diluted with water to obtain the similar final oil (MCT) volume fraction as in the nanocapsules. pH values of all the samples were adjusted to 4.5 using 5 mM acetate buffer.

iii. Thermal stability

Emulsions/suspensions were subjected to thermal treatments to investigate the influence of processing temperatures on their stability. NEI, NEII and suspension samples were diluted with 5 mM acetate buffer (pH 4.5) to obtain the same final oil volume fraction as in the nanocapsules. Samples, 5 mL each, were placed in glass test tubes and incubated in a water bath for 30 min at different temperatures (30–90 °C), followed by cooling to the room temperature and stored for 12 h prior to analysis.

2.8. In-vitro digestion

In-vitro digestion of the emulsions and nanocapsule samples was conducted under simulated gastrointestinal tract (GI) conditions, as described by Liang et al. [24]. Briefly, 1.5 mL of each sample was mixed with 13.5 mL of basal saline solution (140 mM NaCl, 5 mM KCl, and 150 uM BHT) and stirred for 10 min. The pH of the prepared mixture was adjusted to 2.0, using 0.1/1.0 M HCl, immediately before and after the addition of 4.5 mL of simulated gastric fluids (SGF) (composition: 16.5 mg/L pepsin in 0.1 M HCl) to initiate the gastric digestion process. Mixture was incubated at 37 °C for 1 hr under magnetic stirring, followed by the pH adjustment to 7.5 (using 0.1/1.0 M NaOH solution) and addition of 4.5 mL simulated intestinal fluid (SIF) (2.0 mg/mL pancreatin and 12 mg/mL porcine bile extract in PBS, pH 7.5), which initiated the intestinal digestion process. During the 2 hr intestinal digestion process, released free fatty acids (FFAs) were neutralised by adding 0.1 M NaOH to maintain the solution pH at 7.5. The volume of NaOH consumed over time during the digestion process was recorded, and the percentage (%) of FFAs released over time was determined by using the Eq. (1).

\[
\% \text{FFA}^{\text{Released}} = \frac{V_{\text{exp}(\text{NaOH})}}{V_{\text{max}(\text{NaOH})}} \times 100
\]  

(1)

Here, \( V_{\text{exp}(\text{NaOH})} \) is the actual volume of NaOH used to neutralise the FFA released at a given time. \( V_{\text{max}(\text{NaOH})} \) is the theoretical volume of NaOH solution consumed to neutralise the FFAs released; was calculated by using the Eq. (2).

\[
V_{\text{max}(\text{NaOH})} = 2 \left[ \frac{m(\text{MCT}) \times 1,000}{M_w(\text{MCT}) \times C(\text{NaOH})} \right]
\]  

(2)

Here, \( C(\text{NaOH}) \) is the concentration of NaOH used (0.1 M); \( m(\text{MCT}) \) is the mass in grams of MCT (1.0 mL MCT = 0.946 gm) present in the sample. \( M_w(\text{MCT}) \) is the molecular weight of MCT (491 g/mol) calculated by using the Eq. (3).

\[
M_w(\text{MCT}) = \frac{3 \times 1.000 \times M_w(\text{KOH})}{\text{Saponification Value}}
\]  

(3)

Here, \( M_w(\text{KOH}) \) is the molecular weight of KOH (used to express the saponification value). Saponification value used for MCT was 342 mg KOH/g, as provided by the manufacturer.
2.9. Determination of curcumin bioaccessibility

Curcumin bioaccessibility studies of the emulsions and nanocapsule samples were conducted according to the method of Ahmad et al. [25]. Briefly, samples obtained from in-vitro digestion mixture were centrifuged (18,516 × g), (Sorvall ST 40 R, Thermo Fisher Scientific, GmbH, Germany), at 25 °C for 35 min. Samples were separated into three distinct layers, that is, sediment at the bottom, a clear micelle phase in the middle, and a thin oily layer at the top. Aliquots of micelle phase (5 mL) were collected and vortexed with chloroform (5 mL), followed by the centrifugation at 1200 g for 10 min at 25 °C. The bottom layer (chloroform) was collected while the top layer was vortexed again with chloroform (5 mL) and centrifuged (as described) to obtain the residual curcumin. Again, chloroform layer (bottom) was collected and added to the previously collected chloroform layer. Chloroform (containing curcumin) was analyzed by UV-spectrophotometer (Model UV-1600, Mapada Corporation, P.R. China) at the frequency of 416 nm (determined by 2802- UV/VIS spectrophotometer) using chloroform as a reference. The concentration of curcumin in the collected chloroform was determined from a standard calibration curve of absorbance plotted for pure curcumin concentrations (0.5–3.0 µg/mL) in chloroform. Percent (%) bioaccessibility of curcumin was dependent on the concentration of curcumin present in the isolated aqueous (micelle) phase; and calculated according to the following relation.

\[
\text{Bioaccessibility(\%)} = \frac{\text{curcumin in micelle phase}}{\text{curcumin loaded to the formulation}} \times 100
\]  

(4)

2.10. Statistical analysis

Experiments were carried out in duplicates using freshly prepared samples, and results were calculated as mean and standard deviation. Origin pro 8 (OriginLab Corporation, Northampton, MA, USA) was used to generate graphs. Schematic diagram was drawn using Edraw Max® 2014 software, Version 7.9.

3. Results

3.1. Initial characteristics of nanostructures

Freshly prepared curcumin-loaded nanostructures, i.e. NEI, NEII and nanocapsules were characterised for their size, charge and PDI [16,17], as shown in the Table 1. CLSM analysis of nanocapsules was also conducted to understand the multilayer formation process, as shown in Figure 2. Next, these nanostructures were assessed in-vitro for their stability against environmental stress conditions and curcumin bioaccessibility.

3.2. Influence of pH on the droplet diameter

Keeping in view, the isoelectric point (pI) of chitosan (6.3–7.0) and pKa value of CMC (2.0–4.0), we identified a potential pH range of 4.0–6.0 for the formation of stable Chitosan-CMC layer, as depicted in the Figure 3(a). The droplet size of NEI (∼139–147 nm) and nanocapsules (∼159–166 nm) was almost stable under all pH conditions except at pH 7.0, where negatively charged nanocapsules increased by ∼16 nm (Figure 3(b)). Size of NEII droplets also increased slightly (∼156 to 163 nm) when the pH was higher than 4.0. The ζ-potential of NEI droplets stabilised by OSA-modified starch was negative at all pH conditions. Negative charge rapidly decreased with the pH decline
below \( \sim 5.0 \), (Figure 3(c)) which could be due to the proximity of this pH value with the pKa value of carboxyl groups of modified starch.

### 3.3. Influence of salt on droplet size

This set of experiments helped to investigate the influence of varying salt (NaCl) concentrations (0–300 mM) on the nanostructures. OSA-modified starch assisted in stabilising the NEI droplets while starch-chitosan and starch-chitosan-CMC layers protected the NEII and nanocapsules, respectively. In the absence of NaCl, droplet size of NEI, NEII and nanocapsules was \( \sim 143 \text{ nm} \), \( \sim 154 \text{ nm} \) and \( \sim 160 \text{ nm} \), respectively, as shown in

![Figure 2. Confocal (CLSM) images of nanocapsules (a) oily core (red) marked with Nile Red (b) chitosan-CMC shell (green) marked with FITC. (c) Superposition of image a and b (yellowish in colour). (d) inset of image c showing oil droplet (red in colour) encapsulated inside the polymeric shell (yellowish green in colour).](image)

**Table 1.** Mean droplet size, charge and polydispersity index of developed nanostructures.

| Sr. # | Measurement      | NEI               | NEII              | Nanocapsules  |
|-------|------------------|-------------------|-------------------|---------------|
| 1     | Mean droplet size| 142.7 ± 0.85 nm   | 153.8 ± 1.13 nm   | \( \sim 159.85 \text{ nm} \) |
| 2     | \( \zeta \)-potential| \(-39.4 ± 1.84 \text{ mV} \) | 11.45 ± 1.06 \text{ mV} | \(-17.2 \text{ mV} \) |
| 3     | Polydispersity index| 0.150 ± 0.01     | 0.182 ± 0.02     | 0.140 ± 0.01  |
Figure 4. Although, size of all samples was stable up to 250 mM salt concentration, it slightly increased at 300 mM salt concentration for all three samples.

3.4. Influence of heating on droplet size and charge

Depending upon the final use of nanoemulsions and dispersions, it was critical to assess the influence of heat (due to food-processing operations) on their stability. As shown in Figure 5(a), droplet size values for NEI, NEII and suspensions were almost stable when the temperature raised from 30°C to 60°C. Temperature increase from 60°C to 90°C slightly increased the droplet size (~7.0 nm) of NEI samples. Electrical characteristics study of NEI, NEII and nanosuspension revealed that ζ-potential of droplets and nanocapsules was stable at all heating temperatures, as shown in Figure 5(b). After thermally treating from 30°C to 90°C, charge ranged −24.4 to −26.4 mV, 10.4 to 11.1 mV and −15.2 to 17.0 mV for NEI, NEII and nanocapsules (nanosuspension), respectively. These results confirmed that heat treatment did not affect polyelectrolyte layers, and they remained attached to the droplet surface.

3.5. In-vitro digestion

Comparative study of the in-vitro digestibility of lipid droplets in the structured nanoemulsions (NEI, NEII) and nanocapsules was conducted to determine the effect of
deposited polymeric layers on the lipid digestibility. Sample dilutions were carried out in such a way that the final weight of oil in each sample was kept constant at 35.5 mg/1.5 mL. Digestion of lipids refers to the fraction of FFAs released over time due to enzymatic action, as given in Figure 6. In the present study, in-vitro digestibility of oil phase was determined by manually adding 0.1 M NaOH to neutralise the FFA released. Volume of NaOH used was recorded and % FFA released was calculated by Equations 1 and 2. After 2-hour digestion, NEI (having single starch layer) droplets were almost fully digested (% FFA: 133%), as shown in Figure 6.

### 3.6. In-vitro bioaccessibility

In the present study, % bioaccessibility of curcumin from NEI, NEII and nanocapsules was determined after samples were subjected to in-vitro digestion. Digestion mixtures
(Figure 7(a)) of all three samples were ultra-centrifuged to obtain micelles. For NEII and nanocapsules, a very thin oil layer was found at the top, while digestion precipitates were settled down to the bottom as a pellet. Presence of thin oil layer suggested that part of...
curcumin-enriched MCT was not digested. Yellowish-brown colour of pellet indicated that fraction of curcumin was also precipitated.

To determine the curcumin concentration in micelle phase (middle translucent phase), it was washed with chloroform and then curcumin concentration in chloroform was assessed by comparing with the standard calibration curve of absorbance plotted for pure curcumin concentrations in chloroform. The % bioaccessibility of curcumin after in-vitro digestion for single-layered NEI, double layered NEII and triple-layered nanocapsules is shown in Figure 7(b).

4. Discussion

Variation of pH is critical as it may negatively affect the size and charge of nanoparticles, consequently, comprising the overall stability and performance of the designed delivery system. Apparently, the formation of NEI in this study was mainly dependent on the steric hindrance induced by the OSA-modified starch. However, electrostatic attractive forces played a role in the subsequent deposition of chitosan and CMC on NEI droplets. Steric hindrance is independent of pH while electrostatic forces are mainly pH dependent. Therefore, pH variation could alter the interfacial characteristics of NEII and nanocapsules. It is well documented that around or below the pKa values, carboxyl groups start losing their negative charge [11,26]. ζ-potential of NEII (Figure 3) was positive below pH 5.0 due to the chitosan (cationic) deposition on the NEI. With the pH increase (≥ 5.0), the positive charge on NEII droplets was reversed to negative. This could be due to the deprotonation of −NH₃⁺ groups (pI value: 6.3–7.0) of chitosan, as described by Ogawa et al. [27]. Therefore, rise in the pH decreased the positive charge on droplets, concomitantly, reducing the electrostatic attraction between chitosan and OSA-modified starch molecules. This phenomenon might have triggered the detachment of chitosan layer from the surface of NEI droplets. At higher pH (≥ 5.0), reversed negative charge on the NEII droplets was almost like to that of NEI droplets at corresponding pH values. Increase of pH (> 4.0) resulted in a slight aggregation of NEII droplets. Consequently, a small increase in the droplet size (∼ 13 nm) was observed, as shown in Figure 3(b). Nanocapsules retained the negative charge at all pH conditions, besides a steady rise in their negative charge from ∼ −0.5 to −8.0 mV with increasing pH. Additionally, size of nanocapsules constantly increased at pH ≥ 5.0 (Figure 3). These results confirmed that the CMC (outermost layer) molecules could deposit on to the particle surface of NEII at all pH conditions (pH: 3.0–7.0). Gradual increase in the negative charge of nanocapsules at higher pH could be due to the rise of negative charge on CMC molecules (pKa: 2.0–4.0) (Figure 3). Furthermore, higher pH may cause a reduction in positive charge on the chitosan molecules (pI: 6.3–7.0), resulting in the decreased electrostatic attractive forces. Consequently, slight aggregation of nanocapsules was present at higher pH. Nevertheless, nanocapsule based delivery system was resistant to the broad range of environmental pH conditions.

Stability of starch-stabilised nanoemulsion (NEI) can be attributed to the fact that steric hindrance is the major force conferring stability to such systems. However, a slight aggregation at 300 mM NaCl indicated that, besides steric repulsion as a primary source, some other forces were also involved in the stability of NEI. In addition, aggregation of NEI droplets could also be due to the electrostatic screening (ζ-potential decreases) which causes the reduction of repulsive interactions between droplets, as explained by several other researchers [9,28]. Although NEII and nanocapsule suspension were prone to aggregation at higher salt concentrations due to decreased repulsion forces, their droplet size
stability at NaCl concentration of 50-250 mM was interesting. Most probably, the formation of relatively thick polymeric layers of chitosan and CMC around the starch-stabilised NEI droplets contributed towards such stability. Although, chitosan and CMC were deposited primarily under the influence of electrostatic attractive forces, acquired steric stability of chitosan coated NEII droplets and chitosan-CMC coated nanocapsules played a role in their size stability. However, small increase in the size of NEII droplets and nanocapsules was due to slight aggregation at 300 mM NaCl (Figure 4). This could be either due to the significant decrease in the positive charge of NEII droplets attributed by screening effects or due to the desorption of chitosan from the droplet surfaces, as described previously [9]. Charge characteristics (ζ-potential) of all three samples were found unreliable due to the high conductivity (>5 mS/cm) induced by the increased salt (NaCl) concentration.

As reported by other researchers, OSA-modified starch stabilised emulsions are physically less susceptible to heat treatment [11,28,29]. Temperature exceeding 60°C had more obvious effect on NEII samples (droplet size increase: ~23 nm), as shown in Figure 5. On the other hand, nanocapsules were highly stable against such temperature increases (MMD increase: ~4.0 nm). Increase of MMD of NEII droplets could be due to the droplet aggregation under the influence of decreased electrostatic attraction forces between chitosan and OSA-modified starch molecules.

A rapid lipolysis was observed during the first 700 s followed by more gradual FFA release. It normally happens in the presence of intestinal fluids which convert the triglycerides into monoglycerides [24,30]. It was clear that single layer of modified starch failed to effectively inhibit the entry of lipase into the emulsified lipid droplets. On the other hand, % FFA release from the oil droplets coated with starch-chitosan (NEII) and starch-chitosan-CMC layers (nanocapsules) was significantly reduced (Figure 6). These results show that the addition of second and third polymeric layer to oil droplets of NEII and nanocapsules, respectively, acted as a barrier between the lipase and lipid droplet, and made them less susceptible to the lipase digestion. Digestibility of NEII (125.4%) was higher than that of nanocapsules (68.1%) which could be attributed to the (i) lesser number of layers than that of nanocapsules and/or (ii) chitosan polyelectrolyte present on the surface of starch-chitosan coated NEII droplets lose positive charge and become negative at neutral pH, thus, weakening the electrostatic attractive forces between modified starch and chitosan layers. In this state, there is possibility of chitosan molecules either detaching from the starch layer or digested, thereby, exposing the NEII droplets to digesting enzymes. Similar conclusions were drawn for the lipase digestibility of lipid droplets coated by caseinat-chitosan-pectin layers [20].

In addition, our results showed that the digestibility of MCT in NEI and NEII was >100% (Figure 6), thus, contradicting and challenging our calculations. However, increased consumption of NaOH could be due to the additional protons released from the polyelectrolytes (PGU, Chitosan) during the in-vitro digestion process. There is also possibility that number of FFA molecules released from triglycerides digestion was higher than that theoretically expected [31]. Gudipati et al. [32] used citrem, chitosan and sodium alginate to prepare multilayered coatings on the emulsified fish oil droplets; found that single-layered oil droplets were easily digested. On the other hand, digestibility of double and triple-layered oil droplets was controlled due to the limited access of lipase to the droplet core. Several other researchers have confirmed that the addition of multilayer coatings around lipid droplets were helpful to decrease or control the digestion rate [14,20,32,33]. In a more recent study, Espinal-Ruiz et al. [34] concluded that dietary fibres, including chitosan and methyl cellulose, reduced the rate and extent of digestion of
Emulsified corn oil. It was suspected that the reduced digestibility in vitro was due to (i) electrostatic interactions of polysaccharides with oppositely charged species involved in lipid digestion and (ii) the barrier present between lipase and lipid droplets. It was believed that this barrier was formed due to the depletion flocculation and bridging flocculation promoted by methyl cellulose and chitosan, respectively. As polymeric material was used in our work to fabricate triple-layered nanocapsules (Figure 2), these nanocapsules may provide an opportunity to control the lipid digestibility besides protecting and delivering bioactive compound to target site in the human body.

Bioaccessibility of component is the fraction of the component that is released from the ingested material into the juices of the gastrointestinal tract. Released bioactives are trapped in the mixed micelles in the small intestine, and then they are absorbed. Multilayered nanocapsules are considered potential delivery systems for nutraceuticals. In the current research, bioaccessibility study indicated that with the increased number of coating layers, the % bioaccessibility of curcumin increased. For instance, % bioaccessibility of curcumin for the starch coated NEI droplets was minimum (15.25%), although the % FFA released was maximum (> 130%). On the other hand, % bioaccessibility was increased to 23.4 ± 0.8% with the addition of second (chitosan) layer. Similarly, % bioaccessibility was further increased to 29.2 ± 0.8% with the addition of third layer (CMC) (for nanocapsules) even though minimum % FFA was released (68%), as shown in Figure 7(b). Increase of % bioaccessibility with increasing the number of coating layers could be due to the extra protection provided to the curcumin against degradation after it was subjected to simulated digestion conditions.

As nanocapsules are of very small size (about 160 nm), they are less susceptible to mechanical action, churning process of the stomach. Sequential deposition of polyelectrolytes onto the surface of nanoemulsion oil droplets is found to be a good approach as it is versatile and flexible. Several studies have evaluated similar type of nanosystems for their stability in GIT and digestibility using in vitro models under simulated conditions [32,33,35–38], and confirmed that multilayer coatings offered excellent barrier properties and modulated/controlled the process of lipid digestion by hindering the access of enzymes. In the current study, we have prepared curcumin-loaded nanocapsules with oily core, using food-grade biopolymers (polyelectrolytes), i.e. modified starch, chitosan, and carboxymethylcellulose (CMC), by exploiting their electrostatic characteristics. Nanocapsule shell consisted of three different polysaccharide layers. For instance, anionic modified starch formed the inner layer of shell, cationic chitosan constituted the intermediate layer, and an outermost layer was of anionic polysaccharide CMC. We expect that in the presence of the outermost anionic CMC layer, the chitosan-coated droplets will be stable to aggregation at high pH values (pH > 6), as chitosan charge will remain intact. As CMC maintained its anionic character even at lower pH values (pH 3), the outermost layer will offer good stability to nanocapsules at both, higher and lower pH conditions. This phenomenon was confirmed by in vitro digestion model, which indicated that polysaccharide coatings were intact and acted as a barrier to reduce the rate of lipid digestibility. Additionally, modified starch (forming innermost layer) has a certain degree of steric repulsion that may protect the nanostructures from oiling-off, as suggested previously [39].

5. Conclusion

In this research, stability of ultrasound-assisted multilayered nanoemulsions and capsules were investigated in terms of droplet size and potential under varying environmental
stress conditions. In addition, stability of encapsulated curcumin, in terms of concentration, was also investigated under different storage conditions. Single (NEI) and double (NEII)-layered nanoemulsions, and triple-layered nanocapsules, were OSA starch, OSA starch-chitosan and OSA starch-chitosan-CMC, respectively. Particle size of all three samples were almost stable at a broad range of pH (3.0–7.0), salt (0–300 mM) and heating temperature (30–90 °C), except starch-chitosan coated nanoemulsions (NEII) which showed aggregation at higher temperatures (60–90 °C) as droplet size increased from 160 to 188 nm. For all three samples, ζ-potential of the droplets/capsules was affected by the increase in pH; however, droplet size stability was not disturbed due to these changes. Curcumin stability studies revealed that the light was a major factor in the colour degradation of emulsions/suspensions. Introduction of starch-chitosan and starch-chitosan-CMC layers decreased the % FFA release from 133% (for NEI) to 125.4% (NEII) and 68.1% (nanocapsules), respectively, after 2-hour digestion. As prepared nanocapsules retarded the lipid digestion, our system could be helpful to devise the food-based strategies to control the fat-associated health problems, including obesity, heart diseases and hypertension. With increasing the number of layers, the curcumin % bioaccessibility also increased. Nanocapsules offered highest curcumin bioaccessibility (29.2 ± 0.7%) while it was lowest in the case of NEI (15.25 ± 0.6%). This study suggests that the prepared multilayered nanocapsules can be used to encapsulate and deliver the lipophilic bioactives in the nutraceuticals and functional food applications.

Disclosure statement

The authors declare no conflict of interest.

Notes on contributors

Shabbar Abbas is Assistant Professor in Department of Biosciences, COMSATS University, Islamabad. His research work mainly focused on the development of functional food ingredients through encapsulation & nano-techniques.

Dawei Chang is Professor in School of Food and Biological Engineering, Shaanxi University of Science and Technology. His research interests are mainly related to encapsulation and microencapsulation.

Naveeda Riaz is Associate Professor in Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad, Pakistan.

Abid Aslam Maan is Assistant Professor of food engineering in University of Agriculture Faisalabad.

Muhammad Kashif Iqbal Khan is Assistant Professor of food engineering in University of Agriculture Faisalabad.

Ishitaque Ahmad is Lecturer in Department of Dairy Technology, University of Veterinary and Animal Sciences, Lahore, Pakistan.

Suliman A. Alsagaby is Researcher in Department of Medical Laboratories Sciences, College of Applied Medical Sciences, Majmaah University, Majmaah 11932, PO Box 1712, Saudi Arabia.

Ahmed El-Ghorab is Professor in College of Science, Chemistry Department, Jouf University, Sakaka, aljuf, 2014 king Saudi Arabia.

Mazhar Ali is Assistant Professor in Department of Environmental Sciences, COMSATS University Islamabad. His research interests mainly related to environmental sciences.

Muhammad Imran is Professor of food science and technology in University Institute of Diet and Nutritional Sciences, Faculty of Allied Health Sciences, The University of Lahore, Lahore.
Azmat Ullah is Associate Professor of food science and technology in Department of Food Science and Human Nutrition, University of Veterinary and Animal Sciences Lahore.

Tahir Mehmood is Assistant Professor in Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences-UVAS, Lahore, 54000-Pakistan

Muhammad Zeeshan Hyder is Associate Professor in Department of Biosciences, COMSATS University, Islamabad. He has great interest in Bioinformatics and Biotechnology.

Muhammad Sajjad is Assistant Professor in Department of Biosciences, COMSATS University, Islamabad. His research interests mainly focused on plant breeding, genetics and programming languages.

Muhammad Umer is Assistant Professor in Department of Biosciences, COMSATS University, Islamabad. His research mainly focused on Microbiology, Biochemistry and Biofuel Technology.

Asghar Shabbir is Assistant Professor in Department of Biosciences, COMSATS University Islamabad, Islamabad, Pakistan.

Muhammad Inam Afzal is Assistant Professor in Department of Biosciences, COMSATS University, Islamabad, Pakistan. He earned his PhD from University of Lorraine, France. His research mainly focused on Nanomaterials, Encapsulation and Food safety.

ORCID

Azmat Ullah http://orcid.org/0000-0001-9462-150X
Muhammad Inam Afzal http://orcid.org/0000-0002-7338-6237

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