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PEGylated Poly(amidoamine) dendrimer-based dual-targeting carrier for treating brain tumors

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ABSTRACT

A dual-targeting drug carrier (PAMAM-PEG-WGA-Tf) based on the PEGylated fourth generation (G = 4.0) PAMAM dendrimer with transferrin (Tf) and wheat germ agglutinin (WGA) on the periphery and doxorubicin (DOX) loaded in the interior was synthesized and its BBB penetration and tumor targeting properties were explored. DLS and TEM measurements revealed the size of PAMAM-PEG-WGA-Tf was in the range of 14–20 nm. It reduced the cytotoxicity of DOX to the normal cells greatly, while efficiently inhibited the growth rate of the C6 glioma cells. The assay of transport across the BBB showed that PAMAM-PEG-WGA-Tf delivered 13.5% of DOX in a period of 2 h, demonstrating an enhanced transport ratio as compared to the ratio of 8% for PAMAM-PEG-WGA, 7% for PAMAM-PEG-Tf and 5% for free DOX in the same period of time. The accumulation of DOX in the tumor site was increased due to the targeting effects of both Tf and WGA, leading to the complete breakage of the avascular C6 glioma spheroids in vitro.

1. Introduction

As well known, the treatment of brain cancers, such as oligodendrogliomas, astrocytomas and ependymomas remains tremendous challenge for the restrained delivery of therapeutic drugs to reach the tumors [1]. Such limitation arises from (1) an endothelial cell monolayer combined with pericytes and astrocytes, namely, blood–brain barrier (BBB), only allows hydrophobic molecules with MW < 400 Da to pass through it [2], while blocks over 98% small-molecule drugs and almost 100% large-molecule drugs [3] such as recombinant proteins and monoclonal antibodies into the brain; (2) it is difficult to maintain the therapeutic agents mainly accumulating in the tumor site but not to diffuse in the normal tissues due to the short duration time and non-specific binding in vivo. To bridge the requirements of brain cancer therapy, great efforts have been paid to develop various strategies for improving the penetration of drugs across the BBB and the targeting effect to the tumors, including interstitial drug delivery, interacavitary treatments by well-controlled release, lipophilic analogs, prodrugs and various transport systems and carriers. For example, Jiang et al. reported the nanoparticles based on poly (ethylene glycol)-poly (lactic acid) (PEG-PLA) with targeting moieties and fluorescent probe incorporated. They found an increased uptake of the nanoparticles by bEnd.3 cells, a higher accumulation of the nanoparticles in the cortex and striatum, third ventricle and periventricular region and a 2.98 times increase of AUC by the nanoparticles in 24 h [4,5]. Schmidt et al. reported a long-circulating PEG-liposomal glucocorticosteroids. Compared with the healthy control animals, it gave 4.5-fold liposome accumulation in the brain of the (adoptive transfer)-experimental autoimmune encephalomyelitis ((AT)-EAE) rats [6]. Recently, a leptin-derived peptide modified dendrigrafted poly-L-lysine was used as gene vector for brain-targeting delivery [7]. With adjusting the surface charge, PEGylation and brain-targeting ligands linking on the surface, this gene carrier showed good cellular uptake and low cytotoxicity in vitro and in vivo.

Poly(amidoamine) (PAMAM) dendrimers are considered as one of the most promising polymer architectures in biomedical applications in recent years [8,9]. Examples included the encapsulation of therapeutic agents in the interior of dendrimers and attachment of drugs, targeting moieties and functional groups on the surface of dendrimers by covalent bonding or physical absorbing, which afforded the possibility to produce the desired multifunctional nanocarriers for drug delivery.

In the last decades, various PAMAM-based drug carriers have been developed to explore their potential use for cancer therapy.

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PEGylated dendrimers with folic acid as targeting ligand showed enhanced affinity for tumor sites in vitro and in vivo [10–12]. Very recently, Jiang et.al reported a partly PEGylated PAMAM dendrimer linked with doxorubicin (DOX) by an acid-sensitive *cis-aconityl* linkage. They found such a conjugate exhibited rapid drug release rate in vitro, more intracellular uptake in weak acid environment and higher tumor accumulate level in vivo [13]. Although many efforts have been paid to ascertain the potential of PAMAM dendrimers as drug delivery systems in tumor treatment up to now, the reports of dendrimers referred to brain cancer targeting and therapy are still scarce [14–16].

Transferrin (Tf) is a widely known ligand for brain-targeting because transferrin receptor (TfR) is highly expressed on the brain capillaries endothelial surfaces and many malignant cells [17]. With the targeting ligand of Tf, the drug delivery systems come across the BBB without disrupting it through a receptor-mediated transport mechanism to reach the brain and further accumulate in tumor cells [18,19]. A recent study showed that the nanoparticles of PLGA conjugated with Tf displayed higher endocytosis ratio by brain capillary endothelial cells (BCEC). However, the endocytosis was totally inhibited after it pretreated with excess of Tf or a caveolae inhibitor of filipin [20], suggesting a major role of caveolae-mediated transcytosis for Tf-mediated delivery. Additionally, lectins, especially wheat germ agglutinin (WGA) as a newly discovered brain-targeting ligand showed a strong affinity for cerebral capillary endothelium, enhanced binding affinity for malignant tumor cells [21,22] and low toxicity to the normal tissues. Plattner et al. studied the lectin-binding pattern of two typical BBB mimicking cells at the single and monolayer cell level [23]. As a result, WGA exhibited the highest binding strength and specificity to these two cell lines.

Herein, we report a fourth generation (G4) PAMAM dendrimer-based multifunctional nanocarrier with two targeting substances, Tf and WGA, on the dendrimer periphery, and encapsulated DOX, an anticancer drug, in the interior of PAMAM molecules (PAMAM-PEG-WGA-Tf), by which we aim for both overcoming the BBB and ingesting drugs by the brain tumor cells. Various measurements including ^{1}H NMR, UV–vis and SDS-PAGE were used to confirm the structure of carrier, DLS and TEM measurements were performed to detect the morphology and the size of carrier. The targeting effects of drug carriers were estimated on the BBB model in vitro. The cytotoxicity of drug loaded carriers and the inhibitory effect to the avascular C6 glioma cells spheroids were also evaluated in vitro.

2. Materials and methods

2.1. Materials

Wheat germ agglutinin (WGA) was purchased from Medicago AB (Uppsala, Sweden). Methoxy PEG succinimidyl Carbonate ester (mPEG-NHS, Mw = 1000) was purchased from Biomatrik Inc. (Jiaxing, China). Maleimide-PEG-Carbonate-NHS (MAL-PEG-NHS, Mw = 5000) was purchased from NOF Corporation (Japan). Transferrin (Tf), 2-iminothiolane hydrochloride (Traut's reagent), 5, 5-Dithiobis (2-nitrobenzoic acid) (Ellmann's reagent) and sulforhodamine B (SRB) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Other reagents were purchased from Beijing Chemical Reagents (Beijing, China). All reagents were used as received and the solvents were purified according to the general procedures before used.

2.2. Synthesis of dual-targeting dendrimer-based drug carriers

The PEGylated PAMAM dendrimer was prepared by reacting the surface NH_2 groups of PAMAM (G4) with both the PEGs bearing an NHS end group and the PEGs with NHS and MAL groups on both sides of the chain ends. Finally sulfhydryl WGA and Tf were conjugated to the PEGlated PAMAM dendrimer quantificationally by the Michael addition reaction. The PEGlated PAMAM dendrimer with either Tf or WGA as a targeting ligand were also synthesized and tested for evaluating the dual-targeting effects of PAMAM-PEG-WGA-Tf (Fig. 1).

2.2.1. Synthesis of PAMAM dendrimer

G4.0 PAMAM dendrimer (contains 64 primary amino groups on the periphery, Mw = 14,196) was synthesized with a divergent method by repeating the Michael addition of amino groups with methyl acrylate, followed by amidation of the resulting esters with excess ethylenediamine. The crude product was then transferred to a dialysis bag (MWCO 5000) for the purification in deionized water for 48 h to remove the unnecessary byproducts. ^{1}H NMR (D_2O, 300 MHz, ppm): δ 2.25–2.40 (m, $-CH_2CH_2CONH-$); δ 2.45–2.58 (m, $-CH_2CH_2N<$); δ 2.60–2.72 (m, $-NCH_2CH_2CO-$); δ 2.75–2.85 (m, $-CH_2CH_2NH_2$); δ 3.10–3.20 (m, $-CONHCH_2CH_2-$).

2.2.2. PEGylation of PAMAM dendrimer (PAMAM-PEG)

PAMAM-PEG was synthesized by the reaction of the periphery NH_2 groups of PAMAM dendrimer with the NHS-activated PEGs. Briefly, PAMAM G4.0 (5.0 mg, 0.35 μM) and MAL-PEG-NHS (Mw = 5000, 7.0 mg, 1.4 μM) were put into a round glass flask and allowed to stir gently for 15 min in 2 mL deionized water at 20 °C. Then 15 mg (15 μM) of m-PEG-NHS (Mw = 1000) was added to the solution to allow further reacting for 30 min. The resulting product was then passed through a Sephadex G-50 column to remove free PEGs that were completely separated from the product confirmed by Thin Layer chromatography (TLC). The chemical structure and molecular weight of PAMAM-PEG were characterized by ^{1}H NMR measurement using D_2O as the solvent. Yield: 77%, ^{1}H NMR (D_2O, 400 MHz, ppm): δ 2.25–2.40 (m, $-CH_2CH_2CONH-$); δ 2.45–2.55 (m, $-CH_2CH_2N<$); δ 2.68–2.74 (m, $-NCH_2CH_2CO-$); δ 2.95–3.20 (m, $-CONHCH_2CH_2-$ and $-CH_2CH_2NH_2$); δ 3.23 (s, $-CH_2CH_2OCH_3$); δ 3.32–3.75 (m, $-CH_2CH_2O-$); δ 4.06–4.09 (m, $-NHCOOCH_2CH_2-$); δ 6.72 (s, $-COCH=CHCO-$).

2.2.3. Synthesis of thiolated WGA or Tf

Thiolated WGA or Tf was synthesized according to the previous report [14]. Briefly, 1 mL aqueous solution of 2-iminothiolane hydrochloride (1.0 mg, 7.5 μM) was added to 1 mL aqueous solution containing WGA (5.0 mg, 0.12 μM) and allowed to stir slowly for 60 min at 20 °C. Then the mixture was passed a Sephadex G-50 column to remove the impurities. The amount of thiol groups was determined by Ellmann's Reagent [25] with the ratio of 1.5:1 (thiol group to WGA). Tf-SH was obtained in a same manner except the amounts of Tf and 2-iminothiolane hydrochloride were 10 mg (0.12 μM) and 0.7 mg (5.0 μM), respectively.

2.2.4. Synthesis of the targeting carriers

The dual-targeting drug carrier of PAMAM-PEG-WGA-Tf and the carriers modified with either Tf or WGA (PAMAM-PEG-WGA, PAMAM-PEG-Tf) were synthesized using the same procedure. Take PAMAM-PEG-WGA-Tf as an example, 7.4 mg (0.10 μM) of PAMAM-PEG and 4.0 mg (0.1 μM) of WGA-SH were stirred in 10 mL aqueous solution for 2 h at 20 °C, then 8.0 mg (0.1 μM) of Tf-SH was added and the mixture was stirred for another 2 h. The resulting crude product was allowed to run through a Sephadex G-100 column to remove the unconjugated proteins and PAMAM-PEG. SDS-PAGE was used to measure the targeting carriers.

2.3. Particle size and zeta potential measurements

The particle size and zeta potential of the drug carriers were measured using a zeta PALS analyzer (Brookhaven Instruments Corporation, BIC) equipped with a 35 mW solid state laser (660 nm). PAMAM-PEG or PAMAM-PEG-WGA-Tf was dissolved in deionized water at the concentration of 1 mg/mL or 0.5 mg/mL, respectively. Particle size measurements were performed at 35 °C using BIC particle sizing software (9kpsdw32, ver.2.3). Zeta potential measurement was done with BIC PALS zeta potential analyzer software (palsw32, ver.3.43).

2.4. Transmission electron micrograph morphology

Transmission electronic microscopy (TEM) measurements were performed on an H-9000NAR, operating at an acceleration voltage of 100 kV. A drop of PAMAM-PEG or PAMAM-PEG-WGA-Tf solution (concentration: 1.0 mg/mL or 0.5 mg/mL) was placed on carbon-coated Formvar copper grids. After 2 min, the grid was tapped with filter paper to remove the aqueous solution on the surface and air-dried. Negative staining was performed by addition of a drop of 1 wt.% solution of uranyl acetate to the copper grid with the sample.

2.5. Doxorubicin loading

DOX was loaded using an equilibrium dialysis method as described previously [26,27]. 1 mg of DOX hydrochloride was dissolved in 1 mL deionized water and mixed with 10 mg of PAMAM-PEG-WGA-Tf. Then the solution was increased to 5 mL and stirred slowly in dark for 24 h. Then the solution was transferred to a dialysis bag (MWCO 8000) and dialyzed twice against deionized water under strict sink conditions for 10 min to remove free DOX. The encapsulation percentage of DOX was determined by UV–visible scanning spectrophotometer (Schimaduza UV-2101 PC) at 480 nm in deionized water. The encapsulation ratio of DOX for other carriers was determined under the same conditions.

2.6. Cell culture

In this study, murine brain microvascular endothelial cells (BMVECs) and murine C6 glioma cell lines were used. BMVECs were kindly provided by Prof. J.N. Lou