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In vivo Evaluation of Site-specifically PEGylated Chemically Self-assembled Protein Nanostructures

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Abstract

Chemically Self-Assembled Nanorings (CSANs) are made of dihydrofolate reductase (DHFR) fusion proteins and have been successfully used *in vitro* for cellular cargo delivery and cell surface engineering applications. However, CSANs have yet to be evaluated for their *in vivo* stability, circulation and tissue distribution. In an effort to evaluate CSANs *in vivo*, we engineered a site-specifically PEGylated epidermal growth factor receptor (EGFR) targeting DHFR molecules, characterized their self-assembly in to CSANs with bivalent methotrexates (bis-MTX), visualized their *in vivo* tissue localization by microPET/CT imaging and determined their *ex vivo* organ biodistribution by tissue-based gamma counting. A dimeric DHFR (DHFR²) molecule fused with a C-terminal EGFR targeting peptide (LARLLT) was engineered to incorporate a site-specific ketone functionality using unnatural amino-acid mutagenesis. Aminoxy-PEG, of differing chain lengths, was successfully conjugated to the protein using oxime chemistry. These proteins were self-assembled into CSANs with bis-MTX DHFR dimerizers and characterized by size exclusion chromatography and dynamic light scattering. *In vitro* binding studies were performed with fluorescent CSANs assembled using bis-MTX-FITC, while *in vivo* microPET/CT imaging was performed with radiolabeled CSANs assembled using bis-MTX-DOTA [⁶⁴Cu]. PEGylation reduced the uptake of anti-EGFR CSANs by mouse macrophages (RAW 264.7) up to 40% without altering the CSAN's binding affinity towards U-87 MG glioblastoma cells *in vitro*. A significant time dependent tumor accumulation of ⁶⁴Cu labeled anti-EGFR-CSANs was observed by microPET/CT imaging and biodistribution studies in mice bearing U-87 MG xenografts. PEGylated CSANs demonstrated a reduced uptake by the liver, kidneys and spleen resulting in high contrast tumor imaging within an hour of intravenous injection (9.6% ID/g), and continued to increase up to 24 h (11.7% ID/g) while the background signal diminished. CSANs displayed an *in vivo* profile between that of rapidly clearing small molecules and slow clearing antibodies. Thus CSANs offer a modular, programmable and stable protein based platform that can be used for *in vivo* drug delivery and imaging applications.

TOC image

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Introduction

Tumor targeted therapeutic nanoparticles have shown great promise as diagnostic agents, drug delivery vehicles and scaffolds for engineering cell-cell interactions in cancer therapy.^{1–3} To prepare them a variety of synthetic materials, such as polymers and semiconductor metals, and natural materials, such as nucleic acids, polysaccharides, proteins and lipids, have been investigated. Compared to other biomolecules, proteins offer a number of advantages since they display a diversity of functional groups and can be genetically encoded and produced by recombinant DNA technology.⁴ Viral particles, for example, have been successfully used for drug delivery, molecular imaging and immunomodulation.⁵ Expanding on this foundation, the *de novo* design of nanoparticles by either protein-protein interface engineering⁶ or chemically induced dimerization has become an emerging area of interest.⁷ Nevertheless, despite their many potential therapeutic applications, characterization of the *in vivo* stability and behavior of designed protein based nanostructures is still in its infancy.

Our lab has developed a method for engineering highly tunable chemically self-assembled nanorings (CSANs).⁸ By utilizing the principles of protein engineering and knowledge of the strong binding affinity of methotrexate for the enzyme dihydrofolate reductase (DHFR), we have demonstrated that two recombinantly fused DHFRs (DHFR²) can be chemically self-assembled using bivalent methotrexate (bis-MTX) to form robust protein nanorings (Fig. 1).^{8,9} The size of the CSANs has been found to be dependent on the length and composition of the linker between the two DHFRs and the affinity of MTX. When the linker is a single glycine (1DHFR²) predominantly macrocyclic octamers are formed, whereas primarily dimeric CSANs (13DHFR²) are formed with the 13 amino acid linker GLGGGGGLVPRGT. Due to the high binding affinity of MTX and induced protein-protein interactions, the formation of CSANs is characterized by high effective molarities, resulting in high nanoring stability.^{8,7} Unlike other protein nanostructures based solely on protein-protein interface engineering, CSANs can undergo pharmacologic disassembly in the presence of clinically relevant concentrations of trimethoprim, the non-toxic and FDA approved DHFR inhibitor.¹⁰

Recently, we have demonstrated temporal control over the assembly and disassembly of CSANs, which can serve as a platform for the multivalent display of single chain antibodies

(scFv) and peptides, thus also controlling avidity.^{10, 11} CSANs have been successfully used to target CD3 T-cell co-receptors on T-leukemia cells as well as $\alpha_v\beta_3$ integrins, and epithelial cell adhesion molecules (EpCAM) on breast cancer cells.^{12,13, 14} They have also been shown to deliver oligonucleotides and proteins to cancer cells *in vitro*.^{14, 15,16} In addition, CSANs have been employed for the programming of reversible cell-cell interactions.¹³ Further CSANs can be used to construct Prosthetic Antigen Receptors (PARs), a novel non-genetic alternative approach to Chimeric Antigen Receptors (CAR T cell therapy).¹⁷ Despite the immense therapeutic potential, the *in vivo* behavior of CSANs has yet to be defined. Consequently, we have chosen to examine the *in vitro* and *in vivo* stability, delivery, biodistribution and tumor targeting capability of CSANs. Due to high sensitivity of Positron emission tomography (PET), we chose to monitor the *in vivo* behavior of the CSANs using microPET/CT imaging.

Experimental Section

p-Bn-DOTA-SCN was purchased from Macrocyclics. ⁶⁴CuCl₂ was obtained from Department of Medical Physics, University of Wisconsin – Madison, WI, USA. The unnatural amino acid *p*-acetyl phenylalanine was synthesized and characterized as described previously with a minor modification to the final deprotection, refluxing overnight with 6N HCl.¹⁸ The BL21 (DE3) competent cells were purchased from Invitrogen™. Aminoxy-PEG-10 kDa and aminoxy-PEG-20 kDa were purchased from NOF America Corporation. All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO) and used without further purification. Amicon® stirred cells with YM-30 ultrafiltration discs (NMWL 30 kDa) were used in all protein purifications and buffer exchange procedures. All purified proteins were stored in phosphate buffer with 10 % glycerol at –80 °C until used in experiments. RAW246.7 mouse blood macrophage and U87 MG human glioblastoma cell lines were obtained from American Type Culture Collection (ATCC, Rockville MD).

Synthesis of bis-MTX-DOTA

A solution of previously reported DHFR dimerizer,¹² bisMTX-NH₂ (14.3 mg, 12 μ mol) in 0.1 M sodium bicarbonate buffer (1.5 mL, pH 8.5) was added to S-2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (*p*-Bn-DOTA-SCN, 12.4 mg, 18 μ mol) and stirred overnight (18 h) at room temperature. The resulting reaction mixture was loaded on to a Celite® 545 cartridge and purified by reverse phase chromatography on a RediSep Rf-C18 column (43 g) using a Combiflash Rf-200 system (mobile phase gradient 2% – 50 % acetonitrile/0.1% trifluoroacetic acid in water/0.1% trifluoroacetic acid) to obtain the final product, bis-MTX-DOTA as a lyophilized powder (21 mg, 98%). The product was characterized by ¹HNMR and LC-MS. ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.688 (s, 2H), 8.288 (m, 2H), 7.827 (m, 2H), 7.74 (d, *J* = 8 Hz, 4H), 7.408 (m, 2H), 7.211 (m, 2H), 7.194 (m, 2H), 6.827 (d, *J* = 8 Hz, 4H), 4.866 (s, 4H), 4.299 (m, 2H), 3.971 (m, 3H), 3.7 – 3.5 (overlapping m, 12H), 3.240 (s, 6H), 3.1 – 3.0 (overlapping m, 20H), 2.640 (s, 2H); 2.367 (m, 6H), 2.188 (m, 6H), 2.090 (m, 6H), 2.053 (m, 4H), 1.916 (m, 4H), 1.594 (m, 4H), 1.400 (m, 4H), 1.248 (m, 4H), 1.151 (m, 4H). ESI-MS calcd. for C₈₀H₁₁₂N₂₅O₁₈S [M+H]⁺ 1742.8, found 1742.8.

Site-specific PEGylation of DHFR² proteins

Unnatural amino acid, *p*-acetyl phenylalanine incorporated (at M161) anti-EGFR-1DHFR² or 1DHFR² (18–22 μ M) was incubated with 5 – 10 mM PEG-ONH₂ (10 or 20 kDa) at room temperature in phosphate buffer (0.1 M, pH 7.0). The progress of the PEGylation reaction was monitored in a time-dependent manner using SDS-PAGE analysis. More than 95% of the labeling was achieved in approximately 52 hours across all the constructs. The PEGylated proteins were then purified by DEAE anion exchange chromatography. Single PEG labeling of the protein was confirmed by MALDI mass spectroscopy for both 10 and 20 kDa PEG.

Self-assembly and characterization of CSANs

CSANs were prepared by incubating monomeric proteins (5–26 μ M) with 1–3 equivalents of bis-MTX, bis-MTX-DOTA, bis-MTX-DOTA-Cu or bis-MTX-FITC in P500 buffer (50 mM Potassium phosphate, 0.5 M NaCl, 1mM EDTA, pH 7.0) for 30 minutes. Unreacted small molecules were removed by filtration through Amicon centrifugational filter devices [30 kDa MWCO at 12000 rpm for 3 min ($\times$ 3 times)]. All proteins were analyzed by size exclusion chromatography (SEC) by passing through a Superdex G200 column connected to a Beckman Coulter HPLC equipped with a diode array detector using P500 buffer as the mobile phase. The elution was monitored at 280 nm and the relevant protein peak was collected for the DLS analysis. DLS measurements were performed at room temperature (1 mg mL⁻¹ in PBS) on a Brookhaven 90Plus Particle Analyzer (Holtzville, NY) with a 35 mW red diode laser.

In vitro cell binding analysis by flow cytometry

U-87 MG cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 U mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin, and L-glutamine at 37°C in 5% CO₂. Protein samples (5–26 μ M) were incubated with 1.1 equivalent of bis-MTX-FITC in P500 (50 mM Potassium phosphate, 0.5 M NaCl, 1mM EDTA pH 7.0 filtered with 0.22 μ m filter) for 1 hour at room temperature to generate the following four constructs: CSANS, anti-EGFR-CSANs, anti-EGFR-PEG10k-CSANs, and anti-EGFR-PEG20k-CSANs. All four CSANs (1 μ M) were incubated with 1 million EGFR positive U-87 MG cells at 37 °C for 1 hour and subsequently washed three times with PBS. Samples were filtered to remove any cell aggregate prior to analysis by flow cytometry using a BD LSR II Flow cytometer.

Determination of CSAN uptake by Raw 264.7 cells

RAW246.7 mouse blood macrophage cells were cultured in DMEM media supplemented with 10% FBS, 100 U mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin, and L-glutamine at 37°C in 5% CO₂. As previously described, CSANS, anti-EGFR-CSANs, anti-EGFR-PEG10k-CSANs, and anti-EGFR-PEG20k-CSANs constructs were generated using bis-MTX-FITC. Each CSAN construct (1 μ M) was then incubated with 5 million RAW246.7 mouse macrophage cells at 37 °C for 24 hours in a 24 well plate. Macrophage cells were gently scraped off of plates following allotted incubation times and washed 3 times with PBS. The amount of non-specific binding of CSANs to macrophages was quantified using a BD LSR

II flow cytometer to monitor FITC absorbance. The percent uptake was normalized to anti-EGFR-CSANs at 100%.

Preparation of ^{64}Cu labeled CSANs

^{64}Cu Labeling of bis-MTX-DOTA was performed by addition of approximately 50 μL of bis-MTX-DOTA to roughly 10 $\text{mCi}/\mu\text{mol}$ of $^{64}\text{CuCl}_2$ neutralized in 1M sodium acetate buffer (pH 7.0). The reaction mixture was incubated for 30 minutes at 50° C, following which the extent of ^{64}Cu chelation was determined by iTLC. The resulting excess of bis-MTX-DOTA[^{64}Cu] was incubated with 0.5 mg of anti-EGFR-1DHFR², anti-EGFR-1DHFR²-PEG10k, or anti-EGFR-1DHFR²-PEG20k at room temperature for 30 minutes to prepare each respective CSAN construct. Excess ^{64}Cu was bound by addition of 10 mM ethylenediaminetetraacetic acid for 10 minutes and removed, along with excess bis-MTX-DOTA[^{64}Cu], by Bio-spin 6 Tris column. The extent of labeling was once again determined by iTLC and found to be greater than 90% for all three constructs. The specific activity for each construct was approximately 1.75 $\text{mCi}/\mu\text{mol}$.

Small animal PET imaging and evaluation of tissue biodistribution

All *in vivo* animal experiments were performed under a protocol approved by the University of Minnesota Institutional Animal Care and Use Committee in accordance with both federal and institutional regulations. Six weeks old female athymic nude mice (Harlan Sprague Dawley Inc.) were irradiated with 300 cGy to fully suppress any remaining immune activity and following 24 hours were subcutaneously injected with 3 million U-87 MG cells into the right flank. Two weeks following xenograft implant the tumors were roughly 5 to 10 mm in diameter. Mice were intravenously injected with 50 to 170 $\mu\text{Ci}/\mu\text{mol}$ of anti-EGFR-CSANs, anti-EGFR-PEG10k-CSANs, anti-EGFR-PEG20k-CSANs and non-targeted PEG20k-CSANs by tail vein (n = 3), then anesthetized with isoflurane at 4% induction and 1.5% maintenance in oxygen at 1 L min^{-1} flow rate. Static scans were performed at 1, 4 and 24 hours post injection with 15, 30, and 45 minute scan times respectively using a micro-PET rodent scanner (Siemens Inveon preclinical microPET/CT). PET/computed tomographic (CT) co-registered images were acquired by immobilizing the anesthetized mouse on the micro-PET imaging platform for each static PET scan followed by a 5 minute with the micro-CT unit (40 micron resolution). Images were co-registered by a modified Feldkamp algorithm and signals in both the tumor and hind leg muscle were quantified utilizing regions of interest (ROI).

Mice utilized for biodistribution studies were given xenograft tumors identically to those implanted for PET studies. Mice were injected intravenously by tail vein with 170 to 200 $\mu\text{Ci}/\mu\text{mol}$ of anti-EGFR-CSANs, anti-EGFR-PEG10k-CSANs, and anti-EGFR-PEG20k-CSANs and sacrificed at 24 and 48 hours post injection (n =3 for all construct at 24 hours, n =3 for all constructs at 48 hours). The blood, liver, spleen, pancreas, heart, kidney, lung, bone, brain, and tumor were collected and weighed prior to measurement with a gamma ray counter. Decay correction was performed to allow for injected dose per gram of tissue calculation.

Results and Discussion

Synthesis of bis-MTX-DOTA [^{64}Cu]

In order to site specifically and quantitatively radiolabel CSANs, we synthesized a novel DHFR² protein dimerizer by conjugating bis-MTX to the metal chelator, DOTA (Scheme 1). Previously we reported the synthesis of a versatile DHFR² dimerizer with an amine terminus (Bis-MTX-NH₂), which can be conjugated to a variety of amine reactive payloads.¹² In the current study we conjugated *p*-SCN-Bn-DOTA metal chelator to bis-MTX-NH₂ under facile aqueous conditions. The resulting bis-MTX-DOTA was purified by reverse phase flash chromatography and isolated by lyophilization. The LCMS analysis of the product revealed a single peak with the corresponding mass ($[\text{MH}^+] = m/z$ 1742.8) for bis-MTX-DOTA (Fig. S1). Having the metal chelator conjugated to bis-MTX facilitates the chelation of metal radionuclides to the protein dimerizer prior to using it in the self-assembly reaction. This strategy provides a unique non-covalent protein labeling method that avoids non-specific binding of metal ions to proteins. Hence, high radiochemical purities (> 90%) can be achieved for the resulting radiolabeled product.

Due to its moderately long half life we chose ^{64}Cu ($T_{1/2} = 12.7$ hours, β^+ , 0.653 MeV [17.8 %]) as our positron emitter to evaluate the *in vivo* fate and tumor targeting ability of CSANs by PET.¹⁹ The copper binding ability of bis-MTX-DOTA was evaluated with non-radioactive copper as a surrogate for ^{64}Cu . Bis-MTX-DOTA was titrated with CuCl_2 and found to be completely chelated with 5 eq. of Cu^{2+} in three hours at room temperature as determined by HPLC-MS (Fig. S2). After confirming the rapid Cu chelation ability of bis-MTX-DOTA, we followed the general ^{64}Cu labeling conditions from the literature and synthesized the radiolabeled DHFR² dimerizer.²⁰ Bis-MTX-DOTA was incubated with $^{64}\text{CuCl}_2$ in 1M sodium acetate buffer (pH 7.0) at 50° C for 30 minutes to obtain the completely (> 99%) radiolabeled bis-MTX-DOTA[^{64}Cu] as determined by iTLC.

Preparation of anti-EGFR-CSANs

To date we have demonstrated *in vitro* targeting of various cell surface receptors overexpressed on malignant cancer cells with targeted CSANs.^{11, 13, 17} In order to evaluate the tissue distribution and targeting of CSANs *in vivo*, we selected human U-87 MG glioblastoma for a representative mouse xenograft model. Glioblastoma is the most common primary human brain cancer and remains one of the most difficult cancers to treat with current strategies.²¹ About 60% of the primary glioblastomas are characterized with amplified EGFR expression and, recent epidemiological studies have revealed that the EGFR overexpression is of significant prognostic value for predicting survival of glioblastoma patients.²² EGFR is also known to be over-expressed on a variety of other solid cancers such as breast, head and neck, prostate and colorectal cancers.²³ Currently a number of small molecule inhibitors, as well as antibodies that block EGFR activation, are at different stages of preclinical and clinical development.^{24,25} EGFRs are a class of tyrosine kinase receptors that are key modulators of proliferation and death in both normal and malignant cells. Upon native ligand (epidermal growth factor, EGF) binding, the receptors form homo/heterodimers triggering intracellular signaling pathways, which lead to cell survival and proliferation, and subsequently undergo clathrin-mediated endocytosis and

receptor recycling.²⁶ This rapid internalization of the EGFR receptor has been used effectively in drug delivery applications.²⁷ Therefore, as a proof of concept, we designed and constructed anti-EGFR-CSANs to demonstrate their potential in receptor targeted cancer imaging and therapy using EGFR positive U-87 MG mouse xenografts.

Recently, a hexameric peptide (LARLLT) was identified during a campaign to discover EGFR binders by computer-aided design (CAD) and, was successfully shown to target EGFR positive human non-small-cell lung tumors in mice.^{28,29} Hence, we chose to utilize this short targeting peptide with moderate binding affinity by fusing it to the DHFR² protein. A plasmid encoding DHFR² was modified via site directed mutagenesis (QuikChange® Site-Directed Mutagenesis Kit, Stratagene) to express a protein consisting of two *Escherichia coli* DHFR proteins separated by a single glycine, followed by a C-terminal linker (GGSGG) that connects the EGFR targeting peptide, LARLLT (Fig. 2A). The protein was expressed in *E. coli* as a soluble protein using our standard DHFR² expression protocol and purified by methotrexate affinity column chromatography followed by DEAE anion exchange chromatography.¹⁰ The typical isolated yield for the purified anti-EGFR-1DHFR² was found to be around 15–20 mg L⁻¹ of culture.

Self-assembly of the EGFR targeted fusion proteins in to CSANs was achieved by incubating the protein with 1.1 equivalents of bis-MTX or bis-MTX-DOTA for 1 h at room temperature.¹¹ The analysis of the resulting CSANs by SEC confirmed the completion of the self-assembly reaction. The monomeric protein that normally elutes at 33 minutes from the SEC column (Superdex G200, GE Healthcare) disappeared and the peak corresponding to the higher ordered anti-EGFR-CSANs eluted around 20 minutes (Fig. 3). The size of the CSANs were determined by Dynamic Light Scattering (DLS) and found to increase from 4.5 ± 0.5 nm to 15.4 ± 0.8 nm upon oligomerization (Table 1) and was consistent with previously reported values.⁸ Having confirmed the self-assembly of anti-EGFR CSANs we then advanced anti-EGFR-1DHFR² as a programmable platform for *in vivo* tumor targeting (Fig. 4).

Preparation of PEGylated anti-EGFR CSANs

One of the major obstacles in the advancement of targeted delivery of nanoparticles is their rapid clearance from the circulation through hepatic and renal clearance mechanisms.^{30,31} Typically, once injected, circulating nanoparticles are rapidly recognized and taken up by phagocytic cells such as macrophages and Kupffer cells, which comprise the reticuloendothelial system (RES). The RES serves to filter the blood for particles and dead cells, and largely reside within the spleen and liver, thus significantly reducing the delivery of nanoparticles to the target tissue or the tumor.³¹ Renal clearance of the nanoparticles is also a major contributing factor for the rapid loss of the therapeutic or diagnostic nanoparticles from the circulation.³² The resulting elimination of the nanoparticles from the circulation reduces the efficiency of the delivery of the payload to the target tumor site. Indeed, when we performed preliminary *in vivo* biodistribution experiments with radiolabeled CSANs, we observed rapid clearance and, high accumulation of CSANs in excretory organs such as liver, spleen and kidneys in accordance with the observations for other types of nanoparticles (data not shown).

Various strategies have been developed to extend the circulation half-life of therapeutic proteins by incorporating PEGs or PEG mimetics, covalent or non-covalent linking of albumin, and, by fusion to the Fc region of IgG.³³ PEGylation of nanoparticles has been developed to mask nanoparticles thus reducing their recognition by the RES and improving their stealth delivery to the target tissue.³⁴ Further, PEGylation of biotherapeutics has been shown to increase their hydrodynamic volume as well as their circulation half-life.³³ Therefore, we decided to use PEGylation as our first method to overcome the challenges associated with rapid clearance as well as potential immunogenicity.³⁵ Our initial studies revealed that non-specific PEGylation of DHFR² monomers prevented their assembly into CSANs (data not shown). Furthermore, non-specific conjugation resulted in various heterogeneous mixtures of formulations that have proven to be ineffective due to their poor *in vivo* pharmacokinetic and biodistribution properties.^{36,37} To address these issues we chose to prepare site specifically PEGylated anti-EGFR-CSANs. While PEGylation of C-terminal cysteine incorporated DHFR² monomers with maleimido-PEG2k could be self-assembled into CSANs, the PEGylation reaction was incomplete with maximum yields ranging from 60–65%. Our attempts to improve the conjugation reaction proved futile and isolation of the PEGylated product from the unreacted starting material was highly inefficient. (data not shown).

To obtain quantitatively PEGylated DHFR² proteins, we introduced a functional non-natural amino acid into the 1DHFR² via site-directed mutagenesis of the gene encoding target protein (Fig. 2B). The residue Met-161 of anti-EGFR-1DHFR², which is the first residue of the second DHFR unit, was chosen due to its solvent accessibility and distant location from the active site to avoid perturbations on its functional activity. The methionine (161) was replaced with p-acetyl-L-phenylalanine (pAcF) using an orthogonal suppressor aminoacyl-tRNA synthetase/tRNA pair (pEVOL/pAcF) developed by the Schultz laboratory.³⁸ The selection of pAcF was based on its reactivity towards aminoxy groups to form stable oxime linkages that can be used to install additional functionality to the protein of interest.^{39,40,41,42} The mutant protein was expressed in *E. coli* as a soluble protein, and purified using methotrexate affinity column chromatography followed by ion exchange chromatography to obtain the pure protein with an isolated yield of 15 mg L⁻¹. A single band corresponding to the pure protein was observed by SDS-PAGE analysis (Fig. S4). Electrospray ionization mass spectrometry (ESI-MS) revealed a single peak corresponding to the mass of the anti-EGFR-1DHFR²-pAcF (m/z calculated: 37805; observed: 37807.5) and confirmed a > 99% incorporation efficiency for pAcF at Met-161 (Fig. S3–A).

We then prepared protein constructs with increasing hydrodynamic volume by chemically conjugating the anti-EGFR-1DHFR²-pAcF to linear aminoxy-PEGs differing in molecular weight (10 and 20 kDa; Fig. 2C). anti-EGFR-1DHFR²-pAcF or 1DHFR²-pAcF proteins (18–22 μ M) were incubated with an excess of (10 mM) amino-oxy PEG. The progress of the reaction was monitored in a time-dependent manner by removing aliquots of the reaction mixture and preparing SDS-PAGE sample for the analysis. More than 95% of the labeling was achieved within 52 hours of reaction across the constructs. Next, the reaction mixture was loaded onto a DEAE column to remove the unreacted PEG. After un-retained excess PEG was washed off the column, the protein was eluted using a gradient elution buffer as described in the supplemental material. Site-specific conjugation was confirmed by MALDI-

TOF-MS to characterize anti-EGFR-1DHFR²-PEG10k (m/z calculated: 48345; observed: 48347.7, Fig. S3–B) and anti-EGFR-1DHFR²-PEG20k (m/z calculated: 58445; observed: 58447, Fig. S3–C). The PEGylation efficiency was found to be greater than 98% as determined by SDS-PAGE analysis (Fig. S4–C). Similarly, PEGylated versions of non-targeted control protein constructs (1DHFR²-PEG10k and 1DHFR²-PEG20k) were also prepared from 1DHFR²-pAcF and characterized by mass spectroscopy (Fig. S3–E, F).

PEGylated-EGFR targeted and PEGylated-non-targeted 1DHFR² monomeric proteins were self-assembled with bis-MTX-DOTA to form corresponding CSANs and characterized by SEC. Upon incubation of anti-EGFR-1DHFR²-PEG10k and anti-EGFR-1DHFR²-PEG20k with bis-MTX-DOTA, a peak corresponding to a higher order oligomeric structures with increased hydrodynamic diameter was observed eluting within the void volume around 15 min when compared to the monomer which eluted at 26 and 24 minutes for anti-EGFR-1DHFR²-PEG10k and anti-EGFR-1DHFR²-PEG20k (Fig. 5). These results are consistent with our previously observed oligomerization studies of 1DHFR² with bis-MTX.⁸ Moreover, the successive decrease in the retention time on the SEC profile with increasing size of PEG is indicative of the increase in hydrodynamic diameter of PEGylated proteins.

DLS studies were carried out to analyze the size distribution of CSANs prepared with 10 and 20 kDa PEGylated CSANs prepared with bis-MTX (Table 1). The average diameters of monomeric anti-EGFR-1DHFR²-PEG10k and anti-EGFR-1DHFR²-PEG20k proteins were found to be 7.5 ± 0.1 and 9.0 ± 0.5 nm, respectively, a clear increase in average diameter for the non-PEGylated anti-EGFR-1DHFR², which was found to be 4.5 ± 0.5 nm. Similarly, the average diameter of the corresponding non-PEGylated anti-EGFR-CSANs increased from 15.4 ± 0.8 to 16.8 ± 0.5 nm when modified with 10 kDa PEG and 19.6 ± 0.3 nm when modified with 20 kDa PEG. The relatively narrow size distribution of PEGylated and non-PEGylated CSANs observed in DLS studies indicates a relatively homogenous composition of the nanostructures with relatively no aggregate formation (as indicated by no visible particle size formation beyond 100 nm size) (Fig. 6). Similarly, we did not observe significant changes in the average hydrodynamic diameters of the CSANs prepared with bis-MTX-DOTA and EGFR targeted proteins (Fig. S5), thus the appended DOTA chelator has negligible impact on the formation and the size of the CSAN.

In vitro modeling of RES uptake with mouse macrophages

Based on literature data on *in vivo* nanoparticle clearance, a protein based nanostructure with an average hydrodynamic diameter of 15–20 nm should in principle avoid renal filtration and escape the renal clearance.³⁰ On the other hand, a site-specifically PEGylated protein nanostructure with a near homogeneous composition should camouflage the nanostructure and its payload due to the extensive hydration of the PEG and temporarily escape RES recognition.⁴³ An assessment of nanoparticle uptake by macrophages can be a useful method to determine the potential phagocytosis of nanoparticles by the RES.^{44,45} Therefore, we prepared non-PEGylated and PEGylated (10 and 20 kDa) anti-EGFR-CSANs with the fluorescent chemical dimerizer, bis-MTX-FITC for *in vitro* model experiments.¹² The ability of mice macrophages (RAW264.7 cells) to internalize fluorescently labeled CSANs was evaluated during a 24-hour period at 37 °C by flow cytometry. The percentage

(%) uptake was normalized to non-PEGylated anti-EGFR-CSANs at 100% (Fig. 7). A statistically significant decrease in the macrophage uptake by 31% and 47 % was observed upon increasing the size of PEG to 10 and 20 kDa. Thus PEGylation successfully decreased the uptake of CSANs by macrophages. The significant decrease in the *in vitro* macrophage uptake of PEGylated CSANs (10 and 20 kDa PEG) indicated increased shielding of the oligomers from macrophage uptake in the absence of other contributing factors, such as opsonins or antibodies contained in serum.

Characterization of anti-EGFR-CSANs binding

Anti-EGFR-CSANs were shown to specifically bind to the EGFR receptor of interest by use of a cell surface receptor depletion assay. U-87 MG cells were pre-incubated with increasing amounts (0 – 4 μ M) of anti-EGFR-CSANs (prepared with anti-EGFR-1DHFR² and bis-MTX) for 1 h at 37° C followed by treatment with a high affinity phycoerythrin labeled anti-human EGFR monoclonal antibody at a saturating concentration (5 μ M). Cells were subsequently analyzed by flow cytometry to determine the level of monoclonal antibody binding. We were able to observe a 50% decrease in the amount of monoclonal antibody bound to U-87 MG cells, since EGFR undergoes receptor-mediated endocytosis when bound to anti-EGFR-CSANs. These results are consistent with specific binding of the anti-EGFR-CSANs to cell surface expressed EGFR (Fig. S7).

Furthermore, the binding ability of the non-PEGylated and PEGylated anti-EGFR-CSANs with EGFR positive human U-87 MG cells was compared using fluorescent anti-EGFR-CSANs. Cells were incubated with anti-EGFR-CSANs prepared with bis-MTX-FITC at 37° C for 2 h, and the cell binding was quantified. In contrast to non-targeted CSANs, which demonstrated no U-87 MG cell binding, both PEGylated and non-PEGylated anti-EGFR-CSANs were found to bind preferably to U-87 MG cells demonstrating that the binding is mediated by the LARLLT peptide. Additionally both the PEGylated and non-PEGylated anti-EGFR-CSAN constructs bound U-87 MG cells to a similar extent indicating that target cell binding was not hindered by PEGylation. (Fig. 8, Fig. S6).

Biodistribution of ⁶⁴Cu labeled CSANs

To characterize the *in vivo* biodistribution of the anti-EGFR-CSANs, three monomeric constructs were selected for radiolabeling self-assembly. Monomeric anti-EGFR-1DHFR², anti-EGFR-1DHFR²-PEG10k and anti-EGFR-1DHFR²-PEG20k were incubated with three equivalents of bis-MTX-DOTA[⁶⁴Cu] at room temperature to form the relevant ⁶⁴Cu labeled CSAN, followed by purification with a Bio-Spin6 size exclusion column. The labeling efficiencies of the ⁶⁴Cu-CSANs were determined by iTLC and found to be greater than 93%.

Athymic nude mice (n = 3) bearing EGFR overexpressing U-87 MG glioblastoma xenografts were intravenously injected through the tail vein with doses of 170 to 200 μ Ci/ μ mol of each purified ⁶⁴Cu-anti-EGFR-CSANs, ⁶⁴Cu-anti-EGFR-PEG10k-CSANs and ⁶⁴Cu-anti-EGFR-PEG20k-CSANs. The mice were euthanized at 24 h p.i. or 48 h p.i. and organs were harvested and counted for the amount of residual radioactivity using a single-tube well counter. At 24 h, mice treated with non-PEGylated ⁶⁴Cu-anti-EGFR-CSANs showed a significant accumulation of activity in the liver (34.0 ± 6.7 % ID/g), the

spleen (9.4 ± 1.8 % ID/g) and the kidneys (24.4 ± 2.7 % ID/g) while relatively low activity was observed in the tumor (4.9 ± 0.7 % ID/g) and blood (1.1 ± 0.1 % ID/g) (Fig. 9). Mice treated with ^{64}Cu -anti-EGFR-PEG10k-CSANs exhibited an almost 2-fold reduction in accumulated activity in the liver (18.8 ± 2.7 % ID/g) and the spleen (5.5 ± 1.4 % ID/g) and a greater than 3 fold reduction in accumulated activity in the kidneys (8.0 ± 0.9 % ID/g) at 24 h. Radioactivity in the blood (1.5 ± 0.1 % ID/g) and tumor (7.4 ± 0.8 % ID/g) was found to have increased by 28% and 34%, respectively. However, the greatest decrease in accumulated activity within the liver (13.1 ± 0.9 % ID/g), spleen (4.0 ± 0.7 % ID/g) and kidneys (6.4 ± 1.1 % ID/g) was observed with mice treated with ^{64}Cu -anti-EGFR-PEG20k-CSANs at 24 h. In addition, a significant increase in accumulated activity for the tumor (8.0 ± 0.9 % ID/g) and blood (2.0 ± 0.3 % ID/g) was observed with the PEG20 construct. Nevertheless, the tumor to blood ratios remained comparable ($\sim 4.4:1$) across all three constructs at 24 h (Fig. S8).

Biodistribution was analyzed at 48 h p.i. to determine the ability of the CSANs to be retained in circulation and thus enhance tumor targeted accumulation. Although activity levels for the mice treated with ^{64}Cu -anti-EGFR-CSANs were significantly reduced in major RES associated organs (i.e. liver, spleen and kidneys), consistent with tumor targeting activity in the tumor was increased (5.6 ± 0.9 % ID/g) (Fig. S9). Thus, the lower accumulated activity observed for RES associated organs in mice treated with PEGylated CSANs at 24 h p. i. was also observed at 48 h p.i. A minor increase in accumulated activity was observed, however, for the tumor (8.7 ± 1.9 % ID/g with the ^{64}Cu -anti-EGFR-PEG20k-CSANs) at 48 h p. i. Similar to the results for 24 h p. i., the mean tumor to blood ratios remained comparable ($\sim 5.3:1$) across all three constructs (*vide supra*) (Fig. S7). These observations suggests that the targeted PEGylated CSANs have a unique drug delivery profile that enables them to be observed in the target tumor more rapidly than non-targeted CSANs while remaining in circulation for at least 48 h.

To ascertain the stability of the injected ^{64}Cu -anti-EGFR-CSANs *in vivo*, blood samples were collected at 24 h and 48 h. Protein bound and non-protein bound radioactive ^{64}Cu was separated by Bio-spin 6 columns and counted. More than 90% of the ^{64}Cu found in the blood was found to be protein bound (Fig. S10). Since free bis-MTX-DOTA[^{64}Cu] is rapidly eliminated within 30 min after i.v. injection, these results are consistent with the ^{64}Cu -anti-EGFR-CSANs being stable in blood circulation for at least 48 h.

MicroPET/CT Imaging

Our observations from the biodistribution studies were further supported by *in vivo* microPET/CT imaging of the EGFR positive U-87 MG xenografts. In this experiment, mice ($n = 3$) were administered 50 to 70 $\mu\text{Ci}/\mu\text{mol}$ of ^{64}Cu labeled EGFR targeted (^{64}Cu -anti-EGFR-PEG20k-CSAN) or non-targeted CSANs (^{64}Cu -PEG20k-CSAN). Post-injection (p.i.) static microPET/CT scans were performed at 1, 4 and 24 h p.i. to investigate the time dependent biodistribution by quantitative region of interest (ROI) analysis. The delivery of ^{64}Cu -anti-EGFR-PEG20k-CSANs to the tumor was observed within the first hour of intravenous administration and could be visualized with excellent image contrast at 4 h (Fig. 10). The signal intensity for the targeted CSAN was significantly higher ($P < 0.01$) at 4 h p.i.

in the tumor (11.5 ± 0.6 % ID/g) compared to the non-targeted CSAN (8.4 ± 1.3 % ID/g). However, over the 24 h period the signal intensity in tumor for both non-targeted and targeted constructs reached 11.1 ± 1.9 % ID/g and 11.7 ± 0.2 % ID/g respectively. This observation indicated that at longer time points the accumulation of CSANs in tumor was independent of the receptor targeting (Fig. S11). On the other hand, the observed signal intensity in the heart peaked at 1 h p.i. for both constructs and a dynamic intensity of approximately 2% ID/g was maintained after 4 h p.i. suggesting a persistent circulation of CSANs in blood (Fig. S12).

The observed incremental accumulation of the signal intensity in the tumor can be attributed to circulating CSANs in the blood pool. Plateauing of the signal intensities for both targeted and non-targeted CSANs at 24 h p.i also indicated that the Enhanced Permeation Retention effect (EPR) was predominant over targeting at later time points.⁴⁶ A visible decrease in signal intensity for the liver, kidneys and spleen and a remarkable improvement in the signal compared to the background was observable. Therefore, high contrast images of the tumor could be acquired. Consistent with image analysis, the post-imaging *ex-vivo* organ activity measurements at 24 h for the same study revealed a significant increase in tumor accumulation ($8.0 \pm .9$ % ID/g) relative to the kidneys (6.4 ± 1.1 % ID/g) or the liver ($13.1 \pm .9$ % ID/g) (Fig. S13).

In summary, we have demonstrated that chemically self-assembled protein based CSANs are stable and capable of targeting tumor tissue *in vivo*. Similar to findings from studies of other targeted and PEGylated nanoparticles, the amount of accumulation of the nanoparticles by non-targeted tissues can be suppressed by PEGylation. In addition, while the initial kinetics of accumulation in a mouse xenograft tumor is greater with targeted nanoparticles, including CSANs, over time little difference is observed between the tumor accumulation of targeted and non-targeted nanoparticles.⁴⁶ This behavior can be attributed to the prolonged circulation of the nanoparticles in blood.⁴⁷ Consequently, it appears that the relatively flat ring structure of CSANs behaves similar to spheroid based nanostructures with regard to the characteristics governing tumor accumulation, such as the EPR effect.⁴⁸

Radiolabeling of antibodies and other targeting proteins typically depends on conjugation to a chelator of choice at a non-ambient temperature.⁴⁹ We have shown that targeted and radiolabeled CSANs can be prepared by quantitative labeling of the chemical dimerizer before self-assembly, thus avoiding the potential for induced thermal aggregation and denaturation.

Taken together, our results demonstrate that a designed protein based self-assembled nanostructure can exhibit the characteristics required for *in vivo* use, including tissue imaging and potentially drug delivery. The modular and multivalent nature of CSANs allows the targeting ability of ligands with a range of affinities to be evaluated and compared. Moreover, as we have recently demonstrated, the CSANs platform can be used to prepare not only multivalent but bispecific targeting nanoparticles with high specificity,¹⁷ thus further enhancing their potential selective tissue targeting ability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

bis-MTX	bivalent methotrexate
CSANs	Chemically Self-Assembled Nanorings
CAR T cell therapy	Chimeric Antigen Receptors
CAD	computer-aided design
CT	computerized tomography
DHFR	dihydrofolate reductase
DHFR²	two recombinantly fused DHFRs
ESI-MS	electrospray ionization mass spectrometry
EPR	Enhanced Permeation Retention effect
EGFR	epidermal growth factor receptor
EpCAM	epithelial cell adhesion molecules
MTX	methotrexate
pAcF	<i>p</i> -acetyl phenylalanine
% ID/g	Percent Injected dose per gram
PEG	polyethylene glycol
PET	Positron emission tomography
PARs	Prosthetic Antigen Receptors
ROI	region of interest
RES	reticuloendothelial system
scFv	single chain antibodies
SEC	size exclusion chromatography

p*-SCN-Bn-DOTA*S-2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid****References**

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Figure 1.
Chemically induced self-assembly of protein nanorings.

Figure 2. Schematic representations of A) EGFR targeted DHFR² fusion protein and B) Met161 to unnatural amino acid (*p*-acetyl phenylalanine, pAcF) mutated construct of the EGFR targeted DHFR² fusion protein C) general site-specific PEGylation reaction.

Figure 3. Self-assembly of monomeric anti-EGFR-1DHFR² (green trace) with bis-MTX-DOTA to form CSANs (black trace) observed by SEC.

Figure 4.
Schematic representation of programmable and multi-functional CSANs for *in vivo* tumor targeting.

Figure 5. Size exclusion chromatography of anti-EGFR CSANs prepared with bis-MTX-DOTA A) anti-EGFR-1DHFR²-PEG10k B) anti-EGFR-1DHFR²-PEG20k (Black – monomeric protein; Red – Oligomeric CSANs).

Figure 6.
The dynamic light scattering (DLS) analysis: hydrodynamic diameters distribution of CSANs.

Figure 7.

In vitro macrophage uptake of FITC labeled CSANs: Raw 264.7 mouse macrophage cells were incubated with 1 μ M of either non pegylated anti-EGFR-CSANs (grey), anti-EGFR-PEG10k-CSANs (red), or anti-EGFR-PEG20k-CSANs (blue) constructed with bis-MTX-FITC for 24 hours at 37° C. Macrophage cells were monitored by flow cytometry to determine the amount of CSAN uptake. Samples were corrected to non PEGylated anti-EGFR-CSANs at 100%. Statistical significance, * $p < 0.05$.

Figure 8.

Binding of anti-EGFR-CSANs to U-87 MG cells determined by flow cytometry. All treatments were performed with 1 μ M of CSANs for 1 hour at 37°C (n=3).

Figure 9.

Biodistribution ($n = 3$) of anti-EGFR-PEG20k-CSANs (green), anti-EGFR-PEG10k-CSANs (blue), and anti-EGFR-CSANs (red) at 24 hours post intravenous injection in mice bearing EGFR positive U-87 MG flank tumors. Results are reported as percentage injected dose per gram of tissue. Statistical significance, $*p < 0.05$ (two tailed T-test).

Figure 10.

MicroPET/CT images of coronal (C) and sagittal (S) sections of mice bearing U-87 MG xenografts. Static microPET/CT scans were performed 1, 4 and 24 hours following intravenous tail vein injection of ^{64}Cu -PEG20k-CSANs(above) and ^{64}Cu -anti-EGFR-PEG20k-CSAN (below).

Scheme 1.
Synthesis of bis-MTX-DOTA

Table 1

Average hydrodynamic diameters of PEGylated and non-PEGylated CSANs determined by DLS.

Protein construct	Average Diameter (nm) ^a
anti-EGFR-1DHFR ²	4.5 ± 0.5
anti-EGFR-1DHFR ² -PEG10k	7.5 ± 0.1
anti-EGFR-1DHFR ² -PEG20k	9.0 ± 0.5
anti-EGFR-CSANs	15.4 ± 0.8
anti-EGFR-PEG10k-CSANs	16.8 ± 0.5
anti-EGFR-PEG20k-CSANs	19.6 ± 0.3

^a average of 10 measurements