Application of nanoparticle in photodynamic diagnosis for colorectal cancer

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Abstract

Photodynamic medicine is a novel approach for cancer detection and treatment via different photosensitizers and suitable light source. The 5-Aminolevulinic acid (5-ALA) can be converted into protoporphyrin IX (PpⅨ) that can be utilized as a fluorescent probe for tumor detection, however, this biosynthetic pathway can also occur in intestinal normal flora, such as *E. coli*, which inevitably influences the efficacy of tumor detection. In this study, 5-ALA was encapsulated in nanoparticles to prevent uptake by *E. coli*. We mixed chitosan with sodium tripolyphosphate (STPP) to prepare chitosan nanoparticles (CN) — by ionic gelation method. Chitosan nanoparticle loaded with 5-ALA (CN-A) were prepared by the same method. The size and zeta potential of CN and CN-A were measured by transmission electron microscope and zetasizer, respectively. CN and CN-A were both at the range of 60 ~110 nm in size, depending on the pH value of STPP solution and 5-ALA solution. When the pH value of STPP solution and 5-ALA solution increased, the nanoparticle size of CN and CN-A increased. The optimal 5-ALA loading efficiency for CN-A approximated 80%. Bioassy of CN-A for Caco-2 colon cancer cell and E. coli uptake was done, and the result revealed that Caco-2 colon cancer cells could uptake the CN-A and convert the loaded 5-ALA into PpⅨ which emitted red fluorescence when excited by the light with specific wave-length , whereas E. coli showed no such activity. This result implied that chitosan can exclude the influence of normal flora inside the gut and serves as an ideal vector of colon-specific drug delivery system. According to this concept, we designed a novel photodynamic detection system to enhance the accuracy of endoscopic diagnosis for early colorectal cancer.

Keywords：Photodynamic medicine, 5-Aminolevulinic acid, chitosan, and nanoparticles.

1. Introduction

A natural precursor in heme synthesis, 5-aminolaevulinic acid (5-ALA), has been shown to induce accumulation of the photosensitizer protoporphyrin Ⅸ(PpⅨ) in cancer cells [1-2]. On this basic knowledge, 5-ALA has been used clinically in photodynamic therapy of several non-malignant and malignant diseases, including nonmelanoma skin tumors, psoriasis, vulval and vaginal carcinomas in situ, Gorlin's syndrome, cutaneous T-cell lymphoma and tumours of the aerodigestive tract. Additionally, 5-ALA has been found to be a promising agent in diagnosis of cancer due to the fluorescing properties of PpⅨ [3].

Chitosan, a polysaccharide with similar structure to cellulose, comes from the deacetylation of chitin by treating with concentrated alkali solution. There is an important difference between cellulose and chitosan. Chitosan is composed of 2-amino-2-deoxy-β-D-glucan combined with glycosidic linkages. The primary amino groups give special properties that make chitosan more useful in pharmaceutical applications. Compared to other natural polymers, chitosan has a positive charge in basic pH conditions and is mucoadhesive [4]. Chitosan is biocompatible with living tissues since it does not cause allergic reactions and rejection. It possesses antibacterial property and absorbs toxic metals like mercury, cadmium, lead, etc. If degree of deacetylation and molecular weight of chitosan can be modulated precisely, it would be a perfect material for developing micro/nanoparticles. Chitosan has many advantages, including the ability to control the release of active agents and avoidance of the use of hazardous organic solvents while fabricating particles. Furthermore, chitosan is a linear polyamine containing a number of free amino groups that are available for crosslink, and its cationic nature allows for ionic crosslink with multivalent anions. Chitosan also has mucoadhesive character which increases residual time at the site of absorption, and so on [5].

In this study, chitosan nanoparticles loaded with 5-ALA was prepared by ionic gelation method. Characterization of chitosan nanoparticles and loaded efficiency of 5-ALA in nanoparticles were investigated. Prevention of uptake by *E. coli.* was also studied by cell uptake test and microbiology assay.

2. Materials and methods

2.1 Preparation of chitosan nanoparticles loaded with 5-ALA (CN-A)

The preparation of chitosan nanoparticles loaded with 5-ALA is based on an ionic gelation interaction between positively charged chitosan and negatively charged tripolyphosphate or ionized 5-ALA at room temperature.

Chitosan was dissolved to a concentration of 0.5mg/ml in 0.01M acetic acid solution under the pH of 4.0. 5-ALA was dissolved to a concentration of 10 mg/ml in 0.5mg/ml sodium tripolyphosphate solution. Working 5-ALA solutions with concentrations of 0.1 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml and 2.0 mg/ml were made by dilution with 0.5 mg/ml sodium tripolyphosphate solution and the pH were adjusted to 5.0, 6.0, 7.4, 9.0 and 10.0, respectively. Two ml of STPP or 5-ALA STPP PBS solution was dropped slowly in 5 ml of chitosan solution under a flowing rate of 0.5 ml/min. Nanoparticles were used without further purification for all studies.

2.2 Characterization of chitosan nanoparticles and chitosan nanoparticles loaded with 5-ALA

Measurements of particle size and zeta potential of the nanoparticles were performed by dynamic light scattering measurements and laser Doppler electrophoresis, respectively, using a Zetasizer-3000 (Malvern Instruments). The measurement of size was performed at 25℃ at a 90° scattering angle, and it was recorded for 15 min for each measurement. The mean hydrodynamic diameter was generated by cumulative analysis. The measurements of zeta potential were performed using the aqueous flow cell in the automatic mode at 25℃.

2.3 Evaluation of loaded efficiency of 5-ALA in chitosan nanoparticles loaded with 5-ALA

After nanoparticle preparation, loaded efficiency of 5-ALA in nanoparticles was assessed. One ml of nanoparticle solution was added with 0.1 N sodium hydroxide solution and quickly mixed under high vortexing for 60 seconds. Then, by centrifuge the mixing solution was separated into the particle and the suspension which contained free 5-ALA. The concentration of free 5-ALA was determined by TNBSA (2,4,6-Trinitrobenzene Sulfonic Acid) which was a rapid and sensitive assay reagent for the determination of free amino groups. Finally the loaded efficiency of 5-ALA in nanoparticles was calculated by following equation：

$$\text{Loaded efficiency (\%)} = \frac{C_t - C_f}{C_t} \times 100\ \%$$

C_t and C_f were total amount of 5-ALA and free amount of 5-ALA respectively.

2.4 Cell uptake test

Caco-2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % (v/v) fetal bovine serum, 2 mM glutamine, 10 mM non-essential amino acids, 50 IU/ml penicillin and 50 Ag/ml streptomycin, at 37 ℃ and in an atmosphere of 5% CO_2. Growth medium was changed on alternate days until confluent.

Caco-2 cells were seeded into six-well tissue culture plates and maintained for 24 hours. After removing the medium, fresh medium containing CN-A or CN was added into six-well tissue culture plates and incubated for 24 hours to let cells uptake nanoparticles and converted 5-ALA into protoporphyrin IX (PpIX). We removed the medium and washed with phosphate-buffered saline (PBS) three times, Caco-2 cells were immobilized by 10 % formalin and covered by glass slice. Finally Caco-2 cells were observed by fluorescence microscopy in which red fluorescence (635 nm) was induced by green or blue light irradiation with Laser.

2.5 Microbiology assay

E. coli grown on brain heart infusion agar (Difco, Detroit, MI) were transferred into nutrient broth (Difco) at pH 6.5 to a final volume of 25 ml at an initial optical density and were allowed to grow at 37℃ with aeration for 24 hours. Chitosan-5-ALA nanoparticles or chitosan nanoparticles solution were added and were allowed to grow at 37℃ with aeration for 24 hours. A drop of E. coli solution were covered by glass slice and observed by fluorescence microscopy in which red fluorescence (635 nm) was induced by green or blue light irradiation with Laser.

3. Result and discussion

3.1 Physicochemical characterizations of chitosan nanoparticles and chitosan nanoparticles loaded with 5-ALA

Average size and zeta potential of CN and CN-A prepared at various conditions were showed in fig. 1 and table 1. When the pH of STPP solution or 5-ALA solution are 5.0 or 6.0, the nanoparticles are about 60 nm in size, but when the pH of STPP solution or 5-ALA solution is higher than 7.4 and the concentration of 5-ALA in 5-ALA solution is lower than 1.5 mg/ml, the nanoparticles are about 100 nm in size.

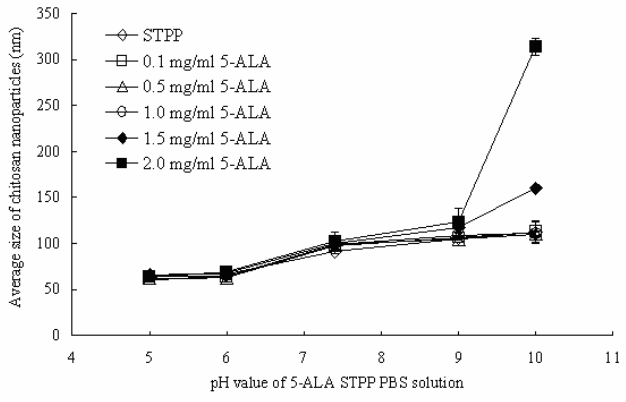

Figure 1. Average size of CN and CN-A.

Zeta potential, that is, surface charge, can greatly influence particle stability in suspension through the electrostatic repulsion between particles. It can also determine nanoparticle interaction in vivo with the cell membrane of bacteria, which is usually negatively charged [6]. Table1 shows that the surfaces of CN or CN-A have a positive charge about 40 mV when the pH of STPP solution or 5-ALA solution is 5.0 or 6.0, while the pH of STPP solution or 5-ALA solution is 7.4 or 9.0, nanoparticles exhibit about 30mV.

Concentration of 5-ALA in STPP PBS solution (mg/ml)	pH value of 5-ALA STPP PBS solution				
	pH=5.0	pH= 6.0	pH= 7.4	pH= 9.0	pH= 10.0
0.0	43.7	46.1	43.5	34.8	22.3
0.1	46.8	33.5	26.1	26.7	23.6
0.5	48.3	38.2	30.5	23.7	28.1
1.0	36.5	34.4	38.5	31.1	25.2
1.5	43.2	42.6	43.4	24.5/	16.0
2.0	40.7	40.9	43.4	28.7	11.1

Table 1. Zeta potential (mV) of various CN and CN-A.

3.2 Evaluation of loaded efficiency of 5-ALA in chitosan nanoparticles loaded with 5-ALA

Loaded efficiency of 5-ALA in CN-A prepared at various conditions were showed in fig. 2. When the pH of 5-ALA solution is 5.0 or 6.0, the loaded efficiency of 5-ALA in CN-A are low. But when the pH of 5-ALA solution is higher than 7.4, the loaded efficiency of 5-ALA is greater than 80 %.

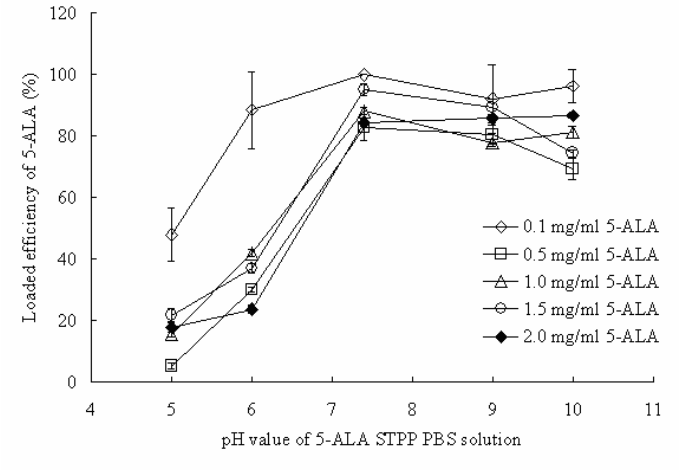

Figure 2. Loaded efficiency of 5-ALA in CN-A.

3.3 Cell uptake test and microbiology assay

Cell uptake and microbiology assay of CN and CN-A prepared at various conditions were showed in fig. 3 and fig.4. Our results show that CN-A can be uptaken by Caco-2 but not *E. coli*, and 5-ALA encapsulated in nanoparticles can be released inside cells but not culture medium. So 5-ALA released inside cells can be converted into PpIX which can be induced by green or blue light irradiation with laser and observed by fluorescence microscopy. So we can suggest that chitosan is ideal natural material for colon-specific drug delivery system.

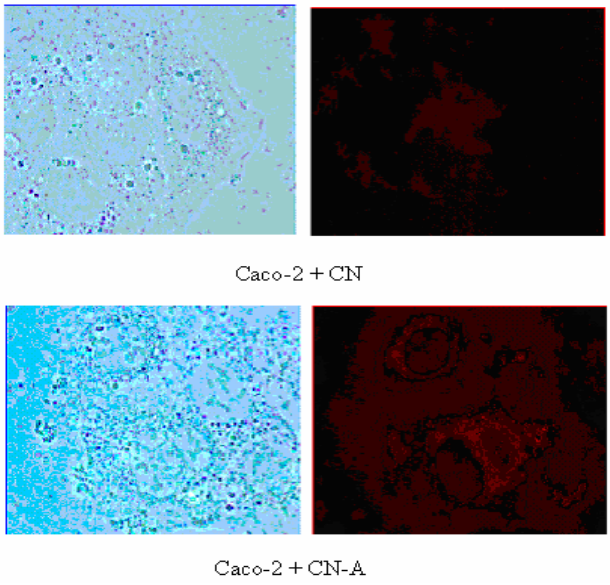

Figure 3. Red fluorescence (635 nm) of pPIX induced by green or blue light irradiation with Laser was observed by fluorescence microscopy.

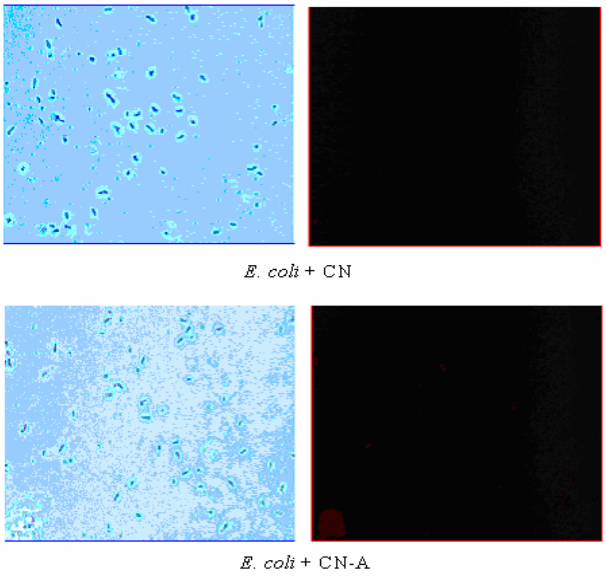

Figure 4. Red fluorescence (635 nm) of pPIX induced by green or blue light irradiation with Laser was observed by fluorescence microscopy.

4. Conclusion

CN and CN-A were both at the range of 60 ~110 nm in size, depending on the pH value of STPP solution and 5-ALA solution. When the pH value of STPP solution and 5-ALA solution increased, the nanoparticle size of CN and CN-A increased. The optimal 5-ALA loaded efficiency for CN-A approximated 80%. Bioassy of CN-A for Caco-2 colon cancer cell and E. coli uptake was done, and the result revealed that Caco-2 colon cancer cells could uptake the CN-A and convert the loaded 5-ALA into PpⅨ which emitted red fluorescence when excited by the light with specific wave-length , whereas E. coli showed no such activity.

Acknowledgments

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