Preparation, in vitro and in vivo evaluation of polymeric nanoparticles based on hyaluronic acid-poly(butyl cyanoacrylate) and D-alpha-tocopheryl polyethylene glycol 1000 succinate for tumor-targeted delivery of morin hydrate

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Abstract

Herein, we describe the preparation of a targeted cellular delivery system for morin hydrate (MH), molecular-weight hyaluronic acid-poly(butyl cyanoacrylate) (HA-PBCA) block copolymer. In order to enhance the therapeutic effect of MH, D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) was added to the HA-PBCA during the preparation process. The MH-loaded HA-PBCA “plain” nanoparticle (MH-PN) and TPGS “mixed” nanoparticles (MH-MNs) were concomitantly characterized in terms of load, particle size, zeta potential, critical aggregation concentration, and morphology. The obtained MH-MNs exhibited a spherical morphology with a negative zeta potential and a particle size less than 200 nm, favorable for drug targeting. Remarkably, the addition of TPGS resulted in about 1.6-fold increase in the drug loading. The in vitro cell viability experiment revealed that MH-MNs enhanced the cytotoxicity of cancer cells compared with MH solution and MH-PNs. Furthermore, blank MNs containing TPGS exhibited low cytotoxic effects against cancer cells without diminishing the viability of normal cells. In addition, the uptake study indicated that MNs resulted in 2.28-fold higher cellular uptake than that of PNs, in A549 human lung adenocarcinoma cells. CD44 receptor competitive inhibition and the internalization pathway studies suggested that the internalization mechanism of the nanoparticles was mediated mainly by the CD44 receptors through a clathrin-depndent endocytic pathway. More importantly, MH-MNs exhibited a higher in vivo antitumor potency and tumor cell apoptosis than did MH-PNs, following intravenous administration to S180 tumor-bearing nude mice. Overall, the results imply that the developed nanoparticles are promising vehicles for the targeted delivery of hydrophilic and/or lipophilic anticancer drugs.

Keywords: anti-tumor effect, hyaluronic acid, TPGS, morin hydrate, nanoparticles
Introduction

Morin hydrate (3,5,7,2′,4′-pentahydroxyflavone) (MH), a naturally occurring bioflavonoid identified in fruits, vegetables, and herbs of the Moraceae family, is emerging as a potent therapeutic drug for maladies, including cardiovascular disease, diabetes mellitus, neurodegenerative disease, cancer, and inflammation. This flavonoid has been reported to induce apoptosis in a hepatocellular carcinoma human cultured prostate cancer cells. Additionally, it inhibits the growth of HL-60 cells and breast resistance protein (ABCG2)-mediated transport. In spite of the research progress made on the pharmacological activity of MH, only two formulation techniques have been developed to improve the bioavailability of water-soluble MH: a self-nanoemulsifying MH delivery system based on the phospholipid complex reported by Zhang et al. and a niosomal dispersion composed of nonionic surfactants (Span 60, SP: Tween 60) developed by our group.

The use of biocompatible polymer in the treatment of various ailments has widely expanded over the past few decades. Hyaluronic acid (HA), a naturally occurring polyanionic unbranched polysaccharide, implicated in several biological functions, such as cell adhesion and motility, cell proliferation and wound healing, and even cancer metastasis. More importantly, the overexpression of HA-binding proteins, such as CD44 and RHAMM, has been found on the cell surface of several malignant tumors and that the interaction of hyaluronan with its cell surface receptors is crucial for tumor progression. These properties and unlike dextran and pullulan, HA has demonstrated a weaker specificity toward haptoglobin receptors, contributing to an enhanced accumulation of HA nanoparticles in tumor sites. Based on HA and its derivatives have been popularly used as active tumor-targeting vehicles for various anti-cancer therapies. The importance of HA has elicited research directed toward developing methodology for its chemical modification and conjugation, mainly by targeting its reactive functionalities, such as carboxylic groups, and the HA reducing end. HA-based brush copolymers have been widely applied to a variety of nanoparticles, whereas HA-based block copolymers are more scarce and appealing. Many groups have already worked on the synthesis of HA block copolymers, via different coupling methods. In this study, we prepared HA-poly(butyl cyanoacrylate) (HA-PBCA) block copolymer, previously reported by us and prepared through redox radical emulsion polymerization. However, polymeric nanoparticles formed from a single polymer often lack multiple functionalities, due to the limitation in the number of building blocks. To overcome this limitation, the polymeric nanoparticles consisting of more than one amphiphilic block aroused great interest recently. Based on the advantages of the mixed polymeric nanoparticles (MNs) and simple polymeric nanoparticles (PNs), a large number of MNs have been designed and found to be their individual constituents. So herein, we report the first attempt on the combination of HA-based copolymer with vitamin E derivatives (Figure 1A).

Figure 1
Schematic illustration of (A) the core–shell structure of MH-PNs and MH-MNs, and (B) accumulation of nanoparticles in tumor sites through the EPR effect, intracellular trafficking pathway, and synergistic antitumor effect of MH and TPGS.

The D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) is a water-soluble derivative of vitamin E. It has been widely applied in the food and drug industry as an absorption enhancer and solubilizer in oral, parenteral, topical, nasal, and rectal/vaginal therapies, and is gaining interest in...
development of novel drug delivery formulations. TPGS is usually mixed with other biodegradable materials in the preparation of nanoparticles, to enhance the aqueous solubility of hydrophobic molecules. TPGS can inhibit the multidrug resistance transporter, P-glycoprotein (P-gp), thus increasing the oral bioavailability of many anticancer drugs. TPGS can also greatly improve the performance of TPGS-emulsified nanoparticles leading to enhanced cellular uptake and in vitro cancer cell mortality, and more desirable in vivo pharmacokinetics. In addition, TPGS has been reported to efficiently inhibit the growth of human cancer cells both in vitro and in vivo. Furthermore, TPGS produces synergistic growth inhibition combination with other anticancer drugs due to its ability to induce apoptosis in tumor cells.

Although there have been many HA-based PNs exploited for drug delivery, this is the first report on formulation of a multifunctional drug delivery system based on HA block copolymer and TPGS. We hypothesized that MNs made of HA-PBCA and TPGS may allow for a better drug encapsulation, stability, and a stronger anticancer activity. Our study focuses on combining functional homopolymers to build a delivery system that can reach the tumor passively through the leaky surrounding vasculature by the permeability and retention effect (EPR) of TPGS, and actively through the binding of HA to the receptors overexpressed by cancer cells or angiogenic endothelial cells (as shown in Figure 1B). TPGS can facilitate the cellular uptake owing to its biodeadhesive properties. In the present work, we describe the preparation of PN and MH-MNs as carriers for targeted delivery of MH. The prepared formulations were characterized for particle size, zeta potential, entrapment efficiency, and drug-loading (DL). The in vitro cellular uptake of MH was investigated receptor-mediated tumor-targeting characteristics, and the cytotoxicity were evaluated against the human adenocarcinoma cell line. Here, the study aimed to unveil whether TPGS could synergistically enhance the cytotoxicity of MH. Finally, the antitumor efficacy of MH-loaded nanoparticles against S180 tumor models was evaluated.

Materials and methods

Materials

Sodium HA (molecular weight [MW] = 10 kDa) was purchased from Zhenjiang Dong Yuan Biotech Co., Ltd (Zhenjiang, People’s Republic of China). Butyl cyanoacrylate (BCA) monomers were obtained from Suncon Medical Adhesive Company (Beijing, People’s Republic of China). MH, TPGS, 3-[4,5-dimethyl-2,5-diphenyl-tetrazolium bromide (MTT), nystatin, and amiloride were purchased from Sigma (St Louis, MO, USA). Propidium iodine (PI) was purchased from Beyotime Biotechnology (Nanjing, Republic of China). All other solvents and chemicals used were of high-performance liquid chromatography (HPLC) and analytical grades.

Cell lines and cell culture

Human lung adenocarcinoma (A549) and human hepatic (L02) cell lines were purchased from Am Culture Collection (ATCC) (Manassas, VA, USA). Cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 100 U/mL streptomycin, 100 U/mL penicillin at 37°C in a humid atmosphere (5% CO₂, 95% air). For subculture, cells were detached with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution, and seeded into new flasks.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4293365/ 2015-3-3
**Synthesis and characterization of HA-PBCA**

HA-PBCA was synthesized as described previously by He et al.\textsuperscript{15} with a little modification. Briefly, it was dissolved in 9 mL of nitric acid (0.2 mol/L) at 40°C and stirred for 10 minutes under nitrogen. Ammonium cerium nitrate (0.8 mmol/L) solution in 1 mL nitric acid (0.2 M) and BCA were successively added under vigorous agitation. Nitrogen bubbling was maintained for 10 minutes. Then the reaction was continued under gentle stirring for another 50 minutes. Reactants were transferred into a dialysis membrane (cutoff [MWCO], 3,500 Da) and were exhaustively dialyzed against water for 3 days. The suspension was lyophilized for 2 days and then stored at 4°C for further use.

**Preparation of MH-PNs and MH-MNs**

MH-PNs and MH-MNs were prepared by a probe-type ultrasonic and dialysis method as previously described by Li et al.\textsuperscript{9} Briefly, 15 mg of HA-PBCA copolymer was dissolved in 3 mL of phosphate-buffered saline (PBS) pH 7.4, then MH in 0.5 mL dimethyl sulfoxide (DMSO) was added dropwise to the polymer suspension at 150 rpm at room temperature; the final mixture was ultrasonicated for 30 minutes in an ice bath. The solution was dialyzed against excess amount of distilled water for 12 hours using a dialysis bag of 3,500 Da, followed by filtration through a 0.45 μm pore-sized microporous membrane and lyophilized. MH-MNs were prepared similarly, except HA-PBCA solution was replaced by a mixture of HA-PBCA (0.5 mg/mL) and TPGS solution in PBS pH 7.4 was used at different weight ratios.

**Critical aggregation concentration (CAC) determination**

The CAC was determined by fluorescence spectroscopy using pyrene as a hydrophobic probe.\textsuperscript{26} Briefly, 6.0×10\textsuperscript{−6} M pyrene solution in acetone was added to a series of 10 mL volumetric flasks, and acetone was subsequently completely evaporated under a gentle nitrogen gas stream for 1 hour at 60°C. HA-PB at different weight ratios, with concentrations ranging from 0.000001 to 1.0 mg/mL, were added to achieve a final pyrene concentration of 6.0×10\textsuperscript{−7} M, followed by sonication for 30 minutes. The solutions were incubated at 50°C for 1 hour and then left to cool down overnight at room temperature. Fluorescence spectra were obtained using a fluorescence spectrophotometer (RF-5301 PC; Shimadzu Corp, Kyoto, Japan) at a wavelength of 390 nm and slit widths of 3 nm for both excitation and emission. The CAC was estimated as the cross point when extrapolating the intensity ratio I338/I333 at low and high concentration regions.

**Morphology, particle size, and zeta potential analysis**

The particle size and polydispersity index were assessed by dynamic light scattering (DLS) (Nanol Particle Size Analyzer; Brookhaven Instruments Corp, New York, NY, USA) at 25.0°C±0.1°C by autocorrelation function at 90°. The mean size and standard error were directly measured by the instrument. The zeta potential of the nanoparticles was determined using a Zeta plus zeta potential analyzer (Brookhaven Instruments Corp) at 25°C. Each experiment was carried out in triplicate. The morphology size distributions were observed by transmission electron microscopy (TEM) under a JEM-2100 E Microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV. A drop of nanoparticle suspension was placed onto a copper grid and left to adhere for 2 minutes. The excess suspension was wicked off with paper, and air-dried before observation.
**Drug-loading and encapsulation efficiency**

The nanoparticles solution was suitably diluted with ethanol (20 times) and sonicated for 30 minutes. Ultrasound Sonicator; Kunshan Ultrasound Instrument Co., Ltd, Kunshan, People’s Republic of China was used, and then centrifuged at 12,000 rpm for 10 minutes at 4°C. The MH content in the supernatant was assayed using Shimadzu LC-2010 CHT system; Shimadzu Corp) equipped with an XTerra MS C18 reversed-ph (250×4.6 mm, 5 μm) used in isocratic mode at 30°C. The mobile phase consisted of a mixture of 10 mM KH₂PO₄ (28:72, v/v) at pH 2 (phosphoric acid) with a flow rate of 1.0 mL/min. Detection was at a wavelength of 260 nm, and the injection volume for samples was 20 μL. The standard curve ranged from 1 to 100 μg/mL, with a correlation coefficient (R²) value of 0.9999. The accuracy was three replicate injections of three different concentrations of MH, and a percent accuracy and relative deviation of repeatability found to be between 99.31% and 100.67% and less than 2%, respectively. Validation studies revealed that the proposed method is suitable for determination of MH concentration in nanoparticle dispersions.

The entrapment efficiency (EE) and DL were calculated using the following equations:

\[
EE(\%) = \frac{\text{Weight of MH in polymeric nanoparticles}}{\text{Weight of MH fed initially}} \times 100
\]

\[
DL(\%) = \frac{\text{Weight of MH in polymeric nanoparticles}}{\text{Weight of polymeric nanoparticles}} \times 100
\]

**Differential scanning calorimetry**

Differential scanning calorimetry (DSC) of the samples was performed using a NETZSCH DSC 204 F1 Phoenix, Germany. Samples were heated from ambient temperature to 300°C at a constant heating rate of 10°C/min in a nitrogen atmosphere.

**In vitro cytotoxicity studies**

In vitro cytotoxicity of the blank nanoparticles was evaluated by the MTT assay. Briefly, A549 and MCF-7 cells were seeded at a density of 5×10³ cells per well in 96-well plates and cultured overnight at 37°C in RPMI-1640 supplemented with 10% FBS. Afterwards, the culture medium was removed, and the cells were incubated for 72 hours in a serum-free medium containing blank nanoparticles at various concentrations. 20 μL of MTT solution (5 mg/mL) was added to each well, and the cells were further incubated for 4 h. The supernatant in the wells was discarded, and 150 μL of DMSO was added to dissolve the substrate. The absorbance in each well was recorded at 570 nm using a microplate reader (Bio-Rad Laboratories, CA, USA). This experiment was performed thrice.

The cytotoxicity of MH-loaded nanoparticles was evaluated by staining nonviable cells with PI27 conducted according to the manufacturer’s protocol. Briefly, cells in 12-well plates were incubated with increasing concentrations of free MH, MH-PNs, or MH-MNs. The culture medium without any fetal bovine serum (FBS) was used as the control. Afterwards, the cells were washed with PBS and harvested by trypsinization. The cells were incubated for 1 h with PBS supplemented with 10% FBS to stop the trypsin action, followed by centrifugation and washing with cold PBS, twice. Then, 20 μL of PI (10 μg/mL in PBS pH 7.4) was added to 200 μL of the cell suspension. The numbers of necrotic cells were immediately evaluated with a flow cytometer (FCM; FACSCalibur™; BD Biosciences, Franklin Lakes, NJ, USA).
In vitro cellular uptake

**Fluorescence microscopy** To evaluate the cellular uptake of PNs and MNs in A549 human lung adenocarcinoma cells, fluorescent probe coumarin-6 (C6) was encapsulated in the nanoparticles, in the same way as MH-loaded nanoparticles. The C6-loading of the two formulations was 0.2%.[28] Moreover, no more than 0.01% of the encapsulated C6 was released from the nanoparticles after 12 hours of incubation in PBS pH 7.4 to guarantee that the fluorescence signal detected in the cells was attributed to C6 encapsulated into the nanoparticles. Cells were seeded in 12-well plates at a density of $2 \times 10^5$ cells/well and incubated for 37°C to allow their attachment. To investigate whether nanoparticles were taken up through HA receptor-mediated endocytosis, cells were incubated with 10 mg/mL of free HA polymer for 1 hour prior to the addition of the medium. The medium was replaced by 0.25 mg/mL of C6-PNs or C6-MNs diluted in serum-free medium (C6 content: 500 ng/mL).[29] After 2, 4, and 6 hours of incubation, the culture media were the same as those used for the fluorescence microscope (IX71; Olympus Corp, Tokyo, Japan)[30] and a Leica scanning microscope (CLSM) (TCS SP5; Leica, Heidelberg, Germany).

**Flow cytometry** The quantitative measurement of C6 fluorescence intensity was conducted using BD FACSCalibur FCM. The intracellular uptake was conducted as described in the previous section. At time intervals, the culture medium was removed and all cell samples were washed with PBS for three times. Cells were harvested by trypsinization and collected by centrifugation. Following supernatant aspiration, cells were washed with PBS twice and then resuspended in 0.5 mL of PBS prior to fluorescent intensity measurements.[31]

Exploring uptake pathways of PNs and MNs using endocytic inhibitors In order to evaluate the effect of endocytic inhibitors on the uptake of the polymeric nanoparticles, A549 cells were preincubated in the following inhibitors: 0.45 M sucrose, 15 μg/mL nystatin, 50 μM amiloride, and 10 mg/mL of free HA polymer for 1 hour prior to the addition of nanoparticles. Subsequently, cells were washed three times with cold PBS (pH 7.4), harvested, collected by centrifugation, and then washed twice with PBS for FCM analysis (BD FACSCalibur). All measurements were performed in triplicate.

In vivo antitumor activity

The in vivo antitumor activity of the nanoparticles was evaluated in a subcutaneous S180 murine sarcoma xenograft model in nude mice. To set up the tumor xenograft model, mice were subcutaneously inoculated with 1.5 × 10^6 cells in 200 μL PBS.[35] When the tumor volume reached approximately 100 mm^3 post-tumor inoculation, mice were randomized divided into six groups (n=7 for each group) as follows: 1) blank PNs; 2) blank MNs; 3) MH drug solution (15 mg/kg); 4) MH-PNs (15 mg/kg); and 6) MH drug solution (15 mg/kg). All groups were injected intravenously via the tail vein (noted as day 0). The therapy was administered at 2-day intervals, with a 1-day interval between two administrations. The rate of survival was calculated based on the Kaplan-Meier plot. Tumor sizes were measured every other day. At 2 days after the last injections, two of each group were randomly chosen and sacrificed to prepare the tumor sections. Tumors were dissected, weighed, and then fixed in 10% formalin. Formalin-fixed tumors were embedded in paraffin, sectioned, stained with hematoxylin and eosin (HE)-stained tumor sections. Furthermore, in vivo tumor growth was investigated by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using an apoptosis detection kit (KeyGen Bio-tech, Nanjing, People’s Republic of China) following the manufacturer’s protocol. The sections were then visualized under a fluorescence microscope (Olympus IX71; Olympus Corp, Tokyo, Japan) and a Leica scanning microscope (CLSM) (TCS SP5; Leica, Heidelberg, Germany).

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each tumor tissue sample, and the apoptotic index was calculated by dividing the number of apoptotic positive cells by the total number of cells.

**Statistical analysis**

All experiments were repeated at least three times. The data are expressed as mean ± SD. Statistics were performed using Student’s t-test, and differences were judged to be significant at \( P<0.05 \).

**Results and discussion**

**Effect of HA/BCA molar ratio on particle size**

Redox radical emulsion polymerization, initiated by a redox system composed of a polysaccharide in the range of 10 to 50 kDa and Ce⁴⁺, first developed by Passirani et al36 has opened a new perspective for the development of new drug delivery systems with versatile properties. The particle size of HA-PBCA reported by He et al was mainly dependent on the MW of HA, the cerium ammonium nitrate concentration, and the HA to BCA ratio.15 It is well known that small micelle size is expected to prevent uptake by the reticuloendothelial system and facilitates extravasation at leaky sites of capillaries, leading to passive accumulation in certain tissues, e.g., tumors.37 However, the particle size reported by the group was 290 nm, which was not favorable for drug-targeting. Therefore, we herein report the preparation of nanoparticles from low-molecular-weight HA (10 kDa), where both the concentration of cerium ammonium nitrate (0.8 mmol/L) and the reaction time (1 hour) were kept constant. As shown in Figure 2, the PBCA nanoparticles prepared at different molar ratios were in the range of 155.7–228.9 nm, with a higher turbidity, indicating that a large number of nanoparticles were formed. Increase in BCA amount did remarkably influence the particle size of the nanoparticles; these results comply with those of a previous study. Therefore, nanoparticles prepared with HA:BCA at a molar ratio of 1:1 were selected to conduct further experiments.

![Figure 2](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4293365/)

**Figure 2**

Effect of HA/BCA molar ratio on particle size.

**Critical aggregation concentration**

Polymeric nanoparticles are prone to deaggregation upon dilution incurred in the biological environment. Therefore, CAC is considered as an important parameter to evaluate nanoparticles stability.39 The PBCA and HA-PBCA/TPGS could be accurately determined from the plot of the intensity ratio \( I_33 \) as a function of the concentration of block copolymers (Figure 3). The CAC value of HA-PBCA was 0.0089 mg/mL, which is comparatively lower than the CAC value of different types of HA-based block copolymers intended for the delivery of anticancer therapeutics, such as HA-poly(lactic-co-glycolic acid) (HA-PCLG) = 0.0089 mg/mL.40 Moreover, HA-PBCA/TPGS exhibited lower CAC value, of 0.00398 mg/mL, with HA-PBCA, due to the π–π interactions induced by the aromatic rings of tocopherol (which has been identified as a CAC-lowering factor).41 These results underlie the high stability and the ability of MNs to maintain their integrity upon extreme dilution in the body.

![Figure 3](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4293365/)

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4293365/
Preparation and characterization of MH-loaded nanoparticles

In this study, self-assembled nanoparticles consisting of biocompatible HA and PBCA block copolymers were formed using a simple ultrasonic method. MH as a model hydrophobic drug was physically incorporated into the inner core of the polymeric nanoparticles. The characteristics of MH-PNs and MH-MNs, including particle size, are summarized in Table 1. We found that the mean diameters of blank PNs and of Mn155.7±5.1 nm and 170.36±2.21 nm, respectively, with a low polydispersity index, of about 0.056±0.130±0.089, respectively. Encapsulation of MH did not significantly increase the average size and distribution of the PNs. As expected, the surface charge of MH-PNs was negative, with a zeta potential of −25.18±0.31, due to the presence of HA in the nanoparticles shell, which would prevent aggregative electrostatic repulsion. The DL and the EE of MH-PNs were about 4.67%±0.71% and 32.61%±1.7%, respectively. Many studies have reported that the major factor influencing both the DL capacity and the interactions between the drug and the PBCA block.42 The relatively low EE of MH is due to the weak interactions between MH and PBCA core leakage of the drug into the dispersion medium during nanoparticle formation.43 Furthermore, it was reported that drug molecules with small molecular weight, such as MH, have a faster diffusion coefficient and may thus escape the forming polymer network, leading to a low EE.44 Aside from low polymer-drug compatibility and interactions, low efficiency may also be due to the high degree of interdigitation chains in the core, which logically reduced the available space for MH and hampered its encapsulation.

| Table 1 |
| Physical characterization of polymeric nanoparticles in PBS pH 7.4 |

The addition of TPGS to HA-PBCA led to a slight decrease in the nanoparticles size (134.73±1.85 nm) and increase in negative zeta potential (−31.72±0.17), thereby confirming the stabilizing ability of TPGS. Interestingly, the addition of TPGS resulted also in a significant increase in DL, proportional with the TPGS feed ratio. The DL of MNs made of HA-PBCA and TPGS at the mass ratio of 1:0.5 (0.2 g of TPGS) was increased by 7.51%±0.29% compared with 4.67%±0.71% for the nanoparticle made of pure HA-PBCA. This was explained by the fact that TPGS possesses a bulky shape and has large surface areas. Such a good emulsifier can emulsify a wide range of hydrophobic drugs, including MH.45 Gao et al. explained the increased EE resulting upon addition of TPGS to nanoparticles by more “loose” and structure because of the aromatic ring of TPGS, which may cause stronger hydrophobic interactions between drugs and polymers.46
Moreover, both MH-PNs and MH-MNs suspensions could be freeze-dried and properly redisperse solution under stirring and gentle sonication conditions. In addition, the MH-loaded nanoparticles exhibited excellent colloidal stability over several months at 4°C, which is crucial regarding the storage stability of the formulated MH. TEM studies (Figure 4B) showed that MH-loaded polymeric nanoparticles (MH-PNs) displayed a uniform spherical morphology. The particle size obtained by TEM was smaller than that obtained by DLS due to the shrinkage of the nanoparticles during the drying process.28

DSC was employed to assess the physical status of MH inside the PNs and the MNs. The DSC thermogram (Figure 4C) of MH-PNs and MH-MNs exhibited endothermic peaks similar to those of blank nanoparticles, suggesting that MH was successfully encapsulated inside the lyophilized nanoparticles.

**In vitro cytotoxicity studies**

Figure 5A depicts the percentage of viable A549 cells incubated with blank nanoparticles for 72 h. Blank PNs had no significant effect on the viability of A549 at concentrations ranging from 1 to 50 μg/mL. However, a small decrease in the cell viability was observed. In contrast, blank MNs achieved a cytotoxic effect than blank PNs (P<0.001) due to the ability of TPGS to trigger the mitochondrial apoptosis in A549 cells.47 To investigate whether blank nanoparticles could affect the viability of the cytotoxicity was assessed in L02 cells by MTT assay for a period of 72 hours (Figure 5B). Both blank PNs and blank MNs did not show a significant toxicity against L02 cells, except at very high concentrations of blank MNs (500 mg/mL). This concentration is, however, much higher than the concentration of the half maximal inhibitory concentration (IC_{50}) of MH-MNs. Together, these findings revealed that TPGS selectively exhibit chemotherapeutic effects against cancer cells without diminishing the cell viability. Previous investigations have also reported the selective effect of TPGS for cancer growth inhibition. A recent study has shown that the selective chemotherapeutic effects of TPGS may possibly be attributed to the downregulation of the antiapoptotic proteins survivin and Bcl-2, which are overexpressed in most cancer cells. However, the exact mechanism responsible for this selectivity is not clear yet.

Previous studies conducted by Manna et al using different cell lines including A549 indicated that morin suppresses the activation of NF-κB and NF-κB-regulated gene expression, leading to an enhancement of apoptosis.51 The tumor specificity of morin was reported to be mainly exerted via both the 2′ and 4′ hydroxyl groups.52 It was further found that morin-induced apoptosis in HL-60 cells was associated with an increase in intracellular reactive oxygen species (ROS).6
Cytotoxicity was evaluated by the PI assay because of the interference of MH with the mitochondrial leading to a false cytotoxicity evaluation by MTT assay. We found that the cytotoxic effect of MH was time-dependent. In compliance with previously published results, the significant apoptotic effect of MH appeared after 72 hours.[4,6] Interestingly, the results shown in Figure 5C demonstrate that MH encapsulated nanoparticles achieved higher cytotoxicity compared with free MH. This can be attributed to the fact that nanoparticles could be more efficiently taken up via CD44 receptor-mediated endocytosis (hence delivery of MH into cells and resulting in an increased cytotoxicity compared with the passive diffusion of free MH). After 72 hours of incubation, the IC$_{50}$ of free MH against A549 cells was 56.23 μM, whereas the IC$_{50}$ values for MH-PNs and MH-MNs were 45.76 μM and 28.89 μM, respectively. Moreover, MH-MNs showed a higher potency compared with MH-PNs. The synergistic antitumor activity of TPGS in the presence of MH may be due to its ability to induce ROS-generation and apoptosis.[54,55] Besides the two above reasons, another factor to the improved cytotoxicity of MH-MNs over MH-PNs and free MH is the ability of both TPGS and MH to inhibit the P-gp protein expressed in A549 cells and reduce drug efflux.[56–58]

**Cellular uptake**

HA-PBCA forms a core–shell structure block copolymer that can self-assemble into polymeric micelles. The advantages of this structure are that hydrophobic MH can be encapsulated in the core, and the shell composed of HA can shield the nanoparticles from the attack of plasma proteins. Moreover, the segment exposed toward the surface of the nanoparticles can target CD44 and RHAMM that are overexpressed in tumor cells. Although MH was reported to display some fluorescence either by itself or by forming metal complexes,[59–61] we found that its fluorescence in A549, HepG2, and HeLa cells at 10 μg/mL concentration range was negligible. MH fluorescence-quenching can be due to interactions with cell components and need further investigation.[62] To address the concept of HA receptor-targeting, C6 as a model molecule was entrapped in the nanoparticles and the endocytosis kinetics were monitored by fluorescence microscopy. C6 fluorescence intensity was further quantified by FCM at different time points. A549 cells overexpressing CD44 receptors were used for cellular uptake evaluation.[63]

As shown in Figure 6A and B, both fluorescence microscopy and CLSM revealed bright green fluorescent signals in the cytoplasm of A549 cells, which was attributed to C6, indicating that C6-incorporated nanoparticles had successfully entered the cells. However, under the same conditions, the C6-loaded nanoparticles displayed remarkably higher intensity compared with free C6. The intracellular uptake was quantitatively analyzed by FCM, as seen in Figure 6C and D. The fluorescence intensities of C6-PNs and C6-MNs were approximately 5-fold and 15.5-fold higher, respectively, than that of free C6 after incubation for 4 hours. These results suggest that C6-loaded nanoparticles internalized into cells via overexpressed CD44 receptors. The fluorescence signal gradually became stronger with extended incubation time, indicating that the intracellular uptake of the nanoparticles increased in a time-dependent manner. Moreover, reports indicate that MH can interact with CD44 mainly through its carboxylates groups, which can engage into polyelectrolyte complexes with the positively charged domain of CD44 receptors.[55,64] Therefore, the high efficacy of cell uptake of nanoparticles in A549 may be due to the fact that the chemical modification of HA at the end of its chains left the carboxylates groups along the polysaccharide chain disposed to interact with the CD44 receptors.

![Figure 6](image_url)

*In vitro evaluation of cellular internalization.*

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4293365/
The higher fluorescence intensity of C6-MNs implied higher intracellular uptake capabilities for them compared with PNs. Indeed, the cellular uptake study showed that mixed nanoparticles with TPGS -fold and 2.28-fold higher cellular uptake compared with the ones without TPGS after 2 and 4 hours incubation, respectively. We have previously demonstrated that TPGS could significantly increase uptake of TPGS-phospholipid-C6 micelles in A549 cells as compared with phospholipid-C6 comp of the TPGS absorption enhancement effect. TPGS-emulsified, drug-loaded PLGA nanoparticle shown higher cellular uptake than emulsified poly(vinyl alcohol) (PVA) nanoparticles, due to biop interactions with the membrane. Zhao and Yung have also demonstrated that the cellular uptake of FOL mixed micelles with TPGS was higher compared with the ones without TPGS. Our in vitro confirmed these previous findings.

To further elucidate the mechanism of internalization, a competitive experiment was performed by cells with free soluble HA prior to incubation with the nanoparticles. As expected, both fluorescence and FCM demonstrated a decrease in the fluorescence intensity in tumor cells pretreated with HA compared with the untreated cells. This result suggests that free HA could competitively bind to CD44 receptors and block the binding of C6-loaded nanoparticles to these receptors. However, the extent of the fluorescence remained unchangeable with or without blocking when cells were incubated with free C6, which was passive diffusion or other energy-dependent mechanism. These observations confirmed that the predominantly trafficked into cells via a receptor-mediated endocytosis pathway.

**Exploring uptake pathways of nanoparticles**

The elucidation of the exact intracellular uptake pathway of the nanoparticles remains challenging as pathways are still insufficiently understood, and the functions of some proteins involved in cellular uptake are still uncertain. The intracellular fate of nanoparticles is ultimately linked with the route of entry dependent on the surface properties of the nanoparticles. Endocytosis represents the major transmembrane mechanism for nanomedicines across the membrane, including two major categories: phagocytosis or “cell eating”, involving uptake of particles larger than 0.5 μm; and pinocytosis or “cell drinking” or and suspensions containing small particles. Pinocytosis can be further subdivided into macropinocytosis-dependent, caveolin-dependent, and clathrin/caveolin-independent pathways.

In order to explore the cellular uptake pathway of PNs and MNs, an uptake inhibition study was carried out by pretreating A549 cells with different chemical inhibitors, each specific for a particular endocytic pathway. Hypertonic sucrose, the most popular inhibitor of clathrin-mediated endocytosis, has been shown to disassemble clathrin lattices at the plasma membrane. Nystatin is a sterol-binding agent that disassembles caveolae and cholesterol in the membrane without affecting the clathrin-dependent internalization. Amiloride is a specific inhibitor of macropinocytosis, by interfering with the Na+ protein in the plasma membrane.

The results of the internalization of C6-PNs and C6-MNs in the presence of different pathway inhibitors are presented in Figure 7. A549 cells incubated with C6-PNs and C6-MNs without inhibitors were used as control groups, with hypertonic sucrose solution reducing the cellular uptake of PNs by 36.84% and 44.93%, respectively. These observations provide strong evidence that the entry of the nanoparticles depends upon clathrin-mediated endocytosis. A slight decrease in the uptake of the nanoparticles after incubation with amiloride was observed, implying that macropinocytosis was partly involved in the internalization process of these nanoparticles. The obtained results suggest that the internalization process occurs via clathrin-mediated endocytosis, while a minor fraction could be internalized via macropinocytosis.
uptake mechanisms of the developed nanoparticles showed good correlations with the previously reported nanoparticles studies. In fact, both pH-responsive HA-g-poly(L-histidine) copolymer micelles conjugated HA-octadecyl (FA-HA-C18) copolymer, developed by Qiu et al and Liu et al respectively, internalized into cells mainly via clathrin-mediated endocytosis.53,77

**Figure 7**
Effect of endocytic inhibitors on the internalization of C6-PNs and C6-MNs in A549 cells (n=3).

**In vivo antitumor activity**

To confirm the antitumor potential of MH-loaded nanoparticles in vivo, the antitumor efficacy was evaluated using the subcutaneous S180 tumor xenograft model. As shown in Figure 8A, saline and blank PNs groups showed a significant increase in tumor growth in S180 tumor-bearing mice over the whole period of the experiment. Mice groups treated with free MH and blank MNs exhibited a higher growth delay in comparison with the control group, which confirmed the antitumor effect of MH and TPGS. Interestingly, significant suppression of the tumor growth was observed during the treatment with MH-PNs and MH-MNs. In addition, MH-MNs exhibited an evidently higher tumor inhibitory effect than other groups.

**Figure 8**
In vivo antitumor efficacy in S180 tumor-bearing nude mice.

**Figure 8B** shows the Kaplan–Meier survival curves of S180 tumor-bearing mice injected with physiological saline, blank nanoparticles, free MH, and MH-loaded nanoparticles. All mice in the saline, blank PNs groups died within 20 days after initial treatment. The survival time of mice treated with blank MNs groups turned out to be longer than that of MH-treated animals. Such superiority could be attributed to the circulation time and the targeting ability of blank MNs toward CD44 receptors, leading to a higher concentration of TPGS in the tumor site compared with MH injection, which was eliminated more rapidly. In contrast, MH-MNs contributed to prolonged survival time of the treated mice, with survival rates of 57% until day 20, respectively.

Besides attenuating the tumor growth, the median survival time of mice treated with MH-PNs and MH-MNs was significantly longer than that of mice treated with physiological saline, blank nanoparticles, and MH-PN groups. The better survival rate of the groups administered with blank MNs and MH-loaded nanoparticles could be attributed to the targeting capability of HA as it increased the binding affinity to CD44 receptors in the tumor site. HA employed has a low molecular weight (10 kDa), long enough to bind to CD44 but too short to bind to hyaluronan receptor for endocytosis (HARE) on liver sinusoidal endothelial cells, thus avoiding clearance by liver.78 The prolonged survival time obtained with MH-MNs over MH-PNs is mainly attributed to the activity of TPGS and to the synergistic combination of active targeting through HA and passive targeting by the EPR effect of TPGS, leading to more accumulation at the tumor site.

The improved antitumor efficacy of the different MH formulations in mice was further confirmed by histopathology analysis. The representative HE-stained tumor sections from the different experimental groups showed a reduction in tumor size and a decrease in the number of tumor cells. These findings are consistent with the in vivo antitumor efficacy results, suggesting that MH-loaded nanoparticles can be a promising therapeutic approach for the treatment of S180 tumor xenografts.
mice are displayed in Figure 8C. The groups injected with saline and blank PNs showed the typical characteristics of tumor, such as closely packed tumor cells. The image showed a massive cancer center after the administration of MH, blank MNs, MH-PNs, and MH-MNs, which presents substantial overall efficient antitumor activity of MH, TPGS, and MH-loaded nanoparticles in vivo. In particular, MH exhibited the most effective antitumor activity due to the synergistic anticancer effect of MH and TPGS combined passive and active targeting effects. The in vivo results were consistent with those obtained in vitro.

To analyze the degree to which MH-loaded nanoparticles induced apoptosis in vivo, we performed an assay on tumor sections. As shown in Figure 8C, tumors from the MH-MNs-treated group exhibit advanced cell apoptosis (TUNEL-positive nuclei are stained in dark brown) compared with the other groups. Quantification of the TUNEL-positive spots (Figure 8D) for tumor sections from the groups treated with blank MNs or MH contained 9.92% and 8.94% positive spots, respectively. The number of TUNEL-positive cells increased to 11.82% in the group treated with MH, and more importantly, to 16.67% in the group treated with MH-MNs, demonstrating that MH-MNs induced advanced cell apoptosis and necrosis in tumor tissue at a much higher level than that observed in the other groups.

Conclusion

In this study, HA-PBCA was synthesized through redox radical emulsion polymerization to encapsulate the anticancer drug MH for specific targeting of CD44-overexpressing cancer cells. TPGS was further conjugated with HA-PBCA during the self-assembly process in order to enhance the therapeutic efficacy of MH, with a high IC50 value. The prepared PNs and MNs displayed a low CAC, a small spherical morphology, and a reasonable DL. MH-MNs exhibited more potent cytotoxic effect towards A549 cells compared with the MH solution, mainly due to the synergistic effect of the combination of TPGS with MH in one delivery system. Furthermore, the newly developed nanoparticulate system demonstrated an excellent cellular uptake efficiency in A549 cells, with mixed nanoparticles showing higher cellular uptake compared with their counterparts with a high IC50 value. Endocytosis inhibition studies revealed that the nanoparticles were internalized mainly through clathrin-mediated endocytosis. Moreover, the intravenous administration of MH-loaded nanoparticle into S180 tumors resulted in a significant reduction of tumor volume. All these results suggest that the combination of MH and TPGS as well as tumor-targeted delivery using HA-based polymeric nanoparticles can be utilized as an effective treatment for cancer.

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Footnotes

Disclosure

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