GSH-responsive curcumin/doxorubicin encapsulated Bactrian camel serum albumin nanocomposites with synergistic effect against lung cancer cells

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Abstract

The aim of this study was to prepare camel serum albumin (CSA) nanoparticles using a self-assembly strategy to co-immobilize curcumin (CCM) and doxorubicin (Dox) which was in favor of combined chemotherapy and biomedical applications of bactrian (Camelus bactrianus) CSA. The constructed CSA nanoparticles with the size around 200 nm displayed a high degree of polydispersity and further encapsulation of CCM and Dox caused no apparent morphological changes to the nanocomposite (CCM/Dox-CSA NPs). The synergistic cytotoxic effect of CCM and Dox on cancer cell A549 was observed with the calculated combination index less than 1.0. Moreover, the release kinetic profile of encapsulated drugs showed a concentration dependence of glutathione (GSH) originating from the GSH used in nanoparticle formation to break the intramolecular disulfide bonds. In vitro cytotoxicity evaluations also revealed that CCM/Dox-CSA NPs showed higher cytotoxicity than that of single drug loaded CSA-NPs, which was also validated by high content screening assay. Taken together, the CCM/Dox-CSA NPs with redox-responsive attributes provided an integrated protein-based combinational drug-delivery matrix to exert synergistic effects.

Keywords: camel serum albumin, nanoparticles, self-assembly, redox-responsive, synergistic effect

Introduction

Nowadays, various types of therapies including chemotherapy, radiation therapy and gene therapy[1–3] have been used to combat cancers. Among them, chemotherapy based on the drug-delivery matrix with good cost-effectiveness and curative effect is frequently used. In particular, combination chemotherapy that involves two or more drugs with improved therapeutic effect has gained considerable CLC number: R734.2, Document code: A
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attention. In principle, drugs binding to different targets may exert different effects and thereby improve the treatment of some drug-resistant cancer cells due to the synergistic anticancer effect. Meanwhile, the enhanced synergistic effect of the combinational design is also desirable for drug administration as the amount of pharmaceuticals is greatly optimized.

Curcumin (CCM) is one of the naturally active polyphenolic compounds from Curcuma longa which has been widely applied in medical practices. Previous pharmacological studies have indicated the multifunctional aspects of CCM such as anti-inflammatory, anti-oxidant and anti-cancer effects. Meanwhile, it can not only take effect by inducing tumor cell death, regulating related signaling pathways, but also increase their chemotherapy sensitivity. Therefore, CCM was listed as the cancer chemoprevention act by National Cancer Research Institute of USA. However, the poor water solubility (<3 μg/mL) and high photosensitivity of CCM may limit its practical application to a great extent.

Doxorubicin (Dox) belongs to anthracycline type anticancer drug which shows a wide-spectrum of cancer treatment including lung cancer, breast cancer, and ovarian cancer. As Dox mainly functions to inhibit the RNA/DNA synthesis, it may cause potent cytotoxic effect. Nevertheless, the lack of selectivity of Dox may inevitably cause serious side effects especially for the cardiac toxicity, which has triggered many researches focused on adoptions of the formulations of drugs.

To improve the efficacy while minimize the adverse effects of pharmaceuticals in cancer treatment, one solution is to encapsulate or entrap these molecules of interest into various delivery systems, which are effective to prevent degradation, to improve the biodistribution and absorption of drugs. Numerous nano-sized delivery matrixes such as nanoparticles, dendrimers, liposomes and metal organic frameworks have been explored. Among these nano-carriers, the spherical nanoparticle has gained growing attention because of its good stability and penetration potential and high loading capability. However, its biocompatibility may remain problematic as the synthesis involves various chemical reagents. Therefore, a bunch of natural biomaterials like gelatin, polyactic acid, and albumin proteins have been tested resulting in desirable low toxicity and good degradability over synthetic polymers.

Serum albumin is one of the most abundant proteins in blood with a molecular weight of 66.5 kDa, which plays an important physiological role in maintaining the plasma osmotic pressure. Additionally, albumin was shown as an important biocargo of a variety of compounds. For example, it is responsible for the transportation of metal ions, lipids and drug molecules. Therefore, serum albumin holds advantages including excellent stability, in vivo degradable nature and scale-up production, which has already been used for clinical practices such as the albumin-based commercial product of Abraxane. Unlike intensively studied bovine or human serum albumin, the structural as well as functional investigations of camel serum albumin are sparse. Previous proteomic studies have revealed that the camel milk proteins are less immunogenic than that of cow milk. Meanwhile, the discovery of heavy-chain only antibody has also proved its favorable biological safety because of its sequence similarity with human.

As so far there has been rare studies on camel serum alumsins (CSA), in this study, we set out to use purified albumin from unique Chinese Bactrian camels (Camelus bactrianus) to prepare protein-based nanoparticles via a self-assembly method to encapsulate both CCM and Dox. The self-assembled CSA spherical nanoparticles were obtained by using the glutathione (GSH) as a reducing agent to break the intramolecular disulfide bonds, resulting in the explosion of hydrophobic regions of alumsins. The therapeutic effect as well as the GSH-responsive drug release behaviors of nanocomposites were investigated.

Materials and methods

Chemicals and reagents

Camel serum albumin was purified from the plasma of camel. Reduced glutathione was from Macklin (Shanghai, China). Curcumin was from Sigma Aldrich. Doxorubicin (Dox) was obtained from Med Chem Express Co., Ltd (Shanghai China). Dimethyl sulfoxide (DMSO) was from Macklin Reagent Company (Shanghai, China). Dulbecco's modified Eagles medium (DMEM) containing 10% fetal calf serum (FCS), gentamicin, L-glutamine, nonessential amino acids, penicillin/streptomycin solution and sodium pyruvate was purchased from Gibco (Carlsbad, CA, USA). Deionized water from Milli-Q50SP Reagent system (Millipore Corporation, MA, USA) was used for the assay.

Preparation of CCM/Dox co-bound CSA nanoparticles

CCM or Dox loaded CSA nanoparticles (NPs) and CCM/Dox co-bound CSA-NPs were prepared by a GSH induced self-assembly method. Briefly, 2.0 mg
CCM or Dox (in 0.5 mL ethanol or water) or 2.0 mg Dox/CCM (1.0 mg each in 0.5 mL ethanol) was mixed before adding CSA solution (40 mg/mL) with 50 mmol/L GSH and incubated at 37 °C for 1 hour. Subsequently, 2.0 mL ethanol was added to the solution to precipitate CSA nanoparticles. The suspension was further kept under stirring for another 1 hour under room temperature. The excessive reagent was removed using a dialysis membrane (cut-off of 12 kDa) under 4 °C for 12 hours and the precipitated NPs in the dialysis bag were then lyophilized and stored at 4 °C. CSA nanoparticles without drugs were prepared as described.

Characterization of prepared nanoparticles

The size and zeta potential of prepared nanoparticles was analyzed on a Malvern Zetasizer (Malvern Instruments Ltd., UK) by dynamic light scattering (DLS) and laser Doppler microelectrophoresis, respectively. Samples were diluted with 0.22 μm filtered ultrapure water prior to the measurement at the scatting angle of 90° at 25 °C. The stability of prepared CCM/Dox co-bound nanoparticles was also evaluated according to the hydrodynamic diameter in a different medium such as deionized water, 0.9% NaCl, 5% glucose and phosphate buffered saline. The nanoparticle solutions were kept at 4 °C and the hydrodynamic diameter was recorded by DLS at different time intervals. Meanwhile, to examine GSH-responsive behaviors of CSA nanoparticles, the constructed nanoparticles were incubated with 20 mmol/L GSH at 37 °C under stirring and both the hydrodynamic diameter and polydispersity index (PDI) were recorded by DLS. Furthermore, the morphological characteristics of prepared nanoparticles were studied using scanning electron microscope (SEM) and transmission electron microscope (TEM).

Considering the relative low water solubility of CCM, the solubility of CCM encapsulated in CSA-NPs had been determined. Briefly, 10 mg of CCM or CCM-CSA-NPs with equivalent amount of CCM had been dissolved in 1.0 mL of deionized water. After vortex for 5 minutes followed by 5-minute sonication, the mixture was centrifuged at 12 000 g for 5 minutes. The CCM in the supernatants was extracted by ethyl acetate and determined by UV-vis spectrometry at 430 nm.

Drug loading and encapsulation efficiency

To determine both the drug loading and encapsulation efficiency of the prepared nanoparticles, lyophilized nanoparticles were redissolved in 5 mL DMSO/H₂O (9 : 1, v/v) solution and sonicated for 30 minutes to extract the drug completely. The level of loaded CCM was determined by UV-vis spectrometry (Hitachi UH5300, Tokyo, Japan) at 430 nm. Considering the overlap of the absorption spectrum for CCM and Dox that may cause deviations of the quantitative analysis, fluorescence spectra of Dox were recorded at the excitation wavelength of 480 nm (RF5301pc, Shimadzu, Kyoto, Japan). The level of loaded CCM and Dox was determined by freshly prepared standard solutions with different concentrations according to the external standard method. Meanwhile, recovery analysis had been further performed by adding standard solutions (50, 100 and 200 μg/mL) into the nanoparticle solutions in order to evaluate the accuracy of determined level of CCM or Dox by either UV-vis or fluorescence spectroscopy.

The drug loading efficiency and encapsulation efficiency were calculated according to the following equation and all the measurements were performed in triplicate. Data were presented as mean±SD (n=3).

\[
\text{Drug loading efficiency (DLE)}=\left(\frac{\text{drug level in nanoparticles}}{\text{total nanoparticles weight}}\right)\times100\% \\
\text{Drug encapsulation efficiency (DEE)}=\left(\frac{\text{drug level in nanoparticles/added level}}{\text{drug level in nanoparticles/total nanoparticles weight}}\right)\times100\%
\]

To study whether the drugs were loaded onto the CSA nanoparticle, absorption spectra of different nanoparticle solutions were recorded by UV-vis spectrophotometer. Conformational changes of CSA were monitored by both UV-vis and fluorescence spectrophotometry.

Determination of the number of the free sulphydryl

To validate the break-down of the intramolecular disulfide bonds and the formation of intermolecular disulfide bonds, the number of exposed free sulphydryl was measured using the Ellman's method according to the manual (BestBio, Shanghai, China). Drug loaded and blank CSA nanoparticles were prepared as described above. Reduced CSA was prepared as follows, 40 mg CSA was dissolved in 1.0 mL deionized water with 50 mmol/L GSH at 37 °C for 1 hour, the solution was taken out for dialysis against deionized water under 4 °C for 12 hours.

Synergistic cytotoxicity of CCM and Dox

To evaluate whether the combinational use of CCM and Dox contributed to the synergistic cytotoxicity, the viability of A549 cells treated by different concentrations of CCM and Dox was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT)-based assay. Briefly, cells were
seeded into the 96-well plate at the density of 1×10^4 cells/well in 0.1 mL DMEM medium containing 10% FBS and 1% penicillin/streptomycin and incubated in 5% CO₂ at 37 °C for 24 hours. Then the medium was replaced by freshly prepared medium containing different concentration of drugs and incubated for 24 hours. The medium was discarded and the cells were washed twice by PBS, then 20 μL MTT solution was added into each well for another 4 hours. Subsequently, the MTT solution was discarded and 100 μL DMSO was added into each well. The absorbance was determined on the microplate reader at 490 nm. The cell viability was calculated from the triplicate samples of each group (n=3) relative to that of control group. The 50% inhibitory concentration value (IC₅₀) was deduced by SPSS 17.0 software (SPSS Inc, Chicago, USA). The combination index (CI) was calculated by CompuSyn software based on the median-effect equation developed by Chou and Talalay[24].

\[
CI = \frac{(D_1)_1 + (D_2)_1}{(D_1)_2 + (D_2)_2}
\]

(1)

In the formula, \((D_1)_1\) or \((D_2)_1\) is the dose of CCM or Dox alone that with the inhibition of x%. In the numerators, either \((D_1)_1\) or \((D_2)_1\) is the portion of CCM or Dox in combination that with the same inhibition of x%. According to the obtained CI, different drug interaction can be obtained. Specifically, CI=1 indicates additive effect in the absence of synergism or antagonism; CI<1 indicates synergism; CI>1 indicates antagonism.

**In vitro cytotoxicity of drug-loaded CSA nanoparticles against A549 cells**

In vitro cytotoxicity of drug-loaded CSA nanoparticles with equivalent concentration of CCM or Dox was determined as described above by MTT assay. The cells were treated with different concentrations of nanoparticles (0, 5, 10, 20, 25, 30, and 40 μg/mL) and then cell viability was calculated according to the method described above.

**In vitro responsive behaviors of CSA-NPs**

The in vitro release of both CCM and Dox from CSA nanoparticles was then investigated. A total of 75 mg prepared nanoparticles was suspended in 2 mL deionized water and dialyzed against 50 mL buffer at 37 °C. Three types of buffers were used for the assay including (I) phosphate-buffered saline (PBS) pH 7.4 corresponding to the physiological condition after intravenous administration, (II) and (III) PBS pH 7.4 containing 5 and 10 mmol/L GSH, respectively. After predetermined time intervals after incubation, 0.5 mL sample was taken out and the absorbance of CCM and Dox was measured spectrophotometrically as described. Considering the acidic micro-environment (pH 5.0) in cancer cells[25], the pH-responsive release behavior of constructed CCM/Dox CSA NP was also studied in the absence or presence of GSH using acetic acid buffers including (I) sodium acetate-acetic acid (NaAc-HAc) pH 5.0 corresponding to acidic condition in cancer cells, (II) and (III) NaAc-HAc pH 5.0 containing 5 and 10 mmol/L GSH, respectively. All the samples were prepared and analyzed in triplicate. The concentration was calculated by external standard method using freshly prepared standard solutions.

**In vitro endocytosis and TUNEL assay**

Qualitative analysis of cell uptake, intracellular distribution of nanoparticles as well as apoptosis of A549 cells were visualized by fluorescence microscope (Nikon, Tokyo, Japan). Briefly, the endocytosis of NPs was visualized according to the fluorescence generated by Dox. Meanwhile, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was performed to evaluate the cell apoptosis. Cells were seeded into the 6-well plate at a density of 1×10^5 cells/well and incubated for 24 hours. Then the medium was discarded and replaced by fresh DMEM medium containing 20 mg nanoparticles (drugs loading in the nanoparticles were comparable with each other) and further incubated for 24 hours. After fixing the cells with 4% paraformaldehyde at room temperature for 30 minutes, each well was washed by PBS for three times. Then the fixed cells were labeled according to the manual of the TUNEL kit (Beyotime Biotechnology, Shanghai, China). Briefly, each sample was reacted with 50 μL TUNEL reagent and the plate was kept in dark at 37 °C for 1 hour. The cells were rinsed with PBS and then were labeled by Hoechst to visualize the nuclei. The intrinsic red fluorescence of Dox as well as the green fluorescence resulted from apoptotic cells were recorded by a fluorescence microscope.

Furthermore, to quantitatively evaluate the apoptosis of A549 cells, TUNEL assay was then performed on the high-content cell quantitative imaging analysis system. The operation was similar with that described above, except that cells were seeded into the 96-well plate at a density of 1×10^4 cells/well. After labeling the nuclei with Hoechst, apoptotic cells were observed and quantitated by Cellomics XTI system (Thermo Fisher Scientific, USA).
Statistical analysis

All data was expressed in the form of mean±standard deviation. The statistical analysis was carried out by using SPSS 17.0 software. Student’s t test and one-way analysis of variance (ANOVA) had been applied to assess the statistical significance between groups. P values <0.05 were considered to be significant.

Results

Preparation and characterization of CCM/Dox CSA-NPs

As shown in Supplementary Table 1 (available online), when the ratio of drug and protein increased from 0.001 to 0.100, the diameter of formed CCM-CSA-NPs had almost no significant change firstly and then gradually increased, while there was no significant change for that of Dox-CSA-NPs. This result had primarily demonstrated that unlike soluble Dox, the highly hydrophobic CCM might play an important role in the formation of CSA-NPs via this self-assembly method.

Owing to the overlap of the absorption spectrum for CCM and Dox UV-vis analysis, fluorescence analysis with good specificity had been applied to determine the level of Dox. For the fixed extraction wavelength 480 nm, there was a significant difference for the emission spectra of CCM and Dox (Supplementary Fig. 1, available online), demonstrating the feasibility to quantify the level of Dox by fluorescence spectrophotometry. Besides, recovery analysis had been performed to evaluate the accuracy of the determined level of CCM or Dox by either UV-vis or fluorescence spectrophotometry and results had been listed in Supplementary Table 2 (available online). As shown, considering the spectrum overlap for CCM, the determined level turned out to be slight higher than the added concentration. However, the recovery ranged from (104.3±2.7)% to (104.3±2.7)%, demonstrating acceptable deviation. While for Dox, the satisfactory recovery varied from (99.8±1.9)% to (101.2±1.3)% had also demonstrated the accuracy of fluorescence spectrophotometry. According to the results outlined in Supplementary Table 3 (available online), the DLE increased with the increase of drug/protein ratio. However, there was a relative sharp decrease of DEE when the ratio reached 0.1, partly resulting from the restricted encapsulation space of CSA. Therefore, we chose 0.05 as the optimized drug/protein ratio for experiments. Meanwhile, previous reports suggested molecular interactions of drug/chemosensitizer are closely related to the weight ratio in nanocarriers with a desirable value of 1 : 1 in this case of CCM/Dox[36]. Therefore, the weight ratio of CCM and Dox was set as 1 : 1 in this study.

As shown in Fig. 1A, a colloidal dispersion of prepared drug-loaded CSA-NPs was visualized after re-dissolving the lyophilized materials indicating its desirable stability. Meanwhile, the hydrodynamic diameters (Fig. 1B and Table 1) of obtained protein NPs were (195.2±7.5) nm, (208.6±10.9) nm, (210.5±9.8) nm, and (215.6±8.2) nm for blank CSA NPs, CCM-CSA NPs, Dox-CSA NPs and CCM/Dox-CSA NPs, respectively. Narrow particle size distribution was observed suggesting the good homogeneity, which was further corroborated by a low PDI value (<0.2) for all samples. All the samples had displayed a negative charge (Fig. 1C and Table 1) in which the average Zeta potential was (−30.8±1.3) mV, (−27.5±2.3) mV, (−24.6±2.2) mV and (−26.2±1.6) mV, respectively.

The morphology of obtained nanoparticles was evaluated using SEM and TEM. As shown in Fig. 1D and E, all NPs exhibited uniform spherical shape and homogeneous distribution with the size of ~200 nm in accordance with the results from DLS. The stability of prepared CSA-NPs was also investigated (Fig. 2A) in different media including deionized water, 0.9% NaCl, 5% glucose and phosphate buffered saline. These results indicated that the particle size almost remained unchanged during the 2 weeks which represented a good stability of prepared CSA-NPs. The calculated DLE and DEE of NPs were listed in Table 2, demonstrating satisfactory DLE and DEE.

The solubility of free CCM and CCM encapsulated in CSA-NPs was determined by UV-vis spectrophotometry and results were displayed in Fig. 2B. The inset was the photographs of prepared CCM solution, indicating that most of the free CCM remained insoluble while enhanced solubility was observed once formulated with CSA-NPs of high solubility. Besides, the calculated solubility of CCM-CSA-NPs was (540.67±35.72) μg/mL, a 140-fold increase compared to that of free CCM [(3.89±0.32) μg/mL], which was promising for practical uses.

During the break-down process of intramolecular disulfide bonds, the hydrophobic region of CSA was exposed in which different drugs could be encapsulated. To examine the self-assembling process of CSA, the number of free sulfhydryl groups was firstly determined by Ellman's method. As shown in Fig. 2C, when incubated with reducing GSH, the number of free sulfhydryl groups in the CSA molecule was dramatically increased from 5.1 to 24.7 and it
dropped to ~6.4 after the formation of nanoparticles comparable with that of native CSA. Moreover, to examine GSH-responsive attributes of CSA nanocomposite, the hydrodynamic diameter of nanoparticles was measured in the absence or presence of 20 mmol/L GSH at 37 °C. When the GSH was added into the solution, the particle diameter decreased while the PDI increased with the incubation time according to measured DLS (Fig. 2D). The diameter decreased from 214 to 106 nm within 24 hours, suggesting the dissociation of nanoparticles into smaller fragments. This observation may verify the redox-triggered responsive behaviors of prepared nanoparticles.

The structural changes of CCM/Dox CSA-NPs

**Fig. 3A** showed the UV-vis spectra of CCM or Dox formulated with CSA NPs. The absorption peaks around 430 and 480 nm corresponding to free CCM and Dox (Supplementary Fig. 2, available online) were observed, respectively. Moreover, for the CCM and Dox co-bound NPs, the UV spectra were also similar with that of CCM/Dox mixed solution at the same weight ratio, in which the characteristic peak of Dox at ~530 nm was also observed. Therefore, it could demonstrate the successful loading of both CCM and Dox into CSA NPs.

The UV-vis spectra of native CSA, GSH-reduced CSA and prepared CSA NPs were also recorded (Fig. 3B). The peak around 256 nm was from Dox validating the existence of Dox in the prepared NPs. Compared with native CSA, there was a slight blue shift in the reduced state, which suggested that during the self-assembly of CSA, the buried tryptophan may be more exposed to the solvent environment[27]. Furthermore, the absorption peak of tryptophan shifted from 275 to 283 nm after NPs formation which confirmed the increased hydrophobicity.

The conformational changes of CSA NPs were also investigated by fluorescence spectroscopy. The intrinsic fluorescence signal of albumin was closely related with its compositional tryptophan (Trp) residues. As shown in Fig. 3C, the $\lambda_{\text{max}}$ of native CSA was ~340 nm and a slight red shift from 340 to 344 nm was observed for reduced form in the presence of GSH. While after the formation of nanoparticles, the $\lambda_{\text{max}}$ shifted toward lower wavelength (337 nm), and

### Table 1 Hydrodynamic diameter, PDI and Zeta potential of prepared CSA-NPs

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Hydrodynamic size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
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<tbody>
<tr>
<td>Blank CSA NPs</td>
<td>195.2±7.5</td>
<td>0.09±0.01</td>
<td>-30.8±1.3</td>
</tr>
<tr>
<td>CCM-CSA NPs</td>
<td>208.6±10.9</td>
<td>0.07±0.03</td>
<td>-27.5±2.3</td>
</tr>
<tr>
<td>Dox-CSA NPs</td>
<td>210.5±9.8</td>
<td>0.12±0.02</td>
<td>-24.6±2.2</td>
</tr>
<tr>
<td>CCM/Dox-CSA NPs</td>
<td>215.7±8.2</td>
<td>0.10±0.04</td>
<td>-26.2±1.6</td>
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</tbody>
</table>

Data represented as mean±SD ($n=3$).
the fluorescence intensity was significantly decreased in the absence of CCM. Besides, the fluorescence quenching of CSA in the presence of CCM confirmed the binding of CCM to CSA [28]. Notably, the superimposable fluorescence spectra of CCM-CSA-NPs with that of CSA-NPs may suggest the role of CCM in the self-assembling of proteins (Supplementary Fig. 3, available online).

**Synergistic cytotoxicity of CCM and Dox**

As shown in **Fig. 4**, CCM alone showed little effect on A549 cells whereas combined use of CCM and Dox exhibited remarkably improved cytotoxicity. The IC_{50} value of single drug or two drugs at different concentrations after 24 hours was calculated based on MTT assay and results were listed in **Table 3**. The IC_{50} values for cells incubated with Dox alone or in combination with CCM at the dosage of 1, 5 and 10 μg/mL (24 hours) were 0.64, 0.46, 0.31 and 0.19 μg/mL, respectively. Similarly, the values for cells incubated with CCM alone or in combination with Dox at 0.1, 0.5 and 1.0 μg/mL (24 hours) were 34.9, 18.1 and 7.79 μg/mL, respectively. To quantitatively examine the synergistic effect of CCM and Dox, combination index (CI) values were calculated by CompuSyn software. Considering the weight ratio of CCM and Dox was 1 : 1 in the NPs, the calculated CI values for the assay was used with the same ratio. Notably, the obtained CI values from both assays were 0.69 and 0.71 respectively, which revealed that the combination of CCM and Dox in the CSA nanocomposite possessed strong synergistic effects on A549 cells.

**Table 2** Drug loading efficiency and drug entrapment efficiency of prepared CSA-NPs (n=3)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>DLE (%)</th>
<th>DEE (%)</th>
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<tbody>
<tr>
<td></td>
<td>CCM</td>
<td>Dox</td>
</tr>
<tr>
<td>CCM-CSA NPs</td>
<td>6.9±0.4</td>
<td>–</td>
</tr>
<tr>
<td>Dox-CSA NPs</td>
<td>–</td>
<td>7.5±0.7</td>
</tr>
<tr>
<td>CCM/Dox-CSA NPs</td>
<td>3.4±0.5</td>
<td>3.8±0.6</td>
</tr>
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</table>

**Fig. 2** Physicochemical properties of prepared drug-loaded CSA nanoparticles. A: Colloidal stability of CCM/Dox-CSA-NPs in different medium at 4 °C; B: Enhanced solubility of CCM in CSA-NPs and the inset showed the solubility of CCM in water (10 mg/mL); C: The number of the free sulfhydryl groups in CSA of different formulations; D: Size and PDI change during incubation with 20 mmol/L GSH in pH 7.4 PBS buffer at 37 °C. All the data are represented as mean±SD (n=3).
In vitro cytotoxicity of drug-loaded CSA NPs against A549 cells

The cytotoxicity of blank CSA-NPs was firstly investigated to evaluate its biocompatibility. As shown in Supplementary Fig. 4 (available online), there was almost no effect on the cell viabilities for A549 cells even at the concentration of NPs up to 500 μg/mL, verifying an ideal biocompatible CSA NPs prepared via the self-assembly method as nano-carrier.

Then the possible synergistic effects against A549 cells of the encapsulated CCM/Dox in the CSA nanoparticles were further evaluated by MTT assay. As shown in Fig. 5, CCM-CSA-NPs showed virtually little effect on the cell viability even at a relative high concentration pointing to the low toxicity of CCM. However, for the Dox-CSA-NPs and CCM/Dox-CSA-NPs, a concentration-dependent decrease in cell viability was observed.

![Optical spectra of CSA-based nanoparticles.](image)

**Fig. 3** Optical spectra of CSA-based nanoparticles. A: UV-vis spectra of prepared CCM-CSA-NPs, Dox-CSA-NPs and CCM/Dox-CSA-NPs; B and C: UV-vis absorption spectra of native CSA, reduced CSA, blank CSA-NP, CCM-CSA-NPs, Dox-CSA-NPs and CCM/Dox-CSA-NPs; D: Fluorescence emission spectra of native CSA, reduced CSA, blank CSA-NP, CCM-CSA-NPs, Dox-CSA-NPs and CCM/Dox-CSA-NPs.

![Cytotoxicity evaluation of CCM and Dox.](image)

**Fig. 4** Cytotoxicity evaluation of CCM and Dox. A and B: Cytotoxicity evaluation of Dox with 0, 1.0, 5.0, and 10.0 μg/mL CCM (A) and CCM with 0, 0.1, 0.5, and 1.0 μg/mL Dox (B) on A549 cells by MTT assay. All the data are represented as mean±SD (n=3).
viability was apparently observed. Compared with Dox-CSA-NPs, CCM/Dox co-bound CSA NPs displayed higher cytotoxicity consistent with results of combination index analysis. The calculated IC_{50} for CCM-CSA NP, Dox-CSA NP and CCM/Dox-CSA NP were 867.28, 32.49 and 26.50 μg/mL, respectively, demonstrating the co-bound CSA were more effective than those single drug-loaded NPs. Taken together, results of both CI values of drugs and cytotoxicity evaluations of drug-loaded CSA NPs have identified a synergistic effect of CCM and Dox.

**In vitro responsive behaviors of CSA-NPs**

As the DLS analysis revealed the redox responsiveness of prepared nanoparticles, the *in vitro* release kinetic files of CCM/Dox from CSA NPs were investigated in the presence of different concentrations of GSH (**Fig. 6A and B**). Unlike free drugs (**Supplementary Fig. 5A**), there was no obvious burst release for all NPs and the drugs were released from CSA NPs in a mainly sustained manner. The relatively rapid drug release at the beginning (~2 hours) was likely attributed to the desorption of drugs at the surface of the formed nanoparticles.

Next, in the presence of 5 mmol/L GSH, the calculated releasing amounts of CCM and Dox over 48-hour incubation were (43.7±2.7)% and (41.6±3.3)%, respectively. At 10 mmol/L GSH, it reached to (60.5±5.2)% and (67.1±4.1)%, respectively. By contrast, only (39.7±4.4)% and (35.2±5.5)% were released in the absence GSH. Furthermore, for the nanoparticles incubated with PBS with 10 mmol/L GSH mimicking the reducing environmental conditions of cytosols, a significantly increased release was observed, demonstrating the prepared CCM/Dox-CSA NPs was a suitable drug delivery system for cellular uptake.

Similar with that of pH 7.4, a burst phase (~51.5%) was observed for the release profile of CCM and Dox in acidic condition (**Supplementary Fig. 5B**). As for drugs encapsulated CSA NP, similar GSH-dependent release behavior was identified (**Fig. 6C and D**). Notably, in the absence of GSH, 39.5% and 36.4% of CCM and Dox was released. By contrast, at 10 mmol/L GSH, it increased to 66.1% and 71.1%, respectively. Although there was little pH-responsiveness for the constructed delivery system, the slightly improved drug release might be attributed to the pH effect on the stability of the formed disulfide bonds.\[29\]

To further explore the release mechanism, the *in vitro* release data were fitted by different models including first order, Higuchi and Korsmeyer-Peppas models. The obtained correlation coefficient was listed in **Table 4**. According to the correlation coefficient, the release of CCM and Dox from CSA-NPs was well fitted by first order model with the higher $r^2$-values. Moreover, based on the $n$ value obtained from Korsmeyer-Peppas model which was less than 0.45, the release mechanism was likely involved with the Fickian diffusion process.\[29\]

**In vitro uptake and synergistic cytotoxicity of CSA-NPs**

The cell uptake and synergistic cytotoxicity of prepared nanoparticles were firstly investigated with a fluorescence microscope. Initially, after 6 hours incubation, red signals resulted from the intrinsic fluorescence of Dox were observed (**Supplementary Fig. 6**), suggesting the cellular uptake of nanoparticles. After 24 hours incubation, considerable amounts of Dox-CSA-NPs and CCM/Dox-CSA-NPs were observed to enter A549 cells judged from the Dox fluorescence intensity. Accordingly, TUNEL assay was employed to visualize the apoptosis induced by different nanoparticles. The cells treated with

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**Table 3** IC_{50} of different combinations and prepared nanoparticles in A549 cells (μg/mL)

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<tr>
<th>Formulation</th>
<th>IC_{50} value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free CCM</td>
<td>34.94</td>
</tr>
<tr>
<td>CCM+0.1 μg/mL Dox</td>
<td>18.12</td>
</tr>
<tr>
<td>CCM+0.5 μg/mL Dox</td>
<td>7.79</td>
</tr>
<tr>
<td>CCM+1.0 μg/mL Dox</td>
<td>N.A.</td>
</tr>
<tr>
<td>Free Dox</td>
<td>0.64</td>
</tr>
<tr>
<td>Dox+1 μg/mL CCM</td>
<td>0.46</td>
</tr>
<tr>
<td>Dox+5 μg/mL CCM</td>
<td>0.31</td>
</tr>
<tr>
<td>Dox+10 μg/mL CCM</td>
<td>0.19</td>
</tr>
<tr>
<td>CCM-CSA-NP</td>
<td>867.28</td>
</tr>
<tr>
<td>Dox-CSA-NP</td>
<td>32.49</td>
</tr>
<tr>
<td>CCM/Dox-CSA-NP</td>
<td>26.50</td>
</tr>
</tbody>
</table>

**Fig. 5** *In vitro* cytotoxicity of CCM/Dox loaded CSA nanoparticles against A549 cells. Cell viability of A549 cells incubated with different concentrations of drug-encapsulated CSA-NPs by MTT assay. All the data are represented as mean±SD (n=3).
CCM-CSA-NPs showed almost none or week green signals while those treated with Dox-CSA-NPs and CCM/Dox-CSA-NPs displayed improved green fluorescence. After 24 hours incubation, the drug-loaded CSA-NPs were absorbed by cancer cells to induce the apoptosis as demonstrated for both Dox-CSA-NPs and CCM/Dox-CSA-NPs. These results were also consistent with the cytotoxicity evaluation.

<table>
<thead>
<tr>
<th>Condition</th>
<th>First order $r^2$</th>
<th>Higuchi $r^2$</th>
<th>Korsmeyer-Peppas $r^2$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CCM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS pH 7.4</td>
<td>0.9863</td>
<td>0.8885</td>
<td>0.9757</td>
<td>0.3820</td>
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<tr>
<td>5 mmol/L GSH in PBS pH 7.4</td>
<td>0.9911</td>
<td>0.88786</td>
<td>0.9170</td>
<td>0.3610</td>
</tr>
<tr>
<td>10 mmol/L GSH in PBS pH 7.4</td>
<td>0.9752</td>
<td>0.9038</td>
<td>0.9242</td>
<td>0.4176</td>
</tr>
<tr>
<td>NaAc-HAc pH 5.0</td>
<td>0.9909</td>
<td>0.9061</td>
<td>0.9366</td>
<td>0.3231</td>
</tr>
<tr>
<td>5 mmol/L GSH in NaAc-HAc pH 5.0</td>
<td>0.9844</td>
<td>0.8994</td>
<td>0.9465</td>
<td>0.3186</td>
</tr>
<tr>
<td>10 mmol/L GSH in NaAc-HAc pH 5.0</td>
<td>0.9829</td>
<td>0.9152</td>
<td>0.9492</td>
<td>0.3719</td>
</tr>
<tr>
<td><strong>Dox</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS pH 7.4</td>
<td>0.9882</td>
<td>0.8651</td>
<td>0.9064</td>
<td>0.4011</td>
</tr>
<tr>
<td>5 mmol/L GSH in PBS pH 7.4</td>
<td>0.9779</td>
<td>0.8774</td>
<td>0.9148</td>
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</tr>
<tr>
<td>10 mmol/L GSH in PBS pH 7.4</td>
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<td>0.9152</td>
<td>0.9211</td>
<td>0.4127</td>
</tr>
<tr>
<td>NaAc-HAc pH 5.0</td>
<td>0.9902</td>
<td>0.8789</td>
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<td>0.3905</td>
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<tr>
<td>5 mmol/L GSH in NaAc-HAc pH 5.0</td>
<td>0.9853</td>
<td>0.8910</td>
<td>0.9472</td>
<td>0.3424</td>
</tr>
<tr>
<td>10 mmol/L GSH in NaAc-HAc pH 5.0</td>
<td>0.9932</td>
<td>0.8951</td>
<td>0.9036</td>
<td>0.3318</td>
</tr>
</tbody>
</table>

Fig. 6 *In vitro* drug release behaviors of CSA-based nanoparticles. A and B: CCM (A) and Dox (B) in PBS buffer (pH 7.4) at 37 °C; C and D: CCM (C) and Dox (D) in NaAc-HAc (pH 5.0) at 37 °C. All the data are represented as mean±SD ($n$=3).
results in which combinational use of CCM/Dox was more effective than the single drug. Furthermore, to quantitate the synergistic cytotoxicity, high content screening assay was performed. As shown in Fig. 7, the percentage of positive cells which met the threefold pre-set in the TUNEL method in the group incubated by CCM/Dox-CSA-NPs were significantly higher than that treated by single drug loaded CSA-NPs. The mean fluorescence intensity also showed the similar results, and more broadly, apoptosis induced by CCM/Dox co-bound CSA-NPs was much higher than that of CCM or Dox alone.

Discussion

Many methods such as desolvation and nano-spray drying have been used to prepare albumin nanoparticles[31]. In this study, we have found, similar with another hydrophobic drug of paclitaxel[32], the CCM concentration dependence on the size of formed protein nanoparticles was also observed, demonstrating the possible hydrophobic interaction between drug molecules and camel albums was associate with the self-assembling of protein nanoparticles. Moreover, based on the measured hydrodynamic diameter of obtained nanocomposites, we have put forward a possible mechanism for the formation of nanoparticles, in which the assembling of CCM-CSA complex was mainly responsive for their formation when adding relatively low amount of CCM. With the addition of CCM, the encapsulation of free CCM as well as the matrix effect resulted from the solvent may lead to a gradually increase for the size of formed nanoparticles. Accordingly, the structural analysis of CSA has also revealed the partial structural change around the tryptophan residue of the protein during the GSH-induced disulfide bonds formation. Meanwhile, the enhanced solubility of CCM (~140

Fig. 7 Cellular uptake and synergistic cytotoxicity of prepared drug-loaded CSA nanoparticles. A: Fluorescence microscopy image of A549 cells incubated with CCM-CSA-NPs, Dox-CSA-NPs and CCM/Dox-CSA-NPs for 24 hours. Nuclei was stained with Hoechst (blue), doxorubicin (red), and nuclei of apoptotic cells TUNEL-positive signal (green); B–D: Quantitative evaluation of the apoptosis of A549 cells, scanning microscopy images of A549 cells (middle), total percentage of positive cells (upper right) and mean fluoresce intensity (bottom right) of cells incubated with prepared CSA-NPs by high-content cell quantitative imaging analysis system. All the data are represented as mean±SD (n=3).
fold) was realized in the composite arising from the excellent solubility of CSA, which is favorable for the practical use of such hydrophobic drug.

Furthermore, CCM and Dox were encapsulated in the CSA-NPs and the synergistic effect on A549 cancer cells was studied. An evident dose-dependent inhibitory effect of prepared NPs on cancer cells was observed, revealing a synergistic effect of combinational use of CCM/Dox. This finding together with the GSH- as well pH-responsive behaviours of protein nanocomposites are desirable for the biomedical applications of CSA-based biocargo.

In summary, a simple and effective self-assembly method was established to encapsulate Dox and hydrophobic CCM into purified CSA nanoparticles without the use of any toxic cross-linking agents. Besides the synergistic effect of CCM and Dox was identified, the cytotoxicity of drug-loaded CSA nanoparticles against A549 cells was investigated to further demonstrate the efficiency of combination therapy in vitro. Notably, as the redox-responsive behavior of prepared nanoparticles was validated by DLS, it was further explored in different buffers at varied concentrations of GSH. Based on results from in vitro apoptosis assay, CCM/Dox co-bound CSA-NPs were more effective against A549 cells, which owns the advantage in avoiding the side effects of doxorubicin to a great extent. Nevertheless, in a long run, further attempts using recombinant CSA should be made by exploring the anti-tumor efficiency in vivo.

Acknowledgments

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