

Colorectal Cancer Cell Detection by Chitosan Nanoparticles Conjugated with Folic Acid

Shu-Jyuan Yang, Jian-Wen Chen and Ming-Jium Shieh*

*Institute of Biomedical Engineering, National Taiwan University, Taipei, Taiwan

Abstract

Endoscopy is a common diagnosis device for the detection of colorectal cancer and there are two methods to improve the diagnostic sensitivity. One is chromoendoscopy, and the other is magnifying endoscopy. Color contrast dye, such as indigo carmine, usually is sprayed on the surface of intestine and can accumulate in the pits and crevices of the mucosa to enhance the identification of the lesion. But this color contrast dye does not have target-specific property. Due to the reason we modified chitosan conjugated with target-specific ligand, such as folic acid, and used this polymer to prepare chitosan nanoparticles (fCN) by ionic gelation method. Then fCN loaded with indigo carmine (fCNIC) by the same method. The size and zeta potential of fCN and fCNIC were measured by transmission electron microscope and zetasizer, respectively. fCN and fCNIC were both at the range of 120-140 nm in size, 20 mV in zeta potential. The loading efficiency of indigo carmine in fCNIC was in the range of 60%-70% depending on the concentration of acetic acid and conjugated ratio of folic acid. Bioassay results of fCNIC adhesion on HT-29 colon cancer cell revealed that the quantity of particles, which adhered onto cells increased with incubation time, and the higher conjugated ratio of folic acid particles had better adhesion effect. These results implied that fCNIC could serve as an ideal vector of colon-specific targeting system. According to this concept, we designed a novel detection system to enhance the accuracy of endoscopic diagnosis for colorectal cancer.

Keywords : chitosan, indigo carmine, folic acid, nanoparticle

1. Introduction

Colorectal cancer is the third common cancer in Taiwan and drives from colonic mucosal neoplastic lesions. Colonic mucosal neoplastic lesions are classified into two groups: polypoid and non-polypoid. Polypoid lesions are easy to detect and not invasive until they are rather large. But non-polypoid lesions can invade the muscularis mucosa and the submucosa even though they are quite small. Muto et al. reported that the non-polypoid lesions were the candidate precursors of colorectal cancer. In order to reduce colorectal cancer risk, detect the non-polypoid lesions early and removal of adenomaous polyps is an effective method [1-3].

Colonoscopy is simple and common method to view colonic imaging, but it is easy to miss the small and flat adenomas. However, these indistinguishable regions can be using a variety of color contrast or absorptive agent to enhance the detail, such as indigo carmine and methylene blue. Indigo carmine dye is commonly used for chromoendoscopy because it is cheap, not absorbed, non-toxicity, and easy to perform. Indigo carmine dye has been performed to emphasize epithelium in the oesophagus. But indigo carmine just only stains the surface of mucosa to emphasize the contrast of the gradation. However, It does not have the ability for targeting specific space. For this reason, we want to develop a carrier loaded with indigo carmine to accumulating in adenomaous polyps specifically and still possess the staining contrast [4].

Chitosan is a polysaccharide polymer, and it is a biocompatible, non-toxicity, biodegradable and antibacterial polymer. Chitosan is a suitable material for constructing nanoparticles because of its reactive amino groups, which make it more possible to chemically modify and conjugate with various ligands or antibodies.

Its cationic amino group can form complexes with negatively charge drug/DNA through a strong electrostatic interaction [5].

Nanoparticles composed of biodegradable polymers have attracted extensive attention because of their potential applications as a drug/DNA delivery carrier. In order to enhance the targeting ability and uptake of the nanoparticles into targeted cells, target-specific ligands, such as folic acid (FA), are used to conjugate to the surface of the polymer carrier. The folate receptor over-expresses on many human cancer cell surfaces, and the targeting ability of drug/DNA can be performed by producing the delivery carrier conjugated with folic acid [6].

The purpose of this study was to synthesis chitosan nanoparticles loaded with indigo carmine and to target to HT-29 cells. In order to improve the ability of nanoparticle carrier to recognize HT-29 cells, chitosan was conjugated with folic acid. The nanoparticles were prepared by ionic gelation method, and the particle size, zeta potential, and loading efficiency of indigo carmine in particles were investigated. The targeting efficiency of the particle against HT-29 was investigated *in vitro*.

2. MATERIALS AND METHODS

2-1. Materials

Chitosan was purchased from Fluka (USA). Indigo carmine was purchased from Aldrich (USA). Folic acid was purchased from TCI (Japan). They were all reagent grade and used without further purification.

2-2. 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 acetic 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. The chitosan was conjugated with different content of folic acid. The molar ratio of folic acid to amino groups were 0.0 (f0), 0.02(f02), 0.04(f04), and 0.06 (f06).

2-3. Preparation of chitosan nanoparticles

Chitosan conjugation with various content folic acid was dissolved into 0.01N and 0.02N acetic acid solution to the concentration of 0.5 mg/ml and pH 4.0. Indigo carmine powder was dissolved into 0.5 mg/ml STPP solution to different concentrations (0.25 mg/ml, 0.5 mg/ml and 0.75 mg/ml) and pH 7.4. Two ml of STPP solution and/or 2 ml of indigo carmine 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, CNIC and fCNIC. The prepared CN, fCN, CNIC and fCNIC suspended in the solution would be directly used in later experiment without further treatment.

2-4. Average particle size and zeta potential

The average particle size and the zeta potential of chitosan nanoparticles were determined by Zetasizer (Malvern Zetasizer 3000 HS). All measurements were performed at 25°C at scattering angle of 90°.

2-5. Loading efficiency of indigo carmine in nanoparticles

100µL NaOH (0.1N) was added into 1ml chitosan nanoparticles solution and vortexed for 1 minute. Then the mixed solution was centrifuged at 14000rpm for 20 minute and read the OD value at 606nm by the UV/Vis spectrophotometer (Cary 50 Conc. Varian, USA). The loading efficiency of indigo carmine 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 indigo carmine and free amount of indigo carmine, respectively.

2-6. Adhesion of nanoparticles on cells

HT-29 cells were seeded on 6-well culture plates. After HT-29 cells cultured for 3 days, the medium was replaced by fresh medium with CNIG or fCNIG addition. HT-29 cells were further cultured for 1 and 3 hours to allow nanoparticles adhere to cells surface. Then the cells were rinsed with PBS and added dimethylsulfoxide (DMSO) 250 μ L to dissolve. And the OD value of mixture was read by an ELISA reader at 618nm.

3. RESULTS AND DISCUSSION

3-1. Average particle size and zeta potential

As the results showed in figure 1 and figure 2, the average particle size increased with the rise of conjugated ratio of folic acid and the concentration of indigo carmine loaded in nanoparticles whatever the concentration of acetic acid was used is 0.01N or 0.02N. When the concentration of indigo carmine was up-to 0.75 mg/ml, the particle size was bigger than the particles prepared in other conditions and the particles were unstable. When the concentration of indigo carmine was 0.25 and 0.5 mg/ml, the particle size was in the range of 100 to 200 nm.

The zeta potential of chitosan nanoparticle was about 20mV (table 1 and 2), and the tendency of the zeta potential value decreases gradually with the rise of conjugated ratio of folic acid. Conjugated with folic acid would consume some amino groups of chitosan and resulted in lower zeta potential.

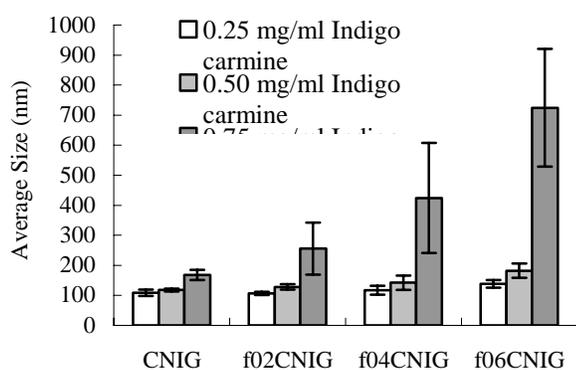


Figure 1. The average particle size of CNIG, f02CNIG, f04CNIG, and f06CNIG loaded with different

concentration of indigo carmine and prepared in 0.01N acetic acid. (n=3)

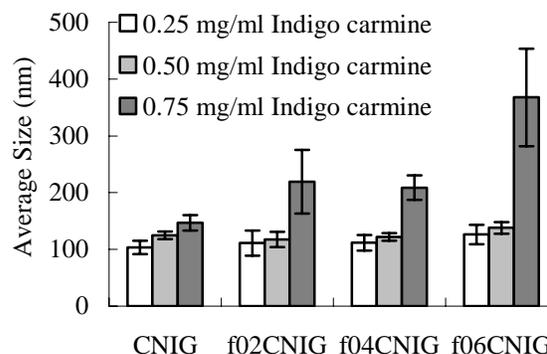


Figure 2. The average particle size of CNIG, f02CNIG, f04CNIG, and f06CNIG loaded with different concentration of indigo carmine and prepared in 0.02N acetic acid. (n=3)

Sample	The concentration of indigo carmine (mg/ml)		
	0.25	0.5	0.75
CNPIG	23.6±0.88	22.6±0.95	21.7±0.62
f02CNIG	21.7±0.78	21.6±0.59	20.8±0.46
f04CNIG	21.7±0.94	20.7±0.36	20.3±0.45
f06CNIG	19.9±0.92	20.0±0.57	19.6±0.6

Table 1. The zeta potential of various chitosan nanoparticles loaded with indigo carmine and prepared in 0.01N acetic acid. (n=3)

Sample	The concentration of indigo carmine (mg/ml)		
	0.25	0.5	0.75
CNPIG	23.3±0.72	22.8±0.86	22.1±0.45
f02CNIG	21.2±0.45	21.2±0.26	20.9±0.56
f04CNIG	21.5±0.17	20.9±0.45	20.9±0.55
f06CNIG	20.9±0.21	20.0±0.55	20.5±0.9

Table 2. The zeta potential of various chitosan nanoparticles loaded with indigo carmine and prepared in 0.02N acetic acid. (n=3)

3-2. Loading efficiency of indigo carmine in nanoparticles

The loading efficiency of indigo carmine in chitosan nanoparticle was below 10 % and decreased when the increasing the concentration of indigo carmine when the concentration of acetic acid was 0.01N (data not

show). However, when the acetic acid concentration was 0.02N, the loading efficiency of indigo carmine could be high to the range of 60 to 70 % (figure 3). Because the chitosan molecules were more stable in acidic solution and carried much positive electricity, the high concentration of acetic acid would let the change of environment pH value be small, and the protonated chitosan molecules could encapsulate much indigo carmine.

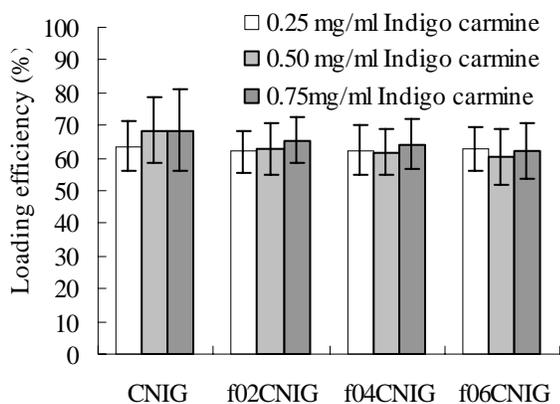


Figure 3. The loading efficiency of indigo carmine in chitosan nanoparticles was prepared in 0.02N acetic acid.

3-3. Adhesion of nanoparticles on cells

The adhesion of nanoparticles on cells was investigated by HT-29 cells, which were incubated in the DMEM medium with CNIG, f02CNIG, f04CNIG, and f06CNIG at 37 °C for 3 hours. As the results showed in figure 4, the content of f06CNIG adhered on HT-29 cells was higher than other chitosan nanoparticles after 3 hours. It exhibited that CNIG conjugated with more folic acid (f06CNIG) had a higher affinity to the folate receptors on HT-29 cell surface.

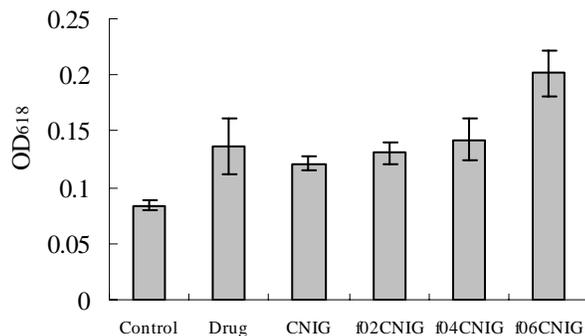


Figure 4. The OD value of indigo carmine adhered on HT-29 cell surface after incubating with CNIG, f02CNIG,

f04CNIG and f06CNIG for 3 hour.

4. Conclusion

In this study, we established a new platform to provide an opportunity to diagnose colorectal cancer through endoscopy. We used chitosan conjugated with folic acid to prepare chitosan nanoparticles which encapsulated with the indigo carmine dye. The average particle size of nanoparticles was between 120nm and 140nm and it is small enough to be uptaken by cancer cell via folate receptor-mediated endocytosis. The zeta potential was about 20mV and the loading efficiency was about 60%. And from adhesion of nanoparticle test, it was found that the f06CNIG had a higher affinity to HT-29 cells and it would be as a novel carrier for specific targeting delivery. Further studies such as tissues test or animal model need to be carried out to prove further targeting efficiency *in vivo*.

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