

## RESEARCH ARTICLE

# Preparation of 5-fluorouracil-loaded Nanoparticles and Study of Interaction with Gastric Cancer Cells

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### Abstract

**Aims:** To prepare 5-fluorouracil (5-Fu) nanoparticles with higher encapsulation efficiency and drug loading, and then investigate interaction with the SGC-7901 gastric cancer cell line. **Materials and Methods:** Prescription was optimized by orthogonal experiments, the encapsulation efficiency and loading capacity were tested by high-performance liquid chromatography, and inhibition of proliferation by 5-Fu nanoparticles and 5-Fu given to cells for 24, 48 and 72 hours was investigated by methyl thiazolyl tetrazolium assay (MTT). In addition, 5-Fu nanoparticles were labeled by fluorescein isothiocyanate (FITC), and absorption into cells was tested by flow cytometry. **Results:** The optimal conditions for preparation were concentrations of 5-Fu of 5mg/ml, of CaCl<sub>2</sub> of 60 mg/ml and of chitosan of 2 mg/ml. With a stirring speed of 1200rpm, encapsulation efficiency of 5-Fu nanoparticles was 55.4±1.10% and loading capacity was 4.22±0.14%; gastric cancer cells were significantly inhibited by 5-Fu nanoparticles in a time and concentration dependent manner, and compared to 5-Fu with slower drug release, in a certain concentration range, inhibition with 5-Fu nanoparticles was stronger. 5-Fu nanoparticles were absorbed by the cells in line with the concentration. **Conclusions:** 5-Fu nanoparticles can inhibit growth of gastric cancer cells *in vitro* to a greater extent than with 5-Fu with good adsorption characteristics, supporting feasibility as a carrier.

**Keywords:** Gastric cancer cells - 5-fluorouracil - nanoparticles - inhibition of proliferation

*Asian Pac J Cancer Prev*, 15 (18), 7611-7615

### Introduction

5-Fluorouracil (5-Fu) has a broad spectrum of activity against solid tumors, especially has a better therapeutic effect on gastrointestinal cancer (Lorca et al., 2005; Yin et al., 2013). However, 5-Fu has heavy toxic side effects, little affinity to tumor cells, a short plasma half-life, so it was administered by intravenous infusion (Grem, 2000; Manar et al., 2012; Hala et al., 2014). Nanoparticles is an colloidal dispersion system with diameters ranging from 10-1000nm which prepared by natural polymers or synthetic polymers (Tsuda, 2010; Fatemeh et al., 2014; Soodabeh et al., 2014). The drug was enfolded or adsorbed on the surface of carriers, nanoparticles has advances in changing the distribution of drugs in the body and the release rate, increasing bioavailability and the permeability of the membrane (Medina et al., 2007; Wang et al., 2009). Recently, in order to reduce 5-Fu associated side effects and improve its therapeutic index by use of incorporation of 5-Fu into particulate carriers (Zhang et al., 2006; Li et al., 2008; Chouhan et al., 2009). We select cationic natural polymer chitosan and anionic sodium alginate as the carriers, prepared 5-Fu nanoparticles use the theory of polyelectrolyte complexation (You et al.,

2006). Encapsulation efficiency as the evaluation index, prescription was optimized by orthogonal experiment, encapsulation efficiency and the drug loading was tested by high-performance liquid chromatography. In order to evaluate the efficacy of the dosage form, inhibition of nanoparticles was investigated by methyl thiazolyl tetrazolium assay (MTT). And 5-Fu nanoparticles were labeled by fluorescein isothiocyanate (FITC) (Huang et al., 2004; Zhang et al., 2008; Arora et al., 2011), the absorption of cells after given labled nanoparticles was tested by flow cytometry, and studied its feasibility as carres.

Recently, there are some researches and reviews about nanoparticles published which have anticancer advantages, especially in the Asian Pacific Journal of Cancer Prevention. Such as Curcumin-loaded nanoparticles are effective to inhibit the growth of human lung cancer with little toxicity to normal tissues (Yin et al., 2013). EGFR antisense encapsulated with PAMAM decreased the level of EGFR mRNA and protein (Najar et al., 2013). Paclitaxel-loaded nanoparticles were associated with increased viability of MCF-7 and B 16F0 cells in comparison to free paclitaxel (Yadav et al., 2014). There are some popular nanoparticle materials for example PLGA, Chitoson, PAMAM dendrimer and so on, in

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which, PLGA are reported very much (Nourazarian, et al., 2012; Mirakabad et al., 2014). Except for organic matter, inorganic materials are also used for nanoparticles, for example, silver nanoparticles, gold nanoparticles, Fe<sub>3</sub>O<sub>4</sub> magnetic nanoparticles (Selin, et al., 2012; Davaran, et al., 2014; Kassas et al., 2014). Some report suggested that the risk of nanoparticles because the enormous difference in size and surface area (Tsuda et al., 2009; 2010).

## Materials and Methods

5-Fu powders (Shanghai Reagent Factory), chitosan (Guoyao Chemical Reagent Co., Ltd.), sodium alginate (Tianjin Yuanhang Chemicals Co., Ltd.), 1640 medium (Invitrogen corporation), trypsin (Sigma), fetal bovine serum (Zhejiang Tianhang bio Technology Co., Ltd.), MTT (Sigma company), dimethyl sulfoxide (Beijing chemical Plant).

### Preparation of 5-Fu nanoparticles and chosen of prescription

Lecithin, ethyl oleate and ethanol were mixed according to a certain mass ratio, add 5-Fu solution 10ml, and then add the sodium alginate solution 3.5ml, stirred 10 minutes (Tianjin Aotesaiensi instrument Co., Ltd., China), CaCl<sub>2</sub> solution 15ml was dropped under slowly stirring for 20 minutes, then put the liquid into the funnel, after stratification, 1% chitosan acetic acid solution dropped into the lower liquid, stirring for 1 hour, 5-Fu nanoparticles get by freeze drying. Encapsulation efficiency as the evaluation index to study the four factors including concentration of 5-Fu, concentration of CaCl<sub>2</sub>, concentration of chitosan and stirring speed to further optimize the prescription by orthogonal experiment.

### Characterization of nanoparticles

The shape of 5-Fu nanoparticles was observed by a transmission electron microscope (TEM, H-7650 Hitachi Co., Japan). Take 0.3g powder of nanoparticles dispersed in 1ml solution, one drop of 5-Fu nanoparticles was added to a copper supported mesh membrane and the excess solution removed with filter paper. Then, 1% phosphotungstic acid was added to the mesh membrane. Excess solution was removed after 5 minutes, the sample dried at room temperature and then examined with the electron microscope. The size and zeta potential of 5-Fu nanoparticles was assessed using a nano-sizer potential analyzer (Zetasizer Nano-ZS90 Malvern Instruments Ltd., UK). The suspension of nanoparticles put in a sample cells, and then put in a measurement cells. The particle size analysis and zeta potential were carried out by nano-sizer potential analyzer.

### In vitro drug release from nanoparticles

In vitro 5-Fu release profiles of 5-Fu nanoparticles were determined as follows. Take 50mg 5-Fu nanoparticles dispersed in 5.0 mL phosphate buffer saline (PBS), and placed into a dialysis membrane bag with a molecular weight of 10 kDa, tied and placed into 50.0 mL

PBS medium. The entire system was kept at 37°C with continuous oscillation frequency. At different times, 3 mL release medium was removed at each time point and 3 mL fresh medium PBS solution was added into the system. The amount of 5-Fu in the release medium was evaluated by liquid phase chromatography (HPLC, waters 2695 Co., USA). All measurements were performed in triplicate.

### Encapsulation efficiency and loading capacity

The encapsulation efficiency and loading capacity of nanoparticles were determined by separation of nanoparticles from the aqueous medium containing nonassociated 5-Fu by ultracentrifugation (Allegra 64R Beckman Coulter Inc., USA) at 28000 r/min for 1h at 4°C. The amount of free 5-Fu in the supernatant was measured by HPLC. HPLC was performed using a The 5-Fu nanoparticles were separated from the aqueous suspension medium through ultracentrifugation. The detective wavelength was set at 265 nm. HPLC analysis of samples was performed using a Kromasil C18 (4.6×250 mm, 5 μm, China). The column temperature was maintained at 30°C. The mobile phase was a mixture of methanol/water (5:95). The flow rate was 0.8mL/min. The 5-Fu encapsulation efficiency (EE) and the 5-Fu loading capacity (LC) of nanoparticles were calculated as follows:

$$EF = [(The\ amount\ of\ 5-Fu - free\ 5-Fu) \div The\ amount\ of\ 5-Fu] \times 100\%$$
$$LC = [(The\ amount\ of\ 5-Fu - free\ 5-Fu) \div Total\ amount\ of\ nanoparticles\ weight] \times 100\%$$

### Inhibition effect of 5-FU nanoparticles

Human gastric cancer cell line SGC-7901 were incubated in 1640 medium at 37°C, 5% CO<sub>2</sub> incubator, with 10% fetal bovine serum (FBS) and 100U/mL penicillin-streptomycin. SGC-7901 cells at logarithmic growth phase (3×10<sup>4</sup> cells/mL) were added to 96-well culture plates (100 μL/well), and then incubated 24h. Then different concentrations of blank nanoparticles, 5-Fu nanoparticles and 5-Fu were added into the wells (100μl/well). Concentration of drugs were 1, 3, 10, 30, 100 μg/ml, and the control group was not administered, each group with six parallel wells, placed in 37°C, 5% CO<sub>2</sub> incubator until the scheduled time. Then 20 μL MTT solution (0.5mg/mL in PBS, pH 7.4) was added to cells (100 μL/well), followed by further incubation for 4 hours. Thereafter, the media was removed and added dimethyl sulfoxide (DMSO) (150 μL/well), mixed by low speed oscillation. The absorbance (OD) was read at 570 nm on microplate reader (Model 680 Bio-rad Co., USA). The inhibition rate was calculated as follows:

$$Inhibition\ rate\ (\%) = [1 - (Administered\ group\ OD \div Control\ group\ OD)] \times 100\%$$

### Preparation of FITC-labeled nanoparticles

The synthesis of FITC-labeled nanoparticles was based on the reaction between the isothiocyanate group of FITC and the amino group of chitosan. Nanoparticles (0.1g) dispersed in aqueous solution, the concentration of nanoparticles is 1g/L, then added 10g/L DMSO solution of FITC, volume ratio of nanoparticle dispersions and DMSO is 3ml: 0.3ml. Reaction in dark for 3 hours, then get FITC-

conjugated fluorescent nanoparticles after centrifugation and washing, keep it from light.

#### Cells uptake of FITC-labeled nanoparticles

SGC-7901 cells at logarithmic growth phase ( $20 \times 10^4$  cells/mL) were added to 6-well culture plates (1mL/well), and then incubated 24h. then different concentrations of FITC-labeled nanoparticles were added into the wells (1ml/well). Concentration of nanoparticles were 10, 30, 100  $\mu\text{g}/\text{ml}$ , and the control group was given free FITC, each group with three parallel wells, placed in  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  incubator for 24h. Then wash the cells with PBS for three times to remove the free drugs, the fluorescence intensity measured by flow cytometry.

#### Statistical analysis

All data were presented as mean $\pm$ standard deviation ( $\bar{x} \pm s$ ), spss statistics as the data analyse software.

## Results and Discussion

#### The results of orthogonal experiment

Encapsulation efficiency as the evaluation index to study the four factors including concentration of 5-Fu, concentration of  $\text{CaCl}_2$ , concentration of chitosan and stirring speed (Table 1). The results of  $L_9$  ( $3^4$ ) orthogonal experiment were shown in Table 2. As it is shown in Table 2, the degree of the factors affect encapsulation efficiency is  $B > C > D > A$ . The optimal reaction conditions is  $A_1 B_2 C_2 D_2$ , that is, the concentration of 5-Fu is 5mg/ml, the concentration of  $\text{CaCl}_2$  60 mg/ml, the concentration of chitosan is 2 mg/ml, stirring speed is 1200rpm.

#### Characterization of nanoparticles

TEM images shown in Figure 1 reveal that the morphology of nanoparticles are spherical shape, The actual diameter of the nanoparticles observed by TEM

was approximately 50-200 nm. The average particle size is 215nm (Figure 2) which detected by nano-sizer potential analyzer, the polydispersity index PDI is 0.164. It was reported that large particles hardly reach the cells, while smaller particles tended to accumulate in the tumor sites due to the enhanced permeability and retention (EPR) effect and a greater internalization was also observed. Zeta potential of nanoparticles is about 0.0534mV.

The size of 5-Fu nanoparticles observed under transmission electron microscope is smaller than the result of nano-sizer potential analyzer detected. May be due to the low zeta potential of nanoparticles, the electrostatic repulsion between particles is small, unstable particles easily reunion, although PDI value is low, it only shows the size distribution of aggregates.

#### In vitro release, encapsulation efficiency and loading capacity

Nanoparticle release occurs by 2 methods: "burst release" and "sustained release". Burst release is the rapid release of a drug from the surface of nanoparticles or diffusion from the polymer matrix. This allows the drug to rapidly reach an effective concentration in the circulation. Sustained release is the slow release of a drug that is entrapped within nanoparticles during nanoparticle bio-degradation. This allows the drug to stay at an effective concentration in the circulation over time. Figure 3 shows the *in vitro* 5-Fu release profiles from nanoparticles in water and PBS (pH=7.4), and the release rate in PBS faster

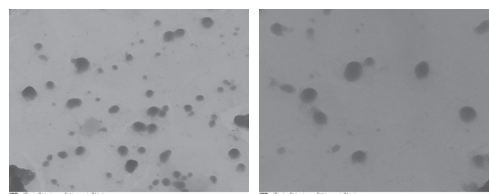


Figure 1. Transmission Electron Micrograph of 5-Fu Nanoparticles

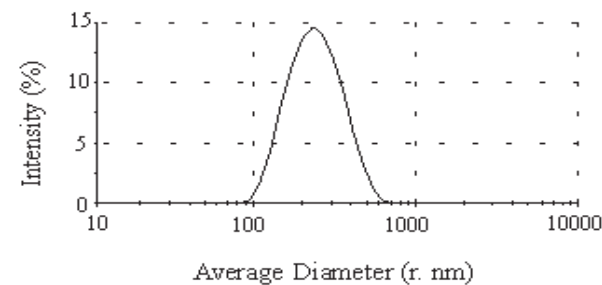


Figure 2. Size Distribution of 5-Fu Nanoparticles

Table 1. Factors and Level

Level	Factors			
	A 5-Fu (mg·ml <sup>-1</sup> )	B CaCl <sub>2</sub> (mg·ml <sup>-1</sup> )	C chitosan (mg·ml <sup>-1</sup> )	D stir speed (rpm)
1	5	45	1.6	900
2	6	60	2	1200
3	7	75	2.4	1500

Table 2.  $L_9$  ( $3^4$ ) Orthogonal Experiment

Experiment number	Factors				EF (%)
	A	B	C	D	
1	1	1	1	1	40.4
2	1	2	2	2	54.22
3	1	3	3	3	36.72
4	2	1	2	3	42.52
5	2	2	3	1	45.3
6	2	3	1	2	37.32
7	3	1	3	2	40.83
8	3	2	1	3	43.7
9	3	3	2	1	42.27
$K_1$	43.78	41.25	40.47	42.66	
$K_2$	41.71	47.74	46.34	44.12	
$K_3$	42.27	38.77	40.95	40.98	
R	2.07	8.97	5.87	3.14	

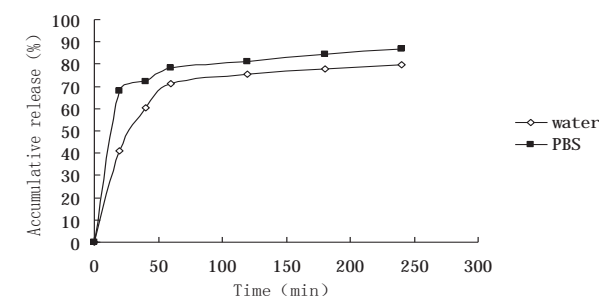
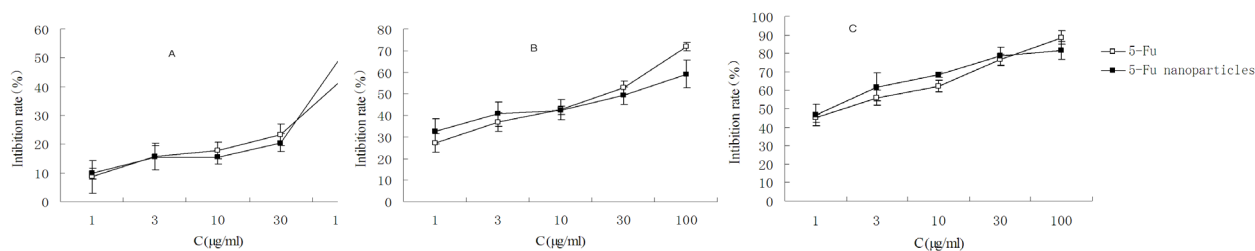


Figure 3. In vitro Release of 5-Fu from Nanoparticles in the Water and PBS



**Figure 4. Inhibition Effect of 5-Fu and 5-Fu NPs.** A) for 24 hours; B) for 48 hours; C) for 72 hours. Data present as mean±SD, n=6. \*P<0.05 represent 5-Fu NPs compared with 5-Fu

**Table 3. OD of Blank Nanoparticles Effect on SGC-7901 Cells for Different Times ( $\bar{x}\pm s$ , n=6)**

Concentration (µg/ml)	OD		
	24h	48h	72h
Control group	0.421±0.058	0.502±0.067	0.727±0.053
1	0.431±0.068	0.518±0.056	0.736±0.020
3	0.436±0.041	0.513±0.060	0.730±0.057
10	0.440±0.052	0.534±0.045	0.745±0.061
30	0.433±0.047	0.556±0.031	0.773±0.088
100	0.451±0.036	0.580±0.064	0.806±0.037

**Table 4. The Results of Flow Cytometry**

Concentration of nanoparticles (µg/ml)	Fluorescence intensity
Control group	26.9±0.1
10	30.2±4.1
30	120.8±3.4
100	293.1±0.1

than in water. The 5-Fu release profiles are composed of burst release and sustained release. The burst release, which resulted in the release of 60% of the 5-Fu, occurred from 0 to 1h. The sustained release occurred from 1 to 5h and resulted in the release of 60% of the 5-Fu. The encapsulation efficiency (EE) of 5-Fu nanoparticles was 55.36±1.10%, and the loading capacity was 4.22±0.14%

In this experiment, we prepared 5-Fu nanoparticles of higher encapsulation efficiency with the natural polymer as the carriers, but loading capacity is low. It might be because of the prescription contains more excipients, but most of them are natural materials, have good safe, so do not affect drug activity. In addition, there are still some shortcomings in detecting the content of samples. In the sample processing, the centrifuge speed is fast, a large centrifugal force may cause drug leakage, resulting in encapsulation efficiency and loading capacity reduce. But this method is simple, it can be used for the determination of nanoparticles in the condensation of minimize the loss of drug,

*Inhibition effect of 5-Fu nanoparticles*

As it is shown in Table 3, different concentrations of blank nanoparticles effect on SGC-7901 cells after 24, 48 and 72 hours, there is no significant difference about OD values compared with the control group (P>0.05). Indicating that drug excipients have no toxic side effects, have good security. However, OD values of each group were higher than control group, indicating that the nanoparticles have an effect on promote cell proliferation (Table 3).

We performed a series MTT assays to compare the inhibition effect of 5-Fu nanoparticles with free

5-Fu. As shown in Figure 4, inhibition effect of 5-Fu and 5-Fu nanoparticles showed time-dependent and concentration-dependent. For 24 hours, inhibition rate of 5-Fu nanoparticles with concentration of 100µg/ml was significantly higher than 5-Fu, inhibition rate of other concentrations of nanoparticles lower or approach to 5-Fu. For 48 hours, inhibition rate of 5-Fu nanoparticles with the concentration of 1µg/ml and 3µg/ml are higher than 5-Fu, and there is the significant differences between 5-Fu nanoparticles and 5-Fu with the concentration of 1µg/ml. But, inhibition rate of 5-Fu nanoparticles with the concentration of 100µg/ml was significantly lower than 5-Fu. For 72 hours, except for inhibition rate of 5-Fu nanoparticles with the concentration of 100µg/ml was significantly lower than 5-Fu, inhibition rate of the other concentrations of 5-Fu nanoparticles were higher than the 5-Fu, and there is significant different between 5-Fu nanoparticles and 5-Fu with the concentration of 3µg/ml and 10µg/ml. These results suggest that, in addition to nanoparticles with the concentration of 100µg/ml, the other concentration of nanoparticles showed the slowly drug release compared to 5-Fu, inhibition rate stronger than 5-Fu along with the time effect on the cells.

MTT assay showed that 5-Fu nanoparticles significantly inhibited the cells, and showed time-dependent and concentration-dependent. However, at 72 hours, in addition to 5-Fu nanoparticles with the concentration of 100µg/ml, the other concentrations of 5-Fu nanoparticles showed stronger inhibition effect than 5-Fu. It indicating that 5-Fu nanoparticles release drugs slower than 5-Fu in a certain range of concentration. But 5-Fu nanoparticles with the concentration of 100µg/ml showed stronger inhibition effect than 5-Fu in the early stage, and weaker than 5-Fu along with the time. May be due to the carrier materials have a certain role in promoting proliferation of cells (Table 3), as a result of the impact of carrier materials, the inhibition rate reduce. In addition, the cell uptake of nanoparticles including membrane transport, adsorption and endocytosis, endocytosis is the main way of particle uptake by cells which the particle size less than 500nm, but the particle size and surface charge are also the main factors affect cell absorption of nanoparticles (Wang et al., 2009; Manar et al., 2012; Soodabeh et al., 2014).

Surface of tumor cells have more negative charge than normal cells, while chitosan can adsorb cells through cationic charge (Zhang et al., 2008), the higher the concentration is, the more electric charge and the stronger the adsorption. Therefore, 5-Fu nanoparticles with the concentration of 100µg/ml showed strong inhibition in the early phase.



### Cellular uptake of nanoparticles

As shown in Table 4, nanoparticles can be absorbed by the cells, and dependent on the concentration of nanoparticles, the higher the concentration of nanoparticles, the stronger the fluorescence intensity. It has been reported that cellular uptake studies performed with fluorescent marker molecules had some limitations. In this study, although all uptake experiments were performed carefully and promptly under light protection and the incubation time was 24 hours, the decrease in the measurement could also result from photobleaching of the FITC and not be related to the decrease in uptake rate.

Flow cytometry results showed that the nanoparticles can be absorbed by the cells, indicating that the nanoparticles as a carrier of 5-Fu is feasible. In addition, fluorescent nanoparticles can also be used for tracer experiments (Zhang et al., 2008), and provide the basis for its targeting study. However, we couldn't sure the fluorescent rate of labeled nanoparticles, so can only do qualitative analysis but quantitative analysis. In addition, we need further study of the way and site of the nanoparticles absorb.

In conclusion, in this study we successfully synthesized 5-Fu nanoparticles, which can inhibit growth of gastric cancer cells *in vitro*, and inhibition of the gastric cancer cells stronger than 5-Fu in a certain concentration range, the nanoparticles can be absorbed by the cells, which provided the feasibility as a carrier of 5-Fu. Therefore, it may be used as a safe and effective novel anti-tumor preparation with profound prospects in clinical application.

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