



Colorectal cancer cell detection by 5-aminolaevulinic acid-loaded chitosan nano-particles

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ABSTRACT

Colorectal cancer is one of the leading causes of malignant death in Taiwan because it often remains undetected until later stages of the disease. In this study, we designed an oral form nano-particle to encapsulate 5-aminolaevulinic acid (5-ALA) to improve the detection of colorectal cancer cells *in vivo*. The nano-particle should escape from bacteria uptake in the gastrointestinal tract which seriously interferes the results of endoscopic observation. In this study, chitosan was mixed with sodium tripolyphosphate (STPP) and 5-ALA to prepare chitosan nano-particles (CN) and 5-ALA loaded chitosan nano-particles (CNA) by adding different pH values and concentrations of 5-ALA solution. The average particle size and zeta-potential of CN and CNA were measured by the Zetasizer-3000. The results revealed that particle size with different zeta-potential could be manipulated just by 5-ALA concentrations and pH values. CNA particles prepared at pH 7.4 and pH 9 of 5-ALA solutions with a concentration higher than 0.5 mg/ml showed a promising loading efficiency of up to 75% and an optimum average particle size of 100 nm. The zeta-potential for CNA was over 30 mV that kept the nano-particle stable without aggregation when stored in suspension solution. Fluorescence microscope examination showed that CNA could be engulfed by Caco-2 colon cancer cells but showed no evidence of being taken up by *Escherichia coli*. This result implies that CNA could exclude the influence of normal flora inside the gut and serves as an adequate tool for fluorescent endoscopic detection of colorectal cancer cells *in vivo*.

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1. Introduction

The incidence of colorectal cancer is increasing in the world, and the prognosis remains poor [1]. In general, survival depends on the degree to which the cancer has spread; the relative survival of colorectal cancer is 50% at

3 years and 40% after 5 years according to statistical inferences that have remained unchanged since the 1960s [2]. If the malignancy can be detected at an early stage, the prognosis is excellent. But small neoplasia or early cancer of the colon are frequently missed during endoscopy, hence a powerful and highly-sensitive tool for the detection of pre-cancerous lesions becomes very important. In this study, we designed a nano-particle to encapsulate 5-ALA for oral administration to detect colorectal cancer cells at an early stage. The nano-particle should escape from bacterial uptake in the gastrointestinal (GI) track that would seriously interfere with the detection results.

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Photo-diagnosis is one of the most promising and non-invasive methods used nowadays to detect malignant or premalignant tissue [3]. To help detection and painting of abnormal tissue regions it usually employs an exogenous chromophore excited by optima light to generate fluorescence to detect the cancer lesions [4].

In the human body, 5-ALA is a precursor during heme group synthesis and it is totally degraded in the cells and converted afterwards to protoporphyrin IX (PpIX). PpIX has different decomposition rates in cancer cells and normal cells, and is therefore one of the photosensitive fluorophores which can be used as a source of fluorescence to detect cancer lesions [5,6]. PpIX in normal cells always maintains a constant level that will be quickly degraded once over the equilibrium level. However, the process or feedback mechanism does not function well in cancer cells, causing PpIX to be degraded at a slow pace; it accumulates to a very high concentration in the cell body. In addition, ferrochelatase, one of the major enzymes to convert PpIX into heme, maintains low activity in cancer cells due to its limited availability of iron that partially contributes to the high concentration of PpIX in those abnormal cells [7]. Generally, PpIX can be totally degraded within 2–4 h in normal cells but 12–24 h in cancer cells [8]. 5-ALA is a hydrophilic and zwitterionic drug that has a poor affinity toward the cell membrane in a physiological environment. In addition, 5-ALA is very easily engulfed by the bacteria in the GI track that leads to misinterpretation of endoscopic observation [9]. Therefore, a carrier is needed to prevent 5-ALA from bacterial uptake and to help it pass through the lipophilic barrier to the cell mitochondria to convert into PpIX as the source for photo-diagnosis [10].

Chitosan is the polymer of 2-amino-2-deoxy- β -D-glucan by glycosidic linkages. The primary amino groups on the molecular chain of chitosan have special properties and make chitosan more useful in pharmaceutical applications. Compared with other biological polymers, chitosan bears positive charges to approach the cell membrane more conveniently and has muco-adhesive properties to prolong the retention time of chitosan in the interested locations [11–14]. Additionally, chitosan is biocompatible and induces no allergic reactions or immuno-rejections. It can be utilized as an adsorbent for toxic metals such as mercury, cadmium, lead, etc. and possesses anti-bacterial properties, which can prevent it from bacterial uptake in the GI track [15]. Furthermore, chitosan is a linear polyamine containing a number of free amino groups which are available for cross-linkages, and its cationic nature also allows for ionic cross-linking with multivalent anions, such as STPP [16]. All the nano-particle preparation and encapsulation can be manipulated in aqueous solution without toxic organic solvents involved.

In this study, the chitosan nano-particles (CN) and chitosan nano-particles loaded with 5-ALA (CNA) were prepared under different conditions by the ionic gelation method. The developed CN and CNA were characterized using the Zetasizer and transmission electron microscope (TEM). The loading efficiency of 5-ALA in CNA was measured by UV-Vis spectrometer and CNA was cultured with *Escherichia coli* to evaluate whether it could escape from the bacterial uptake in the GI track. The content of PpIX

in colorectal cancer cells was also investigated in this study.

2. Materials and methods

2.1. Materials

Chitosan (80% deacetylation and M.W. 10 kDa) and STPP were purchased from Wako Pure Chemical Industries (Japan). 5-ALA was purchased from Fluka (USA). They were all reagent grade and used without further purification.

2.2. Preparation of CN and CNA

The preparation of chitosan nano-particles loaded with 5-ALA was based on the ionic gelation interaction between positively charged chitosan and negatively charged STPP at room temperature [17]. Ionized 5-ALA with negative charge also partially contributed to the gelation interactions in this study. The preparation process is briefly described as follows: 0.05% chitosan solution was prepared by dissolving 0.5 mg chitosan powder in 1 ml 0.01 M acetic acid at pH 4.0. 1% 5-ALA solution was prepared as a stock solution by dissolving 10 mg 5-ALA powder in 1 ml 0.05% STPP solution, where STPP were dissolved in phosphate buffered saline (PBS) beforehand. As known, 5-ALA is a hydrophilic and zwitterionic drug. The loading efficiency of 5-ALA in nano-particles might be altered by changing the pH value of 5-ALA solution so that the pH value of 5-ALA solution during the preparation should play an important factor in loading efficiency. The 1% 5-ALA solution was further diluted to the following concentrations: 0.1, 0.5, 1.0, 1.5 and 2.0 mg/ml by 0.05% STPP solution and adjusted the pH value by NaOH to 5.0, 6.0, 7.4, 9.0 and 10.0, respectively. Two milliliter of STPP solution or 2 ml of 5-ALA solution with different pH values were added to 5 ml of chitosan solution with a peristaltic pump at a flow rate of 0.5 ml/min to prepare the CN and CNA. The final pH values of CN and CNA suspension solutions might change with the pH value of 5-ALA solution addition, but they were all lower than the pK_a of chitosan (Table 1) [18,19]. The prepared CN and CNA suspended in the solution were later used directly without further treatment.

2.3. Particle size and the zeta-potential of CN and CNA

Measurements of particle size and zeta-potential of the nano-particles were assessed on the Zetasizer-3000 (Mal-

Table 1

The final pH values of the CN and CNA suspensions those were determined by adding different 5-ALA concentrations at different pH values into chitosan solution

5-ALA concentration (mg/ml)	pH value				
	5	6	7.4	9	10
0.0	4.11	4.29	4.77	4.90	4.95
0.1	4.14	4.33	4.75	4.96	5.02
0.5	4.18	4.34	4.78	5.09	5.21
1.0	4.27	4.46	4.78	5.24	5.54
1.5	4.30	4.45	4.86	5.57	6.01
2.0	4.31	4.48	4.88	5.72	6.43

vern Instruments) by dynamic light scattering measurements and laser Doppler electrophoresis, respectively. The particle size was measured for 15 min at 25 °C with a 90° scattering angle. The cumulative curve was used to present the mean hydrodynamic diameter. The measurements of zeta-potential were made using the aqueous flow cell in the automatic mode at 25 °C [20]. The measurement of the average particle size was automatically repeated for three times based on the Zetasizer-3000 internal setting; and the zeta-potential of the prepared nano-particle was examined for five times.

2.4. The loading efficiency of 5-ALA in CNA

Loading efficiency of 5-ALA in nano-particles was evaluated by adding 0.1 N NaOH solution into 1 ml of CNA solution and then it was vortexed for 60 s. The mixed solution was centrifuged at a 10,000g force to spin down the particles. The suspension was collected and then added with 2,4,6-trinitrobenzene sulfonic acid (TNBS) as the assay reagent to detect 5-ALA in the suspensions. TNBS assay was performed by using a TNBS kit (Pierce) according to the manufacturer's instructions. In the study, 5-ALA powder was dissolved in reaction buffer (0.1 M sodium bicarbonate, pH 8.5) and diluted to a serial of gradient 5-ALA standard solutions that were used to prepare the calibration curve in the range of 2–20 mg/ml for further analysis. The loading efficiency of 5-ALA in the nano-particles was calculated with the following equation:

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

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

2.5. Transmission electron microscope examination

Carbon coated 200 mesh copper grids were immersed in CN and CNA solutions. The grids were placed on delicate-task-wipers to absorb excess liquid and were dried in a desiccator overnight. The dried copper grids with CN and CNA were examined under the Hitachi TEM H-7500.

2.6. Measurement of PpIX converted from 5-ALA

Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplement with 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 °C and in an atmosphere of 5% CO₂. Cultured medium was changed on alternate days until confluent.

Caco-2 cells (3×10^4) were seeded on 24-well culture plates. After the Caco-2 cells were cultured for 24 h, the medium was replaced with fresh medium with CNA added. CNA solution was concentrated to 10 times by Amicon Ultra (Centrifugal Filter Devices, MILLIPORE) to remove the unloaded 5-ALA beforehand. The Caco-2 cells were further cultured for 24 h to allow the cells facilitation of uptake of nano-particles and to convert 5-ALA to PpIX. In order to determine the effect of serum in the culture medium on the endogenously synthesized PpIX, fresh culture media, both containing 10% (v/v) fetal bovine serum or without, were tested at the same time. After the cultured medium was removed, the plates were washed with PBS three

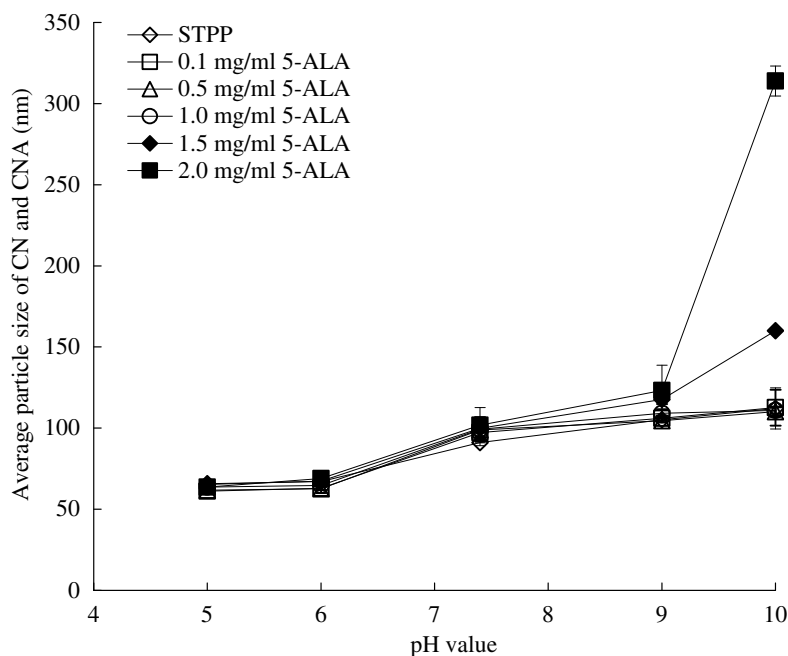


Fig. 1. The average particle size of the CN and CNA those were prepared by adding different 5-ALA concentrations at different pH values into chitosan solution.

times. The PpIX were extracted by using ethyl acetate/acetic acid (3/1 by volume) [10] and the fluorescent intensity of PpIX was determined by the spectrofluorometer (Molecular Device SPECTRA MAX GEMINE XS) with the excitation wavelength of 405 nm and the emission wavelength at 635 nm. The fluorescent intensity was expressed as accumulative percentage.

2.7. Caco-2 cell and human foreskin fibroblast (HFF) uptake test

The glass plate was placed in a Petri dish and seeded with Caco-2 or HFF cells [21]. After Caco-2 or HFF cells were cultured for 24 h, the medium was replaced by fresh medium with 5-ALA or CNA added. Caco-2 or HFF cells were further cultured for 24 h to allow the cells to uptake nano-particles and to convert 5-ALA to PpIX. After the cultured medium was removed, the glass plate was washed with PBS three times. The Caco-2 or HFF cells were fixed by 10% formalin and then examined under a fluorescence microscope in which a green laser was used to excite PpIX to emit red fluorescence.

Table 2

Zeta-potential (mV) of CN and CNA those were prepared by adding different pH values and 5-ALA concentrations into chitosan solution

5-ALA concentration (mg/ml)	pH value				
	5	6	7.4	9	10
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
2.0	40.7	40.9	43.4	28.7	11.1

2.8. Microbiology assay

One *E. coli* colony grown on an agar plate (Difco, Detroit, MI) was transferred to nutrient broth (Difco) at pH 6.5 to a final volume of 25 ml and incubated at 37 °C with aeration for 24 h. 5-ALA and CNA solutions were added and incubated for further 16 h at 37 °C with aeration. The incubated solution was diluted by 10 times with fresh nutrient broth, and the optical density (OD) of *E. coli* at 600 nm was measured by UV-Vis spectrometer to determine the anti-bacterial property of CNA. Furthermore, 50 μ l of the incubated solution containing pure 5-ALA or CNA was spread on the glass plate and then covered with glass for later examination under the fluorescence microscope.

2.9. Statistical analysis

Mean, standard deviation and graphs were used to describe the data. One-way analysis of variance (ANOVA) was made to assess the differences in the fluorescent intensity of PpIX while Caco-2 cells fed with 5-ALA and

Table 3

The abbreviation of developed nano-particles prepared by adding different 5-ALA concentrations at pH 7.4 and 9

5-ALA concentration (mg/ml)	pH value	
	7.4	9
0.0	CN-7.4	CN-9
0.1	CNA01-7.4	CNA0-9
0.5	CNA05-7.4	CNA05-9
1.0	CNA10-7.4	CNA10-9
1.5	CNA15-7.4	CNA15-9
2.0	CNA20-7.4	CNA20-9

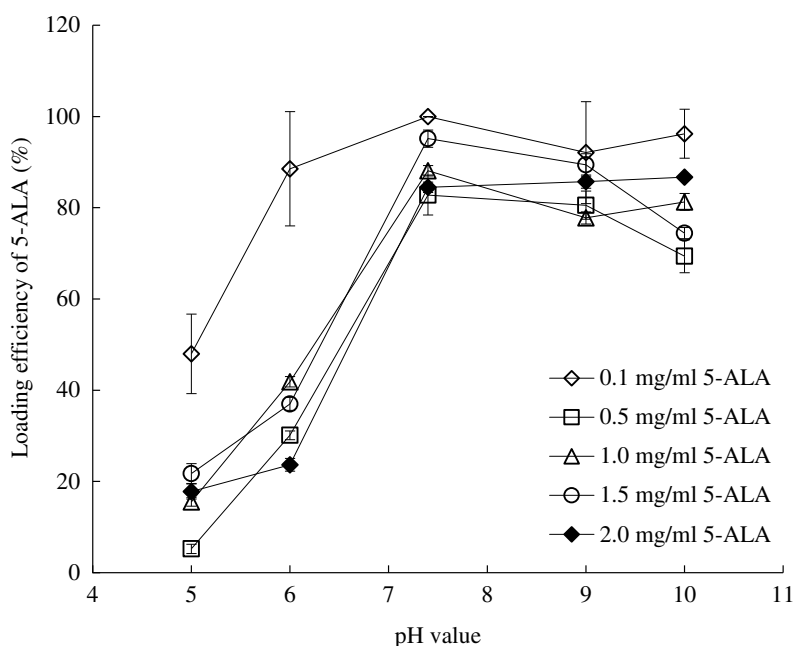


Fig. 2. The loading efficiency of 5-ALA in CNA those were fabricated by adding different pH values and concentrations of 5-ALA solution into chitosan solution.

CNA for 24 h and to determine the differences in the anti-bacterial property while *E. coli*. fed with 5-ALA and CNA for 24 h. All *p* values were two-sided and their significant level was 0.05. SPSS 11.0 was used to conduct all statistical analysis.

3. Results

3.1. Particle size and the zeta-potential of CN and CNA

Fig. 1 shows the average particle size of CN and CNA those were prepared by adding different pH values and concentrations of 5-ALA solutions into chitosan solution. The average particle size of the prepared nano-particle showed no significant difference (about 60 nm) when the pH value of 5-ALA solution was lower than 6. The average particle size was increased with the increment of pH value once pH value over 6. If the pH value was 7.4, the average particle size of the developed nano-particle was about 70–110 nm based on the 5-ALA concentrations. The average particle size showed no statistical difference when the nano-particle was prepared at the 5-ALA concentration in the range of 0.1–1 mg/ml. If the 5-ALA concentration in the solution was over 1.5 mg/ml, the particle size sharply increased especially at high pH conditions. When added 1.5 mg/ml 5-ALA solution with pH value 10 into chitosan solution, the particle size was larger than 160 nm. The particle size was even over 300 nm when added 2 mg/ml 5-ALA solution at pH 10 into chitosan solution.

Table 2 shows the zeta-potential of CN and CNA those were prepared by adding different pH values and concentrations of 5-ALA solutions into chitosan solution. From the results of the zeta-potential analysis, CN and CNA bore a positive charge. When the pH of STPP solution was lower than 7.4, the zeta-potential of CN was kept at the same level in the range of 43–47 mV but it decreased very much once the pH went over 9. If the CN was loaded with 5-ALA, as so-called CNA, the zeta-potential of the CNA decreased with increasing the pH value of 5-ALA solution. The zeta-potential of all the CNA particles prepared at different 5-ALA concentrations was over 30 mV when the pH value of 5-ALA solution was lower than 7.4. It was significantly decreased when the pH was at 9 and 10. The zeta-potential of CNA was even down to 16 and 11 when added 1.5 and 2.0 mg/ml 5-ALA solutions at pH 10 into chitosan solution, respectively.

3.2. The loading efficiency of 5-ALA in CNA

The loading efficiency of 5-ALA in CNA fabricated by adding different pH values and concentrations of 5-ALA solution into chitosan solution were shown in Fig. 2. The loading efficiency of CNA increased with increasing the pH value of 5-ALA solution until pH 7.4 when it reached a plateau. There was no significant difference in loading efficiency when CNA was prepared by adding 5-ALA solutions at a pH value higher than 7.4 into chitosan solution. The loading efficiency was over 75% when the pH was higher than 7.4. When the CNA was prepared at different 5-ALA concentrations, it showed no statistical differences but was slightly increased at the concentration of 0.1 mg/ml. Table 3 summarizes the abbreviations for all the nano-particles prepared at different concentrations and pH values of 5-ALA solution that are relevant in the later analysis and results.

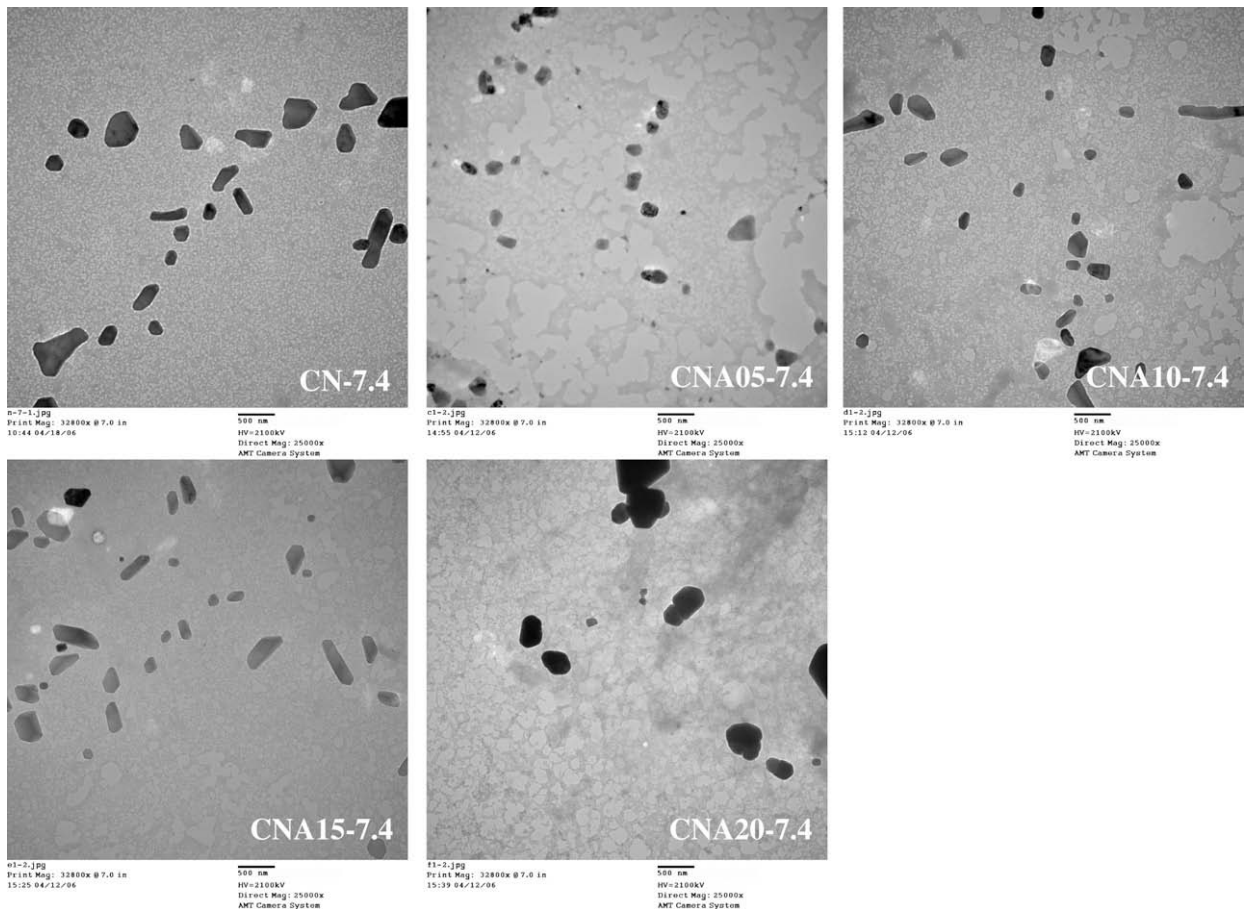


Fig. 3. Photos from TEM for CN and CNA prepared by adding different 5-ALA concentrations with pH 7.4 into chitosan solution. The scale bar in the picture is 500 nm.

3.3. Transmission electron microscope examination

Fig. 3 shows the particle size of CN and CNA prepared by adding different 5-ALA concentrations with pH 7.4 into chitosan solution. The particle size was all in the range of 60–400 nm and independent of the 5-ALA concentrations. Fig. 4 shows the particle size of CN and CNA prepared by adding different 5-ALA concentrations with pH 9 into chitosan solution. The particle size was also in the same range as that prepared at pH 7.4 of 5-ALA solution and showed no correlation with the 5-ALA concentrations. The results are in agreement with the results measured by dynamic light scattering measurements using the Zetasizer-3000 (this data is not shown).

3.4. Measurement of PpIX converted from 5-ALA

Fig. 5 shows the conversion ratio of 5-ALA to PpIX in the Caco-2 cell with a 10% serum supplement. The positive control and negative control in Fig. 5 were the groups with pure 5-ALA feeding and without 5-ALA feeding in the culture medium, respectively. The brightness of the emission light in the positive group was designated as 100%. The relative intensity of the emission light was about 19% in the negative control due to cell self-fluorescence. If fed with CNA15-7.4, the Caco-2 cell showed a very bright red emission light that was higher than that of the positive group. However, the brightness decreased when CNA was prepared at a 5-ALA concentration of 2 mg/ml. When the CNA was prepared by adding pH 9 of 5-ALA solution into chitosan solution and then fed to the Caco-2 cell, the brightness was all lower than that of the positive group. Especially in those groups of CNA prepared in high 5-ALA concentrations, the brightness of the emission light was very close to the negative control.

Fig. 6 shows the conversion ratio of 5-ALA to PpIX in the Caco-2 cell without the serum supplement. If fed with CAN-7.4 prepared with different 5-ALA concentrations, there were no significant differences for the intensity of the red emission light in Caco-2 cells but it was much higher than that of the negative control. When the CAN-9 was prepared with different 5-ALA concentrations and then fed to the Caco-2 cell, the brightness was all lower than that of the positive group but much higher than those of CNA prepared at pH 7.4.

3.5. Caco-2 cells uptake test and microbiology assay

Fig. 7(a) shows the picture that is related with the 5-ALA engulfed by the Caco-2 cell. The 5-ALA was engulfed by Caco-2 cells and converted into PpIX that would be excited by a green laser and emitted red light (Fig. 7(b)). Fig. 7(c) is the picture related with 5-ALA engulfed by *E. coli*. Fig. 7(d) shows a strong emission light due to 5-ALA highly up taken by *E. coli*. That might lead to misinterpretation of endoscopic examination in clinical. Fig. 7(e) is the photo from the fluorescence microscope related with the 5-ALA engulfed by the HFF cell. The red emission light could not be traced under the fluorescence microscope (Fig. 7(f)) because PpIX would be quickly degraded in the normal cell, generally no more than 4 h.

Fig. 8(a) is the photo from the fluorescence microscope related with the CNA engulfed by the Caco-2 cell. The 5-ALA in the CNA was released in Caco-2 cell lysosomes and then converted into PpIX that would be excited by a green laser and emitted red light (Fig. 8(b)). Fig. 8(c) is the picture related with CNA engulfed by *E. coli*. The picture shows no red light emitted due to CNA not staying in the *E. coli*. (Fig. 8(d)). Fig. 8(e) is the photo from the fluorescence microscope related to the CNA engulfed by the HFF cell. The picture shows no observable red light emitted due to

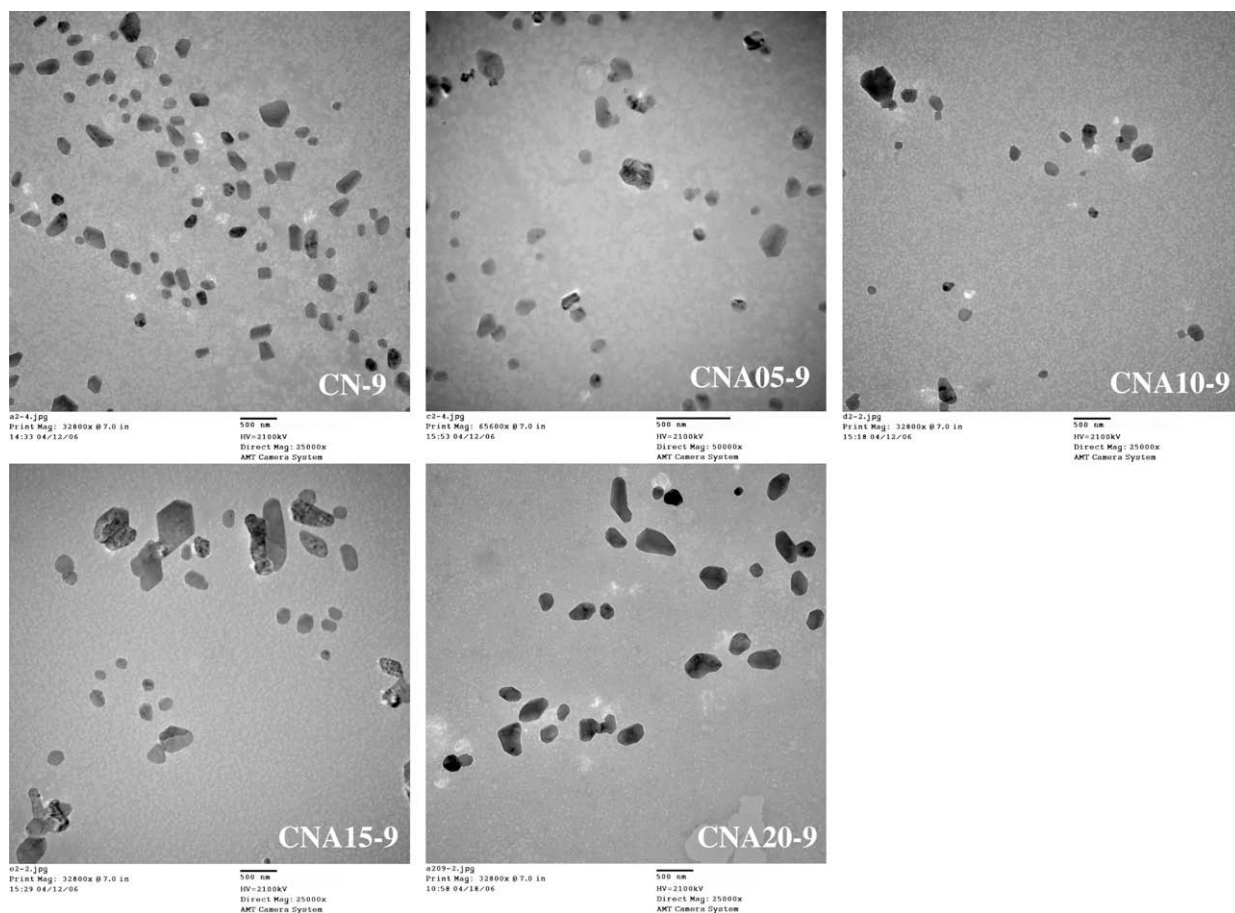


Fig. 4. Photos from TEM for CN and CNA prepared by adding different 5-ALA concentrations with pH 9 into chitosan solution. The scale bar in the picture is 500 nm.

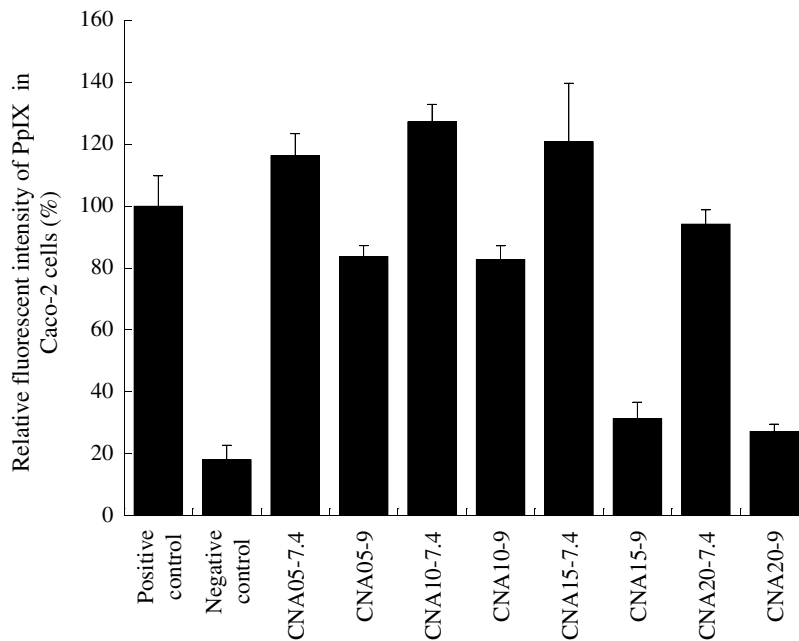


Fig. 5. The relative fluorescent intensity of PpIX in Caco-2 cells cultured in a medium with 10% serum addition. The positive control and negative control were the groups with pure 5-ALA feeding and without 5-ALA feeding in the culture medium, respectively.

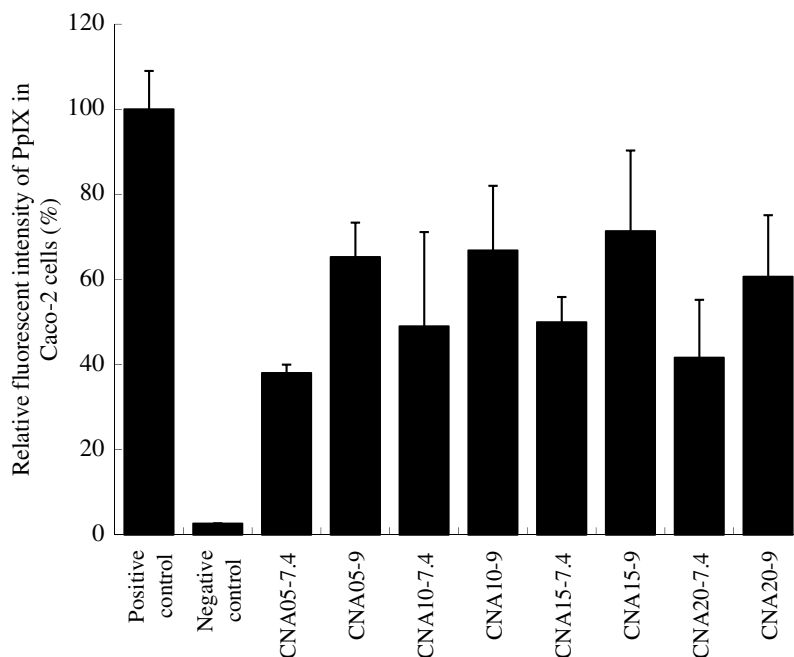


Fig. 6. The relative fluorescent intensity of PpIX in Caco-2 cells cultured in the medium without serum addition. The positive control and negative control were the groups with pure 5-ALA feeding and without 5-ALA feeding in the culture medium, respectively.

the quick degradation rate of PpIX in the normal cell (Fig. 8(f)). We observed in the two pictures that CNA would be engulfed by Caco-2 and HFF cells and that this was not possible for *E. coli*.

Fig. 9 shows the OD_{600nm} of *E. coli* solution fed with pure 5-ALA and CNA. When added with 5-ALA only, the intensity of OD_{600nm} of *E. coli* solution was higher than that of control group. However, when *E. coli* was incubated with nutrient broth containing CNA, the OD_{600nm} intensity was much lower than that of control and 5-ALA groups.

4. Discussion

In this study, we produced the nano-particle by ionic gelation methods. Chitosan was the cationic polymer due to its bearing with lots of the free amino groups that could be quickly gelled once it had contact with polyanions [22]. The average size of the CN and CNA strongly depended on

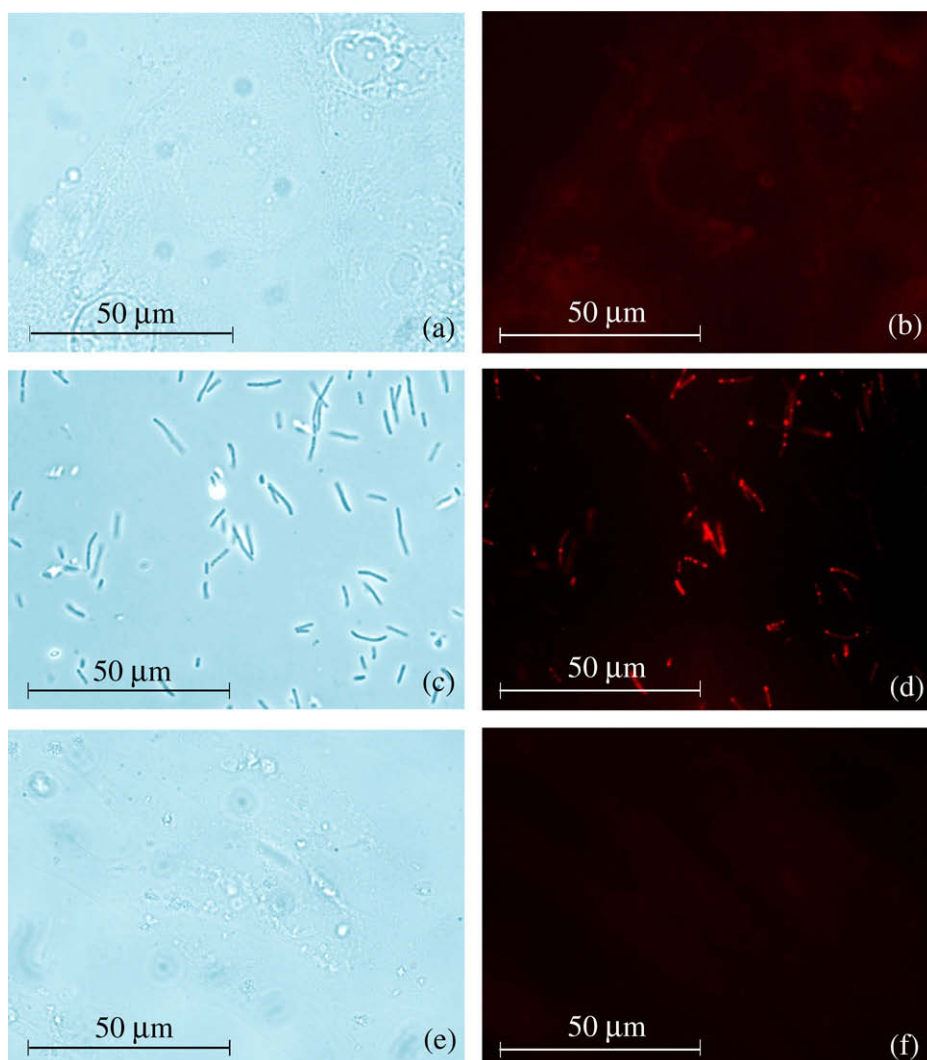


Fig. 7. Red fluorescence of PpIX excited by a green laser and observed under fluorescence microscope. (a) the bright field of Caco-2 cells fed with 5-ALA, (b) the fluorescence picture of Caco-2 cells fed with 5-ALA, (c) the bright field of *E. coli* fed with 5-ALA, (d) the fluorescence picture of *E. coli* fed with 5-ALA, (e) the bright field of HFF cells fed with 5-ALA, (f) the fluorescence picture of HFF cells fed with 5-ALA.

the pH value of STPP solution or 5-ALA solution [23]. Zeta-potential can greatly influence particle stability through the electrostatic repulsion between particles. The greater the zeta-potential resulted in the more stable for a nano-particle in the solution because the charged particles repel one another and therefore overcome the natural tendency to aggregate [24].

As shown in Table 2, the zeta-potential of all the CNA particles prepared at the pH of 5-ALA solution lower than 7.4 was over 30 mV. This provided enough surface charge to stabilize the particles from aggregation while it was significantly decreased when CNA was prepared at a pH of 5-ALA solutions of 9 and 10. The zeta-potential of CNA was even down to 16 and 11 when prepared at pH 10 of 1.5 and 2 mg/ml of 5-ALA concentration, respectively. These results are consistent with the TEM observations (Fig. 3 and Fig. 4). The particle size measured by the Zetasizer-3000 was as

high as 160 and 303 nm when prepared at 1.5 and 2 mg/ml of the 5-ALA concentration at pH 10, respectively, due to the low zeta-potential for aggregation (Fig. 1).

Particle size has been reported to be one of the decisive factors in mucosal and epithelial tissue uptake of nano-particles [22]. The smaller size of nano-particles (about 100 nm) displayed more than a threefold greater uptake compared to larger nano-particles (about 275 nm) and was able to penetrate throughout the sub-mucosal layers for cell uptake [22]. The particle size distribution of nano-particles prepared at pH 7.4 and pH 9 of 5-ALA solutions with a concentration higher than 0.5 mg/ml was in the range of 70–110 nm, which was an optimum condition to prepare the nano-particles for fluorescent endoscopic detection of colorectal cancer in the future.

When CNA was prepared at a pH of 5-ALA solution lower than 7.4, the loading efficiency of 5-ALA in CNA was less

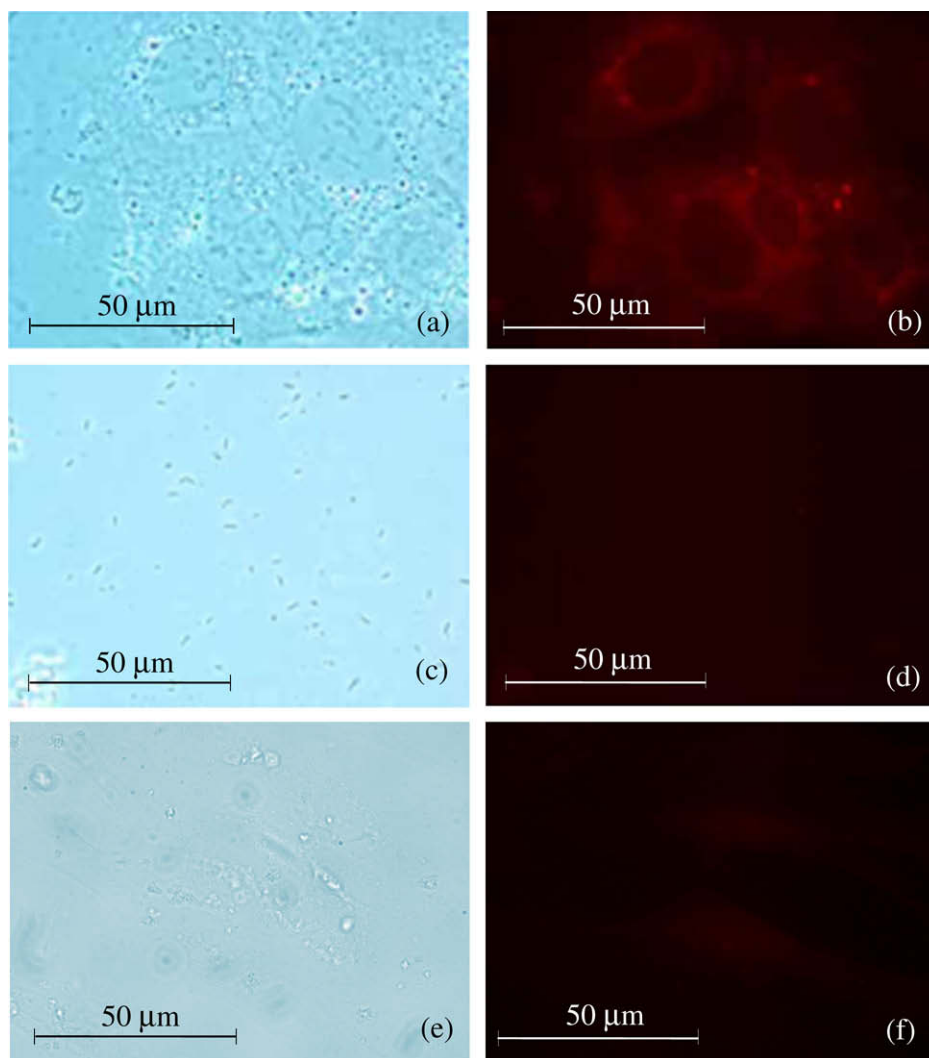


Fig. 8. Red fluorescence of PpIX excited by a green laser and observed under fluorescence microscope. (a) the bright field of Caco-2 cells fed with CNA, (b) the fluorescence picture of Caco-2 cells fed with CNA, (c) the bright field of *E. coli* fed with CNA, (d) the fluorescence picture of *E. coli* fed with CNA, (e) the bright field of HFF cells fed with CNA, (f) the fluorescence picture of HFF cells fed with CNA.

than 40% (Fig. 2). But the loading efficiency was over 80% once the pH of 5-ALA solution was higher than 7.4. These results might be due to the zwitterionic property of 5-ALA. It had two pK_a values at 4.05 and 8.90. According to the Henderson–Hasselbalch equation:

$$pH = pK_a + \log(\text{base/acid})$$

When CNA was prepared by adding a pH of 5 and 6 of 5-ALA solution, the ratio of $[\text{H}_2\text{NCH}_2\text{COCH}_2\text{CH}_2\text{COO}^-]$ to $[\text{H}_2\text{NCH}_2\text{COCH}_2\text{CH}_2\text{COOH}]$ in the reaction mixture was about 1.78 and 2.57, respectively, for the negative charge. The ratio of $[\text{H}_3\text{NCH}_2\text{COCH}_2\text{CH}_2\text{COOH}]$ to $[\text{H}_2\text{NCH}_2\text{COCH}_2\text{CH}_2\text{COOH}]$ in the reaction mixture when 5-ALA solution at pH 5 or pH 6 was added in chitosan solution to prepare CNA was about 3.98×10^4 and 2.75×10^4 , respectively, for the positive charge. From these ratios, most of the 5-ALA molecules in the prepared suspension solution contained

lots of positive charges and reduced the electrostatic attraction between 5-ALA and chitosan that led to a low loading efficiency. When CNA was prepared by adding 5-ALA solution with pH 7.4, 9.0 and 10.0 into chitosan solution, the ratio of $[\text{H}_2\text{NCH}_2\text{COCH}_2\text{CH}_2\text{COO}^-]$ to $[\text{H}_2\text{NCH}_2\text{COCH}_2\text{CH}_2\text{COOH}]$ in the mixture solution was about 5.62, 28.18 and 89.13, respectively, for the negative charge; and the ratio of $[\text{H}_3\text{NCH}_2\text{COCH}_2\text{CH}_2\text{COOH}]$ to $[\text{H}_2\text{NCH}_2\text{COCH}_2\text{CH}_2\text{COOH}]$ was about 1.26×10^4 , 2.51×10^3 , 794.33 for the positive charge, respectively. In these conditions, there were more 5-ALA molecules bearing the negative charge that should have strong electrostatic attraction between the two molecules and achieved high loading efficiency. Moreover, when CNA was prepared at a pH of 5-ALA solution higher than 7.4, excluding 5-ALA molecule itself contained negative charge, the part of positive charge of 5-ALA could interact with chitosan via STPP to achieve high loading efficiency. The loading efficiency was inde-

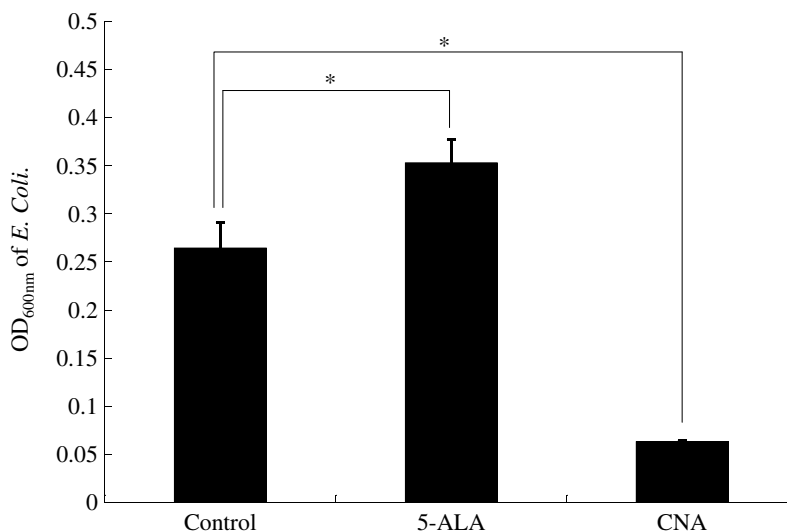


Fig. 9. The OD_{600nm} of *E. coli* solution fed with pure 5-ALA and CNA. * $p < 0.05$.

pendent of 5-ALA concentration. Based on these studies of nano-particle size and loading efficiency, we conclude that a high loading efficiency and large particle size could occur by adding 5-ALA at a pH over 7.4 to chitosan solution.

Fig. 5 shows that CNA-7.4 showed more PpIX accumulation in Caco-2 cells than the positive control group but the result of CNA-9.0 was lower than the positive control group. This difference might be due to the possession of high zeta-potential on CNA-7.4 which could result in the stronger interactions with the Caco-2 cell membrane and the higher efficacy of internalization. When Caco-2 cells were cultured without serum and fed with CNA-7.4 and CNA-9.0 (Fig. 6), the signals of PpIX in these two groups were lower than those cultured in serum supplements. The results might be owing to the lower metabolic activity of the cells to degrade the chitosan at the serum-free medium which resulted in a lower conversion rate of PpIX and poor accumulation of PpIX inside the cell.

In previous studies, 5-ALA was engulfed by cells and bacteria, which would interfere with the detection results of cancer cells. The 5-ALA encapsulated in chitosan showed no evidence of uptake by *E. coli* (Fig. 8) and also suppressed the growth of *E. coli* in this study (Fig. 9). However, the Caco-2 cell showed a strong uptake of the developed CNA nano-particles than 5-ALA (Fig. 7 and Fig. 8). Although the HFF cell could uptake the 5-ALA and CNA and converted 5-ALA into PpIX, the degradation rate of PpIX in the normal cell was quick and would not interfere with the diagnosis. We believe that the cell wall of bacteria bore polycationic amines and subsequently restrained the growth of bacteria [25,26]. The results show that chitosan is an ideal drug carrier for 5-ALA to protect 5-ALA from *E. coli* uptake. However, when chitosan nano-particles are designed as an oral form carrier, it may solve in stomach with pH lower than the pK_a of chitosan [18,19]. Therefore, the coating with pH sensitive polymer, such as poly(acrylic acid), is needed for clinical use.

5. Conclusion

In this study, a chitosan nano-particle was successfully prepared and loaded with 5-ALA that could be taken up by the Caco-2 cell and could escape from being engulfed by *E. coli*. The CNA particles prepared at pH 7.4 and pH 9 of 5-ALA solutions with a concentration higher than 0.5 mg/ml had a better loading efficiency with an adequate particle size. Most of the CNA nano-particles had a zeta-potential higher than 30 mV that provided enough zeta-potential to prevent aggregation. CNA could be engulfed by Caco-2 cells but showed no evidence of uptake by *E. coli*. We conclude that chitosan is an ideal vector for colorectal-specific drug delivery of 5-ALA for fluorescent endoscopic detection of colorectal cancer.

Conflicts of interest

We declare that we have no conflict of interest.

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