Preparation and performance of a pH-sensitive cisplatin-loaded magnetic nanomedicine that targets tumor cells via folate receptor mediation

SHUAI-JUN CHEN[1], HONG-ZHENG ZHANG[1], LIANG-CAI WAN[1], SHAN-SHAN JIANG[1], YI-MING XU[1], FANG LIU[1], TAO ZHANG[1], DONG MA[2] and MIN-QIANG XIE[1]

[1]Department of Otolaryngology Head and Neck Surgery, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong 510282; [2]Department of Biomedical Engineering, College of Life Science and Technology, Jinan University, Guangzhou, Guangdong 510632, P.R. China

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Abstract. The present study aimed to prepare cisplatin (CDDP)-loaded magnetic nanoparticles (MNPs), which target folate receptors via a pH-sensitive release system (FA-PEG-NH-N=MNPs-CDDP). This is of interest for the development of intelligent drug delivery systems that target tumors of the head and neck. The chemical coprecipitation method was used to prepare ferroferric oxide MNPs. These were modified with aldehyde sodium alginate complexed with the chemotherapeutic agent, CDDP on the surface of the nanoparticles. Double hydrazine-poly(ethylene glycol; PEG) was also prepared by attaching the carboxyl group of hydrazine-folate on one side of the double hydrazine-PEG, obtaining folate-hydrazine-PEG-diazenyl. This binds the aldehyde group of sodium alginic acid on the MNP to enclose CDDP, in order that it is sequestered within the carrier. This method obtained a pH-sensitive, FA-modified CDDP-loaded MNP (FA-PEG-NH-N=MNPs-CDDP), which acts as an intelligent tumor targeting drug delivery system. The mean size of the MNPs was ~10.2±1.5 nm, the mean hydrodynamic diameter detected by laser particle sizing instruments was 176.6±1.1 nm, and the ζ-potential was -20.91±1.76 mV. The CDDP content was 0.773 mg/ml, the iron content was ~1.908 mg/ml and the maximum saturation magnetization was 16.3±0.2 emu/g. The current study produced a pH-sensitive FA-modified CDDP-loaded MNP that is stable and exhibits magnetic responsiveness, which releases CDDP in a low pH environment.

Correspondence to: Dr Min-Qiang Xie, Department of Otolaryngology Head and Neck Surgery, Zhujiang Hospital, Southern Medical University, 1838 North Guangzhou Avenue, Guangzhou, Guangdong 510282, P.R. China
E-mail: xiemingqing_l@163.com

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Introduction

Cisplatin (CDDP) is a common broad-spectrum antitumor therapeutic agent used in clinical settings. It is recommended in the treatment of a number of types of cancer, including melanoma, cancer of the head and neck, non-small cell lung cancer, liver cancer, cervical cancer and nasopharyngeal carcinoma (1). However, as with numerous cytotoxic anti-tumor therapeutic agents, CDDP results in toxic side effects, as it also affects non-cancerous cells. CDDP is also unstable in the body, further limiting its efficacy in clinical use (2). In order to increase the curative effect and reduce the toxic side effects of CDDP, targeted drug delivery aims to selectively treat a tumor area or enable therapeutic agents to enter tumor cells, thus reducing the therapeutic agent concentration within healthy tissue. Previous studies regarding anticancer targeted drug-delivery systems have aimed to establish methods of targeting tumor tissue; however, the present study investigates how to deliver a greater concentration of therapeutic agent into tumor tissue upon reaching the tumor location.

Tumor characteristics, such as rapid metabolism and cell division, result in a slightly acidic environment (3). The pH-sensitive magnetic nanoparticles (MNPs) deliver the therapeutic agent to the tumor using the difference in physiological pH between the healthy tissue and tumor tissue. This increases the concentration of therapeutic agent in the tumor and improves the bioavailability (3). The use of pH-sensitive, targeted therapeutic agent release in the treatment of malignant tumors has progressed, however, it has been observed that certain pH-sensitive MNPs induce therapeutic agent release outside of tumor cells (3). The therapeutic agents released outside of cell then spreads to healthy tissue and reduces the target specificity of the therapeutic agent. Under normal conditions, the use of a receptor and a ligand allows specific positioning of the ligand, and separation by normal biological function is non-toxic and non-immunogenic (3). Low molecular weight ligands, including folic acid (FA), vitamin B1 (thiamin) and sugars, attached to or coating the MNP facilitate active targeting (4). Furthermore, peptides, proteins and antibodies improve the MNP efficacy.

The method of combining a pH-sensitive carrier with pH-sensitive chemical bonds was adopted in the present study, according to a previous study (5). The majority of malignant tumor cells exhibit positive FA expression, therefore, a novel pH-sensitive FA-modified MNP drug delivery system may target cancer cells and deliver the therapeutic agent by combining highly efficient endocytosis, targeted drug delivery and pH-sensitive release of the therapeutic agent.

The aim of the present study was to introduce pH sensitivity to a drug delivery system, optimize its preparation and characterize its physicochemical properties, in order to elucidate the mechanism of targeting by assessing CDDP uptake by tumor cells.

Materials and methods

Reagents. Sodium alginate (molecular weight, ~60,000; viscosity, 2 cps) was obtained from Qingdao Crystal Rock Biology Development, Co., Ltd (Qingdao, China). Analytically pure poly(ethylene glycol) (PEG; molecular weight, 2,000) and triethylamine was sourced from Shanghai Crystal Pure Reagent Co., Ltd. (Shanghai, China). Analytically pure FA was obtained from Guangdong Guanghua Chemicals Factory Co., Ltd. (Guangdong, China). Nitrophenyl chloroformate hydroxylamine hydrochloride, acetic acid, dimethylsulfoxide (DMSO), polymer film-lined copper net, paraformaldehyde, Prussian blue, Neutral Red, Gluteraldehyde was sourced from Southern Medical University (Guangzhou, China). Acros Organics™ dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). , sodium periodate, $FeCl_3 \cdot 6H_2O$ and $FeSO_4 \cdot 4H_2O$ were purchased from Guangzhou Chemical Reagent Factory. CDDP was obtained from Shandong Qilu Pharmaceutical Factory (Shandong, China). Phenanthroline was obtained from Tianjin No. 1 Chemical Reagent Factory (Tianjin, China), and *o*-phenylenediamine from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). N,N-dimethylformamide was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). CNE-2 and HNE-1 were obtained from Shanghai Institutes for Biological Sciences (Shanghai, China). RPMI-1640 and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific Inc. (Shanghai, China). Phosphate-buffered saline (PBS) was obtained from Wuhan Boster Biotechnology Co., Ltd. (Wuhan, China). MTS kits were obtained from Promega Corporation (Beijing, China). ELISA kits were obtained from Takara Biotechnology Co., Ltd (Dalian, China).

Instruments and equipment. Transmission electron microscopy (TEM) was performed using a TecnalTM G2 Polara (J&R Scientific instruments Co., Ltd., Guangzhou, China) and a ZetaPALS ζ-potential analyzer (Brookhaven Instruments, Corporation, Holtsville, NY, USA). An MPMS XL-7VSM magnetometer (Quantum Design, Inc., San Diego, CA, USA), Alpha 1-2LD vacuum freeze drier (Martin Christ Gefriertrocknungsanlagen, GmbH, Osterode am Harz, Germany), DZF-6201 vacuum drying oven (Shanghai Yiheng Technology Co., Ltd., Shanghai, China), THZ-100 constant temperature shaking incubator (Shanghai Yiheng Technology Co., Ltd.) and a UVIKON923 ultraviolet (UV)-visible spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT, USA) were used during the present study.

Preparation of PEG with hydrazine and FA conjugation. The method of PEG attachment to basic hydrazine was conducted as described in a previous study (6). Dried PEG (20 g) was dissolved in 60 ml anhydrous methylene chloride, and added into 40 ml anhydrous methylene chloride solution containing 6.05 g nitrophenyl chloroformate and 12.5 ml anhydrous triethylamine. This was performed drop by drop in an ice-water bath, the resulting molar ratio of PEG, nitrophenyl chloroformate and triethylamine was 1:3:9. Spin steaming was used to remove the majority of the dichloromethane solvent following a 12-h reaction in the dark. Anhydrous ether was added to precipitate the PEG activated by nitrophenyl chloroformate, this was then vacuum dried.

The PEG activated by nitrophenyl chloroformate was dissolved in 100 ml dichloromethane, and 29.1 ml water and 29.1 ml hydrazine solution was added, the final molar ratio of PEG and hydrazine was 1:30. Spin steaming was performed to remove the majority of the dichloromethane solvent following the reaction at room temperature. Anhydrous ether was added to precipitate the PEG modified by hydrazine and this was vacuum dried.

FA-PEG-$NHNH_2$ was prepared as previously described (7). FA (0.88 g) was dissolved in anhydrous DMSO,0.84 g DCC and 0.44 g NHS were added, and the reaction proceeded at room temperature for 4 h with continuous mixing. Double hydrazine (4 g) was added to the PEG [PEG2000$(NHNH_2)_2$], continuously mixed and the reaction proceeded for 8 h. Following the reaction, 120 ml double-distilled water (ddH_2O) was added, the undissolved solid was removed by filtering and the product (FA-PEG-$NHNH_2$) was freeze-dried.

Preparation of aldehyde sodium alginate (ASA)-modified CDDP-loaded Fe_3O_4 MNPs. ASA was prepared according to a previous study by Laurienzo *et al* (8) and coprecipitation was used to prepare the ASA-modified CDDP-loaded Fe_3O_4 MNPs, as described previously (9). The product (molecular weight, ~14,000) was then dialyzed (TXDJZ-60; Solarbio Technology Co., Ltd., Beijing, China) for three days in double distilled water to remove unattached CDDP.

Preparation of ASA-CDDP-modified MNPs, decorated with FA and PEG-hydrazine. FA-PEG-$NHNH_2$ (0.3 g) was dissolved in 10 ml ddH_2O and added to 30 ml ferrofluid (Wenzhou JingCheng Chemical Co., Ltd., Wenzhou, China) containing 0.5 g ASA-modified CDDP-loaded MNPs. According to the previously described method of hydrazone bond formation (10), anhydrous acetic acid was used to create an acidic environment and obtain a pH value of 5. The reaction was conducted with continuous mixing, at room temperature under the protection of nitrogen for 48 h. The solution was dialyzed in distilled water for three days (molecular weight, 5,000-14,000) and subsequently freeze-dried.

Detection of particle size, shape and dispersity of the magnetic core. A sample of the drug delivery system, FA-PEG-NH-N=MNPs-CDDP was prepared by dilution to 6-300 mg/ml, and the sample was dropped on a polymer

film-lined copper net. Subsequent to slow drying, the sample was deposited with a layer of carbon film (thickness, 10-20 nm) and observed by TEM.

Detection of saturation magnetization and evaluation of magnetic responsiveness. At 25˚C, a hysteresis curve of the sample was determined between -10 and 10kOe. Samples of FA-PEG-NH-N=MNPs-CDDP (4 ml) were added to two 5-ml centrifuge tubes, one was placed into a constant magnetic field and the distribution of the MNPs was observed following 2 h.

Detection of hydrodynamic diameter and ζ-potential. FA-PEG-NH-N=MNPs-CDDPs, ASA-MNPs and CDDP-MNPs were diluted to a certain concentration prior to analysis (iron concentration, 0.02 mg/ml prior to testing). The analysis was conducted on the ZetaPALS ζ-potential and laser particle size analyzer. A scattering angle of 90˚ and temperature of 25˚C was used; the analysis was repeated to obtain an mean value of three experiments. The aim was to evaluate the change in MNP size during preparation.

Observing MNP stability. The CDDP-loaded MNPs were placed in a 4˚C refrigerator for 2 months, and their shape and any changes in CDDP loading capacity were subsequently observed.

Detection of iron content and CDDP loading capacity. The present study used the phenanthroline method, as previously described (11), to detect the iron content in the FA-PEG-NH-N=MNPs-CDDP. The *o*-phenylenediamine colorimetric method (12) was used to detect the CDDP content in FA-PEG-NH-N=MNPs-CDDP (13).

Observation of cell targeting by iron staining. Prussian blue staining can be conducted to demonstrate the MNPs taken up into the cell via formation of ferric ferrocyanide, by the reaction of Fe^{3+} with ($K_4Fe(CN)_6$), where iron is present. Two nasopharyngeal carcinoma cell lines were used in the present study, CNE-2, which is folate receptor-negative and HNE-1, which is folate receptor-positive. RPMI-1640 culture medium was used to prepare a single-cell suspension and, following digestion and centrifugation at 1,000 x g, the concentration of the cells was adjusted to $2x10^4$ cells/ml. The solution was inoculated in a 24-well plate (well volume, 500 μl) and the HNE-1 cells were cultured for 24 h in RPMI-1640 complete medium without FA. Following 24 h of adherent growth, the medium was removed and FA-PEG-NH-N=MNPs-CDDP and MNPs were diluted with RPMI-1640 without FA and cultured for 6 h; the concentration of CDDP was 5, 10 and 20 μg/ml according to the iron content. The culture media was removed from the wells, the MNPs were collected and washed with PBS. The cells were fixed in 4% paraformaldehyde solution for 30 min at room temperature and washed with ddH_2O. Prussian blue dye was added and the samples were placed in the dark at room temperature for 30 min. Neutral red dye was added as a contrast dye following washing with ddH_2O and an inverted microscope was used for observation and to obtain images.

Observation of cell targeting by TEM. Following culture of the HNE-1 cells with MNPs and FA-PEG-NH-N=MNPs-CDDP, the condition of the cells following the uptake of MNPs was observed by TEM. A single-cell suspension of HNE-1 cells was produced with RPMI-1640 culture medium without FA. Following digestion and centrifugation, cells ($5x10^4$ cells/ml) were inoculated in 6-well plates (well volume, 2 ml). The cells were cultured for 24 h, subsequent to this, FA-CDDP-FA-PEG-NH-N=MNPs-CDDP (5, 10 and 20 μg/ml depending on iron content) and MNPs diluted in RPMI-1640 complete medium without FA were added and cultured for 6 h. The medium was collected and washed with PBS to remove any remaining MNPs. The cells were trypsinized and the cell suspension was centrifuged for 5 min at 1,000 x g, following which the supernatant was discarded. Gluteraldehyde (2.5%) was added to the cell pellet and the cells were fixed at 4˚C. Centrifugation at 1,000 x g was repeated and the supernatant discarded, 1% osmic acid was added and dehydration was conducted using a gradient of acetone concentrations. The cells were embedded in epoxy resin and thin sections (40-50 nm) were cut and observed by TEM following staining with uranyl acetate and lead citrate to observe the uptake of MNPs by the cells.

Detection of cytotoxicity of FA-PEG-NH-N=MNPs-CDDP with the MTS assay. Pancreatin was used to digest HNE-1 cells in the logarithmic phase and RPMI-1640 medium was used to prepare a single-cell suspension without FA, containing 10% FBS. The suspension (200 μl) was inoculated on a 96-well plate at a density of $3x10^4$ cells/ml. The plates were incubated overnight at 37˚C in an atmosphere of 5% CO_2.

Each culture plate was comprised of the treatment [pure CDDP, FA-PEG-NH-N=MNPs-CDDP (pH 6.5 and 7.4), and the MNPs group], control (culture medium without any treatment) and blank control (culture medium without cells) wells. The treatment concentrations were 0.5, 1, 2, 4, 8 and 25 μg/ml CDDP (three wells per concentration), and the concentration in the MNP group was determined by the iron content. The culture plates were incubated for 24 and 48 h at 37˚C in 5% CO_2 with saturated humidity. Following incubation of the cells, 20 μl MTS/phenazine methosulfate was added to the wells and incubated for 3-4 h. Following 10 sec of agitation to mix the color, ELISA was used to detect absorbance at a wavelength of 490 nm.

The half maximal inhibitory concentration was calculated for the different concentrations administered to HNE-1 cells for 24 and 48 h using probit analysis. In addition, the inhibition ratio at each concentration was calculated as follows: Inhibition ratio (%) = control group A_{490} - treatment group A_{490}/control group A_{490}. Where all groups had been normalized to zero.

Statistical analysis. Data are expressed as the mean ± standard deviation. SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform statistical analysis. Factorial analysis, one way analysis of variance, least significant difference, Student-Newman-Keuls and Dunnett's method were used to analyze the data from the present study. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Confirmation of product. UV-visible spectroscopy was conducted (Fig. 1). $PEG(NHNH_2)_2$ demonstrated no marked

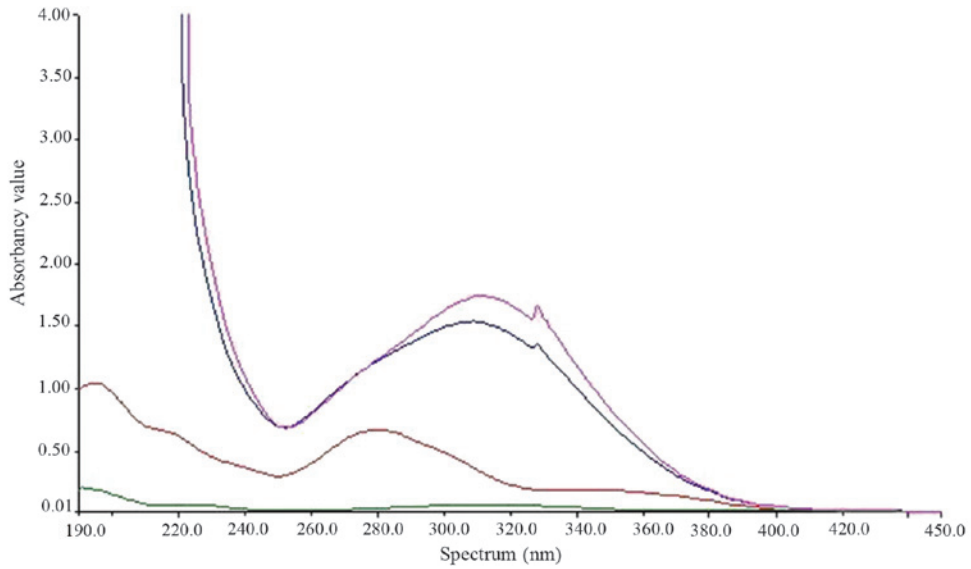

Figure 1. Ultraviolet-visible spectroscopy demonstrates cisplatin and iron content in FA-PEG-NH-N=MNPs-CDDP. The green curve shows PEG; the red curve shows folic acid with a peak at 280 nm; the blue and purple curves show FA-PEC-NH-N=MNPs-CDDP measured twice.

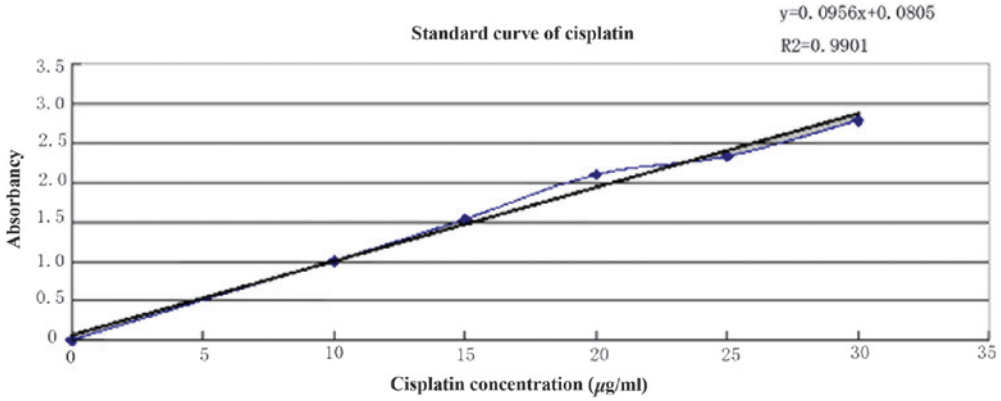

Figure 2. Standard curve of cisplatin concentration.

absorption peak and FA exhibited an absorption peak at 280 nm. FA-NH_2NH-PEG-$NHNH_2$ demonstrated a conjugate absorption peak at 310 nm demonstrating the bond between FA and PEG $(NHNH_2)_2$. However two of the absorption curves are the same, demonstrating that the method of forming the product is reproducible and practical.

In the UV mapping of FA-PEG-NH-N=MNPs-CDDP, the absorption curves were disordered (causing MNPs to appear black), which was caused by interference between 200 and 300 nm.

CDDP and iron content detection in the CDDP carrier. CDDP concentration was determined using the *o*-phenylendiamine method and the standard curve is presented in Fig. 2. The regression equation is as follows: y=0.0956x+0.0805, R^2 =0.9901 and the CDDP concentration was calculated as 0.773 mg/ml. The concentration of iron was determined using the phenanthroline method and the standard curve is presented in Fig. 3. The regression equation is as follows: y=0.1963x+0.0052, R^2 =0.9992, the iron concentration was calculated as 1.908 mg/ml.

Shape and structure of MNPs. The freeze-dried powder of the pH-sensitive FA-modified CDDP-loaded MNPs is uniformly black; when scattered into distilled water ultrasound processing produced a uniform colloidal solution.

The shape of the MNPs was observed by TEM (Figs. 4 and 5). The two MNPs were round with a mean particle size of 10.2±1.5 nm.

Saturation magnetization. The maximum saturation magnetization of FA-PEG-NH-N=MNPs-CDDP was 16.3±0.2 emu/g. The coercive force was zero (Fig. 6), indicating that the MNPs have good superparamagnetism and magnetic responsiveness.

Fig. 7 presents the two centrifuge tubes containing pH-sensitive FA-modified CDDP-loaded MNPs. The MNPs located to the tube wall closest to the magnet, with the solution becoming light and transparent; this transparency increased over time. However, in the centrifuge tube away from the magnet, the MNPs remained evenly scattered indicated by the black solution. When the centrifuge tubes were removed from the magnet, the MNPs rapidly scattered throughout the solution becoming black within 5 sec. This further the good

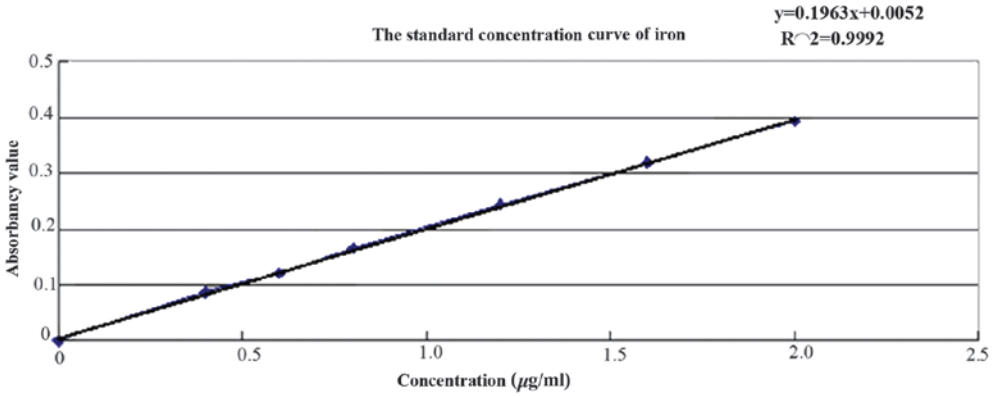

Figure 3. Standard curve of iron concentration.

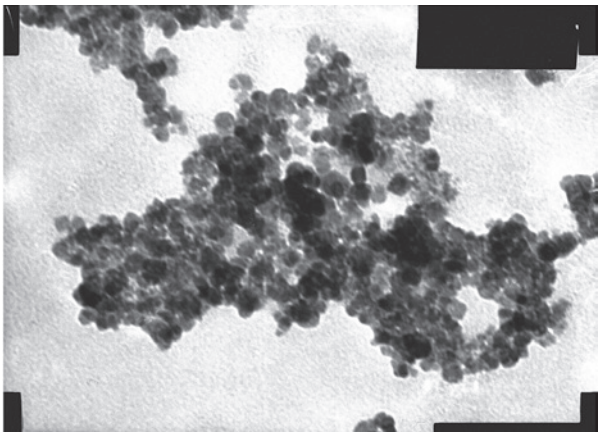

Figure 4. Transmission electron microscopy of cisplatin-loaded magnetic nanoparticles (magnification, x20,000).

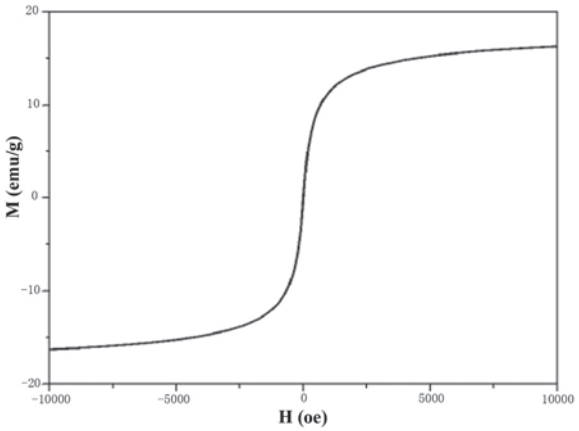

Figure 6. Hysteresis curve of FA-PEG-NH-N=MNPs-CDDP. FA, folic acid; PEG, poly(ethylene glycol); MNPs, magnetic nanoparticles; CDDP, cisplatin; M, saturation magnetization.

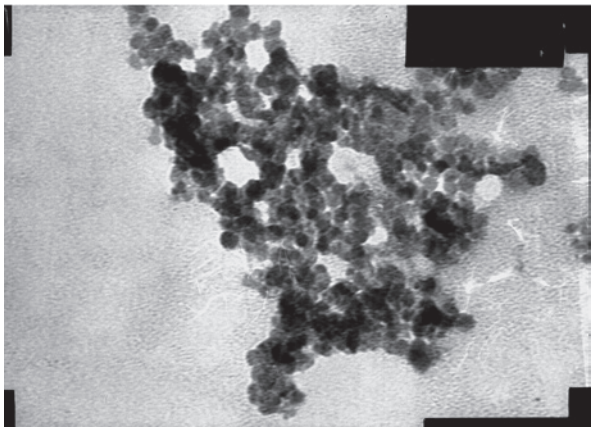

Figure 5. Transmission electron microscopy of the drug delivery system, FA-PEG-NH-N=MNPs-CDDP (magnification, x20,000). FA, folic acid; PEG, poly(ethylene glycol), MNPs, magnetic nanoparticles; CDDP, cisplatin.

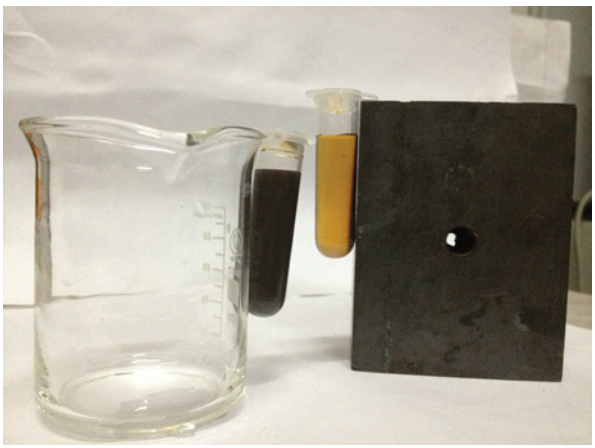

Figure 7. Magnetism of FA-PEG-NH-N=MNPs-CDDP. FA, folic acid; PEG, poly(ethylene glycol); MNPs, magnetic nanoparticles; CDDP, cisplatin.

magnetic responsiveness and superparamagnetism of the MNPs.

Particle size and ζ-potential. A laser particle analyzer was used to detect dynamic light scattering and ascertain the particle size of FA-PEG-NH-N=MNPs-CDDP. The products produced in the preparation process, ASA-MNPs and CDDP-MNPs, served as a comparison. The results demonstrate that the mean hydrodynamic diameter of FA-PEG-NH-N=MNPs-CDDP was 176.6±1.1 nm and the ζ-potential was -20.91±1.76 mV. The hydrodynamic diameter of the ASA-MNPs was 198.9±3.4 nm and the ζ-potential was -29.04±2.17 mV. The hydrodynamic diameter of CDDP-MNPs was 153.4±1.8 nm and the

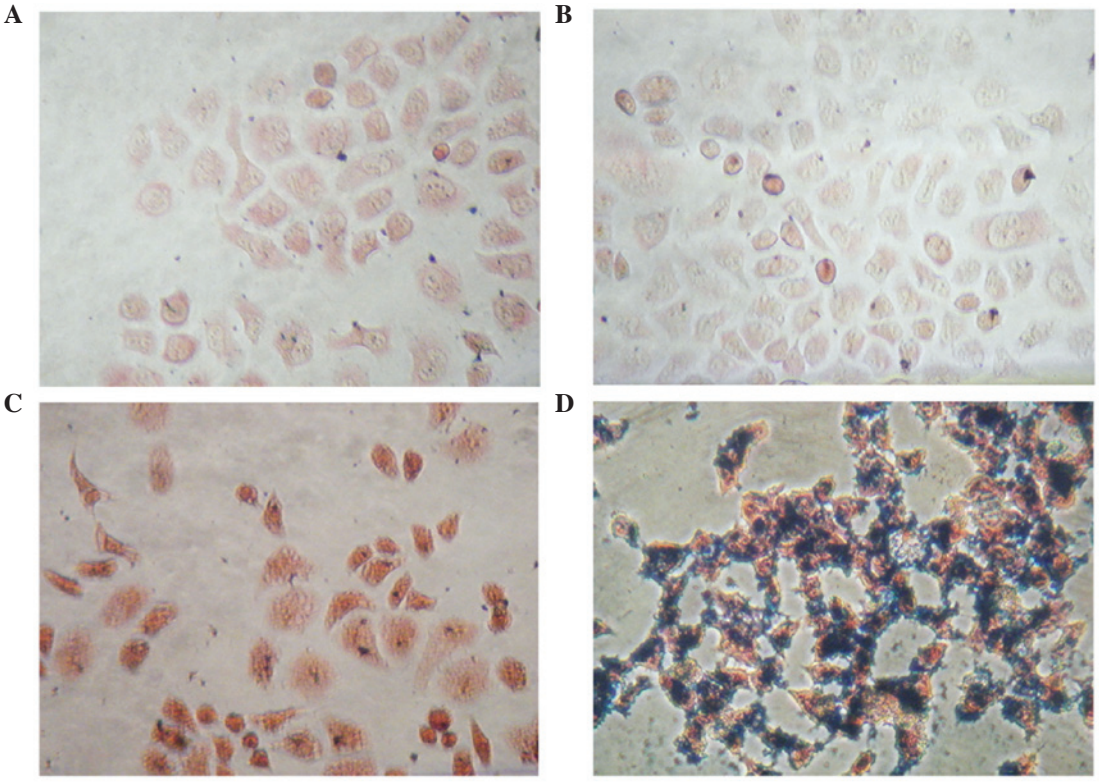

Figure 8. Prussian blue staining in CNE-2 cells treated with (A) MNPs, (B) FA-PEG-NH-N=MNPs-CDDP. Prussian blue staining in HNE-1 cells treated with (C) MNPs and (D) FA-PEG-NH-N=MNPs-CDDP. MNP, magnetic nanoparticle; FA, folic acid; CDDP, cisplatin. Magnification, x200.

ζ-potential was -25.08±0.96 mV. All the results demonstrate logarithmic distribution.

Stability. Sediment was observed at the bottom of the tubes following removal of the solution, which increased with the concentration of pH-sensitive FA-modified CDDP-loaded MNPs in the solution. However, following ultrasound processing, the sediment was evenly scattered in the liquid.

The stability of the MNPs was analyzed two months following production by assessing the concentration of CDDP and iron in the solution that the MNPs were stored in (Table I). No significant changes in concentration were observed (P>0.05), suggesting high stability.

Prussian blue staining. The PEG-NH-N=MNPs-CDDP and MNPs were cultured at different concentrations with CNE-2 (folate receptor-negative) and HNE-1 (folate receptor-positive) cells for 6 h. Prussian blue was added and following culture with FA-PEG-NH-N=MNPs-CDDP, only the HNE-1 cells demonstrated a positive result (blue staining; Fig. 8)

TEM. TEM demonstrated black high density MNPs located within HNE-1 cells following a 6-h culture with FA-PEC-NH-N=MNPs-CDDP. A high density shadow is located inside the pinocytotic vesicle; furthermore a pseudopod extending from the surface of the cell membrane to take up MNPs. This was not observed in the normal cells (Figs. 9-11).

Fig. 11 demonstrates two magnification strengths, but this phenomenon is apparent in the normal cell, however above the high density particle also can not be seen in HNE-1 cell following co-culture MNPs and HNE-1 for 6 h.

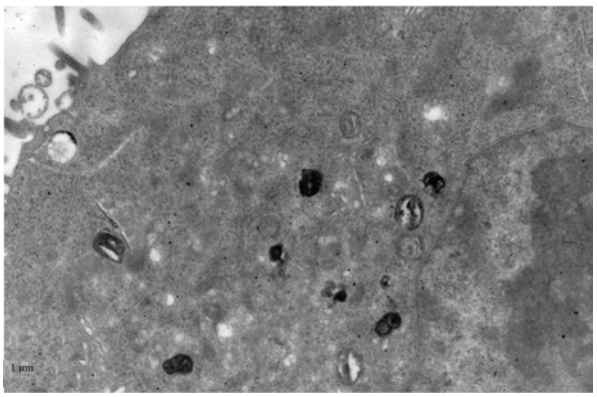

Figure 9. Transmission electron microscope image of untreated HNE-1 cells (magnification, x5.800).

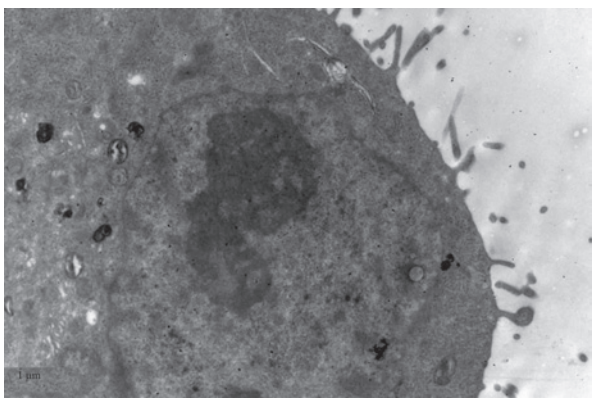

Figure 10. Transmission electron microscope image of HNE-1 cells treated with magnetic nanoparticles (magnification, x5,800).

Table I. Stability of FA-PEG-NH-N=MNPs-CDDP.

Component	Concentration (μg/ml)		T-value	P-value
	0 months	2 months		
CDDP	0.191±0.010	0.190±0.002	0.234	0.826
Iron	0.366±0.008	0.376±0.013	-0.906	0.416

FA, folic acid; PEG, poly(ethylene glycol); MNP, magnetic nanoparticle; CDDP, cisplatin.

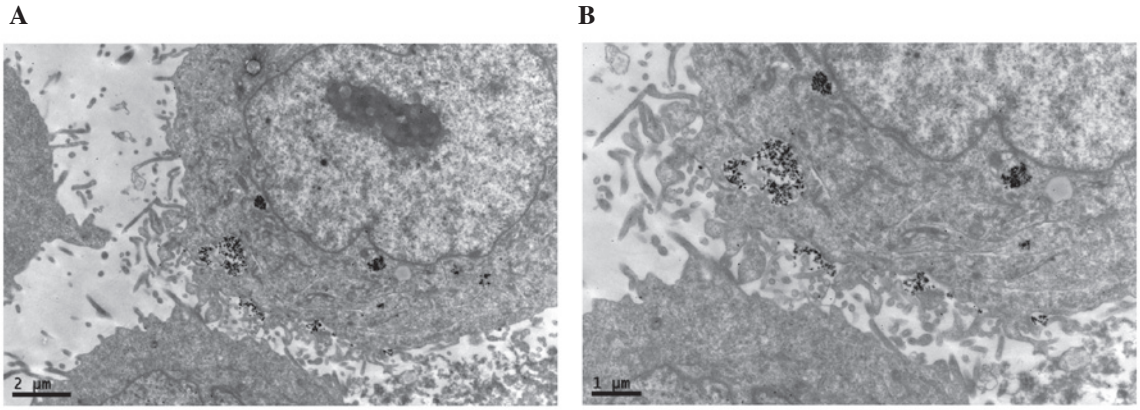

Figure 11. Transmission electron microscope images of HNE-1 cells treated with FA-PEG-NH-N=MNPs-CDDP. Magnification: (A) x5,800 and (B) x13,500. FA, folic acid; PEG, poly(ethylene glycol); MNPs, magnetic nanoparticles; CDDP, cisplatin.

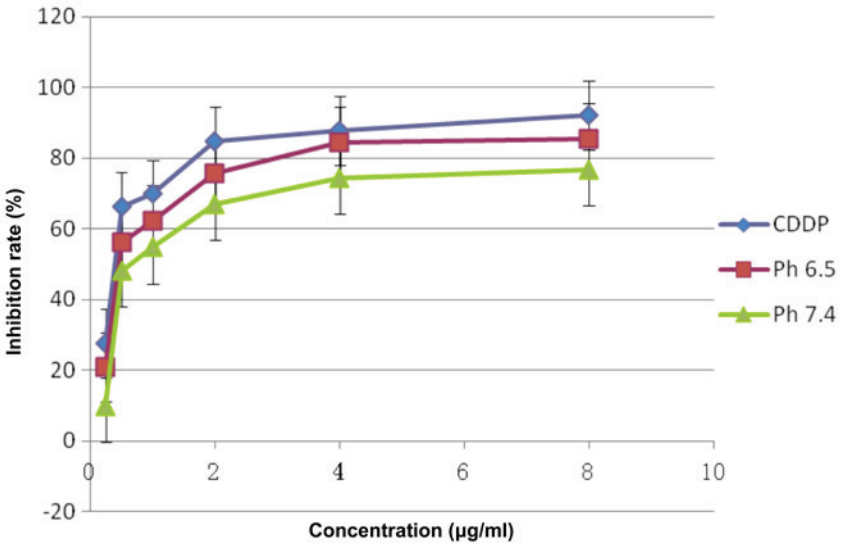

Figure 12. Inhibition ratio of HNE-1 cells treated with different concentrations of CDDP for 24 h. After 24 h, the inhibition rate for cells treated with pH 6.5 or 7.4 FA-PEG-NH-N=MNPs-CDDP were lower when compared with pure CDDP, with greater inhibition in the pH 6.5 group when compared with the pH 7.4 FA-PEG-NH-N=MNPs-CDDP-treated cells. CDDP, cisplatin.

Detection of cell inhibition by MTS assay. The pure CDDP group, and the treatment groups treated with pH 6.5 or 7.4 FA-PEG-NH-N=MNPs-CDDP demonstrate markedly different inhibition ratios. The differences are dose dependent (F=11,325.296; P<0.0001; Figs. 12 and 13). Inhibition of HNE-1 cells occurred regardless of whether the pH value of FA-PEG-NH-N=MNPs-CDDP was 7.4 or 6.5, or whether the culture period was 24 or 48 h; however, inhibition was dose- and time-dependent (P<0.05). The empty carrier, ASA-modified MNPs, exerted little influence on the inhibition of HNE-1 cells (P>0.05). Furthermore, following 24-h culture, the inhibition ratio for cells treated with pH 6.5 or 7.4 MNPs were lower when compared with pure CDDP, with greater inhibition in the pH 6.5 group when compared with the pH 7.4 MNP-treated cells (Fig. 12). However, following culture for 48 h, Figure 13 demonstrates that differences in

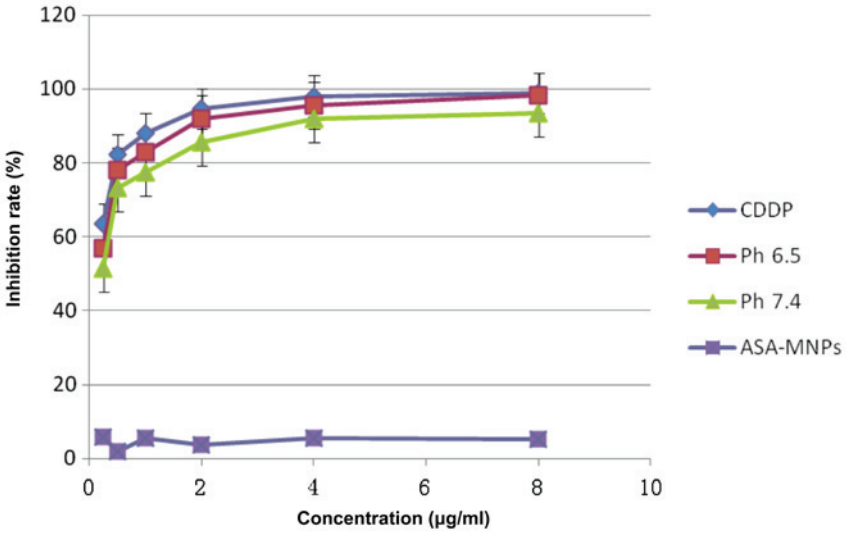

Figure 13. Inhibition ratio of HNE-1 cells treated with different concentrations of CDDP for 48 h. After 48 h, the inhibition rate for cells treated with pH 6.5 or 7.4 FA-PEG-NH-N=MNPs-CDDP were lower when compared with pure CDDP, with greater inhibition in the pH 6.5 group when compared with the pH 7.4 FA-PEG-NH-N=MNPs-CDDP-treated cells. CDDP, cisplatin; ASA, aldehyde sodium alginate; MNPs, magnetic nanoparticles.

inhibition ratios in the pH 7.4 and 6.5 groups reduce although remain statistically significant.

Discussion

CDDP is one of the most effective antitumor chemotherapeutic agents for head and neck cancer. However, CDDP has numerous limitations, including, rapid clearance from the blood, formation of irreversible binding to plasma proteins, it readily hydrolyzes, transforming into reverse CDDP, and causing marked toxicity, loss of antitumor activity and drug resistance.

The present study used a carrier MNP loaded with CDDP to attempt to overcome these limitations and improve the efficacy of treatment with CDDP. pH-sensitive FA-modified CDDP-loaded MNPs were produced. The current study characterized various properties of the drug-delivery system. The freeze-dried powder is black and uniform and when scattered in double distilled water, generates a uniform colloidal solution following ultrasound processing. TEM was used to observe the MNP, which appeared uniform and regular with a mean particle size of 10.2±1.5 nm; dynamic light scattering was used and indicated that the hydrodynamic diameter was 176±1.1 nm, these results indicate the MNPs would be suitable as an active targeting nano-drug-delivery system. The ζ-potential of FA-PEG-NH-N=MNPS-CDDP was -20.22±1.36 mV indicating good stability. A solution containing the CDDP-loaded MNPs also demonstrated good stability, with no marked sedimentation or flocculation occurring over the 2-month observation period at a constant temperature. The CDDP loading capacity and iron content did not significantly change, which further indicated the stability of the product. In addition, it exhibited good superparamagnetism and magnetic responsiveness under a constant magnetic field. MNPs were observed on the tube wall closest to a magnetic field, however became evenly scattered following removal from the magnetic field. Its maximum saturation magnetization, as detected by magnetic curve, was 16.3±0.2 emu/g and the residual magnetism was 0. The concentration of CDDP being transported was 0.773 mg/ml and the iron concentration was 1.908 mg/ml. In addition, the MNPs could be filtered from water by ultrafiltration, enabling acquisition of a high concentration of CDDP.

The dynamic light scattering of pH-sensitive FA-modified CDDP-loaded MNPs measured by laser particle analyzer was 176.6±1.1 nm and the ζ-potential was -20.91±1.76 mV. The particle size as measured by TEM was 10.2±1.5 nm. The particle size obtained with the dynamic light scattering test was greater than that observed by TEM. There are two possible factors resulting in this, firstly dynamic light scattering is more sensitive in measuring larger particles, and, secondly when TEM samples are prepared, due to the volatilization of solvent, the nanometer polymer particle may have collapsed while those undergoing dynamic light scattering analysis are in a solution maintaining their shape (12,14). The dynamic light scattering results of particle size demonstrate that FA-PEG-NH2N=H-CHO-MNPs-CDDP particles had a mean hydrodynamic diameter of 176.6±1.1 nm, the ASA-MNPs had a mean of 198.9±3.4 nm, but that of the CDDP-loaded MNPs (CDDP-MNPs) was 153.4±1.8 nm. These are produced during preparation of the final product. The size of CDDP-MNPs is markedly smaller than ASA-MNPs, possibly due to CDDP complexing with sodium alginate, which results in the linear molecule that was stretched becoming coiled, thus, reducing the hydrodynamic diameter. The pH-sensitive FA-modified CDDP-loaded MNP is formed by wrapping FA-PEG-NHNH2 around the surface of CDDP-MNPs resulting in a larger particle size, although it remains smaller than ASA-MNPs. The ζ-potential of the pH-sensitive FA-modified CDDP-loaded MNPs was -20.91±1.76 mV. The particles in the solution are mutually rejected due to their negative charge, indicating a good colloid stability, however, the negative potential is lower than ASA-MNPs (-29.04±2.17 mV) and CDDP-MNPs (-25.08±0.96 mV). This may be due to the wrapping of the outer layer by FA-PEG-NHNH2 decreasing the carboxyl groups. Therefore, it is not as stable as CDDP-MNPs, but has good stability.

The present study aimed to elucidate the distribution of MNPs within cells following uptake. Only the HNE-1 cells (folate receptor-positive) cultured with FA-PEG-NH-N=MNPs-CDDP demonstrated staining with Prussian blue dye. As the blue stain is due to ferrous ferricyanide precipitation formed by ferric acid and potassium ferrocyanide under acidic conditions, this result indicates that FA-PEG-NH-N=MNPs-CDDP culture is the only method by which MNPs are able to enter the nasopharyngeal carcinoma cell lines. The entry to the cell is mediated by the FA and the FA receptor. The FA-modified MNPs were observed in the HNE-1 cells under TEM following culture for 6 h. The particle diameter is comparable with those prepared above. But above phenomenon are all not seen in normal cells following culture of pure MNPs and HNE-1 for 6 h.

The results of the present study demonstrate that a pH-sensitive FA-modified CDDP-loaded MNP can enter HNE-1 cells due to their upregulated expression of folate receptors on the cell surface. Entering the cell allows CDDP to be released in the low pH environment within the cell.

A pH-sensitive therapeutic agent release system has been demonstrated to be effective in cancer therapy. As the therapeutic agent-loaded MNPs are not released in surrounding tissue, side effects of the therapeutic agent on normal tissue are reduced. Upon reaching the tumor tissue and entering cells by targeting the folate receptors, the MNPs are taken up by endosomes with a pH value between 4.5 and 6.5, this releases CDDP from the MNP. The hydrazone bond between the dianzyl PEG and sodium alginate is hydrolyzed in acidic conditions resulting in the FA-dianzyl detaching from the MNP, which exposes CDDP. This also breaks the bond between sodium alginate and CDDP, releasing CDDP and allowing it to target the DNA. However, when the MNPs are in the blood and normal tissue with a higher pH of 7.4, the hydrazone bond remains stable and CDDP is not released.

The MTS assay demonstrated that administration of CDDP, either directly or via the FA-modified CDDP-loaded MNPs, inhibited the HNE-1 cell proliferation. Following a 24-h culture, the inhibition ratio with each treatment group [FA-PEG-NH-N=MNPs-CDDP (pH 6.5) and FA-PEG-NH-N=MNPs-CDDP (pH 7.4)] increased in a dose- and time-dependent manner. The pH 6.5 delivery group exerted greater inhibition effects than the pH 7.4 group, however, CDDP alone produced greater inhibitory ratio. This suggests that the MNPs require longer to allow the therapeutic agent to dissociate inside the cell and do not reach the concentration achieved by CDDP alone. As MNP (pH 6.5) exerts a greater inhibitory effect, the results suggest the importance of the hyrazone bond in pH sensitivity. However, following 48 h, the inhibition ratio of pure CDDP is similar to the experimental MNP groups, pH 6.5 and 7.4. The longer incubation time may be important in this. The similar values for the different pH groups may be due to the fall in pH following endocytosis, despite the environment being a higher pH in the pH 7.4 group, releasing CDDP to affect the cell. The present study demonstrated that the blank MNPs, at the same concentration, did not influence the growth of HNE-1 cells at 24 or 48 h indicating that MNPs alone were not cytotoxic.

In conclusion, the CDDP content of the pH-sensitive, FA-modified CDDP-loaded MNPs was 0.773 mg/ml, the iron content was 1.908 mg/ml; the mean particle diameter of magnetic nuclei, as observed by TEM, was ~10.2±1.5 nm, the mean hydrodynamic diameter detected using a laser particle analyzer was 176.6±1.1 nm. The ζ-potential was -20.91±1.76 mV and the maximum saturation magnetization intensity was 16.3±0.2 emu/g. These results suggest that the pH-sensitive FA-modified CDDP-loaded MNPs have good stability, magnetic responsiveness and superparamagnetism The hydrazone bond can be hydrolyzed at a low pH, exposing and releasing CDDP. The present study provides a foundation for further investigation into targeted treatment of malignant tumors, particularly nasopharyngeal cancer.

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