A cascade targeting strategy for brain neuroglial cells employing nanoparticles modified with angiopep-2 peptide and EGFP-EGF1 protein

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1. Introduction

As a large proportion of society ages, the risk of central nervous system (CNS) diseases has increased annually and has become one of the most serious health threats. According to US statistical data informed 1999, the incidence of CNS diseases was as high as that seen for cancer and cardiovascular diseases combined. Currently, there are 1.5 billion people worldwide afflicted with CNS diseases, and the number will increase to 1.9 billion by 2020 [1]. The existence of a blood brain barrier (BBB) shadows the diagnosis and therapy of CNS diseases, however brain targeting delivery systems bring new hope. Brain targeting delivery systems can transport drugs directly to the brain to treat CNS diseases, and potentially at the same time, imaging agents loaded into the delivery system could afford more information about a diseased brain. Brain targeting delivery systems mediated by receptors, such as nanoparticles (NPs), liposomes and polymersomes modified with transferrin, lactoferrin, transferrin receptor monoclonal antibody-OX26, have been the most effective [2–5]. Unfortunately, these systems facilitated brain targeting but there was no region or cell type specificity. Thus drugs utilized with these systems were distributed into normal regions or non-target cells of brain and may cause serious side effects, undermining the treatment and also decreasing the drug amounts reaching target regions or cells. Therefore it is essential to develop a delivery system that precisely targets particular cells, enzymes or proteins with a lower distribution to the non-target region or cells. As the main component of brain, neuroglial cells are involved in the majority of CNS diseases, including Alzheimer's disease, Parkinson's disease, stroke, pain, epilepsy, etc [6–10]. Here we utilized neuroglial cells as a model to evaluate the effects of a new cascade targeting system.
The first barrier to targeting brain neuroglial cells is the BBB. It has been identified that low-density lipoprotein receptor-related protein (LRP) expressed on the BBB could mediate specific ligand transport across the BBB [11]. Demeule et al. reported on a family of peptides called angiopep, derived from the Kunitz domain of aprotinin. One of these, angiopep-2 (TFFYGSGRKNNKFLTEY, molecular weight 2.4 kDa), possessed a higher BBB penetration capability [12]. As a small targeting ligand, angiopep-2 was effectively used in brain targeting research by several groups [13,14]. In our previous study, angiopep-2 modified doxorubicin-loaded liposomes significantly increased the median survival time of mice with brain tumors (in press). Here, we employed angiopep-2 as a first stage targeting ligand to transport the NP into brain.

A second stage ligand was needed to deliver the system to neuroglial cells after penetrating the BBB. Tissue factor (TF) could bind with coagulation factor VII (FVII) and initiate the coagulation cascade that results in thrombus [15,16]. EGFP-EFG1 is a fusion protein derived from FVII which retains the specific TF binding capacity but does not cause coagulation [17]. Recently, Mei et al. employed EGFP-EFG1 as a targeting moiety and conjugated it with NP. This confirmation showed an ideal targeting effect on thrombus [18], however TF was highly expressed in neuroglial cells while minimally expressed in endothelial cells [19]. Therefore, EGFP-EFG1 was excellent for a second stage targeting ligand delivering NP to neuroglial cells.

In this study, we established a new cascade targeting delivery system, using both angiopep-2 and EGFP-EFG1 modified NP (AENP), to deliver the NPs into brain neuroglial cells with precision. Several methods were employed to identify the targeting effect, including in vivo cellular uptake, ex vivo imaging, fluorescent in situ hybridization and transmission electron microscopy.

2. Materials and methods

2.1. Materials

The EGFP-EFG1 fusion protein was expressed from E. coli BL21 cells as described previously [17]. Angiopep-2 was synthesized by the Chinese Peptide Company (China). Methoxy poly(ethylene glycol)-poly(-caprolactone) (MPEG-PCL) [Mw: 3 k-15 k], R-carboxyl poly(ethylene glycol)-poly(-caprolactone) [HOOC-PEG-PCL] [Mw: 3.4 k-15 k], and maleimide poly(ethylene glycol)-poly(-caprolactone) [MAL-PEG-PCL] [Mw: 3.4 k-15 k] were synthesized as previously described [2]. G-carboxy-ε-dimethylaminopropy-N-ε-carboxymethylamide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) was obtained from Sigma (USA). Fe3O4 nanoparticles, 10 nm, were fabricated as previously described [20]. 1,1′-Dioctadecyl-3,3,3′-tetramethylindocarbocyanine iodide (DiR), a near-infrared dye, was obtained from Biotium (CA). Normal donkey serum, AMCA-conjugated anti-rabbit IgG was purchased from Jackson (USA).

Sheep polyclonal to von willebrand factor (VWF) was purchased from Abcam (UK). Rabbit anti mouse GFAP was obtained from Boster (China). Dulbecco’s Modified Eagle Medium (high glucose) cell culture medium (DMEM), plastic cell culture dishes, and plates were purchased from Corning Incorporation (USA). Fetal bovine serum (FBS) was purchased from Gibco (CA). All the other chemicals were analytical reagent grades, purchased from Sinopharm Chemical Reagent (China).

Sprague–Dawley (SD) rats (male, 4–5 weeks, 180–200 g) were purchased from the Shanghai Slac Laboratory Animal Co, Ltd (China) and maintained under standard housing conditions. All animal experiments were carried out in accordance with protocols evaluated and approved by the ethics committee of Fudan University.

2.2. Preparation and characterization of NP

NPs were prepared by single emulsion methods described previously. Briefly, 28 mg of MPEG-PCL, 1 mg of COOH-PEG-PCL, and 1 mg of MAL-PEG-PCL were dissolved with 1 mL dichloromethane (DCM), and then added into 5 mL of 0.6% sodium cholate hydrate solution. The mixture was pulse sonicated for 75 s at 200 W with the tube immersed in an iced water bath. Then the emulsion was applied to rotary evaporator to remove the DCM. The NP could be condensed to a fixed concentration by ultrafiltration at 4000 g. For the EGFP-EFG1 conjugation, the carboxyl unit of NPs was activated by EDC and NHS in pH 6.0 MES buffer for 0.5 h. The MES buffer was then replaced by pH 7.4 PBS using Hitrap desalting column and 0.5 mg of EGFP-EFG1 in 1 mL of pH 7.4 PBS was added into the NP suspension and stirred for 4 h, in the dark. For the angiopep-2 conjugation, 50 μg of angiopep-2 was added and stirred for 6 h, in the dark. The product was then applied to a sepharose CL-4B column to remove the unconjugated EGFP-EFG1 and angiopep-2, and the AENP was collected.

Angiopep-2 modified NP (ANP) was prepared as above without the addition of EGFP-EFG1.

EGFP-EFG1 modified NP (ENP) was prepared as above without the addition of angiopep-2.

Curamin-6-loaded AENP was prepared as above except the materials were dissolved in 1 mL of 30 μg/mL curamin-6 in DCM at the beginning. DiR-loaded AENP was prepared as above except the materials were dissolved by 1 mL of 500 μg/mL DiR in DCM at the beginning. Fe3O4 loaded AENP was prepared as above except the materials were dissolved by 1 mL of 3 mg/mL Fe3O4 particles in DCM at the beginning.

The morphology of NP was determined by transmission electron microscope (TEM) (H-600, Hitachi, Japan) following negative staining with 2% sodium phosphotungstate solution. The particle size was determined by dynamic light scattering (DLS) using a Zeta Potential/Particle Sizer NICOMP™ 380 ZLS (PSS/NICOMP PARTICLE SIZE SYSTEM, Santa Barbara, USA). The in vitro release of curamin-6- and DiR-loaded NPs was performed in pH 7.4 and pH 7.0 PBS, respectively, the cumulate release percentage of both was lower than 0.1% in three days.

2.3. In vitro cell uptake of NPs

Neuroglial cells were separated from new born SD rats as described previously [21] and cultured in 10 cm dishes in DMEM containing 15% FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL). The brain capillary endothelial cell line, bEnd.3 was maintained in 10 cm dishes in DMEM containing 10% FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL).

For qualitative analysis, neuroglial cells and bEnd.3 cells were seeded into 24-well plates with 2 × 104 cells/well. After 24 h, cells were preincubated with Hank’s Balanced Salt Solution (HBSS) for 5 min, and then each well was incubated with 1 mL of 250 μg/mL curamin-6-loaded NP, ANP, AENP or ENP for 1 h. Wells were then rinsed four times with PBS and the fluorescence intensity was directly observed by fluorescence microscope (Leica, Germany).

For quantitative analysis, neuroglial cells and bEnd.3 cells were seeded into 6-well plates with 1 × 105 cells/well. Cells were treated as noted above and then harvested and resuspended in 0.5 mL PBS after centrifugation. The fluorescence intensity was detected by FACS Aria Cell Sorter (BD, USA).

2.4. Ex vivo imaging

Twelve male SD rats were randomly divided into four groups: NP group, ANP group, AENP group and ENP group. Each rat was injected with 100 μg DiR contained within a different kind of NP (as determined by the assigned group) via the tail vein. At 0.5 h, 2 h and 4 h post injection, one rat from each group was sacrificed for brain sampling. The fluorescence intensity of brains was determined by MAESTRO in vivo imaging system (Cri, USA).

Fig. 1. Transmission electron microscopy of NP that negatively stained by 2% sodium phosphotungstate solution. The bar is 100 nm.
Fig. 2. Qualitative (A) and quantitative (B, C) measurement of in vitro uptake of coumarin-6-loaded NP, ANP, AENP and ENP by neuroglial cells and bEnd.3 cells. The bar is 100 μm. Quantitative measurement of in vitro uptake of coumarin-6 6-loaded NP (a, e), ANP (b, f), AENP (c, g) and ENP (d, h) by neuroglial cells (a, b, c, d) and bEnd.3 cells (e, f, g, h). The negative control of neuroglial cells (i) and bEnd.3 cells (j) were showed. C was conclusion of quantitative uptake measurement.

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2.5. Fluorescent in situ hybridization

Four male SD rats were randomly divided into four groups as described above. Each rat was injected with 5 μg coumarin-6 contained within a different kind of NP (as determined by the assigned group) through the tail vein. Two hours after injection, all rats were anesthetized and then heart perfusion with saline followed by 4% paraformaldehyde. The brains were removed and fixed in 4% paraformaldehyde overnight. Then the brains were placed in a 15% sucrose PBS solution for 24 h, and then the solution was replaced with a 30% sucrose PBS solution for another 24 h. The brains were then embedded in Tissue Tek® O.C.T. compound (USA) and frozen at −80 °C. Consecutive frozen sections of 5 μm thicknesses each were prepared with a cryotome Cryostat (Leica, CM 1900, Germany).

Endothelial cells of the frozen sections were stained by VWF and neuroglial cells were stained by GFAP [22]. Briefly, after rinsing with Tris buffered saline (TBS) (pH 7.2) for 10 min, sections were incubated in 0.5% Triton X-100 in TBS for 60 min at room temperature (RT) followed by incubation with a Blocking Solution (10% donkey serum + 0.5% Triton X-100 in TBS) for 1.5 h at RT. Then the sections were incubated with sheep polyclonal VWF (1:100) and rabbit polyclonal GFAP (1:200) diluted in 5% donkey serum containing 0.5% Triton X-100, 2.6. Transmission electron microscopy

The ultrastructures were embedded in an epoxy resin, and then cut into ultra-thin sections with an ultramicrotome. The ultrastructures were generally spherical and uniform, particle sizes were corresponded with the mean particle size determined by DLS (Fig. 1).

3. Results

3.1. Characterization of NP

The intensity mean particle size of the NPs was 126.0 nm, polydispersity index was 0.168. After conjugation with EGFP-EGF1 and/or angiopep-2, the particle sizes were slight increased, and were measured as 157.3 nm, 130.3 nm and 154.4 nm for ANP, ENP and AENP respectively. Loading with coumarin-6, DiR or Fe3O4 particles did not obviously affect the particle size either, which were 124.4 nm, 122.7 nm and 133.1 nm respectively. The particles were generally spherical and uniform, particle sizes were corresponded with the mean particle size determined by DLS (Fig. 1).

3.2. In vitro cell uptake

The neuroglial cells and bEnd.3 cells could uptake the 6-coumarin loaded NP, ENP, AENP and ANP at different capacities (Fig. 2). The fluorescence intensity of the NP was the lowest observed in both cell types. In neuroglial cells, the fluorescence intensity of ENP and AENP was higher than that of NP, but the fluorescence intensity of ANP was almost the same as NP, indicating that EGFP-EGF1 could enhance the uptake of NP by neuroglial cells while angiopep-2 failed. In bEnd.3 cells, the fluorescence intensity of ENP was the same as NP, while that of ANP and AENP was higher than that of NP and ENP, suggesting the angiopep-2 could increase the uptake of NP while EGFP-EGF1 could not.

The quantitative results indicated almost the same results as the fluorescence imaging (Fig. 2). The amount of ENP taken up by neuroglial cells was 0.74 times higher than NP uptake, while ANP was only 0.22 times higher than NP uptake. However in bEnd.3 cells, ANP uptake was much higher than that of ENP, about 1.70 times and 1.45 times as that of NP, respectively. As a result of the targeting capacity of angiopep-2 and EGFP-EGF1, the AENP uptake by both neuroglial and bEnd.3 cells were much higher than that of NP, 0.41- and 1.08-fold greater, respectively.

3.3. Ex vivo imaging

The fluorescence intensity seen in whole brain tissue was different among different kinds of particles (Fig. 3). The
fluorescence intensity of NP and ENP was obviously lower than that of ANP and AENP, especially at 0.5 h and 2 h post injection, indicating that angiopep-2 could facilitate the transport of NP into brain, but the EGFP-EGF1 could not. The fluorescence intensity of AENP varied at different time points. Cumulative intensity was achieved at 2 h, which suggested that the transport of AENP into brain was balanced with the elimination of AENP.

3.4. Fluorescent in situ hybridization

The fluorescence distribution in the brain tissue was different among different kinds of particles (Fig. 4). In both endothelial and neuroglial cells, there was no obvious NP distribution in the brain, suggesting that the NP alone could not be delivered into the brain. However ANP was obviously distributed in the region with many endothelial cells but slightly distributed in the region that most were neuroglial cells. Different from ANP group, it was found that AENP obviously co-localized with neuroglial cells, indicating AENP was targeting to neuroglial cells. But in ENP group, the same as NP, there was little distribution of particles in brain, suggesting ENP could not penetrate BBB to targeting neuroglial cells.

3.5. Transmission electron microscopy

Particles could be observed in different parts of the neuroglial cells (Fig. 5). There was also particle that partly dispersed (Fig. 5B), which indicated the NP could be destroyed in the cell and release its cargo. These results further demonstrated that AENP could be transported into neuroglial cells.
4. Discussion

Several studies have previously demonstrated on the utility of dual targeting delivery systems [24–27]. However, these systems employed two brain targeting moieties to enhance the targeting effect however there was no inter-facilitation between the two targeting moieties. Thus these systems were “luster bombs” rather than “multistage rockets” and were ultimately simply double the traditional delivery system. Here we described the development of a true cascade targeting delivery system: AENP.

The cellular uptake results suggested the angiopep-2 was only targeted to endothelial cells while EGFP-EGF1 was only targeted to neuroglial cells. Thus they provided two ideal ligands to evaluate the effect of a cascade targeting delivery system, because individually they could not enter both endothelial and neuroglial cells. In the ex vivo imaging, fluorescence intensity of AENP and ANP was significantly higher than that of NP and ENP, indicated the first stage targeting of AENP was successful. It was re-confirmed the EGFP-EGF1 alone could not penetrate the BBB and there was no obvious increase in fluorescence intensity of ENP over that seen with NP alone. The fluorescent in situ hybridization demonstrated AENP distribution in the neuroglial cells but not in the endothelial cells, indicating AENP could precisely target brain neuroglial cells. The particles in neuroglial cells were further identified by transmission electron microscopy.

In our cascade targeting strategy, EGFP-EGF1 can be replaced by many other ligands that specifically recognize diseased regions, cells, and even proteins, all of which could be used for disease diagnosis and therapy. For example, there is significant Aβ protein aggregation seen in the brain with Alzheimer’s disease. An RNA aptamer, called β55, recognizes the aggregated αβ protein [28], and this could serve as a second stage targeting ligand for diagnosis and therapy of Alzheimer’s disease.

In addition to brain diseases, diseases such as hepatic cancer and kidney cancer which were considered strong related with liver cancer stem cells and RLIP76 protein [29,30], are also candidates for treatment with this dual target cascade system. In these diseases, the precise targeting to the aimed cells or proteins was also important for the diagnosis and therapy. Our cascade targeting strategy could be introduced to these areas by replacing the angiopep-2 with liver or kidney targeting ligands and employing special cells or proteins for second stage targeting.

5. Conclusions

We have developed a new cascade targeting strategy, and evaluated the strategy for targeting brain neuroglial cells using several methods. Replacing EGFP-EGF1 and angiopep-2 with other ligands may extend the utility of the system to diagnose and treat organ diseases beyond brain.

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