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The effectiveness of a magnetic nanoparticle-based delivery system for BCNU in the treatment of gliomas

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ABSTRACT

This study describes the creation and characterization of drug carriers prepared using the polymer poly-[aniline-*co*-*N*-(1-one-butyric acid) aniline] (SPAnH) coated on Fe_3O_4 cores to form three types of magnetic nanoparticles (MNPs); these particles were used to enhance the therapeutic capacity and improve the thermal stability of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a compound used to treat brain tumors. The average hydrodynamic diameter of the MNPs was 89.2 ± 8.5 nm and all the MNPs displayed superparamagnetic properties. A maximum effective dose of 379.34 μg BCNU could be immobilized on 1 mg of MNP-3 (bound-BCNU-3). Bound-BCNU-3 was more stable than free-BCNU when stored at 4 °C, 25 °C or 37 °C. Bound-BCNU-3 could be concentrated at targeted sites *in vitro* and *in vivo* using an externally applied magnet. When applied to brain tumors, magnetic targeting increased the concentration and retention of bound-BCNU-3. This drug delivery system promises to provide more effective tumor treatment using lower therapeutic doses and potentially reducing the side effects of chemotherapy.

1. Introduction

Malignant glioma is a serious disease. Patients treated using surgical resection, radiotherapy and chemotherapy survive, on average, for 6–24 months post-treatment [1–3]. The chemotherapeutic agent BCNU (also known as carmustine) has been commercialized for the treatment of malignant brain tumors [4]. Although it improves patient survival, its efficiency is limited by side effects such as myelosuppression, hepatic toxicity, and pulmonary fibrosis [5,6]. Furthermore, BCNU hydrolyzes readily with increasing temperature, resulting in a short half-life in the human body [7,8]. Such drawbacks can be overcome in part by developing a carrier to enhance the concentration of the drug specifically at the tumor site. For example, biodegradable polymer fiber carriers have been studied for their ability to deliver BCNU over longer periods, improve therapeutic efficacy and reduce toxicity [7,8]. Similarly, microelectromechanical devices [9] and polymeric microchips [10] have been designed to release complex profiles of multiple substances to enhance the efficiency of anticancer drug therapies.

MNPs coated with a shell of hydrophilic polymers provide high-capacity functionalized surfaces capable of binding enzymes [11], inhibiting aggregation, and increasing stability in aqueous solution by preventing undesirable interactions caused by electrostatic repulsive forces between the particles and hydrophilic surfaces [12,13]. They can be used for a variety of biomedical applications, such as contrast-enhancing agents for magnetic resonance imaging (MRI) [14,15], magnetically guided drug targeting [16–18], enhancing enzyme stability (which normally is limited by conformational restrictions imposed by binding of the enzyme to carriers [11,19,20]) for repeated use, and magnetic diagnosis to detect tumor cells in blood or bone marrow by modifying the surfaces with different specific proteins or antibodies [21]. They also exhibit high drug-loading capacities and good stability in aqueous solutions due

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to their hydrophilic polymer shells with high-capacity functionalized surfaces, and have biocompatibilities with cells and tissues when used for drug delivery [22–26].

The biocompatibilities of conducting polymers such as polyaniline (PAn) and polypyrrole have been demonstrated both *in vivo* and *in vitro* [27–29], and such compounds have been applied to tissue engineering [28], biosensor–human interfaces [30,31], etc. However, their poor solubility limits their use. Thus, we have enhanced the utility of PAn by modifying it to SPAnH to act as a hydrophilic conducting polymer. Three types of MNPs with different ratios of SPAnH shell to Fe_3O_4 core were prepared and their magnetizations and drug capacities were investigated. Immobilizing BCNU on these particles enhanced its thermal stability, and allowed it to be targeted actively to brain tumors using an external magnetic field. The drug-loading capacity, activity, stability, and *in vitro* cytotoxicity of bound-BCNU were also investigated, as well as its effect on gliomas *in vivo*.

2. Materials and methods

2.1. Materials

Succinic anhydride was purchased from MP Biomedicals. Aluminum chloride and 1-methyl-2-pyrrolidone (NMP) were purchased from ACROS. Aniline monomer, ammonium peroxydisulfate (98%), hydrochloric acid (37%), sodium hydroxide, iron (II) chloride, and iron (III) chloride were purchased from Merck. NMP and the aniline monomer were distilled under reduced pressure before use. 1-Ethyl-3-(3-dimethylaminepropyl)carbodiimide hydrochloride (EDC), 2-(*N*-morpholino)ethanesulfonic acid hydrate (MES), sulfanilamide, *N*-(1-naphthyl)ethylenediamine, sodium bicarbonate, XTT sodium salt, phosphate-buffered saline (PBS), and trypsin were purchased from Sigma. *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS) was purchased from Fluka. BCNU was obtained from Boehringer Ingelheim.

Rat C6 glioma cells were obtained from Professor Chia-Rui Shen of the Graduate Institute of Medical Biotechnology, Chang Gung University in Taiwan. Human umbilical vein endothelial cells (HUVEC) were purchased from the Bioresource Collection and Research Center in Taiwan. Endothelial cell growth supplement (ECGS) was purchased from Millipore. Medium 199 (M199), RPMI Medium 1640 and Hank's balanced salt solution (HBSS) were purchased from Gibco. Fetal bovine serum (FBS) was purchased from Biological Industries. Gentamycin, penicillin, and streptomycin were purchased from MDBio. The LIVE/DEAD Viability/Cytotoxicity Kit and minimum essential medium were purchased from Invitrogen. Deionized (DI) water was used in all experiments.

2.2. Preparation of MNPs coated with SPAnH

Detailed procedures for the synthesis of poly[aniline-*co*-sodium *N*-(1-one-butyric acid) aniline] (SPAnNa) and Fe_3O_4 were reported previously [32]. MNPs were generated by covering Fe_3O_4 cores with three different concentrations of SPAnH. Briefly, two mL of Fe_3O_4 (10 mg/mL) were reacted with 0.8 mL of SPAnNa (1.85, 3.7 or 4.9 mg/mL), and the mixtures were doped slowly by addition of 0.5 M HCl. Acid doping of SPAnNa induces formation and aggregation of SPAnH, encapsulating the Fe_3O_4 to form MNP-1, MNP-2 and MNP-3, respectively. The MNPs were separated from the solution using a strong magnet, washed with DI water, and sonicated continuously until the solution became neutral. One mL of MNPs (5 mg/mL) was loaded into sample vessels and a 0.3-T magnet was used to attract the particles for 30 min. The attracted MNPs were vacuum-dried for 48 h at 80 °C.

2.3. Characterization of MNPs

The hydrodynamic sizes of MNPs were measured by dynamic light scattering (ZEN3600 Zetasizer, Malvern Instruments Ltd., Malvern, UK). The magnetizations of Fe_3O_4 and MNPs were measured using a superconducting quantum interference device (SQUID; MPMS-7, Quantum Design, San Diego, CA, USA) at 25 °C and ±4-kOe applied magnetic field. Fourier transform infrared (FT-IR) spectroscopy (TENSOR 27, Bruker Optics, Billerica, MA, USA) was recorded by the KBr sample holder method. The concentrations of carboxyl groups on the surface of MNPs were measured by the Toluidine Blue O method [33]. The ratios of the SPAnH shells to the Fe_3O_4 cores were analyzed by inductively coupled plasma optical emission spectrometry (720-ES, Varian Inc., Palo Alto, CA, USA).

2.4. Immobilization of BCNU

EDC (24 mg) and sulfo-NHS (50 mg) were dissolved in 2 mL of 0.5 M MES in the dark. A 0.2-mL aliquot of this solution was mixed with 0.2 mL of MNP-1, MNP-2, or MNP-3 (10 mg/mL) at 25 °C and sonicated for 30 min in the dark. After separation, MNPs were washed with 0.8 mL of 0.1 M MES, resuspended in 0.2 mL of MES, and mixed with 0.1 mL of BCNU at 10 °C. The solutions were mixed by sonicating for 0.5 h, and then shaken for another 2.5 h at 10 °C. MNPs with BCNU immobilized on the surface were separated from the solution, washed with pure alcohol and DI water, washed again with DI water to remove residual alcohol, and dispersed in 0.2 mL DI water.

To calculate the ratio of BCNU immobilized on the different MNPs (i.e., bound-BCNU-1, bound-BCNU-2, and bound-BCNU-3), samples were analyzed by high-performance liquid chromatography (HPLC) on a SUPELCOSIL™ LC-18 column (4.6 × 250 mm) using an L-2130 pump and an L-2400 UV-detector (Hitachi). The mobile phase was a 40:60 (v/v) mixture of DI water and methanol with a flow rate of 2 mL/min; data were measured at 270 nm.

2.5. Stability assay for bound-BCNU

The activities of bound-BCNU-1, bound-BCNU-2, and bound-BCNU-3 were analyzed using the Bratton–Marshall assay [1,34]. Forty μL of each bound-BCNU solution were reacted with 80 μL of sulfanilamide solution (5 mg/mL in 2 M HCl) at 50 °C for 45 min and then cooled to room temperature. One hundred μL of the above solution and 10 μL of Bratton–Marshall reagent (3 mg/mL of *N*-(1-naphthyl)ethylenediamine in DI water) were reacted for 1 min and absorbances were measured at 540 nm using a Genesys 20 spectrophotometer (Thermo Scientific). The stabilities of free-BCNU and bound-BCNU stored at 4 °C, 25 °C and 37 °C for 1–30 days were also analyzed using the Bratton–Marshall assay.

2.6. DNA interstrand crosslinking

To confirm the cytotoxic effects of BCNU, an ethidium bromide fluorescence assay was used to measure the level of DNA interstrand crosslinking in C6 cells [35]. The cells were exposed initially to different concentrations (5–30 μg/mL) of free-BCNU or bound-BCNU-3 and incubated for 8 h. After incubation, ~1×10^6 cells were collected by centrifugation at 5000 rpm for 6 min at 8 °C and resuspended in PBS. Forty μL of the cell suspension were incubated for 15 min at 4 °C with 200 μL of lysis buffer. After lysis, the cell pellets were separated by centrifugation at 5000 rpm for 6 min and the suspension incubated with 25 μL of heparin (500 IU/mL) for another 20 min at 37 °C, followed by the addition of 1 mL of ethidium bromide solution. The mixture was heated for 5 min at 100 °C to denature the DNA, and then cooled in an ice bath for 6 min to renature it. Fluorescence was measured with excitation and emission wavelengths of 525 and 580 nm, respectively. The percentage of DNA interstrand crosslinking (C) was calculated using following equation:

$$C = \{[(f_a/f_b)_t - (f_a/f_b)_u]/[1 - (f_a/f_b)_u]\} \times 100\%$$

where f_b and f_a are the fluorescence before and after heat denaturation for treated (t) and untreated (u) cells.

2.7. In vitro cytotoxicity assay

HUVEC were cultured in M199 supplemented with 2.2 mg/mL sodium bicarbonate, 10% FBS, 50 μg/mL gentamycin, 50 μg/mL penicillin, 50 μg/mL streptomycin, 25 U/mL heparin and 30 μg/mL ECGS at 37 °C and 5% CO_2. Approximately 10,000 cells (i.e., 150 μL of a suspension of 6.67×10^4 cells/mL) were placed in each well (coated with 1% gelatin) of a 96-well culture plate and incubated in a humidified chamber at 37 °C and 5% CO_2 for 48 h. Fifty μL of different concentrations of MNP-3 in M199 were added and the culture continued. Cell cultures were also performed in the presence of an 800-G magnetic field applied beneath the culture plate. Cell proliferation was determined by counting after 48 h.

C6 cells were cultured in RPMI 1640 supplemented with 2.2 mg/mL sodium bicarbonate, 10% FBS, 50 μg/mL gentamycin, 50 μg/mL penicillin, and 50 μg/mL streptomycin at 37 °C and 5% CO_2. Approximately 10,000 cells (i.e., 150 μL of a suspension of 6.67×10^4 cells/mL) were placed in each well of a 96-well culture plate and incubated in a humidified chamber at 37 °C and 5% CO_2 for 24 h. Fifty μL of different concentrations of free-BCNU, MNP-3 or bound-BCNU-3 in medium were added and the cultures continued. Cell cultures were also performed in the presence of an 800-G magnetic field applied beneath the culture plate. Cell proliferation was determined by counting after 48 h.

Before counting, the culture medium was removed and cells were incubated in 120 μL of XTT for 3 h. After the reaction, 100 μL of XTT solution were sampled from each well and transferred to a 96-well counting dish. The cytotoxicity of bound-BCNU-3 on C6 cells *in vitro* was evaluated by measuring the OD at 490 nm using an ELISA reader.

In a separate series of experiments, 2 mL of C6 cells (10,000 cells/mL) were plated in 35-mm diameter plates and cultured in a humidified chamber at 37 °C and 5% CO_2 for 48 h. One hundred μL of MNP-3 (4.5 mg/mL) or different concentrations of bound-BCNU-3 in RPMI 1640 medium were added and the incubations continued for 1–6 days or 8 h, respectively. The medium was removed, cells were washed with 1 mL of HBSS and 1 mL LIVE/DEAD reagent was added. After 30 min, the reagent was removed and the cells washed again with HBSS. Cytotoxicity was monitored using a TCS SP2 confocal spectral microscope (Leica).