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1 **Controlling the Burst Release of Amorphous Drug-Polysaccharide Nanoparticle Complex**
2 **via Crosslinking of the Polysaccharide Chains**

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Abstract

High-payload amorphous drug-polysaccharide nanoparticle complex (or nanoplex in short) represents a new class of supersaturating drug delivery systems intended for bioavailability enhancement of poorly-soluble drugs. Not unlike other nanoscale amorphous formulations, the nanoplex exhibits fast dissolution characterized by a burst drug release pattern. While the burst release is ideal for supersaturation generation in the presence of crystallization inhibitor, it is not as ideal for passive targeting drug delivery applications in which the nanoplex must be delivered by itself. Herein we developed nanoplex exhibiting controlled release via crosslinking of the polysaccharide chains onto which the drug molecules were electrostatically bound to. Curcumin and chitosan were used, respectively, as the drug and polysaccharide models with amine-reactive disuccinimidyl tartrate as the crosslinking agent. The crosslinked nanoplex exhibited improved morphology (i.e. smaller size, more spherical, and higher uniformity) that signified its more condensed structure. A twenty-fold reduction in the initial burst release rate with a three-fold reduction in the overall dissolution rate was obtained after crosslinking. The slower dissolution was attributed to the more condensed structure of the crosslinked nanoplex that enhanced its dissociation stability in phosphate buffered saline. The reduction in the dissolution rate was proportional to the degree of crosslinking that was governed by the crosslinker to amine ratio. The crosslinking caused slight reductions in the payload and zeta potential of the nanoplex, but with no adverse effect on the cytotoxicity. This proof-of-concept study successfully demonstrated the use of polysaccharide crosslinking to control the drug release from high-payload amorphous drug nanoplex.

Keywords: amorphization; drug-polysaccharide complex; supersaturating drug delivery system; curcumin nanoparticles; chitosan

31 **List of abbreviations**

32	AUC	area under the curve
33	BCS	biopharmaceutics classification system
34	CHI	chitosan
35	CUR	curcumin
36	DMEM	Dulbecco's modified Eagle's medium
37	DMSO	dimethyl sulfoxide
38	DR	dissolution rate
39	DST	disuccinimidyl tartrate
40	EDTA	ethylenediaminetetraacetic acid
41	EPR	enhanced permeability and retention
42	FESEM	field emission scanning electron microscope
43	FTIR	Fourier transform infrared spectroscopy
44	HPLC	high performance liquid chromatography
45	HPMC	hydroxypropylmethylcellulose
46	M_{DST/NH_2}	molar ratio of DST to NH_2
47	MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
48	NHS	N-hydroxysuccinimide
49	OD ₅₅₀	optical density at 550 nm
50	OD ₆₀₀	optical density at 600 nm
51	PBS	phosphate buffered saline
52	PCS	photon correlation spectroscopy
53	PVP	polyvinylpyrrolidone

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1. Introduction

Amorphous drug nanoparticles have been established as a highly effective bioavailability enhancement strategy of poorly soluble drugs by virtue of their ability to generate high apparent solubility of such drugs not obtainable in their crystalline counterparts [1]. Herein drug nanoparticles are defined as nanoparticles of the active ingredient itself, hence distinguishing them from nanocapsules in which the active ingredient is encapsulated in nanoparticle carriers typically made up of lipids or polymers. The high apparent solubility, which is higher than the thermodynamic saturation solubility, is attributed to the generation of supersaturated drug concentration afforded by the metastable state of the amorphous form of the drug nanoparticles [2].

While the conventional supersaturating drug delivery system in the form of microscale amorphous solid dispersion is also capable of generating high apparent drug solubility [3], the superiority of the supersaturation generation of the amorphous drug nanoparticles has been demonstrated in both *in vitro* [1] and *in vivo* [4-6] studies. The superior supersaturation generation of the amorphous drug nanoparticles is attributed to the fast dissolution velocity afforded by the nanoscale size, which in turn suppresses the solution-mediated crystallization of the amorphous solid phase upon dissolution [1].

Conventionally, amorphous drug nanoparticles are prepared by incorporating polymeric stabilizers having high glass transition temperatures, such as hydroxypropylmethylcellulose (HPMC) and polyvinylpyrrolidone (PVP), to occupy the high-energy sites of the drug nanoparticles immediately after their formation [7]. The roles of the polymeric stabilizers are twofold, i.e. (1) to inhibit the post-nucleation growth of the drug nanoparticles and (2) to suppress the crystallization propensity of the amorphous form during storage. Using this nanoscale amorphization strategy, various nanoparticle precipitation methods (e.g. antisolvent [1], pH-shift [6], ultrasonic [4], and evaporative [8] precipitations) have been employed in the presence of polymeric stabilizers to prepare amorphous drug nanoparticles.

Recently, we developed a new class of amorphous drug nanoparticles in the form of drug-polysaccharide nanoparticle complex (or nanoplex in short) by self-assembly electrostatically-driven complexation between drug molecules and oppositely charged polysaccharides [9]. In this nanoscale amorphization strategy, the stabilization was accomplished by restricting the molecular mobility of the drug molecules by means of physical binding with the polysaccharide chains. As the nanoplex was held together primarily by electrostatic binding, it readily dissociated in

salt solution, such as phosphate buffered saline (PBS), resulting in supersaturation generation [9]. Using itraconazole as the model poorly-soluble drug, the itraconazole nanoplex was shown to exhibit superior colloidal and amorphous state stabilities, as well as prolonged supersaturation, compared to the nano-itraconazole prepared by the more conventional high-energy site occupation strategy [9].

Amorphous drug nanoparticles prepared by both strategies were known to produce the “spring and parachute” supersaturation profile characteristic of the amorphous form upon dissolution in the presence of crystallization inhibitors (e.g. HPMC, PVP) [1, 9]. Initially, the drug was released in a burst pattern to produce the supersaturation peak (i.e. “spring”), followed by its gradual decline (i.e. “parachute”), resulting in a sustained supersaturation level before it eventually reached the thermodynamic saturation solubility [2]. In the absence of crystallization inhibitors, however, the rate of decline in the supersaturation level has been shown to increase with increasing burst release rates, resulting in a smaller area under the curve (AUC) for the supersaturation versus time profile, hence lower drug bioavailability *in vivo* is to be expected [10].

While the crystallization inhibitors can always be incorporated in oral solid dosage forms of **amorphous drug nanoparticles** intended for systemic delivery, there are therapeutic applications that would require the **amorphous drug nanoparticles** to be delivered locally by parenteral routes to the disease sites as stand-alone nanoparticles, instead of their granulated solid dosage form. Examples include (1) passive targeting of tumor tissues in anticancer therapies via the well-known enhanced permeability and retention (EPR) effect of nanoparticles [11], and (2) passive targeting of microbial pathogens embedded in a thick mucus (e.g. lung, eye) using mucus-penetrating antimicrobial nanoparticles [12]. For such applications, the **amorphous drug nanoparticles** ideally exhibit a slower burst release rate in the absence of crystallization inhibitors in order to maintain sufficiently high local bioavailability.

To the best of our knowledge, however, **amorphous drug nanoparticles** exhibiting controlled release functionality have not been developed before. In contrast, numerous studies have reported microscale amorphous solid dispersions exhibiting controlled drug release intended for oral systemic delivery, where the controlled release was achieved by varying the type and amount of the polymeric stabilizers used [13, 14]. While there were several studies that reported controlled drug release from amorphous nanofiber drug dispersion prepared by electrospinning

[15, 16], the nanofibers were not classified as **amorphous drug nanoparticles** because only their diameters were in the nanoscale, while their lengths were in the millimeter scale.

Herein we presented the development of amorphous drug nanoplex exhibiting controlled release functionality achieved by crosslinking of the polysaccharide chains onto which the drug molecules were bound. **The crosslinking was performed on the nanoplex prepared from the basic formulation immediately after its preparation.** We hypothesized that **the** crosslinked polysaccharide chains would make the nanoplex structure more condensed, which in turn would slow down the escape of **the** drug molecules upon their dissociation from the polysaccharide chains in the dissolution medium.

Curcumin - a natural flavonoid isolated from turmeric plants classified as Biopharmaceutics Classification System (BCS) Class IV compound [17] - was used as the model poorly-soluble drug owed to its well-established therapeutic properties ranging from anti-inflammatory and anticancer to antimicrobial and antioxidant [18]. Moreover, curcumin as a weakly acidic compound was readily ionized upon dissolution in base [19] (Fig. 1), thus making it ideal for transformation to the nanoplex by the drug-polysaccharide electrostatic complexation. Chitosan was used as the polysaccharide because (1) it was readily ionized in weak acid to produce opposite charge to that of curcumin (Fig. 1) [20], and (2) it contained amine (NH₂) functional groups that could be crosslinked upon the introduction of an amine-reactive crosslinking agent, such as disuccinimidyl tartrate (DST), **which is** classified as homobifunctional N-hydroxysuccinimide (NHS) esters [21].

The objective of the present work was to investigate the effects of the molar ratio of DST to the NH₂ group of chitosan in the nanoplex on the (1) physical characteristics (i.e. size, zeta potential, shape, payload) of the crosslinked nanoplex produced, (2) dissociation stability of the crosslinked nanoplex in PBS as compared to the unmodified nanoplex, and (3) *in vitro* dissolution time-profile of the crosslinked nanoplex under sink condition. In addition, the *in vitro* cytotoxicity of the crosslinked nanoplex towards human lung epithelium cells was characterized to examine whether the crosslinking had any adverse effect on the nanoplex's cytotoxicity. Lung epithelium cells were selected as the model cell because we envisioned that the amorphous curcumin-chitosan nanoplex could potentially find its application in passive-targeting therapy of respiratory infections.

2. Materials and Methods

2.1. Materials

Materials for nanoplex preparation and characterization: Chitosan (CHI) having molecular weight (MW) of 50-190 kDa and 75-85% deacetylation, potassium hydroxide (KOH), sodium chloride (NaCl), sodium phosphate (Na_3PO_4) glacial acetic acid, ethanol, PBS (pH 7.4), and potassium bromide (KBr) were purchased from Sigma-Aldrich (Singapore). Curcumin (CUR) at 98% purity and disuccinimidyl tartrate (DST) were purchased from Alfa Aesar (USA) and Thermo Fisher Scientific (USA), respectively. **Materials for cytotoxicity test:** A549 adenocarcinomic human alveolar basal epithelial cells were purchased from ATCC (USA). 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution, penicillin-streptomycin, dimethyl sulfoxide (DMSO), and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT, 98% purity) were purchased from Gibco (Canada), PAA Laboratories (Austria), Sigma-Aldrich (Singapore), and Alfa Aesar (UK), respectively. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from HyClone (Thermo Scientific, USA).

2.2. Methods

2.2.1. Preparation of **basic** CUR-CHI nanoplex

The **basic** CUR-CHI nanoplex was prepared at the optimal pH (i.e. 4.4) and charge ratio of CHI to CUR (i.e. 0.8) previously determined in Nguyen, Yu, Kiew and Hadinoto [22]. CUR having pK_a of 8.4, 9.9, and 10.5 [19] was fully deprotonated upon dissolution in 0.1 M KOH at pH 13 to form the negatively charged CUR⁻ having charge density of 8.14×10^{-6} mol-charge/mg. CHI having pK_a of 6.5 [20] was protonated upon dissolution in 1.2% (v/v) acetic acid to form the positively charged CHI⁺ with charge density of 4.46×10^{-6} mol-charge/mg assuming 80% deacetylation of CHI.

Briefly, equal volumes of the CUR (5.0 mg/mL) and CHI (7.3 mg/mL) solutions were mixed and then vortexed for 10 s to produce soluble CUR-CHI complex, which subsequently formed aggregates driven by hydrophobic interactions among the bound CUR molecules (Fig. 2). The CUR and CHI solutions were mixed immediately after their preparation to minimize the alkaline degradation of CUR. The aggregated complex later precipitated out to form the CUR-CHI nanoplex upon reaching a critical aggregate concentration. The resultant nanoplex suspension was ultrasonicated for 25 s (CV24 probe sonicator, Sonics, USA) after which the suspension was washed by two cycles of centrifugation ($14,000 \times g$ for 25 min) and rinsing. Lastly, the washed nanoplex suspension was re-suspended in deionized water for the crosslinking step.

2.2.2. Crosslinking of **the basic** CUR-CHI nanoplex

The free NH₂ groups of CHI in the basic nanoplex were crosslinked with each other upon the addition of amine-reactive DST via the formation of amide linkages between DST and the free NH₂ (Fig. 2). Herein the free NH₂ groups were defined as those that did not form electrostatic binding with CUR. As a homobifunctional crosslinker, one DST molecule formed covalent bonding with two NH₂ molecules. Thus, the end products consisted of the crosslinked CUR-CHI nanoplex and two NHS molecules (Fig. 2).

Briefly, the washed nanoplex suspension was added to a reaction buffer made up of 0.4 mM Na₃PO₄ and 3 mM NaCl, while DST was dissolved separately in DMSO at 30 mM. The DST solution was then added to 1 mL of the nanoplex suspension at different molar ratios of DST to the total NH₂ groups of CHI in the nanoplex (i.e. M_{DST/NH_2} = 0.2, 0.4, 1.0, and 2.0). A sample calculation on the M_{DST/NH_2} determination was provided in the Supporting Information. The mixed solution wrapped in aluminum foil was incubated at room temperature for 30 min. Afterwards, the crosslinked nanoplex suspension was washed by two cycles of centrifugation (14,000×g for 25 min) and rinsing, followed by its re-suspension in deionized water. The crosslinking experiment was carried out in triplicates using three independent batches of the basic nanoplex.

2.2.3. Physical characterizations

The size and zeta potential of the crosslinked nanoplex suspension were characterized by photon correlation spectroscopy (PCS) using Brookhaven 90 Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corporation, USA) at 25°C. The nanoplex suspension was diluted 100 times in deionized water prior to the PCS measurement. The morphology of the crosslinked nanoplex was characterized by Field Emission Scanning Electron Microscope (FESEM) using JSM-6700F (JEOL, USA). Dry powders of the nanoplex prepared by freeze drying were used for the FESEM after sputter coating them with platinum.

The payload, which was defined as the ratio of CUR mass in the nanoplex to the total nanoplex mass (i.e. CUR + CHI + DST), was characterized by dispersing a known mass of the nanoplex powders in 80% (v/v) aqueous ethanol solution to fully dissolve CUR in the nanoplex. Afterwards, the mass of the dissolved CUR was determined by UV-Vis spectrophotometry using UV Mini-1240 (Shimadzu, Japan) at the optimal CUR absorbance wavelength of 423 nm. The presence of CUR after crosslinking was checked by Fourier Transform Infrared Spectroscopy (FTIR) using Spectrum One (Perkin-Elmer, USA). The FTIR was performed between 400 and 4500 cm⁻¹ at 1 cm⁻¹

spectral resolution for the native CUR, native CHI, and for both the basic and crosslinked CUR-CHI nanoplexes. The FTIR pellets were prepared by die pressing of 4 mg of the samples and 100 mg KBr at 10 tons for 1 min.

2.2.4. Dissociation stability

The dissociation stability of the crosslinked nanoplex in PBS was evaluated in triplicates by two methods. First, the turbidity of the nanoplex suspension upon addition to PBS was characterized, where higher turbidity, which denoted the presence of particulates in the suspension, indicated a lower degree of nanoplex dissociation (i.e. less dissolution). Second, the amount of CUR remaining in the nanoplex after its addition to PBS was quantified, where a larger amount of CUR remaining denoted a lower degree of nanoplex dissociation. The dissociation stability tests were performed at a CUR concentration below its saturation solubility in PBS (i.e. 4.15 µg/mL [22]) to ensure that CUR precipitates did not contribute to the measurements.

Briefly, 7 µL of the crosslinked nanoplex suspension was added to 4 mL PBS and subsequently vortexed for 20 s. The turbidity of the resultant suspension was immediately characterized using UV-Vis spectrophotometry from the optical density at 600 nm (OD₆₀₀). In a separate experiment, the nanoplex suspension in PBS was centrifuged at 14,000×g for 3 min after which the amount of CUR in the supernatant was quantified by UV-Vis spectrophotometry at 423 nm. The percentage amount of CUR remaining in the nanoplex was then calculated from Eq. (1).

$$\% \text{ CUR remaining} = \left(1 - \frac{\text{Mass of CUR in supernatant}}{\text{Payload} \times \text{Mass of Nanoplex}}\right) \times 100\% \quad (1)$$

2.2.5. Dissolution time-profile

The crosslinked nanoplex suspension was added to 10 mL PBS under sink condition in a shaking incubator at 37°C. At specified time points over 20 min, 1 mL aliquot was withdrawn and replaced with the same volume of fresh PBS. The amount of CUR in the aliquot after filtered in 200-nm syringe filter was determined by High Performance Liquid Chromatography (HPLC) using Agilent 1100 (Agilent Technologies, Singapore) with ZORBAX Eclipse Plus C18 column (250×4.6 mm and 5-µm particle size). The detection wavelength in the HPLC was set at 423 nm and 90% (v/v) aqueous ethanol solution at 1 mL/min was used as the mobile phase, resulting in CUR's retention time of 2.5 min. The dissolution time-profile of the nanoplex was characterized from three replicates using the dissolution rate (% DR) defined in Eq. (2).

$$\% \text{ DR} = \frac{\text{Mass of CUR recovered in the dissolution medium}}{\text{Initial mass of CUR in the nanoplex prior to dissolution}} \times 100\% \quad (2)$$

As CUR was widely known to undergo hydrolytic degradation in a physiological pH environment, including in PBS [23], the rate of degradation of the native CUR in PBS was characterized using the same initial concentration of CUR as the one used in the nanoplex's dissolution. Briefly, the native CUR was dissolved in 10 mL PBS under sink condition after which the solution was incubated in a shaking incubator at 37°C. At specific time points over 20 min, 1 mL aliquot was withdrawn and replaced with fresh PBS of the same volume. The amount of CUR in the aliquot was subsequently determined by HPLC from which the rate of CUR degradation as a function of time was derived. To take into account the CUR degradation in the dissolution time-profile, corrected % DR was reported in which the amount of CUR that had degraded was added to the numerator of Eq. (2).

2.2.6. Cytotoxicity

The A549 cells were cultivated in a 24-well plate using DMEM supplemented with 10% (w/v) fetal bovine serum and 1% (w/v) penicillin-streptomycin solutions, followed by 24-h incubation at 37°C in 5% CO₂ incubator, resulting in cell density of 10⁵ cells/well. Afterwards, the nanoplex suspension containing 40 µg/mL of CUR was added to the cells and they were subsequently incubated for 24 h at 37°C in 5% CO₂ incubator. The wells containing only the cells and supplemented DMEM were used as the control. Next, the medium was replaced with serum-free DMEM containing 100 µL of MTT dye solution (i.e. 0.5% w/v in PBS) after which the cells were incubated for 4 h. Afterwards, 2 mL DMSO was added to the 24-well plate under gentle shaking to fully dissolve the formazan crystals produced by the viable cells via enzymatic reduction of MTT. Subsequently, the formazan concentration was determined by optical density measurement at 550 nm (OD₅₅₀) using UV-Vis spectrophotometry. The cytotoxicity was reported in terms of the cell survival percentage relative to the control, which was calculated from the ratio of OD₅₅₀ of the cells to that of the control. The cytotoxicity test was performed with two replicates each for the nanoplex (i.e. basic and crosslinked) as well as for the native CUR dissolved in 0.1 M KOH.

3. Results

3.1. Physical characteristics of the crosslinked CUR-CHI nanoplex

The crosslinking resulted in a significant reduction in the nanoplex size from 536 ± 42 nm for the basic nanoplex to 306 ± 14 nm for the crosslinked nanoplex prepared at M_{DST/NH_2} = 0.2 (Fig. 3A). The size reduction was intensified at higher M_{DST/NH_2} , decreasing the nanoplex size to 247 ± 6 nm at M_{DST/NH_2} = 2.0. The decrease in the nanoplex size was accompanied by a decrease in the zeta potential from 21.8 ± 1.8 mV for the basic nanoplex to

15.8 $\pm$ 0.5 mV for the crosslinked nanoplex prepared at M_{DST/NH_2} = 0.2 (Fig. 3A). The zeta potential was further decreased, albeit slightly, with increasing M_{DST/NH_2} to reach 13.0 $\pm$ 0.7 mV at M_{DST/NH_2} = 2.0.

The CUR payload was not greatly affected by the significant reduction in size, where the payload decreased from 85.1 $\pm$ 3.7% before crosslinking to 78.5 $\pm$ 1.7% after crosslinking at M_{DST/NH_2} = 0.2 (Fig. 3B). Not unlike the trends in the size and zeta potential, the payload continued to decrease, albeit slightly, with increasing M_{DST/NH_2} to reach 74.2. $\pm$ 1.8% at M_{DST/NH_2} = 2.0. The crosslinking also resulted in more spherical nanoplex with improved uniformities in both size and shape as can be seen from the FESEM images in Figs. 4B-C. The FESEM image of the basic nanoplex (Fig. 4A) also revealed that the basic nanoplex was prone to interparticle fusion, which in turn contributed to the larger size detected by the PCS measurement as well as its more irregular shape.

In addition to the payload, the presence of CUR in the crosslinked nanoplex was further verified by FTIR, which showed the appearance of the characteristics bands of CUR at 1626, 1508, and 1272 cm^{-1} in the FTIR spectra of the crosslinked nanoplex (Fig. 5). These bands, which also appeared in the spectra of the native CUR and the basic nanoplex, corresponded to the stretching vibrations of the C=C-C_{ring}, C=O, and enol C-O bonds of CUR, respectively [24]. The broad band at 1591 cm^{-1} , which distinctly appeared in the nanoplex's spectrum, but not in the native CUR's, corresponded to the bending of the NH₂ group of CHI [25] in the nanoplex. However, the presence of the amide bonds (-CONH-) formed between CHI and DST after crosslinking was not evident from the FTIR spectrum of the crosslinked nanoplex due to the low DST content of the nanoplex. These amide bands typically appeared at 1635 (amide I) and 1565 (amide II) cm^{-1} corresponding to the C=O stretching and N-H bending, respectively [26].

3.2. Dissociation stability of the crosslinked CUR nanoplex

The basic nanoplex readily dissociated upon its dissolution in PBS resulting in the release of CUR as evident from the low turbidity of the nanoplex suspension ($\text{OD}_{600} \approx 0.02$) (Fig. 6A) and less than 40% of CUR remaining in the nanoplex (Fig. 6B). In comparison, the crosslinked nanoplex exhibited higher dissociation stability as reflected by the higher turbidity and higher % CUR remaining, which both increased with increasing M_{DST/NH_2} , hence signifying the achievement of a higher degree of crosslinking at higher M_{DST/NH_2} . Specifically, the turbidity was increased from $\text{OD}_{600} \approx 0.08$ at M_{DST/NH_2} = 0.4 to $\text{OD}_{600} \approx 0.13$ at M_{DST/NH_2} = 2.0 (Fig. 6A), while the % CUR

remaining in the nanoplex was increased from $68.5 \pm 3.9\%$ to $84.5 \pm 4.0\%$ (Fig. 6B). The high % CUR remaining signified that the release of CUR was successfully suppressed at $M_{DST/NH_2} = 2.0$.

3.3. Dissolution time-profile of the crosslinked CUR-CHI nanoplex

In the first 5 min of dissolution under sink condition, the higher dissociation stability of the crosslinked nanoplex led to a slower CUR release rate as reflected in the lower % DR compared to that of the basic nanoplex (Fig. 7A). Not unexpectedly, the % DR was found to be lower at higher M_{DST/NH_2} attributed to the higher degree of crosslinking. The % DR, however, ceased to increase after 5 min and began to either slowly decrease or reach a plateau due to the hydrolytic degradation of CUR in PBS. Under the sink condition investigated here, the rate of CUR degradation was determined to be constant as a function of time at $0.132 \pm 0.011 \mu\text{g/mL}\cdot\text{min}$ for the first 15 min of dissolution, resulting in approximately 50% loss of CUR (data not shown). The rate of CUR degradation was found to greatly diminish and became negligible after 15 min for the amount of CUR and PBS used in this study.

To evaluate the true effect of crosslinking on the nanoplex's dissolution rate, the % DR was corrected by taking into account of the amount of CUR that had degraded after its dissolution. The corrected % DR of the basic nanoplex was shown to reach $77.5 \pm 2.3\%$ after 5 min, where approximately 50% of the CUR was released within 15 s of dissolution, thus signifying a rapid burst release rate (Fig. 7B). Subsequently, the corrected % DR of the basic nanoplex remained at $\approx 78\%$ from 5 to 20 min denoting minimal CUR release in that period.

For comparison, the corrected % DR of the crosslinked nanoplex prepared at $M_{DST/NH_2} = 0.4$ reached $70.3 \pm 2.2\%$ after 5 min, where approximately 50% of the CUR was released after a significantly longer period (i.e. 1 min versus 15 s) (Fig. 7B). Subsequently, the corrected % DR increased slightly to $76.2 \pm 1.2\%$ after 20 min. The slow-down of the nanoplex's dissolution rate after crosslinking was intensified at higher M_{DST/NH_2} equal to 2.0, where the corrected % DR after 5 min was $51.3 \pm 2.3\%$ with approximately 50% of the CUR was released only after 5 min. This was followed by a gradual increase in the corrected % DR to reach $82.4 \pm 1.7\%$ after 20 min (Fig. 7B). For both the basic and crosslinked nanoplexes, the corrected % DR reached a plateau at $\approx 75\text{-}80\%$ after 20 min.

3.4. Cytotoxicity

The crosslinking of the CUR-CHI nanoplex with DST was not found to have any adverse effect on the toxicity of the nanoplex towards the A549 cells even at the highest M_{DST/NH_2} investigated (Fig. 8). At $40 \mu\text{g/mL}$ CUR, the cell survivals after exposure to the nanoplex were in the range of 66.9 ± 4.2 to $73.0 \pm 4.5\%$, which were comparable

to the cell survival ($61.3 \pm 3.8\%$) after exposure to the native CUR of the same concentration. The slightly higher cell survival after exposure to the nanoplex was likely attributed to the fact that CUR was not fully released from the nanoplex as observed from the dissolution time-profile results, resulting in less CUR presence compared to when the native CUR was tested.

4. Discussion

As hypothesized, the results showed that crosslinking of the CUR-CHI nanoplex with DST made the nanoplex structurally more condensed as manifested in the smaller, more uniform, and more spherical size after crosslinking. This was attributed to the bound CHI chains in the nanoplex being pulled closer together upon crosslinking. The more condensed structure of the crosslinked nanoplex also made it less prone to interparticle fusion, where the interparticle fusion was postulated to be caused by hydrophobic interactions among the surface-bound CUR molecules originated from different nanoplexes. The more condensed structure was believed to provide increased enclosure to the bound CUR molecules, resulting in less inter-drug hydrophobic interactions. Therefore, the weaker interparticle fusion tendency of the crosslinked nanoplex contributed to its smaller and more uniform size.

As the crosslinked nanoplex size continued to decrease with increasing M_{DST/NH_2} , it suggested that increasing M_{DST/NH_2} could enhance the degree of crosslinking, even at M_{DST/NH_2} values above unity where there was excess DST. Thus, the continuous decrease in the zeta potential of the crosslinked nanoplex with increasing M_{DST/NH_2} was not unexpected considering that more DST formed amine linkages with the free NH_2 groups of CHI due to the higher degree of crosslinking. This resulted in fewer free NH_2 groups available to serve as charge stabilizer of the nanoplex, resulting in lower zeta potential, hence lower colloidal stability of the crosslinked nanoplex. Furthermore, the fact that the payload was decreased slightly at higher M_{DST/NH_2} suggested that the higher degree of crosslinking at higher M_{DST/NH_2} came at the expense of increased loss of CUR from the nanoplex, where some of the NH_2 groups of CHI, which initially formed electrostatic binding with CUR (i.e. non-free NH_2 groups), formed covalent bonding with DST instead, resulting in the disengagement of CUR from the nanoplex.

The amorphous state, small size, and high payload of the basic nanoplex had been shown in our previous study [27] to result in rapid drug dissolution that was responsible for its excellent supersaturation generation. For the crosslinked nanoplex, the more condensed structure made it less prone to dissociation in highly ionic condition of PBS as the enclosed CUR molecules were less affected by the charge shielding effect of salt, thereby they did not

disengage as quickly from the CHI chains. As a result, CUR was released more slowly from the crosslinked nanoplex, despite its smaller size, which typically led to faster dissolution. Specifically, the time needed to reach 50% dissolution of the payload was prolonged by twenty folds from 15 s for the basic nanoplex to 5 min for the crosslinked nanoplex prepared at the highest M_{DST/NH_2} investigated, whereas the time needed to reach the maximum dissolution of the payload was prolonged by three folds from 5 min to 15 min.

Using CUR as the model drug, we acknowledged that the reduction in the dissolution rate in terms of the absolute time was not substantial enough as both the basic and crosslinked nanoplexes still released the bulk of their payload in less than 20 minutes. The use of CHI of low MW likely played a role in contributing to the fast CUR release as it provided less resistance for the dissociated CUR molecules to escape from the swollen CHI gels. Nevertheless, our study had successfully demonstrated the proof-of-concept of controlling the burst release rate from drug-CHI nanoplex by performing crosslinking of the CHI chains. The same concept can be applied to other poorly-soluble acidic drugs to produce multifold reductions in their dissolution rates from the nanoplex. For the practical applications, the crosslinking should be combined with (i) the use of CHI of higher MW and/or (ii) surface coating to equip high-payload amorphous drug nanoparticles with built-in controlled release functionality.

5. Conclusion

Controlled drug release from amorphous drug-CHI nanoplex was successfully achieved by means of crosslinking of the CHI chains using amine-reactive DST. The typical burst release of drug from the nanoplex, which may not be desirable in certain therapeutic applications involving passive targeting, was suppressed by multifold after crosslinking. The slower dissolution rate was attributed to the higher dissociation stability of the crosslinked nanoplex in the highly ionic condition of the dissolution medium. The reduction in the burst release was proportional to the degree of crosslinking in the nanoplex which itself could be enhanced by increasing the ratio of DST to NH_2 of CHI. In addition to the controlled release, the crosslinked nanoplex exhibited improved morphology (i.e. smaller size, higher sphericity, and better uniformity) attributed to its more condensed structure compared to the unmodified nanoplex. The crosslinking, however, led to slight reductions in the payload and colloidal stability of the nanoplex. Lastly, the crosslinking did not have any adverse effect on the cytotoxicity of the nanoplex, which remained comparable to that of the native drug.

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416 **Figure captions**

- 417 Fig. 1 Chemical structures of curcumin (CUR) and chitosan (CHI) and their ionizations in weak base and acid,
418 respectively
419
- 420 Fig. 2 Schematics of the CUR-CHI nanoplex formation (left) followed by the subsequent crosslinking of the
421 free NH_2 groups of CHI in the nanoplex using N-hydroxysuccinimide (NHS) esters - disuccinimidyl
422 tartrate (DST) - as the amine-reactive crosslinking agent (right)
423
- 424 Fig. 3 Effects of the ratio of DST to NH_2 group of CHI (i.e. $M_{\text{DST}/\text{NH}_2}$) on the (A) size, zeta potential and (B)
425 payload of the nanoplex produced after crosslinking
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- 427 Fig. 4 FESEM images of the (A) basic nanoplex and (B) crosslinked nanoplex prepared at $M_{\text{DST}/\text{NH}_2}$ equal to 0.2
428 and (C) 2.0
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- 430 Fig. 5 FTIR spectra of the native CHI, the native CUR, the crosslinked nanoplex prepared at $M_{\text{DST}/\text{NH}_2}$ equal to
431 2.0, and the basic nanoplex
432
- 433 Fig. 6 Effects of the ratio of DST to NH_2 group of CHI (i.e. $M_{\text{DST}/\text{NH}_2}$) on the (A) turbidity of the nanoplex
434 suspension and (B) % CUR remaining in the nanoplex upon its addition to PBS
435
- 436 Fig. 7 (A) Dissolution time-profiles of the basic nanoplex ("DST 0.0") and the crosslinked nanoplex prepared at
437 $M_{\text{DST}/\text{NH}_2}$ equal to 0.4 ("DST 0.4") and 2.0 ("DST 2.0"); (B) Corrected dissolution time profiles after
438 taking into account CUR hydrolytic degradation in PBS
439
- 440 Fig. 8 Cytotoxicity of the native CUR, basic nanoplex, and crosslinked nanoplex prepared at $M_{\text{DST}/\text{NH}_2}$ equal to
441 0.4, 1.0, and 2.0 towards the lung epithelium cells. Note: "NP" and "CR" stand for nanoplex and
442 crosslinked, respectively

Fig. 1

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Fig. 2

Fig. 2 Schematics of the CUR-CHI nanoplex formation (left) followed by the subsequent crosslinking of the free NH₂ groups of CHI in the nanoplex using N-hydroxysuccinimide (NHS) esters - disuccinimidyl tartrate (DST) - as the amine-reactive crosslinking agent (right)

Fig. 3

Fig. 3 Effects of the ratio of DST to NH_2 group of CHI (i.e. $M_{\text{DST}/\text{NH}_2}$) on the (A) size, zeta potential and (B) payload of the nanoplex produced after crosslinking

Fig. 4

Fig. 4 FESEM images of the (A) basic nanoplex and (B) crosslinked nanoplex prepared at M_{DST}/NH_2 equal to 0.2 and (C) 2.0

Fig. 5

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Fig. 6

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Fig. 7

Fig. 7 (A) Dissolution time-profiles of the basic nanoplex (“DST 0.0”) and the crosslinked nanoplex prepared at M_{DST/NH_2} equal to 0.4 (“DST 0.4”) and 2.0 (“DST 2.0”); (B) Corrected dissolution time profiles after taking into account CUR hydrolytic degradation in PBS

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