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<td><strong>Author(s)</strong></td>
<td>Lim, Li Ming; Tran, The-Thien; Wong, Jerome Jie Long; Wang, Danping; Cheow, Wean Sin; Hadinoto, Kunn</td>
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Amorphous Ternary Nanoparticle Complex of Curcumin-Chitosan-Hypromellose

Exhibiting Built-In Solubility Enhancement and Physical Stability of Curcumin

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The low aqueous solubility of curcumin (CUR) had greatly limited the clinical efficacy of CUR therapy despite its well-known potent therapeutic activities. Previously, we developed amorphous nanoparticle complex (nanoplex) of CUR and chitosan (CHI) as a solubility enhancement strategy of CUR by electrostatically-driven drug-polyelectrolyte complexation. The CUR-CHI nanoplex, however, (1) lacked a built-in ability to produce prolonged high apparent solubility of CUR in the absence of crystallization-inhibiting agents, and (2) exhibited poor physical stability during long-term storage. For this reason, herein we developed amorphous ternary nanoplex of CUR, CHI, and hypromellose (HPMC) where HPMC functioned as the crystallization inhibitor. The effects of incorporating HPMC on the (1) physical characteristics and (2) preparation efficiency of the CUR-CHI-HPMC nanoplex produced were investigated. Compared to the CUR-CHI nanoplex, the HPMC inclusion led to larger nanoplex ($\approx 300-500$ nm) having lower zeta potential ($\approx 1-15$ mV) and lower CUR payload ($\approx 40-80\%$), albeit with higher CUR utilization rates ($\approx 100\%$) attributed to the CUR interactions with both CHI and HPMC. The CUR-CHI-HPMC nanoplex’s physical characteristics could be controlled by varying the HPMC to CHI ratio in the feed. Subsequently, the CUR-CHI-HPMC and CUR-CHI nanoplexes were examined in terms of their (1) storage stability, (2) dissolution characteristics in simulated gastrointestinal fluids, and (3) in vitro solubility enhancement. The results showed that the CUR-CHI-HPMC nanoplex exhibited superior (i) amorphous state stability after twelve-month storage, (ii) dissolution characteristics, and (iii) solubility enhancement in simulated gastrointestinal fluids, with minimal cytotoxicity towards human gastric epithelial cells.

Keywords: colloidal drug carrier; chitosan; hypromellose; nanoparticles; curcumin bioavailability
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<tr>
<th>No.</th>
<th>Abbreviation</th>
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<tr>
<td>50</td>
<td>List of abbreviations</td>
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<tr>
<td>51</td>
<td>ASD</td>
<td>amorphous solid dispersion</td>
</tr>
<tr>
<td>52</td>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>53</td>
<td>C</td>
<td>supersaturated concentration of CUR</td>
</tr>
<tr>
<td>54</td>
<td>C&lt;sub&gt;Sat&lt;/sub&gt;</td>
<td>saturation solubility of CUR</td>
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<td>55</td>
<td>CE</td>
<td>complexation efficiency</td>
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<tr>
<td>56</td>
<td>CHI</td>
<td>chitosan</td>
</tr>
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<td>57</td>
<td>CUR</td>
<td>curcumin</td>
</tr>
<tr>
<td>58</td>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
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<td>59</td>
<td>DSC</td>
<td>differential scanning calorimetry</td>
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<td>field emission scanning electron microscope</td>
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<td>61</td>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
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<tr>
<td>62</td>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>63</td>
<td>HPMC</td>
<td>hypromellose</td>
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<td>64</td>
<td>M&lt;sub&gt;HPMC/CHI&lt;/sub&gt;</td>
<td>mass ratio of HPMC to CHI</td>
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<td>65</td>
<td>PXRD</td>
<td>powder x-ray diffraction</td>
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<tr>
<td>66</td>
<td>R&lt;sub&gt;CHI/CUR&lt;/sub&gt;</td>
<td>charge ratio of CHI to CUR</td>
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<td>67</td>
<td>SGJ</td>
<td>simulated gastric juice</td>
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<tr>
<td>68</td>
<td>SIJ</td>
<td>simulated intestinal juice</td>
</tr>
<tr>
<td>69</td>
<td>TGA</td>
<td>thermal gravimetric analysis</td>
</tr>
<tr>
<td>70</td>
<td>UV-Vis</td>
<td>ultraviolet visible</td>
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<td>71</td>
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<td>United States Pharmacopeia</td>
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1. Introduction

The potent therapeutic activities of curcumin (CUR) - a natural polyphenol isolated from turmeric - as anti-inflammatory, antioxidant, antimicrobial, and anticancer agents have been well established, resulting in the vast application of CUR as oral dietary supplements [1, 2]. The low aqueous solubility of CUR (<< 1 mg/mL) and the consequential poor oral bioavailability, however, have greatly limited its true therapeutic potential clinically [3]. Numerous solubility enhancement strategies of CUR have therefore been proposed, for example, via amorphization [4, 5], chemical conjugation [6, 7], encapsulation [8, 9], cyclodextrin inclusion complex [10, 11], and nanonization [12, 13]. Among these strategies, amorphization and nanonization represent the more feasible strategies for large-scale implementation attributed to (i) the high CUR payload of their products, (ii) their organic solvent-free preparation, and (iii) less intricate preparation techniques [3, 14].

To take advantage of the solubility enhancement afforded by amorphization and nanonization, our group previously developed a solubility enhancement strategy of CUR that combined amorphization and nanonization principles in the form of amorphous nanoparticle complex of CUR and chitosan (CHI) [15]. First, the metastable amorphous state of the CUR-CHI nanoparticle complex (or nanoplex in short) enabled it to generate highly supersaturated CUR concentration upon dissolution, resulting in high apparent solubility of CUR. The high apparent solubility would lead to enhanced CUR bioavailability if the high supersaturation level could be maintained over a time period sufficient for CUR absorption across the gastrointestinal lumen [16, 17].

Second, owing to its nanoscale size, the amorphous CUR-CHI nanoplex exhibited superior supersaturation generation in vitro to microscale amorphous solid dispersion (ASD) of CUR, which represented a well-established amorphization strategy of CUR [18]. The superior supersaturation generation of the CUR-CHI nanoplex was attributed to the faster dissolution rate afforded by its nanoscale size [18]. Furthermore, the CUR-CHI nanoplex also exhibited superior characteristics to the ASD of CUR and other CUR solubility enhancement strategies due to its (1) simple, fast, and cost-effective preparation involving only mixing of CUR and CHI solutions under ambient condition, and (2) high CUR utilization rate (> 90%) resulting in minimal CUR wastage [15].

Nevertheless, the current CUR-CHI nanoplex formulation exhibits two major drawbacks. First, while the CUR-CHI nanoplex could generate a high supersaturation level, the supersaturation level rapidly decreased in the absence of crystallization-inhibiting agents in the dissolution medium [15]. The CUR-CHI nanoplex thus did not possess a
built-in capability to generate a prolonged high supersaturation level necessary for bioavailability enhancement. From the product formulation perspectives, the crystallization-inhibiting agents must thus be added in the subsequent formulation step, namely during the oral solid dosage form preparation of the nanoplex. This approach is less than ideal because the supersaturation generation of the CUR-CHI nanoplex becomes dependent on the dissolution rate of the crystallization inhibitors from the solid dosage form, which itself is influenced by a myriad of factors [19, 20].

Second, the CUR-CHI nanoplex exhibited poor physical stability during long-term storage in its dry-powder form, where parts of its amorphous form underwent crystallization, resulting in its diminished solubility enhancement capability [18]. Moreover, the amorphous state stability of the CUR-CHI nanoplex remained lacking even when it was stored as intimate mixtures with crystallization inhibitors [18]. The amorphous state stability of the nanoplex could only be maintained when it was stored in the presence of a large amount of crystallization inhibitors and other adjuvants, resulting in undesirably low nanoplex contents (< 30%) [21].

To address these drawbacks, the present work aimed to incorporate hypromellose - a well-established polymeric crystallization inhibitor [22] - into the CUR-CHI nanoplex early at the nanoplex formation step, rather than at the solid dosage formulation step as previously done. Herein we hypothesized that the presence of hypromellose at the nanoscale would provide the CUR-CHI nanoplex with a built-in capability (1) to prolong its high supersaturation level upon dissolution and (2) to enhance its long-term physical stability.

The first objective of the present work was to investigate the feasibility of forming amorphous ternary nanoplex of CUR-CHI-hypromellose via the same preparation principle (i.e. drug-polyelectrolyte complexation) used in the CUR-CHI nanoplex preparation. In this technique, the electrostatic interaction between ionized drug molecules (i.e. CUR) and oppositely charged polyelectrolytes (CHI) resulted in the formation of soluble CUR-CHI complexes that subsequently aggregated due to inter-CUR hydrophobic interactions. The complex aggregates then precipitated out of the solution to form the CUR-CHI nanoplex upon reaching a critical aggregate mass [15].

In this regard, hypromellose had been known to readily form aggregates with non-ionic molecules via hydrophobic and hydrogen bond interactions [23, 24]. In fact, hydrogen bond interactions between hypromellose and CUR had been reported when they were formulated as ASD of CUR [25, 26]. We postulated that a similar kind
of interactions between hypromellose and CUR occurred during the nanoplex formation, resulting in the formation of amorphous ternary nanoplex.

The second objective of the present work was to examine the optimal formulation of the CUR-CHI-hypromellose nanoplex and compare it against the CUR-CHI nanoplex in terms of their (1) physical stability during long-term storage, (2) dissolution characteristics in simulated gastrointestinal fluids, and (3) in vitro supersaturation generation. The optimal formulation of the CUR-CHI-hypromellose nanoplex was determined by investigating the effects of (i) mass ratios of hypromellose to CHI and (ii) charge ratios of CHI to CUR on the physical characteristics and preparation efficiency of the CUR-CHI-hypromellose nanoplex produced. In addition, we also examined the cytotoxicity of the CUR-CHI-hypromellose nanoplex towards human gastric epithelial cells NCI-N87 to evaluate its potential in vivo applications as oral dietary supplement.

2. Materials and Methods

2.1. Materials

Curcumin (CUR) (95% curcuminoid) was purchased from Alfa Aesar (Singapore). Chitosan (CHI) (50-190 kDa, 75-85% deacetylation), hypromellose (hydroxypropylmethylcellulose, HPMC) (26 kDa), potassium and sodium hydroxides (KOH, NaOH), potassium dihydrogen phosphate (KH$_2$PO$_4$), sodium chloride (NaCl), hydrogen chloride (HCl), glacial acetic acid (AA), and ethanol were purchased from Sigma-Aldrich (Singapore). The materials and methods for the cytotoxicity test were provided in the Supplementary Materials.

2.2. Methods

2.2.1. Preparation of CUR-CHI-HPMC nanoplex

The CUR-CHI-hypromellose nanoplex (hereinafter referred as CUR-CHI-HPMC nanoplex for brevity) was prepared at two different charge ratios of CHI to CUR ($R_{\text{CHI/CUR}}$) (i.e. 0.7 and 1.0) at a fixed CUR concentration of 5 mg/mL. The effects of the mass ratios of HPMC to CHI ($M_{\text{HPMC/CHI}}$) in the feed solution were investigated in the range of 0.33 to 2.0 and 0.23 to 1.4, respectively, for $R_{\text{CHI/CUR}} = 0.7$ and 1.0. The sample calculation for $R_{\text{CHI/CUR}}$ was provided in the Supplementary Materials. Briefly, CHI was dissolved in 1.2% (v/v) aqueous AA solution (pH 2.7) at either 6.4 mg/mL ($R_{\text{CHI/CUR}} = 0.7$) or 9.1 mg/mL ($R_{\text{CHI/CUR}} = 1.0$). CHI having $pK_a$ of 6.5 [27] was protonated upon dissolution in AA to form cationic CHI molecules. Separately, HPMC was dissolved in 0.1M KOH (pH 13) at different concentrations (i.e. 2-12 mg/mL) depending on the desired $M_{\text{HPMC/CHI}}$. Afterwards, CUR powder was
added to the HPMC solution in 0.1M KOH at 5 mg/mL, where CUR subsequently dissolved as CUR having pKₐ of 8.4, 9.9, and 10.5 [28] was fully deprotonated at pH 13 to form anionic CUR molecules.

Next, the (CUR+HPMC) solution was mixed immediately upon its preparation with equal volume of the CHI solution to minimize alkaline degradation of CUR, resulting in mixed solution exhibiting pH 4.4. On this note, the CUR degradation rate at pH 13 expressed as the % CUR loss was reported in two separate studies to be equal to approximately 50% after 48 h and 67% after 20 h with negligible losses after 1 h [28]. Hence, CUR degradation in the (CUR+HPMC) solution prior to its mixing with the CHI solution was minimal.

Afterwards, the resultant CUR-CHI-HPMC nanoplex suspension was ultrasonicated for 15 s at 20 kHz (VC 505, Sonics, USA). The nanoplex suspension was then washed by two cycles of centrifugation (14,000×g) for 10 min to remove excess CUR, CHI, and HPMC. The washed nanoplex suspension was re-dispersed in deionized water for characterizations. Two types of control runs, i.e. (1) (CUR+HPMC) solution in 0.1M KOH added at equal volumes to 1.2% AA solution without CHI (i.e. Control #1), and (2) HPMC solution in 0.1M KOH without CUR added at equal volumes to CHI solution in 1.2% AA (i.e. Control #2), were carried out. The CUR-CHI nanoplex was prepared at Rₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜₜ০
The amorphous state of the nanoplex was verified by powder x-ray diffraction (PXRD) using D8 Advance X-ray Diffractometer (Bruker, Germany) performed between 10° and 70° (2θ) with a step size of 0.02°/s. The thermal behavior of the nanoplex was examined by thermal gravimetric analysis (TGA) (Pyris Diamond TGA, PerkinElmer, USA) and differential scanning calorimetry (DSC) (DSC 822E, Mettler Toledo, USA). The TGA analysis was performed at heating rate of 10°C/min between 25°C and 400°C, while the DSC analysis was performed at heating rate of 2°C/min between 25°C to 300°C. The FTIR, PXRD, TGA, and DSC analysis were also performed for the native CUR, CHI, and HPMC.

### 2.2.3. Preparation efficiency

The CUR utilization rate was characterized in quadruplicates by the complexation efficiency (CE) defined in Eq. 1. The mass of CUR that formed the nanoplex was determined from the difference between the initial mass of CUR added and the mass of CUR recovered in the supernatant after centrifugation of the nanoplex suspension. The amount of CUR in the supernatant was determined by UV-Vis spectrophotometer as previously described. The overall production yield was determined from Eq. 2, where the mass of the nanoplex produced was determined in triplicates from the dry mass of the washed nanoplex suspension after 24-h freeze drying.

\[
CE(\%) = \frac{\text{Mass of CUR that formed CUR–CHI–HPMC nanoplex}}{\text{Initial mass of CUR added}} \times 100
\]

\[
Yield(\%) = \frac{\text{Mass of CUR–CHI–HPMC nanoplex produced}}{\text{Initial masses of CUR, CHI, and HPMC added}} \times 100
\]

### 2.2.4. Dissolution rates under sink condition

The dissolution rates of the nanoplex under sink condition were characterized in triplicates in simulated gastric juice (SGJ) and simulated intestinal juice (SIJ) prepared according to the United States Pharmacopoeia (USP) specifications (Test Solutions, USP 35, NF 30) as summarized previously in [29, 30]. Specifically, the SGJ (without pepsin) was prepared from 0.2% (w/v) aqueous NaCl solution adjusted to pH 1.2 by adding 1.0M HCl, whereas the SIJ was prepared from 0.9% (w/v) aqueous KH₂PO₄ solution adjusted to pH 6.8 by adding 0.2M NaOH. The sink condition was defined according to the USP specification as a condition in which the drug saturation solubility in the dissolution medium was at least three times larger than the drug concentration used in the dissolution tests [31].

Briefly, the nanoplex was added at ¼ of the saturation solubility of CUR (Cᵣₑₐₖ) to a dialysis bag immersed in 100 mL of SGJ (or SIJ) placed in a shaking incubator maintained at 37°C. Next, 1 mL aliquot was withdrawn and
syringe filtered using 0.22-μm pore size at specific time points over a one-hour period, while 1 mL fresh SGJ (or SIJ) was added back to the dissolution vessel as replenishment. Afterwards, the aliquot was centrifuged at 14,000×g for 3 min and the amount of CUR in the supernatant was determined by high performance liquid chromatography (HPLC) using Agilent 1100 (Agilent Technologies, USA) at detection wavelength of 423 nm [28]. 80% (v/v) aqueous ethanol solution was used as the mobile phase at 1.0 mL/min in ZORBAX Eclipse Plus C18 column (250 x 4.6 mm, 5 μm particle size), resulting in the CUR’s retention time of ≈ 2.5 min.

The dissolution test was also carried out for the native CUR. Prior to the dissolution tests, C_{Sat} of CUR in SGJ and SIJ were determined by incubating excess CUR in the SGJ and SIJ in a shaking incubator at 37°C for 48 h. Afterwards, the solution was centrifuged to remove the undissolved CUR. The amount of CUR in the supernatant, which represented C_{Sat}, was quantified by HPLC as previously described. The C_{Sat} values of CUR in the SGJ and SIJ were determined to be equal to 3.1 and 4.9 μg/mL, respectively.

**2.2.5. Supersaturation generation**

The supersaturation generation of the nanoplex in the SIJ was characterized in terms of the ratio of the supersaturated CUR concentration (C) to C_{Sat} (i.e. C/C_{Sat}). Briefly, the nanoplex was added in excess at 15×C_{Sat} to 100 mL SIJ placed in a shaking incubator maintained at 37°C. Next, 400 μL aliquot was withdrawn and syringe filtered using 0.22-μm pore size at specific time points over 24 h. The aliquot was immediately diluted tenfold with fresh SIJ to prevent CUR precipitation from the supersaturated solution. The amounts of CUR in the aliquot were then determined by HPLC as previously described.

**2.2.6. Physical stability testing**

The nanoplex was freeze dried immediately after preparation for 24 h (Alpha 1-2 LDPlus, Martin Christ, Germany) at -52°C and 0.05 mbar. The powders were stored in an open container inside a desiccator for three months under accelerated storage condition (i.e. 40°C and 75% relative humidity), which was approximately equivalent to twelve-month storage under normal condition (i.e. 25°C and 60% relative humidity) [32]. The 75% relative humidity was generated inside the desiccator by placing an open container of saturated NaCl solution at 40°C. After three months, the amorphous state of the nanoplex powders was examined by PXRD.

**3. Results and discussion**
3.1. Preparation of CUR-CHI-HPMC nanoplex

3.1.1. Effects of \( M_{\text{HPMC}/\text{CHI}} \) at \( R_{\text{CHI}/\text{CUR}} = 1.0 \)

The preparation of the CUR-CHI-HPMC nanoplex was first attempted at \( R_{\text{CHI}/\text{CUR}} = 1.0 \) at which there were equal charges of cationic CHI available for complexation with anionic CUR to ensure a high CUR utilization rate. The effects of varying \( M_{\text{HPMC}/\text{CHI}} \) on the size and zeta potential of the nanoplex were presented in Fig. 1A. In the absence of HPMC (i.e. \( M_{\text{HPMC}/\text{CHI}} = 0 \)), the CUR-CHI nanoplex exhibited size of \( 140 \pm 16 \) nm and zeta potential of \( 12.8 \pm 2.1 \) mV, where the positive zeta potential signified the presence of CHI on the nanoplex surface acting as colloidal stabilizer. Upon the inclusion of HPMC, the size of the nanoplex produced, which was later verified to be the CUR-CHI-HPMC nanoplex, increased to \( \approx 300-350 \) nm for \( 0.23 \leq M_{\text{HPMC}/\text{CHI}} \leq 0.91 \). The size increased further to \( \approx 470-500 \) nm at \( M_{\text{HPMC}/\text{CHI}} = 1.14 \) and 1.37.

The zeta potential, on the other hand, was found to gradually decrease with increasing \( M_{\text{HPMC}/\text{CHI}} \) to reach as low as \( \approx 1-2 \) mV at \( M_{\text{HPMC}/\text{CHI}} \geq 0.91 \) (Fig. 1A). While the lower zeta potentials observed at larger nanoplex sizes were not completely unexpected, the sharp decrease in the zeta potential from \( 13.1 \pm 1.2 \) mV at \( M_{\text{HPMC}/\text{CHI}} = 0.23 \) to \( 1.3 \pm 0.5 \) mV at \( M_{\text{HPMC}/\text{CHI}} = 0.91 \), when the nanoplex size hardly changed from \( 370 \pm 10 \) nm to \( 341 \pm 4 \) nm, suggested that a different factor, other than the size, must have contributed to the lower zeta potential. Significantly, the CUR payload was also found to decrease upon the inclusion of HPMC from \( 91 \pm 5\% \) (w/w) for the CUR-CHI nanoplex (\( M_{\text{HPMC}/\text{CHI}} = 0 \)) to \( 83 \pm 3\% \) at \( M_{\text{HPMC}/\text{CHI}} = 0.23 \) (Fig. 1B). The CUR payload continued to decrease with increasing \( M_{\text{HPMC}/\text{CHI}} \) to reach \( 42 \pm 5\% \) at \( M_{\text{HPMC}/\text{CHI}} = 1.37 \).

For comparison, the two control runs (i.e. Control #1 and Control #2) resulted in no precipitated products. This suggested that the interactions between CUR and HPMC in Control #1, and between CHI and HPMC in Control #2, did not lead to precipitation. Therefore, the nanoplex produced in the above must have been attributed to the complexation between CUR, CHI, and HPMC. As the complexation was performed at constant CUR and CHI concentrations, the sharp decreases in both the zeta potential and the CUR payload with increasing \( M_{\text{HPMC}/\text{CHI}} \) provided an indication that HPMC had been successfully incorporated into the nanoplex.

To elaborate, the constant CEs at nearly 100\% observed at \( 0.23 \leq M_{\text{HPMC}/\text{CHI}} \leq 0.91 \) (Fig. 1B) indicated that the amount of CUR in the nanoplex was fixed, despite the decrease in the CUR payload from \( 83 \pm 3\% \) at \( M_{\text{HPMC}/\text{CHI}} = 0.23 \) to \( 51 \pm 4\% \) at \( M_{\text{HPMC}/\text{CHI}} = 0.91 \). The lower CUR payload was thus caused by the increased amount of CHI.
and/or HPMC in the nanoplex, which also explained for the aforementioned size increase in the presence of HPMC. However, if the lower CUR payload had been merely caused by the increased CHI content (i.e. HPMC was not present in the nanoplex), the zeta potential should have increased due to the cationic nature of CHI.

The fact that the zeta potential decreased with decreasing CUR payload hinted at successful incorporation of HPMC into the nanoplex, where the increased presence of the non-ionic HPMC was responsible for the lower zeta potential. Furthermore, the trend in the CUR payload as a function of $M_{\text{HPMC}/\text{CHI}}$ suggested that the HPMC content of the CUR-CHI-HPMC nanoplex could be manipulated by varying $M_{\text{HPMC}/\text{CHI}}$ in the feed. Excessive $M_{\text{HPMC}/\text{CHI}}$, however, resulted in lower CEs of 73 ± 6% and 86 ± 1% at $M_{\text{HPMC}/\text{CHI}} = 1.14$ and 1.37, respectively. The excessive presence of HPMC during the complexation thus led to lower CUR utilization rates and consequently lower production yields.

The overall production yields of the CUR-CHI-HPMC nanoplex at $R_{\text{CHI}/\text{CUR}} = 1.0$, however, were low at ≈ 16-28% (w/w) across $M_{\text{HPMC}/\text{CHI}}$, which signified wastage of CHI (Fig. 1B). Therefore, the CUR-CHI-HPMC nanoplex preparation at $R_{\text{CHI}/\text{CUR}} = 0.7$ was investigated next aimed at improving the production yield. The effects of $M_{\text{HPMC}/\text{CHI}}$ on the nanoplex’s size, zeta potential, CUR payload, and CE were also re-examined.

### 3.1.2. Effects of $M_{\text{HPMC}/\text{CHI}}$ at $R_{\text{CHI}/\text{CUR}} = 0.7$

Without HPMC, the CUR-CHI nanoplex prepared at $R_{\text{CHI}/\text{CUR}} = 0.7$ exhibited size, zeta potential, and CUR payload of 227 ± 20 nm, 15.9 ± 2.2 mV, and 81 ± 3%, respectively (Fig. 2). Upon the inclusion of HPMC, the nanoplex size increased to ≈ 320-370 nm in the range of $M_{\text{HPMC}/\text{CHI}}$ investigated, while the zeta potential and CUR payload gradually decreased with $M_{\text{HPMC}/\text{CHI}}$ to reach as low as 1.37 ± 0.3 mV and 43 ± 3%, respectively, at $M_{\text{HPMC}/\text{CHI}} = 2.00$. The CEs were relatively constant at nearly 100% for $0.33 \leq M_{\text{HPMC}/\text{CHI}} \leq 1.33$ before the excessive presence of HPMC at $M_{\text{HPMC}/\text{CHI}} = 1.67$ and 2.00 resulted in lower CUR utilization rates of 75 ± 5% and 62 ± 9%, respectively (Fig. 2B).

The impacts of the HPMC inclusion on the size, zeta potential, CUR payload, and CE of the nanoplex were thus found to be similar between $R_{\text{CHI}/\text{CUR}} = 1.0$ and 0.7. For the reasons elaborated earlier, the sharp decreases in both the zeta potential and the CUR payload observed at $0.33 \leq M_{\text{HPMC}/\text{CHI}} \leq 1.33$, while the CE and size were minimally affected, signified that HPMC had also been successfully incorporated into the nanoplex at $R_{\text{CHI}/\text{CUR}} = 0.7$. The production yields across $M_{\text{HPMC}/\text{CHI}}$, nevertheless, were only slightly higher at ≈ 22-42% (Fig. 2B). Decreasing
the RCHI/CUR further, however, had been found to result in low CUR utilization rates for the CUR-CHI nanoplex [15], hence it was not pursued here.

3.2. Characterizations of the CUR-CHI-HPMC nanoplex

3.2.1. FESEM and colloidal stability

In this section, further characterizations of the CUR-CHI-HPMC nanoplex were presented using the nanoplex prepared at RCHI/CUR = 0.7 and MHPMC/CHI = 0.67 as the representative sample (CUR payload = 68 ± 6%). The nanoscale size of the CUR-CHI-HPMC nanoplex was verified by FESEM where the FESEM image showed the appearance of nanoparticles in the size range of 100 to 300 nm with roughly spherical shapes (Fig. 3A), which were similar to the CUR-CHI nanoplex morphology presented in Nguyen, Yu, Kiew and Hadinoto [15]. Despite its relatively low zeta potential (12.9 ± 2.0 mV), the CUR-CHI-HPMC nanoplex exhibited good colloidal stability after its preparation as reflected by the minimal variations in its size and zeta potential over 24 h (Fig. 3B).

3.2.2. FTIR

First, the presence of CUR in both the nanoplexes were verified by the appearance of the characteristics bands of the native CUR at 1626, 1508, and 1272 cm\(^{-1}\) in the FTIR spectra of the CUR-CHI-HPMC and CUR-CHI nanoplexes (Fig. 4A). These bands corresponded to the stretching vibrations of the C=C-C\(_{\text{ring}}\), C=O, and enol C-O bonds of CUR, respectively [10]. Second, the presence of HPMC in the CUR-CHI-HPMC nanoplex was evident from the appearance of the characteristic band of HPMC at 1080 cm\(^{-1}\) corresponding to the vibration of the glycosidic C-O-C bond of HPMC [33]. This C-O-C band, which did not appear in the FTIR spectra of the native CUR, CHI, and CUR-CHI nanoplex, was shifted slightly from 1050 cm\(^{-1}\) when compared to the FTIR spectrum of HPMC likely due to the CUR-HPMC interaction (Fig. 4A).

Third, the CUR-CHI complexation was evident in the FTIR spectra of both the nanoplexes by the disappearance of the stretching NH\(_2\) band of CHI at 3360 cm\(^{-1}\) [34] and the reduced intensity (or disappearance in the case of the CUR-CHI-HPMC nanoplex) of the phenolic OH band of the native CUR at 3500 cm\(^{-1}\) (Fig. 4B), which were resulted from the electrostatic interaction between the charged amine group of CHI and phenol group of CUR [15]. Fourth, the CUR-HPMC interaction was reflected in the FTIR spectrum of the CUR-CHI-HPMC nanoplex by (1) the aforementioned disappearance of the phenolic OH band of CUR at 3500 cm\(^{-1}\) as it formed hydrogen bond with the C-O-C group of HPMC, in addition to its interaction with the NH\(_2\) group of CHI, and (2) the
reduced intensity of the OH group of HPMC at 3410 cm$^{-1}$ due to its hydrogen bond interaction with the C=O group of CUR [25, 26].

3.2.3. PXRD and DSC

The amorphous state of the CUR-CHI-HPMC nanoplex was verified by PXRD, which showed the appearance of low-intensity peaks in its PXRD pattern, as opposed to the sharp crystalline peaks observed with the native CUR (Fig. 5A). After the three-month accelerated storage, the CUR-CHI-HPMC nanoplex remained in its amorphous state as reflected by the appearance of broad amorphous halos in its PXRD pattern. In contrast, the CUR-CHI nanoplex after the three-month accelerated storage exhibited high-intensity peaks in its PXRD pattern at 2$\theta \approx$ 8 and 16, which were not present before storage, indicating the occurrence of amorphous-to-crystalline transformation. The higher physical stability of the CUR-CHI-HPMC nanoplex was attributed to the reduced molecular mobility of the amorphous CUR as a result of its hydrogen bond interaction with HPMC that was known to be highly stable.

The superior physical stability of the CUR-CHI-HPMC nanoplex was also evident from its DSC thermograph, which did not show the presence of an endothermic crystalline melting point peak at 178°C that was observed with the native CUR (Fig. 5B). This signified the absence of amorphous-to-crystalline transformation in the CUR-CHI-HPMC nanoplex. In contrast, the DSC thermograph of the CUR-CHI nanoplex showed the glass transition peak at 100°C, followed by two endothermic peaks at $\approx$ 160°C and 170°C likely attributed to the crystalline melting points of both CUR and CHI. On this note, the endothermic peaks at $\approx$ 145-150°C observed in the DSC thermographs of the CHI and HPMC were attributed to the melting points of the crystalline parts of the mostly amorphous polymers, as verified by PXRD (Fig. S1 of the Supplementary Materials). On this note, the TGA thermographs showed that the nanoplexes had largely decomposed above 280°C (Fig. S2 of the Supplementary Materials), hence the DSC thermograph was not analyzed above 280°C.

3.3. Dissolution characteristics in SGJ and SIJ

The dissolution of the CUR-CHI-HPMC nanoplex was characterized using the nanoplexes prepared at $M_{\text{HPMOCCHI}} = 0.67$ and 1.33 ($R_{\text{CHICUR}} = 0.7$) as the representative samples because they possessed distinct CUR payloads, hence distinct HPMC contents, of 68 ± 6% and 48 ± 7%, respectively. In the SGJ, the CUR-CHI
nanoplex rapidly dissolved achieving nearly 100% dissolution after 2 min, in contrast to the native CUR that hardly dissolved due to low solubility of CUR at acidic pH (Fig. 6A). Hence, the amorphization of CUR by complexation with CHI resulted in its fast dissolution in the SGJ, which was less than desirable as it might lead to less CUR available for absorption in the intestines.

The incorporation of HPMC into the nanoplex was able to slow down CUR dissolution in the SGJ slightly likely attributed to the aforementioned hydrogen bond interaction between CUR and HPMC. The slowdown was intensified with increasing HPMC content, where 86 ± 1% and 70 ± 2% of CUR were released after 15 min from the CUR-CHI-HPMC nanoplexes prepared at M_{HPMC/CHI} = 0.67 and 1.33, respectively (Fig. 6A). The CUR-CHI-HPMC nanoplex, nevertheless, exhibited similar CUR dissolution rates in the SII as the CUR-CHI nanoplex with 87 ± 6% dissolution after 5 min, compared to nearly zero dissolution for the native CUR after the same period (Fig. 6B). On this note, the decline in the % CUR dissolution after reaching the peak value was caused by the well-known hydrolytic degradation of CUR in physiological pH environment [36].

3.4. Supersaturation generation

In agreement with our hypothesis, the CUR-CHI-HPMC nanoplex prepared at M_{HPMC/CHI} = 0.67 exhibited superior supersaturation generation to the CUR-CHI nanoplex (Fig. 7). Specifically, the CUR-CHI nanoplex produced a maximum achievable supersaturation level of \((11.5 \pm 0.8)\times C_{Sat}\) after 10 min, which then decreased sharply to \((4.5 \pm 0.8)\times C_{Sat}\) after 2 h due to the crystallization of the supersaturated solution, before it eventually settled at \(\approx 1\times C_{Sat}\) after 8 h. For comparison, the CUR-CHI-HPMC nanoplex produced a maximum achievable supersaturation level of \((9.5 \pm 0.4)\times C_{Sat}\) after 20 min, which then decreased slowly to \((7.7 \pm 0.3)\times C_{Sat}\) after 2 h. The supersaturation level of the CUR-CHI-HPMC nanoplex remained high at \((6.2 \pm 0.8)\times C_{Sat}\) after 8 h and it settled at \(\approx 1\times C_{Sat}\) only after 24 h (data not shown).

The supersaturation time-profile of the CUR-CHI-HPMC nanoplex thus exhibited a larger area-under-the-curve (AUC) than the CUR-CHI nanoplex at 58 ± 2 versus 28 ± 2 μg/mL·h over 8 h, respectively. The larger AUC in the supersaturation time-profile of the CUR-CHI-HPMC nanoplex signified its superior CUR solubility enhancement capability. Similar to the role of HPMC in the supersaturation generation of amorphous drugs [37], the superior supersaturation time-profile exhibited by the CUR-CHI-HPMC nanoplex was attributed to the presence of swelling HPMC layer on the nanoplex surface upon dissolution that limited further uptake of the liquid from the dissolution
medium to the nanoplex. This led to the suppression of the solution-mediated crystallization of the amorphous solid phase of the nanoplex, which in turn enabled it to generate a high supersaturation level over a prolonged period.

3.5. Cytotoxicity

The safety of the CUR-CHI-HPMC nanoplex for its potential \textit{in vivo} applications was demonstrated from its minimal cytotoxicity towards the human gastric epithelial cells at two different CUR concentrations, i.e. low (1 μg/mL) and high (10 μg/mL) (Fig. 8). These concentration values represented the typical range of CUR concentrations used in the \textit{in vivo} studies of CUR for gastrointestinal diseases [38, 39]. Specifically, the percentages of viable cells after exposure to the CUR-CHI-HPMC nanoplex were equal to 99.7 ± 0.5% and 68.8 ± 3.1% at CUR = 1 and 10 μg/mL, respectively. The native CUR also demonstrated non-cytotoxicity at these concentrations (Fig. 8). The non-cytotoxicity of CHI and HPMC at their corresponding low to high concentrations in the nanoplex (i.e. 0.29 to 2.9 μg/mL for CHI and 0.17 to 1.7 μg/mL for HPMC) were also demonstrated (Fig. 8).

4. Conclusions

Amorphous ternary nanoplex of CUR-CHI-HPMC exhibiting superior CUR solubility enhancement and physical stability to the amorphous CUR-CHI nanoplex was successfully prepared. First, compared to the CUR-CHI nanoplex, the incorporation of HPMC into the nanoplex resulted in larger nanoplex (≈ 300-500 nm) having lower zeta potential (≈ 1-15 mV) due to the non-ionic nature of HPMC and lower CUR payload (≈ 40-80%). The impacts of the HPMC inclusion on the nanoplex’s physical characteristics could be controlled by varying the $M_{\text{HPMC}/\text{CHI}}$ in the feed. Second, the incorporation of HPMC resulted in higher CUR utilization rate (nearly 100%) than that achieved in the CUR-CHI nanoplex owed to the CUR interactions with both CHI and HPMC. The CUR utilization rate and production yield were nevertheless minimally affected by $M_{\text{HPMC}/\text{CHI}}$ though excessive presence of HPMC at $M_{\text{HPMC}/\text{CHI}}$ larger than unity led to their lower values. Third, the CUR-CHI-HPMC nanoplex exhibited superior dissolution characteristics and supersaturation generation in simulated gastrointestinal fluids compared to the CUR-CHI nanoplex that boded well for its bioavailability enhancement capability. Fourth, the CUR-CHI-HPMC was found to be non-cytotoxic towards the human gastric epithelial cells. Lastly, the long-term amorphous state stability exhibited by the CUR-CHI-HPMC nanoplex further established it as a highly promising CUR solubility enhancement strategy.

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**Figure captions**

Fig. 1  Effects of M\textsubscript{HPMC/CHI} on the (A) size, zeta potential and (B) CUR payload, CE, and yield of the CUR-CHI-HPMC nanoplex prepared at R\textsubscript{CHI/CUR} = 1.0

Fig. 2  Effects of M\textsubscript{HPMC/CHI} on the (A) size, zeta potential and (B) CUR payload, CE, and yield of the CUR-CHI-HPMC nanoplex prepared at R\textsubscript{CHI/CUR} = 0.7

Fig. 3  (A) FESEM image and (B) colloidal stability of the CUR-CHI-HPMC nanoplex prepared at R\textsubscript{CHI/CUR} = 0.7 and M\textsubscript{HPMC/CHI} = 0.67 as the representative sample

Fig. 4  FTIR spectra of the CUR-CHI-HPMC nanoplex, CUR-CHI nanoplex, native CUR, CHI, and HPMC in the wavenumber range of (A) 700 to 2100 cm\textsuperscript{-1} and (B) 2300 to 2700 cm\textsuperscript{-1}

Fig. 5  (A) PXRD patterns and (B) DSC thermographs of the CUR-CHI-HPMC nanoplex, CUR-CHI nanoplex, native CUR, CHI, and HPMC

Fig. 6  Dissolution time-profiles of the CUR-CHI-HPMC nanoplex, CUR-CHI nanoplex, and native CUR in the (A) SGJ and (B) SIJ, where 0.67 and 1.33 indicated the M\textsubscript{HPMC/CHI} used to prepare the CUR-CHI-HPMC nanoplexes

Fig. 7  Supersaturation generation of the CUR-CHI-HPMC nanoplex prepared at R\textsubscript{CHI/CUR} = 0.7 and M\textsubscript{HPMC/CHI} = 0.67 in comparison with that of the CUR-CHI nanoplex

Fig. 8  Cell viability after exposures to the CUR-CHI-HPMC nanoplex, native CUR, native CHI, and native HPMC at two different concentrations
Fig. 1

A) Graph showing the relationship between HPMC/CHI (w/w) ratio and size and zeta potential of particles.

B) Graph showing the relationship between HPMC/CHI (w/w) ratio and CE, yield, and payload of particles.
Fig. 2

A) 

B)
Fig. 3

A)

B)

![Graph showing size and zeta potential changes over time](image)

- **Size** (nm)
- **Zeta potential** (mV)

- Time (h):
  - 0
  - 3
  - 6
  - 24
Fig. 4

A) CUR-CHI-HPMC nanoplex
   CUR-CHI nanoplex
   Native CUR
   CHI
   HPMC

B) CUR-CHI-HPMC nanoplex
   CUR-CHI nanoplex
   Native CUR
   CHI
   HPMC
Fig. 5

A) Native CUR

CUR-CHI-HPMC nanoplex (0 month)

CUR-CHI-HPMC nanoplex (12 months)

CUR-CHI nanoplex (0 month)

CUR-CHI nanoplex (12 months)

B) Heat Flow (endo down)

Heat Flow (endo down)

Heat Flow (endo down)

Native CUR

CUR-CHI-HPMC nanoplex

CUR-CHI-HPMC nanoplex

CUR-CHI-HPMC nanoplex

CUR-CHI nanoplex

CUR-CHI nanoplex

CUR-CHI nanoplex

CUR-CHI nanoplex

CUR-CHI nanoplex

CHI

CHI

CHI

HPMC

HPMC

HPMC

HPMC

Temperature (°C)

Temperature (°C)

Temperature (°C)

Temperature (°C)
Fig. 6

A) % CUR dissolution in SGJ (w/w)

- Native CUR
- CUR-CHI nanoplex
- CUR-CHI-HPMC nanoplex (0.67)
- CUR-CHI-HPMC nanoplex (1.33)

Time (h)

B) % CUR dissolution in SIJ (w/w)

- Native CUR
- CUR-CHI nanoplex
- CUR-CHI-HPMC nanoplex (0.67)
- CUR-CHI-HPMC nanoplex (1.33)

Time (h)
Fig. 7

Supersaturation ($C/C_{\text{Sat}}$) in SIJ

- CUR-CHI nanoplex
- CUR-CHI-HPMC nanoplex (0.67)