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Real-time monitoring of magnetic drug targeting using fibered confocal fluorescence microscopy

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ABSTRACT

Magnetic drug targeting has been proposed as means of concentrating therapeutic agents at a target site and the success of this approach has been demonstrated in a number of studies. However, the behavior of magnetic carriers in blood vessels and tumor microcirculation still remains unclear. In this work, we utilized polymeric magnetic nanocapsules (*m*-NCs) for magnetic targeting in tumors and dynamically visualized them within blood vessels and tumor tissues before, during and after magnetic field exposure using fibered confocal fluorescence microscopy (FCFM). Our results suggested that the distribution of m-NCs within tumor vasculature changed dramatically, but in a reversible way, upon application and removal of a magnetic field. The m-NCs were concentrated and stayed as clusters near a blood vessel wall when tumors were exposed to a magnetic field but without rupturing the blood vessel. The obtained FCFM images provided *in vivo in situ* microvascular observations of m-NCs upon magnetic targeting with high spatial resolution but minimally invasive surgical procedures. This proof-of-concept descriptive study in mice is envisaged to track and quantify nanoparticles *in vivo* in a non-invasive manner at microscopic resolution.

1. Introduction

Magnetic drug targeting has been shown to be a promising method of concentrating therapeutic agents at a target site, and allowing higher drug doses to be administered while still being tolerated by patients [1–3]. A number of studies have demonstrated that magnetic nanoparticles (MNPs) can be magnetically targeted to tumor sites and the targeting effect has been assessed by various techniques, including optical imaging, magnetic resonance imaging (MRI) and histology studies (Prussian blue staining) [4–11]. These studies have suggested that a higher concentration of magnetic therapeutic agents can be achieved upon the application of an external magnetic field. However, the magnetic behaviors of MNPs in blood vessels and tumor microcirculation have not been investigated. The above mentioned imaging methods cannot offer sufficient resolution to show how MNPs travel through blood vessels and accumulate in tumors, whereas histological studies can only provide *ex vivo* information at post-mortem in a non-dynamic way. Some studies have been carried out using *ex vivo* artery models or mathematical simulation to characterize the MNPs behaviors in blood vessels and/or surrounding tissues, but no *in vivo* studies have been performed so far [12–15].

Here we visualized the magnetic capture of magnetic polymeric nanocapsules (*m*-NCs) within blood vessels and tumor tissues in real-time *in vivo* before, during and after magnetic field exposure. The magnetic targeting efficacy of m-NCs was firstly quantified by gamma counting and this was further confirmed by direct imaging of the magnetic targeting process in blood vessel on the microscopic scale. This proof-of-concept descriptive study in mice is envisaged to track and quantify nanoparticles *in vivo* in a non-invasive manner with microscopic resolution.

2. Materials and methods

2.1. Materials

'Ferrofluid' magnetic oil (oleic acid-coated superparamagnetic iron oxide nanoparticles (SPIONs) with diameter of 10 nm, suspended in kerosene at 10^{17} particles per mL) was purchased from Magnacol Ltd. (UK). Soybean lecithin (Epikuron 140 V) was a kind gift from Cargill Pharmaceuticals (USA). Polyoxyethylene-bis-amine (NH_2-$PEG_{3.5\ kDa}$-NH_2) was purchased from JENKEM (USA). D/L-lactide/glycolide copolymer 75/25 ($PLGA_{18\ kDa}$-COOH) was purchased from Purac Biomaterials (the Netherlands). Tween® 80, nitric acid, methanol, dimethylsulphoxide (DMSO) and dichloromethane were obtained from Fisher Scientific Ltd. (UK). Fluorescein isothiocyanate–dextran (FITC-Dextran, average molecular weight 2000 kDa), sodium chloride, diethylene triamine pentaacetic acid (DTPA), castor oil,

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ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) and Sephadex® G-75 were purchased from Sigma Aldrich (UK). 1,1-dioctadecyltetramethyl indotricarbocyanine Iodide (DiR) was obtained from Cambridge Bioscience (UK). Advanced RPMI-1640 media, penicillin-streptomycin 100×, 0.25% Trypsin-EDTA with phenol red, Glutamax™ supplement, phosphate buffered saline PBS (10×, pH 7.4) and phosphate buffered saline PBS (1×, pH 7.4) were obtained from Gibco, Invitrogen (UK). Fetal bovine serum (FBS) was obtained from First-Link Ltd. (UK). Pentobarbital sodium (Euthatal®) was obtained from Merial (UK). PD-10 desalting column was obtained from GE Healthcare Life Sciences (UK).

2.2. Preparation and characterization of m-NCs and m-NC-DiR

The magnetic polymeric nanocapsules (*m*-NCs) were prepared by single emulsification/solvent evaporation method [1]. $PLGA_{18\ kDa}$-$PEG_{3.5\ kDa}$-NH_2 and $PLGA_{18\ kDa}$-$PEG_{3.5\ kDa}$-DTPA were synthesized as described in our previous work [16,17]. Briefly, $PLGA_{18\ kDa}$-$PEG_{3.5\ kDa}$-NH_2 (12.5 mg, 10% w/w $PLGA_{18\ kDa}$-$PEG_{3.5\ kDa}$-DTPA was incorporated for m-NCs to be used for radiolabeling), castor oil (75 mg), soybean lecithin (25 mg) and increasing amounts of SPIONs (0, 0.5, 2.5 or 10 mg) were dissolved in 2.5 mL dichloromethane. DiR was incorporated into m-NCs formulation at 0.5% w/w DiR/castor oil for fibered confocal fluorescence microscopic (FCFM) imaging. The organic phase was poured into an aqueous phase (5 mL) containing Tween® 80 (20 mg) as a hydrophilic surfactant. The resultant dispersion was emulsified by ultrasonication using a probe sonicator (Soniprep 150, UK) at 15 μ amplitude for 180 s in an ice bath. Organic solvents were then evaporated in a chemical fume hood for 20 min. The final volume of the m-NC suspension was adjusted to 5 mL. The obtained *m*-NC suspension was concentrated using a rotary evaporator (Buchi, Switzerland) by 10 (FCFM imaging) or 20 times (gamma counting), yielding 25 and 50 mg/ml of polymer, respectively.

2.3. Size and zeta potential measurements

The hydrodynamic size (*Z*-Average), polydispersity index (PDI) and zeta potential of NCs and m-NCs were determined by NanoZS (Malvern Instrument, UK) at 25 °C using disposable square polystyrene cuvettes (for size and PDI) or disposable capillary cells (for zeta potential) (Malvern Instrument, UK). The *Z*-Average diameter and polydispersity index were measured in water and presented as the average value of three measurements, with 15 runs within each measurement. The zeta potential was also measured in water and presented as the average value of three measurements, with 20–25 runs within each measurement. The mean and standard deviation (SD) of size and zeta potential were calculated for each sample.

2.4. Determination of SPION encapsulation efficiency in m-NCs

m-NCs were prepared with increasing loadings of SPIONs (0, 0.38, 1.84 and 7.02% w/w SPION/NC) and purified by size exclusion chromatography (Sephadex® G-75 column, size exclusion chromatography) to remove any un-encapsulated SPIONs. The Fe content was determined by inductively couple plasma mass spectrometry (ICP-MS, Perkin Elmer SCIEX ICP mass spectrometer, ELAN DRC 6100, USA). For ICP-MS measurements, Fe standards (Leeman Labs Inc., USA) were prepared in 20% nitric acid to obtain a standard curve in the range of 10–10,000 parts per billion with respect to Fe. m-NCs were digested in 2 mL of nitric acid in Falcon™ 15 mL conical centrifuge tubes (Fisher Scientific, UK) and incubated overnight at 50 °C. The resulting solution was diluted by 10 times with water before the measurements.

2.5. Determination of DiR encapsulation efficiency in m-NCs

The encapsulation efficiency of DiR in m-NCs was assessed using a UV/fluorescence spectrometer (Varian, Cary Eclipse, Australia). Prior to quantification, m-NC-DiR suspensions were purified by a PD-10 desalting column (size exclusion chromatography) and eluted in PBS buffer to remove any free DiR. The m-NC suspensions before and after purification were diluted in DMSO (1/19, v/v) to rupture the NC structure. The excitation/emission wavelengths for the detection of DiR were 740/785 nm. The encapsulation efficiency was expressed as the percentage of the encapsulated dye to the total amount of DiR added to the formulation. All measurements were performed in triplicate and expressed as mean ± SD ($n = 3$).

2.6. Radio-labelling of m-NCs and serum stability studies

To radio-label the m-NCs with indium-111, m-NCs were prepared as described in the previous section except that $PLGA_{18\ kDa}$-$PEG_{3.5\ kDa}$-DTPA was included at 10% (w/w) of the total polymer content. The m-NC suspension (250 μL, 50 mg/mL of polymer) was incubated with 2 M ammonium acetate (one ninth of the reaction volume, pH 5.5), to which 1 MBq of $^{111}InCl_3$ (Mallinckrodt, UK) was added for gamma counting. The reaction was kept at room temperature for 30 min with intermittent vortexing every 10 min. Upon completion, the radiolabelling reaction was quenched by the addition of 0.1 M EDTA chelating solution (one twentieth of the reaction volume). $^{111}InCl_3$ alone was subjected to the same labelling reaction conditions and used as a control.

The m-NC-^{111}In was passed through PD-10 columns before injecting into animals to exchange the ammonium acetate buffer (pH 5.5) with PBS (pH 7.4) and remove free ^{111}In-EDTA. The m-NCs-^{111}In (~150 μL per injection dose, 25 mg/mL of polymer) were collected from the column and spotted on instant thin layer chromatography (iTLC) strips which were then developed in 0.1 M ammonium acetate containing 50 mM EDTA as a mobile phase. Strips were allowed to dry before being developed and counted quantitatively using a cyclone phosphor detector (Packard Biosciences, UK) to ensure no free ^{111}In-EDTA present in the injected solution.

2.7. Animal studies and tumor inoculation

All animal experiments were performed in compliance with the UK Home Office (1989) Code of Practice for the Housing and Care of Animals used in Scientific Procedures. CT26 murine colon carcinoma cells (CT26, ATCC®, CRL-2638TM) were cultured in Advanced RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 1% L-glutamine, 1% penicillin-streptomycin and 10% fetal bovine serum (FBS), in 5% CO_2 and 95% air, at 37 °C. The harvested CT26 cells were suspended in PBS solution (pH 7.4). A total of 1×10^6 cells in 20 μL were injected subcutaneously and bifocally at the hind foot of female BALB/c mice aged 4–6 weeks (Harlan, UK). After inoculation, the tumor volume was measured on day 8 and then every other day using a digital caliper and calculated using Eq. (1) [2]

$$\text{Tumor volume } (\text{mm}^3) = (4/3) * \pi\,(A/2)^2 * (B/2) = 0.52A^2B \quad (1)$$

where *A* and *B* represent the width and the length of the tumors, respectively. All experiment were carried out (m-NCs administration) when the tumor volume reached approximately 500 mm^3.

2.8. Magnetic targeting setup in vivo

Disk-shaped nickel-coated neodymium iron boron ($Nd_2Fe_{14}B$) magnets (Magnet Expert Ltd., Tuxford, UK) were used for the *in vivo* magnetic drug targeting studies. That was an 8 mm diameter, 5 mm thick, N42 grade magnet (product code F324), which had a reported field strength of 0.43 Tesla (T) and a reported 'vertical pull' parameter (a