Encapsulation of Frankincense Essential Oil by Microfluidic and Bulk Approaches: A Comparative Study

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Abstract: Herbs and hand-made herbal remedies have been long used for disease control, treatment, and health improvement. Evidence suggests that a single herb's compounds can have synergistic functions with probably no adverse effect. Unfortunately, the traditional administration of essential oil (EO) does not follow a regulatory dose regimen, causing reduced bioavailability due to EO evaporation or denaturation in harsh biological environments. Hence, encapsulation can help enhance EO stability and dose dependence. Here, chitosan nanoparticles (ChNPs) were used to encapsulate frankincense essential oil (FEO) to preserve its remarkable therapeutic effects. Also, traditional bulk preparation methods were compared with microfluidic with central aqueous stream configuration (CAS). The results indicated the higher quality of microfluidic-based nanoparticles with uniform, spherical, and separated morphologies. The size of bulk and microfluidic nanoparticles ranged from 86 to 118 nm, the surface charge varied from 12.5 to 19.5 for different amounts of FEO, and the as-prepared colloid remained stable for three days. Narrow size distribution (PDI ≤ 0.28) and high encapsulation efficiency (EE ≥ 99%) were achieved using the microfluidic process compared to the bulk method (PDI = 0.74, EE ≤ 90%). The microfluidic nanoparticle also indicated a burst release rate greater than the bulk method (≤ 30%). It was concluded that the high-performance microfluidic approach provided higher quality and more control over nanoparticle properties.

Keywords: Chitosan nanoparticle, Ionic gelation, Microfluidic flow-focusing device, Poly(methyl methacrylate) chip, Herbal medicine

Introduction

With the failure of new therapies and their invasive approaches against various diseases, such as microbial and genetic disorders, medicinal plants have recently become attractive alternatives for synthetic drugs. These alternatives have a long history of being applied for treatment, diagnosis, or general healthcare improvement [1]. Herbalists believe that enhanced therapeutic potency and no side effect can be achieved if the entire herb compounds are administered without purification, which seems to be the prominent advantage of herbs compared to synthetic drugs [2]. However, achieving patient compliance and dose-dependent administration using plant extract or essential oil is easy. These natural alternatives contain all active ingredients of herbs separated from inert compounds by physical routes. It is usually more convenient to prescribe and use plant extracts or essential oils that are more absorbable without digestion. Essential oils and extracts are also used as preserving or flavoring agents in foods and cosmetics [3]. In this context, it is imperative to apply antimicrobial extracts to preserve food products during chilled storage [4,5]. Hence, using herbal treatments, also known as phyotherapeutics, is a practice that presents promising tools to improve medical performance against incurable diseases, such as new coronavirus (COVID-19) through different administration routes, i.e., topical or inhalation [6]. Frankincense is a resin secreted from Genus Boswellia plants in semi-arid lands. Terpenoids and phenolic compounds are the most-appeared chemicals in frankincense essential oil (FEO), with remarkable therapeutic effects that have been documented by herbalists and health investigators [7]. Frankincense essential oil (FEO) is anti-cancer, antimicrobial, antioxidant, anti-arthritic, anti-asthma, immunostimulatory, anti-inflammatory, and hypoglycemic [8]. Various administration procedures have been introduced for FEO, including aromatherapy, gastrointestinal route, and topical application [9]. Like other essential oils, FEO is inherently volatile at ambient temperature and chemically unstable upon exposure to air or harsh biological conditions, e.g., gastric acid and enzyme interaction [10], leading to its deficient therapeutic potential and several side effects. Hence, it is suggested to study new drug delivery systems.

Chitosan nanoparticles (ChNPs) are well-known drug delivery vectors that benefit simultaneously from chitosan biopolymer and nano-sized structure properties. Thanks to its nontoxicity, biodegradability, antimicrobial and nature-derived characteristics, chitosan is widely used in multiple industries, from food to biomedical sectors [11]. Chitosan nanoparticles can enhance the bioavailability of drugs by crossing through bio-barriers while preserving their initial active potentials [12]. Studies have indicated enhanced
antioxidant and antimicrobial properties of EOs against fungi, gram-positive and gram-negative bacteria upon their encapsulation in ChNPs [13,14]. Kavaz et al. demonstrated that Cyperus articulates rhizome EO-loaded ChNPs had a stronger cytotoxic effect against MB-231 breast cancer cells after 48 hours compared to unloaded EO [15]. Various procedures have been used to develop ChNPs, such as ionotropic gelation, microemulsion, emulsification solvent diffusion (nanoprecipitation), polyelectrolyte, and reverse micelle. However, there are multifold concerns associated with organic solvent and unreacted crosslinkers in biomedical fields [11]. In this regard, the Sotelo-Boyás team developed limb EO-loaded ChNPs using the nanoprecipitation method for antimicrobial purposes giving rise to nanoparticles with bimodal size distribution in 6-18 nm and 100-300 nm [16]. All methods mentioned above can be linked to the bulk polymerization method based on size variation and non-uniformity in particle quality and the significant amounts of valuable reactant [17].

Microfluidic is a novel technique for improving the performance of traditional methodologies in biology and engineering. By controlling the liquid parameters in micro-scale, the microfluidic approach offers ultrafine monodisperse nanoparticles with an enhanced reproducibility not achievable by bulk methods [18]. Several studies have reported the micro- and nano-encapsulation of plant extractions or terpenoids using microfluidic methods, although the encapsulation of all EO compounds has been rarely addressed [19,20]. A review of previous studies shows that the microfluidic method has not been employed to prepare ChNPs containing multiple plant-derived chemicals, such as EOs or herbal extracts.

Thus far, microfluidic nanoparticles have been widely compared with bulk methods for incorporation of various chemical or natural drugs [21-23]; however, this comparison has not yet been performed in the case of EOs. So, this study aims to encapsulate beneficial FEO into ChNPs using bulk and microfluidic approaches for comparative characterization of nanoparticles. To this end, quantitative analysis was performed by the thermogravimetric analysis (TGA), X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR) and Dynamic Light Scattering (DLS) techniques for thermal and molecular properties as well as colloidal stability measurement. The essential oil's encapsulation efficiency (EE) and loading capacity (LC) were assessed using UV-vis spectroscopy. Also, the Field-Emission Scanning Electron Microscopy (FESEM) images were used for size and topographical measurement. Finally, in vitro release profile was plotted, and the release model was determined.

**Experimental**

**Materials**

Medium molecular weight chitosan and phosphate buffer saline tablets were purchased from Sigma-Aldrich St. Louis, MO, USA. Tween 80, dichloromethane, sodium tripolyphosphate (TPP), and acetic acid 100 % were supplied by Merck & Co Inc., Kenilworth, NJ, USA. Ethanol 90 % was provided from Kimiagar Toos, Mashhad, Iran. Finally, the Indian frankincense of *Boswellia serrata* was purchased from a local medicinal plants shop in Semnan, Iran. The chemicals were applied with no further purification.

**Methods**

**Essential Oil Extraction**

FEO was extracted by the traditional hydrodistillation method using a Clevenger apparatus. In a typical procedure, 200 g frankincense resin was ground and added to a 5000 ml round-bottom flask. Then, distilled water was added at the ratio of 10:1 ml/g. The flask was then connected to the Clevenger set-up. After two hours of boiling, the entire EO content floating on top of aqueous distillates was collected for further analysis.

**Preparation of FEO-loaded ChNPs**

**Bulk Nanoparticle Synthesis**

Two-step bulk synthesis was performed according to Hosseini *et al.* [24] with minor modifications. At the first step, 10 ml of chitosan solution 1 % (w/v) was prepared by dissolving chitosan powder in an aqueous phase of 1 % (v/v) acetic acid solution, followed by overnight stirring at room temperature. After centrifugation for 30 min at 4500 rpm, the supernatant was passed through a 0.45 μm syringe filter to remove possible impurities. Then, 45 mg of Tween 80 was added and agitated for 2 h at 45 °C. Different amounts of FEO (8, 16, and 32 %) were separately dissolved in dichloromethane to make 1 ml of the organic phase, then added dropwise to the chitosan solution under proper agitation rate (11000 rpm) in the ice bath. The agitation continued for more than 10 min till reaching a milky emulsion solution. Next, 10 ml of the TPP solution 0.4 % (w/v) was dropwise added and agitated continuously for another 10 min under ice bath conditions. Finally, centrifugation was performed to collect nanoparticles at 4500 rpm for 30 min at 4 °C.

**Microfluidic Nanoparticle Synthesis**

The protocol reported by Pessoa *et al.* [25] was followed for microfluidic nanoencapsulation with CAS flow configuration. The direct synthesis was performed using a flow-focusing microfluidic chip with specific dimensions engraved by micro-milling on a polymethyl methacrylate (PMMA) sheet (Figure 1(A)).

Chitosan was dissolved in aqueous acetic acid 1 % (v/v) under overnight agitation 10 ml, 0.05 % (w/v). Ten mg of Tween 80 was subsequently added and stirred for 2 h at 45 °C. Different amounts of FEO 8, 16, and 32 % were separately dissolved in dichloromethane to make 0.2 ml of the organic phase, then added dropwise to chitosan solution during agitation under ice bath conditions. Homogenization
continued for a further 10 min to achieve a stable emulsion. Then, 40 ml of TPP solution 0.1 % (w/v) was also prepared. Chitosan and the TPP solution flow were connected to the side channels, and an aqueous acetic acid solution 1 % flowed through the main channel (Figure 1(A)). Considering Q as volumetric flow rate (μl/min), an optimum flow rate ratio of 1.15 (Q_{CHI}+Q_{TPP})/Q_{WATER} was chosen with 20 μl/min for the main flow and 13 μl/min for the sheath flow. Suspensions were collected from the outlet for two hours while the temperature was kept low using an ice pack to preserve FEO before encapsulation. Finally, centrifugation was conducted at 4500 rpm for 30 min at 4 °C to collect nanoparticles.

According to three different concentrations of FEO and three repeats, twelve specimens for bulk, including blanks, and nine specimens for microfluidic were considered for analysis. In both procedures, nanoparticles were washed twice and probe sonicated at the frequency of 100 Hz for 4 min and alteration of 1 sec (on and off). Samples were freeze-dried at -70 °C for three days, if necessary.

**Instrumental Analysis**

Gas chromatography-mass spectrometry (GC-MS) analysis was performed for FEO characterization using Trace MS analyzer (ThermoQuest-Finnigan; Waltham, MA, USA) equipped with a DB-1 fused silica column (60 m×0.25 mm (inner diameter), film thickness=0.25 μm). The oven temperature was raised from 60 °C to 250 °C at a rate of
5 °C/min, and then held at 250 °C for 40 min; transfer line temperature was 250 °C. The quadrupole mass spectrum was scanned over the 45-465 amu with an ionizing voltage of 70 eV and an ionization current of 150 μA. with helium gas carrier.

The constituents of the oil were identified by calculation of their retention indices under temperature-programmed conditions on a DB-1 column for n-alkanes \((C_n-C_{14})\) and the oil under the same conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those reported in the literature. Quantitative data were obtained from FID area percentages without the use of correction factors.

The sizes of particles, distribution, and agglomeration were measured by DLS analysis using a NANOPHOTON 90-246V (Sympatec; Clausthal-Zellerfeld, Germany) at the wavelength of 623 nm. Two ml of particle suspension (16 % FEO) were taken after sonication and before being dried.

Zeta potential of the bulk sample with different amounts of FEO (8, 16, and 32 %) was measured after drying and resuspension in deionized water by SZ-100z Zeta potential analyzer (Horiba; Kyoto, Japan).

The shape and morphology of particles were evaluated by FESEM (Tescan; Brno, Czech Republic). About 0.5 mg of dried bulk and microfluidic samples (16 % FEO) were suspended in 2 ml of deionized water using a low-rate shaker for 5 min. Then, 50 μl of samples was placed on a laboratory slide and dried at room temperature. The samples were coated by a thin layer of platinum using a sputter-coater, and the required microscopic images were taken.

The FTIR analysis was performed to evaluate the encapsulation of FEO by analyzing the functional groups. FEO, chitosan powder, FEO-loaded ChNPs (16 %), and unloaded particles obtained by bulk preparation were analyzed at the wavenumber range of 400-4000 cm\(^{-1}\) and the resolution of 4 cm\(^{-1}\) by a spectrometer supplied by PerkinElmer Inc., Waltham, MA, USA.

The thermal stability of FEO and bulk nanoparticles was explored by the TGA analyzer- STA503 supplied by BAHR, Hüllhorst, Germany. The analysis was conducted on 1 ml of FEO and 5 mg of bulk nanoparticles (8, 16, 32 % and unloaded particle) taken after freeze-drying under inert argon gas from 25 to 600 °C at a heating rate of 10 °C/min.

The XRD patterns were recorded over a 20 range of 5-50 ° using XRD-D8 supplied by Bruker Co; Karlsruhe Germany. Then, 3 mg of chitosan powder as well as unloaded and FEO-loaded nanoparticles (16 %) from the bulk method were tested.

For encapsulation efficiency (%EE) assessment, the free drug content was measured before washing the particles. 2 ml ethanol was added to 10 ml of loaded particle suspension after synthesis and homogenized by vigorous shaking. After 30 min of high-rate centrifugation at 4 °C, the supernatant was analyzed by UV-visible Nanodrop Spectrophotometer supplied by Thermo Scientific, Wilmington, DE, the USA at 334 nm. Then, deionized water, ethanol, and dichloromethane were used as blank samples. The free drug was measured using a calibration curve of 99.5 % adaptation \((y = 0.0544x)\), and EE% was obtained using equation (1):

\[
EE(\%) = \frac{\text{initial amount of FEO} - \text{unloaded amount of FEO}}{\text{initial amount of FEO}}
\]

Loading capacity of ChNPs was also measured using EE data and equation (2):

\[
LC(\%) = \frac{\text{total amount of loaded FEO}}{\text{weight of dried nanoparticles}}
\]

In vitro release studies were accomplished at pH 7.4 in 24 hours for bulk and microfluidic samples. 2 ml of elution solution (60 % PBS and 40 % ethanol) was added to a microtube containing 8 mg of dried loaded nanoparticles. All microtubes were put into Erlenmeyer and incubated at 37 °C under gentle agitation for 24 hours. At specific time intervals, samples were centrifuged at 4500 rpm for 5 min. Then, 5 μl of supernatant was analyzed by a UV-visible nanodrop at 334 nm in which ethanol, dichloromethane, and deionized water were used as blank.

Results and Discussion

### FEO Characterization

The FEO obtained by hydrodistillation was very light yellow in color and perfumery odor. The GC-MS chromatogram indicated 18 organic compounds in FEO with most monoterpenes, while previous studies report up to 80 compounds differentiated based on extraction procedures, frankincense species, environmental factors, time of gum picking, and storage conditions [26]. According to Table 1, higher percentages belong to N-octyl acetate and octanol with no recorded therapeutic effect, acting as the solvent for the other compounds [27]. The literature describes α-thujene and α-pinene as major EO compounds obtained by hydrodistillation, while the former has no registered therapeutic effect [28,29]. The therapeutic properties of compounds are also summarized in Table 1.

Positive effects can overlap between the compounds, demonstrating signs of synergistic capabilities. However, some chemicals probably reduce the side effects of the other chemicals, i.e., anti-inflammatory and sedative products facilitate anti-tumor function and cause more patient compliance as traditional medicine claims [30]. All terpenoids are volatile and sensitive to unwanted reactions. Moreover, anti-inflammatory, anti-tumor, and antioxidant compounds

### References

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can be more efficient if encapsulation overpasses the biological barriers.

Table 1. Main chemical constituents and therapeutic effects of FEO reported in the literature

| Chemical name                  | Conc. (%) | Therapeutic effect | Reference |
|-------------------------------|-----------|--------------------|-----------|
| α-Pinene                      | 0.38      | -                  | [64,65]   |
| Methylanisole                 | 0.26      | -                  |           |
| p-Cymene                      | 0.08      | -                  | [66]      |
| Limonene                      | 1.07      | -                  | [67]      |
| 1,8-Cineol                    | 0.19      | -                  | [68]      |
| E-β-Ocimene                   | 0.47      | -                  | [69]      |
| 1-Octanol                     | 4.87      | -                  |           |
| Linalool                      | 0.5       | -                  | [70]      |
| 2,3,3-trimethyl-1,4-pentadiene| 0.5       | -                  |           |
| n-Octyl acetate               | 84.99     | -                  |           |
| Geranial                      | 0.18      | -                  | [71-73]   |
| Geranyl acetate               | 0.21      | -                  | [74]      |
| Decyl acetate                 | 0.17      | -                  |           |
| (E-)Nerolidol                 | 2.44      | -                  | [75,76]   |
| β-Elemene                     | 0.43      | -                  | [77]      |
| Cemberene                     | 1.92      | -                  | [78]      |
| Incensole                     | 0.47      | -                  | [79-81]   |
| Incensole acetate             | 0.87      | -                  | [80]      |

*The colored symbols representing functions: red anti-inflammatory, blue sedative, green anti-tumor, yellow anti-microbial, brown anti-oxidant, pink anti-depressant, orange anti-diabetic.

Shape, Size, and Morphology of ChNPs

Figures 1(B) and (C) show the shape and morphology of bulk and microfluidic loaded nanoparticles (16 % FEO). Both loaded particles were spherical and uniform. They remained stable after the preparation and freeze-drying process. Nevertheless, agglomeration was observed in the bulk sample even after sonication impacting pharmacokinetic behaviors, such as biological distribution, elimination rate, and release profile. Lack in precisely controlling the mixing process in bulk preparation resulted in aggregated particles. Simultaneous processes of nucleation, growth and agglomeration uncontrolled, also ended up with high batch to batch variation of physicochemical properties of these particles. But microfluidic nanoparticles were completely separated and exhibited a more uniform size distribution than their bulk counterparts, which can be attributed to the lower concentration of precursors and the use of the CAS synthesis procedure under mild reaction conditions. Here, because of faster mixing time, nucleation and growth process can be isolated as a function of channel length and particle agglomeration could be avoided [31]. CAS also prevented channel clog, usually by forming microfiber-like structures instead of the particle inside the channels [25]. Furthermore, microfluidic offered higher mono-dispersity (PDI=0.27) rather than the bulk method (PDI=0.74) according to the DLS results (Figure 2(A, B)).

As obtained by DLS, the average hydrodynamic size was 86 nm and 118 nm for bulk and microfluidic nanoparticles. However, the microfluidic method's tunable flow rate can decrease the particle size compared to the bulk. Besides, freeze-drying seemed mostly responsible for the agglomeration of bulk samples since no agglomeration was observed in

![Figure 2](image-url) (A, B) DLS results of size distribution for (A) bulk and (B) microfluidic nanoparticle (16 % FEO); (C, D, E) zeta potential of bulk ChNPs nanoparticles containing different amount of FEO; (C) 8 %, (D) 16 %, and (E) 32 %.
DLS results [32]. This agglomeration can be attributed to the higher surface energy of bulk nanoparticles due to non-optimized precursors and harsh reaction conditions. Finally, the dry particle size was measured in the range of 30 to 70 nm from FESEM photographs in both samples adapted to the nanoscale. The report of Liu et al. [33] well agrees with our results as they found that the microfluidic method is more appropriate in reaching narrow size distribution and uniform shape. Using their flow-focused microfluidic platform, all three polymeric nanocarriers (PLGA, HPCS, and AcDX) were smaller than those obtained through bulk preparation. Tahir et al. [34] used the same platform to synthesize lipid-polymer hybrid nanoparticles with particle sizes ranging from 191.8-302 nm, higher than the nanoparticles synthesized via bulk nanoprecipitation (187.9-203.3 nm). However, lower PDI belonged to microfluidic, which is fully in agreement with this study. Streck et al. [35] obtained smaller microfluidic PLGA nanoparticles with higher PDI than the bulk method, while the nanoprecipitation method was employed. They reported higher ratio between aqueous and organic phases led to less agglomeration and lower PDI in bulk procedure compared to microfluidic with a lower ratio.

Zeta Potential

In this study, surface charge evaluation contributed to the clarification of release profile and colloidal stability measurement. As shown in Figure 2(C-E), surface charges of all three EO-loaded bulk nanoparticles were in a positive range showing the positive nature of chitosan even in the neutral environment, as mentioned in the literature [36]. Surface charges of 13.5, 12.5, and 19.5 mv were obtained for loaded-nanoparticle (8, 16, and 32 %, respectively), which increased from +13.5 to +19.5 with an increasing amount of FEO. This indicates that the particles’ surfaces are positively charged. A minimum Zeta potential of ±30 mv for electrostatic stabilization and ±20 mv for the combination of electrostatic and steric stabilization is desirable [36]. In the recent work, Zeta potential was absolutely below 30 mv for bulk nanoparticles and did not improve the colloidal stability. However, no aggregation was observed in three days, completely in agreement with the observation of Onyebuchi et al., for preparation of Ocimum Gratissimum EO-loaded ChNPs [37]. This indicates an increased dispersity of FEO, which has no solubility in water.

Higher amounts of FEO probably hinder or decrease the crosslinking density of amine groups of chitosan with TPP and expose a higher positive charge on the surface [38]. However, some investigations reported a deducted charge in a higher amount of EO [39,40]. According to the negatively charged cell membrane, endocytosis is more likely in these nanoparticles, although elimination by macrophage is unavoidable [41]. The positive charge can also improve the antibacterial function of FEO [40]. Altogether, according to the low Zeta potential and insignificant Zeta difference between the samples due to the neutral charge of octyl acetate as the major compound, it cannot generally affect on release profile and colloidal stability of samples.

FTIR

Successful encapsulation was shown by identification of the functional groups. Figure 3 indicates the characteristic peaks of chitosan at 3323 cm$^{-1}$ (OH and NH-stretching), 2883 cm$^{-1}$ (CH-stretching), 1650 cm$^{-1}$ (amide I), 1588 cm$^{-1}$ (amide II and N-H bending), 1429 cm$^{-1}$ (OH-bending), 1082 cm$^{-1}$ (C-O-C stretching) and 570 cm$^{-1}$ (glucose ring stretching) [27,39,42].

In the case of dichloromethane-loaded (unloaded) particles, the peak of N-H bending shifted from 1588 to 1538 cm$^{-1}$, and a new peak appeared at 1252 cm$^{-1}$ (P=O and P=O), reflecting the desired interaction of chitosan and TPP moieties. FEO also showed individual peaks at 2932 cm$^{-1}$ (C-H stretching) for alkane, 1769 cm$^{-1}$ (C=H stretching) for carboxylic acid and other esters, 1469 cm$^{-1}$ (C-H bending) for methylene, and (S=O stretching) for sulfonamides which are known as antibacterial and antiparasite compounds, 1235 cm$^{-1}$ (C-N stretching) for aromatic amine, 1042 cm$^{-1}$ (CO-O-CO stretching) for anhydride and 722 cm$^{-1}$ for aromatic C-H bond [39,43,44]. According to Figure 4, all of the peaks mentioned above appear in the loaded sample spectra at about the same wavelength showing successful encapsulation with no undesirable interaction between the particle and FEO [45].

Thermal Analysis

TGA was performed to evaluate the thermal stability of FEO before and after encapsulation (Figure 4). Moreover, the degradation temperature (T_d) corresponds to the maximum mass loss, which is clearly demonstrated as a peak in the DTG thermogram [46]. The FEO graph shows one-step weight loss (two overlapping peaks) starting at 100 °C (peak at 164 °C). Similar results were reported in the

Figure 3. FTIR spectra of FEO, chitosan powder, unloaded and loaded ChNPs with 16 % FEO.
literature for other EOs [14,46]. The weight loss of unloaded ChNPs appeared in three major thermal events. The first one at 60-120 °C corresponds to the evaporation of bonded water, while the second one at 220-280 °C (peak at 254 °C) is for decomposition of ChNPs [47]. The third one at 300-370 °C (peak at 331 °C) should be ascribed to the vaporization of dichloromethane existing in the sample. Nonetheless, Hosseini et al. reported that the third peak is related to chitosan crosslinked with TPP [24]. After the encapsulation of FEO, the peak of dichloromethane disappeared, and no peak was manifested for EO in all FEO concentrations. As ChNPs thermograms indicated the same percentage of weight loss around 250 °C (almost 25 %) and no peak was found corresponding to T_d of EO, FEO elimination seems to be extended and occurred gradually with polymer weight loss above 250 °C. The interaction of lipophilic FEO might explain this with ChNPs or specifically Tween 80 emulsifier. This possibility can be relevant to the slight displacement of the FEO amine peak (at 3500 nm) in the FTIR, indicating a weak oil-particle interaction that optimistically increases the encapsulation efficiency.

Jahed et al. [48] stated that the -NH bending peak shift from 1600 to 1570 after oil incorporation probably represents the interaction between chitosan and EO. Moreover, the thermal stability of encapsulated FEO was significantly enhanced by at least 1.5 fold which is in agreement with the findings reported by Karimirad [49] Kujur [46] and Hadidi [14]. Song et al. [50] reported that by incorporating Mandarin essential oil into ChNPs, thermograms remained unchanged because adding essential oil did not affect the molecular mobility of chitosan chains. Finally, less than 50 % of the ChNPs' weight remained at 600 °C, which can be related to the polymer residues and minerals like TPP [49].

XRD Patterns

Figure 5 shows the XRD pattern of chitosan before and after encapsulation of EO. Chitosan showed two sharp overlapping peaks at 2θ of 11 ° and 20 °, attributed to hydrated crystals and the orthorhombic unit cell arrangement, respectively [43,46,48]. Nevertheless, no peak was found at 2θ of 11 ° for unloaded and loaded bulk diffractogram because of hydrogen bond formation between chitosan and TPP. The broader peak of ChNPs at 2θ of 20 ° along with much lower intensity represents the destruction of chitosan crystalline structure by TPP crosslinking reaction [46].

As the width of the XRD peak is inversely related to crystallite size, the broadened peak usually results from a smaller crystallite size in ChNPs [51]. Additionally, the peak shifting toward a higher angle (19 to 21) after loading the EO indicated encapsulation, as Shetta et al. [13] reported. Equal peak height and width of loaded and unloaded samples indicated that EO did not affect the arrangement of ChNPs packing structure, confirming the FTIR results. In a similar study, Hadidi et al. [52] also reported that the characteristic peaks of hyssop EO disappeared after encapsulation in the isolate nano-complex of chitosan-pea protein, confirming a successful encapsulation. Some investigations reported a completely non-crystalline structure for unloaded ChNPs with a flat pattern while the crystallinity increased after oil incorporation showing a new peak at higher angles which could be attributed to the interaction of ChNPs with the oil compounds [14,53].

Encapsulation Analysis

Encapsulation efficiency (EE) and loading capacity (LC) of bulk and microfluidic samples are summarized in Table 2.
A high amount of EO was encapsulated in both procedures (more than 80%), while higher percentages belonged to microfluidic samples. Highly efficient encapsulation can be attributed to the weak oil-particle interaction and highly efficient emulsification that make ChNPs perfectly suited for encapsulation of hydrophobic drugs. Ahmadi [54] and Natrajan [55] also reported a highly efficient encapsulation of EO (more than 70%) in chitosan-TPP and chitosan-alginate using bulk preparation methods. Ahmadi et al. [54] also claimed that EE% is pH-dependent, and higher efficiency is achieved by decreasing the pH of chitosan solution. According to Table 2, encapsulation efficiency is inversely correlated to the concentration of FEO because of multiple reasons. First of all, the low amount of FEO causes fewer side interactions resulting in a higher contact area and crosslinking degree of chitosan with TPP. Second, limited capacity is offered by nanoparticles for encapsulation and hence increasing amount of FEO ends up in saturation of encapsulated oil and thus higher free herbal compounds. Furthermore, as some cargo molecules are loosely absorbed superficially to the nanoparticles at higher FEO concentrations, detachment is possible during nanoparticles collection i.e. by centrifugation. This is in full agreement with finding of Amiri et al. in the field of food preservation [56]. Interestingly, the EE of microfluidic-based nanoparticles was slightly higher than that of the conventional bulk method. Actually, poor control over fluid flow is represented by the bulk mixing method leading to rapid aggregation. However, in the case of microfluidic, the synthesis process was well-controlled, and the mixing timescale was shorter than the aggregation timescale ($\tau_{\text{mix}} < \tau_{\text{agg}}$) allowing more effective stabilization of nanoparticles and uniform size [21]. Hence drug encapsulation of bulk preparation was not as efficient as microfluidic although the higher amount of EE may be attributed to the larger size of microfluidic nanoparticles.

However, the LC of bulk nanoparticles indicated a reverse trend compared to EE in the range of 10.3-38.2% when the initial EO content was in the range of 8-32%, denoting the high space capacity of ChNPs for the encapsulation of FEO (Table 2). Our results about LC agree with the observation of Amiri [39] and Esmaeili [42] and disagree with the findings of Mohammadi et al. [57], who observed a constant LC with a negligible depletion with increasing the EO content.

### Table 2. EE and LC of FEO-loaded ChNPs prepared by bulk and microfluidic methods

| Preparation method | FEO concentration | EE (%) | LC (%) | EE (%) | LC (%) | EE (%) | LC (%) |
|--------------------|--------------------|--------|--------|--------|--------|--------|--------|
|                    | 8 %                |        |        | 16 %   |        | 32 %   |        |
| Bulk               |                    | 90.4   | 10.3   | 88.6   | 20.2   | 85     | 38.2   |
| Microfluidic       |                    | 99.1   | -      | 97.8   | -      | 93.3   | -      |

### Cumulative Release

The in vitro release profiles of FEO from the nanoparticles obtained using different amounts of EO were evaluated at pH 7.4 for the bulk and microfluidic methods (Figure 6), aiming to investigate the release mechanism and kinetics of FEO. Generally, release from drug-loaded nanoparticles occurs by multiple mechanisms, including diffusion, desorption, surface erosion, and disintegration [58]. According to Figure 6, FEO release from microfluidic and bulk ChNPs can be described as a two-step biphasic process, representing an initial burst release for the first 6 hours followed by a subsequent slower release. At the first step, FEO was released up to 60% and 83% for bulk and microfluidic nanoparticles, respectively. The initial burst release can be assigned to rapid inflation of ChNPs, as well as the release of the FEO absorbed on the surface or encapsulated in top layers, as they require low energy for desorption from the surface and the high dissolution rate of the polymer near the surface [38,47,59]. In the next step, the long-delayed release
can be assumed to be due to the diffusion of the EO from the core of the nanoparticles into the chitosan matrix. The release rate of FEO is very low and graph is so-called plateau in this region. Further release of FEO requires the swelling and destruction of the ChNPs.

The release profile depends on EO concentration, and lower cumulative releases (17 % and 43 % for bulk and microfluidic, respectively) were observed at a higher FEO concentration of 32 % after 24 hours (Figure 7). This may result from a higher amount of dichloromethane included in ChNPs with a lower amount of FEO. As dichloromethane is practically more volatile than FEO, solvent evaporation at 37 °C probably increased the particle pore size and accelerated the release rate compared with samples containing higher FEO dosages. Hesami et al. [45] argued that cumulative release is a function of particle size, reporting that particle size decreases with decreasing amount of Pistacia atlantica hulls’ essential oil, and a higher surface-to-volume may result in the fast release of EO. Other researches also stated that EO release from ChNPs is pH dependent as at low pH, by increasing the hydrogen ion concentration in the environment, amine groups of the chitosan chains increases and causes repulsive force. So, the structure of ChNPs would be weakened and further release of EO would happened [39]. However, microfluidic ChNPs exhibited higher cumulative release despite being larger than bulk, probably due to a lack of agglomeration and higher surface area, resulting in more interaction with elution solution. Majedi et al. [60] observed that decreasing the compactness of microfluidic ChNPs as we go from the smallest flow ratio (polymeric to water stream) to bulk mixing accelerated the release rate. Thus, the release rate of bulk mixing should be higher, which is against the recent study's finding.

To achieve a characteristic release profile from a drug delivery system, it is necessary to know the complicated mechanism of mass transport and predict quantitatively release kinetics. This study used four commonly used kinetic models of zero-order, first-order, Higuchi, and Korsmeyer-Peppas to fit the release data. The formulations and kinetic parameters related to the models are shown in Table 3. As shown, Q is the cumulative release of FEO at time t while A, K, and n are constant, kinetic constant, and diffusion constant, respectively [74]. The Higuchi formulation better fitted the release profile of the bulk samples (correspondence of 97.3 to 99.1 %), indicating a Fickian diffusion process [61]. In the case of microfluidic samples, the Korsmeyer-Peppas equation offered better fitting (correspondence of 97.3 to 98.9 %). This model is also used when the release mechanism is unknown. When n ≤0.43, Fick diffusion is the dominant mechanism of release; when 0.43< n<0.85, implying a non-Fickian anomalous release wherein both diffusion and bulk erosion are involved in the release mechanism; when n ≥0.85, the main mechanism is bulk erosion [61]. As shown in Table 3, diffusion exponent (n) is less than 0.43 for bulk ChNPs showing a Fickian model of release, while an anomalous release behavior was observed for microfluidic nanoparticles through the Korsmeyer-Peppas equation. This observation can be due to the agglomeration-free and completely separated nanoparticles represented by the microfluidic approach, leading to more interaction with the elution solution and simultaneous mechanisms of bulk erosion and diffusion.

Almeida et al. [62] reported that the best correlation coefficient is related to Korsmeyer-Peppas and Higuchi release models for EO-loaded PLGA nanoparticles prepared by the emulsification/solvent diffusion method. Due to n value and Higuchi kinetic, diffusion was recognized as a dominant release mechanism for citral, one of EO's main compounds. Another study performed the incorporation of streptokinase in chitosan nanoparticles by ionic gelation using bulk and microfluidic methods [63]. The authors reported that the best fitting was obtained from the Higuchi model for bulk and microfluidic, confirming the diffusional release of streptokinase from ChNPs.

### Table 3. Kinetic parameters of different mathematical models for release behaviors of FEO-loaded ChNPs

| Mathematical model         | Formulation | 8 % (EO) | 16 % (EO) | 32 % (EO) | 8 % (EO) | 16 % (EO) | 32 % (EO) |
|----------------------------|-------------|----------|-----------|-----------|----------|-----------|-----------|
| Zero-order                 | Q = A + Kt  | 96.8     | 95.7      | 97.3      | 92.5     | 92        | 93.6      |
| First-order                | Q = A ∗(1−exp(−Kt)) | 95.8 | 94.7      | 95.8      | 87       | 84.2      | 81.8      |
| Higuchi                    | Q = A ∗Kt^n | 98       | 97.3      | 99.1      | 97.7     | 97.2      | 96.7      |
| Korsmeyer-Peppas           | Q = A ∗Kt^n | 97.5     | 97.4      | 98.6      | 98.9     | 98.2      | 97.3      |

### Conclusion

In this study, FEO-loaded chitosan nanoparticles were successfully produced using microfluidic and traditional bulk methods. Larger sizes with narrower distribution belonged to microfluidic ChNPs with more efficient encapsulation dependent on EO concentration. Improving the thermal stability was confirmed by a TGA thermogram indicating
the capability of ChNPs to preserve volatile and highly reactive compounds of FEO. In vitro release data indicated a two-step biphasic process confirmed to Higuchi and Korsmeyer-Peppas mathematical models for bulk and microfluidic approaches, respectively. The results showed the superiority and high performance of state-of-the-art direct-synthesis microfluidic for encapsulating essential oils compared to the conventional bulk method. Nonetheless, limitations related to channel blockage and fluid leakage remain.

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**Conflict of Interest**

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

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