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Cationic Quaternized Chitosan Nanoparticles for C-phycocyanin Delivery to Hela Cells

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Abstract: In this study we synthesize C-PC/QAC nanoparticles using cationic quaternary ammonium chitosan (QAC) as substrate material loaded C-phycocyanin (C-PC) by ionic-gelation method. The prepared NPs was about 120nm size with spherical morphology, the entrapment efficient was 62%, and the drug-loading amount was 20%. The NPs exhibited a pH sensitive realease of C-PC *in vitro*. The drug-NPs were proved to be hemocompatible and safe. In conclusion, QAC increased bioavailability and anticancer property of C-PC.

Keywords: Cancer Therapy· Drug Delivery· C-phycocyanin·Quaternary Ammonium Chitosan· Nanoparticles

1. INTRODUCTION

Nano-drug is a hot-spot in the field of medicine, which can overcome the limitations of conventional drug delivery systems[1]. With the development of nanoparticle technology, the study of nanoparticle as drug carrier has attracted broad attention. Cervical cancer is one of the first discovery of cancer and seriously threatening life and health among females worldwide[2]. C-phycocyanin (C-PC) is an important natural photosynthetic pigment with many advantages such as safety, non-toxicity, high hydrophilcity and good dyeing force. Its absorption peak at 620nm is strongest[3, 4]. Li bing's studies found that C-PC has anti-tumor and immune-enhancing effect[5]. However, its application in medical field is limited by the poor stability. Therefore, nano-drugs provide a good choice to set up an efficient delivery for C-PC. Quaternary ammonium chitosan (QAC), a kind of chitosan derivative, had an attention in drug delivery application[6]. It can load C-PC to form nanoparticles and achieve slow-release effect.

2. MATERIALS AND METHODS

2.1. Materials

Quaternary ammonium chitosan (Mwt:10-20kDa; deacetylation degree ≥95% and degree of substitution ≥90%) was procured from Beijing huamaike Bio-Tech Co.Ltd., China. C-phycycoanin was purchased from Taizhou Binmei Biotechnology Co., Ltd., China. Human cervical cancer cells (Hela) and fresh human blood were provided by the Affiliated Hospital of the Medical College of Qingdao University. Methyl thiazolyl tetrazolium (MTT) and DAPI were purchased from Beijing Solarbio Science & Technology. RPMI1640 medium and febal bovine serum (FBS) were from HyClone Company. All other chemicals used were of analytical grade.

Cell culture

Hela cells was maintained in RPIM 1640 supplemented with 10% FBS and 0.5% antibiotics. The cells were incubated at 37℃ with 5% CO_2.

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2.2 Methods

2.2.1 Preparation of C-PC/QAC-NPs

The C-PC/QAC-NPs were made by the simple ionic-gelation with crosslinking agent tripolyphosphate (TPP)[7, 8]. Briefly, 10% TPP was added dropwise into the aqueouos solution of QAC (0.1 wt%) pre-incubated with C-phycocyanin (1.0mg/ml) till the solution became turbid under constant stirring. Drug loaded nanoparticles were separated by centrifugation for 15min at 12000 rpm and further lyophilized.2.2.2 Drug encapsulation efficiency (DE) and drug loading rate (DL).

The spectrophotometer was used to measure the DE and DL of C-PC. The nanoparticles solution was centrifuged for 15 min at 12000 rpm at 4℃, and the supernant was detected by spectrophotometer. The absordance density of the drug in 650 nm and 615 nm was measured by ultraviolet spectrophotometer[9]. The formula C(c-pc) = (OD615-0.474OD650)/5.34 was used to calculate C-PC concentration. The EE and LE values were calculated by using the following equations (Eqs. (1) and (2))

$$DE(\%)= \frac{C_1-C_2}{C_1} \times 100\% \quad (1)$$

$$DL(\%)= \frac{(C_1-C_2)V}{W_{nps}} \times 100\% \quad (2)$$

where C_1 is the total C-PC concentration in the NPs solution, C_2 is the C-PC concentration in the supernatant, V is the total volume of prepared NPs solution, and W_{nps} is the total amount of NPs.

2.4 Evaluation of nanoparticles

The particle size and surface morphology of NPs was analyzed using Scanning Electron Microscope (SEM/JEOL JSM-840 Analytical Scanning Electron Microscope).

2.5 *In vitro* drug release studies

In vitro C-PC release from C-PC/QAC-NPs was determined by dialysis method under two different pH5.6 and 7.4 at 37 ºC as described in literature. The prepared NPs suspension was centrifuged at 12000 rpm for 15 min and the collected pellets were redispersed in 5mL of PBS, then filled in a dialysis tubes, and put in a beaker containing 30 ml PBS (pH5.4 and 7.4) at 37ºC with gentle stirring in a shaking incubator. The release studies were carried at fixed time intervals: 1, 2, 4, 8, 12, 24, 48h up to 10 days. At proper time intervals, 1ml of release media was removed and replaced with fresh PBS. The amount of C-PC released at different time intervals was quantified at 615 nm and 650 nm using UV-vis spectroscopy. The ratio of the amount of C-PC released form the NPs and the total amount of C-PC in the NPs was used to calculate the cumulative release percentage.

2.6 *In vitro* cytotoxicity assay

MTT assay was used to measure cytotoxicity of the prepared NPs on the proliferation of Hela cells. The cells were seeded in 96-well plates (1×10^4 cells/ well) in 100ul medium. After incubated overnight, the cells were treated with 100ul of samples (C-PC, C-PC/QAC-NPs) with equivalent C-PC final concentration 1, 10 and 100 µg/ml and bare QAC-NPs (1, 10 and 100 µg/ml) for 24h. Untreated cells and 1% (v/v) Triton-X-100 acted as positive control and negative control, respectively. Subsequently, 20µl of MTT solution (5 mg/ml) was added for another 4 hours. Finally, 100µl of dimethyl sulfoxide (DMSO) was added to each well. The absorbance of each well at 490nm was measured by enzyme immunoassay. *In vitro* cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{OD_{sample}}{OD_{control}} \times 100\% \quad (3)$$

where OD_{sample} and $OD_{control}$ were the absorbance of drug treated and untreated cells. Repeat five parallel experiments for each group.

2.7 Hemolysis assay *in vitro*

Hemolytic activity of C-PC, QAC-NPs and C-PC/QAC-NPs was evaluated according to the previous protocol. Fresh blood from human volunteers was added into the tubes with acid citrate dextrose

(ACD), diluted by physiological saline, and centrifugated in order to obtain the red blood cells (RBCs). After washing and diluting, the RBCs suspension were added to C-PC, C-PC/QAC-NPs and QAC-NPs solution. Then incubated at 37 ºC for 2h. PBS and Trixon X-100 (10g/L) were used as negative and positive controls, respectively. After incubation, the RBCs were centrifuged at 3000rpm for 10 min. Free hemoglobins in the supernatant were measured spectrophoto-metrically using Beckman Coulter Elisa plate reader at 540nm. Hemolysis ratio (HR) of RBCs was calculated based on the equation[10].

$$HR\ (\%) = \frac{OD_{sample} - OD_{negative\ control}}{OD_{positive\ control} - OD_{negative\ control}} \times 100 \quad (4)$$

2.9 Statistics analysis

The data were expressed as mean ± SD. A Student's t-test was used to determine the significance. Data were considered to be statistically significant at $P<0.05$. SPSS software was used for statistical analysis.

3 Results

3.1 Synthesis of C-PC loaded QAC nanoparticles

C-PC/QAC-NPs were obtained as a result of chemical cross-linking with triphosphoric acid (TPP). The preincubation of C-PC with QAC was carried out to facilitate electrostatic interaction between the anionic C-PC and cationic QAC in neutral conditions.

3.2 Structural features of C-PC/QAC-NPs

SEM image revealed that the mean particle size of C-PC/QAC-NPs was about 120nm with spherical morphology (Fig 1).

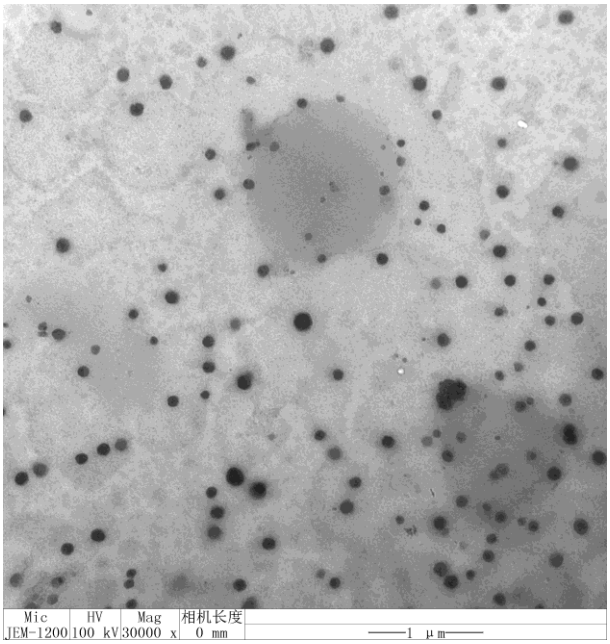

FIG.1. Morphology and particle size of QAC-NPs by SEM.

3.3 Drug entrapment efficiency and drug loading efficiency

The drug entrapment efficiency in NPs was measured by spectrophotometer at 650nm and 615nm of C-PC in the supernatant after completely extraction of C-PC from NPs in PBS. Then the total mass of NPs was weighted. Finally, the drug entrapment efficiency and loading efficiency were about 62% and 20%, respectively.

3.3 *In vitro* drug release profile of C-PC/QAC-NPs

The C-PC released from nanoparticles was measured in

PBS. The results showed that C-PC/QAC-NPs got an initial burst in the first 6h and a slow-release pattern as shown in Figure 2. The release rate of C-PC from C-PC/QAC-NPs was 60% in the first 24h cumulatively. The release pattern could attribute to rapid release of surface adsorbed C-PC and sustaining release of entrapped C-PC from NPs with QAC degradation.

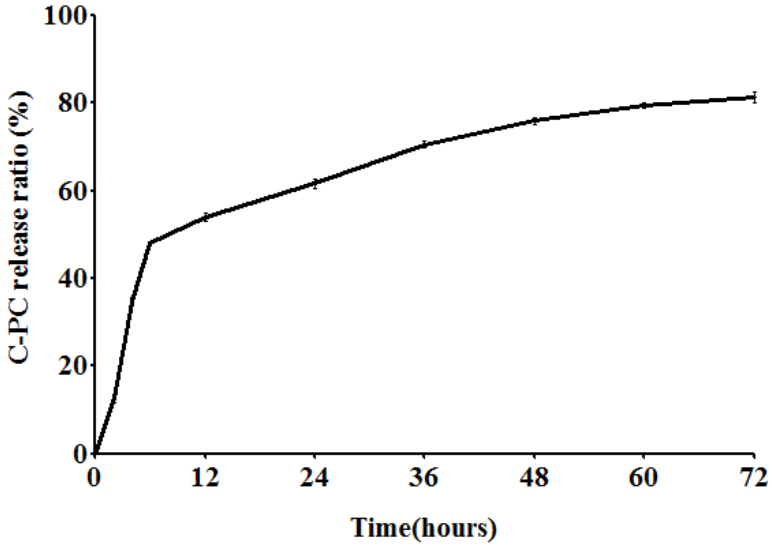

FIG.2. The release rate of C-PC from C-PC/HACC-NPs

3.4 Cytotoxicity studiesg

The cytotoxic effects of different forms and concentrations of C-PC on Hela cells were detected by MTT assay (Figure 3). The results showed that all forms C-PC could inhibit the growth of Hela cells. Meanwhile, C-PC and C-PC/QAC-NPs induced more toxicity than bare QAC-NPs. The highest concentration (100ug/ml) of C-PC and C-PC/QAC-NPs showed that 40% more toxiticy than bare QAC-NPs on Hela cells. Thus, it is clearly evident that C-PC/QAC-NPs enhanced the anticancer effect.

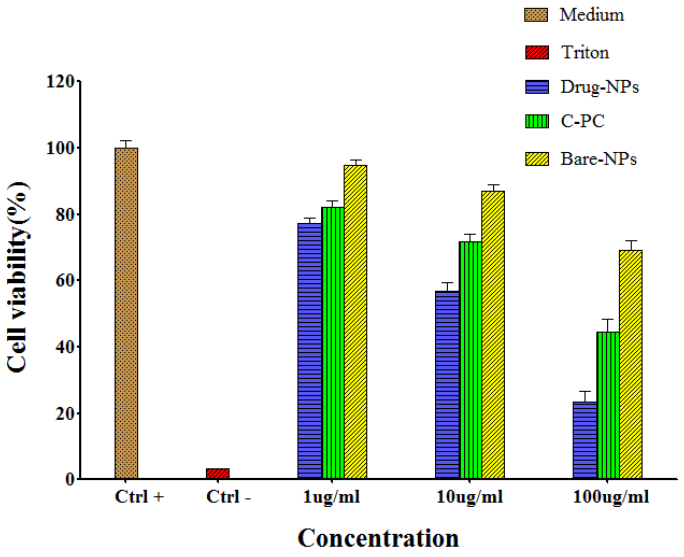

FIG3. Cell viability of Hela cells treated with different forms and concentrations of C-PC.

3.5 Hemolysis assay

It is necessary to make sure the blood compatibility of the drug-NPs, because the drug will finally enter into blood vessels. The hemolysis assay was carried out based on the previous protocol. The optical photographs of blood samples showed clear plasma compared to Triton group (100% hemolysis) with red plasma due to lysed RBCs (Fig 4A). The percentage of hemolysis were less than 5%, the critical safe hemolytic ratio for biomaterials according to ISO/TR 7406[11]. The results indicated that C-PC/QAC-NPs are hemocompatible.

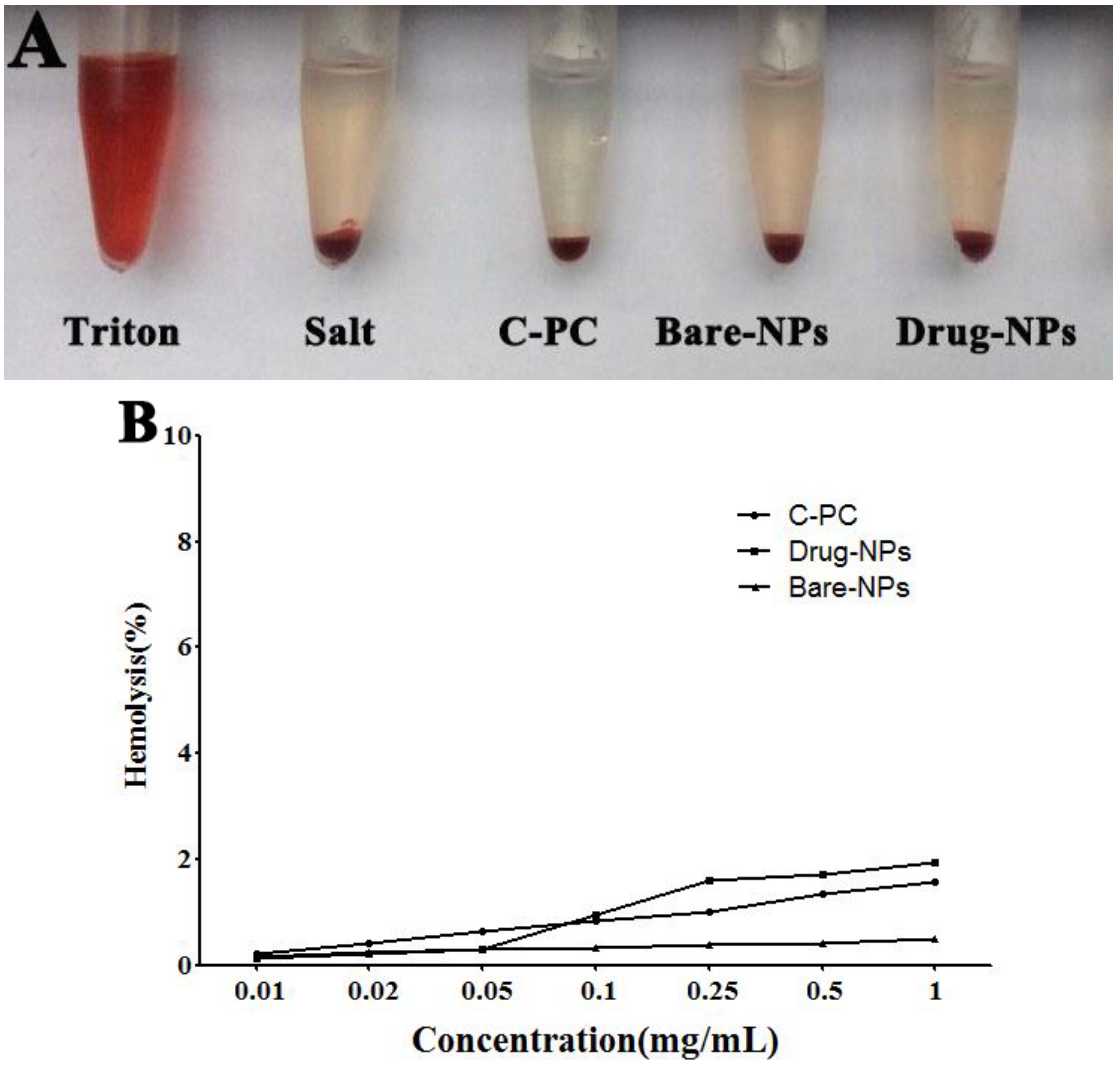

FIG. 4. The hemolysis of RBCs. (A) Photographs of RBCs hemolysis in the presence of C-PC, Bare-NPs, Drug-NPs; (B) Hemolysis percentage of RBCs treated by C-PC, bare-NPs, drug-NPs.

4. Discussions

Drug delivery system based on nanoparticle has been become a hot-spot in the field of medicine, which have advantages of high drug-loading efficiency, low adversely effect and sustaining release[12]. Thus, nanoparticles can significantly enhance the drugs to tumor sites and improve their efficacy.

Quaternary ammonium chitosan (QAC), a water soluble chitosan derivative, had an attention in drug delivery application with nontoxic, excellent water solubility and biocompability[13]. In recent years, chitosan derivatives were chosen as a good candidate

for drug controlled carrier. Anitha prepared N,O-CMC nanoparticals loading 5-FU used in treatment of breast cancer[14]. Li made O-CMC nanoparticals loading chelerythrine through crosslink emulsification process using glutaraldehyde as linkage reagent.

We prepared C-PC/QAC-NPs by ionic crosslinking method and using TPP as linkage reagent. SEM was used to analyze size distribution and surface morphology. Ultraviolet spectrophotometer was applied to assess encapsulation efficiency, drug-loading rate and drug release. The results showed that the mean particle size of C-PC/QAC-NPs were 120nm with spherical morphology. The drug encapsulation efficiency was 60% and drug loading efficiency was 20%. Meanwhile, C-PC/QAC-NPs had significant slow-release effect.

MTT assay was adopted to determine the effects of C-PC, QAC and C-PC/QAC-NPs on the growth of HeLa cells. It is obvious that all drugs could inhibit the proliferation of HeLa cells and had dose-independent effect, especially C-PC/QAC-NPs. Hemolysis assay indicated that C-PC/QAC-NPs are hemocompatible and safe.

The next step of the study is to link a targeted specific ligands of CD59（CD59sp）on the surface of QAC in order to prepare novel nanoparticals loading C-PC[15]. Making use of the target effect of CD59sp, C-PC is targetly delivered to cancer cells to realize tumor targetting therapy. The study offer a new approach to tumor targeting therapy with wide exploitation and application prospects.

5. Conclusion

C-phycocyanin loaded quaternary ammonium chitosan were obtained by ionic gelation method. The prepared NPs was about with spherical morphology using SEM. *In vitro* drug release showed an initial burst release and sustained release. The C-PC/QAC-NPs showed cytotoxic effects to Hela cells. The C-PC/QAC-NPs were proved to be hemocompatible and safe. In conclusion, QAC increased bioavailability and anticancer property of C-PC. Thus, QAC-NPs were a potential drug delivery system for C-PC into cancer cells.

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Conflicts of Interest

None.

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