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Supporting Information

for

Development of a novel ionic liquid-curcumin complex to enhance its solubility, stability, and activity

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Contents

1. Nomenclature and Abbreviations

Abbreviation	Name/description
CCM	Curcumin
IL	Ionic Liquid
CCM-IL complex	Curcumin-Ionic Liquid complex
[Ch][Ole] IL	Choline Oleic Acid Ionic Liquid
Ole-Na	Oleic Acid Sodium Salt
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulfoxide

2. Materials and Methods

2.1 Chemicals

Curcumin (CCM), HPLC grade methanol, acetonitrile, cyclohexane, dimethyl sulfoxide (DMSO) and silver oxide was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Choline Chloride, Oleic Acid and Oleic Acid Sodium Salt (Ole-Na) were procured from Chameleon Guaranteed Reagent, Japan. The choline oleic acid ionic liquid ([Ch][Ole] IL) used in this study was synthesized following the procedure of a previously reported method.[1] A cell counting kit, WST-8, and Hoechst 4432 solution were purchased from Dojindo Laboratories, Inc. (Kumamoto, Japan). Eagle's minimum essential medium (EMEM), fetal bovine serum (FBS), penicillin, streptomycin, OPTI-MEM were purchased from Gibco, Life Technologies Corp. (USA). Trypsin/1, 2-ethylenediaminetetraacetic (EDTA) and Dulbecco's phosphate buffered saline (D-PBS) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Milli-Q purification system from Millipore (Molsheim, France) used to prepare de-ionized and ultrapure water throughout the experiment. All other chemicals were analytical grade.

2.2 HepG2 and Fibroblast Cell Lines

The human liver carcinoma cell (HepG2 cell) and normal human cell (fibroblast) were procured from RIKEN cell bank (Tsukuba, Japan). HepG2 and fibroblast cells were cultured in EMEM medium and MEM medium, respectively, supplemented with 10 % FBS and 0.1% of antibiotic-antimitotic.

2.3 Chromatographic Conditions

The concentrations of CCM from different samples (stability and release study) were estimated by using a HPLC-system consisting of a pump (PU-4180), a photo diode array (PDA) detector

(MD-4010), an auto sampler (AS-4050) and a column oven controller (CO-4060) with a interface box (LC-NetII/ADC) and a bottle stand (BS-4000-1) (JASCO International Co. Ltd., HachioJi, Tokyo 193-0835 Japan). The mobile phase was consisted of acetonitrile and Milli-Q water at a ratio of 60:40 (v/v) and delivered at a flow rate of 1.0 mL/min. The chromatographic separation was performed on an Inertsil ODS-3 column (4.6×250 mm, 5 µm, GL Sciences Inc., Shinjuku-ku, Tokyo, 163-1130, Japan) at the wavelength of 445 nm. A linear calibration curves was established within the range from 1.0 µg/mL to 5.0 µg/mL (correlation coefficient, $R^2 = 0.9998$) for analysis of CCM in different sample of stability and release study.

2.4 Preparation of CCM-Ole-Na and CCM-IL Complexes

CCM was dissolved in acetone at a concentration of 20 mg/mL. Then 50 µL of the prepared solution was mixed with 100 µL of methanol. To this solution, 0.85 mL of cyclohexane was added to obtain an oil phase. A volume of 4 mL aqueous phase containing either Ole-Na or [Ch][Ole] IL solution (1 mg/mL in Milli-Q water) was added to the oil phase. An oil-in-water (o/w) emulsion was developed in the presence of aqueous phase using a Polytron homogenizer (Kinematica, Bohemia, NY, USA) at 16,000 rpm for 2 min that was immediately frozen in liquid nitrogen for 25-30 min. Then, it was freeze-dried to form a solid CCM-Ole-Na or CCM-IL complexes.

2.5 Characterizations of CCM-Ole-Na and CCM-IL Complexes

At first, the hydrodynamic size, polydispersity index (PDI), and zeta potential of CCM-Ole-Na and CCM-IL complexes were measured by dynamic light scattering (DLS: Zetasizer Nano ZS, Malvern Instruments Ltd., USA). Briefly, CCM-Ole-Na and CCM-IL complexes were dispersed in 1 mL of Milli-Q water at concentration of 1 mg/mL of CCM. Then, 0.2 mL of dispersed solution was further diluted to 1 mL by Milli-Q water. The resultant dispersion was used for DLS measurement. Transmission electron micrograph (TEM) analysis was performed as follows: 2 µL of the resultant dispersion was placed on a copper TEM grid supported by carbon film, incubated for 2 min, and additional solution was absorbed by a filter paper. Then, 2 % uranyl acetate solution in water was used for staining the deposited complexes. Finally, the samples were imaged on a TEM-2010 (JEOL, Tokyo, Japan), operated at an accelerationg voltage of 120 kV. The encapsulation efficiency (EE) of CCM by Ole-Na or [Ch][Ole] IL were determined by a filtration method. Briefly, the freeze dried CCM-Ole-Na or CCM-IL complexes were dispersed in Milli-Q water. The suspension was filtrated by using a membrane filter (0.2 µm). The unloaded CCM should be removed by the filtration process. Finally, samples from un-filtrated and filtrated portion were injected in HPLC system for analysis with appropriate dilution with mobile phase. The EE (%) and Yield (%) were calculated as follows:

$$EE\ (\%) = \frac{Content\ of\ CCM\ in\ filtraed\ portion}{Content\ of\ CCM\ in\ unfiltarted\ portion} \times 100\%.$$

$$Yield\ (\%) = \frac{Total\ CCM\ entraped\ in\ final\ product}{Total\ CCM\ added\ at\ initial} \times 100\%.$$

2.6 Solubility of CCM-Ole-Na and CCM-IL Complexes in Milli-Q

The solubility study was performed by adding an excessive amount of CCM-Ole-Na and CCM-IL complexes in a glass vial containing 1.0 mL of Milli-Q water. As a control, excessive amount of free CCM was added in 10 % DMSO in Milli-Q. After addition, the suspension were prepared by shaking and rotating gently the vial for 2 min to obtain a dispersed solution. The undissolved CCM remained as precipitates, which were removed by high centrifugation (15,000 rpm, 5 min). The amount of solubilized drug in supernatant was diluted with mobile phase, mixed well by sonication, filtrated through a 0.22 μm filter (Milliopore Millex-LG, PTFE, Fisher Scientific, USA) and analyzed using a HPLC-system (JASCO International Co. Ltd., HachioJi, Tokyo, Japan) at 445 nm. Following a literature validation method with slight modification, the supernatants were diluted with acetonitrile at 50:50 ratio and content of CCM were estimated using a calibration curve prepared by reference standards.

2.7 Stability Study of CCM-Ole-Na and CCM-IL Complexes

To perform the stability study, CCM-Ole-Na and CCM-IL complexes containing 0.5 mg/mL of CCM were dissolved in Milli-Q water. Similarly, 0.5 mg/mL of CCM was dispersed in 10 % DMSO in Milli-Q as a control. The dispersions were observed visually to check whether formation of precipitations or clear solution. For the chemical stability, content of CCM was quantitatively analyzed at different time intervals. Briefly, after 0, 10, 20, 30, 60, 90, 120, 180, and 240 min of initial dispersion, the solution was withdrawn, diluted by acetonitrile at a 50:50 ratio, mixed well by vortex, filtrated through a 0.22 μm filter and analyzed using the HPLC system as described above. The experiment was carried out at room temperature. The content of CCM in each time point was calculated using the calibration curve derived from reference standard. Finally, a graph of concentration (mg/mL) vs time (min) was developed to calculate the half-life ($t_{1/2}$) of CCM using the following equation:

$$Half\text{-}life,\ t_{1/2} = \frac{0.693}{k}\ ,\ where,\ rate\ constant,\ k = -2.303 \times slope$$

2.8 In-Vitro Release Profile of CCM from CCM-Ole-Na and CCM-IL Complexes

To investigate the release profile, CCM-Ole-Na and CCM-IL complexes containing 1.0 mg of CCM were dispersed in 2 mL of Milli-Q water and 0.5 mL of the solution was put in a dialysis membrane (Spectra/Por 3 molecular porous membrane tubing, MWCO: 3.5 KD, Spectrum laboratories, Inc. USA). It was immersed in 25 mL of receiving phase as a release media containing simulated intestinal fluid (SIF, pH 1.2), simulated gastric fluid (SGF, pH 7.0) and phosphate buffered saline (PBS, pH 7.4) with 50 % ethanol. As a control, 0.5 mL same concentration of free CCM in DMSO was immersed in PBS pH 7.4 containing 50 % ethanol. The experiment was operated in an incubator containing a shaker at 37 °C with 50 rpm for 24 h. According to previous report, 50 % ethanol in release media was used to maintain the sink condition. After 0.5, 1, 2, 4, 8, 12 and 24 h of incubation, 0.5 mL of release media was withdrawn and replaced with equal volume of pre-warmed same media. The amount of CCM released in receiving phase was estimated using HPLC-analysis, as previously described. The release amount of CCM at different time interval was calculated as follows:

$$CCM\ Release\ (\%) = [(A_x \times 25\ mL) / (A_o \times 0.5\ mL)] \times 100\ \%$$

Where, A_x and A_o represent the area of CCM obtained from HPLC analysis of the receiving phase at x h from initial during dispersion in Milli-Q water, respectively.

2.9 In-Vitro Cytotoxicity of CCM-Ole-Na and CCM-IL Complexes

At first, a 96-well plate (CELLSTAR, Greiner Bio-One gmnH, Germany) containing 5,000 of cells was incubated overnight at 37 °C and 5 % CO_2 in an incubator (Espec Corp., BNS-110, Japan). After that, the medium was withdrawn and the CCM-Ole-Na and CCM-IL complexes dispersed in 100 µL of Opti-MEM at different concentration ranging from 1-25 µg/mL were added into the wells. Free CCM solution in Opti-MEM, in which DMSO was added to dissolved free CCM that was kept < 0.5 mg/mL in final solution, at the same concentrations as a control. The incubation was performed in triplicates for each sample including the control. After 24 hour incubation, the cells were washed twice with D-PBS and again diluted by WST-8 solution and incubated for 3 h at 37 °C. Finally, the absorbance at 450 nm was measured using a microplate reader (iMark, Bio-Rad, USA). Cell viability was calculated as the percentage of exposed cells that remained viable over controls as follows:

$$Cell\ Viability\ (\%) = (A_{exposed} / A_{control)} \times 100\ \%$$

Where, $A_{exposed\ and}$ $A_{control}$ represent the absorbance of exposed and control cells, respectively. The data were collected from at least three independent experiments and presented as mean with the standard deviation.

3. Tables and Figures

Table S1. Encapsulation efficiency, yield, half-life ($t_{1/2}$) and conductivity of CCM encapsulated by Ole-Na or [Ch][Ole] IL in water.

Surfactant	Encapsulation Efficiency (%)	Yield (%)	Half-life [$t_{1/2,}$ hour]	Conductivity [mS/cm]
Ole-Na	96.4 ± 1.5	86.5 ± 3.9	4.3 ± 1.1	0.011 ± 0.010
[Ch][Ole] IL	97.9 ± 1.2	92.8 ± 2.7	1.3 ± 0.2	0.098 ± 0.010

Data are shown as mean ± standard deviation, n=3.

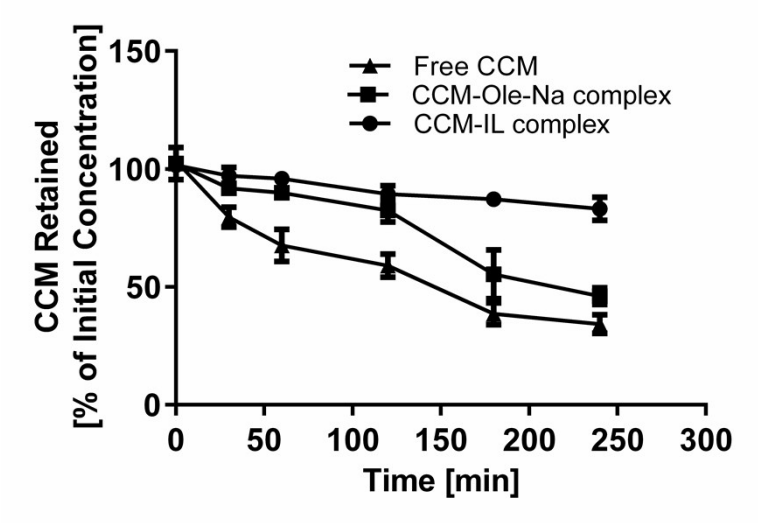

Fig. S1 Stability of curcumin in CCM-Ole-Na and CCM-IL complex in water

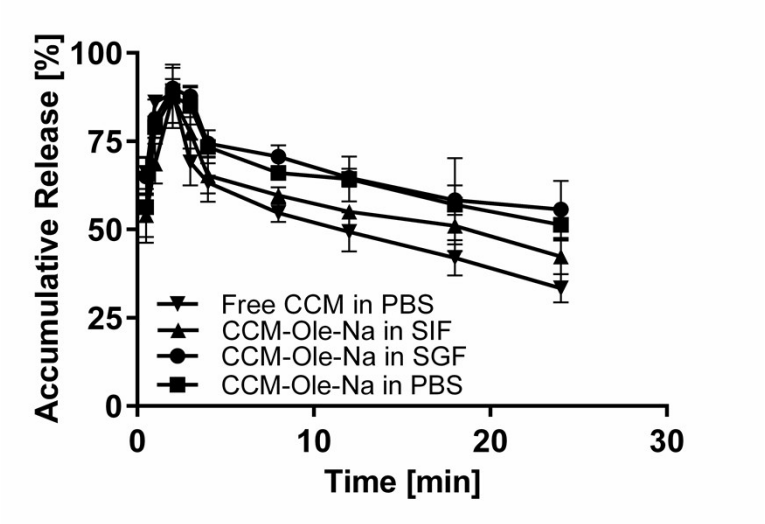

Fig. S2 Release profile of CCM-IL complex in different pH of buffered solutions. Data are shown as mean ± standard deviation, n=3.

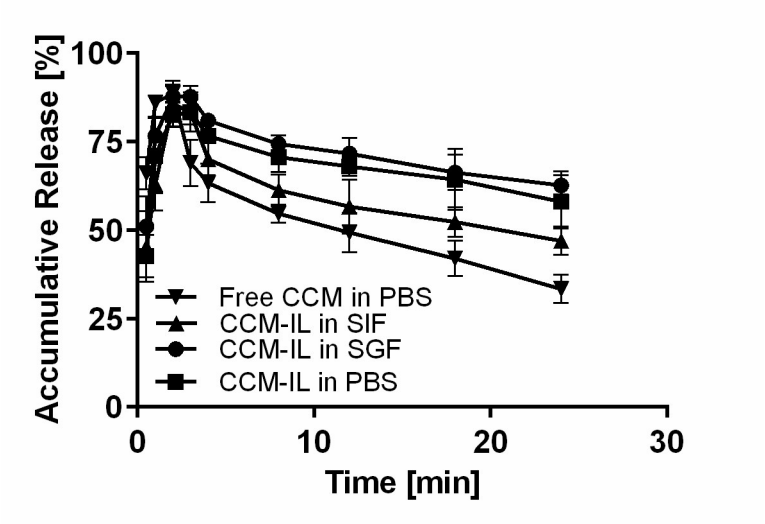

Fig. S3 Release profile of CCM-Ole-Na complex in different pH of buffered solutions. Data are shown as mean ± standard deviation, n=3.

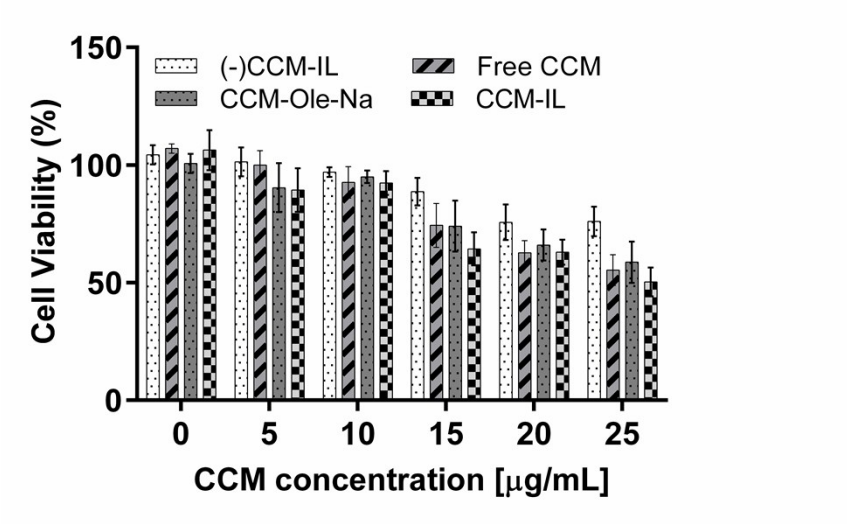

Fig. S4 *In-vitro* cytotoxicity of CCM-Ole-Na or CCM-IL complexes in fibroblast cells. Data are shown as mean ± standard deviation, n=3.

4. References

(1) Mu, L.; Shi, Y.; Ji, T.; Chen, L.; Yuan, R.; Wang, H.; Zhu, J. Ionic Grease Lubricants: Protic [Triethanolamine][Oleic Acid] and Aprotic [Choline][Oleic Acid]. *ACS Appl. Mater. Interfaces* **2016**, *8* (7), 4977–4984.