A Novel Detection of Early Colorectal Cancer by Chitosan Nanoparticles Conjugated with Folic Acid

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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), and in cancer cells which serves as a fluorescent probe for tumor detection. In this study, folic acid was conjugated onto chitosan molecules via covalent bond for specific target, then 5-ALA was encapsulated in chitosan nanoparticles to prevent the uptake by E. *coli*. Chitosan nanoparticles (CN) and folic acid conjugated chitosan nanoparticles (fCN) were prepared by ionic gelation. Then the nanoparticles loaded with 5-ALA (CNA and fCNA) were prepared by the same method. The size and zeta potential of nanoparticles were measured by transmission electron microscope and zetasizer, respectively. The particle size was at the range of 90-110 nm and the loading efficiency of 5-ALA was at the range of 80%-90% depending on the concentration of 5-ALA prepared and the percentage of amino groups left in chitosan. Bioassy results of CNA and fCNA for HT-29 colon cancer cell uptake revealed that HT-29 colon cancer cells could uptake CNA and fCNA, and the content of PpIX converted from 5-ALA depended on the modified percentage. This result implied that fCNA could 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, folic acid and nanoparticle.*

1 INTRODUCTION

The incidence of colorectal cancer is increasing in the world, and the prognosis of colorectal cancer remains poor.[1] In general, survival depends on the degree to which the cancer has spread, and if the malignancy can be detected at early stage, the prognosis is excellent. But small neoplasia or early cancer of the colon are frequently missed during endoscopy, hence a powerful and high-sensitive tools for the detection of cancerous lesions is important in clinical practice.

Photo-diagnosis is one of promising and non-invasive methods to detect malignant or premalignant tissue.[2] Photo-diagnosis generally uses fluorescence to help detection and painting of abnormal tissue regions, in which exogeneous chromophore is excited by optima light to generate fluorescence to detect the cancer lesions.[3]

5-ALA is a precursor during heme group synthesis in human body, and totally degraded in cells after converted to protoporphyrin IX (PpIX). PpIX is one of photosensitive fluorophore which can be used as fluorescence source to detect cancer lesion due to the different decomposition rate of PpIX in cancer cells and normal cells.[4,5] Generally, PpIX can be totally degraded within 2 - 4 hours in normal cells but 12 - 24 hours in cancer cells.[6] 5-ALA is easily engulfed by the bacteria in gastrointestinal (GI) track that leads to miss interpretation of endoscopic observation. Therefore, a carrier is needed to prevent 5-ALA from bacteria uptake and to enhance 5-ALA passing through the lipophilic barrier and entering the mitochondria to convert into PpIX as the photo source for photo-diagnosis.[7]

Chitosan is a natural polymer. Compared with other biological polymers, chitosan bears positive charge to approach cell membrane conveniently and has muco-adhesive property to prolong the retention time of chitosan in the interested locations.[8,9] Additionally, chitosan is biocompatible and induces no allergic reactions or immuno-rejections. It can be utilized as an adsorbent for toxic metals and possesses antibacterial properties, which can prevent from bacteria uptake in GI track.[10] Furthermore, chitosan is a linear polyamine containing a number of free amino groups which are available for crosslink, and its cationic nature also allows for ionic crosslink with multivalent anions.[11]

Since cell surface receptors for folic acid are generally over-expressed on human cancer cells. Folic acid and its conjugates can enter cancer cells by receptor-mediated endocytosis, therefore, folic acid-conjugated molecules could display high cancer cell specificity. [12]

In this study, we designed a folic acid-conjugated nanoparticle to encapsulate 5-ALA for oral administration to detect the colorectal cancer cell.

2 MATERIALS AND METHODS

2.1 Conjugation of folic acid on chitosan

A solution of folic acid and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) in anhydrous DMSO was prepared and stirred at room temperature until EDC and folic acid were well dissolved and mixed. The mixture was then added slowly to a solution of 0.5 % (w/v) chitosan in acetate acid (pH 4.7). The resulting mixture was stirred at room temperature in the dark for 16 h to react. It was dripped with diluted aqueous NaOH to let polymer precipitate and dialyzed first against phosphate buffer pH 7.4 for 3 days and then against water for 4 days. The chitosan conjugated with folic acid was isolated by lyophilization.[13]

2.2 Determination of percentage of modified amino groups on chitosan

0.5 ml of 0.05% chitosan conjugated with folic acid solution was prepared by dissolving 0.5 mg chitosan conjugated with folic acid in 1 ml 0.01M acetic acid solution and added with 2,4,6-Trinitrobenzene sulfonic acid (TNBSA) as assay reagent to detect the remnant amino groups on chitosan molecules. The percentage of modified amino groups on chitosan was calculated by following equation：

$$\text{Modified percentage (\%)} = \frac{I_t - I_f}{I_t} \times 100\ \%$$

where I_t and I_f were the absorbable intensity of chitosan and chitosan conjugated with folic acid, respectively.

2.3 Preparation of CN 、fCN 、CNA and fCNA

Preparation of chitosan nanoparticle is based on ionic gelation interaction between positively charged chitosan and negatively charged tripolyphosphate at room temperature.[14] Ionized 5-ALA with negative charge also partially contributes to the gelation interactions. The preparation process was described briefly as follows： 0.05% chitosan solution was prepared by dissolving 0.5 mg chitosan powder or chitosan conjugated with folic acid in 1 ml 0.01M acetic acid solution at pH 4.0. One % 5-ALA solution was prepared as stock solution by dissolving 10 mg 5-ALA powder in 1 ml 0.05% STPP solution. The 1% 5-ALA solution was further diluted to the following concentrations: 0.5 mg/ml, 1.0 mg/ml and 1.5 mg/ml by 0.05% STPP solution. Two ml of STPP solution and/or 2 ml of 5-ALA solution were added in 5 ml of chitosan solution or chitosan conjugated with folic acid solution by peristaltic pump at a flow rate of 0.5 ml/min to prepare CN 、fCN 、CNA and fCNA. The prepared CN 、 fCN 、 CNA and fCNA suspended in the solution would be directly used in later experiment without further treatment.

2.4 The particle size and zeta potential of CN 、fCN 、CNA and fCNA

The particle size and zeta-potential of CN 、 fCN 、 CNA and fCNA were measured on Zetasizer-3000 (Malvern Instruments) by dynamic light scattering measurements and laser Doppler electrophoresis, respectively. Particle size was measured for 15 mins at 25°C with 90° scattering angle. The cumulative curve was used to present mean hydrodynamic diameter. The measurements of zeta-potential were performed using the aqueous flow cell in the automatic mode at 25°C.[15]

2.5 The loading efficiency of 5-ALA in CNA and fCNA

The loading efficiency of 5-ALA was evaluated by adding 0.1 N sodium hydroxide solution into 1 ml of CNA solution and vortexed for 60 seconds. The mixed solution was centrifuged at 10000 g force to spin down the particles. The suspension was collected and then added with 2,4,6-Trinitrobenzene sulfonic acid (TNBSA) as assay reagent to detect 5-ALA in the suspensions. The loading efficiency of 5-ALA in nanoparticles was calculated by following equation：

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

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

2.6 Transmission electron microscope

Carbon coated 200 mesh copper grids were immersed in CN 、 fCN 、 CNA and fCNA solution by tweezers and wavered slowly. The grids were placed on delicate-task-wipers to absorb excessive liquid and dried in a desiccator overnight. The dried copper grids with CN and CNA were examined under Hitachi TEM H-7500.

2.7 HT-29 colon cancer cells uptake test

The glass plate was placed in Petri dish and seeded with HT-29 cells. After HT-29 cells were cultured for 24 hours, the medium was replaced by fresh medium with CNA and fCNA additions. HT-29 cells were further cultured 12 hours to allow cells to uptake nanoparticles and convert 5-ALA to PpIX. The cultured medium was removed, and then the glass plate was washed with PBS three times. HT-29 cells were fixed by 10 % formalin and examined under fluorescence microscopy in which green laser was used to excite PpIX to emit red fluorescence.

3 RESULTS AND DISCUSSIONS

3.1 Determination of percentage of modified amino groups

Figure 1 revealed that the percentage of modified amino groups of chitosan molecules decreased with the decreasing molar ratio of folic acid to amino groups, and unexpectedly the lowest modified percentage reached to 26%. These results didn't fit with the molar ratio of folic acid to amino groups which we planned. It was assumed that the mole of amino groups was calculated from the average molecular weight about 150 KD, and many amino groups were estimated in this case, therefore, false modified percentage was obtained. Because the lower molar ration of folic acid to amino groups had amino groups enough to prepare the stable nanoparticles, the ratios of 0.02 and 0.06 were chosen to prepare the folic acid-conjugated nanoparticles.

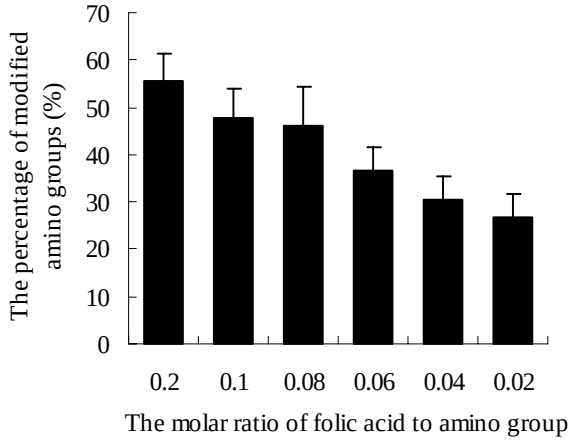

Figure 1. The percentage of modified amino groups of chitosan.

3.2 The average particle size and zeta potential of CN、fCN、CNA and fCNA

The average particle size and zeta-potential of CN、fCN、CNA and fCNA were showed in Table 1. The average size of nanoparticles was about 100 nm and the chitosan with higher modified percentage would get the larger particle size. The zeta potential of nanoparticles was higher than 21 mV but the chitosan with higher modified percentage would get the lower value. This result suggested that f06CN、f06CNA05 and f06CN15 had more amino groups modified and lower amino groups left to bear positive charge.

3.3 The loading efficiency of 5-ALA in CNA and fCNA

The loading efficiency of 5-ALA in CNA and fCNA were showed in Figure 2. The loading efficiency of 5-ALA in nanoparticles was at the range of 80-90% and the chitosan with higher percentage of modified amino groups would get lower loading efficiency. It was proposed that since more amino groups were conjugated with folic acid, there were few amino groups left to react with 5-ALA and consequently resulted in low loading efficiency.

Sample	Average size (nm)	Zeta potential (mV)
CN	102.4±8.74	25.0±1.84
CNA05	92.0±3.66	25.0±2.34
CNA15	96.0±4.82	24.4±1.78
f02CN	100.7±7.79	23.7±1.69
f02CNA05	101.7±9.29	24.2±1.56
f02CNA15	97.6±11.90	22.6±1.71
f06CN	116.9±21.72	22.3±1.05
f06CNA05	113.1±6.53	21.6±1.10
f06CNA15	105.6±5.43	21.0±1.40

Table 1：The average size and zeta potential of various nanoparticles prepared at different conditions.

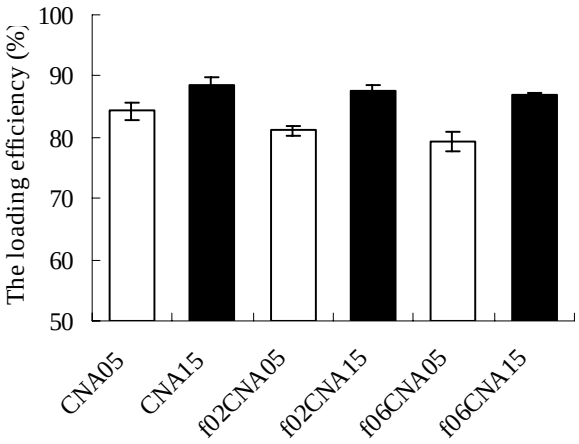

Figure 2. The loading efficiency of 5-ALA in CNA and fCNA

3.4 Transmission electron microscope

Figure 3 showed the photos from TEM for CNA and fCNA prepared at various 5-ALA concentrations. From this figure, it was found that the particle size increased with the increasing modified percentage of amino groups of chitosan, and these results conformed to the results of average particle size.

3.5 HT-29 colon cancer cells uptake test

The photo images of red fluorescence of PpIX excited by green laser under fluorescence microscope were showed in figure 4. From this figure, the chitosan with higher modified percentage loaded with 5-ALA could be up-taken by HT-29 cells likely. This result might be due to the fact that folic acid conjugated-chitosan molecules entered cancer

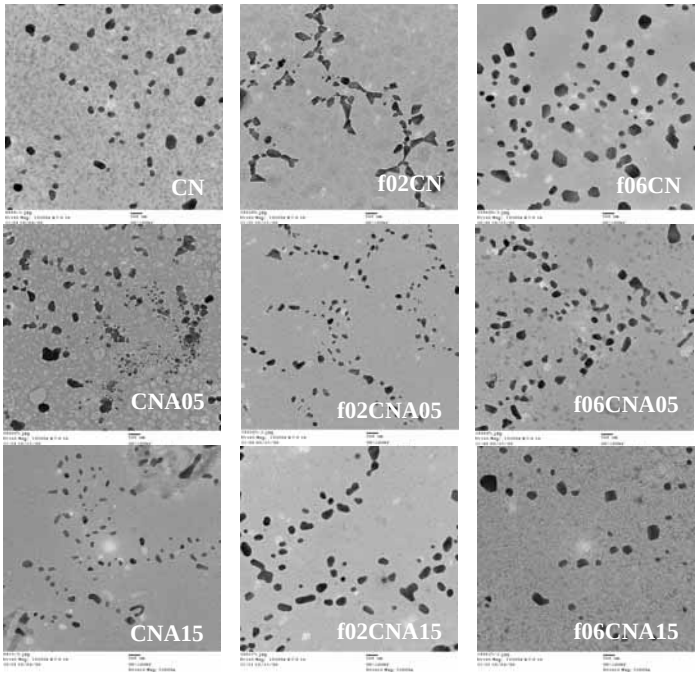

Figure 3. Photos from TEM for CNA and fCNA prepared at various 5-ALA concentrations. The scale bar in the picture is 500 nm.

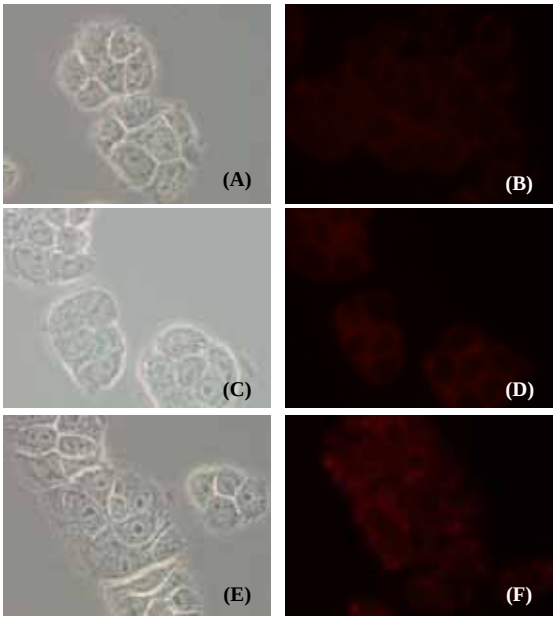

Figure 4. Red fluorescence of PpIX excited by green laser and observed under fluorescence microscope. (A) the bright field of HT-29 cells fed with CNA15, (B) the fluorescence picture of HT-29 cells fed with CNA15, (C) the bright field of HT-29 cells fed with f02CNA15, (D) the fluorescence picture of HT-29 cells fed with f02CNA15, (E) the bright field of HT-29 cells fed with f06CNA15 and (F) the fluorescence picture of HT-29 cells fed with f06CNA15.

cells effectively by receptor-mediated endocytosis and resulted in large production of PpIX .

4 CONCLUSSION

Via simple reaction, folic acid could be conjugated onto chitosan molecules by covalent bond and the modified percentage of amino groups in chitosan increased with the increasing ratio of folic acid to amino groups. By ionic gelation, chitosan nanoparticles or folic acid-conjugated chitosan nanoparticles were prepared and the average sizes were about 100 nm. The loading efficiency of 5-ALA in particle was about 80-90% and decreased with the increasing modified percentage of amino groups in chitosan. The results of HT-29 cells uptake test showed that the chitosan with higher modified percentage of amino groups was a suitable material as 5-ALA drug carrier.

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