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Assessment of oral bioavailability of nanocapsules loaded-curcumin *in-vivo*

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Abstract

Curcumin, a hydrophobic polyphenol found in rhizome of turmeric (curcuma longa) is one of the generally used spice in Asian countries. It has been found through studies that it has numerous health promoting properties and is proved to have curative properties as it possesses antioxidant and anti-inflammatory properties. Due to low oral bioavailability, decreased solubility, quick metabolism and removal from body, it is challenging to achieve maximum benefits from it. This study was aimed to enhance curcumin oral bioavailability by making use of nanoencapsulation technique. Biodegradable nanocapsules were prepared by consecutive addition of food grade polyelectrolytes including OSA-modified starch (PGU), as an emulsifier, chitosan and sodium-carboxymethylcellulose by ultrasonication technique using primary nanoemulsion as template. Results showed that the mean droplet diameter of nanocapsules was 160.57 ± 1.06 nm, the average PDI was 0.14 ± 0.01 and average charge was recorded as -24.43 ± 0.49 mV. Microscopy results showed that the nanostructures were spherical in shape having mean droplet diameter below 200nm. The nanocapsules assessment as a carrier in enhancing the *in vivo* oral bioavailability of curcumin was made; however, further studies and better tools are needed to clearly know the potential of developed nanosystem.

Keywords: Curcumin; Polyelectrolytes; Nanoemulsion; Sonication; Nanocapsules

1. Introduction

Bioactive compounds are naturally occurring extra nutritional components present in numerous food sources. They play significant role in improvement of human health and their role in controlling physicochemical reactions in the body is extensively studied. In order to acquire maximum benefits from these compounds, new range of compounds called Nutraceuticals are introduced which besides providing nutrition; provide health benefits such as prevention of degenerative ailments such as cancer [1]. Therefore, the interest in using these products as a preventive measure against numerous diseases has increased dramatically in the recent times hence, the regular consumption of bioactive compounds has gained great importance to prevent and treat diseases for which consumers favor the oral administration compared to other approaches it is less costly, consumer friendly and allow simple dosing plan.

Curcumin is a bioactive compound present in rhizome of turmeric and displays a large number of health benefits. It has anti-inflammatory, antioxidant, anti-aging, antiviral and anti-carcinogenic properties [2-4]. Numerous studies have been carried out in order to unveil its proficiency in treatment and prevention of a number of health issues [5, 6]. Curcumin is proved to be non-toxic to human upon consumption in larger amounts [7, 8].

However, like most other bioactive compounds, curcumin has very low bioavailability [9]. The bioavailability of curcumin is negatively affected by decreased solubility (11ng/mL in water), least permeation, rapid metabolism and

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removal from the body [10]. After being absorbed by small intestine, curcumin breaks down into its metabolites. Curcumin glucuronide and curcumin sulfate are major metabolites [11].

According to numerous studies, bioavailibity of curcumin has increased upon encapsulation in different materials [12]. Lately, many study groups reported improvement in bioavailability and bioaccessibility of curcumin after encapsulation and delivery via nano-systems [13]. Nanotechnology has gained great importance in the fabrication of efficient delivery system for bioactive compounds in which nanometer sized structures possessing unique properties due to their small size and large surface area are fabricated [14, 15]. Nanotechnology has positive impact on food science as it improves food texture, taste, processability and increased shelf life [16]. Among wide variety of nanostructures, nanoemulsions are found to be more efficient in the delivery of bioactive compounds [17]. A number of advantages are offered by nanoemulsions including increased bioaccessibility and bioavailability, improved encapsulation efficiencies [15].

On the other hand, these nanoemulsions are thermodynamically unstable hence to overcome the issue and improve its stability, researchers attempted to add ultrathin polymeric layers to the nanodroplets making multilayered droplets [18]. Multilayered nanocapsules are found to have increased permeability for cells resulting in enhanced cellular uptake. They are synthesized from biodegradable materials; they are biologically suitable with body cells and tissues [19]. Efficient encapsulation of bioactive compound is carried out by nanoencapsulation system as the compound is solubilized in the core of polymer hence protecting it from degradation and lowers tissue irritation due to presence of polymeric shell [20, 21].

Generally, the evaluation of functional properties of prepared nanostructures is carried out by *in-vitro* and *in-vivo* studies. However, the *in-vitro* models could not mimic complexity of actual GIT hence *in-vivo* studies were conducted using animal models for the determination of bioavailability of bioactive compound [22, 23]. *In-vivo* studies provided better understanding of interaction and mechanism of bioactive compound in the body [24]. The present study was conducted with the aim to prepare curcumin-loaded polymeric nanocapsules, its characterization and to assess its oral bioavailability through rat models.

2. Material and methods

2.1. Materials for Nanofabrication

MCT (NEOBEE 1053) was a caprylic/capric triglyceride (Composition: C8: 55%, C10: 44%) and obtained from Stepan Specialty Products Company (Maywood, NJ, USA). Curcumin (catalog number 820354) was supplied by Merck (Darmstadt, Germany) (≥70.0% pure, with ≤20.0% of dimethoxy-curcumin and ≤5.0% of bisdemethoxycurcumin). Purity Gum Ultra (PGU), OSA-modified starch, was a gift from Ingredion EMEA (Manchester, UK) and supplied through Rafhan Maize Products, Faisalabad, Pakistan. Low molecular weight chitosan (Aldrich cat. 448869, degree of deacetylation: > 85%, made from shrimp shells) was purchased from Sigma-Aldrich Company Ltd. Carboxymethyl cellulose (Na-CMC, Mw- 90,000 kDa, Sigma-Aldrich: 419273), a biodegradable polymer with 50-200 cps viscosity for 4% w/v aqueous solution. Other chemicals/reagents used were of analytical grade and obtained from Merck (Darmstadt, Germany). Doubly distilled water was used for all solutions and in nanoemulsion preparation.

2.2. Materials In vivo Studies

Twelve rats (Sprague Dawley) of 8-12 weeks age were purchased and acclimatized at animal house, National Institute of Health (NIH), Islamabad.

2.3. Methods

Emulsifier (PGU) solution was prepared by dispersing 1.5% w/v of amorphous PGU in warm water at 45°C followed stirring for 30 minutes. Acetate buffer (0.1M, 4.5pH) solution was prepared by dissolving 3.15 g of sodium acetate in water followed by the addition of glacial acetic acid. Acetate buffer solution was used to prepare 0.075% (w/v) chitosan solution by overnight stirring.

CMC dispersion (0.1%w/v) was prepared by dissolving CMC in distilled water and stirred for 1 minute.

2.4. Preparation of nanostructures

Oil phase was prepared by dispersing curcumin (6mg/mL) in MCT oil for which oil was preheated at 100°C for 5 min and curcumin was added in to it and stirred for further 2 minutes by hot plate stirrer as demonstrated by [25]. Curcumin

enriched MCT oil was cooled down at room temperature and stored in dark place for further experiments. For the preparation of aqueous phase, 1.5%w/v of emulsifier was dissolved at 50°C for 30 minutes using magnetic stirrer.

Firstly, 5mL oil phase and 95mL aqueous phase were homogenized coarsely using a high-speed blender (Yellow Line DI basic homogenizer) at 13500 rpm for 2 minutes to prepare coarse emulsion which was followed by sonication (Ultra sonic processor model CV18 of Cole Parmer Company) at 20 kHz operating frequency for 10 minutes. The temperature of sample was maintained at 45°C by cold water.

2.5. Preparation of multilayer nanoemulsion

Primary nanoemulsions NEI was used as a template for the preparation of nanocapsules by sequential deposition of chitosan and CMC polyelectrolytes. NEII were prepared by the addition of Chitosan dispersion (10 mL) drop wise into the 20mL of NEI, and coarsely homogenized for 5 min. It was further ultrasonically homogenized for 7 minutes to acquire the double layered nanoemulsions or NEII. Nanocapsules were prepared by adding 6.66 mL of CMC solution dropwise in 20 mL of NEII and homogenized for 5 min using high speed blender followed by sonication for 3 min to obtain triple layered nanocapsules suspension. Flow diagram of the whole preparation process of nanocapsules suspension is given in Figure 1.
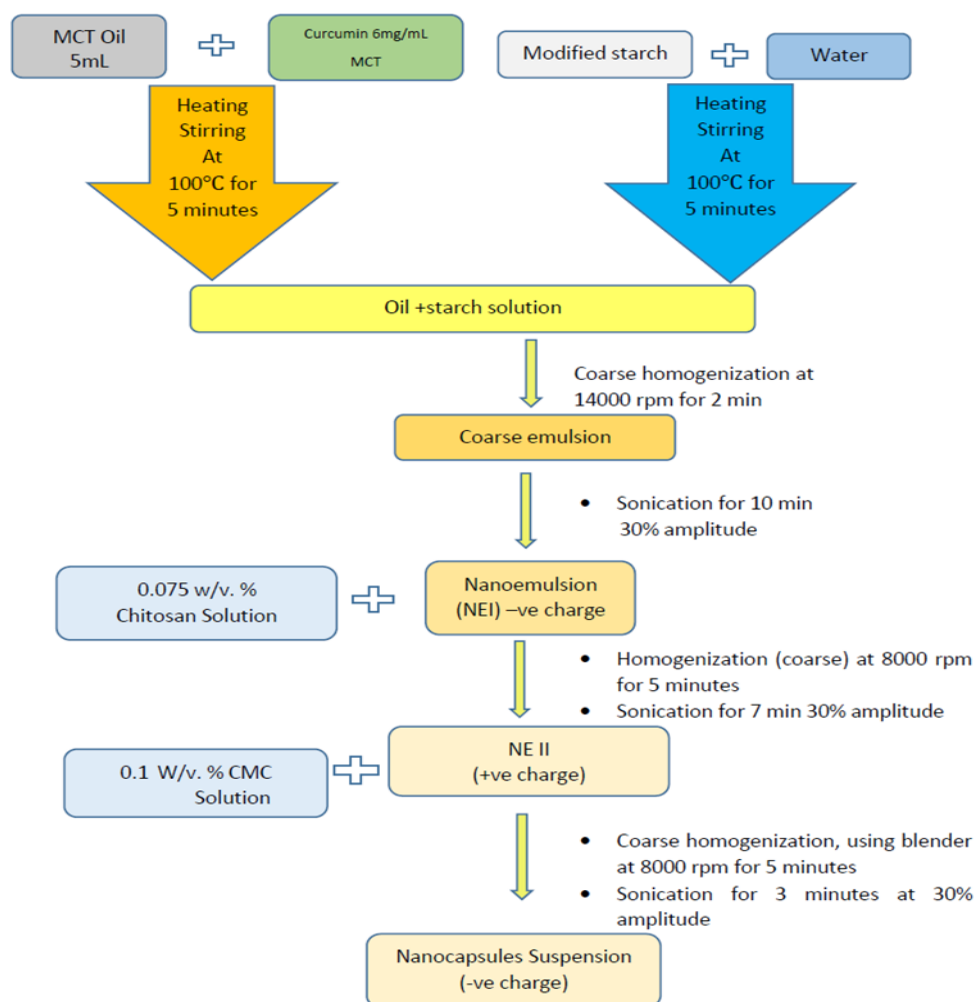

Figure 1 Flow diagram of the preparation of nanocapsules

2.6. Measurement of droplet size and zeta potential

Mean droplet diameter (MDD) and polydispersity index and charge of nanostructures investigated in triplicate by using Zetasizer (Malvern Instruments, Ltd., UK) equipped with dynamic light scattering technology [18]. Samples were diluted 200-folds using purified water. Surface charge of the nanostructures was checked by measuring electrophoretic mobility at 25 °C. The size (MDD) was expressed in nm and charge in mV [25].

2.7. Microscopy

Microscopy was conducted for nanostructures using light microscope (NIKON, model YS 100). Samples were observed at magnifying power of 10X and 40X at room temperature.

2.8. Transmission electron microscopy (TEM)

Electron microscope, JOEL JEM-7000 transmission electron microscope was employed at accelerating voltage of 80kV for morphological study of prepared nanocapsules. Briefly, nanocapsule suspension were put on the carbon coated copper grid and stained with 2% phosphotungstic acid and dried at room temperature before analysis [26].

2.9. *In vivo* Studies

Twelve Sprague Dawley (250-300g, 8-12weeks) rats were used to determine the oral bioavailability of curcumin [27]. Rats were divided in 4 groups under 12h light/dark cycle at 22-26℃ and 50% humidity. After a week of adaptation, rats were fasted overnight and a total of 16mL of control (CMC solution) NE I, NE II, and nanocapsules (4mL each) given at intervals of 30 minutes, were administered in their stomach. Rats were anesthetized with chloroform after 30min. and sacrificed for blood collection. Blood samples for plasma were collected in heparinized vials.

2.10. Plasma separation

Blood samples were centrifuged at 4000 g for 10 minutes at 4 °C to separate the plasma. Plasma samples were stored at −20 °C for further analysis.

Extraction: Curcumin was extracted from plasma samples by adding 0.1 mL acetonitrile into 0.1 mL plasma and centrifuged at 4000 rpm for 5 min. The supernatant was collected and subjected to further analysis by HPLC [28].

2.11. HPLC

HPLC analysis was performed using Shimadzu LC-20A (Kyoto, Japan) HPLC system, consisting the UV–Visible indicator supplied with C8 column (4.6x 150mm, 3μm) with guard column (4.6x20mm, 3μm), kept at 35℃. The mobile phase was composed of two components: (A) 10mM of ammonium acetate buffer solution with adjusted pH 4.5 and (B) acetonitrile. The rate of flow was set to be 1.5mL/minute whereas, the UV-detector was adjusted at 426 nm. Its elution was gradient elution starting with 95% A and 5% B, to be changed t0 55% A and 45% B at 20 min then changed to 5% A and 95% B at 33 min [28].

2.12. Statistical Analysis

All statistical analysis was done using MS-EXCEL-2013 package. All measurements were done for at least three replicates. Results are presented as means ± standard deviation of means.

3. Results

The curcumin nanoemulsion and nanocapsules prepared by coarse homogenization followed by ultra-sonication treatment were characterized for their size (mean droplet diameter or MDD), polydispersity index (PDI) and surface charge using Zetasizer ZS nano (Malvern Instruments, Ltd., UK). Furthermore, their morphological studies were conducted by conventional light microscopy and TEM. Finally, blood plasma was attempted to determine through high performance liquid chromatography (HPLC) for curcumin concentration.

3.1. The MMD, PDI and Zeta Potential

Mean droplet diameter (MDD) is the average of hydrodynamic size of a droplet or particle while polydispersity index (PDI) is the measure of distribution of molecular mass in a sample of polymer. It measures the deviation from the uniformity of a dispersion. Zeta Potential is the charge present on the surface of the droplets.

The MDD, PDI and Zeta Potential NE I, NE II and Nanocapsules are summarized in Table 1.

Table 1 MDD, PDI and Zeta potential of primary, secondary and multilayered nanoemulsions

Sample	MDD	PDI	Zeta-Potential
NE I	142.97 ± 2.51	0.18 ± 0.01	-36.13 ± 1.70
NE II	151.03 ± 5.76	0.16 ± 0.02	+09.30 ± 0.49
NE III	160.57 ± 1.06	0.14 ± 0.01	-24.43 ± 0.49

MDD: Mean droplet diameter, PDI: Polydispersity Index, NE: Nanoemulsion

3.2. Curcumin Loaded Single-Layer Nanoemulsion (NE I)

The sample containing curcumin-loaded nanostructures was analyzed in triplicate and the result was recorded in terms of MDD. Each sample was represented by an individual peak, and their MDD was found to be 144.8 ± 2.51 nm (Figure-2). The average PDI of these samples was recorded as 0.18 ± 0.01. The surface charge on the starch stabilized nanodroplets was negative. Results were recorded in triplicate and the average negative charge present on the droplets was determined as -36.13±1.70 mV, as shown in Figure-3.
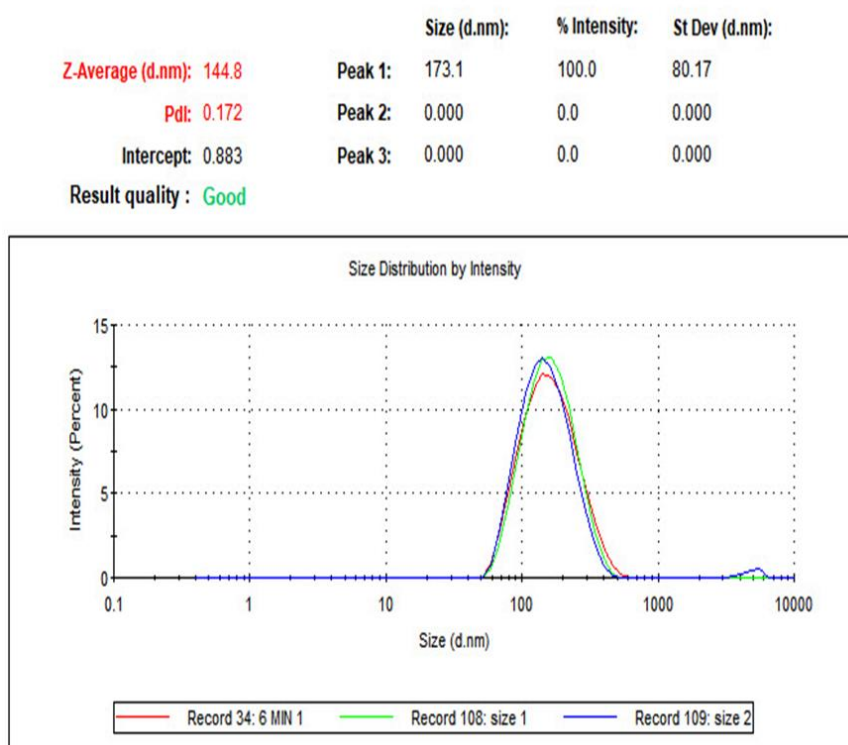
Figure 2 The MDD and PDI of curcumin-loaded nanodroplets (NE I)

3.3. Curcumin Loaded Double-Layer Nanoemulsion (NE II)

The sample was examined three times and result was presented in MDD. The MDD of the sample droplet was found to be 151.03 ± 5.76 nm and the average PDI was recorded as 0.16 ± 0.02 (Figure-4). There was a net positive charge on the surface of double layer nano-droplet. The average positive charge present on the droplets was 9.3 ± 0.49 mV (Figure-5).

	Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -38.1	Peak 1: -38.1	100.0	6.43
Zeta Deviation (mV): 6.43	Peak 2: 0.00	0.0	0.00
Conductivity (mS/cm): 0.00796	Peak 3: 0.00	0.0	0.00

Result quality : Good
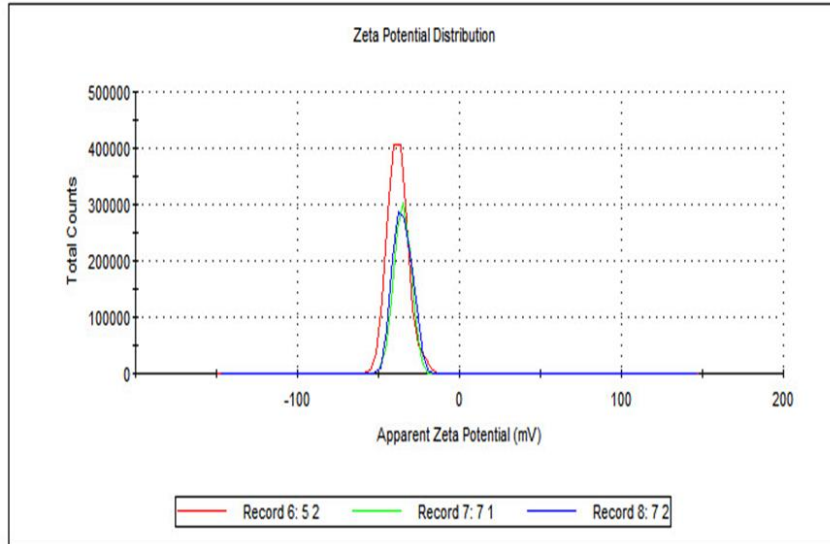
Figure 3 Charge present on the primary nano-droplets NE I

	Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 154.5	Peak 1: 181.7	100.0	76.22
Pdl: 0.139	Peak 2: 0.000	0.0	0.000
Intercept: 0.883	Peak 3: 0.000	0.0	0.000

Result quality : Good
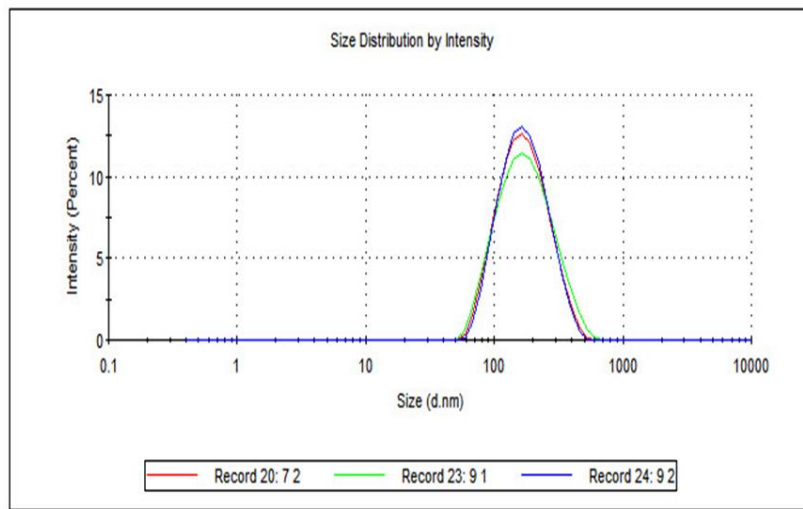
Figure 4 The MDD and PDI of secondary nano-droplets

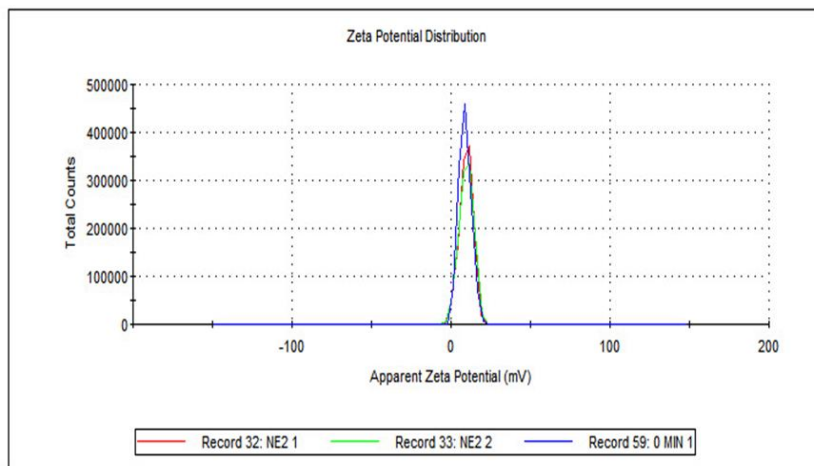|		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): 8.74	Peak 1:	8.74	100.0	3.80
Zeta Deviation (mV): 3.80	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.0362	Peak 3:	0.00	0.0	0.00
Result quality : Good				

Figure 5 Charge present on the surface of secondary nano-droplets

3.4. Curcumin Loaded Nanocapsules (NE III)

The MDD of nanocapsules was recorded as 160.57 ± 1.06 nm. The mean PDI value of these nanocapsules was 0.14 ± 0.01 (Figure-6). The net surface charge on the nanocapsules was negative. The average zeta potential was -24.43 ± 0.49 mV, as presented in Figure-7.

		Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 160.4	Peak 1:	189.0	100.0	76.57
Pdl: 0.144	Peak 2:	0.000	0.0	0.000
Intercept: 0.905	Peak 3:	0.000	0.0	0.000
Result quality : Good				
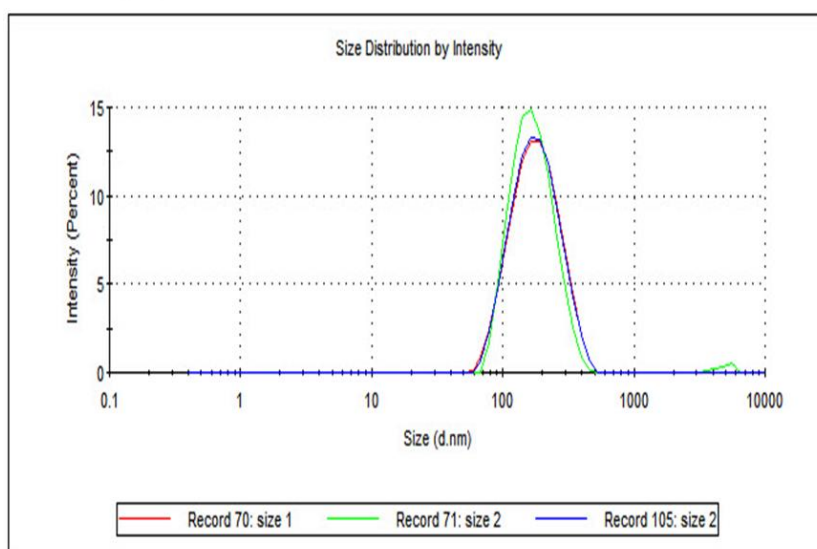
Figure 6 MDD and PDI of nanocapsules

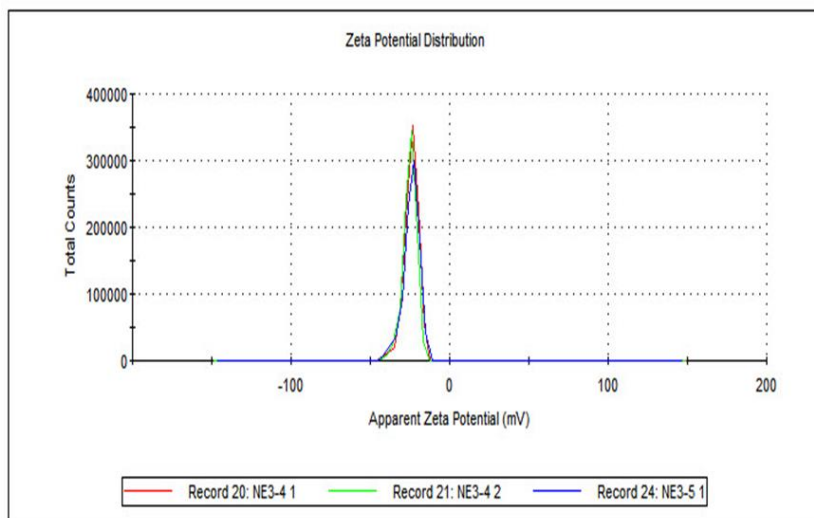|	Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -24.2	Peak 1: -24.2	100.0	4.68
Zeta Deviation (mV): 4.68	Peak 2: 0.00	0.0	0.00
Conductivity (mS/cm): 0.0256	Peak 3: 0.00	0.0	0.00

Result quality : Good

Figure 7 Charge present on the nanocapsule surface

3.5. Microscopy

Microscopy of the samples was done in order to visualize the nanodroplets and nanocapsules and study their morphology.

3.6. Light Microscopy

Light microscopy of the coarse emulsion, nanoemulsions and nanocapsules was conducted using compound microscope. Only coarse emulsions were visible under simple microscope due to their micron (Figure-8a). Nanoemulsions and nanocapsules could not be seen through light microscopy as nanoscale material is difficult to observe (see Figure 8b, c and d).

A   B

| C | D |
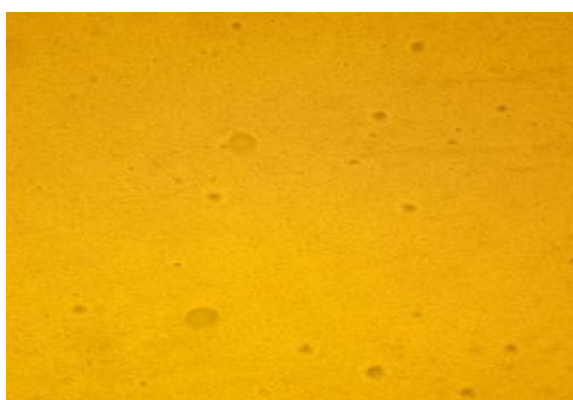
Figure 8 A- Light microscopy of coarse emulsion; B- Light microscopy of NE I; C- Light microscopy of NE II
D- Light microscopy of NE III

3.7. Transmission Electron Microscopy (TEM)
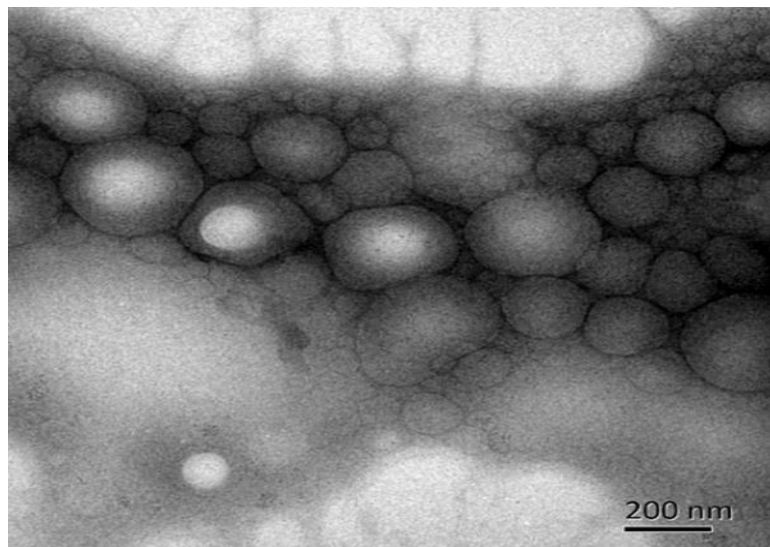
Figure 9 TEM image of nanocapsules showing the morphology and the core material

The morphology of nanocapsules was determined through TEM analysis and according to it, the average number of the nanocapsules had lower than 200 nm sizes and these core-shell structures are spherical in shape. The core material was also visible through the thick outer layer as core part appeared to be lighter and the outer layers appeared darker in color.

3.8. *In-vivo* Absorption Study

Quantification of curcumin present in blood plasma was conducted by HPLC method in order to assess the absorption of curcumin loaded in nanocapsules. In the first step, standard chromatogram was obtained for pure curcumin compound, as shown in Figure 12a.

Sample Name: Standard 250 ppm   Vial Type: STD1
Injection from this vial: 1 of 1   Volume: 10.0 ul
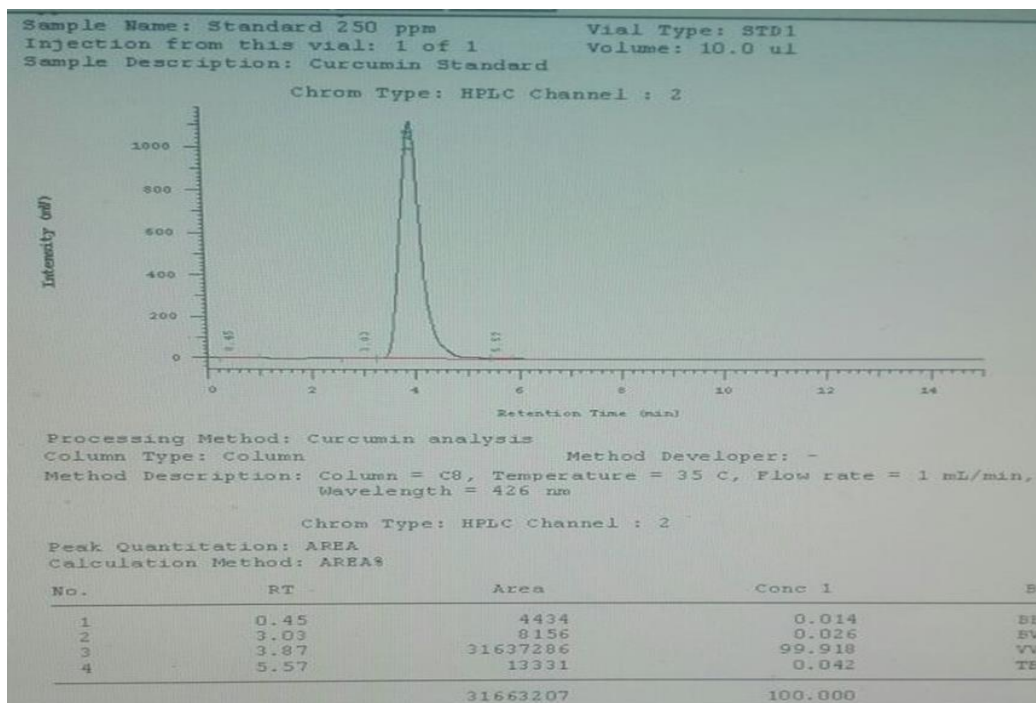Sample Description: Curcumin Standard

Chrom Type: HPLC Channel : 2

Processing Method: Curcumin analysis
Column Type: Column   Method Developer: -
Method Description: Column = C8, Temperature = 35 C, Flow rate = 1 mL/min,
                    Wavelength = 426 nm

Chrom Type: HPLC Channel : 2

Peak Quantitation: AREA
Calculation Method: AREA%

No.	RT	Area	Conc 1	BC
1	0.45	4434	0.014	BB
2	3.03	8156	0.026	BV
3	3.87	31637286	99.918	VV
4	5.57	13331	0.042	TBI
		31663207	100.000	

Figure 10a A chromatogram showing standard curcumin HPLC profile

alyzed Date and Time: 03/29/2018   Reported Date and Time: 03/29,
                05:19 PM   05:58
ocessed Date and Time: 03/29/2018
                05:27 PM
ta Path: C:\Win32app\D2000HSM\samples\DATA\0858\
ocessing Method: Curcumin analysis
stem (acquisition): Sys 1   Series: 0858
plication(data): Samples   Vial Number: 1
mple Name: Sample: P-NE-I   Vial Type: STD1
jection from this vial: 1 of 1   Volume: 10.0 ul
ample Description: Curcumin Standard

Chrom Type: HPLC Channel : 2

Processing Method: Curcumin analysis
Column Type: Column   Method Developer: -
Method Description: Column = C8, Temperature = 35 C, Flow rate = 1 mL/m
                    Wavelength = 426 nm

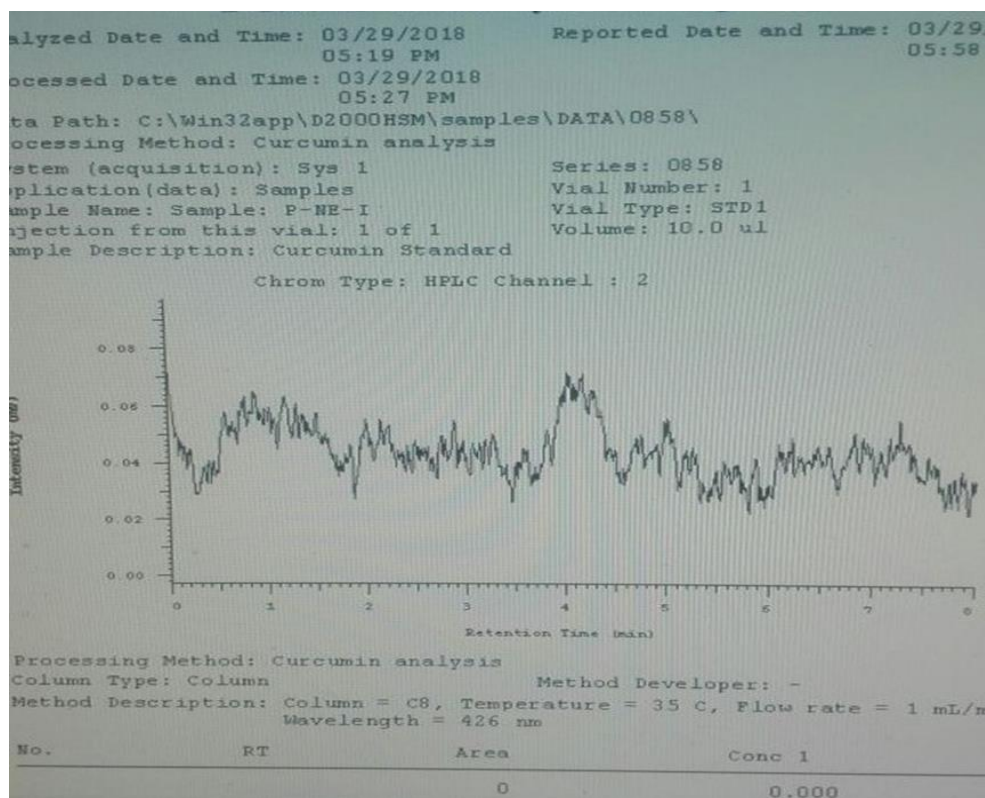| No.	RT	Area	Conc 1
		0	0.000

Figure 10b Chromatogram of plasma sample obtained from a rat which was orally administered with curcumin loaded nanocapsule suspension

As no clear chromatographic peaks (in the area of interest) were obtained in the case of blood plasma obtained from the rats administered (orally) with NEI and NEII, their results are excluded from the current study.

4. Discussion

Curcumin, an active component of turmeric that has been used as a food spice and homeopathic medication since long. It found to have preventive and curative properties in numerous diseases due to its antioxidant and anti-inflammatory properties [29]. Due to several challenges like low bioavailability, solubility, rapid metabolism and quick removal from the body its potential benefits cannot be achieved.

In this study, nanotechnology was used to overcome the issue related to curcumin. Firstly, o/w nanoemulsions were prepared by ultrasonication and were further used as template for nanocapsule fabrication. Numerous studies suggest that nanostructures with size range of 20-200nm have increased stability against physical destabilization [30-32]. In our study, the prepared nanostructures had size below 200nm.

The mean diameter of nanocapsules was recorded 160.57 ± 1.06 nm which was in accordance with [18] in which the mean diameter of nanocapsule was 159.85 ± 0.92 nm. Likewise, Mishra et al. [33] prepared stable multilayered nanoemulsion using layer-by-layer technology, and particle size were in range of 90.8 to 167.8 nm. Many studies found that stability of encapsulated compound against oxidation was improved in multilayer nanoemulsions [34].

The PDI value below 0.2 is considered reliable as it indicates the uniformity in size of nanostructures. The nanostructures prepared in our study had average PDI value ranging from 0.14 ± 0.01 to 0.18 ± 0.01.

The surface charge (expressed in mV) on the nanostructures is an essential parameter in maintaining stability of the system by their electrostatic behavior. In case, particles are highly positive/negative, strong repulsion will occur and if they are weakly charged, coalescence occurs, hence declining system's stability. Zanotto-Filho et al. [22] prepared multilayer nanocapsules having the zeta potential value of -9.56 with enhanced efficiency. The mean charge value on the particles prepared in our study was recorded as -24.43 ± 0.49 mV which is somewhat higher that recorded by Abbas et al., 2015, i.e., -17.2 ± 0.35 mV [22]. Similar results were recorded by Mishra et al. [33] and Langella et al. [35] with the zeta potential values of 22.9 to 31.01 mV.

TEM gives information on the morphology of the prepared nanostructures i.e. size, shape, and wall thickness [36]. According to the TEM results, the particles had spherical shape and outer shell could be differentiated form inner core. Spherical shape provides surface area and mobility hence assisting in absorption of bioactive compound being loaded.

HPLC was conducted to assess the oral bioavailability of encapsulated bioactive.

However, a compound peak was obtained (Figure 10b), which indicates that there was interference of other compounds (usually proteins) as well. As a result, the quantification of curcumin was almost impossible. However, the presence of curcumin in the plasma samples of rat is indicated by the chromatograph. Nanocapsules helped in raising the level of curcumin in the blood. Curcumin was not detected from plasma of rats which were fed on NEI and NEII by this method.

The discussion is concluded with statement that "curcumin could not be detected using conventional methods as probably it was reduced and converted into metabolites and its actual concentration became too low for detection.

5. Conclusion

In this study, nanostructures including curcumin-loaded polymeric nanocapsules using high energy method (ultrasonication) were successfully prepared. As all three type of nanostructures were prepared from food grade material, they could be good candidates as a vehicle for the oral delivery of nutrients including curcumin. Due to poor water-solubility and chemical stability, curcumin bioavailability is significantly compromised. In our study, only nanocapsules (triple-layered structures) showed increased oral bioavailability of curcumin. However further study is recommended to exactly quantify the curcumin concentration in blood plasma.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

Statement of ethical approval

Animals used in this study were handled, according to ethical guidelines of COMSATS University Islamabad, Pakistan.

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