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Tunable Lipidoid-Telodendrimer Hybrid Nanoparticles for Intracellular Protein Delivery in Brain Tumor Treatment

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Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor in humans and, despite surgical resection, chemotherapy, and radiotherapy, there is no cure.^[1] The blood–brain barrier (BBB) is a notorious obstacle to macromolecular therapeutics and small-molecule drugs being able to access primary brain tumors.^[2] The ability to efficiently deliver highly potent therapeutics to the tumor bed is crucial to improve GBM treatment. One effective way to bypass the BBB is by convection-enhanced delivery (CED), which is a continuous local infusion of therapeutics. Nanocarriers when delivered by CED show an increased distribution volume and a longer therapeutic retention within the tumor mass.^[1a,3] Significant efforts have been made toward the application of nanocarrier delivery of small-

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Supporting Information

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molecule drugs by CED.^[3a-c] However, the efflux proteins highly expressed on the BBB efficiently pump chemodrugs out of the central nervous system, decreasing the effectiveness of such treatments.^[1a] CED infusion of protein toxins has been previously used for GBM treatment but has been associated with significant toxicity toward normal brain tissue as well as systemic toxicity and has yet to show an increased survival benefit in clinical trials.^[4] As nanoparticle-based delivery of chemotherapeutics through CED for treatment of GBM showed increased retention and improved treatment efficacy,^[3a-c] nanocarriers will likely improve the effectiveness of cytotoxins in GBM therapy.

Numerous proteins and peptides have been approved for clinical use.^[5] The majority of these protein therapeutics target the extracellular receptors highly expressed on pathologic cells, especially for cancers. Diphtheria toxin (DT) is a highly potent protein toxin: one molecule kills one cancer cell.^[4a,6] The nonspecific cytotoxicity of this protein limits its application in tumor therapies. To curb the nonspecific toxicity, immunotoxins have been developed by fusing the cancer specific targeting ligands with the toxin devoid of its native receptor-binding domain.^[4b] However, the potential immunogenicity of these toxins as well as the high mutation rate of cancer-specific targets have been challenging for clinical applications.^[7] Truncated DT, like DT₃₉₀, with the deletion of the targeting domain is not cell permeable and nontoxic to normal cells.^[6a,d] By encapsulating it into nano-particles (NPs), truncated DT can be delivered into cancer cells to become an efficient therapeutic for GBM treatment by CED infusion.

The development of efficient nanocarriers for intracellular protein delivery with minimum protein denaturation is not a trivial task.^[8] Promising nanocarriers mainly include liposomes,^[9] nanogels,^[10] polymeric NPs,^[8c,11] and inorganic nanomaterials.^[12] Some of them have been investigated for intracellular protein delivery in treating breast cancer^[9c] and lung cancer.^[13] Cationic lipid-like materials, termed lipidoids, exhibit excellent efficacy for intracellular delivery of genes and proteins in vitro and in vivo.^[9c,14] A cationic lipidoid made from 1,2-epoxyhexadecane and *N,N'*-dimethyl-1,3-propanediamine (**Scheme 1** and Figure S1–S3) has been identified through a combinatorial approach for efficient protein delivery.^[9c] However, the large particle sizes (≈ 130 nm) and the positive zeta potential of such lipidoid NPs stabilized by the mixture of cholesterol, lipids and PEGylated lipids may limit intratumoral diffusion.^[1a] Although many lipid-polymer hybrid NPs have been widely studied for drug and gene delivery,^[15] very few examples were reported to employ amphiphilic polymers (except for PEGylated lipids) to stabilize lipid nanostructures. Wang and coworkers applied poly(ethylene glycol)-*block*-polylactide (PEG-PLA) to efficiently stabilize a cationic cholesterol-containing lipid via a single-step nanoprecipitation method for siRNA delivery.^[15d] However, the capability of PEG-PLA in stabilizing cationic lipids was not optimum, evidenced by the unsheltered surface charge (zeta potential +35 mV) and the limited lipid content (10 wt%) to form monodispersed particles with the size of ≈ 100 nm. It is still challenging to fabricate small lipidoid-polymer hybrid nanocarriers with a neutral surface charge and high intracellular protein delivery efficiency, which are needed^[1a] for efficient DT₃₉₀ delivery to GBM cells. We have developed a unique telodendrimer system by precise conjugation of various building blocks on a terminal of polyethylene glycol (PEG) to generate an amphiphilic linear-dendritic copolymer system.^[16] The peptide

chemistry used in telodendrimer synthesis allows for structure-based nanocarrier design to optimize the in vivo drug delivery.^[16,17] The modular design of the functionally segregated telodendrimer has been demonstrated to allow for precise control of size, structure, and surface charge of the nanocarriers.^[18] The multivalent hydrophobic periphery of the dendritic portion in telodendrimer enables it to form stable complexes with other hydrophobic materials, similar to PEG-distearoylphosphoethanolamine for liposome post-modification.^[19] We hypothesize that the precisely engineered telodendrimers are promising to stabilize the cationic lipidoid to form small and stable NPs by single-step assembly of lipidoid-telodendrimer binary mixtures, based on their structural and architectural compatibility.

We have synthesized a series of telodendrimers with diverse compositions and architectures to optimize drug delivery as shown in Scheme 1.^[16] The structure characterizations of these telodendrimers have been reported in our previous publications.^[16] As the first generation of telodendrimer, PEG^{5k}CA₈ consisting of a linear PEG (M_w 5 kDa) and eight amphiphilic cholic acids (CAs) as peripheral groups^[16a] was first applied to form hybrid NPs with the lipidoid. The resultant lipidoid-PEG^{5k}CA₈ NPs (50/50, lipidoid/telodendrimer, L/T) have an average hydrodynamic diameter (D_h) of 56 nm (**Table 1**; Figure S4, Supporting Information) determined by dynamic light scattering (DLS). However, the zeta potential of these NPs is over +11 mV, indicating less sufficient PEG shielding and superficial lipidoid partition. This may be due to poor compatibility of the amphiphilic CA with the hydrophobic domains of the lipidoids. Moreover, large aggregates formed after loading of a model protein, bovine serum albumin (BSA). The exposed positive charges may induce crosslinking of NPs by interacting with the negatively charged BSA, thereby causing the large aggregation. Cholesterol (CHO) naturally interacts with lipids to regulate plasma membrane properties and is often used for liposome formation.^[14a] With this in mind, PEG^{5k}CHO₈ containing 8 CHO groups^[16b] (Scheme 1) was used to prepare hybrid NPs, and the properties of lipidoid-PEG^{5k}CHO₈ NPs at an L/T ratio of 50/50 were evaluated. As expected, the zeta potential of lipidoid-PEG^{5k}CHO₈ NPs was reduced close to neutral and the particle size was measured to be 66 nm (Table 1). Although the zeta potential was improved with the proper particle size of the blank NP, multiple peaks appeared in the D_h distribution after BSA loading. The overwhelming hydrophobicity of cholesterol may lead to aggregation. In addition, the lipidoid-PEG^{5k}CHO₈ NPs exhibited a strong hemolytic activity (Figure S5, Supporting Information), mainly because of the membrane activity of CHO groups and the charge interactions.

To further optimize the stability of the lipidoid-telodendrimer (L-T) binary hybrid NPs for protein loading, a second generation telodendrimer PEG^{5k}CA₄CHO₄[16b] (Scheme 1) was tested, which has the mixed peripheral groups: 4 CA groups and 4 CHO groups on the α - and ϵ -positions of lysine, respectively. The facial amphiphilic CA has shown to be critical in stabilizing the hydrophobic components in telodendrimers for hydrophobic drug delivery.^[16b,18] CHOs are tethered on a longer side chain of lysine for efficient interaction with the lipidoid. As a result, NPs generated by the lipidoid and PEG^{5k}CA₄CHO₄ (50/50 of L/T) showed monodispersed particle sizes of $\approx$ 68 nm with low zeta potential of 3.4 mV and a low hemolytic activity (Figure S5, Supporting Information). After BSA loading, the

particle size increased to ≈ 85 nm and zeta potential decreased to -0.3 mV. The improved properties are a testament to the synergistic combination of the amphiphilic CA and hydrophobic CHO groups in stabilizing the lipidoid complex. Furthermore, a functionally segregated third generation telodendrimer PEG^{5k}CA₄-L-CHO₄[16b] with segregated CA and CHO domains by a triethylene glycol linker (Scheme 1) was rationally designed to define the functions of the subunits of telodendrimer to stabilize the NP, which may bind to the lipidoid more favorably. As a result, the lipidoid-PEG^{5k}CA₄-L-CHO₄ (50/50 of L/T) NPs exhibit improved physical properties both before and after BSA loading (Table 1), including smaller particle sizes (43 and 56 nm, before and after protein loading, respectively), slightly negative zeta potential (-0.5 and -0.6 mV, before and after protein loading, respectively), minimized hemolytic activity (Figure S5, Supporting Information), and reduced cytotoxicity (Figure S6, Supporting Information). In comparison with the hybrid PEG^{5k}CA₄CHO₄, PEG^{5k}CA₄-L-CHO₄ has a functionally segregated three-layer structure when interacting with the lipidoid: the hydrophobic peripheral CHO groups may interact with the hydrophobic tails of the lipidoid; the intermediary amphiphilic CA moiety is able to interact with the polar head groups of the lipidoid via hydrogen bonding; in addition, the facial amphiphilic CA mediates the difference in polarity between the hydrophobic core and the hydrated PEG outer layer, therefore endowing the NPs with improved stability and a small size. Overall, lipidoid-PEG^{5k}CA₄-L-CHO₄ hybrid system is the most promising nanocarrier for intratumoral and intracellular delivery of therapeutic proteins.

The particle size and zeta potential of the lipidoid-PEG^{5k}CA₄-L-CHO₄ NPs are greatly affected by L/T ratios. As shown in **Figure 1a,b** and Figure S7 (Supporting Information), the D_h and zeta potential increased with increasing lipidoid content. At 80% lipidoid content, we observed a large particle size and positive zeta potential. The sizes of the NPs with no more than a 50% lipidoid content only slightly increased after BSA loading (Figure S8, Supporting Information), while the size of the NP with an 80% lipidoid increased to over 110 nm after BSA loading. The spherical NPs were visualized by transmission electron microscopy (TEM) (Figure 1c–e) with average particle sizes (Figure 1f–h) in reasonable agreement with the DLS analysis. After BSA loading at 10% in mass ratio, the average sizes of 30/70 and 50/50 L/T NPs remained as small as 23 ± 7 and 28 ± 11 nm, respectively, as observed by TEM (Figure S9, Supporting Information). By contrast, the average particle size of 80/20 NPs increased to 73 ± 58 nm with irregularly shaped large aggregates over 100 nm after BSA loading (Figure S9, Supporting Information). The zeta potential slightly declined after BSA loading. While 80/20 NPs still showed undesirable positive zeta potential after BSA loading. The doping of telodendrimers to the lipidoid systems effectively reduced their hemolytic activity and cytotoxicity, especially for the hybrid NPs with lipidoid content at 50% or below. (Figure S10 and S11, Supporting Information).

The L-T binary hybrid NPs can form complexes with proteins mainly based on electrostatic and hydrophobic interactions. The loading capacity of the NPs for a model protein, fluorescein isothiocyanate-labeled BSA (FITC-BSA), was semi-quantitatively determined using agarose gel electrophoresis,^[20] where free protein and protein-loaded NPs can be separated based on their differences in size and charge (**Figure 2a**). The protein loading

capacities were calculated to be 11%, 12%, and 13% for 30/70, 50/50, and 80/20 NPs, respectively. The gel was then stained by Coomassie blue (Figure 2b), which revealed good agreement with the fluorescence analysis. A protein-loading ratio of 10% in the NPs (1/10 of protein/NP by weight) was therefore chosen for further studies. In addition, the blank nanocarrier could also be slightly stained by Coomassie blue through hydrophobic effects and they were found to be trapped in the original wells by their large particle sizes. Other proteins and peptides, such as insulin and pH low insertion peptide (pHLIP),^[21] can also be efficiently loaded in the L-T hybrid NPs (Figure S12, Supporting Information).

To mimic the physical characteristics of the brain, a 0.6 wt% agarose gel has been previously applied to evaluate the distribution volume of the tested therapeutics.^[22] Such agarose gel was also used as a matrix to study the diffusion of our protein nanoformulations. The free FITC-BSA and the nanocomplexes were dissolved in such melted agarose solutions, respectively, and homogenous gels were formed with cooling before immersing into phosphate buffered saline (PBS). The released FITC-BSA was monitored by fluorescent measurement of the PBS solution. Free FITC-BSA released quickly from the gels with $\approx 50\%$ proteins detected in PBS within 2 h (Figure 2c). By contrast, slower release profiles were observed for FITC-BSA nanoformulations with 50% released at 8, 48, and 120 h for the NPs at L/T ratios of 30/70, 50/50, and 80/20, respectively. The electrophoresis analysis for the released supernatant revealed that the majority of the released proteins were still loaded in the NPs (Figure S13, Supporting Information), which explained the size effects in controlling release rates of NPs with different sizes (Figure 2c). It was expected that the release rate decreased with the increasing particle sizes along with the increasing L/T ratio. Among these nanocomplexes, the NP at a 50/50 L/T ratio exhibits a sustained and continuous release profile, which is promising for achieving efficient intratumoral diffusion and sustained residency for efficient brain tumor treatment.

U87 GBM and HT-29 colon cancer cells were incubated with free FITC-BSA and FITC-BSA-loaded NPs, respectively. As the confocal laser scanning microscopy (CLSM) images revealed in **Figure 3a**, the cellular uptake efficiency for free FITC-BSA in U87 cells was very low. By encapsulating FITC-BSA in NPs (50/50 of L/T), the cellular uptake was significantly increased (Figure 3b). As shown in Figure 3c and Figure S14 and S15 (Supporting Information), the cellular uptake in both cell lines increased with the increasing content of lipidoid in the NPs. Importantly, the NP with a 50/50 L/T ratio exhibited sufficient cell uptake for efficient therapeutic delivery.

DT₃₉₀ is a truncated diphtheria toxin with the deletion of the cell targeting domain to eliminate nonspecific cytotoxicity.^[4a,6d] DT₃₉₀ can be loaded efficiently in the lipidoid-PEG^{5k}CA₄-L-CHO₄ NPs with loading capacities of 11–12%, as detected in the gel retention assay (Figure S16, Supporting Information). Different GBM cell lines including U87, LN229, and U138 were exposed to free DT₃₉₀ and DT₃₉₀-loaded NPs at different L/T ratios for cytotoxicity evaluation. As expected, free DT₃₉₀ was nontoxic to all those cell lines (Figure 3d and Figure S17, Supporting Information). In comparison, DT₃₉₀ delivered by these lipidoid-PEG^{5k}CA₄-L-CHO₄ NPs exhibited dose-dependent cell killing (Figure 3d and Figure S17, Supporting Information). The potency of DT₃₉₀ correlated with increasing lipidoid content and increasing cell uptake efficiency of the NPs. For U87 cells (Figure 3d),

DT₃₉₀-loaded hybrid NPs at L/T ratios of 50/50 and 80/20 have significantly enhanced protein cytotoxicities with IC₅₀ values of 194 and 24 ng mL⁻¹, respectively. The blank NPs were detected to be nontoxic in the corresponding concentrations studied in Figure 3d as shown in Figure S10 and S17 (Supporting Information).

The high potency of DT₃₉₀ loaded in lipidoid-PEG^{5k}CA₄-L-CHO₄ NPs at L/T ratios of 50/50 and 80/20 in killing GBM cells in vitro warrants further in vivo evaluation for GBM treatment. A near-infrared fluorescence dye-labeled BSA (Cy5-BSA) was used as a model protein to study the intracranial distribution of proteins delivered by L-T NPs via CED infusion in mice bearing orthotopic human U87 GBM tumors. The tumor-bearing mice were sacrificed 24 h after a 7 d CED infusion had been completed and the brains were harvested. On a cryostat, 20 μm slices were made for microscopic analysis. No apparent fluorescent signals could be detected in the brain sections of the animal treated with free Cy5-BSA due to the leakage of small-sized free proteins from the brain over the course of the CED infusion (upper row in **Figure 4a**). Cy5-BSA in the NPs at an L/T ratio of 80/20 was also observed to have weak fluorescent signal (lower row in Figure 4a), which may be due to the restricted diffusion and possible backflow at the injection site for the NPs with large particle sizes.^[1a] In addition, macrophages that infiltrate into GBM may accelerate the clearance of such NPs with positive charges and large particle sizes.^[23] In comparison, Cy5-BSA-NP (50/50 of L/T) could effectively diffuse into and accumulate at the brain tumor site (middle row in Figure 4a). A significantly higher protein concentration at the microscopic level was also observed (middle row in Figure 4b), owing to the neutral surface charge and small particle size (≈56 nm by DLS), which proved to be the appropriate nanocarrier to deliver DT₃₉₀ for GBM tumor treatment. The biodistribution of therapeutics with and without our NPs was further studied in tumor-bearing mice employing pHLIP-Alexa750-DOX as a model therapeutic. The results in Figure S18 (Supporting Information) confirmed the improved retention of therapeutics in brain tumor by encapsulation within our NPs, and no signal of pHLIP-Alexa750-DOX was detected in the normal organs.

The DT₃₉₀-loaded lipidoid-PEG^{5k}CA₄-L-CHO₄ NPs (50/50 of L/T, 1/10 of protein/NP), noted as DT₃₉₀-NPs (50/50), were selected for in vivo brain tumor treatment by CED infusion in an intracranial mouse model of GBM. Free DT₃₉₀, BSA-loaded NPs, and PBS were selected as comparisons. The infusion started on day 9 after the intracranial cell implantation. The CED infusion lasted for 7 d. Tumor progression was observed via bioluminescent imaging for all groups by day 17, one day after CED infusion had been completed, and similar bioluminescent signals were observed for all tumors. However, on day 24, the tumor in the nanotherapeutic group had a significantly smaller volume on bioluminescence imaging when compared to the control groups all of which showed similar rates of tumor growth (**Figure 5a,b**). Animals were sacrificed when the disease progression required the animals to be sacrificed due to weight loss greater than 20% of original weight or neurologic decline. Whole brains were harvested and examined by histological studies after sacrifice. Hematoxylin and eosin (H&E) staining revealed a reduced density of tumor cells in animals treated with DT₃₉₀-loaded NPs presumably due to the shrinkage and/or clearance of apoptotic cells (Figure 5c). In the control groups PBS, BSA-NP, and DT₃₉₀, dense tumor masses were observed. The terminal deoxynucleotidyl transferase dUTP nick

end labeling (TUNEL) staining also identified a significant increase of apoptotic cells (green in Figure 5d) in the tumors of the animals treated with DT₃₉₀-NPs (50/50) compared with other control groups. These results suggested that a significant brain tumor growth inhibition had been achieved by the intracranial CED infusion of DT₃₉₀-NPs (50/50) by inducing tumor cell apoptosis.

To the best of our knowledge, this is the first attempt to apply a lipidoid-polymer binary hybrid nanocarrier for intracellular protein delivery for brain tumor treatment. In summary, we have established a facile strategy to create precisely engineerable lipidoid NPs by employing rationally designed telodendrimers as building blocks for intracellular delivery of therapeutic proteins. This strategy provides a facile and efficient approach to control the physicochemical properties of the lipidoid NPs, which overcomes a fundamental problem for lipidoid-polymer systems in fabricating small and neutrally charged NPs with high efficiency for intracellular protein delivery. By optimizing the NPs to have a sub 100 nm particle size with a neutral zeta potential, the intratumoral distribution of the payload proteins has been significantly improved. Efficient delivery of active proteins into the intracellular space of cancer cells by means of CED results in efficient brain tumor growth inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

a) Particle size and (b) zeta potential of lipidoid-PEG^{5k}CA₄-L-CHO₄ NPs at different L/T ratios without or with BSA in PBS. c–e) TEM images and (f–h) particle size analysis of lipidoid-PEG^{5k}CA₄-L-CHO₄ NPs at L/T ratios of (c,f) 30/70, (d,g) 50/50, and (e,h) 80/20.

Figure 2.

a,b) Loading abilities of lipidoid-PEG^{5k}CA₄-L-CHO₄ NPs at different L/T ratios (30/70, 50/50, and 80/20) for FITC-BSA determined by an agarose gel retention assay under (a) SYBR Green and (b) Coomassie Blue modes. (1) Lanes 1–7 are free FITC-BSA, (2) FITC-BSA-loaded 30/70 NPs, (3) FITC-BSA-loaded 50/50 NPs, (4) FITC-BSA-loaded 80/20 NPs, (5) 30/70 NPs, (6) 50/50 NPs, and (7) 80/20 NPs, respectively. The feed mass ratio of protein/NP is 1/5. c) Release profiles of free FITC-BSA and FITC-BSA loaded in lipidoid-PEG^{5k}CA₄-L-CHO₄ NPs at different L/T ratios from agarose gels.

Figure 3.

a) CLSM images of U87 cells incubated with free FITC-BSA, and (b) FITC-BSA-incorporated lipidoid-PEG^{5k}CA₄-L-CHO₄ NPs (5/50 of L/T). The cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). c) Mean fluorescence intensity in cells after incubation with free FITC-BSA, and FITC-BSA-incorporated lipidoid-PEG^{5k}CA₄-L-CHO₄ NPs at different L/T ratios. d) Cell viability assay for free DT₃₉₀, and DT₃₉₀-incorporated lipidoid-PEG^{5k}CA₄-L-CHO₄ NPs with different L/T ratios at a DT₃₉₀ loading ratio of 1/10 (protein/NP).

Figure 4.

Distributions (a) in brain sections containing U87 tumors indicated by dotted circles and (b) in intracranial U87 tumors at the cellular level of free Cy5-BSA, and Cy5-BSA incorporated in lipidoid-PEG^{5k}CA₄-L-CHO₄ NPs with different L/T ratios (50/50 and 80/20) at a loading ratio of 1/10 (protein/NP). The cell nuclei in (b) were stained with DAPI (blue).

Figure 5.

a) Typical bioluminescence images of mice injected with intracranial U87 tumors treated with different formulations at different days after the cell intracranial implantation. b) DT₃₉₀-incorporated NP delivery suppresses tumor growth on mice injected with intracranial U87 tumors. * $P < 0.05$ as compared to each control group. Histological images of the (c) H&E and (d) TUNEL immunofluorescence staining assays for intracranial U87 tumor tissues after treatment with PBS, BSA-incorporated NPs, free DT₃₉₀, and DT₃₉₀-incorporated NPs. The cell nuclei in (d) were stained with DAPI (blue).

Scheme 1.

Chemical structures of the lipidoid, and four telodendrimers with varying architectures and compositions containing CA and/or CHO groups.

Table 1

Properties of the lipidoid-telodendrimer NPs (50/50 of L/T) without and with proteins.

Telodendrimer	D_h [nm] ^{a)}	ζ [mV] ^{a)}	D_h' [nm] ^{b)}	ζ [mV] ^{b)}
PEG ^{5k} CA ₈	56 ± 4	11.4 ± 1.6	MP	2.6 ± 1.1
PEG ^{5k} CHO ₈	66 ± 5	0.6 ± 0.5	MP	-1.1 ± 0.7
PEG ^{5k} CA ₄ CHO ₄	68 ± 5	3.4 ± 0.5	85 ± 8	-0.3 ± 0.6
PEG ^{5k} CA ₄ -L-CHO ₄	43 ± 4	-0.5 ± 0.5	56 ± 7	-0.6 ± 0.1

^{a)} Obtained without BSA. D_h : hydrodynamic diameter; ζ : zeta potential;

^{b)} Acquired with BSA (1/10 of protein/NP). D_h' : hydrodynamic diameter after BSA loading; ζ : zeta potential after BSA loading; MP: multiple peaks.