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Enhanced accumulation of theranostic nanoparticles in brain tumor by external magnetic field mediated *in situ* clustering of magnetic nanoparticles

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ABSTRACT

Magnetic iron oxide nanoparticles (MIONs) have received much attention due to their unique properties such as ferromagnetic and superparamagnetic characters. These magnetic properties enable the broad use of MIONs in biomedical applications including magnetic resonance imaging (MRI), magnetically guided delivery, and hyperthermal therapy. In particular, magnetic field guided delivery systems have shown promising potential in the development of targeted drug delivery systems for brain tumors. This system facilitates the extravasation and accumulation of MIONs within the brain tumor under external magnetic field. However, the practical use of MIONs is highly limited due to the large physical size of MIONs required for the sufficient retention and accumulation of particles in the brain tumor. This study aims to enhance the accumulation and retention of MIONs in the brain tumor by *in situ* formation of large clusters of MIONs. To achieve this goal, MIONs with core size of 100 nm were modified with free thiol end groups by conjugating bi-functional poly(ethylene glycol) (NHS-PEG-SH). It is expected that the prepared MIONs-PEG-SH remain stable during the systemic circulation. When the circulating MIONs-PEG-SH are exposed to the external magnetic field applied to the brain tumor, the local concentration of MIONs-PEG-SH can be increased and subsequent interactions among MIONs induce a disulfide bond formation. As a result, *in situ* formation of the large clusters of MIONs allows enhanced accumulation and retention of MIONs in a rat brain tumor model. Moreover, when doxorubicin is loaded onto the MIONs, the biodistribution of doxorubicin at brain tumor site is highly enhanced, suggesting their potential use in theranostic applications.

Introduction

Brain tumors are one of the most hard-to-cure tumors with a very abysmal prognosis. Due to its unique pathophysiological conditions separating the systemic blood circulation from brain parenchyma, the delivery of chemotherapeutic drugs and cancer imaging agents is highly limited by the blood–brain barrier (BBB) [1,2]. Although there are some reports on the delivery of anticancer drug molecules across the BBB, clinically effective drug concentration is often difficult to achieve at the brain tumor site [3,4]. Thus, there are unmet needs for the development of novel approaches to enhance drug delivery to the brain tumor and maintain the effective drug concentration for antitumor therapy.

Magnetic iron oxide nanoparticles (MIONs) have unique magnetic properties which allow them to be utilized in various biomedical applications including T2 contrast imaging agents for magnetic resonance imaging (MRI), magnetically guided delivery platforms for targeted therapy, and heat generating nanoparticles under magnetic field for hyperthermia treatment [5–7]. In particular, targeted drug delivery systems utilizing MIONs have shown great potential in delivering therapeutic or diagnostic molecules to tumor sites under the guidance of external magnetic field. For *in vitro* and *in vivo* application, the surface of MIONs is

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often modified with various biocompatible and water soluble polymers to improve the stability of colloidal dispersion in aqueous solution, while co-encapsulating various therapeutic or diagnostic molecules within the dispersion system. Due to their high surface to volume ratio, large amount of molecules can be loaded or conjugated onto the surface of MIONs. Therefore, there have been numerous approaches using surface-engineered MIONs for the delivery of therapeutic or diagnostic molecules [8–10]

Previously, an external magnetic field-mediated brain tumor targeting has been reported using MIONs [11–14]. According to the studies, the external magnetic field applied to the brain tumor tissue induced extravasation of MIONs from blood vessels resulting high accumulation of MIONs. To achieve better magnetic field-mediated brain tumor targeting efficiency, the core size of MIONs needs to be optimized. In this regard, it is suggested that large MIONs in the order of micron can enhance the tumor targeting efficiency since they can be accumulated and retained more in the brain tumor in response to the external magnetic field. However, the larger particles are rapidly removed from the blood by reticuloendothelial system (RES), which severely restricted their further application *in vivo*. Therefore, there is an unavoidable size limit of MIONs. To achieve sufficient magnetic targeting efficiency with small MIONs, the long circulating MIONs are prepared by conjugating polyethylene glycol (PEG) onto the surface of MIONs. The PEGylated MIONs show a considerable targeting efficiency to the brain tumor under a magnetic guidance owing to their longer blood half-life as compared to that of bare MIONs. However, there still needs another strategy to further enhance the accumulation and retention of MIONs in the tumor site.

In this study, enhanced accumulation and retention of MIONs in the brain tumor tissue is attempted by the *in situ* formation of clusters of MIONs under external magnetic field. Since the tumor tissue can be located away from the source of magnetic field, the strategy enhancing the magnetic responsiveness of MIONs is especially important. Many studies have reported that the clusters of MIONs show better magnetic susceptibility response to the external magnetic field compared to the single MIONs [15,16]. Therefore, it is assumed that the clustering of MIONs at the targeted site will further enhance the accumulation and retention of MIONs in the tumor tissue. To achieve this goal, PEG-modified MIONs with free end thiol groups are prepared by conjugating bifunctional PEG onto the surface of MIONs. The prepared PEG-modified MIONs with free thiol group (MIONs-PEG-SH) are expected to generate clusters of MIONs under exposure to an external magnetic field by forming disulfide binds among particles through a thiol–thiol interaction. As a result, the MIONs-PEG-SH can show superior magnetic response as compared to that of the MIONs with bare PEG molecules (MIONs-mPEG). Since the MIONs are well-known T2 contrast agents of MRI, the accumulation of MIONs within the brain tumor tissue can be imaged *in vivo*. In addition to the MR imaging, doxorubicin (DOX), one of the well-known anticancer drugs, was loaded onto the surface of the MIONs-PEG-SH as a therapeutic agent. Enhanced accumulation of DOX in brain tumor is achieved by MIONs-PEG-SH, confirming the high potential of the developed MIONs-PEG-SH as a theranostic system.

Materials and methods

Materials

Starch-stabilized fluid MAG-D magnetic nanoparticles (Fe_3O_4) were purchased from Chemicell GmbH (Berlin, Germany). Amine-reactive methoxy polyethylene glycol succinimidyl carbonate (NHS-mPEG, MW 5000) and heterofunctional amine and thiol-reactive polyethylene glycol generating free thiol after deprofection (OPSS-PEG-NHS, MW 5000) were purchased from JenKem Technology USA Inc. (Plano, Texas). Epichlorohydrin, NH_4OH (30% ammonia), concentrated HCl, NaOH, dithiothreitol (DTT) were obtained from Sigma-Aldrich (St. Louis, Missouri).

Preparation of starch-stabilized MIONs conjugated with thiolated PEG or methoxy PEG

Starch-stabilized magnetic iron oxide nanoparticles (MIONs) with amine functional units were prepared as previously described [11,12]. Briefly, two milliliters of starch-stabilized MIONs (core size: 100 nm) were mixed with 2.6 ml of NaOH (6 M) for 20 min. After 20 min, 1.3 ml of epichlorohydrin was added, and the reaction mixture was shaken overnight. The reacted solution was purified by dialysis (MWCO 12 k) against deionized-distilled water (DDW) for 24 h. Two milliliters of concentrated NH_4OH were added to the purified MIONs for 24 h, and subsequent dialysis against DDW (MWCO 30 k). The prepared amine-functionalized, starch-stabilized MIONs were conjugated with either mPEG-NHS (5 k) or OPSS-PEG-NHS (5 k) via simple reaction of NHS esters with amines in 1X PBS condition (pH 7.5). The unreacted PEGs were removed by using ultrafiltration technique (MWCO 100 k). The hydrodynamic size and concentrations of the MIONs were measured by using dynamic light scattering (ZetaSizer Nano ZS90, Malvern, UK) and inductively couple plasma optical emission spectroscopy (ICP-OES, Optima DV 2000 spectrometer, Perkin Elmer, Waltham, MA), respectively.

Preparation of Doxorubicin-loaded MIONs-PEG-SH (MIONs-PEG-SH/DOX)

The MIONs-mPEG and MIONs-PEG-SH were dispersed in 1X PBS solution (pH 7.5) at a concentration of 2 mg Fe/ml. Equal volume of DOX solution with various concentration (0.2–1 mg/ml) was added to the dispersed MION solution, and subsequent reaction for 24 h. The unloaded DOX was removed by ultrafiltration using Amicon Ultra-4 (MWCO 100 k). After purification, the supernatants were collected and their UV absorptions at 485 nm were measured to determine the DOX loading efficiency. The DOX loading efficiency (DLE; μg Dox/mg Fe) were calculated according to the following equation.

$$DLE = \frac{Dox_{\text{added}} - Dox_{\text{supernatant}}}{Iron_{\text{ultrafiltrated}}}$$

Preparation of a rat model of 9L-glioma brain tumor

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

Male Fisher 344 rats (120–150 g) were utilized to induce intracerebral brain tumors as reported previously [12,17]. In brief, 9 L-glioma cells were cultivated in T175 flask using DMEM with 10% FBS and 1% antibiotics. The grown cells were detached using trypsin-EDTA, washed several times with PBS. After final washing step, the cells were re-suspended in serum free DMEM medium (10^4 cells/μl). Rats were anesthetized using ketamine/xylazine mixture and their pain during the surgery was controlled with buprenorphine. After making small hole in the skull, 10 μl of the prepared 9 L cell suspension was injected into the right forebrain at a depth of 3 mm beneath the skull. The hole in the skull was filled with bone wax, and all the surgical area was cleaned with 100% ethanol and iodine to prevent extracerebral extension of the tumor. The tumor volume was monitored using MATLAB image processing

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