A simple strategy to enhance the in vivo wound-healing activity of curcumin in the form of self-assembled nanoparticle complex of curcumin and oligochitosan

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1 **A Simple Strategy to Enhance the *In Vivo* Wound-Healing Activity of Curcumin in the**
2 **form of Self-Assembled Nanoparticle Complex of Curcumin and Oligochitosan**

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Abstract

While the wound healing activity of curcumin (CUR) has been well-established, its clinical effectiveness remains limited due to the inherently low aqueous CUR solubility, resulting in suboptimal CUR exposure in the wound sites. Previously, we developed high-payload amorphous nanoparticle complex (or nanoplex) of CUR and chitosan (CHI) capable of CUR solubility enhancement by drug-polyelectrolyte complexation. The CUR-CHI nanoplex, however, exhibited poor colloidal stability due to its strong agglomeration tendency. Herein we hypothesized that the colloidal stability could be improved by replacing CHI with its oligomers (OCHI) owed to the better charge distribution in OCHI. The effects of key parameters in drug-polyelectrolyte complexation (i.e. pH, salt inclusion, CUR concentration, and OCHI/CUR charge ratio) on the physical characteristics and preparation efficiency of the CUR-OCHI nanoplex produced were investigated. The *in vivo* wound healing efficacy of the CUR-OCHI nanoplex and its cytotoxicity towards human keratinocytes cells were subsequently examined. The results showed that CUR-OCHI nanoplex exhibited prolonged colloidal stability (72 h versus <24 h for the CUR-CHI nanoplex). At the optimal condition, the CUR-OCHI nanoplex (without ultrasonication) exhibited size, zeta potential, and CUR payload of ≈ 140 nm, 20 mV, and 78% (w/w), respectively. The nanoplex preparation was simple yet robust at nearly 100% CUR utilization rate. The CUR-OCHI nanoplex exhibited superior wound healing efficacy to the native CUR with wound closure of >90% after 7 days versus 9 days for the native CUR, attributed to its generation of high CUR concentration in the wound sites and lower cytotoxicity.

Keywords: wound healing agent; curcumin; chitosan; oligochitosan; nanoparticle complex

50 **List of abbreviations**

51	AA	acetic acid
52	C _{Sat}	saturation solubility of CUR
53	CHI	chitosan
54	CUR	curcumin
55	DLS	dynamic light scattering
56	DMSO	dimethyl sulfoxide
57	DMEM	Dulbecco's modified Eagle's medium
58	FESEM	field emission scanning electron microscope
59	FTIR	Fourier transform infrared spectroscopy
60	HPLC	high performance liquid chromatography
61	HPMC	hydroxypropyl methylcellulose
62	MT	Masson's trichrome
63	MTT	(3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
64	MW	molecular weight
65	OCHI	oligochitosan
66	PBS	phosphate buffered saline
67	PDI	polydispersity index
68	R _{OCHI/CUR}	charge ratio of OCHI to CUR
69	UV-Vis	ultraviolet visible

70

1. Introduction

The wound healing activities of curcumin (CUR) - a natural polyphenol extracted from turmeric - has been well established attributed to its anti-inflammatory and antioxidant properties [1, 2]. The cutaneous wound healing process is generally divided into three phases, i.e. (1) hemostasis and inflammation, (2) proliferation, which includes collagen production, angiogenesis, and granulation tissue formation, and (3) maturation, which include collagen remodeling, apoptosis, and epithelialization leading to scar tissue formation [3]. The application of CUR as a wound-healing agent has been shown to result in improved inflammatory response, reduced oxidative stress, increased fibroblast production, enhanced granulation tissue formation, and faster wound contraction and epithelialization [1, 4].

The application of CUR for wound healing has been found to be most effective via the topical administration route as it provides direct access to the wound sites [5]. Moreover, oral administration of CUR results in poor systemic bioavailability of CUR due to its low aqueous solubility and first-pass metabolism [6]. Numerous topical formulations of CUR in the form of wound dressing and topical gels have been developed, where CUR is physically or molecularly dispersed in various polymer or lipid matrices to form CUR-loaded fibers, films, microemulsion, and hydrogel [7-9]. The efficacy of topical CUR formulations, however, remain far from optimal due to the low aqueous solubility of CUR ($< 10 \mu\text{g/mL}$) and low chemical stability of CUR due to its hydrolytic degradation in physiological environments [10]. These two factors inevitably lead to low CUR concentration in the wound sites [5].

For this reason, nanoscale formulations of CUR in which CUR is encapsulated in polymer/lipid nanoparticle carriers have been extensively investigated for wound healing applications [11]. The CUR nanoformulations are capable of enhancing CUR dissolution owed to the large specific surface areas afforded by the nanoparticle carriers. The CUR nanoformulations also improve chemical stability of CUR attributed to the protection provided by the encapsulating carrier matrix. Furthermore, CUR nanoformulations improve tissue permeation of CUR and enable sustained release of CUR over a prolonged period beneficial to the wound healing process [11].

A wide range of CUR nanoformulations for topical wound-healing application have been developed ranging from polymer nanocapsules [12-14] and liposomes [15, 16] to polymeric micelles [17, 18] and electro-spun nanofibers [19, 20]. While they have been demonstrated to be superior in *in vivo* animal studies to the native (raw) CUR formulations, a closer look at these CUR nanoformulations reveals that (1) they require highly intricate preparation steps which may not be readily scalable from the current laboratory scale, and (2) they exhibit low CUR payloads ($< 15\%$ w/w) due to

the low solubility/miscibility of CUR in the encapsulating matrix. While the low CUR payload may not constitute a problem in animal studies, the low CUR payload would lead to high dosage requirement upon clinical application in larger human wounds. The high dosage requirement in which a majority of the dosage is composed of the carrier materials not only would increase the risk of adverse side effects, but also increase economic burden to the patients.

Previously, we developed high-payload CUR nanoformulation ($\approx 80\%$ w/w) in the form of amorphous nanoparticle complex of CUR and chitosan (CHI) [21]. The metastable amorphous state of the CUR-CHI nanoparticle complex (or nanoplex in short) enabled it to generate a highly supersaturated CUR concentration upon its dissolution, resulting in high apparent solubility of CUR. For wound healing applications, the high CUR payload and supersaturation generation capability of the CUR-CHI nanoplex bode well for its ability to produce high CUR concentration in the wound sites. Moreover, the presence of CHI in the nanoplex brings additional benefits for its wound healing applications as CHI itself is known as a wound healing promoter attributed primarily to its hemostatic and antimicrobial activities [22, 23].

The CUR-CHI nanoplex was prepared by a simple method based on electrostatically-driven drug-polyelectrolyte complexation, which involved only mixing of ionized solutions of CUR and CHI under ambient condition [21]. While the high efficiency ($> 90\%$ CUR utilization) and scaled-up production of the CUR-CHI nanoplex preparation were successfully demonstrated [24], the existing nanoplex had a strong tendency to agglomerate upon its formation. As a result, an ultrasonication step was necessary to disperse the microscale agglomerates to produce CUR-CHI nanoplex in the size range of 200 to 300 nm [21]. Furthermore, the existing nanoplex exhibited diminished colloidal stability in which it would begin to agglomerate again after a prolonged static period (< 24 h). Consequently, the nanoplex ought to be transformed to its dry-powder form shortly after its preparation to preserve its original size [21, 24].

For its application as a wound-healing agent, the strong agglomeration tendency of the existing CUR-CHI nanoplex is less than desirable as it would nullify the aforementioned benefits afforded by nanoscale formulations. Moreover, the agglomeration would limit the clinical effectiveness of the nanoplex to only for few hours after its aqueous re-suspension from the dry powders. In this regard, we found previously that the colloidal stability of the CUR-CHI nanoplex improved slightly when the CHI's molecular weight (MW) was increased from 50-190 to 310-375 kDa [25]. Nevertheless, the higher colloidal stability was obtained at the expense of the individual nanoplex size that increased to ≈ 500 -600 nm. Furthermore, the ultrasonication step remained needed.

Recognizing the strong influence of MW of CHI on the nanoplex's colloidal stability and size, we herein investigated the use of chitosan oligomers (or oligochitosan in short) to prepare colloiddally stable CUR nanoplex and simultaneously to remove the need for the ultrasonication step in the process, which could be time consuming for larger production scales as our previous study showed [24]. Oligochitosan (OCHI) - a short-chain CHI formed by hydrolysis cleavage of the CHI's glycosidic bonds - has emerged as an attractive alternative to CHI owed to its higher aqueous solubility and lower viscosity while exhibiting similar bioactivities as CHI [26]. Moreover, the use of OCHI for wound healing applications has also been demonstrated [27, 28]. Herein we postulated that the better charge distribution of OCHI owed to its more uniform MW and shorter chain length would lead to the formation of CUR-OCHI nanoplex having higher colloidal stability without jeopardizing the nanoplex size.

The objectives of the present work were twofold. First, we aimed to investigate the effects of four key parameters in drug-polyelectrolyte complexation, namely (1) pH, (2) salt concentration, (3) CUR concentration, and (4) charge ratio of OCHI to CUR on the CUR-OCHI nanoplex's (i) physical characteristics (i.e. size, zeta potential, CUR payload) and (ii) preparation efficiency (i.e. CUR utilization rate, overall yield). The outcomes of this study were used to evaluate the robustness of the CUR-OCHI nanoplex preparation while simultaneously used to determine the optimal preparation condition. Second, we aimed to examine the CUR-OCHI nanoplex prepared at the optimal conditions in terms of its (1) colloidal stability, (2) supersaturation generation, (3) *in vitro* cytotoxicity towards human epidermal cells, and (4) *in vivo* wound healing efficacy in comparison to that of the native CUR.

2. Materials and methods

2.1. Materials

Materials for nanoplex preparation: CUR ($\geq 95\%$ curcuminoid) was purchased from Alfa Aesar (Singapore) and OCHI (MW = 2.7 kDa, 80% deacetylation) was prepared by gamma Co-60 ray irradiation as previously described in Dzung, et al. [29]. CHI (MW = 90-140 kDa, 80% deacetylation), potassium hydroxide (KOH), glacial acetic acid (AA), phosphate buffered saline (PBS, pH 7.4), sodium chloride (NaCl), and hydroxypropyl methylcellulose (HPMC) were purchased from Sigma Aldrich (Singapore). **Materials for cytotoxicity and wound healing tests:** human epidermal keratinocytes cells (HaCaT) were purchased from American Type Culture Collection (ATCC, USA). Penicillin-streptomycin, formaldehyde, dimethyl sulfoxide (DMSO), trichrome stain kit, and (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT, 98% purity) were purchased from Sigma—

Aldrich (Singapore). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from HyClone (ThermoScientific, USA).

2.2. Methods

2.2.1. Preparation of CUR-OCHI nanoplex

The CUR-OCHI nanoplex was prepared by the drug-polyelectrolyte complexation technique starting at the same preparation condition used previously for the CUR-CHI nanoplex [21]. Briefly, ionized CUR solution (CUR^{3-}) with charge density of 8.14×10^{-6} mol-charge/mg was prepared by dissolving CUR at 5 mg/mL in 0.1 M KOH ($\text{pH} > \text{pK}_a$ of CUR) [30]. Oppositely charged OCHI solution (OCHI^{1+}) with charge density of 4.46×10^{-6} mol-charge/mg was prepared by dissolving OCHI at 9.12 mg/mL in 1.0% (v/v) AA solution ($\text{pH} < \text{pK}_a$ of OCHI) [31].

Next, equal volumes of the CUR and OCHI solutions (1 mL each) were mixed under gentle stirring immediately after their preparation to minimize the alkaline degradation of CUR. The charge ratio of OCHI to CUR ($R_{\text{OCHI/CUR}}$) at this condition was equal to 1.0 denoting equal amounts of CUR and OCHI charges were available for complexation. Sample calculations for the charge density and $R_{\text{OCHI/CUR}}$ were provided in the Supplementary Materials. The CUR-OCHI nanoplex produced was washed by two cycles of centrifugation ($14,000 \times g$ for 10 min) to remove excess CUR and OCHI after which the washed nanoplex was re-suspended in deionized water for characterizations.

The effects of (1) pH that influenced the degree of ionization, (2) salt inclusion and its charge screening effect, (3) CUR concentration, and (4) $R_{\text{OCHI/CUR}}$ on the CUR-OCHI nanoplex preparation were investigated by varying, respectively, (1) the AA concentration used to dissolve OCHI from 0.5 to 1.4% (v/v), (2) NaCl concentration from 0 to 500 mM, (3) CUR concentration from 1 to 9 mg/mL, and (4) $R_{\text{OCHI/CUR}}$ from 0.6 to 1.4. On this note, the resultant pH of the mixed (CUR+OCHI) solution at these AA concentrations were in the range of 4.31 to 5.75.

For comparison, the CUR-CHI nanoplex was prepared at its optimal condition as determined previously in Nguyen, et al. [21] and not repeated here for brevity. The effects of ultrasonication on the nanoplex's size and zeta potential were investigated for both the CUR-OCHI and CUR-CHI nanoplexes by subjecting them to ultrasonication (VC 505, Sonics, USA) at 20 kHz for 25 s (pulse on/off every 5 s).

2.2.2. Physical characterizations of CUR-OCHI nanoplex

The size, polydispersity index (PDI), and zeta potential of the CUR-OCHI nanoplex were characterized by dynamic light scattering (DLS) after 100× dilution using Zetasizer Nano ZS (Malvern Panalytical, UK). The nanoplex's morphology was examined by field emission scanning electron microscope (FESEM) (S-4800, Hitachi, Japan). The CUR payload, which was defined as the amount of CUR contained per unit mass of the CUR-OCHI nanoplex, was determined by dissolving a known mass of the nanoplex in 80% (v/v) aqueous ethanol solution. The amount of CUR in the ethanol solution was subsequently determined by UV-Vis spectrophotometer (UV Mini 1240, Shimadzu, Japan) at the optimal absorbance wavelength of 423 nm. The presence of CUR in the nanoplex and its complexation with OCHI was verified by Fourier transform infrared spectroscopy (FTIR) (FT/IR-4600, JASCO, Japan) performed between 500 and 4000 cm⁻¹ at 4 cm⁻¹ spectral resolution. The FTIR analysis was also performed for the native CUR, native OCHI, and physical mixture of CUR and OCHI.

The CUR utilization rate was characterized according to Eq. (1) in which the mass of CUR that formed the CUR-OCHI nanoplex was determined first by filtering the nanoplex suspension before the washing step using 3-μm pore size syringe filter to remove CUR precipitates that did not form the nanoplex. The filtered nanoplex suspension was then centrifuged at 14,000×g for 10 min to remove excess OCHI in the supernatant. The amount of CUR in the sedimented pellet was determined by UV-Vis spectrophotometer at 423 nm after dissolution in 80% ethanol (v/v) solution. The overall production yield was characterized according to Eq. (2) in which the mass of the nanoplex formed was determined from the freeze-dried mass of the nanoplex suspension after the washing step.

$$\text{CUR utilization rate (\% w/w)} = \frac{\text{Mass of CUR that formed CUR - OCHI nanoplex}}{\text{Initial mass of CUR added}} \times 100 \quad (1)$$

$$\text{Overall yield (\% w/w)} = \frac{\text{Mass of CUR - OCHI nanoplex formed}}{\text{Initial masses of CUR and OCHI added}} \times 100 \quad (2)$$

2.2.3. Colloidal stability and supersaturation generation

The colloidal stability of the CUR-OCHI nanoplex prepared at the optimal condition was evaluated by incubating 10 mL of the nanoplex suspension in a shaking incubator held at room temperature over a 96-h period. The nanoplex suspension used in this study was obtained from re-suspending the dry-powder nanoplex after freeze drying. At specific timepoints, 1 mL aliquot of the nanoplex suspension was withdrawn and replaced with the same volume of deionized water. The size and zeta potential of the nanoplex suspension in the aliquot were determined by DLS as

previously described. The colloidal stability was characterized from the fluctuations in the size and zeta potential in which minimal fluctuations denoted good colloidal stability.

The supersaturation generation of the CUR-OCHI nanoplex prepared at the optimal condition was characterized from the ratio of the supersaturated CUR concentration generated by the nanoplex upon its dissolution to the thermodynamic saturation solubility of CUR (C_{Sat}). Briefly, the CUR-OCHI nanoplex suspension was added in excess at $20 \times C_{\text{Sat}}$ to 100 mL PBS in a shaking incubator maintained at 37°C ($C_{\text{Sat}} = 4.15 \mu\text{g/mL}$). Afterwards, 400 μL aliquot was withdrawn and syringe filtered using 0.2- μm pore size filter at specific time points over a 24-h period. The aliquot was then immediately diluted ten times with fresh PBS to prevent CUR precipitation from the supersaturated solution.

The CUR concentration in the aliquot was determined by High Performance Liquid Chromatography (HPLC) using Agilent 1100 (Agilent Technologies, USA) at detection wavelength of 423 nm. Aqueous ethanol solution 80% (v/v) was used as the mobile phase at 1.0 mL/min in ZORBAX Eclipse Plus C18 column, resulting in the CUR retention time of approximately 2.5 min. The effect of having HPMC - a well-established crystallization-inhibiting agent - in the dissolution medium on the supersaturation generation was examined at HPMC = 2 mg/mL

2.2.4. Cytotoxicity

The HaCaT cells were cultivated in a 24-well plate containing DMEM supplemented with 10% (w/v) fetal bovine serum and 1% (w/v) penicillin–streptomycin solutions. The cells were incubated at 37°C in 5% CO₂ incubator for 24 h to produce cell density of 10⁴ cells/well. The CUR-OCHI nanoplex suspension was then added to the HaCaT cells at two CUR concentration levels, i.e. 0.25 mg/mL (high) and 0.05 mg/mL (low), where the CUR concentrations were chosen to be similar to the values used in the *in vivo* wound healing study. The microplate wells containing only the cells and growth medium were used as the control run. The exposed cells were then incubated for further 24 h at 37°C in 5% CO₂ incubator. Afterwards, the medium was replaced with serum-free DMEM containing 100 μL of MTT dye solution in 0.5% (w/v) PBS) and the cells were incubated for further 4 h.

Subsequently, 2 mL DMSO was added to each well to dissolve the formazan crystals formed by MTT enzymatic reduction of the viable cells. The formazan concentration in the well was then determined by optical density measurement at 550 nm (OD₅₅₀) using absorbance microplate reader (IRE 96, France). The cell viability was reported as the ratio of OD₅₅₀ of the treated cells to OD₅₅₀ of the control run. The cytotoxicity tests were also performed for the

native CUR (also at 0.25 and 0.05 mg/mL) and for the native OCHI at concentrations corresponding to the amount of OCHI in the CUR-OCHI nanoplex.

2.2.5. Wound healing efficacy

Male mice *Mus musculus var. Albino* (18-22 g) were anesthetized with ketamine solution at 2 mg/kg dose and the dorsal area of the mice was shaved. Afterwards, 8-mm diameter circular full-thickness skin dorsal wounds were created using surgical scissors. The mice were randomly divided into three groups, namely (1) mice treated with the CUR-OCHI nanoplex at [CUR] = 0.25, 0.10, 0.05, and 0.01 mg/mL (n = 6 each), (2) mice treated with the native CUR at [CUR] = 0.05 mg/mL as the positive control (n = 6), and (3) untreated mice as the negative control (n = 6).

For the mice undergoing treatment, 200 μ L of the CUR-OCHI nanoplex suspension (or 200 μ L aqueous dispersion of the native CUR for the positive control) was administered intradermally to the wounds using sterile syringe. Digital photographs of the wounds were taken and the wound healing efficacy was characterized from the reduction in the wound area after 3, 5, 7, 9, and 12 days. The animals were sacrificed at the end of the study (i.e. 22 days). This animal study was approved by the animal study's ethics committee at the University of Medicine and Pharmacy of Ho Chi Minh City.

Histological analysis of the scar tissue was performed after 22 days when the healed tissue had become stable. The scar size was measured and the extend of collagen deposition in the healed tissues was examined by the Masson's Trichrome (MT) staining. Briefly, the scar tissues on Day 22 were immediately fixed upon collection in 10% (v/v) formaldehyde solution in PBS at 4°C for 24 h. Afterwards, the scar tissues were embedded in paraffin tissue block and sectioned at 5- μ m thickness using Tissue-Tek Cryo3 microtome (Sakura, The Netherlands). The sectioned tissues were stained with MT stain and were analyzed under light microscope.

2.2.6. Statistical analysis

The reported values for the CUR-OCHI nanoplex preparation and characterization were expressed as mean $\pm$ standard deviation from a minimum of three independent replicates, whereas the reported mean values for the *in vivo* wound healing efficacy were based on six independent replicates. The statistical significance was evaluated by one-way ANOVA with post hoc Duncan test using SPSS software ($p < 0.05$).

3. Results and discussion

3.1. CUR-OCHI nanoplex preparation

3.1.1. Comparison with the CUR-CHI nanoplex

In the drug-polyelectrolyte complexation technique, the anionic CUR molecules formed electrostatic binding with the oppositely charged OCHI molecules to form soluble CUR-OCHI complex. The soluble CUR-OCHI complex then formed aggregates among themselves owed to the hydrophobic interactions between the bound CUR molecules. Upon reaching a critical aggregate mass, the complex aggregates precipitated out of the solution to form the CUR-OCHI nanoplex. Herein we compared the basic formulation of the CUR-OCHI nanoplex prepared at [CUR] = 5 mg/mL, AA = 1.0% (w/v) (corresponding to the preparation pH of 4.54), and $R_{\text{OCHI/CUR}} = 1.0$ without NaCl with the optimal formulation of the CUR-CHI nanoplex prepared at the same condition.

Fig. 1 (A) The size and zeta potential of the CUR-OCHI and CUR-CHI nanoplexes prepared at the same condition with and without the ultrasonication step; (B) FESEM image of the CUR-OCHI nanoplex

Without the ultrasonication step, the DLS results showed that the CUR-OCHI nanoplex was significantly smaller at 103 ± 3 nm (PDI = 0.29 ± 0.02) compared to 698 ± 11 nm (PDI = 0.31 ± 0.03) for the CUR-CHI nanoplex (Fig. 1A), where the larger CUR-CHI nanoplex size was caused by its aforementioned strong agglomeration tendency. Both nanoplexes exhibited similar zeta potentials of 21 ± 2 and 23 ± 1 mV, respectively, where the positive zeta potentials denoted the presence of cationic OCHI or CHI on the nanoplex's surface. The CUR-OCHI nanoplex size was verified by FESEM that showed the presence of well-dispersed spherical particles in the size range of 50 to 100 nm (Fig. 1B).

With the ultrasonication step, the CUR-CHI nanoplex size was reduced approximately twofold to 284 ± 19 nm denoting the breakup of its agglomerates, while its zeta potential was minimally affected (Fig. 1A). In contrast, the CUR-OCHI nanoplex size remained relatively the same at 95 ± 1 nm after ultrasonication, while its zeta potential was reduced slightly to 16 ± 1 mV (Fig. 1A). Significantly, the present results showed that the use of OCHI in place of CHI enabled us to remove the ultrasonication step from the CUR nanoplex preparation. The lower agglomeration propensity of the CUR-OCHI nanoplex upon its formation was attributed to the better charge distribution of OCHI as a result of its uniform MW and shorter chain length.

Importantly, the CUR-OCHI nanoplex also exhibited a high CUR utilization rate at $93 \pm 1\%$ (w/w) and a high CUR payload of $86 \pm 2\%$ (%) that were comparable to that of the CUR-CHI nanoplex at $92 \pm 1\%$ and $84 \pm 4\%$, respectively. On a side note, the present results also reaffirmed our previous finding in Yu, et al. [25] that the CUR nanoplex size decreased with decreasing MW of CHI used for the complexation with CUR, as the use of low MW OCHI in the present work resulted in the formation of even smaller CUR nanoplex.

3.1.2. Effects of pH and salt concentration

The effects of the preparation pH on the size and zeta potential of the CUR-OCHI nanoplex were investigated at $5.75 \geq \text{pH} \geq 4.31$ while keeping [CUR] and $R_{\text{OCHI/CUR}}$ at 5 mg/mL and 1.0, respectively. The results showed that the CUR-OCHI nanoplex was successfully produced at different pH with minimal variations in its size (≈ 140 -160 nm), PDI (≈ 0.29 -0.34), and zeta potential (≈ 22 -26 mV) for pH up to 5.13 (Fig. 2A). At pH 5.75, however, microscale agglomerates of the CUR-OCHI nanoplex (≈ 3000 nm) were formed likely caused by the lower degree of ionization of OCHI at higher pH resulting in the lower colloidal stability of the nanoplex produced. The effect of pH on the CUR

utilization rate was found to be minimal, where the CUR utilization rates remained constant at $\approx 85\text{-}90\%$ independent of pH, which consequently resulted in constant CUR payloads at $\approx 85\%$ (Fig. S1A of the Supplementary Materials).

The effects of adding NaCl to the complexation medium on the size, zeta potential, and CUR utilization rate were found to be minimal either (Figs. S1B-C of the Supplementary Materials). The results of this study, which was performed at fixed pH of 4.54, suggested that the electrostatic binding between the CUR and OCHI molecules was not adversely affected by the charge screening effect incurred in the presence of NaCl. The subsequent studies were thus performed in the absence of NaCl.

Fig. 2 The effects of (A) pH and (B) CUR concentration on the size and zeta potential of the CUR-OCHI nanoplex produced

3.1.3. Effects of CUR concentration and $R_{OCHI/CUR}$

The effects of CUR concentration were investigated at $1 \geq [\text{CUR}] \geq 9 \text{ mg/mL}$ while keeping the pH and $R_{\text{OCHI/CUR}}$ constant at 4.54 and 1.0, respectively. The results showed that the CUR-OCHI nanoplex size increased with increasing CUR concentration from $62 \pm 5 \text{ nm}$ ($\text{PDI} = 0.25 \pm 0.04$) at $[\text{CUR}] = 1 \text{ mg/mL}$ to $258 \pm 54 \text{ nm}$ ($\text{PDI} = 0.09 \pm 0.02$) at $[\text{CUR}] = 9 \text{ mg/mL}$ (Fig. 2B). The larger nanoplex produced at higher CUR concentration was not unexpected because besides the electrostatic binding, the nanoplex formation was also attributed to the cooperative binding of the free CUR molecules to the already formed CUR-OCHI complex via inter-CUR hydrophobic interactions [21]. The increased presence of the free CUR molecules at higher CUR concentration led to a higher degree of CUR cooperative binding, resulting in the larger nanoplex.

The zeta potential was nevertheless minimally affected by the size increase and remained at $\approx 22\text{-}26 \text{ mV}$ with the exception of the CUR-OCHI nanoplex prepared at $[\text{CUR}] = 1 \text{ mg/mL}$, whose zeta potential decreased slightly to $19 \pm 1 \text{ mV}$ (Fig. 2B). The CUR utilization rate, on the other hand, gradually increased with increasing CUR concentration from $83 \pm 2\%$ at $[\text{CUR}] = 1 \text{ mg/mL}$ to $97 \pm 1\%$ at $[\text{CUR}] = 9 \text{ mg/mL}$ attributed to the abovementioned increase in the degree of CUR cooperative binding at higher CUR concentration (Fig. S1D of the Supplementary Materials). Next, the effects of $R_{\text{OCHI/CUR}}$ were investigated at $0.6 \geq R_{\text{OCHI/CUR}} \geq 1.4$ by varying the amount of OCHI at a fixed mass of CUR (i.e. 5 mg/mL) while also keeping the pH fixed at 4.54.

The results showed that the size and zeta potential of the CUR-OCHI nanoplex were minimally affected by the variation in $R_{\text{OCHI/CUR}}$, where they lay in the narrow range of $\approx 125\text{-}150 \text{ nm}$ ($\text{PDI} = 0.30\text{-}0.34$) and $\approx 19\text{-}22 \text{ mV}$, respectively (Fig. 3A). The CUR utilization rates were also minimally affected by $R_{\text{OCHI/CUR}}$, where they remained at $\approx 91\text{-}95\%$, therefore suggesting that the increased presence of OCHI charges at higher $R_{\text{OCHI/CUR}}$ did not necessarily lead to an increased amount of CUR transformed to the nanoplex (Fig. S1E of Supplementary Materials).

The overall production yield, however, gradually decreased with increasing $R_{\text{OCHI/CUR}}$ from $54 \pm 2\%$ (w/w) at $R_{\text{OCHI/CUR}} = 0.6$ to $37 \pm 1\%$ (w/w) at $R_{\text{OCHI/CUR}} = 1.0$ and $26 \pm 1\%$ (w/w) at $R_{\text{OCHI/CUR}} = 1.4$ (Fig. 3B). The trend in the yield as a function of $R_{\text{OCHI/CUR}}$ was not unexpected as the more abundant presence of OCHI in the feed solution at higher $R_{\text{OCHI/CUR}}$ had been shown earlier not to lead to higher CUR utilization rates. The CUR payload, on the other hand, varied as a function of $R_{\text{OCHI/CUR}}$ despite the relatively constant CUR utilization rate. Specifically, the CUR payload gradually increased from $76 \pm 1\%$ at $R_{\text{OCHI/CUR}} = 0.6$ to reach the peak value at $86 \pm 2\%$ at $R_{\text{OCHI/CUR}} = 1.0$, followed by its gradual decrease to $68 \pm 3\%$ at $R_{\text{OCHI/CUR}} = 1.4$ (Fig. 3B).

Fig. 3 The effects of $R_{OCHI/CUR}$ on (A) the size and zeta potential and (B) CUR payload and overall production yield of the CUR-OCHI nanoplex

As the constant CUR utilization rate indicated that the amount of CUR transformed to the nanoplex was relatively unchanged, the lower CUR payloads of the nanoplexes prepared at $R_{OCHI/CUR} < 1.0$ and $R_{OCHI/CUR} > 1.0$ signified an increased composition of OCHI in the nanoplex. In this regard, the increased OCHI composition in the nanoplex at $R_{OCHI/CUR} > 1.0$ could be explained simply by its more abundant presence in the feed solution. Whereas the increased OCHI composition at $R_{OCHI/CUR} < 1.0$ was postulated to be caused by a higher degree of CUR-OCHI interactions attributed to a lower degree of repulsive inter-chain interactions among the OCHI chains at lower $R_{OCHI/CUR}$.

The results presented in Sections 3.1 demonstrated the robustness of the CUR-OCHI nanoplex preparation, where the nanoplex's physical characteristics and preparation efficiency were shown to be not overly sensitive to the deviations in the preparation conditions from the known workable condition. The CUR concentration and $R_{OCHI/CUR}$

were determined to be the governing parameters via their strong influences on (1) the nanoplex size and (2) CUR utilization rate for the CUR concentration, and on (1) the CUR payload and (2) production yield for $R_{\text{OCHI/CUR}}$.

The optimal preparation condition was subsequently determined to be at $[\text{CUR}] = 5 \text{ mg/mL}$ and $R_{\text{OCHI/CUR}} = 0.8$ attributed to the small nanoplex size ($140 \pm 7 \text{ nm}$) produced and high CUR utilization rate ($93 \pm 1\%$), while keeping the production yield ($42 \pm 1\%$) and CUR payload ($78 \pm 1\%$) in the acceptable range. On this note, higher CUR concentration led to larger nanoplex, while lower CUR concentration led to lower CUR utilization rate. Similarly, higher $R_{\text{OCHI/CUR}}$ resulted in lower production yield, while lower $R_{\text{OCHI/CUR}}$ resulted in lower CUR payload.

Fig. 4 (A) FTIR spectra of the CUR-OCHI nanoplex, native CUR, physical mixture of CUR and OCHI, and raw OCHI, (B) Supersaturation generation time-profiles of the CUR-OCHI nanoplex

3.2. Characterizations of the optimal CUR-OCHI nanoplex

3.2.1. FTIR

The presence of CUR in the CUR-OCHI nanoplex and its complexation with OCHI were verified by FTIR analysis presented in Fig. 4A. The peaks at 1626, 1601, 1511, and 1282 cm^{-1} in the FTIR spectrum of the native CUR, which corresponded to the stretching of the $\text{C}_{\text{ring}}\text{-C}=\text{C}$, aromatic $\text{C}=\text{C}$, $\text{C}=\text{O}$, and aromatic C-O groups, respectively [32], were used for the CUR identification. The appearance of these peaks in the FTIR spectrum of the CUR-OCHI nanoplex reaffirmed the presence of CUR in the nanoplex. The CUR-OCHI complexation in the nanoplex was evident from the disappearance of the enolic COH peak at 1429 cm^{-1} [32] in the CUR-OCHI nanoplex spectrum, as a result of its electrostatic complexation with the charged amine group of OCHI. This peak was otherwise present in the spectrum of the native CUR and the physical mixture of the native CUR and OCHI.

3.2.2. Supersaturation generation

In the absence of HPMC in the dissolution medium, the CUR-OCHI nanoplex attributed to its amorphous state was capable of generating a supersaturation level as high as $\approx 4.2 \times C_{\text{Sat}}$ that was achieved rapidly in less than 5 min after dissolution (Fig. 4B). The fast supersaturation generation was attributed to its nanoscale size that led to its fast dissolution rate. The supersaturation level, nevertheless, gradually decreased to $\approx 2.9 \times C_{\text{Sat}}$ and $2.3 \times C_{\text{Sat}}$ after 30 min and 1 h, respectively, due to the crystallization of the supersaturated CUR solution, before it eventually decreased to $1 \times C_{\text{Sat}}$ after 12 h (data not shown). The supersaturation generation exhibited by the CUR-OCHI nanoplex represented at least twofold enhancement in the CUR solubility that boded well for the nanoplex's ability to produce a high CUR concentration in the wound sites.

The supersaturation generation of the CUR-OCHI nanoplex was greatly enhanced by having HPMC in the dissolution medium to inhibit crystallization of the supersaturated CUR solution. In the presence of HPMC, the CUR-OCHI nanoplex exhibited a maximum supersaturation level of $\approx 15 \times C_{\text{Sat}}$ achieved 2 h after dissolution (Fig. 4B). The supersaturation level then gradually decreased to $\approx 11 \times C_{\text{Sat}}$ after 8 h before it eventually settled at $1 \times C_{\text{Sat}}$ after 24 h (data not shown). Thus, the CUR concentration in the wound sites, hence its wound healing activity, could be greatly enhanced by co-administering the nanoplex with aqueous HPMC solution.

3.2.3. Colloidal stability

Unlike the CUR-CHI nanoplex, the CUR-OCHI nanoplex was found to exhibit high colloidal stability in its aqueous suspension form during prolonged storage up to 72 h, despite their similar zeta potential (≈ 20 mV). Specifically, the CUR-OCHI nanoplex size varied minimally in the range of ≈ 100 -170 nm (not statistically significant) in the first 24 h (Fig. 5A). The nanoplex size only started to increase after 48 h to ≈ 233 nm and continued to increase to ≈ 280 nm after 72 h denoting the agglomeration among the individual nanoplex. The agglomeration intensified after 96 h, resulting in the formation of microscale agglomerates (> 1000 nm).

Not unexpectedly, the zeta potential was observed to gradually decrease to ≈ 16 mV and 9 mV after 72 and 96 h, respectively, signifying the reduction in the electrostatic repulsion forces among the nanoplex leading to the agglomeration (Fig. 5A). The higher colloidal stability of the CUR-OCHI nanoplex indicated that the nanoplex would remain functional over a prolonged period (i.e. 72 h). Clinically, the prolonged stability of the CUR-OCHI nanoplex meant that the same dosage could be used for multiple treatments, hence reducing the economic burden to the patients.

Fig. 5 (A) colloidal stability of the CUR-OCHI nanoplex during prolonged storage, (B) *in vitro* cytotoxicity of the CUR-OCHI nanoplex towards human epidermal cells (HaCaT)

3.2.4. Cytotoxicity

The CUR-OCHI nanoplex was shown to exhibit minimal cytotoxicity towards the HaCaT cells, where the % cell survivals were equal to $89.6 \pm 1.6\%$ and $99.4 \pm 3.6\%$ at the high and low CUR concentrations investigated, respectively (Fig. 5B). Likewise, OCHI was found to be non-cytotoxic towards the HaCaT cells with nearly 100% cell survivals at the two CUR concentrations investigated (Fig 5B). Significantly, the cytotoxicity of the CUR-OCHI nanoplex was lower than that of the native CUR, whose % cell survivals were equal to $70.2 \pm 2.2\%$ (high CUR) and $78.2 \pm 2.0\%$ (low CUR) (Fig. 5B). These results signified that the CUR complexation with OCHI resulted in a lower safety risk for the CUR therapy, whose true toxicology profiles remained in question, despite its numerous therapeutic applications [33].

3.3. Wound healing efficacy

The results of the *in vivo* study on mice ($n = 6$) showed that the wound healing efficacy of the groups treated with the CUR-OCHI nanoplex were considerably higher than the group treated with the native CUR. Specifically, after 3 days, the wound areas of the groups treated with the CUR-OCHI nanoplex were reduced to approximately 32-38% of its original size depending on the CUR dose (i.e. 0.01-0.25 mg/mL) (Fig. 6A). At the same CUR dose of 0.05 mg/mL, the wound area of the group treated with the native CUR was equal to $49 \pm 3\%$ compared to $32 \pm 2\%$ for the group treated with the CUR-OCHI nanoplex.

In fact, the group treated with the native CUR did not demonstrate after 3 days a statistically significant difference in its wound area reduction from the untreated group (Fig. 6A). The native CUR, nevertheless, began to exhibit superior wound healing rate to the untreated group from Day 5 onwards (Fig. S2 of the Supplementary Materials). The slow wound healing effect of the native CUR was likely caused by its slow dissolution rate in the wound site due to its poor aqueous solubility. On this note, the exact wound sizes from the different treatment groups observed on Days 0, 3, 5, 7, 9, and 12 were presented in Table S1 of the Supplementary Materials.

The superior wound healing efficacy of the CUR-OCHI nanoplex to the native CUR was further demonstrated on the subsequent days. Specifically, the % wound areas were equal to $8 \pm 1\%$ (Day 7) and $2 \pm 0.6\%$ (Day 9) for the CUR-OCHI nanoplex's groups versus $15 \pm 1\%$ (Day 7) and $8 \pm 1\%$ (Day 9) observed for the native CUR's group

(Figs. 6B, 6C). The photographs of the wounds taken on Day 9 from the different treatment groups clearly showed the superior wound healing efficacy of the CUR-OCHI nanoplex in which a well progressively healed wound was observed (Fig. 6D). The superior wound healing efficacy of the CUR-OCHI nanoplex to the native CUR could be attributed to (1) its ability to produce high CUR concentration in the wound sites via its supersaturation generation, (2) lower cytotoxicity towards the keratinocytes cells, and plausibly (3) the additional wound healing activity afforded by the presence of OCHI in the nanoplex.

Fig. 6 The % wound areas of the different treatment groups on (A) Day 3, (B) Day 7, and (C) Day 9 calculated with respect to the original wound area on Day 0 (i.e. 50 mm²); (D) Photographs of the wounds from the untreated group and the groups treated by the native CUR and CUR-OCHI nanoplex (both at 0.05 mg/mL) on Day 0 and Day 9

In terms of the optimal CUR dose, the initial variations in the wound healing efficacy as a function of the CUR dose were found to be statistically insignificant until Day 5. After a longer period of 9 days, however, CUR doses of 0.05 mg/mL and 0.10 mg/mL were found to produce the highest wound healing efficacy with % wound area equal to $2 \pm 0.6\%$ and $4 \pm 0.7\%$, respectively. These % wound areas were significantly smaller compared to $7 \pm 0.7\%$ and $8 \pm 0.3\%$ observed at CUR doses of 0.25 mg/mL and 0.01 mg/mL, respectively, after the same period. In this regard, the lower wound healing efficacy at CUR dose = 0.25 mg/mL was believed to be caused by the increased cytotoxicity of CUR towards the keratinocytes cells at higher CUR concentrations, whereas the lower efficacy at 0.01 mg/mL was caused by a lower CUR exposure in the wound sites.

Fig. 7 (A) Light microscopy image of the scar tissue after MT staining for collagen for the group treated with the CUR-OCHI nanoplex at 0.05 mg/mL, (B) dimensions of the scar tissue for the different treatment groups (Day 22)

Next, the histological analysis of the scar tissue formed after the CUR-OCHI nanoplex treatment at 0.05 mg/mL showed a good extent of collagen deposition in the tissues (Fig. 7A). The skin structure also appeared almost completely recovered to its pre-wound state. Furthermore, the size of the scar tissue for the group treated with the CUR-OCHI nanoplex was significantly smaller with length $\times$ width of $(3.2 \pm 0.2 \text{ mm} \times 1.7 \pm 0.1 \text{ mm})$ compared to $(4.9 \pm 0.5 \text{ mm} \times 1.8 \pm 0.3 \text{ mm})$ for the untreated group and $(4.9 \pm 0.3 \text{ mm} \times 1.7 \pm 0.1 \text{ mm})$ for the group treated with the native CUR (Fig. 7B). Thus, the present results showed that the CUR-OCHI nanoplex led to superior *in vivo* wound area reduction rate as well as scarring process compared to the native CUR.

4. Conclusions

The present work successfully demonstrated the preparation, characterizations, and *in vivo* wound healing application of the CUR-OCHI nanoplex with the following key findings. First, in agreement with our hypothesis, the use of OCHI, in place of CHI, in the self-assembly complexation with CUR resulted in the formation of CUR nanoplex exhibiting high colloidal stability attributed to OCHI's better charge distribution. The use of OCHI also removed the need for the ultrasonication step to obtain CUR particles in the nanoscale range, hence further simplified the CUR nanoplex preparation process. Second, the robustness of the CUR-OCHI nanoplex preparation was demonstrated from which the CUR concentration and $R_{\text{OCHI/CUR}}$ were identified as the governing parameters through their influences on the nanoplex size, CUR payload, and preparation efficiency. At the optimal preparation condition, the CUR-OCHI nanoplex exhibited size and zeta potential of $\approx 140 \text{ nm}$ and 20 mV , respectively, and $\approx 78\%$ CUR payload. The nanoplex was efficiently prepared at $\approx 93\%$ CUR utilization rate and $\approx 42\%$ overall production yield. Third, the CUR-OCHI nanoplex exhibited superior *in vivo* wound healing efficacy to the native CUR with multifold improvement in the wound closure rates, which was primarily attributed to (1) its ability to produce highly supersaturated CUR concentration in the wound sites and (2) its lower cytotoxicity towards the human keratinocytes cells. In conclusion, the present results successfully established the CUR-OCHI nanoplex as a highly attractive delivery vehicle of CUR for wound healing applications owed to its simple, yet robust, preparation, high CUR payload, and superior wound healing efficacy to the native CUR.

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470 **6. Declaration of interest**

471 None.

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- 556

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Supplementary Materials

1. Sample calculations for charge density and $R_{\text{OCHI/CUR}}$

Chemical	MW (g/mol)	No. of charge per molecule	Charge density (mol·charge/mg)
CUR	368.4	3	$3/(368.4 \times 10^3) = 8.14 \times 10^{-6}$
OCHI monomer with 80% deacetylation	179.2	1	$0.8 \times 1/(179.2 \times 10^3) = 4.46 \times 10^{-6}$

Thus, at $[\text{CUR}] = 5 \text{ mg/mL}$, the amount of $[\text{OCHI}]$ needed to obtain $R_{\text{OCHI/CUR}} = 1.0$ was calculated as follow:

$$[\text{OCHI}] = 1.0 \times 8.14 \times 10^{-6} \text{ mol} \cdot \text{charge/mg} \times 5 \text{ mg/mL} / (4.46 \times 10^{-6} \text{ mol} \cdot \text{charge/mg}) = 9.12 \text{ mg/mL}$$

2. Effects of different preparation conditions on the CUR-OCHI nanoplex's characteristics

Fig. S1 (A, C, D, E) The effects of pH, NaCl inclusion, CUR concentration, and $R_{OCHI/CUR}$, respectively, on the CUR utilization rates; (B) the effect of NaCl inclusion on the size and zeta potential of the CUR-OCHI nanoplex produced

3. Wound healing rates of the different treatment groups

Fig. S2 % wound areas relative to the original wound size of the different treatment groups on Days 5 and 12

Table S1 The wound diameters of the different treatment groups as a function of time (n = 6)

Time (days)	Curcumin concentration (mg/mL)					
	Control (-)	Control (+)	0.25	0.10	0.05	0.01
0	7.96 ± 0.04 ^a	7.93 ± 0.03 ^a	7.96 ± 0.05 ^a	8.00 ± 0.04 ^a	8.01 ± 0.05 ^a	7.87 ± 0.17 ^a
3	5.73 ± 0.13 ^a	5.53 ± 0.14 ^{ab}	4.71 ± 0.24 ^{bc}	4.76 ± 0.14 ^{bc}	4.51 ± 0.12 ^c	4.87 ± 0.29 ^{bc}
5	4.49 ± 0.10 ^a	4.11 ± 0.11 ^{ab}	3.69 ± 0.13 ^{ab}	3.64 ± 0.07 ^{ab}	3.37 ± 0.14 ^b	4.17 ± 0.42 ^{ab}
7	3.46 ± 0.16 ^a	3.07 ± 0.08 ^{ab}	2.87 ± 0.06 ^{abc}	2.56 ± 0.12 ^{bc}	2.29 ± 0.11 ^c	2.82 ± 0.21 ^{bc}
9	2.49 ± 0.11 ^a	2.23 ± 0.08 ^{ab}	2.09 ± 0.07 ^{ab}	1.60 ± 0.10 ^{bc}	1.20 ± 0.12 ^c	2.21 ± 0.30 ^{ab}
12	1.16 ± 0.08 ^a	0.77 ± 0.06 ^a	0.77 ± 0.07 ^a	0.29 ± 0.10 ^b	0.16 ± 0.10 ^b	0.91 ± 0.14 ^a

The Control (+) refers to the wounds treated with the native CUR, whereas Control (-) refers to the untreated wounds

Note: Any two mean values on the same row that are presented with the same superscript letters indicate that the two mean values are not statistically significant from each other