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Magnetic tumor targeting of β -Glucosidase immobilized iron oxide nanoparticles

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Abstract

Directed enzyme/prodrug therapy (DEPT) has promising application for cancer therapy. However, most current DEPT strategies face shortcomings such as the loss of enzyme activity during preparation, low delivery and transduction efficiency in vivo, difficult to be monitored. In current study, a novel magnetic directed enzyme/prodrug therapy (MDEPT) was set up by conjugating β -Glucosidase (β -Glu) to aminated, starch-coated, iron oxide magnetic iron oxide nanoparticles (MNP), abbreviated as β -Glu-MNP, using glutaraldehyde as the crosslinker. This β -Glu-MNP was then characterized in detail by size distribution, zeta potential, FTIR spectra, TEM, SQUID and magnetophoretic mobility analysis. Compared to free enzyme, the conjugated β -Glu on MNP remained $85.54\% \pm 6.9\%$ relative activity and showed much better temperature stability. The animal study results showed that β -Glu-MNP display preferable pharmacokinetics characteristic in relation to MNP. With adscititious magnetic field on the surface of tumor, a significant quantity of β -Glu-MNP was selectively delivered into a subcutaneous tumor of glioma-bearing mice. Remarkably, the enzyme activity of the delivered β -Glu in tumor lesions showed as high as 20.123 ± 5.022 mU/g tissue with 2.14 of tumor/non-tumor of β -Glu activity.

Keywords

β -Glucosidase; magnetic iron oxide nanoparticles; magnetic directed enzyme/prodrug therapy; 9L-glioma

1. Introduction

Cancer is a major public health problem in the world, responsible for almost 30% of all deaths each year [1]. Chemotherapy has been used to treat cancers for more than five

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decades with varying success. Conventional chemotherapeutics, however, raise concerns of side-effects due to narrow therapeutics indexes and relatively poor selectivity for neoplastic cells. A potential strategy to overcome these limitations is the use of prodrugs and prodrug-activating enzymes [2].

Directed enzyme prodrug therapy (DEPT) has been investigated as a means to improve tumor selectivity of therapeutics. This strategy comprises the delivery of prodrug-activating enzymes or their encoding genes to the tumor before administering a prodrug. The tumor-targeted enzyme converts the prodrug to an active anticancer drug, achieving high concentrations in the tumor and sparing normal tissue. Carriers for enzymes or their encoding DNA include antibodies (ADEPT) [3], viruses (VDEPT) [4] and lectin (LEAPT) [5]. The limitations of bioactive carriers such as antibodies are loss of enzyme activity, the scarcity of tumor-specific antigens, poor delivery efficiencies, the immunogenicity of the carrier, difficulty of monitoring, uncontrollable phagocytosis and pharmacokinetics characteristics [6]. Gene expression carriers face more problems, such as the poor transduction efficiency, the inefficient expression of enzymes from the targeted DNA, also the difficulty of monitoring. Enzyme stability is an additional factor that must be considered [6].

A technique used often, in chemical and enzyme engineering, to stabilize enzymatic activity is the immobilization of the enzyme on a solid matrix [7, 8]. Enzyme immobilization has been shown to not only improve enzyme stability, but also improve shelf life and convenience of separation, recovery and reuse of the enzyme[9]. Additionally, it may be possible to preferentially accumulate the enzyme in tumors if the immobilization matrix is itself targeted to tumors.

A variety of matrices, such as colloidal gold and magnetic nanoparticles, have been utilized to immobilize enzymes. Most of these, however, were for chemical engineering purposes, and little attention has been paid to their biomedical applications [10] due to the poor *in vivo* stability of the carries or enzymes [11, 12].

Magnetic nanoparticles are novel and interesting nancarriers as they have been shown to deliver a variety of micromolecules and macromolecules to the tumors [13–15]. Their magnetic responsiveness and satisfactory biocompatibility enable them to be extremely useful for magnetic targeting and MR-monitoring [16]. Especially, continuous accumulation of magnetic nanoparticles in tumor tissue by EPR effect increase its application possibility for MDEPT strategy [17,18]. Our laboratory has studied the utility of magnetic nanoparticles and magnetic targeting on the *in vivo* delivery of biomacromolecules to brain tumors in a glioma-bearing rat model [19]. In order for this strategy to be successful, however, the stability and delivery efficiency of the carrier, along with the attached enzyme, must to be improved.

β -glucosidase (β -Glu) is a typical prodrug-activating enzyme which can hydrolyze the glucosidic bond of compounds such as linamarin and amygdalin to release hydrogen cyanide, which can effectively kill tumor cells by inhibiting cytochrome c oxidase in the mitochondria. The initial use of β -Glu for DEPT goes back as early as 1998 [20]. Due to

above-mentioned shortcomings of DEPT, however, β -Glu/prodrug therapy did not show promising efficacy *in vivo* and caused serious toxic effects [21, 22]. Non-specific toxicity of hydrogen cyanide to normal cells/tissues can be minimized by administering the prodrug only after the accumulation of the enzyme in tumor tissue and its clearance from blood circulation.

In this study, we try to conjugate covalently β -Glu to MNP. The conjugation efficiency and enzyme activity were accessed. The pharmacokinetics and magnetic tumor targeting behaviors of the β -Glu-MNP were also studied, identifying possibility of reaching satisfactory accumulation of targeted β -Glu in a subcutaneous tumor model by magnetic target and ERP effect, confirming unique advantage of MDEPT correspondence with other DEPT strategy.

2. Materials and methods

2.1 Materials

All materials were obtained from commercial suppliers and used without further purification, unless otherwise noted. Starch-coated, fluidMAG-D magnetite (Fe_3O_4) nanoparticles were purchased from Chemicell[®] GmbH (50 mg/mL, with magnetic core of about 100 nm, Berlin, Germany). β -Glu, extracted from sweet almonds with molecular weight of 135 kDa, was bought from Fisher Scientific (MP Biomedicals, LLC, Solon, USA). Lacey carbon film-coated copper grids were obtained from Ted Pella (Redding, CA). Iron standard (1000 mg Fe/L) and yttrium internal standard (1000 mg Fe/L) were purchased from GFS Chemicals (Ohio, USA). Glutaric dialdehyde solution (50%, w/v) in water, 4-Nitrophenyl β -D-glucopyranoside (Glc β Np), sodium phosphate (mono- and di-basic), epichlorohydrin, concentrated ammonium hydroxide (NH_4OH , containing 30% ammonia), sodium hydroxide pellets (NaOH) were all obtained from Sigma-Aldrich (St. Louis, MO). Deionized water ($\text{DI H}_2\text{O}$) used in syntheses was obtained from a Milli-Q A10 Biocel water purification system (Millipore, Billerica, MA).

2.2 Preparation of β -Glu-MNP

2.2.1 Cross-linked, aminated starch MNP—As shown in Figure 1, fluidMAG-D (“D-MNP”) was cross-linked and aminated via the modification of the coating starch moieties by epichlorohydrin and concentrated ammonium hydroxide (30%, w/v), respectively [12]. Briefly, 1.5 mL of D-MNP suspension (50 mg Fe/mL) was incubated with 2.0 mL of 6M NaOH for 15 min, followed by dropwise addition of 1.0 mL of epichlorohydrin. The mixture was then incubated for 24 h at 25°C with shaking. Dialysis was carried out to purify the cross-linked product using a 10,000 MWCO Slide-A-Lyzer dialysis cassette (Thermo Scientific, Rockford, IL). The purified crosslinked D-MNP was then incubated with 2.5 mL of concentrated NH_4OH (30%, w/v) for 24 h at 25°C with shaking. The cross-linked, aminated product (DN-MNP) was also purified by dialysis and then concentrated using a Dynal magnetic separator (Invitrogen, Carlsbad, CA) to 30–40 mg Fe/mL.

2.2.2. Conjugation of β -Glu on aminated D (DN-MNP)— β -Glu was conjugated onto DN-MNP using a modified glutaraldehyde method [23]. Briefly, 160 μL of glutaraldehyde

water solution (50%, w/v) was added into 8.0 mL of 0.15M phosphate buffer saline (PBS, pH 8.0). The DN-MNP particles (40mg) were resuspended in 4.0 mL of 0.15 M PBS (pH 8.0) and then added dropwise to the above prepared glutaraldehyde solution with shaking. The mixture was then incubated for 3 h at room temperature (RT) with stirring. The glutaraldehyde conjugated nanoparticles (Glutaral-MNP) were concentrated by magnetic separation and washed with deionized water five times to remove the excess glutaraldehyde. The purified Glutaral-MNP was then resuspended in 4.0 mL of PBS (0.15 M, pH 8.0) and divided equally into eight aliquots. Different amounts of β -Glu (0–25 mg) was mixed with each part of the Glutaral-MNP suspension, respectively, and incubated for 3 h at RT with stirring. The β -Glu conjugated particles (β -Glu-MNP) were then purified by centrifugation (15000 r.p.m., 20 min $\times$ 5) with a 5417 C centrifuge (Eppendorf, Germany). After each centrifugation cycle, the particles were redispersed by sonication for 2 min of sonication using a Sonifier sonicator operated at 10% amplitude (Branson, Danbury, CT) at 25°C. The amount of conjugated enzyme on the particle was determined by the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific Inc., Rockford, IL) [19].

2.3 Characterization of β -Glu-MNP

2.3.1. Particle size and zeta potential of MNP—Z-average, intensity-weighted size (hydrodynamic diameter) distribution and zeta potential of MNP suspensions were measured by dynamic light scattering (DLS). The measurements were carried out in triplicate from very dilute MNP suspensions in DI H₂O using a Nano ZS90 particle-sizer (Malvern, Worcestershire, UK).

2.3.2 Fourier transform infrared (FTIR) analysis of β -Glu-MNP— β -Glu-MNP suspension was lyophilized prior to collection of the infrared spectra (IR) using a Fourier transform infrared spectroscopy. Each sample (β -Glu, D-MNP and β -Glu-MNP) was mixed and grounded with potassium bromide (KBr) to yield a 1–3 wt% sample, and then pressed into ~1 mm thick discs. IR spectra were acquired via a Spectrum BX FTIR spectrometer (Perkin Elmer, Waltham, MA).

2.3.3 Transmission electron microscopic images—Samples were prepared by applying dilute particle suspensions onto Lacey carbon film-coated copper grids followed by ambient drying. Scanning Transmission Electron Microscopy-Bright Field (STEM-BF) imaging were carried out with a JEOL-2010F analytical electron microscope (Tokyo, Japan) operated in STEM mode at an accelerated voltage of 200 kV.

2.3.4 Magnetization measurement—Magnetization properties of magnetic nanoparticles were assessed using an MPMS-XL Superconducting Quantum Interference Device (SQUID) magnetometer (Quantum Design Inc., San Diego, CA). Fluid samples were mounted in capsules and analyzed at varying DC magnetic field (0–20000 G) at 25°C.

2.3.5 T₂ relaxivity (R₂) measurement of β -Glu-MNP—The magnetic resonance R₂ of D-MNP and β -Glu-MNP was measured in 1% agarose gel using the same animal imaging system described in Section 2.5.3 with iron concentration range of 0 ~ 0.30 mM. In brief, 96-well plate was cut into 3 $\times$ 3 wells with the size of 2.54 cm (L) $\times$ 2.54 cm (W) $\times$ 1.27 cm

(H) to fit the coil for rat MRI. Nanoparticle suspensions (0, 0.025, 0.050, 0.075, 0.100, 0.150, 0.200, 0.250 and 0.300 mM Fe) with 1% agarose were heated for 35 seconds (for dissolution of agarose), mixed well and added to each well immediately. After gelation, T2-weighted images of the samples were acquired using a multi-slice fast spin echo sequence with the sets as: 1-mm-thick $\times$ 13 slices; echo time (TE) of 30 or 60 ms. And R_2 was calculated according to the following equation:

$$R_2 \cdot [Fe] + \frac{1}{T_2^0} = \frac{1}{T_2([Fe])} = \frac{\ln[SI_1(TE_1, [Fe])/SI_2(TE_2, [Fe])]}{TE_2 - TE_1}$$

Where, T_2^0 and $T_2([Fe])$ are the proton T2-weighted relaxation times in pure water and the nanoparticle suspension, respectively; $SI_1(TE_1, [Fe])$ and $SI_2(TE_2, [Fe])$ are the signal intensities of the nanoparticle suspension acquired with effective echo times of TE_1 (30 ms) and TE_2 (60 ms), respectively.

2.3.6 Iron content measurement—Throughout the course of synthesis, iron content was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) using an Optima DV 2000 spectrometer (Perkin Elmer, Waltham, MA) [24]. Briefly, 10 μ L of sample was digested in 2 mL of concentrated HCl and brought to a total volume of 5 mL with DI H₂O to a concentration of 1–5 mg Fe/L. Samples were analyzed in triplicate at 238.204 nm. Dilutions of an iron standard (0–10 mg Fe/L) and yttrium internal standard were employed for the calibration of the system.

2.3.7 Magnetophoretic mobility—Magnetophoretic mobility of the nanoparticles was measured by monitoring the change of iron concentration of particle suspension with magnetic separation time according to a previously described protocol [25]. Briefly, samples were well dispersed into DI H₂O to get an initial iron concentration of 0.5 mg Fe/mL. After that, each sample was placed into a 1 cm $\times$ 1 cm quartz cuvette fixed on the inner plate of Dynal magnetic separator (Invitrogen, Carlsbad, CA). 10 μ L of the suspension was taken at pre-set time intervals for iron content measurement by ICP-OES. The migration half-time ($t_{1/2}$), defined as the time required to reduce the Fe concentration by 50%.

2.4 Characterization of immobilized β -Glu

2.4.1 Enzyme activity assay—Glc β Np was used here as the substrate to measure β -Glu activity [26]. Briefly, 0.1 mL of enzyme solution was added to 4.9 mL of citrate-phosphate buffer (0.1 mol/L, pH 6.0) containing Glc β Np with a final concentration of 5.0 mmol/L, followed by incubation at 37°C. A 0.5 mL aliquot was withdrawn at pre-set intervals and added to 1.0 mL of carbonate buffer (0.2 mol/L, pH 10.2). After centrifugation at 15000 r.p.m. $\times$ 20 min, absorbance of the liberated p-nitrophenol, with a molar extinction coefficient of 18300 mol⁻¹·cm⁻¹, in the supernatant was assayed by spectrophotometry at 405 nm. One β -Glu activity unit (1 U) was defined as the quantity of enzyme required for hydrolysis of 1 μ mol of the substrate (Glc β Np) per minute under the testing conditions.

2.4.2 Kinetics study of β -Glu—In order to investigate the activity of the enzyme on the nanoparticles, the kinetics of free and conjugated β -Glu were determined by using Glc β Np as the substrate in the range of 0 to 4 mM.

Michaelis-Menten parameters for free β -Glu and β -Glu-MNP obtained by fitting of reaction ratio versus substrate concentration data on a Lineweaver-Burke plot. The apparent Michaelis constant (K_m), reflecting the binding efficiency of the enzyme with the substrate, was determined based on the following equation:

$$\frac{1}{\nu} = \frac{K_m}{V_m} \cdot \frac{1}{[S]} + \frac{1}{V_m}$$

2.4.3 Effects of temperature on the free and immobilized β -Glu activities—

Aliquots of 100 μ L of the free and immobilized β -Glu were incubated in a water bath for 30 min at various temperatures (23°C, 37°C, 40°C, 45°C, 50 °C, 55 °C and 60 °C). The residual enzyme activities of the samples were then determined through the β -Glu activity assay. The maximum value of enzyme activities was defined as 100%.

To evaluate the stability of immobilized β -Glu in physiological condition, 100 μ L of PBS (150 mmol/L, pH 7.4) containing free or immobilized β -Glu was incubated in water bath for various time periods (0 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 7.5 h, 10 h and 14h) at 37°C. The same activity assay mentioned above was carried out to measure the residual activities of β -Glu (free or immobilized).

2.5 In vivo studies

All animal experiments were conducted according to protocols reviewed and approved by the University of Michigan Committee on Use and Care of Animals (UCUCA).

2.5.1 Pharmacokinetics study of β -Glu-MNP—C57 mice (20 – 25 g, Harlan, Indianapolis, IN) were anesthetized by intraperitoneal injection of ketamine/xylazine mixture (87/13 mg/kg body weight). D-MNP and β -Glu-MNP suspensions were diluted to 4 mg Fe/mL in cell culture grade PBS (pH 7.4) and administered through the lateral tail vein at a dose of 12 mg Fe/kg, the same dose as that used in previous studies [12]. Blood collection was performed by cardiac puncture. 100 μ L of blood samples were collected at pre-set time intervals (n = 3 per set) into 0.5 mL Eppendorf tubes spiked with 10 μ L of heparin solution (5000 U/mL). Samples were immediately centrifuged at 5000 r.p.m. $\times$ 10 min to separate the plasma from whole blood. Plasma samples (30 μ L) were added to ESR tubes using a microsyringe attached to a 12 inch long Teflon syringe needle and stored at –80 °C until analyzed.

2.5.2 Induction of subcutaneous tumor model—The magnetic targeting of β -Glu-MNP was evaluated in nude mice inoculated with 9L-glioma cells subcutaneously. Briefly, 9L-glioma cells (Brain Tumor Research Center, University of California, San Francisco) were cultured in 100 mm culture flasks to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL

of penicillin, 100 mg/mL of streptomycin and 0.29 mg of L-glutamine at 37 °C in a humidified atmosphere of 5% CO₂. Prior to implantation, cells were harvested and resuspended in serum free DMEM at a concentration of 4×10^7 cell/mL. The cell suspension (50 μ L) was implanted in the right flank of the anesthetized mouse. Magnetic targeting experiments were conducted when tumor volumes reached about 100–200 mm³.

2.5.3 Magnetic targeting and magnetic resonance imaging (MRI) imaging of β -Glu-MNP in vivo—Briefly, a small cylindrical ferromagnet magnet (NdFeB, 9 mm in diameter,) was mounted to the pole of a tandem three DYOYO-52 permanent magnets (K&J magnetic, Inc., USA) to get a magnetic field strength of about 320 mT. The tumor-bearing mice were anesthetized through intraperitoneal injection of ketamine/xylazine mixture (87/13 mg/kg body weight) and placed on a platform with their tumors positioned directly on the pole face of the small magnet. Each mouse was then injected with nanoparticle suspension at a dose of 12 mg Fe/kg through the lateral tail vein and retained in the magnetic field for 1 h.

MRI experiments were conducted as previously described with modification [27]. Images were acquired on a 30-cm horizontal-bore, 7T Direct Drive small animal imaging system (Varian, Palo Alto, CA). Animals were maintained at 37°C inside the magnet with a thermostated circulating water bath. T2-weighted images were acquired prior to and 1 h post-magnetic targeting. The signal intensities (SI) of MR images were normalized using ImageJ to calculate the average SI decrease in tumor lesions caused by the accumulation of nanoparticles. After imaging, the mice were euthanized and tissue samples taken immediately after the MRI scan. The tumor tissue was carefully separated from the normal tissue and kept at –80 °C for further analysis.

2.6 Ex vivo analysis of tissue samples

2.6.1 Quantitative iron content analysis using electron spin resonance (ESR) spectroscopy—Quantitative iron content analysis of plasma samples and tumor tissue samples was conducted using ESR as described previously [27]. Briefly, ESR spectra were acquired through an EMX ESR spectrometer (Bruker, Billerica, MA) equipped with a liquid nitrogen cryostat. The acquisition parameters were: 9.2GHz for resonant frequency; 20 mW for microwave power; and –128 °C for temperature. The combination of receiver gain (1×10^4 or 1×10^5) and modulation amplitude (1G or 5G) varied depending on both the type of sample and observed spectra intensity. The double integral (DI) values were obtained from spectra by using WinEPR software (Bruker, Billerica, MA). Control (no exposure to MNP) tumor tissue and plasma samples showed negligible DI when compared to test samples and, therefore, background correction was not necessary. Calibration standards were prepared by applying dilutions of known-iron-concentration MNP stock to the bottom of ESR tubes with a microsyringe coupled to a 12 inch long Teflon syringe needle.

The area under the plasma concentration versus time profiles (AUC) was estimated numerically by a linear trapezoidal integration method.

2.6.2 Analysis of β -Glu activity in tumor tissue samples— β -Glu activity in excised tissue samples was assayed by a modified spectrophotometric method, using Glc β Np as the

substrate, as described previously [27] to minimize the background of endogenous β -Glu activity. Tissue samples (~100 mg) were homogenized at 0 °C in 50 μ L lysis buffer (Tropix Inc., Bedford, MA) using a pestle tissue grinder. The homogenates were diluted with 950 μ L of 100 mmol/L buffer HEPES (pH 7.0) containing 100 mmol/L KCL and 1 mmol/L MgSO_4 . Sonication for 5 seconds at 30% amplitude was employed to disperse the samples in HEPES buffer. Enzyme activity of the homogenates was determined at 37°C using the method described in Section 2.4.1. Because of the relatively low enzyme activity of the excised tumor and influence of endogenous enzyme, the time of β -Glu reaction with substrate was extended to 10h in 37 °C. According to literature [28] and above relation between temperature and activity change of immobilized β -Glu, immobilized enzyme maintained more activity (88.83%) than that of free enzyme (only 51.67%) after 10h incubation at 37 °C, which can further reduce the error caused by the endogenous enzyme.

To account for background enzyme activity caused by endogenous enzymes, β -Glu activity in tissue samples from the control group was also measured. The background enzyme activity was subtracted from the enzyme activity of the tissue samples from the experimental group to acquire the net activity caused by the targeted β -Glu.

2.7 Statistical analysis

Data are presented as mean $\pm$ SD throughout this study, unless indicated otherwise. SPSS 16.0 statistical software package (SPSS Inc., Chicago, IL) was used for data analysis. β -Glu activities in excised tumor tissues were compared using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. A p-value of <0.05 was considered as statistically significant difference.

3. Results and disscussion

3.1 Size distribution and zeta potential

Starch-coated MNPs have shown their potential application in magnetic targeting in previous studies [12]. The hydroxyl functional groups on the coating starch enabled successfully cross-linking with epichlorohydrin and further introduction of amine groups to coating. As a homo-crosslinker of two amine groups, glutaraldehyde can not only crosslink β -Glu to the particle surface (one amine group from β -Glu, the other amine group from MNP), but also crosslink two particles (two amine groups from two different particles) by Schiff's base formation (Figure 1). The resulting β -Glu-MNP was characterized by measuring the hydrodynamic size distribution and surface charge density (Table 1). The crosslinking and amination of parent D-MNP particles resulted in an increase of the particle size from 101.8 ± 1.2 nm to 137.2 ± 2.1 nm. Zeta potential (in DI H_2O) measurments suggested the satisfactory amination of D-MNP to DN-MNP based on a positive shift in zeta potential from -1.7 ± 0.8 mV to $+39.6 \pm 1.6$ mV.

Following modification with glutaraldehyde, the average hydrodynamic diameter of Glutaryl-MNP increased to 233 ± 4.1 nm (Tab. 1). The Glutaryl-MNP showed the expected negative shift in zeta potential from $+39.6 \pm 1.6$ mV to -8.99 ± 1.2 mV (Tab. 1). The zeta potential, however, shifted back to a positive value of 18.5 ± 1.3 mV after the conjugation of

β -Glu, indicating another favorable Schiff's base formation between amine groups on the enzyme and the free aldehyde groups on the Glutaral-MNP. β -Glu immobilization also resulted in a slight increase of the average hydrodynamic diameter from 233 ± 4.1 nm to 251.4 ± 8.1 nm (Table 1). The increase in particle size and the variations in particle zeta potential followed the pattern expected for the successful synthesis of β -Glu-MNP from parent D-MNP.

3.2 Content and activity of conjugated β -Glu on magnetic particles

With the β -Glu/MNP feed ratio of (mg protein/mg Fe $\times$ 100%) increasing from 0.12 to 3.86, the weight percent of β -Glu/MNP (mg protein/mg Fe $\times$ 100%) increased from 0.36% to 7.31% (Figure 2). The enzyme activity of conjugated β -Glu increased along with the increase of the feed ratio and showed the best activity of 1.17 U/mg Fe with a feed ratio of 1.45 β -Glu/MNP (mg/mg Fe). As the feed ratio was increased further to 3.86 mg β -Glu/mg Fe MNP, however, the activity of the immobilized β -Glu decreased rapidly to approximately 0.76 U/mg Fe. At lower feed ratios, there is enough particle surface area for the conjugation of β -Glu. The enzyme activity increased accordingly with the increase of the feed ratio. However, too much amount of enzyme conjugated to the particles made the particle surface very "crowded" and resulted in adverse protein-protein interactions. As a result, the activity of conjugated enzyme decreased rapidly. Based on the fact that enzyme activity is much more important than the amount of conjugated enzyme, a feed ratio of 1.45 was finally selected for all further evaluation of the β -Glu-MNP.

Furthermore, with the optimized feed ratio of 1.45, 85.54% of enzyme activity was maintained after immobilization, indicating the satisfactory stability of β -Glu during the chemical conjugation process and the suitability of using glutaraldehyde as the crosslinker.

3.3 FTIR, TEM, SQUID, T_2 relaxivity and magnetic mobility of β -Glu-MNP

As shown in Figure 3, two additional peaks (2934.8 cm^{-1} and 1543.1 cm^{-1}) were visible in the FTIR spectra of β -Glu-MNP compared to that of D-MNP. These peaks are assigned to the amide bond ($-\text{CONH}-$) of β -Glu, suggesting the successful conjugation of β -Glu onto the MNP.

TEM images indicated that D-MNP morphology (larger coated cores comprised of multiple smaller iron oxide domains) was conserved throughout the synthesis steps (Figure 4). These images also reconfirmed the particle size results measured by DLS, especially the increase of the particle size during the glutaraldehyde crosslinking.

Examination of the magnetic properties of β -Glu-MNP revealed that they exhibit superparamagnetic behavior, as the induced magnetization curves displayed neither hysteresis nor remanent magnetization (Figure 5(a)). A high saturation magnetization of about 60 emu/g Fe suggested that magnetic targeting of the protein-loaded nanoparticles should be feasible.

The magnetophoretic mobility test showed that the migration half-time ($t_{1/2}$) of β -Glu-MNP was even shorter (2 min) than that of D-MNP (4 min), as shown in Figure 5(b), which proved that β -Glu-MNP is very sensitive to the magnetic field.

The T2-weighted relaxivity of MNP was also measured by fast spin echo sequence. Results showed that R_2 of nanoparticles increased from $118.3 \text{ s}^{-1} \text{ mM}^{-1}$ (D-MNP, $r^2 > 0.99$) to $164.2 \text{ s}^{-1} \text{ mM}^{-1}$ (β -Glu-MNP, $r^2 > 0.99$). The increase of R_2 indicated the mean size increase of the nanoparticles after the glutaraldehyde crosslinking and enzyme conjugation, which is consistent with the results of DLS measurements.

3.4 Enzymatic activity of β -Glu-MNP

The corresponding K_m values, a measure of the enzyme's affinity for the substrate, and maximum reaction rate (V_{max}), are summarized in Figure 6(a) and Table 2.

The immobilized β -Glu showed a similar K_m (2.27 mmol/L) to that (2.23 mmol/L) of free β -Glu, showing that the immobilized enzyme maintained the affinity for the substrate Glc β Np. The maximum reaction rate of β -Glu-MNP was found to be $23.7 \text{ }\mu\text{mol/minute/mg}$, which is about 73% of that of free enzyme ($32.4 \text{ }\mu\text{mol/minute/mg}$). The K_m of β -Glu immobilized on MNP is about 4-fold lower than the literature reported value ($9.4 \pm 1 \text{ mmol/L}$) for β -Glu immobilized on microgels [29]. This finding underscores the value of MNP as carriers for enzymes [29, 30]. The lower reaction rate of immobilized β -Glu was most likely due to enzyme inactivation, the change of enzyme structure, and/or the cross linking of enzyme caused by glutaraldehyde.

The enzymatic activities of both the free β -Glu and β -Glu-MNP show a strong dependence on temperature with decreasing activity at higher temperatures, as showed in Figure 6(b). It was found that the immobilized β -Glu exhibited better enzymatic activity than the free β -Glu along with increasing of temperature. At 50°C , the immobilized β -Glu exhibited 78.25% relative activity, while the free β -Glu showed 45.48% relative activity. It could be concluded that the immobilized β -Glu showed a better thermoresistance, consistent with previous studies [31], which might be due to the covalent conjugation of the enzyme molecule onto the amine-functionalized magnetic nanoparticles as well as diffusional limitations of the immobilized molecules.

The enzymatic stability of β -Glu and β -Glu-MNP were also evaluated at the physiological body temperature of 37°C . As seen in Figure 6(c), β -Glu-MNP showed greater retention of enzymatic activity, compared to β -Glu, with increasing incubation time at 37°C . For example, approximately 89% of initial activity was retained by β -Glu-MNP after 10 hours while that of β -Glu dropped to only 52% of the initial, which enables immobilized β -Glu to be more suitable for in vivo study than that of free enzyme. The better thermoresistance of immobilized β -Glu also helps it to maintain its activity in vivo.

3.5 In vivo pharmacokinetics and tumor accumulation of β -Glu-MNP

The promising *in vitro* results prompted us to examine the pharmacokinetic behavior of β -Glu-MNP *in vivo*. Analysis of nanoparticle concentration in plasma samples following intravenous administration revealed the rapid clearance of D-MNP from circulation. Only 14.4% of the dose ($14.78 \text{ }\mu\text{g Fe/ml}$) was left in the circulation at 20 min post-administration, consistent with previous studies [27]. β -Glu-MNP displayed slightly increased blood stability with more than 34.3% of the dose ($36.49 \text{ }\mu\text{g Fe/ml}$) still left in the circulation at 20

min post-administration. The total plasma exposure is equal to the area-under-the-curve (AUC) of the plasma concentration-time profiles. The plasma AUC (Figure 7) for β -Glu-MNP ($2791 \pm 234 \mu\text{g Fe/mL}\cdot\text{min}$) was approximately 2.7-fold ($p < 0.001$) greater than that of the D-MNP ($1031 \pm 123 \mu\text{g Fe/mL}\cdot\text{min}$).

In fact, the plasma pharmacokinetics results coincided with that of literature reported urokinase covalently conjugated on magnetic nanoparticles [32]. Therefore, the conjugation of β -Glu on MNP can result in enhanced *in vivo* stability of MNP. According to some published reports, half-life of enzyme-loaded antibody is much shorter than that of free antibody *in vivo* [33] due to the serious immunogenicity reactions caused by the enzyme conjugation. In terms of this aspect, MNP could be used as a better carrier than antibody for enzyme conjugation and *in vivo* delivery.

MRI analysis showed the accumulation of β -Glu-MNP in tumor lesion at 60 minutes post-administration (Figure 8). Importantly, the region of accumulation spatially corresponded to the location of the tumor lesion (hyperintense region on the T2-weighted prescan image taken prior to particle administration), confirming a markedly augmented accumulation of β -Glu-MNP selectively at the tumor site. The images further indicated a greatly enhanced accumulation of β -Glu-MNP with magnetic targeting, compared to animals to which particles were administered without magnetic targeting. The accumulation of β -Glu-MNP in tumor site was then relatively quantified by normalizing the MR images using ImageJ. The SI of the brightest area of the images (abdominal cavity) was normalized as 14000 arbitrary unit (a.u.). Accordingly, the normalized mean SIs of the tumor sites were 12389 a.u. (targeting, pre-injection), 6352 a.u. (targeting, post-injection), 12174 a.u. (non-targeting, pre-injection) and 10932 a.u. (non-targeting, post-injection), respectively. The mean SI decrease of the tumor with magnetic targeting (6037 a.u.) was about 5-fold higher than that of non-targeting tumor (1242 a.u.).

This targeted delivery of β -Glu-MNP was further quantified by *ex vivo* analysis of MNP concentration in excised tumor tissue samples taken 1 hour after particle administration. As shown in Figure 9, a 3.9-fold higher nanoparticle concentration ($p < 0.01$) was detected in tumor lesion of mice administered with β -Glu-MNP ($289 \pm 58 \text{ nmol Fe/g tissue}$) compared with those injected with D-MNP ($74 \pm 30 \text{ nmol Fe/g tissue}$). For mouse with normalized body weight of 20 g, the accumulations of MNP in tumor tissues are 6.7% injected dose/g tumor (β -Glu-MNP) and 1.7% injected dose/g tumor (D-MNP). Moreover, magnetic targeting resulted in significantly greater accumulation of MNP in tumors for both β -Glu-MNP and D-MNP, compared with non-targeted groups ($20.0 \pm 5.0 \text{ nmol Fe/g tissue}$ and $9.2 \pm 3.2 \text{ nmol Fe/g tissue}$, respectively). Interestingly, the tumor accumulation quantity of non-targeting β -Glu-MNP was higher than that of non-targeting D-MNP. The reason may be that better plasma pharmacokinetics characteristic of β -Glu-MNP resulted in the apparently enhanced permeability and retention (EPR) effect.

It should be pointed out that magnetic targeting is a multifaceted process. The capture of MNP in tumor lesion was not only determined by AUC, but also determined by magnetic response, interparticle interaction and even the tumor microenvironment in varying degrees.

As a result, β -Glu-MNP showed 2.7-fold better AUC than that of D-MNP, but 3.9-fold better tumor accumulation than that of D-MNP.

Even if the MNP are successfully delivered to tumor lesion, the conjugated β -Glu may fall off or be destroyed in the circulation system. So a more relevant index for evaluating therapeutic potential is the β -Glu activity in the excised tumors. Tissue samples from control mice, to which no β -Glu was administered, showed an endogenous background β -Glu activity of 17.6 mU/g tissue. The result (Figure 10) showed that a 3.84-fold higher ($p < 0.001$) β -Glu activity was found with magnetic targeting of β -Glu-MNP (20.1 ± 5.0 mU/g tissue) compared to the non-targeted group (5.2 ± 1.9 mU/g tissue). This was also significantly greater ($p < 0.001$) than the increase in activity found in animals administered free β -Glu (6.8 ± 2.8 mU/g tissue). So, enzyme activity of the accumulated β -Glu in targeted tumor tissues could be as high as the order of mU/g tissue, which was remarkably higher than that of the order of μ U/g tissue reported previously [18].

The current study just showed proof-of-concept data about the potential application of D-MNP based MDEPT strategy for selective delivery of enzyme. Considering the potential toxicity issue caused by endogenous β -Glu activity when the prodrug is intravenously administered in the future, at least two major studies will be done in the future: 1) the optimization of β -Glu-MNP (e.g., PEGylation and higher enzyme conjugation) to improve its tumor accumulation as well as ratio of β -Glu activity in tumor to normal tissue, which will greatly resolve the potential toxicity issue caused by the endogenous β -Glu; 2) injection of a prodrug (linamarin/amygdalin) to assess and confirm the therapeutic effect of this MDEPT strategy. The potential toxicity issue caused by the endogenous β -Glu will be further neutralized by injection of cystathionase γ -lyase [34] or rhodanese [35] when necessary.

Currently, the further studies of this MDEPT strategy are on-going in our group, including PEGylation of MNP for better plasma stability, better conjugation for higher enzyme activity and efficacy study of the optimized MDEPT system, etc. At this moment, it's difficult to know the exact enzyme activity needed to treat a tumor. However, based on current reports of enzyme-prodrug strategies, enzyme activity with the order of mU/g tissue should be very effective for tumor growth inhibition/elimination.

4. Conclusion

In this work, The β -Glu was covalently conjugated with iron oxide nanoparticles by glutaraldehyde method. The enzyme activity of immobilized β -Glu was highest while the feed ratio of enzyme/Fe (mg/mg) was 1.45. The immobilized β -Glu had similar affinity and maintained 85.54% residue enzyme activity compared with free enzyme. The immobilized β -Glu also showed a better thermoresistance than that of free enzyme.

After the immobilization of enzyme, β -Glu-MNP (Area Under Curve (AUC): 2791.33 ± 234.34 μ g Fe/mL $\cdot$ min) also displayed preferable plasma pharmacokinetics than that of parent starch-coated particles D-MNP (AUC: 1031 ± 123.23 μ g Fe/mL $\cdot$ min). Moreover, selective magnetic subcutaneous tumor targeting ($t = 1$ h) of β -Glu-MNP (12 mg Fe/kg) was

confirmed in a mouse model of 9L-glioma subcutaneous tumor by MRI image and ESR quantitative analysis. Most importantly, the ability to deliver immobilized β -Glucosidase at high milliunit-range tumor concentration was apparently higher than the results reported previously.

The encouraging data shown in current study was as a result of (1) the short distance between subcutaneous tumors and the magnets, (2) the satisfactory stability of β -Glu during the whole process (chemical conjugation, reaction with substrate and in vivo delivery), (3) the relatively high strength of the magnetic field (300 mT) used in this study and (4) the satisfactory magnetic response of β -Glu-MNP. And these results offered a promising perspective for further studying of this MDEPT system. Optimization and efficacy study of this MDEPT system are currently on-going in our group.

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Figure 1.
The schematic synthesis of β -Glu-MNP.

Figure 2.

The activity (black) and amount (blue) of β -Glu immobilized on the MNP surface under different feed ratios (β -Glu/Fe, mg/mg). The amount of immobilized β -Glu increased according to the increase of feed ratio. However, immobilized β -Glu had maximum activity of 1.169 U/mg Fe with feed ratio of 1.446.

Figure 3.
FTIR spectra of D-MNP (black), β -Glu-MNP (red) and β -Glu (green).

Figure 4. Scanning Transmission Electron Microscopy-Bright Field (STEM-BF) images of (a) D-MNP and (b) β -Glu-MNP.

Figure 5.

(A) Magnetization properties of β -Glu-MNP and D-MNP via Superconducting Quantum Interference Device (SQUID) analysis. (B) Magnetophoretic mobility of the magnetic nanoparticles. The Fe concentrations of nanoparticle suspensions represent the amount of the nanoparticles not captured by the magnetic field.

Figure 6.

Activity characterisation of free and immobilized β -Glu. (a) Lineweaver-Burk plot analysis of the enzymatic kinetics of free β -Glu and β -Glu immobilized on magnetic nanoparticles. (b) Effect of temperature on the enzymatic activity of free and immobilized β -Glu. (c) Effect of incubation time on the enzymatic activity of free and immobilized β -Glu at 37° .

Figure 7.

Plasma concentration-time profiles of D-MNP and β -Glu-MNP. Data expressed as Mean $\pm$ SD, n = 4. Inset: corresponding area under the concentration-time curve (AUC) of D-MNP and β -Glu-MNP.

Figure 8.

Representative MRI images of mice bearing 9L-glioma subcutaneous (flank) tumors (12 mg Fe/kg): MRI images of mouse taken prior to β -Glu-MNP administration (a) and at 60 min post-administration with magnetic targeting (b); MRI images of control mouse taken prior to β -Glu-MNP administration (c) and at 60 min post-administration without magnetic targeting (d).

Figure 9. Quantitative ESR analysis of nanoparticle concentrations in targeted and no-targeted tumor tissue of mice administered with β -Glu-MNP or D-MNP. Data expressed as Mean $\pm$ SD, n=4.

Figure 10.

Activity of β -Glu in the tumor lesion of the mice administered with β -Glu-MNP or β -Glu (control). Tumors of magnetically-targeted mice administered with β -Glu-MNP exhibited significantly higher β -Glu activity than any other analyzed samples ($p < 0.001$), confirming the selective tumor targeting of β -Glu-MNP.

Table 1

Particle size distributions and zeta potential changes in reaction procedure.

MNP	Hydrodynamic Diameter (nm)	Zeta Poterntial (mV)
D-MNP	101.8±1.2	-1.7±0.8
DN-MNP	137.2±2.1	+39.6±1.6
DN-MNP-glutaral	233±4.1	-8.99±1.2
β-Glu-MNP	251.4±8.1	+18.5±1.3

Samples measured very dilute in DI H₂O @ 25 ° for DLS measurements. For all expertiments, n = 3.

Table 2Activity of β -Glu immobilized to MNP.

Enzyme	K_m (mmol/L)	V_{max} (μ mol/ minute.mg)
Free β -Glu	2.23 $\pm$ 0.2	32.4 $\pm$ 2.8
Immobilized β -Glu	2.27 $\pm$ 0.1	23.7 $\pm$ 2.3

For all experiments, n = 3.