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Affinity-Controlled Protein Encapsulation into Sub-30 nm Telodendrimer Nanocarriers by Multivalent and Synergistic Interactions

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Abstract

Novel nanocarriers are highly demanded for the delivery of heterogeneous protein therapeutics for disease treatments. Conventional nanoparticles for protein delivery are mostly based on the diffusion-limiting mechanisms, e.g., physical trapping and entanglement. We develop herein a novel linear-dendritic copolymer (named telodendrimer) nanocarrier for efficient protein delivery by affinitive coating. This affinity-controlled encapsulation strategy provides nanoformulations with a small particle size (<30 nm), superior loading capacity (>50% w/w) and maintained protein bioactivity. We integrate multivalent electrostatic and hydrophobic functionalities synergistically into the well-defined telodendrimer scaffold to fine-tune protein binding affinity and delivery properties. The ion strength and density of the charged groups as well as the structure of the hydrophobic segments are important and their combinations in telodendrimers are crucial for efficient protein encapsulation. We have conducted a series of studies to understand the mechanism and kinetic process of the protein loading and release, utilizing electrophoresis, isothermal titration calorimetry, Förster resonance energy transfer spectroscopy, bio-layer interferometry and computational methods. The optimized nanocarriers are able to deliver cell-

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Appendix A. Supplementary data

Supplementary data related to this article can be found at XXXX.

impermeable therapeutic protein intracellularly to kill cancer cells efficiently. *In vivo* imaging studies revealed cargo proteins preferentially accumulate in subcutaneous tumors and retention of peptide therapeutics is improved in an orthotopic brain tumor, these properties are evidence of the improved pharmacokinetics and biodistributions of protein therapeutics delivered by telodendrimer nanoparticles. This study presents a bottom-up strategy to rationally design and fabricate versatile nanocarriers for encapsulation and delivery of proteins for numerous applications.

Graphical abstract

Keywords

nanoparticles; telodendrimers; multivalent interactions; synergistic effects; affinity-controlled encapsulation; protein delivery

1. Introduction

Proteins and peptides are used to treat an array of human diseases.[1- 2] Modification of the pharmacokinetics of proteins by delivery systems can enhance their therapeutic efficacy. PEGylation of protein has a long-standing history of efficiently prolonging circulation time, increasing stability and reducing the immunogenicity of protein therapeutics.[3- 4] Physical encapsulation of proteins into micro/nanoparticles has been intensively studied for systemic or local administration.[5-8] It is promising to dramatically enrich the arsenal of therapeutic proteins, if efficient vehicles can be developed for intracellular delivery of cell-impermeable functional proteins. Aside from costly recombinant proteins fused with targeting domains[9], the intracellular delivery of proteins by cell-penetrating peptides and cationic polymers/liposomes has been widely studied.[10- 11] The advancement of these vehicles are mainly hindered by their positive surface charges, that usually cause cytotoxicity and are also subjected to fast *in vivo* clearance by nonspecific phagocytosis in the reticuloendothelial system (RES).[12] Another important concern in protein delivery is to maintain protein structure and activity. For example, the oil/water emulsion technique[13] for the encapsulation of proteins into polymeric micro/nanoparticles has potential to cause the loss of protein activity to some extent.[14] Encapsulation of proteins in aqueous environments, such as in hydrogels[15- 16], represents an efficient way to sustain protein structure and activity, which is however restricted to the applications of local controlled protein release.

Recently, protein-binding moieties have been introduced into hydrogel systems for further control of protein release based on the affinitive binding and dissociation.[17–19]

Affinity-controlled protein release platforms rely on the noncovalent interactions (e.g., electrostatic, hydrophobic and hydrophilic interactions) between ligands (e.g., peptides, proteins or aptamers) and payload proteins.[19] However, such well-defined interactions are hard to be implanted in polymer nanocarriers for protein encapsulation, due to the monotonic feature of conventional polymer chemistries. Alternatively, Xu and coworkers have demonstrated that the structures and properties of both charged head groups and hydrophobic tails in the positively charged surfactant are important for efficient protein absorption and intracellular delivery using these surfactant nanoparticles (NPs) stabilized by phospholipids/cholesterol and polyethylene glycol-lipid (PEG-lipid).[20] It would be beneficial to further optimize the surface charge and particle size of such NPs (zeta potential ~8 mV, ~130 nm) for *in vivo* protein delivery. Many studies have demonstrated that small NPs (<30 nm) with neutrally charged surfaces allow for deep tissue penetration and promote efficient *in vivo* therapeutic delivery.[21–25] Several nanogel systems with small particle sizes[26–28] have been developed for protein encapsulation, which normally request post-crosslinking to stabilize the NPs. These chemical processes may cause complications in quality control and *in vivo* applications. Stable encapsulation of protein therapeutics into NPs with small particle sizes via a “green” process turns out to be a non-trivial task.

Well-defined protein surface structures warrant the fine-tuning of the affinitive moieties in nanocarriers for optimum protein delivery. Linear-dendritic block copolymer is a well-defined system that enables feasible control of its chemical composition, functional groups and colloidal behavior for drug delivery.[29–33] A PEG-*b*-dendritic poly(benzyl ether) has been reported to selectively interact with glycan moieties on a protein to enhance its enzymatic activity by providing a hydrophobic depot for substrates.[34, 35] In contrast to this, the linear PEG-polystyrene polymer consistently decreases protein activity.[35] Multivalent guanidinium-containing, hydrophilic linear-dendritic polymers have been developed to interact with proteins as molecular glues or to form hydrogels.[36, 37] However, such linear-dendritic copolymers with purely hydrophobic or cationic dendrons have not been reported for *in vivo* protein delivery, which may be due to the poor efficiency/stability or the known toxicity of the cationic materials. The design of a novel linear-dendritic scaffold to target both polar/charged and hydrophobic features on protein surfaces may provide a promising solution for the encapsulation of bioactive proteins in NPs with desired physical properties.

Inspired by the precise cooperative and/or multivalent interactions[38–40] in biological systems, we present a versatile nanoplatform, a telodendrimer (a linear-dendritic copolymer), to precisely integrate oligo-electrolytes and hydrophobic biocompatible moieties in a well-defined manner using stepwise peptide chemistry[41, 42]. We hypothesize that the multivalent charged and hydrophobic functionalities built in the telodendrimers will enhance protein-binding affinity via synergistic effects: (i) Dendritic hybrid functionalities maximize the opportunity to interact with the heterogeneous protein surface/groove. (ii) Multivalent interactions can dramatically increase the binding affinities. (iii) The charge interactions between telodendrimer and protein will be enhanced and stabilized by the

decreased polarity of the surroundings[43-44] because of the closely adjacent hydrophobic moieties in the telodendrimers. In this study, we engineered the ionic strength, charge density and the structure of the hydrophobic groups in telodendrimers to fine-tune the binding affinity of protein-nanocarrier and to study the mechanism for protein loading and release. The optimized nanocarriers were further evaluated for both intracellular and systemic delivery of protein therapeutics.

2. Materials and methods

2.1. Materials

Monomethylterminated poly(ethylene glycol) monoamine hydrochloride (MeO-PEG-NH₂·HCl, M_w : ~5 kDa) was purchased from Jenkem Technology. (Fmoc)Lys(Fmoc)-OH and Fmoc-Lys(Boc)-OH were obtained from AnaSpec Inc. Fmoc-Arg(Pbf)-OH was purchased from Novabiochem. Cholesteryl chlorofomate was purchased from Alfa Aesar. CellTiter 96® AQueous MTS reagent powder was purchased from Promega. Heptadecanoic acid was purchased from Acros. Cy5NS succinimidyl ester was purchased from AAT Bioquest, Inc. Diisopropyl carbodimide (DIC), *N*-hydroxybenzotriazole (HOBt), D- α -tocopherol succinate, trifluoroacetic acid (TFA), fluorescein isothiocyanate isomer I (FITC), bovine serum albumin (BSA, M_w 66.5 kDa, isoelectric point 5.4), acetic anhydride, *N*-hydroxysuccinimide (HOSu), rhodamine B isothiocyanate (RBITC), methyl chlorooxoacetate, polyethylenimine (PEI, branched, M_w 25 kDa), and other chemical reagents were purchased from Sigma-Aldrich. Dialysis membrane with 3,500 M_w cut off was purchased from Spectrum Laboratories, Inc. Lysozyme (M_w 14.3 kDa, isoelectric point 11.0) was purchased from MP Biomedicals, LLC. Bovine insulin (M_w 5.8 kDa, isoelectric point 5.7) was purchased from Gemini Bio-Products. WT pH low insertion peptide conjugated with near infrared dye (Alexa 750) and drug (doxorubicin), which is named as pHLIP-Alexa750-DOX (M_w ~6 kDa, isoelectric point ~4), was provided by Prof. Ming An of Department of Chemistry at SUNY Binghamton University, details can be found in the previous publication[45]. Granulocyte-colony stimulating factor (G-CSF, M_w 18.8 kDa, isoelectric point 5.7) was provided by Prof. Li-Ru Zhao of Department of Neurosurgery at SUNY Upstate Medical University, which was originally purchased from Amgen. Recombinant green fluorescent protein (GFP, M_w 28.2 kDa, isoelectric point 6.0) was provided by Prof. Stewart N. Loh of Department of Biochemistry and Molecular Biology at SUNY Upstate Medical University. Truncated diphtheria toxin (DT₃₉₀, M_w 42.5 kDa, isoelectric point 5.1) and DTEGF (a construct consisting of a truncated diphtheria toxin, a seven-amino-acid linker, and a human epidermal growth factor, M_w 49.3 kDa, isoelectric point 4.9) were offered by Prof. Walter A. Hall of Department of Neurosurgery at SUNY Upstate Medical University and Prof. Daniel A. Vallera of the University of Minnesota, details can be found in the previous publication[46].

2.2. Telodendrimer synthesis

The telodendrimers with four guanidine groups containing heptadecanoic acids, cholesterol and D- α -tocopherol succinates, which are named as PEG^{5k}(Arg-L-C17)₄, PEG^{5k}(Arg-L-CHO)₄ and PEG^{5k}(Arg-L-VE)₄, respectively, were synthesized using a solution-phase condensation reaction starting from MeO-PEG-NH₂·HCl (5 kDa) via stepwise peptide

chemistry. The partition coefficient (LogP) values for C17, CHO and VE small molecules are 7.56, 7.47 and 8.78, respectively, which are predicted online at <http://www.molinspiration.com/cgi-bin/properties>. The procedure was performed as follows: (Fmoc)Lys(Fmoc)-OH (3 eq.) reacted with the N terminus of PEG using DIC and HOBt as coupling reagents until a negative Kaiser test result was obtained, indicating completion of the coupling reaction. PEGylated molecules were precipitated through the addition of the cold ether and then washed with cold ether twice. Fmoc groups were removed by the treatment with 20% (v/v) 4-methylpiperidine in dimethylformamide (DMF), and the PEGylated molecules were precipitated and washed three times by cold ether. White powder precipitate was dried under vacuum. One coupling of (Fmoc)Lys(Fmoc)-OH and one coupling of Fmoc-Arg(Pbf)-OH were carried out respectively upon the removal of Fmoc groups to generate an intermediate of dendritic poly(amino acid) terminated with four Pbf groups and four Fmoc groups on one end of PEG. Then four PEG linker molecules (M_w : 470) were coupled to the amino groups upon the removal of Fmoc groups with 20% (v/v) 4-methylpiperidine in DMF. After the removal of Fmoc groups, the polymers were coupled with heptadecanoic acid, cholesteryl chlorofomate, or D- α -tocopherol succinate. The Pbf protecting groups were consecutively removed via the treatment with 50% TFA in dichloromethane (DCM) to yield PEG^{5k}(Arg-L-C17)₄, PEG^{5k}(Arg-L-CHO)₄ and PEG^{5k}(Arg-L-VE)₄ (Scheme S1). These resulting telodendrimers were dissolved in deionized water, dialyzed against deionized water for 2 days, and then dried by lyophilization. The synthesis procedure of the telodendrimers with eight guanidine groups containing heptadecanoic acids, cholesterol and D- α -tocopherol succinates, which are noted as PEG^{5k}(ArgArg-L-C17)₄, PEG^{5k}(ArgArg-L-CHO)₄ and PEG^{5k}(ArgArg-L-VE)₄, respectively, is similar with that for the telodendrimers with four guanidine groups, the only difference is to couple Fmoc-Arg(Pbf)-OH to the amino groups of the arginines before coupling the PEG linker molecules (Scheme S2). The synthesis procedure for the telodendrimers with eight amino groups containing heptadecanoic acids, cholesterol and D- α -tocopherol succinates, noted as PEG^{5k}(LysLys-L-C17)₄, PEG^{5k}(LysLys-L-CHO)₄ and PEG^{5k}(LysLys-L-VE)₄, respectively, is similar with that for the telodendrimers with eight guanidine groups, the only difference is to couple twice Fmoc-Lys(Boc)-OH to the amino groups of the polylysine before coupling the PEG linker molecules (Scheme S3). The telodendrimer with eight oxalic acid groups containing heptadecanoic acids and cholesterol, named as PEG^{5k}(OAOA-L-C17)₄ and PEG^{5k}(OAOA-L-CHO)₄, were synthesized based on PEG^{5k}(LysLys-L-C17)₄, PEG^{5k}(LysLys-L-CHO)₄, respectively, by coupling with methyl chlorooxoacetate, and the hydrolysis reaction of the product was carried out in a 1/9 (v/v) of water/methanol mixture in the presence of 0.2 M of lithium hydroxide to yield PEG^{5k}(OAOA-L-C17)₄ and PEG^{5k}(OAOA-L-CHO)₄ (Scheme S4).

The telodendrimer with guanidine groups and without hydrophobic groups (named as PEG^{5k}Arg₄Ac₄, Scheme S6) was synthesized from acetic anhydride and a chemical intermediate 4 in Scheme S1. The Fmoc groups of chemical intermediate 4 were first removed by the treatment with 20% (v/v) 4-methylpiperidine in DMF, and the polymer was then coupled with acetic anhydride using triethylamine as a deacid reagent. The Pbf protecting groups were consecutively removed via the treatment with 50% TFA in DCM to yield PEG^{5k}Arg₄Ac₄.

The telodendrimers containing cholesterol and/or cholic acid groups (named as PEG^{5k}CHO₈, PEG^{5k}CA₄CHO₄, and PEG^{5k}CA₄-L-CHO₄, respectively, Scheme S6) were also synthesized using a solution-phase condensation reaction starting from MeO-PEG-NH₂·HCl (~5 kDa) via stepwise peptide chemistry. The synthesis and characterization of these telodendrimers have been reported separately[42] and, therefore, are not repeated here.

2.3. Telodendrimer characterization

¹H NMR spectra for telodendrimers were recorded on a Bruker AVANCE 600 MHz spectrometer at 25 °C. The concentration of the telodendrimers was kept at 5 × 10⁻⁴ M for NMR measurements. The solvent residual peak was used as reference (DMSO-*d*₆: 2.50 ppm). The numbers of hydrophobic groups in the telodendrimer molecules were calculated based on the average integration ratio of the peaks of the methyl protons (~0.84, ~0.65 and ~0.83 ppm for C17, CHO and VE, respectively) and the methoxy proton of PEG at ~3.24 ppm in ¹H NMR spectra in DMSO-*d*₆. A local phase correction was first performed to bring the signal of methoxy proton of PEG at ~3.24 ppm to the baseline level to eliminate the signal comes from the adjacent water signal at ~3.30 ppm. PEG methoxy peak was integrated as three protons (value of 3.00). Then, a global phase correction was performed and the peak areas were integrated for the signature groups introduced to telodendrimer. The formulas of the telodendrimers were calculated based on the relative peak integration to methoxyl integration on PEG. It should be noted that the apparent integration of methoxyl signal on PEG is higher than three protons after the global phase correction, due to the elevated baseline by the neighboring water peak. MALDI-TOF MS spectrum was recorded on a Bruker REFLEX-III instrument (linear mode) using α-cyano-4-hydroxycinnamic acid as a matrix. The spectrum smooth and baseline correction were performed for the raw MS data, and the peak molecular weight (*M*_p) and polydispersity index (PDI) were obtained based on the baseline-corrected MS spectrum. Dynamic light scattering (DLS) studies were performed using a Zetatrac (Microtrac Inc.) instrument, and the area-based mean particle sizes were presented. Each sample was measured for three times with an acquisition time of 30 seconds at room temperature. The data were analyzed by Microtrac FLEX software and the values were reported as the means for each triplicate measurements. Zeta potential measurements were carried out on a Malvern Nano-ZS zetasizer at room temperature. TEM images were taken on a JEOL JEM-2100 HR instrument operating at a voltage of 200 kV. The samples were stained by uranyl acetate.

2.4. Determination of critical micelle concentration (CMC)

Telodendrimers were dissolved in phosphate buffered saline (PBS). The initial micelle solution was diluted with PBS to obtain the required solutions ranging from 0.39 to 200 µg/mL. A known amount of Nile red in methanol was added to a series of vials. After methanol was evaporated under vacuum, a measured amount of polymer solutions were added to each vial to obtain a final Nile red concentration of 1 µM. The mixture solutions were left to shake overnight in the dark and at room temperature. The fluorescence emission intensity was measured using a microplate reader (Synergy 2, BioTek Instruments, Inc.) at the wavelength of 620 nm with excitation at 543 nm. CMCs were determined at the intersection of the tangents to the two linear fitting of the curve of the fluorescence intensity as a function of the log concentration of the telodendrimers.

2.5. Encapsulation of proteins in telodendrimer nanoparticles (NPs)

The proteins, peptides, or protein mixtures were dissolved in PBS, and the telodendrimers in PBS were quickly added into protein solution. The proteins were encapsulated by the telodendrimers through electrostatic interaction, hydrogen bonding, and hydrophobic-hydrophobic interaction. All protein/telodendrimer (P/T) ratios used in this study are mass ratios.

2.6. Fluorescently labeled proteins and telodendrimers

FITC-labeled BSA (named FITC-BSA) was prepared by mixing 3 mg of FITC dissolved in 0.3 mL of DMSO with 10 mL of BSA aqueous solution (10 mg/mL) in the presence of 0.1 M of NaHCO_3 being stirred. After 24 h, the reaction mixture was dialyzed against deionized water in the dark for one week to remove the unreacted FITC molecules, and dried by lyophilization.

FITC-labeled insulin (noted as FITC-insulin) was synthesized as follows: FITC was dissolved in acetone (0.5 mg in 200 μL) and added dropwise to a 10 mL solution containing the appropriate amount of insulin dissolved in PBS contained 200 μM EDTA. After 24 h, the reaction mixture was dialyzed against deionized water in the dark for one week to remove the unreacted FITC molecules and then lyophilized.

FITC-labeled lysozyme (noted as FITC-lysozyme) was prepared by mixing 0.5 mg of FITC dissolved in 0.1 mL of DMSO with 15 mL of 3.3 mg mL^{-1} of lysozyme in PBS. After 24 h, the reaction mixture was dialyzed against deionized water in the dark for one week to remove the unreacted FITC molecules, and dried by lyophilization.

RB-labeled BSA (named RB-BSA) was prepared by mixing 10 mg of RBITC dissolved in 0.5 mL of DMSO solution with 20 mL of BSA aqueous solution (13 mg/mL) in the presence of 0.1 M of NaHCO_3 while being stirred. After 24 h, the reaction mixture was dialyzed against deionized water in the dark for one week to remove the unreacted RBITC molecules and then lyophilized.

Cy5-labeled GCSF (named Cy5-GCSF) was prepared by mixing 0.06 mg of Cy5NS succinimidyl ester dissolved in 0.5 mL of DMSO solution with 9 mL of GCSF aqueous solution (0.1 mg/mL) while being stirred in the presence of 0.1 M of NaHCO_3 . After 24 h, the reaction mixture was dialyzed against deionized water in the dark for one week to remove the unreacted dye molecules and then lyophilized.

Cy5-labeled insulin (named Cy5-insulin) was prepared as follow: Cy5NS succinimidyl ester was dissolved in DMSO (0.5 mg in 200 μL) and added dropwise to a 10 mL solution containing the appropriate amount of insulin dissolved in PBS contained 200 μM EDTA. After 24 h, the reaction mixture was dialyzed against deionized water in the dark for one week to remove the unreacted dye molecules and then lyophilized.

FITC-labeled $\text{PEG}^{5k}(\text{ArgArg-L-C17})_4$ (named FITC- $\text{PEG}^{5k}(\text{ArgArg-L-C17})_4$) was prepared by mixing 0.4 mg of FITC dissolved in 0.2 mL of DMSO with 1 mL of $\text{PEG}^{5k}(\text{ArgArg-L-C17})_4$ aqueous solution (10 mg/mL) in the presence of 0.1 M of NaHCO_3 . After 24 h, the

reaction mixture was dialyzed against deionized water in the dark for one week to remove the unreacted FITC molecules, and dried by lyophilization.

2.7. Agarose gel retention assay

Samples in loading buffer (30% glycerol aqueous solution) were loaded into agarose gel (1.5% wt) in Tris-acetate-EDTA (TAE) buffer. The gel tray was run for 2 h at a constant current of 20 mA. The gel was then stained with 1% coomassie blue (30 min) then allowed to destain overnight. The gel was imaged by a Bio-Rad Universal Hood II Imager (Bio-Rad Laboratories, Inc.) under SYBR Green and Coomassie Blue modes. The loading capacity and loading efficiency of the NPs were semi-quantitatively calculated from the Adj. Vol. (Int.) of the fluorescence bands for unloaded FITC-labeled proteins using the Image Lab 3.0 software. For near-infrared dye-labeled protein or peptide, the gel was imaged by an IVIS-50 imaging system.

2.8. Isothermal titration calorimetry (ITC)

ITC was performed on VP-ITC (MicroCal, LLC) with 1.4 mL cell at 295 rpm stirring at 37 °C. All samples were prepared in PBS (1×) and degassed for 5 min before use. Protein sample was dialyzed against PBS overnight before the ITC measurement, and the concentration was calculated from the UV-vis spectrum based on the molar extinction coefficient of 43,824 M⁻¹cm⁻¹ for BSA at 279 nm. Titrations were performed by injecting BSA solution (137 μM) into the calorimetric sample cell containing 30 μM of telodendrimers using 1 step of 1.5 μl injection and another 30 steps of 5 μl injections and 300 sec pauses between injections to allow the solution to reach equilibrium. Titration of protein into blank buffer was performed for reference. Heats of injections were calculated using Microcal analysis package for Origin 7.0.

2.9. Förster resonance energy transfer (FRET) studies

RB-BSA and FITC-PEG^{5k}(ArgArg-L-C17)₄ were used to prepare FRET NPs. Equal volumes of 2 mg/mL of FITC-PEG^{5k}(ArgArg-L-C17)₄ solution and 2 mg/mL of RB-BSA solution were mixed then stirred overnight. Concentrated BSA solutions were then added into the above mixtures to reach final BSA concentrations from 0 to 40 mg/mL. The fluorescence spectra with a range from 480 to 640 nm at different time points excited by 439 nm were recorded using a microplate reader (BioTek Synergy 2). The FRET ratio was calculated by the formula of $[100\% \times I_{584} / (I_{584} + I_{528})]$, where I_{584} and I_{528} were the fluorescence intensities of at 584 and 528 nm, respectively. FITC-BSA and RB-BSA were also used as a pair for FRET study.

2.10. Bio-layer interferometry (BLI)

The kinetics of protein-telodendrimer interactions was measured at 37 °C by BLI on an Octet-Red 96 (ForteBio). Streptavidin biosensors (ForteBio #18–5019) were prewetted in 40 mg/mL of BSA solution for 900 s, incubated in the same solution for 900 s, washed in PBS for 480 s, and transferred to wells containing telodendrimers at concentrations ranging from 75 to 600 nM in PBS for 900 or 1800 s (association). Dissociation at each studied concentration was carried out in either PBS or BSA solution (5 or 40 mg/mL) for 1800 s.

The association constant (k_{on}) and the dissociation constant by competition within 40 mg/mL BSA solution (k_{off}) were obtained by globally fitting the BLI data to a 1:1 model algorithm using Octet software.

2.11. Docking calculation using Molecular Operating Environment (MOE)[47]

The protein crystal structure of BSA was downloaded from Protein Data Bank (PDB ID: 4F5S). It was visualized in MOE viewer and all the chains except chain A, including ligands and waters, were deleted. Hydrogen atoms were added and the whole structure was minimized by MMFF94x force field and charges. Induced Fit protocol was selected to set the side chains of BSA free during the docking process. All surface atoms of BSA were chosen as the active sites manually. The ligand molecules (their chemical structures as shown in Scheme S5) were placed in the sites with the Triangle Matcher method and ranked with the London dG scoring function. The 100 best poses were retained and further refined by energy minimization in the pocket, followed by rescoring with the GBVI/WSA dG scoring function. All negative docking binding energies of each ligand were averaged, and the average binding energy was used as the final binding energy for comparison and analysis.

2.12. Hemocompatibility assay

One milliliter of fresh blood from healthy human volunteers was collected into 5 mL of PBS solution in the presence of 20 mM EDTA. Red blood cells (RBCs) were then separated by centrifugation at 1000 rpm for 10 min. The RBCs were washed three times with 10 mL of PBS and resuspended in 20 mL of PBS. Diluted RBC suspension (200 μ L) was mixed with NP PBS solutions at serial concentrations (10, 100, and 500 μ g/mL) by gentle vortex and incubated at 37 °C for 0.5 h, 4 h, and overnight, respectively. The mixtures were centrifuged at 1,000 rpm for 5 min, and then the free of hemoglobin in the supernatant was determined by measuring the absorbance at 540 nm using a UV-vis spectrometer. Incubations of RBCs with Triton-100 (2%) and PBS were used as the positive and negative controls, respectively. The percent hemolysis of RBCs was calculated using the following formula:

$$\text{RBC hemolysis} = \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{negative control}})}{(\text{OD}_{\text{positive control}} - \text{OD}_{\text{negative control}})} \times 100\% \quad (1)$$

2.13. Cell culture and MTS assays

The human glioblastoma multiforme (GBM) cell line U87 was obtained from the Brain Tumor Laboratory of SUNY Upstate Medical University originally purchased from ATCC (Manassas, VA, USA). The colon cancer cell line HT-29 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The U87 cells were cultured in DMEM medium and HT-29 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μ g/mL streptomycin at 37 °C using a humidified 5% CO₂ incubator. Cells were seeded in 96-well plates at a density of 3000 cells/well 24 h prior to the treatment. Various formulations of proteins with different dilutions were added to the plate and then incubated in a humidified 37 °C, 5% CO₂ incubator. After 72 h incubation, a mixture solution composed of CellTiter 96 Aqueous

MTS, and an electron coupling reagent, PMS, was added to each well according to the manufacturer's instructions. The cell viability was determined by measuring absorbance at 490 nm using a microplate reader (BioTek Synergy 2). Untreated cells served as the control. Results were shown as the average cell viability $[100\% \times (OD_{\text{treat}} - OD_{\text{blank}}) / (OD_{\text{control}} - OD_{\text{blank}})]$ of triplicate wells. The cells were also treated with blank NPs in PBS at different dilutions and incubated for a total of 72 h to evaluate NP-related toxicity.

2.14. Cellular uptake

The cellular uptake and intracellular trafficking of the protein-incorporated NPs were determined by fluorescence microscopy. FITC-BSA was used as a model protein. HT-29 and U87 cells were seeded in a chamber slide with a density of 5×10^4 cells per well in 350 μL of medium and cultured for 24 h. The original medium was replaced with free FITC-BSA and FITC-BSA-loaded NPs at a final FITC concentration of approximately 1.5 $\mu\text{g/mL}$ at 37 °C. After a 3 h incubation, the cells were washed three times with PBS (1 $\times$) and fixed with 4% paraformaldehyde for 10 min at room temperature, and the cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The slides were mounted with cover slips and cells were imaged with a Nikon FV1000 laser scanning confocal scanning microscope.

2.15. Animals and subcutaneous tumor model

Female athymic nude mice (NCRNU-Sp/Sp), at age 8 weeks, were purchased from Taconic Biosciences (Germantown, NY). All animals were kept under pathogen-free conditions according to Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines and were allowed to acclimatize for at least 4 days prior to any experiments. All animal experiments were performed in compliance with institutional guidelines and according to protocol approved by the Committee for the Humane Use of Animals of State University of New York Upstate Medical University. Subcutaneous tumor xenograft mouse models were established by injecting 1×10^7 HT-29 cancer cells in 100 μL of a mixture of PBS and Matrigel (1:1, v/v) subcutaneously at the right flank in female nude mice.

2.16. Fluorescence animal imaging and biodistribution for subcutaneous tumor model

Nude mice with subcutaneous tumors of an approximate 8 to 10 mm diameter were subjected to *in vivo* near-infrared fluorescence optical imaging. At different time points post injection of free protein and protein-incorporated telodendrimer NPs (1/5 of protein/telodendrimer by weight), mice were anesthetized and optically scanned using an IVIS-200 small animal imager (PerkinElmer) at Cy5.5 excitation and emission channels. The mice were anesthetized by isoflurane gas before and during each imaging. Animals were euthanized by CO₂ overdose after the last *in vivo* imaging. Tumors and major organs were excised for *ex vivo* imaging. The associated fluorescence intensities were determined by Living Image software (Caliper Life Sciences) using operator-defined regions of interest measurements.

2.17. Intracranial orthotopic GBM tumor model

8-week old female athymic nude mice purchased from Taconic Biosciences (Germantown, NY) were anesthetized with ketamine/xylazine (80 mg/kg: 5 mg/kg) through an intraperitoneal injection. The mice were then placed in a stereotactic head frame from Kopf Instruments (Tujunga, California, USA). Using aseptic technique, a midline skin incision was made using a scalpel. A burr hole using a Foredom Microdrill (Bethel, CT, USA) was created 0.5 mm anterior to bregma and 2.5 mm lateral of midline. A 25G needle was used to puncture the dura. Using a Hamilton needle (Reno, NV, USA), approximately 5×10^4 of U87 tumor cells (in 1 μ L of PBS) were delivered 3.0 mm deep via the stereotactic head frame. Using a microsyringe pump controller, the injection occurred over the course of 5 minutes, the needle left in place for 5 minutes, and then the needle was slowly retracted. Veterinary grade surgical glue was used to close the skin.

2.18. Bioluminescence imaging of orthotopic intracranial tumors

In vivo bioluminescence imaging studies were carried out using IVIS 50 (PerkinElmer) for brain tumor tracking. For imaging, mice with intracranial U87 tumor were anesthetized with isoflurane and simultaneously D-luciferin potassium salt was administered intraperitoneally at a dose of 3.75 mg/mouse. Mice were imaged 7 days after tumor implantation to ensure tumor growth.

2.19. Convection-enhanced delivery (CED) administration

After tumor growth from the U87 injections had been confirmed on bioluminescence imaging, micro-osmotic pumps were used for an intracranial peptide distribution study on post injection day 8. Free peptide and peptide-incorporated telodendrimer NPs (100 μ L peptide solution at a concentration of 100 μ M for each mouse, 1/5 of peptide/telodendrimer by weight) were infused at a rate of 0.5 μ g/hr over 7 days via micro-osmotic pumps from ALZET (Cupertino, CA, USA). The micro-osmotic pumps were sterilely loaded with 100 μ L formula the evening prior to insertion per manufacturer instructions and allowed to prime overnight at 37 °C. The animals were anesthetized with ketamine/xylazine (80 mg/kg: 5 mg/kg) through an intraperitoneal injection. Using aseptic technique, the original incision was opened and the original burr hole identified. The tip of the micro-osmotic pump was inserted into the original burr hole at a depth of 3.0 mm, placing the tip approximately at the center of the tumor. A pocket was tunneled on the dorsum of the mouse for the pump reservoir to be placed in. The skin was closed with veterinary grade surgical glue. After the 7 days of CED, the pump was removed as it can interfere with subsequent bioluminescent imaging procedures. 48 h after CED, the mice were sacrificed, and the brain tumors and major organs were excised for *ex vivo* imaging. The associated fluorescence intensities were determined by Living Image software. The removed brains were flash frozen with liquid nitrogen and stored at -80 °C. The frozen tissue was then gradually warmed to -20 °C just prior to sectioning on a cryostat. The brains were sectioned at 20 μ m and the sections then mounted. The sliced brain tumor tissues were analyzed using a Zeiss Axioskop upright fluorescence microscope equipped with a digital camera (Carl Zeiss Microimaging, Inc., Thornwood, NY).

3. Results and discussion

3.1. Telodendrimer synthesis and protein encapsulation

Telodendrimers with multiple hybrid functionalities were synthesized starting from a MeO-PEG-NH₂ ($M_w \sim 5$ kDa) via peptide chemistry (Schemes S1–S4). The formula of telodendrimer was denoted as PEG^{5k}(CF-L-HF)₄ (Fig. 1), where “CF” represents charged functionalities, e.g., cationic guanidine-based arginine (Arg) and amino-based lysine (Lys), or anionic oxalic acid (OA); “L” means a flexible linker[48], and “HF” represents hydrophobic functionalities, such as heptadecanoic acid (C17), cholesterol (CHO) and D- α -tocopherol (VE). The molecular weights of the telodendrimers determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) are very close to their theoretical values (Table 1 and Fig. S1). In addition, the polydispersity of telodendrimers, analyzed by MALDI-TOF MS (Table 1), maintained the same as the monodispersed starting PEG (polydispersity index, PDI = 1.01), indicating that the well-defined dendritic structures were synthesized on the terminus of PEG chains. The proton nuclear magnetic resonance (¹H NMR) spectra for the telodendrimers recorded in deuterated dimethyl sulfoxide (DMSO-*d*₆) also confirmed their well-defined chemical structures with the calculated formula being close to the designed structures (Table 1 and Fig. S2–S12). Telodendrimers self-assembled into monodispersed nanoparticles (NPs) with hydrodynamic diameters (D_h) of 11–32 nm (Fig. 2a, S13 and S14, and Table 2) in phosphate buffered saline (PBS) determined by dynamic light scattering (DLS). Neutral zeta potential for these NPs was detected (Fig. 2b and Table 2) indicating efficient PEG shielding. The telodendrimers containing C17 or CHO self-assemble into spherical micelles while the VE-containing telodendrimers tend to form both spherical micelles and nanofibers as shown in the transmission electron microscopy (TEM) images (Fig. 2c–e). The critical micelle concentrations (CMCs) of the telodendrimers in PBS range from 1.14 to 2.68 μ M, which was determined by a fluorescent method[49] employing Nile red as a probe (Table 1 and Fig. S15–S17). The NP sizes generally decrease with increasing protein/telodendrimer (P/T) mass ratios detected by DLS (Fig. 2a). Accordingly, nanofibers observed by TEM for blank PEG^{5k}(ArgArg-L-VE)₄ disappear after BSA loading. The protein-loaded telodendrimer NPs are generally spherical in shape with uniform size distributions (Fig. 2f–h). The zeta potential of protein-loaded NPs gradually decreases with increasing P/T ratio (Fig. 2b). The protein-loaded NPs have excellent stability in size as colloids in PBS for storage over 2 months (Table 2 and Fig. S18).

As a dynamic micelle system, free telodendrimers (<CMC) can coat the surface of protein; and protein also can absorb on the surface of micelles (>CMC). The disappearance of nanofibers and reduced particle sizes after protein loading indicate the reassembly of nanoconstructs during protein encapsulation as illustrated in Fig. 2i. Native agarose gel electrophoresis was used to separate the free and the loaded proteins based on the particle sizes and charge effects. Protein loading in the telodendrimer NPs follows the charge selectivity (Fig. S19–S21), i.e., negatively charged bovine serum albumin (BSA) can be loaded in the telodendrimer NPs with cationic guanidine or amino groups, while positively charged lysozyme forms complexes with OAOA telodendrimers. The electrophoresis results in Fig. 2j indicate that the telodendrimers have super high loading capacities (>50% of NPs

by weight) for fluorescein isothiocyanate-labeled BSA (FITC-BSA). The increased charge density increases protein loading efficiency and aliphatic C17 contributes to higher BSA loading efficiency than CHO and VE, given the same charged groups (Fig. 2j). To study the protein binding stoichiometry and binding affinity with telodendrimers, isothermal titration calorimetry (ITC) experiments were performed. BSA solution in PBS was titrated into PEG^{5k}(ArgArg-L-VE)₄ telodendrimer solution in PBS at 37 °C (Fig. 2k). An exothermic process was observed in Fig. 2k with a binding constant (K_d) of 2.2 μ M. The number of telodendrimer to BSA in the equilibriums are fitted to be ~9/1, which corresponds to a P/T mass ratio of 0.75/1 in NPs, consistent with the high protein loading capacity as observed in the electrophoresis assay.

Existing vehicles for protein and gene delivery usually have difficulty achieving good *in vivo* performance due to their poor physical properties along with their high cytotoxicity. For example, the highly charged polyethylenimine (PEI) may crosslink proteins to form large aggregates (Fig. S22); the low protein loading capacity and poor colloidal stability of lipid-like NPs may restrict their applications (Fig. S23). In comparison, our telodendrimer NPs possess small and stable particle sizes, neutral surface charge, and high protein loading capacities, which are promising platforms for *in vivo* applications of protein delivery.

3.2. Mechanistic studies for protein loading and release

Förster resonance energy transfer (FRET) experiments were used to probe the molecular proximity[50] during protein loading. When the mixture of FITC-PEG^{5k}(ArgArg-L-C17)₄ and rhodamine B-labelled BSA (RB-BSA) is excited at the excitation wavelength of FITC at 439 nm, a significant FRET signal was recorded at the emission wavelength of RB at 584 nm (Fig. 2l), indicating the close proximity of protein and telodendrimer within one NP. The mixture of RB-BSA and FITC-BSA in solution doesn't yield an apparent FRET effect (Fig. 2m), due to the isolation of these soluble proteins. The addition of non-fluorescent PEG^{5k}(ArgArg-L-C17)₄ to this mixture gradually increased the FRET ratio with the increasing telodendrimer concentration, which approached equilibrium at a P/T mass ratio of 1/3 (Fig. S24a), this can be explained by the absorption-reassembly process for protein loading. When the telodendrimer content is low, single layer telodendrimer-coating on the individual protein may occur prominently, therefore preventing FRET efficiency. With increased micelle concentrations, two or more FRET proteins have chance to attach to the surface of a micelle and are co-encapsulated into the reassembled NP, resulting in efficient FRET. We further utilized the FRET effect to study the protein release profile from the nanocomplex upon protein exchange by serum protein. Different amounts of blank BSA were added in the complex solutions of FITC-PEG^{5k}(ArgArg-L-C17)₄ and RB-BSA. The FRET ratio slightly decreased with increased BSA concentrations (Fig. S24b), indicating that some payload proteins can be released in the presence of a high concentration of free BSA, which was also confirmed by the agarose gel retention assay (Fig. S25). Notably, only a small amount of the cargo proteins were exchanged and the majority of the cargo proteins were still associated with telodendrimers even when incubated with high concentrations of serum proteins (Fig. S25). We would like to emphasize that the protein release rate by serum protein exchange can vary for different cargo proteins and can be tailored by engineering the relative binding affinity of telodendrimer to the cargo protein.[19] For example, insulin, a

pH-low insertion peptide (pHLIP-Alexa750-DOX)[45] conjugated with doxorubicin and a near infrared dye Alexa750, and granulocyte-colony stimulating factor (GCSF) loaded in the telodendrimer NPs were very stable against BSA incubation at a biological concentration of 40 mg/mL (Fig. S26–S29), indicating strong binding affinity between these cargo proteins and telodendrimers.

In the above gel electrophoresis study, various hydrophobic functionalities in the telodendrimers were observed to affect BSA loading capacity and efficiency, which may be correlated with their protein binding affinity. Therefore, we applied bio-layer interferometry (BLI)[51] to study the kinetics of protein-telodendrimer binding to illustrate the structure-property relationship in BSA encapsulation. A streptavidin-conjugated BLI sensor was used to mimic BSA, based on their similarities in isoelectric points and molecular weights to study protein-telodendrimer interactions. Biosensors were pre-wetted in BSA solution to block the nonspecific binding sites, then incubated in telodendrimer solutions for association followed by dissociation at 37 °C (Fig. 3a). The BLI binding signals increased with the time in a concentration dependent manner. Interestingly, the proceeding dissociation of telodendrimers from the biosensors in PBS incubation was very slow (Fig. 3b), indicating strong binding affinity between protein and telodendrimer. However, the dissociation rate was too slow in PBS and not sufficient for dissociation constant (k_{off}) fitting[52]. BSA, the most abundant protein in blood plasma, was observed to accelerate telodendrimer dissociation in a concentration dependent manner (Fig. 3b). A sustained dissociation profile was observed even in a BSA solution at 40 mg/mL, which is a biologic serum protein concentration. A series of associations at different telodendrimer concentrations (72–600 nM, <CMC) were conducted followed by dissociations in 40 mg/mL of BSA solution (Fig. 3c–e). It should be pointed out that the telodendrimer dissociation in BSA solution should be mainly driven by the direct BSA “attacking” on the biosensor surface, which is evidenced by the BSA concentration dependent dissociation observed in Fig. 3b. If the BSA in solution only serves as a scavenger to absorb the telodendrimers dissociated from the sensors, the dissociation would also be a slow process similar to that in an extremely diluted solution in PBS (Fig. 3b). Therefore, the k_{off} fitting in BSA solution can’t reflect the thermodynamic dissociation rate of telodendrimers. Instead, it reflects the exchange rate and binding affinity of telodendrimers to BSA. The addition of BSA in dissociation solution invalids the classic equilibrium binding constant analysis by $K_D = k_{\text{off}}/k_{\text{on}}$ in comparing the binding affinity of different telodendrimers. Therefore, we apply the association constant k_{on} to compare the binding affinity of different telodendrimers. We define the dissociation constant in 40 mg/mL BSA solution as k_{off}' (Fig. 3f). The hydrophobic species in the telodendrimers were observed to affect the k_{on} and k_{off}' : PEG^{5k}(ArgArg-L-VE)₄ exhibited a slower binding than C17- and CHO-containing ones and also a slower dissociation through BSA competition (Fig. 3c–f), which may indicate the slower protein release for PEG^{5k}(ArgArg-L-VE)₄ nanoformulation by serum protein exchange in blood circulation. In addition, the BLI analysis reveals a significantly higher saturated signal for VE-containing telodendrimer than that for C17- and CHO-containing ones (Fig. 3c–e). It indicates that more VE-telodendrimers are needed to stably coat BSA, which is correlated with the relatively lower protein loading capacity for VE-telodendrimers (Fig. 2j).

BLI studies for telodendrimers at the concentrations close to and higher than CMC were also conducted. Although the similar concentrations of the free telodendrimers existed in the systems, the association rates increased significantly proportional to the concentration. It indicates that micellar NPs are also involved in the surface coating directly, therefore increasing the association rate (Fig. S30). Interestingly, the saturated signals above CMC were similar to the bindings close to CMC, indicating a similar thickness of telodendrimer coating in equilibrium irrespective of coating with either monomer ($< \text{CMC}$) or NP ($> \text{CMC}$). In addition, no significant dissociations were observed in PBS, indicating no micelle coating on the equilibrium surface, which was observed to dissociate from the surface easily in PBS in our previous study.[53] These observations tell us that telodendrimer micelles may adhere on the protein-coated surface above CMC and then spread out on the surface to form a monolayer coating driven by the thermodynamics. This is in line with the absorption-reassembly mechanism for protein encapsulation based on the size/morphology and FRET studies.

3.3. Synergistic combination of electrostatic and hydrophobic interactions

Different subunits of telodendrimers (shown in Scheme S5) were virtually docked to the surface of BSA via Molecular Docking. The docking results indicate the average binding energy decreases significantly when multivalent and hybrid functionalities are built in the subunits (Fig. 4a), suggesting the combination of the multivalent charge and hydrophobic interactions is crucial for protein binding. The docking result also suggests the protein surface can be efficiently covered by $(\text{ArgArg-L-CHO})_4$ ligand molecules with a nearly uniform ligand distribution (Fig. S31). To test the synergistic combination of charge and hydrophobic interactions further in experiments, a charged-only telodendrimer of $\text{PEG}^{5k}\text{Arg}_4\text{Ac}_4$ with the *N*-terminal acetylation instead of hydrophobic moieties (Scheme S6, and Fig. S32 and S33), and an arginine-protected hydrophobic-only $\text{PEG}^{5k}(\text{Arg}(\text{Pbf})\text{-L-CHO})_4$ were prepared as controls for BSA loading. The gel retention assay (Fig. 4b) indicated that these telodendrimers with multivalent single type of moieties were not sufficient for BSA encapsulation. Only the hybrid $\text{PEG}^{5k}(\text{Arg-L-CHO})_4$ could retard proteins from migration in an electric field. In addition, we attempted to use another three typical drug-loading telodendrimers[42] with amphiphilic or hydrophobic dendritic segments to load BSA (Scheme S6). As a result, these NPs didn't show any BSA loading capability as shown in the electrophoresis assay (Fig. S34). BLI was employed to further characterize the protein binding properties of these telodendrimers. $\text{PEG}^{5k}(\text{Arg-L-CHO})_4$ triggered a fast association (Fig. 4c), although with a weaker binding when compared to the doubly charged $\text{PEG}^{5k}(\text{ArgArg-L-CHO})_4$. Without hydrophobic groups to anneal telodendrimer on the protein surface, the charged-only $\text{PEG}^{5k}\text{Arg}_4\text{Ac}_4$ was unable to stably bind to proteins on the sensors presumably due to the highly dynamic charge interactions in PBS (Fig. 4d). Hydrophobic-only $\text{PEG}^{5k}(\text{Arg}(\text{Pbf})\text{-L-CHO})_4$ was observed to associate with protein in BLI studies, although with a much slow association rate (small k_{on}) especially at low concentrations due to the lack of fast attracting charges (Fig. 4e). A thicker layer of $\text{PEG}^{5k}(\text{Arg}(\text{Pbf})\text{-L-CHO})_4$ were observed to deposit on the BLI biosensor, which may be due to the nonspecific hydrophobic absorption. At the same time, the dissociation in BSA was also very slow, indicating weaker protein interaction. Accordingly, no apparent FRET was observed when $\text{PEG}^{5k}\text{Arg}_4\text{Ac}_4$ or $\text{PEG}^{5k}(\text{Arg}(\text{Pbf})\text{-L-CHO})_4$ were added into the

mixture of RB-BSA and FITC-BSA in contrast to the strong FRET induced by PEG^{5k}(Arg-L-CHO)₄ (Fig. 4f–h), indicating weak BSA interactions with these two telodendrimers.

The above observations evidence the synergistic combination of hydrophobic and charge interactions in protein encapsulation, wherein the charged functionality plays an attracting role to accelerate protein coating while the hydrophobic functionality plays an annealing function for telodendrimers to deposit on protein surfaces. The presence of hydrophobic moieties in telodendrimer decreases the polarity around charge interactions between telodendrimer and protein, therefore it may further stabilize the “salt bridges” in the nanocomplex by decreasing the static dielectric constants of surroundings,[43–44] which is well-known to protein structures.[54] We also introduced amphiphilic riboflavin (Rf) into telodendrimers, PEG^{5k}(ArgArg-L-Rf)₄, intending to match both polar and nonpolar surfaces of protein in addition to the charge interactions. However, the resultant telodendrimers showed poor protein encapsulation properties in agarose gel electrophoresis assays (data not shown). It was likely due to reduced hydrophobic interactions and increased polarity at the P-T interface which weaken the charge interactions.

3.4. Biocompatibility and intracellular delivery of active proteins

The stable PEG shell and the slightly negative surface zeta potential render the telodendrimer micelles nontoxic at the concentrations about 100 µg/mL. These telodendrimers are completely inert in the hemolytic assays up to 500 µg/mL for 24 h incubation with red blood cells (Fig. S35–S37). In comparison, a traditional intracellular delivery vehicle PEI has high cytotoxicity (the half maximal inhibitory concentration, IC₅₀: 2 µg/mL) and induces severe hemolysis even at a low concentration of 10 µg/mL (Fig. S38). Telodendrimers are composed of all biocompatible components constructed via peptide bonds, which can be hydrolyzed by peptidase *in vivo*. In addition, telodendrimers have low molecular weights less than 10 kDa. They can be eliminated by renal clearance once dissociated from proteins or micelles.

When in circulation, the PEG shell can shelter positive charges efficiently to avoid nonspecific binding to cell membranes. However, the presence of guanidiniums in the telodendrimers may trigger cell uptake in close proximity of micelle to plasma membrane, for example, in cell culture mediums or in tumor sites where the NPs are concentrated by the enhanced permeability and retention (EPR) effects[23]. The kinetic approaching of NPs to plasma membrane is able to deplete the dynamic PEG sheltering and expose some positive charges, therefore increasing membrane adhesion of NPs and inducing endocytosis. To test this hypothesis, FITC-BSA was used to probe the intracellular trafficking of proteins delivered by telodendrimer nanocarriers in HT-29 colon cancer cell cultures (Fig. 5a–d). FITC-BSA could hardly enter cancer cells spontaneously (Fig. 5a), but when loaded in PEG^{5k}(ArgArg-L-HF)₄ NPs, FITC-BSA was translocated into the cytoplasm (Fig. 5b). VE-telodendrimer showed the highest efficiency for intracellular delivery, which may be correlated with an increased net charge density of NPs due to a thicker PEG^{5k}(ArgArg-L-VE)₄ coating (Fig. 3e). Lysine-containing PEG^{5k}(LysLys-L-HF)₄ have less efficiency for intracellular protein delivery when compared to PEG^{5k}(ArgArg-L-HF)₄ (Fig. 5c), because of the weaker ionic strength. Further, the reduced arginine number in PEG^{5k}(Arg-L-HF)₄ NPs

leads to a weaker capability for intracellular delivery (Fig. 5d), which is due to a reduced charge density.[55]

Except for above mentioned model proteins and peptides, green fluorescent protein (GFP) and DTEGF (a fusion toxin consisting of a truncated diphtheria toxin and a human epidermal growth factor) can also be effectively encapsulated in the telodendrimer NPs (Fig. 5e and S39a). After being loaded in the NPs, the GFP can maintain its fluorescent property in solution (Fig. S40), indicating an intact protein structure. In addition, the encapsulated GFP showed significantly stronger fluorescence on agarose gel electrophoresis than free GFP (Fig. 5e). This indicates the protection of the protein structure against stress in electric field by charge neutralization. On the other hand, the protein structure of free GFP may be deformed during electrophoresis assay, thereby showing reduced fluorescence intensity. DTEGF can target U87 human glioblastoma cells and cause cytotoxicity.[46] After being loaded in telodendrimer NPs, it exhibited similar IC_{50} values to that for the free DTEGF on U87 cells (Fig. S39b), suggesting the complete maintenance of protein bioactivity in telodendrimer nanoformulations. This indicates a “green” process for *in situ* protein encapsulation without interrupting protein structures.

Truncated diphtheria toxin (DT₃₉₀) is a potent protein that induces cell death by inhibiting protein synthesis, but only if it is delivered into the cytoplasm.[46] Free DT₃₉₀ is nontoxic (Fig. 5f) because it is impermeable to the plasma membrane. When DT₃₉₀ is loaded in PEG^{5k}(Arg-L-HF)₄ NPs, cytotoxicity is only obvious at a high protein concentration of 150 nM due to inefficient intracellular delivery. In contrast to this, DT₃₉₀ exhibits potent cancer cell killing with IC_{50} around 30 nM after loaded in PEG^{5k}(ArgArg-L-HF)₄ NPs with improved intracellular delivery. Telodendrimer micelles has shown to be nontoxic under the above tested concentrations (Fig. S35 and S36). During a confocal laser fluorescence microscopy (CLSM) imaging study by counterstaining the endosome/lysosome (green) of a U87 cell (the inset in Fig. 5f), we revealed the co-localization of RB-BSA (red) loaded in PEG^{5k}(ArgArg-L-VE)₄ NPs within the endosome/lysosome after 3 h of incubation. Furthermore, significant amounts of protein had already escaped from the endosomal compartment, as indicated by the red fluorescence throughout the cell, which is essential for therapeutic proteins to be functional intracellularly.

3.5. Improved tumor targeting and retention

The finding of stable protein encapsulation *in vitro* warrants further evaluation of the fate of protein nanotherapeutics *in vivo*. Small animal imaging was performed to study the systemic protein delivery and the biodistribution of protein-loaded telodendrimer NPs. The near-infrared dye-labeled insulin (Cy5-insulin) was selected as a model protein to study the *in vivo* biodistribution and tumor targeted protein delivery. The free Cy5-insulin and the Cy5-insulin-loaded nanoformulation were injected through the tail vein into the nude mice bearing xenograft HT-29 colon cancers. *In vivo* animal imaging indicated that the proteins loaded into PEG^{5k}(ArgArg-L-VE)₄ telodendrimers could target xenograft HT-29 colon cancer efficiently (Fig. 6a). In contrast, the free protein injections experienced fast clearance, nonspecific distribution and low tumor uptake. The *ex vivo* imaging (Fig. 6b) and semi-quantitative biodistribution analysis (Fig. 6c) clearly showed that the highest signal was

observed in tumors of animals treated with the nanoformulations. Healthy organs showed very low fluorescence signals in the animals treated with nanoformulations. The accumulation of the nano-sized protein formulations at tumor sites is believed to be due to the prolonged circulation of NPs, which selectively diffuse through leaky blood vessels in solid tumors known as the EPR effect.[23] Free proteins showed weak signals in healthy organs and the tumor due to fast clearance.[56] When compared to VE- and CHO-containing telodendrimers, PEG^{5k}(ArgArg-L-C17)₄ nanoformulation exhibited a less effective *in vivo* tumor targeting effect (Fig. S41). This may be related to the faster protein release rate of the PEG^{5k}(ArgArg-L-C17)₄ nanoformulation in biological serum protein concentration as shown by BLI (Fig. 3c). The xenograft model of A549 lung cancer was also used for the study of systemic protein delivery to tumor by telodendrimer NPs, which showed a similar result with the xenograft model of HT-29 colon cancer (Fig. S42).

Aside from the systemic delivery, local infusion of therapeutics is an alternative approach adopted in treating certain diseases, particularly useful for brain tumors, because the blood-brain barrier prevents the therapeutics from reaching the cancerous cells in systemic administration.[57] The small size and neutral surface charge of our NPs are beneficial to achieve large volume distribution in brain tumor tissues during local administration. In addition, protein concentration in the interstitial fluid of solid tumors is lower than in blood, [58] which slows down protein release induced by protein exchange, therefore further prolonging the residence of the nanoformulation in tumor tissue. To test this hypothesis, pHLIP-Alxa750-DOX was used as a model peptide to study the intracranial distribution of peptides encapsulated in PEG^{5k}(ArgArg-L-C17)₄ NPs via intratumoral convection-enhanced delivery (CED)[57, 59] infusion in mice bearing orthotopic tumors of the human glioblastoma cell line U87, that had been transfected with firefly luciferase gene. Mice were imaged 7 days after tumor cell implantation, tumor growth was confirmed after administering D-luciferin intraperitoneally (Fig. 6d). The tumor-bearing mice were sacrificed 48 hours after the completion of a 7-day CED infusion and the brains were harvested. *Ex vivo* imaging of the brain and glioblastoma tumor showed a large volume distribution and significantly improved retention of the peptide when loaded in telodendrimer NPs (Fig. 6e), and no signal of pHLIP-Alxa750-DOX was detected in the healthy organs (Fig. S43). A higher doxorubicin (conjugated on the peptide) concentration at the microscopic level was also observed (Fig. 6f), confirming improved therapeutic retention.

4. Conclusions

In this study, we have developed a novel telodendrimer nanoplatform for effective affinity-controlled encapsulation and delivery of proteins and peptides through the synergistic combination of electrostatic and hydrophobic interactions. The well-defined and modularly tailored structure of the telodendrimer allows for free engineering of its functionalities to optimize protein loading efficiency and to fine-tune the physical properties of the nanocomplex. The optimized telodendrimers form sub-30 nm NPs with efficient cell-penetrating capability and superior protein loading capacities, which can efficiently deliver proteins to xenografted tumors by EPR effect and improve retention of peptides in an orthotopic brain tumor. Our studies support a mechanism of protein encapsulation via protein absorption onto micelles followed by the reassembly of nanocarriers. The

multivalent synergistic combination of charged and hydrophobic moieties in a telodendrimer is novel and critical for stable protein encapsulation and delivery. Sufficient density of charged guanidine groups in the telodendrimers increases the dynamics of polymer micelles and the guanidine groups may serve an approaching function for rapid protein binding. It also enables efficient membrane transport of proteins. The hydrophobic groups in the telodendrimers may serve an annealing function to stabilize the protein binding in aqueous solutions. The structures of the hydrophobic moieties are critical for the protein loading/release behaviors as well as the *in vivo* performance of the protein nanoformulations. Combinatorial library of telodendrimers can be designed based on the structure of a protein guided by computational chemistry, which allows for the fine-tuning of the hydrophobic functional moieties as well as the charge groups to optimize protein loading capacity and stability for efficient *in vivo* protein delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Schematic illustration of telodendrimer structures and protein encapsulation.

Fig. 2.

(a,b) Particle size (a) and zeta potential (b) of telodendrimers before and after BSA loading. The error is for standard deviation ($n = 3$). (c–h) TEM images and particle size analysis for PEG^{5k}(ArgArg-L-C17)₄ (c,f), PEG^{5k}(ArgArg-L-CHO)₄ (d,g), and PEG^{5k}(ArgArg-L-VE)₄ (e,h) NPs before (c–e) and after (f–h) BSA loading (P/T = 1/3, w/w). (i) Proposed process for *in situ* protein encapsulation. (j) Loading ability of telodendrimer NPs for FITC-BSA determined by an agarose gel retention assay. The feed mass ratio is 2/1 (P/T). (k) Calorimetric titration of PEG^{5k}(ArgArg-L-VE)₄ with BSA at 37 °C in PBS. (l,m) Fluorescence emission spectra (λ_{ex} 439 nm) of RB-BSA, FITC-PEG^{5k}(ArgArg-L-C17)₄ and their mixture (l) and the mixture of RB-BSA and FITC-BSA without/with PEG^{5k}(ArgArg-L-C17)₄ (m).

Fig. 3.

(a) Schematic illustration of the association in telodendrimer solution (left) and dissociation in BSA solution (right) for the streptavidin-coated biosensors prewetted with BSA solution. (b) Kinetics for association in PEG^{5k}(ArgArg-L-C17)₄ solution (500 nM) and dissociation in PBS and BSA solutions of different concentrations. (c–e) Kinetics for association in PEG^{5k}(ArgArg-L-C17)₄ (c), PEG^{5k}(ArgArg-L-CHO)₄ (d), and PEG^{5k}(ArgArg-L-VE)₄ (e) solutions (75–600 nM) and dissociation in BSA solutions (40 mg/mL). (f) Summary of BLI results.

Fig. 4.

(a) Average docking energy of different subunits with BSA (average of 100 docking runs). (b) Loading efficiency of telodendrimers for FITC-BSA determined by an agarose gel retention assay. The feed mass ratio is 1/3 (P/T). (c–e) Kinetics for association in telodendrimer solutions and dissociation in BSA solution (40 mg/mL). (f–h) Fluorescence emission spectra of mixture of RB-BSA and FITC-BSA without/with telodendrimers of PEG^{5k}(Arg-L-CHO)₄ (f), PEG^{5k}Arg₄Ac₄ (g), and PEG^{5k}(Arg(Pbf)-L-CHO)₄ (h) in PBS with 439 nm excitation.

Fig. 5.

(a–d) CLSM images of HT-29 cells incubated at 37 °C for 3 h with free FITC-BSA (a), and FITC-BSA loaded in PEG^{5k}(ArgArg-L-HF)₄ (b), PEG^{5k}(LysLys-L-HF)₄ (c), and PEG^{5k}(Arg-L-HF)₄ (d) NPs (P/T = 1/3, w/w). The cell nuclei were stained with DAPI (blue). (e) Loading ability of telodendrimer NPs for GFP determined by an agarose gel retention assay. The feed mass ratio is 1/3 (P/T). (f) Cell viability assay on U87 cells after a 72 h continuous incubation at 37 °C for free DT₃₉₀, and DT₃₉₀-loaded telodendrimer NPs. The error is for standard deviation (*n* = 3). Inset in (f) is a CLSM image of a representative U87 cell incubated at 37 °C for 3 h with RB-BSA-loaded PEG^{5k}(ArgArg-L-VE)₄ NPs. The cell nuclear and endosome/lysosome were stained with DAPI (blue) and LysoTracker (green), respectively.

Fig. 6.

(a–c) Systemic delivery. (a,b) *In vivo* (a) and *ex vivo* (b) animal images of the HT-29 colon cancer bearing nude mice xenograft models (#A1 and #A2) after tail vein injection of free Cy5-insulin, and Cy5-insulin-loaded PEG^{5k}(ArgArg-L-VE)₄ NPs at a loading ratio of 1/5 (P/T, w/w). The black circles in (a) indicate the tumor sites. (c) Quantitative analysis of the *ex vivo* tumor and organ uptake. (d–f) Local delivery. (d) *In vivo* bioluminescence images for brain tumor tracking in the mice (#B1 and #B2) injected with intracranial U87 tumors taken using the Photon Imager system at day 7 post injection. (e,f) Distributions in

intracranial U87 tumors determined by *ex vivo* imaging (e) and at the cellular level determined by microscopy (f) at 48 h after CED of free pHLIP-Alexa750-DOX, and pHLIP-Alexa750-DOX-loaded telodendrimer NPs at a loading ratio of 1/5 (peptide/telodendrimer, w/w). The cell nuclei in (f) were stained with DAPI (blue).

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Table 1

Characterization and molecular properties of the telodendrimers.

telodendrimer	Theo. M_w^a	M_p by MS^b	PDI by MS^b	formula by NMR ^c	CMC (μM) ^d
PEG ^{5k} NH ₂	N/A	5,100	1.01	N/A	N/A
PEG ^{5k} (Arg-L-C17) ₄	8,040	8,053	1.01	PEG ^{5k} (Arg-L-C17) _{3,8}	2.68
PEG ^{5k} (Arg-L-CHO) ₄	8,681	8,237	1.01	PEG ^{5k} (Arg-L-CHO) _{3,0}	1.32
PEG ^{5k} (Arg-L-VE) ₄	9,081	8,734	1.01	PEG ^{5k} (Arg-L-VE) _{3,2}	1.35
PEG ^{5k} (ArgArg-L-C17) ₄	8,665	8,667	1.01	PEG ^{5k} (ArgArg-L-C17) _{3,7}	1.45
PEG ^{5k} (ArgArg-L-CHO) ₄	9,306	9,310	1.01	PEG ^{5k} (ArgArg-L-CHO) _{3,5}	1.38
PEG ^{5k} (ArgArg-L-VE) ₄	9,706	9,729	1.01	PEG ^{5k} (ArgArg-L-VE) _{3,7}	1.14
PEG ^{5k} (LysLys-L-C17) ₄	8,457	8,184	1.01	PEG ^{5k} (LysLys-L-C17) _{3,7}	1.47
PEG ^{5k} (LysLys-L-CHO) ₄	9,098	8,473	1.01	PEG ^{5k} (LysLys-L-CHO) _{3,0}	1.38
PEG ^{5k} (LysLys-L-VE) ₄	9,498	8,892	1.01	PEG ^{5k} (LysLys-L-VE) _{3,5}	1.62
PEG ^{5k} (OAOA-L-C17) ₄	9,017	N/D	N/D	PEG ^{5k} (OAOA-L-C17) _{4,0}	1.66
PEG ^{5k} (OAOA-L-CHO) ₄	9,658	N/D	N/D	PEG ^{5k} (OAOA-L-CHO) _{3,5}	1.52

^aTheoretical molecular weight.

^bPeak molecular weight (M_p) and polydispersity index (PDI) acquired by MALDI-TOF MS analysis.

^cThe values of the subscripts in the molecular formulae are calculated by based on the average integration ratio of the peaks of the methyl protons (~0.84, ~0.65 and ~0.83 ppm for C17, CHO and VE, respectively) and the methoxy proton of PEG at ~3.24 ppm in 1H NMR spectra in DMSO-*d*₆.

^dMeasured by fluorescent method using Nile Red dye as a probe. N/A: not applicable. N/D: not detectable.

Table 2

Zeta potential and particle size of the telodendrimers before and after loading of proteins.

telodendrimer	zeta potential (mV) ^a	D_h (nm) ^a	zeta potential (mV) with protein ^b	D_h (nm) with protein ^b	D_h (nm) with protein after storage ^c
PEG ^{5k} (Arg-L-C17) ₄	-4.6±0.7	11±3	-5.1±0.7	10±4	11±3
PEG ^{5k} (Arg-L-CHO) ₄	-4.1±0.5	27±8	-4.3±0.3	22±7	15±5
PEG ^{5k} (Arg-L-VE) ₄	-3.2±1.2	25±9	-4.4±0.3	19±9	22±8
PEG ^{5k} (ArgArg-L-C17) ₄	-2.4±0.6	17±4	-3.4±0.3	13±4	14±4
PEG ^{5k} (ArgArg-L-CHO) ₄	-4.0±0.1	18±4	-4.4±0.1	12±4	15±5
PEG ^{5k} (ArgArg-L-VE) ₄	-1.2±0.8	32±13	-3.0±0.6	18±8	27±9

^a Obtained in PBS at a concentration of 1 mg/mL.

^b Obtained in PBS at a telodendrimer concentration of 1 mg/mL with a BSA/telodendrimer ratio of 1/3 by weight.

^c Obtained in PBS at a telodendrimer concentration of 1 mg/mL with a BSA/telodendrimer ratio of 1/3 by weight after a storage of 2 months. For the zeta potential data, the value behind '±' is the standard deviation in multiple measurements. For the DLS data, the value behind '±' describes the half-peak width of the size distribution.