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Substantiating *In Vivo* Magnetic Brain Tumor Targeting of Cationic Iron Oxide Nanocarriers via Adsorptive Surface Masking

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Abstract

Cationic magnetic nanoparticles are attractive as potential vehicles for tumor drug delivery due to their favorable interactions with both the tumor milieu and the therapeutic cargo. However, systemic delivery of these nanoparticles to the tumor site is compromised by their rapid plasma clearance. We developed a simple method for *in vivo* protection of cationic nanocarriers, using non-covalent surface masking with a conjugate of low molecular weight heparin and polyethylene glycol. Surface masking resulted in an 11-fold increase in plasma AUC and a 2-fold increase in the magnetic capture of systemically injected nanoparticles in orthotopic rodent brain tumors. Overall, the described methodology could expand the prospective applications for cationic magnetic nanoparticles in magnetically-mediated gene/drug delivery.

Keywords

drug delivery; cationic magnetic nanoparticles; magnetic targeting; brain tumors; surface masking

1. Introduction

Superparamagnetic nanoparticles, composed of an iron oxide core and a biocompatible polymeric coating, have been widely studied as a platform for localized drug delivery to tumors via magnetic targeting[1]. Magnetic targeting is a strategy to retain magnetically responsive nanoparticles at the tumor site by an external magnetic field[2]. Although magnetically-mediated nanoparticle retention is mainly enabled by the iron oxide core, the significance of nanoparticle surface properties in drug/gene magnetic delivery cannot be overstated. The surface properties mediate the interaction of the nanocarrier with both the therapeutic cargo [3,4] and the physiological milieu[5]. One particularly important surface property is the surface

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charge of the nanoparticles. A positive surface charge has been shown to confer nanoparticles with several drug/gene delivery advantages over anionic and neutral counterparts. Modification of nanoparticle surface with polycations was demonstrated to enable efficient compacting of DNA/siRNA[3,6], enhancement of cell internalization[7] and strong avidity to anionic proteoglycans in tumor vasculature [8,9]. However, cationic nanoparticles suffer from a serious drawback which limits their application *in vivo*. Upon administration into systemic circulation, cationic nanoparticles attract opsonising proteins, which leads to rapid plasma clearance of the nanocarriers[5,10].

Short nanoparticle plasma half-life constitutes a major obstacle for the application of magnetic targeting to tumors located in hard-to-reach areas of the body. Commonly performed magnetic targeting procedures involve the magnetic capture of nanoparticles administered locally to superficial tissues or subcutaneous tumors[2,11]. For realization of this procedure, the targeted tumor must lie near the body surface to allow for direct injection of the nanoparticles. However, direct injection is often hindered by poor tumor accessibility and the risk of damage to the surrounding normal tissue. For example, brain tumors are typically embedded deep within the brain parenchyma, which is often compromised by direct brain intervention[12,13]. To this regard, vascular administration of magnetic nanoparticles seems to offer a safer alternative. Yet, following intravenous administration, magnetic nanoparticles have to be passively delivered to the tumor vasculature for subsequent magnetic capture[14]. The amount of agent available for tumor uptake over time after systemic administration is known to be directly proportional to the area under its plasma concentration-time curve (AUC)[15]. Hence, the negligible AUC of cationic nanoparticles greatly reduces the chance for their magnetic accumulation within the tumor tissue following systemic administration.

Surface modification with polyethylene glycol (PEG), termed PEGylation, has been shown to increase the AUC for many nanoparticulate systems[16]. Surface PEGylation is most commonly performed via covalent chemical coupling[16]. However, covalent modification of cationic nanoparticles often results in a permanent, partial neutralization of the positive charge, reducing the benefits of cationic nanocarriers with respect to enhanced drug/gene loading [17] and cell interaction at the target site[14]. Moreover, the reaction conditions required for chemical surface modification could affect the integrity and biological functionality of the encapsulated drug/gene compounds.

Here we report an alternative, non-covalent method for surface PEGylation of cationic nanoparticles, based on a reversible, electrostatic surface masking process. A novel anionic surface masking agent, composed of low molecular weight heparin (LMWH) conjugated to PEG, was developed in this work. LMWH are highly anionic oligosaccharides containing a high density of negatively charged carboxylic and sulfate groups [18]. It was hypothesized that electrostatic complexation between the anionic LMWH-PEG conjugate and the positively charged nanoparticle surface (Figure 1) would result in surface PEGylation of the nanoparticle, enhancing its plasma AUC and subsequently substantiating its tumor delivery via magnetic capture.

2. Materials and Methods

2.1 Synthesis and purification of LMWH-PEG conjugate

LMWH-PEG was synthesized using a two-step process. First, heparin (Grade I-A, Sigma) was partially depolymerized to form an aldehyde-containing terminal anhydromannose residue. Activated LMWH oligosaccharides were then coupled to amine-bearing polyethylene glycol (mPEG-NH₂) by reductive amination.

2.1.1 Preparation of aldehyde-bearing LMWH (LMWH-CHO)—Nitrous acid cleavage of heparin was carried out as previously described[19] with minor modifications. Briefly, heparin (0.5 g) was dissolved in 150 ml of Milli-Q (Millipore, Billerica, MA) deionized water (DW) and the solution cooled on ice. Sodium nitrite (NaNO_2 , 20 mg) was added to the solution, and 0.5M HCl then used to adjust the solution to pH 2.7. The reaction mixture was stirred on ice for 2 hours and then the reaction terminated by adjusting the pH to 7.0 with NaOH. Heparin depolymerization products were fractionated using two consecutive ultrafiltration steps with 3kDa and 5kDa cut-off membranes to screen for oligosaccharides with the desirable molecular weight of 3–5 kDa. The lyophilized product was analyzed for presence of aldehydes using the dinitrosalicylic acid method[20] and was found to contain 0.25 μmol aldehydes/mg heparin, equivalent to about one aldehyde group per heparin chain.

2.1.2 Coupling of LMWH-CHO and mPEG-NH₂—Coupling of the aldehyde-bearing LMWH-CHO and primary amine-bearing mPEG-NH₂ (10kDa, Nektar Therapeutics, Huntsville, AL) was carried out by reductive amination. Briefly, LMWH-CHO (20mg, 5 μmol CHO) and mPEG-NH₂ (15 μmol) were dissolved in 10 ml borate buffer (50mM, pH 9.5). Sodium cyanoborohydride (NaCNBH_3 , 250 μmol) was then added to the reaction mixture for *in situ* reduction of the imine bond[21]. After 24 hours of incubation at 37°C, additional 250 μmol of NaCNBH_3 was added and the solution was stirred for another 24 hours.

2.1.3. Purification of LMWH-PEG—The conjugate was purified in three steps. First, the reaction mixture was subjected to ultrafiltration with 5kDa cut-off membrane (Millipore) to remove unreacted LMWH. The concentrated retentate was then further purified from unreacted PEG by anion-exchange with a High-Q column (BioRad Laboratories, Hercules, CA). The elution was performed with 0 – 2M NaCl step-gradient at a flow rate of 1 ml/min and followed by UV detection at 214 nm. The fraction eluted with 1M NaCl (the salt concentration required for elution of LMWH) was collected for further purification. This fraction was loaded on an Altima C8 (250 × 4.6 mm, 5 μm) reverse phase column equilibrated with 0.1% trifluoroacetic acid in DW. The remnants of the unreacted LMWH were removed with the flow through at a flow rate of 1 ml/min and the column-adsorbed species were eluted with acetonitrile gradient (0–70% acetonitrile in DW). The fraction desorbed from the column with 40% acetonitrile in DW was collected. After solvent evaporation, the residue was redissolved in DW and analyzed for the presence of Heparin and PEG using Azur A[22] and Barium-Iodine[23] spectrophotometric assays, respectively.

2.2 GPEI/LMWH-PEG complexation studies

Cationic magnetic nanoparticles were prepared by attaching polyethyleneimine (PEI) chains to pendant carboxylic groups of fluidMAG-ARA magnetic nanoparticles (Chemicell®, Germany) using the well-established EDC-coupling method[24]. The modified particles were termed GPEI.

Complexes were formulated by mixing GPEI solutions (3 mg Fe/ml, 50 μL) with LMWH-PEG conjugate or free LMWH at 0 – 6.4% w/w (LMWH/GPEI). Resulting solutions were diluted to 200 μL with deionized water (DW) and stirred at RT for 15 minutes. GPEI/LMWH-PEG complexes were removed from solution with a magnetic separator. Isolated complexes were resuspended in DW and analyzed for particle size distribution and zeta potential using Nicomp 380 particle sizer (Nicomp, Santa Barbara, CA). Residual amounts of free conjugates in the supernatant were determined with the Azur A assay[22].

2.3 Evaluation of nanoparticle stability *in vitro*

Size stability of conjugate protected and free GPEI nanoparticles was studied as a function of time in protein-rich medium using dynamic light scattering (Nicomp, Santa Barbara, CA).

GPEI complexes with LMWH or LMWH-PEG, formulated as described above at 0 – 6.4% w/w LMWH/GPEI, or free GPEI, were mixed with reduced serum medium (2 ml, Opti-Mem I, Invitrogen). Samples were incubated in a cuvette at ambient temperature and particle size distribution was analyzed at 5 minute intervals for 30 minutes.

2.4 Pharmacokinetic analysis

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

The pharmacokinetics of GPEI and GPEI/LMWH-PEG complexes were studied in male Fisher 344 rats weighting 200–250 g ($n = 4$ for each groups). The nanoparticles were administered intravenously via tail vein and blood samples taken through the cannulated carotid artery. All animals were initially anaesthetized by intraperitoneal injection of ketamin/xylazine mixture (87/13 mg/kg body weight). The left carotid artery of the animals was exposed by blunt dissection and ligated rostrally to occlude the flow. Polyethylene tubing (PE-10, BD Corp., Franklin Lakes, NJ) was inserted caudally via a small incision in the arterial wall and secured in place by ligation. The intracarotid catheter was flushed with Heparin flush solution (Hepflush-10, 10 USP Units/ml, Abraxis Pharmaceutical products, IL) and clamped. Tail veins of the animals were cannulated with a 26-gauge angiocatheter (AngiocathTM, BD Corp., Franklin Lakes, NJ). The nanoparticle suspension in PBS was administered to rats via the tail vein catheter at a dose of 12 mg Fe/kg body weight. Blood samples (100 μ L) were collected from the cannulated carotid artery in Eppendorf tubes (0.5 ml) spiked with Heparin solution (10 μ L, 5,000 USP Units/ml). Samples were acquired before and serially after nanoparticle administration at pre-set time intervals for 30 minutes. Plasma fractions were immediately separated by centrifugation (3 minutes at 7000g) and stored at -80°C .

2.5 Tumor implantation

Intracerebral 9L tumors were induced in male Fisher 344 rats weighting 125–150 g according to a previously described procedure[25]. Briefly, rat 9L-glioma cells (Brain Tumor Research Center, University of California, San Francisco) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, 100 μ g/mL streptomycin and 0.29 mg of L-glutamine at 37°C in a humidified atmosphere of 5% CO_2 . Prior to implantation, cells were grown to confluency in 100 mm culture dishes, harvested and resuspended in serum free DMEM at a concentration of $\sim 10^5$ cells/ μ L. A 1-mm hole was drilled in the right skull of the animals, 1 mm anterior to the bregma and 5 mm lateral to the midline. The cell suspension (10 μ L) was then implanted at a depth of 3 mm into the right forebrain through the burr hole. The surgical field was cleaned with 70% ethanol and the burr hole was filled with bone wax (Ethicon Inc., Summerfield, NJ) to prevent extracerebral extension of the tumor. The tumor volume of the animals was monitored with MRI beginning on day 10 after cell implantation to select tumors between 70 and 90 μ L for magnetic targeting experiments.

2.6 Magnetic targeting

Magnetic targeting was carried out according to our previously reported procedure[26]. Briefly, anaesthetized animals were positioned on a platform with their head subjected to 0.4 T magnetic field[26]. GPEI or GPEI/LMWH-PEG complexes were administered intravenously at a dose of 12 mg Fe/kg via the cannulated tail vein ($n = 4$ for each group). Animals were retained in the magnetic field for 30 minutes after nanoparticle administration.

2.7 Magnetic resonance imaging (MRI)

MRI experiments were performed on an 18-cm horizontal-bore, 7 Tesla Varian Unity Inova imaging system (Varian, Palo Alto, CA). Animals were anesthetized with 1.5% isoflurane/air mixture and imaged using a 35-mm-diameter quadrature RF head coil (USA Instruments Inc, OH). Animals were maintained at 37°C inside the magnet using a thermostated circulating water bath. To monitor nanoparticle accumulation in brain tumors, transverse relaxation rate maps (R_2) were constructed according to our previously reported procedure[26]. Briefly, T_2 -weighted MRI scans of rat brain were acquired using a multi-slice fast spin echo sequence with the following parameters: repetition time (TR) = 4 s, field of view = 30×30 over 128×128 matrix, slice thickness = 1 mm, slice separation = 2 mm, number of slices = 13, four signal averages per phase encoding step. Two consecutive sets of T_2 -weighted images with effective echo time (TE) of either 30 or 60 ms were collected before administration of nanoparticles (pre-scans) and immediately after magnetic targeting. R_2 relaxation rate maps were calculated from resulting signal intensities using the following equation:

$$R_2 = \frac{1}{T_2} = \frac{\ln[S_1(TE_1)/S_2(TE_2)]}{TE_2 - TE_1} \quad [1]$$

where $S_1(TE_1)$ and $S_2(TE_2)$ are the signal intensities acquired with effective echo times TE_1 and TE_2 , respectively.

Image analysis was performed with Matlab 7.1 software (The MathWorks, MA). To infer the nanoparticle accumulation within tumor lesions, tumor-circumscribing regions of interest (ROIs) were manually drawn on the R_2 maps. Mean signal intensities of the ROIs were calculated to compare transverse relaxation rates before nanoparticle administration and after magnetic targeting. The change in R_2 relaxation rate caused by the presence of nanoparticles within the outlined ROIs was expressed as a percent change of the baseline R_2 value.

2.8 EPR analysis

Nanoparticle concentrations were determined by EPR spectroscopy as previously reported [26]. Briefly, ESR spectra of the samples were acquired using an EMX ESR spectrometer (Bruker Instruments Inc., Billerica, MA) equipped with a liquid nitrogen cryostat. The acquisition parameters were: resonant frequency: ~9.2GHz, microwave power: 20mW, temperature: 145K, modulation amplitude: 5G and receiver gain of 5×10^4 and 5×10^3 for tissue and plasma samples, respectively. The double integral of the ESR spectra of tissue/plasma samples was calculated to quantify the nanoparticles. Calibration curves were constructed with nanoparticle solutions of known iron concentrations. The data were corrected for the background tissue absorption using control tissue samples from the animals not exposed to the nanoparticles or plasma samples collected prior to the nanoparticle injection.

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

2.9 Statistical Analysis

Data are presented as mean $\pm$ SD, unless indicated otherwise. Nanoparticle concentrations in tumor and contra-lateral brain tissues of complex-protected and non-protected GPEI were compared using unpaired student t-test. A p-value of <0.05 was considered statistically significant.

3. Results

3.1 Preparation and Characterization of LMWH-PEG conjugate

LMWH-PEG conjugates were synthesized by end-to-end, site-specific attachment of LMWH to PEG. This procedure was specifically selected in order to avoid the loss of carboxyl and sulfate moieties along the heparin backbone, which could potentially lead to reduced heparin avidity towards the positively charged nanoparticle surface. To generate a terminal attachment site on the heparin molecules, unfractionated heparin was first partially depolymerized with nitrous acid (Section 2.1.1) to form LMWH with a 2,5-anhydromannose terminal residue. This residue possesses a reactive aldehyde group, which was used to couple LMWH to the primary amine of mPEG-NH₂ by reductive amination (Section 2.1.2).

The resulting conjugate was purified using several sequential steps (Section 2.1.3). Purification chromatograms are shown in Figure 2. As evident from Figure 2A, free PEG (Fig. 2A: a) exhibited only weak association with the anion-exchange ($-N^+(CH_3)_3$) matrix and could be eluted with 0.2 M NaCl. In contrast, free LMWH (Fig. 2A: b) was found to bind strongly to the cationic matrix and its elution required 1 M NaCl. Thus, anion-exchange step was used to remove the free PEG from the mixture of LMWH-PEG and remnants of free LMWH (Fig. 2A: c). Subsequent C8 reverse phase separation (Fig. 2B) was used to ensure removal of free LMWH remnants from the conjugate solution. Hydrophilic LMWH did not bind to the C8 matrix (Fig. 2B: b) and thus eluted with the flow through. In contrast, the PEG-containing conjugate, purified of free PEG, exhibited affinity to the C8 matrix (Fig. 2B: c). Overall, the chromatograms of Figure 2 qualitatively reveal formation of the LMWH-PEG species. Furthermore, the molar ratio of LMWH to PEG in the purified conjugate was found to be approximately 1:1 (data not shown), further confirming successful end-to-end coupling.

3.2 *In vitro* GPEI/LMWH-PEG complexation and stability analysis

GPEI iron oxide nanoparticles, surface-modified with short polyethyleneimine (PEI - MW~1200 Da) chains, were chosen as the model to represent cationic magnetic nanocarriers. GPEI exhibited a zeta potential of +25.2 mV and hydrodynamic diameter of 228 nm (polydispersity, PDI = 0.2), as assessed by dynamic light scattering.

Complexation of GPEI nanoparticles with the LMWH-PEG conjugate was examined at different weight ratios of these two components. Properties of the nanoparticle surface were assessed by utilizing two techniques: 1) determination of ζ -potential, and 2) analysis of the residual amount of free conjugate in the supernatant following removal of GPEI/LMWH-PEG complexes from solution with a magnetic separator. As seen in Figure 3, zeta potential of the nanoparticles exhibited an inverse linear dependence on the amount of added conjugate ($R^2 = 0.98$, Figure 3A), indicating electrostatic adsorption of the anionic LMWH-PEG to the cationic GPEI nanoparticle surface. For example, addition of the conjugate at 3.2% w/w (LMWH/GPEI) resulted in two-fold reduction in surface charge ($\zeta = +13.4$ mV) compared with free GPEI ($\zeta = +25.2$ mV). In agreement with the ζ -potential data, amounts of residual conjugate in the supernatant were found to increase with increasing amount of added conjugate (Figure 3B). Steadily low amounts of surplus conjugate were detected in the supernatant with addition of conjugate to GPEI up to a ratio of about 5% w/w. Further addition up to about 6% w/w ratio resulted in an abrupt increase of conjugate level in the supernatant, corresponding well with charge neutralization of the GPEI surface ($\zeta = +1.3$ mV) and indicating saturation of the GPEI surface with the charge masking LMWH-PEG. Nevertheless, complexation did not significantly alter the hydrodynamic diameter of conjugate-masked GPEI in deionized water (243 nm, PDI = 0.1) from that of free GPEI (228 nm, PDI = 0.2).

To examine whether complexation with the LMWH-PEG conjugate would yield protection to the positively charged GPEI nanoparticles in a protein-rich environment, we evaluated *in vitro* stability of the complexes, and free nanoparticles, in medium containing reduced serum (Opti-Mem I, Invitrogen). We incubated complexes of varying GPEI/LMWH-PEG ratios with the medium and monitored changes in particle size over time using dynamic light scattering. As displayed in Figure 4, native, non-protected GPEI particles were prone to aggregation and, after only 5 minutes of incubation with the medium, particle size shifted from about 228 nm (GPEI diameter in water) to the micron range. Complexation of the GPEI with free LMWH and PEG, at individual concentrations equivalent to that in the 3.2% w/w LMWH/GPEI complex, did not reverse the aggregation pattern observed for the unprotected GPEI (middle lane of Figure 4). In contrast, nanoparticles complexed with the LMWH-PEG conjugate at concentrations of 3.2% w/w (LMWH/GPEI) and higher maintained their original size distribution in the protein-rich medium for at least 30 minutes. Hydrodynamic diameters determined for 3.2% w/w (LMWH/GPEI) complexes were 243 nm (PDI = 0.13) and 265 nm (PDI = 0.15) after 5 and 30 minutes of incubation in the medium, respectively.

3.3 *In vivo* pharmacokinetics and tumor accumulation of complex-masked GPEI

The promising *in vitro* results prompted us to examine the pharmacokinetic behavior of the complexes *in vivo*. The 3.2% weight ratio of LMWH-PEG and GPEI was used for complex formulation in pharmacokinetic studies because of the previously observed *in vitro* stabilization effect. Analysis of nanoparticle concentration in plasma samples following intravenous administration revealed that complexation with LMWH-PEG significantly improved the pharmacokinetic profile of GPEI (Figure 5), as the plasma AUC (Figure 5; inset) for GPEI/LMWH-PEG complexes ($164.8 \pm 5.5 \mu\text{g Fe/ml} \cdot \text{min}$) was found to increase by 11-fold ($p < 0.001$) over that of the native, non-protected GPEI particles ($14.8 \pm 9.3 \mu\text{g Fe/ml} \cdot \text{min}$).

We next assessed whether improvement in AUC could enhance magnetic entrapment of the conjugate-protected GPEI in tumor lesion of glioma-bearing rats. Accumulation of magnetic nanoparticles in tumor lesions was monitored with magnetic resonance imaging (MRI). Iron oxide nanoparticles are known to enhance transverse relaxation rate of protons (R_2), and thus manifest their presence at a given spatial location by a local increase in R_2 . R_2 maps were calculated from sets of T_2 -weighted MRI images, acquired before nanoparticle administration and after magnetic targeting (Section 2.7). Figure 6 presents representative R_2 maps of glioma regions of interest (ROIg) overlayed on anatomical T_2 -weighted brain images for rats administered with GPEI and GPEI/LMWH-PEG complexes. In the group of complex-protected GPEI (Fig. 6A), a $15.2 \pm 2.5\%$ increase in R_2 mean pixel intensity above the baseline value was observed within the ROIg after magnetic targeting reflecting nanoparticle accumulation in the tumor lesion. In contrast, a mean R_2 change of only $1.3 \pm 0.7\%$ was seen in tumors of rats administered with the native, non-protected GPEI (Fig. 6B). No significant difference ($p = 0.121$) in the baseline mean R_2 values of the ROIg was observed between the groups administered with the complex-protected ($14.3 \pm 0.8 \text{ s}^{-1}$) and the non-protected GPEI ($15.3 \pm 0.9 \text{ s}^{-1}$).

To quantify the extent of nanoparticle accumulation in the brain of GPEI and GPEI/LMWH-PEG injected rats, excised tissue samples of tumor and normal brain were assayed for nanoparticle concentration (Section 2.8). As evident from Figure 7, a 2-fold higher nanoparticle concentration ($p < 0.01$) was detected in tumor lesions of rats administered with GPEI/LMWH-PEG complexes ($14.7 \pm 1.2 \text{ nmol Fe/g tissue}$) compared to those injected with the non-protected GPEI ($7.3 \pm 0.8 \text{ nmol Fe/g tissue}$). Interestingly, this increase in surface-protected nanoparticle accumulation in the tumor of GPEI/LMWH-PEG administered rats was not accompanied by any detectable increase in the normal brain tissue, as accumulation in the contra-lateral brain was not significantly different between the surface-protected and the non-protected GPEI

groups ($p = 0.617$). To this regard, surface protection appeared to also result in an improved targeting selectivity of nanoparticles towards the tumor lesion.

4. Discussion

Magnetically responsive nanoparticles present an attractive platform for tumor delivery of therapeutic drugs and genes via magnetic targeting. To realize the benefits of magnetic targeting for hard-to-access tumor lesions, such as brain tumors, which are not clinically amenable to direct intervention, intravenous administration of nanoparticles is required. With vascular administration, pharmacokinetic properties of the nanoparticles gain critical importance for magnetic targeting as they control the passive delivery of the nanoparticles to tumor vasculature for subsequent local magnetic capture. Nanoparticle pharmacokinetic behavior is, in turn, largely influenced by the surface characteristics of the nanocarriers. For example, previous studies revealed that, while negatively charged iron oxide nanoparticles of 22 nm hydrodynamic diameter exhibited a plasma half-life of ~40 min, positively charged particles of the same size had a half-life of only 1–2 minutes[5]. Although surface cationization reduces pharmacokinetic performance of nanoparticles, a positive surface charge is highly advantageous for tumor delivery of therapeutics as it facilitates efficient compacting of anionic macromolecules (e.g. DNA, siRNA)[3,6] and promotes nanoparticle association with tumor vasculature[8]. Hence, it was the goal of this work to overcome the pharmacokinetic drawback of cationic magnetic nanoparticles and thus enable their passive delivery to tumor vasculature. To this regard, a novel approach for reversible masking of cationic surfaces with anionic LMWH-PEG surface masking agent was developed in this study.

The surface masking activity of LMWH-PEG was explored using GPEI as the representative cationic magnetic nanoparticle carrier. Without surface protection, the magnetic targeting procedure failed to accumulate GPEI in brain tumor lesions following intravenous administration. In previous work, we successfully entrapped slightly negative, starch-coated magnetic nanoparticles (G100) in brain tumor lesions using the same magnetic targeting approach employed in the current study[26]. Since GPEI exhibited similar magnetic properties to those of G100, tumor delivery failure of GPEI could not be attributed to the lack of magnetic interaction with the external magnetic field. On the other hand, pharmacokinetic analysis revealed a negligible arterial AUC of systemically administered GPEI ($14.8 \pm 9.3 \mu\text{g Fe/ml} \cdot \text{min}$). Low arterial AUC reflected a low exposure of brain tumor vasculature to GPEI, which likely contributed to the failure of GPEI magnetic entrapment within the tumor lesion.

To improve passive access of GPEI to the tumor vasculature, it is essential to reduce its clearance from the vascular compartment. Rapid plasma clearance of cationic nanoparticles has been attributed to their interaction with negatively charged plasma proteins and subsequent aggregation[27,28]. In agreement with previous reports, our *in vitro* studies also revealed rapid aggregation of GPEI in serum-containing medium. This aggregation, however, could be completely prevented using our surface masking strategy with LMWH-PEG. Interestingly, aggregation could be avoided with a conjugate/nanoparticle ratio even as low as 3.2% w/w. At this ratio the nanoparticle surface still maintained cationic character as the corresponding zeta potential was +13.4 mV. Thus, complete charge neutralization was deemed not essential for surface protection with LMWH-PEG.

The most exciting finding of this study was that the *in vitro* surface masking strategy could be successfully translated to the *in vivo* setting. A dramatic 11-fold improvement in AUC was achieved by surface masking of the GPEI surface. Interestingly, this AUC enhancement was obtained in the presence of a relatively high positive charge ($\zeta = +13.4 \text{ mV}$) on the surface of the LMWH-PEG protected nanoparticles; a finding consistent with the *in vitro* results. The fact that surface protection with LMWH-PEG can be achieved even in the presence of a net

positive surface charge is greatly advantageous for drug delivery. Available positive surface charges, not consumed by electrostatic binding with LMWH-PEG, could potentially be utilized for loading of genes and certain protein drugs by electrostatic complexation.

The surface-protection methodology for AUC enhancement enabled us to evaluate whether an increase in vascular presentation of the nanoparticles affects the extent of their magnetic capture within the tumor. Indeed, our results demonstrated 2-fold increased tumor accumulation of GPEI with the increase in AUC. While these results provide a proof-of-concept demonstration of the feasibility of enhanced nanoparticle accumulation in the tumor by surface masking, it is noteworthy that the increase in tumor accumulation did not seem to exhibit 1:1 correlation with AUC, as an 11-fold AUC increase resulted in only 2-fold increase in tumor capture. It should be pointed out that magnetic targeting is a multifaceted process. Parameters other than AUC, including tumor extravasation, magnetic response, interparticle interaction and interaction with the tumor microenvironment are likely to play a role in tumor capture of magnetic nanoparticles. Studies are underway to assess how these parameters might be affected by masking agent composition and conjugate loading density to further improve nanoparticle capture within the tumor tissue.

5. Conclusions

Results presented demonstrate that surface shielding of cationic nanoparticles with LMWH-PEG conjugates is a plausible strategy to enhance plasma AUC and, more critically, the magnetic entrapment of cationic nanoparticles in brain tumor lesions following systemic administration. Our surface shielding methodology is both facile and versatile for further optimization of AUC, as surface charge and PEG density can be readily regulated by varying the amount of adsorbed LMWH-PEG conjugate. Since the described electrostatic complexation process is reversible, it is anticipated that LMWH-PEG conjugates would gradually dissociate from GPEI at the target site due to competitive binding of the negatively charged proteoglycans on the tumor cell membranes. Positive surface charges, thus unshielded, could then associate strongly with the tumor vasculature, yielding continued MRI visibility, or contribute to enhancement of cellular uptake of nanocarriers and their drug cargo. Overall, we believe that our non-covalent, surface masking methodology can expand the potential clinical applications of cationic magnetic nanoparticles, in tumor monitoring and magnetically-mediated gene/drug delivery, by enhancing their systemic accessibility to the tumor vasculature.

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Figure 1.
Surface masking of cationic magnetic nanoparticles with LMWH-PEG conjugates.

Figure 2. Chromatograms obtained during purification of LMWH-PEG (c) as compared to free PEG (a, control) and free LMWH (b, control) on (A) High-Q anion exchange column and (B) C8 reverse phase column.

Figure 3.

Complexation of GPEI with LMWH-PEG conjugates. (A) Inverse linear dependence ($R^2=0.98$) of nanoparticle ζ -potential (pH=5.5) on the amount of added LMWH-PEG conjugate suggests charge masking of GPEI cationic surface by ionic complexation. (B) Dependence of the residual LMWH concentrations in the supernatant on the amount of added conjugate indicates saturation of the GPEI surface with the LMWH-PEG conjugate and confirms complexation between these two species. The data set was fitted to a Boltzmann sigmoidal function ($R^2=0.995$) using the non-linear curve-fitting module of OriginPro 7.5 software (OriginLab Corp., Northampton, MA).

Figure 4.

In vitro stability of GPEI complexed with LMWH-PEG conjugates (test) or free LMWH and PEG (i.e. the non-conjugated control) in reduced serum medium. Particle size was measured after 5 and 30 minutes of incubation at room temperature. Equal concentrations of the complexing agents (free LMWH + PEG and LMWH-PEG conjugate) were used in control and test experiments: 3.2% w/w LMWH/GPEI and 1:1 molar ratio of PEG:LMWH.

Figure 5.

Plasma concentration-time profiles for GPEI nanoparticles in rats following intravenous administration of GPEI (■) with and (○) without LMWH-PEG complexation. Data points represent Mean $\pm$ SD, n = 4. Inset: corresponding area under the concentration-time curve (AUC) of GPEI with (hash bar) and without (solid bar) LMWH-PEG.

Figure 6. Representative R_2 maps (msec^{-1}) of the tumor region (color) before nanoparticle administration (baseline) and after magnetic targeting (post-targ) in rats injected with (A) GPEI/LMWH-PEG, and (B) GPEI. R_2 maps are superimposed on the corresponding T_2 -weighted MRI images of the rat brain.

Figure 7. Quantitative EPR analysis of nanoparticle concentration in excised tumor and contra-lateral brain tissue of rats administered with GPEI/LMWH-PEG and GPEI. Data expressed as Mean $\pm$ SD, n = 4.