Fabrication of amorphous curcumin nanosuspensions using \(\beta\)-lactoglobulin to enhance solubility, stability, and bioavailability

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**A R T I C L E   I N F O**

**A B S T R A C T**

Curcumin has low aqueous stability and solubility in its native form. It also has a low bioavailability which presents a major barrier to its use in fortifying food products. The aim of this work was to reduce the size of curcumin crystals to the nanoscale and subsequently stabilize them in an amorphous form. To this end, amorphous curcumin nanosuspensions were fabricated using the antisolvent precipitation method with \(\beta\)-lactoglobulin (\(\beta\)-lg) as a stabilizer. The resulting amorphous curcumin nanosuspensions were in the size range of 150–175 nm with unimodal size distribution. The curcumin particles were amorphous and were molecularly dispersed within the \(\beta\)-lg as confirmed by differential scanning calorimetry (DSC) and X-ray diffraction (XRD) studies. The solubility of the amorphous curcumin nanosuspension was enhanced ∼35-fold due to the reduced size and lower crystallinity. Among the formulations, the amorphous curcumin nanosuspensions stabilized with \(\beta\)-lg and prepared at pH 3.4 (\(\beta\)-lg-cur 3.4), showed maximum aqueous stability which was >90% after 30 days. An in vitro study using Caco-2 cell lines showed a significant increase in curcumin bioavailability after stabilization with \(\beta\)-lg.

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1. Introduction

In recent years, the food industry has shown an increased desire to incorporate nutraceuticals with properties beneficial to health into food products. Curcumin (diferuloylmethane) is one such yellow hydrophobic polyphenol derived from the herb turmeric (Curcuma longa) [1]. Unfortunately, this compound cannot be readily incorporated into the food products due to its aqueous insolvency which leads to its degradation and trifling bioavailability [1,2].

In the past, several attempts have been made to improve the stability and bioavailability of curcumin using various types of delivery systems and carrier materials (lipsids, polymers, carbohydrates, etc.) [3–6]. Unfortunately their application in food matrix is compromised or minimal due to the factors like toxicity of the delivery systems e.g. carrier material, surfactants, etc., uncorroborated cost to benefit ratio, difficulty in availability of raw materials with required quality, quantity, etc. [7]. Overall, it would be useful if a system could be developed which increases solubility and bioavailability of curcumin which is specifically suitable to use in food products. In this regard recently very few efforts have been made to fabricate curcumin–\(\beta\)-cyclodextrin complex [8,9] and protein nanoparticles [10] to use these nanocarriers in food products. However, further research is highly warranted to discover new types of carrier materials and methods to increase solubility and stability of curcumin in order to fortify their application in food, pharma and cosmetic application.

In this observe, nanonization technique has been used to produce nanosized curcumin particles with an intention of increasing curcumin solubility, stability and bioavailability. To achieve nanonization, antisolvent precipitation method which is a well-accepted method for the production of food-grade nanoparticles and crystals was chosen [11]. However during the antisolvent precipitation, newly formed nanoparticles used to have increased surface Gibbs free (\(\Delta G\)) energy and particle growth tendency by coagulation and condensation which leads to recrystallization. This recrystallization processes compromise the advantage of higher saturation solubility and bioavailability, and faster dissolution rate associated with amorphous state of molecules in comparison to crystalline state of the same molecule [12]. Curcumin which exists in amorphous form initially after precipitation, gets converted into large crystals as time proceeds due to aggregation [13]. Hence to get the maximum solubility and bioavailability, it is necessary to prevent the recrystallization process.
In this regard, to avoid the particle aggregation by reducing the interfacial tension, selection of suitable stabilizer is of utmost importance [14]. In this stair, food proteins are well suited for the desired application due to their non-toxicity and safe history of human consumption. Above or below the isoelectric point, proteins exhibit amphiphilic nature and hence they act as an efficient surfactant. In addition, recent studies have shown to increase the stability, solubility and antioxidant activity of curcumin when either entrapped or after binding with food proteins such as β-lg and bovine serum albumin [15,16]. Further, due to β-lg stability in acidic pH and the presence of β-lg specific receptors in small intestine which will facilitate to achieve our goal of increasing the oral bioavailability of curcumin, β-lg has been selected over other proteins as a stabilizer [17].

Therefore the objective of the study was to: (a) fabricate an amorphous curcumin nanosuspension, using β-lg as stabilizer, (b) study the stability of these nanosuspensions in a model beverage system, and (c) study the in vitro bioavailability of the nanosuspensions using Caco-2 cell lines. This study is novel because of the fabrication of amorphous curcumin nanosuspensions and the avoidance of undesired recrystallization by molecularly dispersing the nanosuspension within β-lg. For the first time we have applied nanonization technique to incorporate bioactive compounds into liquid foods.

2. Materials and methods

2.1. Materials

Curcumin (>95% pure) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Protein β-lg was procured from Davisco Foods International, Inc. (Le Sueur, MN, USA). The protein content was 98.0% and 93.4% on a dry basis and as a percentage of total weight, respectively. The fat, ash, and moisture contents were reported to be 0.1%, 1.9%, and 4.7%, respectively. All other chemicals were of HPLC or analytical grade.

2.2. Preparation of β-lg stabilized amorphous curcumin nanosuspensions

Solutions of antisolvent consisting of water and a hydrophilic surfactant (β-lg; native or denatured; 2 mg/mL) with different pH values (3.4, 5.4 and 7.04) were prepared. Denaturation was done to expose the hydrophobic moieties of the β-lg and pH was adjusted to acidic and basic pH to induce conformational changes in the β-lg. In earlier studies, changes in the pH and heat induced transformation in the basic protein structure are known to affect the curcumin binding with β-lg [15,16]. Sodium azide aqueous solution (0.002%, w/w) was added to the β-lg solution to avoid the bacterial contamination. Both original and denatured β-lg solutions were stored at <4°C for maximum 7 days. The solvent phase was prepared by dissolving curcumin in ethanol (4.0 mg/mL).

To begin with, both solvent and antisolvent phases were cooled below 4°C and then the solvent phase was added to the antisolvent phase under magnetic stirring. The resulting nanosuspension was further processed using a probe-type ultrasonicator (SON-1 VCV130, Sonics and Materials Inc., Newton, CT, USA). Finally, the solvent was evaporated under reduced pressure at 30°C using a rotary evaporator. For the production of amorphous curcumin nanosuspensions, the various processing parameters that were studied were stirring speed (200–1500 rpm), temperature (5–25°C), sonication power (20–40%) and solvent to antisolvent ratio (1:10–1:30). Freshly prepared samples were lyophilized using 1% trehalose as a cryoprotectant. All samples were produced in at least triplicate.

2.3. Particle size, zeta potential and morphology analysis

The average particle diameter, size distribution, and zeta potential (surface charge) of the nanosuspensions were measured, after suitable dilution, using a zeta-potential and particle size analyzer (DelsaNano, Beckman Coulter, Inc., Fullerton, CA, USA) at 25°C with a scattering angle of 165°. Both the size and surface charge measurements were performed in at least triplicate (n ≥ 3) and the reported results are the means of the readings.

The morphology of the lyophilized samples of curcumin nanosuspension was studied using a low vacuum scanning electron microscope (S-350 N, Hitachi Science System, Ltd., Ibaraki, Japan). Each sample was fixed on the surface of a V1 mica disk (20 mm diameter) of the highest quality grade using double-sided tape and then gold-coated under vacuum using a sputter coater. Finally, observations were made under an accelerating voltage of 15.0 kV.

2.4. Differential scanning calorimetry (DSC)

The crystallinity profiles of the samples were determined using DSC (DSC 200 F3, Netzsch-Gerätebau GmbH, Selb, Germany). For the DSC measurements ~4 mg of freeze dried samples were weighed accurately into a standard aluminum pan and then hermetically sealed with a standard aluminum lid. An empty aluminum pan served as a reference. All samples were heated from 25°C to 90°C at a heating rate of 10°C/min.

2.5. X-ray diffraction (XRD)

Powder X-ray diffraction (PXRD) studies of the raw curcumin and lyophilized powders of curcumin nanosuspension, curcumin nanosuspension stabilized by native and denatured β-lg at different pH values (3.4 and 7.04) and curcumin nanosuspension without any stabilizers were carried out using a high resolution X-ray diffractometer (X’Pert PRO MPD, PANalytical, Almelo, Netherlands). The X-ray diffractometer was operated at a scan rate of 4°C/min for 2 h between 5° and 40°.

2.6. Short term stability study

Freshly prepared curcumin nanosuspension formulations stabilized with native β-lactoglobulin (β-lg) or denatured β-lactoglobulin (DB-β-lg) fabricated at different pH (3.4 and 7.04) i.e. (β-lg-cur 3.4, β-lg-cur 7.04, DB-β-lg-cur 3.4, and DB-β-lg-cur 7.04) and a curcumin nanosuspension without β-lg were selected for short-term stability studies. The stability of curcumin nanosuspensions was assessed by tightly sealing them in glass containers and keeping them at 4°C for 1 month. The mean particle size and curcumin content were considered to be indicators of formulation stability and were evaluated at predetermined time intervals (0, 7, 15, and 30 days) using HPLC as described previously [1]. Briefly, an Agilent-1200 HPLC System controlled by Chem Station software (Hewlett-Packard, Wilmington, DE, USA) equipped with an analytical C18 column (Zorbax Eclipse XDB-C18, 4.6 mm × 150 mm, 5 μm packing) was used for the detection of curcumin. The mobile phase consisted of methanol, acetonitrile, and 5% acetic acid at a ratio of 35:55:10 (v/v/v). The flow rate was 0.8 mL/min. The stability of curcumin was calculated using the formula:

\[
\text{Curcumin stability(%) = } \frac{\text{curcumin detected}}{\text{initial curcumin added}} \times 100
\]
2.7. Short term stability study in a model beverage

A model beverage system was prepared and fortified with amorphous curcumin (β-lg-cur 3.4) and then compared to native and nanosized curcumin without β-lg with respect to curcumin stability. The beverage system was prepared as described previously with slight modification [18]. To begin with, sucrose (8 g), vitamin C (0.1 g), and citric acid (0.15 g) were dissolved in 100 mL of water. Later, native curcumin, crystalline curcumin nanosuspension, or amorphous curcumin nanosuspension (curcumin 100 µg/mL) were mixed homogeneously into the model beverage system. The stability of the curcumin in the system was evaluated at predetermined time intervals (0, 15, and 30 days) using HPLC in the manner described above.

2.8. Bioavailability study of β-lg stabilized amorphous curcumin nanosuspensions

A bioavailability study was conducted in the manner described previously [19]. Briefly, Caco-2 human intestinal cells were grown using Dulbecco’s modified Eagle’s medium (DMEM, Gibco Rockville, MD, USA) with 10% fetal bovine serum (FBS, Gibco, NE, USA), 1% nonessential amino acids (Sigma), 1% penicillin (Gibco), and 0.1% gentamicin (Gibco). Between passage numbers 32–36 the cells were seeded in a 12-transwell plate and incubated in a humidified atmosphere of 95% air and 5% CO2 at 37 °C. The cellular transport of curcumin nanosuspension with and without β-lg and curcumin in its original form was assessed when the cells reached confluence (2–3 weeks) and the transendothelial electrical resistance (TEER) value was higher than 350 Ω as measured using a Millicell ERS-2 system (Millipore, New Bedford, MA, USA). Each formulation was diluted to an appropriate concentration using cell culture medium and administered to apical Caco-2 cells which were then incubated at 37 °C for 3 h. The basal medium was then collected for the detection of curcumin at regular time intervals (1 and 3 h). Bioavailability was calculated using the formula:

\[
\text{Bioavailability} \% = \left( \frac{\text{curcumin (basal)}}{\text{curcumin (apical)}} \right) \times 100
\]

where curcumin (apical) is the amount of curcumin added to the apical side of culture plates and curcumin (basal) is the amount of curcumin detected in the basal side of the culture plate after intestinal transport of curcumin.

3. Results and discussion

3.1. Preformulation studies

To investigate the effect of the critical processing parameters, a series of preformulation studies were conducted where the size and polydispersity index (PDI) of the nanosuspension was measured soon after the preparation. In our preliminary experiments, we decided to investigate the effect of stirring speed, solvent to antisolvent ratio, and temperature on nanocrystals formation. To begin with, the stirring speed was varied from 200 to 1500 rpm using a magnetic stirrer. As the stirring speed was increased from 200 to 1500 rpm, a considerable decrease in size (5050–2638 nm) and PDI (1.6–0.6) was observed (Fig. 1). This may be due to the increased rate of diffusion between the phases and enhanced mass transfer leading to the more rapid formation of a homogeneous supersaturation state resulting in the rapid nucleation and formation of smaller particles [11, 20].

In the next experiments, the stirring speed was kept at 1500 rpm, and temperature was varied from 5 to 40 °C. Particle size increased considerably (1405–2825 nm) as the temperature was increased from 5 to 40 °C. This may be due to the decrease in viscosity of the antisolvent phase which increases the movement of the solute (curcumin) resulting in increased particle coagulation and agglomeration. In addition, the solubility of curcumin increased as temperature is increased which resulted in the reduction of supersaturation resulting in a reduced nucleation rate and therefore the formation of larger particles [21, 22].

The ratio of solvent to antisolvent is another important parameter which is known to affect the particle’s properties. In this regard, the solvent to antisolvent ratio was varied from 1:10 to 1:30. As shown in Fig. 1, as the antisolvent volume increased from 1:10 to 1:25, a decrease in particle size was observed (4373–1416 nm). A further increase in antisolvent volume did not affect the particle size significantly. This may be either due to increased diffusion distance for the solutes which decreases crystal growth or due to decrease in the time required to reach supersaturation [20, 23].

Finally, to obtain nanocrystals smaller than 200 nm with a narrow size distribution, ultrasonic power was applied for 15 min. As the ultrasonic power was increased from 20% to 40%, the size of the curcumin nanocrystals decreased significantly from 335 to 156 nm. Interestingly, at 20% sonication power, the curcumin nanocrystals were bimodal in distribution, whereas at 30 and 40% power they were unimodal. This may be due to the formation of large temperature and pressure gradients caused by the collapsing of the bubbles generated by the high-intensity ultrasound which are capable of breaking the curcumin crystals. From the above preformulation data, 1500 rpm stirring speed, 5 °C temperature, 1.25 solvent to antisolvent ratio and 40% amplitude sonication power were selected for further formulation development.

3.2. Physicochemical characteristics of amorphous curcumin nanosuspensions

The physicochemical characteristics of the curcumin nanosuspensions are listed in Table 1. The size of the particles in the curcumin nanosuspension without any stabilizer was 385.3 ± 70 nm with a PDI of 0.2 ± 0.1. Interestingly, in the preformulation study where the size of the nanocrystals was studied soon after preparation, a similar formulation without β-lg had a size of 156 ± 15 nm, whereas in a formulation in which the size was measured after 2–3 h, the size was 385.3 ± 70 nm. This is in agreement with an earlier study that showed a time dependent increase in the size of curcumin crystals due to aggregation of formed nanocrystals [13]. The use of β-lg as a stabilizer, both in its native form and in denatured form at pH 3.4 and 7.04, significantly reduced the size of the nanocrystals to 150 ± 20 nm. This contradicts earlier results which showed an increase in the solubility of a solute in antisolvent system leading to a decreased supersaturation (S) value and nucleation rate and thereby increasing the size of the nanocrystals [20]. This can be explained by the fact that, even though β-lg increased the solubility of curcumin in the antisolvent it also efficiently inhibited the aggregation of newly formed particles. It does this by forming a barrier around the newly formed particles rather than increasing the nucleation rate or super saturation velocity. This barrier decreases the surface Gibbs free (ΔG) energy and therefore reduces the surface tension. Even though more quickly reaching supersaturation and a quicker nucleation rate are associated with a reduction in particle size, excessive nucleation sometimes leads to particle aggregation by enhancing particle collision. In support of the above explanation, when β-lg was used as stabilizer at pH 5.5 – which is close to its isoelectric point in both its native and in its denatured form – the mean particle size increased significantly to >2000 nm [24]. This is in agreement with earlier studies which have shown similar aggregation of nanoparticles near the isoelectric point of the proteins at which the protein charge remains zero [25]. For all formulations except the formulation at pH 5.5, PDI remained within the
acceptable range (<0.5). The solubility of native curcumin increased from 0.39 ± 0.05 µg/mL to 2.27 ± 0.13 µg/mL when converted into a crystalline nanosuspension. The solubility of curcumin further increased by up to ~35-fold in an amorphous curcumin nanosuspension compared to a crystalline curcumin nanosuspension as shown in Table 1. Among the amorphous curcumin nanosuspensions, no significant difference was observed.

From the above experiment it was evident that (a) β-lg stabilized the curcumin nanosuspension via electrostatic stabilization and (b) to obtain small nanocrystals using the antisolvent precipitation method in addition to increasing the supersaturation value (S) and ensuring long-term stability, it is of primary importance to avoid the aggregation of newly formed particles. Generally in β-lg there are three binding sites for curcumin i.e., at the calyx, surface cleft and in the monomer/monomer interface [16]. In the β-lg-curf 3.4 formulation, curcumin binds to β-lg on either the surface cleft or the monomer/monomer interface due to non-availability of calyx at a pH <7 due to the closure of the EF loop which acts as a lid and determines the accessibility of the hydrophobic core of β-lg for binding [15]. In the β-lg-curf 7.04 formulation, curcumin will bind to β-lg on all the three binding sites. However, in the denatured form, at both acidic and basic pH, curcumin is known to bind to only the surface cleft or the monomer/monomer interface due to the reduced calyx binding site.

The β-lg-curf and Dβ-lg-curf nanosuspensions were negatively charged (~53 and ~51 mV) at basic pH (Table 1). In acidic conditions, the surface charge of β-lg-curf and Dβ-lg-curf nanosuspension was positive (~+51 and ~+45 mV respectively). Due to the formation of a highly unstable nanosuspension at pH 5.5 we were unable to measure the surface charge for this sample.

The retention of curcumin concentration was determined soon after the fabrication of the nanosuspensions. A curcumin nanosuspension fabricated without any stabilizer showed a maximum loss of ~30% (Table 1). Among the curcumin nanosuspensions stabilized with the β-lg, the sample at pH 3.4 showed the maximum initial concentration of >90%. This may be due to the degradation of curcumin and the partial loss to the surface of glassware in aqueous solution.

Since some initial loss of curcumin was observed in all of the formulations, lyophilization was carried out to assess the long term stability. Trehelose was used as a cryoprotectant. Trehelose was used at 1%, 3%, and 5% (w/w) concentrations and samples without cryoprotectant served as control. Trehelose was added in the required concentration; samples were uniformly mixed and

Table 1
Mean droplet size, zeta-potential, polydispersity index (PDI), solubility and curcumin concentration of amorphous curcumin nanosuspensions stabilized by β-lg.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Solubility (µg/mL)</th>
<th>Mean droplet size (nm)</th>
<th>PDI</th>
<th>ZP (mV)</th>
<th>Curcumin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cur-crystals</td>
<td>2.3 ± 0.1*</td>
<td>385.3 ± 71*</td>
<td>0.2 ± 0.1</td>
<td>nd*</td>
<td>69 ± 3*</td>
</tr>
<tr>
<td>β-lg-curf 3.4</td>
<td>30.9 ± 1.0*</td>
<td>149.7 ± 20*</td>
<td>0.2 ± 0.2</td>
<td>51 ± 2</td>
<td>100 ± 0.8*</td>
</tr>
<tr>
<td>β-lg-curf 7.04</td>
<td>29.7 ± 1.2*</td>
<td>153.0 ± 15*</td>
<td>0.2 ± 0.1</td>
<td>~53 ± 5</td>
<td>99 ± 0.3*</td>
</tr>
<tr>
<td>β-lg-curf 5.5</td>
<td>nd*</td>
<td>1962.7 ± 149*</td>
<td>0.6 ± 0.3</td>
<td>nd*</td>
<td>nd*</td>
</tr>
<tr>
<td>Dβ-lg-curf 3.4</td>
<td>31 ± 4.2*</td>
<td>142.3 ± 18*</td>
<td>0.2 ± 0.2</td>
<td>45 ± 7</td>
<td>94 ± 0.2*</td>
</tr>
<tr>
<td>Dβ-lg-curf 7.04</td>
<td>28.7 ± 0.05*</td>
<td>171.3 ± 16*</td>
<td>0.4 ± 0.1</td>
<td>~51 ± 4</td>
<td>96 ± 0.3*</td>
</tr>
<tr>
<td>Dβ-lg-curf 5.5</td>
<td>nd*</td>
<td>2738.3 ± 42*</td>
<td>0.7 ± 0.4</td>
<td>nd*</td>
<td>nd*</td>
</tr>
</tbody>
</table>

Each experiment was repeated at least thrice (n ≥ 3). Means with common letters are not significantly different at P ≤ 0.05 according to Duncan’s multiple range test (DMRT) in their respective column.

* nd: not determined; solubility of native curcumin: 0.39 ± 0.05 µg/mL.

Fig. 1. Effect of process variables such as (A) rotation speed, (B) fabrication temperature, (C) solvent to antisolvent ratio and (D) sonication power on formation of amorphous curcumin nanosuspension. Each experiment was repeated at least thrice (n ≥ 3). Means with common letters are not significantly different at P ≤ 0.05 according to DMRT.
lyophilized. A fluffy, yellow intact cake was observed after complete lyophilization. These lyophilized powders were reconstituted using water and used for size and PDI measurements. A significant difference was observed between the size of the nanosuspensions before and after lyophilization in formulations in which trehalose was not used as a cryoprotectant (Fig. 2). Neither hydrophobicity nor a conformational change in the protein has any effect on the activity of trehalose. Since there was no significant change in the size between different concentrations of trehalose, 1% trehalose was selected for use during the lyophilization of all samples which were used for further physicochemical characterization.

3.3. Crystallinity and morphology of amorphous curcumin nanosuspensions

The effect of β-lg on the thermal characteristics and crystallinity of curcumin was then examined. Fig. 3A shows that the DSC curves of pure curcumin and the curcumin suspensions exhibited a single endothermic peak at 179.7 and 176.8 °C, respectively, which was in accordance with previously published results [26]. However, in the case of the crystalline curcumin nanosuspension, a decrease in crystallinity was observed compared to native curcumin powder as evidenced by the significant decrease in enthalpy from 210.3 J/g in native curcumin to 45.97 J/g in crystalline curcumin nanosuspension. In the case of β-lg stabilized curcumin nanosuspensions, similar endothermic peaks were absent. This indicates that: (a) curcumin after precipitation initially existing in the form of amorphous particles was able to maintain its amorphous status by avoiding particle growth even after a time period that is otherwise long enough for crystallization to occur, and (b) curcumin particles are being molecularly dispersed within the β-lg structure. Thus it can be concluded that curcumin in the β-lg stabilized nanosuspensions was in an amorphous state whereas in its original form and in curcumin nanosuspension without β-lg it was in a crystalline state.

As shown in Fig. 3B, curcumin in its pure form exhibited significant crystallinity as evidenced by the diffraction peaks between 2θ values of 5° and 30°. A curcumin nanosuspension without β-lg also exhibited diffraction peaks between 2θ values of 5° and 30° although with less intensity. β-lg stabilized formulations did not show any peaks indicating that curcumin is molecularly dispersed within β-lg and is successfully avoiding the formation of crystals. XRD results again confirmed the DSC data and showed that β-lg stabilized curcumin was in an amorphous state rather than its original crystalline state irrespective of β-lg conformation or pH. Crystallinity reduced according to the following trend: native curcumin > curcumin crystalline nanosuspension > β-lg stabilized amorphous curcumin nanosuspension.

SEM images of the curcumin nanosuspension without β-lg showed extensive aggregations with a needle-like morphology (Fig. 4A). This is in agreement with earlier results which have shown that even though curcumin is initially precipitated as an amorphous nanocrystals with a size of <100 nm, over time they undergo unidirectional aggregation and form needle-like crystals and increase in size [13]. However, in curcumin nanosuspensions stabilized by β-lg, these needle-like structures were not present; instead, lath-like structures were observed indicating the successful incorporation of these amorphous curcumin nanocrystals into the molecular structures of β-lg soon after their formation. This means they avoid aggregation and the corresponding formation of needle-like crystals and the increase in particle size that was generally observed in curcumin without any surfactant (Fig. 4B). This is in clear agreement with our preformulation study and physicochemical characterization which showed that β-lg had a significant effect on the morphology of curcumin nanocrystals post-precipitation.

3.4. Short term stability study of amorphous curcumin nanosuspensions

A study of short term stability was conducted to investigate the effect of the amorphous form on the nanosuspension stability. As summarized in Fig. 5A, no significant changes in the mean particle size and the PDI of the curcumin nanosuspension stabilized with β-lg was observed after 30 days of storage. The curcumin nanosuspension formulation without any stabilizer showed a significant increase in size from day 0 to day 1 (430 ± 106 to 1025 ± 135 nm) and from day 1 to day 7 (1025 ± 135 to 1328 ± 171 nm). An additional slight increase in size was observed from day 7 to day 30. This may be attributed to the lack of suitable surfactant to prevent the aggregation of newly formed nanocrystals due to increased surface Gibbs free energy [86].

Fig. 5B shows the stability of curcumin in various formulations. It could be observed that in curcumin nanosuspension without any stabilizer, curcumin stability decreased significantly with remarkable variation from 70 ± 5% on day 0 to 8 ± 5% on day 30. This is in agreement with earlier results which have shown that curcumin degrades under physiological conditions in vitro [27]. Compared to the curcumin nanosuspension without β-lg, curcumin nanosuspensions with β-lg showed increased curcumin stability. Our findings correlated well with earlier studies which have shown that enhanced solubility results from enhanced stability against hydrolytic degradation. Among the curcumin formulations stabilized with β-lg, native β-lg stabilized nanosuspensions have been shown to be more stable as compared to denatured β-lg stabilized nanosuspensions. Among the denatured β-lg stabilized formulations, a significant amount of curcumin was degraded from day 1 to day 30. This may be due to the weak interaction between curcumin and the β-lg caused due to the conformational changes which takes place during denaturation. This may result in the separation of curcumin and β-lg resulting in curcumin degradation. This is in agreement with earlier results which have also shown a similar decrease in the binding efficiency of curcumin to β-lg after denaturation [15].

Among the native β-lg stabilized formulations, β-lg-cur 3.4 formulation showed better stability compared to β-lg-cur 7.04, where >90% of curcumin remained after 30 days. This is interesting and also contradicts earlier studies which have shown that: (a) proteins are not good stabilizers at acidic pH due to their existence as monomers [28,29] and (b) due to the decrease in EF loop size at acidic pH, curcumin does not enter into the hydrophobic core of the β-lg and this results in reduced curcumin and β-lg interaction [15]. This may be due to: (a) the stability of β-lg at an acidic pH, and (b) since, curcumin was nanoized, it successfully entered the calyx with minute opening of the EF loop which acts as a lid and determines the accessibility of the hydrophobic core. The stability of curcumin in various formulations followed the trend:
Fig. 3. (A) DSC thermograms and (B) XRD spectra of (a) native curcumin, (b) curcumin nanosuspension without β-1g, (c) β-1g-cur 3.4, (d) β-1g-cur 7.04, (e) Dβ-1g-cur 3.4, and (f) Dβ-1g-cur 7.04.

Fig. 4. Scanning electron micrographs of (A) curcumin nanosuspension without β-1g and (B) β-1g-cur 3.4.
β-lg-cur 3.4 > β-lg-cur 7.04 > Dβ-lg-cur 7.04 > Dβ-lg-cur 3.4 > curcumin suspension. The higher stability of the β-lg-cur 3.4 formulation indicates the suitability of these systems for use in beverages with an acidic pH such as fruit juices (pH 3.2–3.8) and carbonated drinks (pH 2.8–3.7) [24].

3.5. Short term stability study in beverage application

The formulation that showed the maximum aqueous stability (β-lg-cur 3.4) was chosen to fortify the model beverage system. As shown in Fig. 6A, after 30 days curcumin stabilized with β-lg showed the maximum stability of ~89%. The other two formulations (native curcumin and curcumin nanosuspension without β-lg) showed a curcumin stability of ~28% and 44%, respectively which is significantly lower than the β-lg stabilized counterpart. Interestingly, the curcumin crystalline nanosuspension showed a greater stability when used in a beverage system compared to normal water (Figs. 5B and 6A). This may be due to the presence of the antioxidant vitamin C in the beverage system which might have arrested the degradation of curcumin in the aqueous system. For the first time we have successfully used a nanonization technique to fortify a beverage system. This will be useful for fortifying non-fat beverage systems with curcumin and, if possible, other nutraceuticals. As shown in aqueous stability data, curcumin nanosuspensions stabilized by β-lg have shown resistance to gravitational separation which further shows its appropriateness for commercial product development.

3.6. Bioavailability study

Since stabilizing the curcumin nanosuspension with β-lg-cur 3.4 led to an improvement in stability, solubility and product quality, the effect on oral bioavailability was studied using Caco-2 cells. As shown in Fig. 6B, the bioavailability of curcumin was ~2.7% after 3 h incubation which is in agreement with earlier studies which have shown limited bioavailability of curcumin both in vivo and in vitro [30]. Among the nanosuspensions, the amorphous curcumin nanosuspension had a bioavailability of ~4% after 3 h incubation and the simple crystalline nanosuspension had a bioavailability of ~3.1%. This shows that there is a significant increase in curcumin bioavailability after nanonization specifically after the fabrication of an amorphous curcumin nanosuspension. Though, nanonization increased the bioavailability of curcumin, it was not significant without β-lg. The main reason is the aggregation of curcumin nanocrystals soon after the precipitation as evident from our current study (Section 3.2) and earlier study from other research group [13]. This will reduce the permeability and solubility of curcumin crystals. The degree of curcumin bioavailability in the different forms followed the following trend: amorphous curcumin nanosuspension > crystalline curcumin nanosuspension > native curcumin.

These results are in line with earlier results which have shown an increased permeation of curcumin across the Caco-2 cells after solubilization [31]. As shown in Table 1, the solubility of curcumin was increased ~35-fold after stabilization using β-lg. However, this result also contradicts the earlier results which have shown decreased curcumin cell permeation after binding with proteins.
such as albumin, whey protein isolate (WPI) at a binding constant of \(10^{-5}\) M\(^{-1}\) [5]. There are two possible reasons for this. Firstly, earlier studies have shown the binding constant of curcumin with proteins is pH dependent. At pH 7.4, curcumin is known to bind to proteins such as β-lg with a binding constant of the order \(10^{4} - 10^{5}\) M\(^{-1}\) and, with this binding constant, proteins are known to hinder the cell permeation capacity of solubilized curcumin [5]. However, in our case the pH of the formulation was 3.4 and at this pH the binding constant of curcumin with β-lg was known to be \(<10^{3}\) M\(^{-1}\) which is significantly less than binding constant at pH 7.4 and this binding constant is known to be ineffective at hindering the curcumin cell permeation [5,15]. The second possible reason for our results contradicting previous work is that Caco-2 cells are known to process lipocalin-interacting-membrane-receptors (LMIR) which are known to internalize intact β-lg which can also aid transport of the curcumin across the cells. This type of receptor is absent for other type of proteins like WPI, albumin, etc. In support of our data, earlier studies have shown that compared to albumin, β-lg crossed the Caco-2 monolayers more rapidly [32]. From the above data, it can be hypothesized that curcumin first gets internalized due to the presence of LMIR receptors and then dissociate from the β-lg and transfers to the other side of the monolayer leading to increased bioavailability.

4. Conclusion

In this study, β-lg in its native and denatured forms was used to stabilize curcumin nanosuspensions. Physicochemical characterization of these nanosuspensions confirms the existence of the curcumin in an amorphous form rather than its original crystalline form. Among the formulations, the β-lg-cur 3.4 formulation has shown decreased particle size and increased solubility, stability and cell permeability compared to both curcumin in its native form and a curcumin nanosuspension prepared without β-lg as a stabilizer. The development of a method for the preparation of an amorphous curcumin nanosuspension is an important step toward incorporating the ayurvedic compound curcumin into functional beverages.

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