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Therapeutic Anti-Methamphetamine Antibody Fragment-Nanoparticle Conjugates: Synthesis and *In Vitro* Characterization

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Abstract

Treatments specific to the medical problems caused by methamphetamine (METH) abuse are greatly needed. Towards this goal, we are developing new multivalent anti-METH antibody fragment-nanoparticle conjugates with customizable pharmacokinetic properties. We have designed a novel anti-METH single chain antibody fragment with an engineered terminal cysteine (scFv6H4Cys). Generation 3 (G3) polyamidoamine dendrimer nanoparticles were chosen for conjugation due to their monodisperse properties and multiple amine functional groups. ScFv6H4Cys was conjugated to G3 dendrimers via a heterobifunctional PEG crosslinker that is reactive to a free amine on one end and a thiol group on the other. PEG modified dendrimers were synthesized by reacting PEG crosslinker with dendrimers in a stoichiometric ratio of 11:1, which were further reacted with three-fold molar excess of anti-METH scFv6H4Cys. This reaction resulted in a heterogeneous mix of G3-PEG-scFv6H4Cys conjugates (dendribodies) with three to six scFv6H4Cys conjugated to each dendrimer. The dendribodies were separated from the unreacted PEG modified dendrimers and scFv6H4Cys using affinity chromatography. A detailed *in vitro* characterization of the PEG modified dendrimers and the dendribodies was performed to determine size, purity, and METH-binding function. The dendribodies were found to have identical affinity for METH as the unconjugated scFv6H4Cys in saturation binding assays, whereas the PEG modified dendrimers had no affinity for METH. These data suggest that an anti-METH scFv can be successfully conjugated to a PEG modified dendrimer nanoparticle with no adverse effects on METH binding properties. This study is a critical step towards preclinical characterization and development of a novel nanomedicine for the treatment of METH abuse.

INTRODUCTION

The socioeconomic impact of methamphetamine (METH) abuse is of great concern worldwide. Due to its multiple sites of action in the brain and other vital organs,¹ it is difficult to attenuate the detrimental effects of METH using a site specific receptor

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antagonist or agonist. Currently, there are no FDA approved medications to treat METH addiction. The available therapies are mainly supportive and involve behavior modification. METH specific antibody-based medications that act as pharmacokinetic (PCKN) antagonist by reducing the concentration of METH in the brain and other crucial organs have shown promise as a potential therapeutic. Anti-METH monoclonal antibodies (mAb) alter the disposition of METH in the body thus reduce the associated medical complications.² They significantly shorten the duration of METH-induced locomotor activity³ and also reduce or block METH self-administration in preclinical rat models.⁴

There are currently two forms of anti-METH mAbs in preclinical testing: a long-acting IgG and an extremely short-acting single chain variable fragment (scFv).⁵ The prolonged serum half-life of IgG is largely attributed to the neonatal Fc receptor (FcRn) mediated recycling pathway.⁶ In this, the interaction between the fragment crystallizable (Fc) region of IgG and the FcRn exposes IgGs to pH changes that could potentially alter critical molecular interactions in antigen binding sites. Indeed, *in vivo* inactivation of some anti-METH mAbs have been reported,⁷ but whether this involves the FcRn pathway remains to be determined. Since anti-METH mAbs do not depend on Fc region interactions for complement binding, this domain could be removed with no loss of METH binding function. Thus scFv6H4 without the FcRn binding domain was designed by joining the heavy and light chain variable domains of the parent anti-METH mAb6H4. This new antibody fragment was one-sixth the size of an intact IgG and capable of altering the *in vivo* disposition of METH within one minute of administration.⁸ The scFv can be produced more economically and the protein dose required to deliver the same number of anti-METH binding sites is one-third of the IgG. However, the small molecular size of monomeric scFv6H4 (~27 kDa) leads to its rapid clearance from the blood stream. This PCKN property could be advantageous for treating acute drug overdose but not chronic METH abuse.

Numerous research groups in the field of oncology and neurology have reported conjugating drugs and proteins to nanoparticles as a way to improve efficacy, imaging or targeting.^{9–11} Nanotechnology provides a unique platform for customizing pharmacological properties like efficacy and PCKN. One particularly useful innovation in this field is a class of molecules called dendrimers.¹² In Greek, dendrimer means “tree-like” referring to the branched structure that increases in density with each round of synthesis, or generation (G1, G2, G3, etc.; Figure 1). Dendrimer particles are 1–10 nm in diameter and have excellent monodispersity. They possess the potential to carry multiple functional groups that can be used for protein coupling, as well as a “payload” in the interior of the molecule.¹³

We envisioned that the PCKN of an anti-METH antibody fragment could be customized and optimally controlled by conjugating it to a dendrimer delivery system. The increase in molar mass of the nanoparticle could reduce the clearance of the conjugated antibody fragment from the body by reducing its potential for glomerular filtration (molecular weight cut off ~50 kDa), and restrict its volume of distribution to the vasculature. This novel prototype anti-METH antibody fragment-conjugated to dendrimer nanoparticles (dendribodies) could also exhibit increased efficacy due to multivalency, improved residence time, and reduced antigenicity compared with the native antibody fragment. As a first step toward testing this hypothesis, in this paper we describe the synthesis rationale, chemistry optimization, analysis of reaction intermediates, and *in vitro* functional analysis of a novel anti-METH scFv-dendrimer conjugate.

MATERIALS AND METHODS

Chemicals and drugs

Enzymes and *Escherichia coli* strains were purchased from Invitrogen (Carlsbad, CA). [³H]-N-Methyl-1-phenylpropane-2-amine (METH) (39 Ci/mmol) labeled at two metabolically stable sites on the aromatic ring structure was obtained from the National Institute on Drug Abuse (Bethesda, MD) after synthesis at the Research Triangle Institute (Research Triangle Park, NC). G3 PAMAM dendrimers were purchased from Dendritic Nanotechnologies, Inc. (Mt. Pleasant, MI). All other reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

Cloning and large-scale expression of anti-METH scFv6H4Cys

General molecular and genetic techniques used for plasmid construction and transformation of scFv6H4Cys were performed as described in Sambrook and Russell.¹⁴ A carboxyl terminus cysteine was engineered immediately following the His6 tag of scFv6H4 by DNA synthesis to create scFv6H4Cys in pUC57 vector (Genscript, Piscataway, NJ). The pUC57 scFv6H4Cys plasmid was transformed into *E. coli* strain DH5α (Invitrogen) for amplification and plasmid maintenance. The gene product was then restricted using EcoRI and XbaI, gel purified, and ligated into the matching sites in cloning vector pPICZαA (Invitrogen). Ampicillin resistant colonies containing pPICZαA were selected on plates containing LB agar and ampicillin. DNA was isolated and the integrity of the transformed product was confirmed by restriction digestion and DNA sequencing (University of Arkansas for Medical Sciences DNA Core Sequencing Facility). After sequence confirmation, the plasmid was linearized with SacI and used to transform *Pichia pastoris* strain X33 by electroporation according to manufacturer's instructions (Invitrogen).

Zeocin-resistant colonies containing the scFv6H4Cys cDNA were selected on agar plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol and 2% agar] containing 100 µg/ml Zeocin. Colonies that exhibited high Zeocin resistance were tested for scFv6H4Cys expression. In brief, colonies were picked from a freshly streaked plate and grown for 24 hr in a 5 ml starter culture of BMGY [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base, 4 × 10⁻⁵% biotin, and 1% (v/v) glycerol] at 30°C. Then 500 µl of this starter culture was inoculated in 50 ml of BMGY and cultured for additional 18 hr. The cells were harvested and resuspended in 500 ml BMMY [BMGY with 0.5% methanol (w/v) substituted for glycerol] to induce protein expression. The cultures were grown at 30°C with shaking overnight and methanol was fed to a final concentration of 0.5% (w/v) at 24, 48 and 72 hr. To harvest and analyze total scFv6H4Cys expression, the samples were centrifuged at 20,000 *g* for 10 min, and the supernatant was analyzed using SDS-PAGE and Coomassie dye G-250 based GelCode Blue staining (Pierce Thermo Scientific).

ScFv6H4Cys purification and formulation

The scFv6H4Cys expression product was purified as described for scFv6H4¹⁵ using an AKTA explorer 100 FPLC system and a nickel sepharose IMAC HiPrep FF16/10 column (GE Healthcare, Piscataway, NJ). In brief, the column was equilibrated with 5 column volumes of binding/wash buffer (20 mM Sodium phosphate buffer, pH 7.4, 500 mM NaCl, 10 mM imidazole) and eluted in a single step with the elution buffer (binding/wash buffer with 500 mM final concentration of imidazole). The fractions were collected, pooled, buffer exchanged and concentrated into scFv administration buffer (15 mM Sodium phosphate buffer, 150 mM NaCl, pH 7.5) using 7 or 20 ml Pierce® concentrators with 9 kDa molecular weight cut off (MWCO) (Pierce Thermo Scientific). The protein was characterized by SDS-PAGE, Western blot, and N-terminal sequencing (Midwest Analytical Inc, St. Louis, MO).

Synthesis of PEG₂₄ modified G3 PAMAM dendrimers

A working stock of 0.1 mM G3 PAMAM dendrimers **1** (see Scheme 1) was prepared in phosphate buffered saline (PBS) (2.683 mM KCl, 1.47 mM KH₂PO₄, 136.893 mM NaCl, 8.101 mM Na₂HPO₄) (MP Biomedicals LLC, Solon, OH) containing 1 mM EDTA. The heterobifunctional crosslinker with NHS ester and maleimide groups with PEG **2** spacer arms (NHS-PEG₂₄-Mal) was added in 5, 10, 15 and 20 fold molar excess to the dendrimer working stock (e.g., 2 mM crosslinker to 0.1 mM dendrimer). The G3 dendrimer **1** and PEG₂₄ **2** conjugation reactions were set up in stoichiometric ratios such that a single PEG₂₄ molecule had 5, 11, 16 or 32 amine groups of dendrimer available for interaction. The reaction was incubated at room temperature (RT) for 2 hr in the dark with gentle shaking. After incubation, excess of crosslinker and leaving NHS group were removed using protein desalting spin columns (Thermo Fisher Scientific) equilibrated with conjugation buffer (50 mM Sodium phosphate buffer, pH 6.4, 150 mM NaCl, 2 mM EDTA).

Characterization of PEG₂₄ modified G3 PAMAM dendrimers

PEG₂₄ modified G3 PAMAM dendrimers **3** were characterized using UV spectrometry, gel electrophoresis, and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). A Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) was used to obtain UV spectra. Reducing SDS-PAGE analysis was conducted using 4–12% acrylamide Bis-Tris NuPage gels (Invitrogen) according to the manufacturer's instruction. Samples were heated with sample loading buffer containing dithiothreitol (DTT) at 70–80°C for 10 min and were run at 200 V for 35 min. The gel was stained with Coomassie based GelCode Blue for 1 hr at RT and destained overnight in water. For PEG-specific iodine staining, the gel was soaked in 5% barium chloride solution for 10 min and placed in 0.1 M iodine solution until a brown color developed.¹⁶ MALDI-TOF mass spectra were obtained on a Bruker Ultraflex II TOF-TOF (Arkansas Statewide Mass Spectrophotometry Facility). A 1 M solution of 2,5-dihydroxybenzoic acid in 90% methanol with 0.1% formic acid (MALDI matrix) was mixed with the various reaction ratios of PEG₂₄ modified dendrimer **3** samples in a 1:1 ratio (v/v) and 1 µL of the mixture was deposited onto the MALDI target. Mass spectra were acquired in the linear, positive ion mode and externally calibrated with a protein standard mixture.

Synthesis and optimization of PEG₂₄ modified G3 PAMAM dendrimer **3** and scFv6H4Cys **4** conjugation reactions

The individual parameters of the dendrimer **3** reaction (*i.e.*, reaction ratios of PEG modified dendrimers **3** to scFv6H4Cys **4**, type and concentration of reducing agent, pH, temperature, EDTA, and NaCl concentration) were optimized to obtain the maximum dendrimer **3** yield. The reaction was performed in conjugation buffer at pH 6.2, 6.4, 6.6, 6.8, 7.0 and 7.2. The conjugation reaction was then set at 10°C, 25°C and 37°C to determine the optimum temperature. To ensure that the scFv6H4Cys's **5** thiol groups remained in their reduced state, various concentrations of two reducing agents TCEP (tris-2-carboxyethyl phosphine) and DTT were explored. The scFv6H4Cys was treated with freshly prepared 2, 4, 6, 8 and 10 mM DTT or TCEP equal to 2, 5, or 10 fold the protein concentration. These reduction reactions were allowed to proceed for 2 hr at RT in dark with shaking. DTT treated scFv6H4Cys **5** was desalted twice to ensure complete removal of DTT as confirmed by Ellman's assay (Thermo Fisher Scientific) using Pierce® desalting spin column into the conjugation buffer. The PEG₂₄ modified dendrimers **3** were reacted with a three molar excess of reduced scFv6H4Cys **4**. This reaction was allowed to proceed for 2 hr at RT in the dark with shaking to produce dendrimers.

Characterization of PEG₂₄ modified G3 PAMAM dendrimers and scFv6H4Cys conjugates (dendribodies)

Dendribodies **5** were characterized using SDS-PAGE, SEC, and Western blot. Gel electrophoresis was performed as described for PEG₂₄ modified dendrimers **3** and was used to assess the efficiency of crosslinking. Western blot was used to detect the presence of scFv6H4Cys **4** in the higher molecular weight dendribody **5** bands. The gel was electrophoretically transferred to a polyvinylidene fluoride membrane using a XCell SureLock™ Mini-Cell Western blotting kit (Invitrogen) as per manufacture's instruction. The membranes were then blocked with 10% non-fat dry milk overnight at 4°C and incubated for 2 hr at RT with 1:5000 mouse monoclonal anti-FLAG® M2-alkaline phosphatase antibody (Invitrogen) made in 0.2% bovine serum albumin (Biorad Laboratories Inc., Hercules, CA). Following incubation, the membrane was washed with tris-buffered saline (+0.1% Tween-20, three times with five minutes per rinse). Bands were identified by exposing the membrane to 5-bromo-4-chloro-3-indolyl-phosphate in conjunction with nitro blue tetrazolium for colorimetric detection.

Purification of the dendribodies **5** from the unreacted PEG₂₄ modified G3 PAMAM dendrimers **3** and scFv6H4Cys **4**

The dendribodies **5** were purified by immobilized metal affinity chromatography (IMAC) using an AKTA Explorer 100 fast protein liquid chromatography system and a 1 ml HiTrap IMAC FF column. In brief, the column was equilibrated with five column volumes of binding/wash buffer (containing 10 mM imidazole) at 1 ml/min. The dendribody reaction mixture containing the unreacted PEG₂₄ modified dendrimers **3**, unconjugated scFv6H4Cys **4** and dendribodies **5** was loaded onto the column. The dendribodies **5** were eluted from the column with the elution buffer (binding/wash buffer plus 470 mM imidazole, pH of 7.5) and collected as 0.5 ml fractions. The elution profile was monitored by UV absorbance at 220, 254, and 280 nm wavelengths. Fractions were analyzed by SDS-PAGE and the fractions enriched in dendribodies **5** were pooled and concentrated into PBS using 3 kDa MWCO Vivaspin concentrators (GE Healthcare).

Saturation binding assay for determination of K_D values for METH

Rapid equilibrium dialysis (RED) (Pierce Thermo Scientific) device was used to perform the saturation binding assay. The Teflon base plate was washed in 20% v/v ethanol for 10 min. After ethanol removal, the base plate was thoroughly rinsed three times with water and allowed to dry. RED inserts with a nominal MWCO of 6–8 kDa were used. A concentration of scFv6H4Cys **4** or dendribodies **5** that was found to bind 20% of a 50,000 dpm [³H]-METH solution was added to the sample chamber. This protein concentration was determined using a titration binding assay where serial dilutions of scFv6H4Cys **4** or dendribodies **5** were allowed to bind a constant concentration of [³H]-METH. For total binding, 100 µl of PBS and 200 µl of increasing concentrations of [³H]-METH were added into the buffer chamber. For non-specific binding, 100 µl of PBS was replaced by 100 µl of 10 µM unlabeled METH as a competitor for [³H]-METH. After loading, the plates were sealed with self-adhesive plastic sealing sheets to prevent evaporation and incubated overnight at RT on an orbital shaker with gentle shaking. After equilibrium was reached (18 hr), a 50 µl aliquot from both the sample and buffer sides, was added to 5 ml of scintillation fluid, vortexed and was quantified using liquid scintillation spectrophotometry.

Data analysis and statistics

Data represent mean ± standard error of the mean (S.E.M) for the number (n) of experiments indicated in parentheses. The saturation binding data was analyzed by non-linear curve fitting using Graphpad PRISM® 5.0d software.

RESULTS

Overall design of dendribody synthesis

Generation 3 polyamidoamine (G3 PAMAM) dendrimers **1** consist of a diaminobutane core that has undergone three generations of synthesis, doubling the number of functional groups with each progressive generation. It was used as the supporting scaffold of the dendribody **5** (Figure 1A) as it has a maximum number of 32 amine terminal groups, which can be utilized for conjugation with a variety of homo- and heterobifunctional crosslinking agents. We chose a heterobifunctional PEG crosslinker that is reactive to amines via an *N*-hydroxysuccinimide (NHS) ester at one terminus and to a free sulfhydryl group via a maleimide group at the opposite terminus. PEG chains of varying size (690, 856, 1270, 3400 and 5000 Da) were investigated for their ability to form dendribodyes. However, only 1270 Da PEG with 24 ethylene oxide repeats (PEG₂₄) (Figure 1B) resulted in successful dendribody formation.

Sulfhydryl crosslinking was used to conjugate the anti-METH antibody fragment to dendrimers because it has been shown to be effective for site-specific coupling of proteins to non-protein molecules, such as detection probes, PEG, and liposomes.^{17–19} Our design goal was to utilize the selective reactivity of the cysteine to the maleimide group of a heterobifunctional PEG linker. The scFv inherently possess cysteines that form intrachain disulfide bonds in the variable light and heavy chains, however conjugating to these internal cysteines could disrupt the structure of the antibody and ligand binding. Thus, we engineered an scFv with a cysteine at the carboxy-terminus of the DNA coding sequence to create scFv6H4Cys (Figure 1C).

The general steps to assemble the dendrimer-PEG-scFv6H4Cys conjugate are represented in Scheme 1. Briefly, the first step was to crosslink the NHS-moiety of the heterobifunctional PEG₂₄ **2** to the amine terminal groups of the G3 PAMAM dendrimer **1**, followed by removal of unreacted dendrimers and PEG₂₄ by desalting chromatography (Scheme 1). Step two was to treat the carboxy-terminal cysteine of the scFv6H4Cys **4** with DTT reducing agent followed by a desalting step (Scheme 1). We found the later step to be important for two reasons. First, it reduces any disulfide mediated scFv dimerization or any potential disulfide interactions that occurred during scFv production in the presence of sulfhydryl-containing amino acids or peptides in the cell growth media. Second, the desalting step serves as a quick method of buffer exchange and removes the small adducts and DTT, both of which could compete and interfere with the maleimide reaction. Once the PEG₂₄ modified dendrimer **3** and DTT treated scFv6H4Cys **4** were desalted and buffer exchanged, they were combined. This allowed the free sulfhydryl groups of the scFv6H4Cys to covalently link to the maleimide groups of the PEG modified dendrimer **3** forming the final dendribody **5** product. Scheme 1 represents a single scFv conjugated to the PEG modified dendrimer **3** for illustration purposes. However, higher order conjugates are possible and this was the goal of the optimization experiments presented in this paper.

PEG modification of G3 PAMAM dendrimers

In order to control the degree of PEG modification of the dendrimers, the crosslinking reaction of NHS-PEG-maleimide **2** to G3 PAMAM dendrimer **1** was performed with PEG₂₄:G3 stoichiometric ratios of 5:1, 11:1, 16:1 and 32:1. In this reaction, an amide bond is formed when the NHS-ester reacts with a terminal amine of the dendrimer, releasing a NHS cyclic group. Hydrolysis of the NHS-ester is a major competing reaction of the NHS-ester acylation reaction, since a NHS group is released by both reacting with primary amines and by hydrolysis. Thus, the extent of NHS hydrolysis or reaction with primary amines in solution can be determined by measuring the absorbance of the NHS-ester group at 260 nm.

Dendrimers and PEG₂₄ do not exhibit UV absorption at this wavelength. This difference in spectral absorption allowed us to follow the efficiency of PEG modification of dendrimers at increasing stoichiometric reaction ratios (Figure 2A) as well as during desalting. The NHS group and unreacted PEG₂₄ **2** were removed to increase the efficiency of later steps of the dendrimer **5** synthesis.

Following desalting and spectrophotometric analysis, the degree of PEG modification of dendrimer was further analyzed by SDS-PAGE. The amine-terminated dendrimers **1** exhibit a net positive charge and do not migrate into standard polyacrylamide gels, even in the presence of SDS. However, they can be analyzed if the SDS-PAGE is conducted with reversed polarity.²⁰ We found that the PEG₂₄ **2** alone migrated into SDS-PAGE gel, but it could not be detected with Coomassie-based stains. However, the new PEG modified dendrimer **3** migrated into SDS-PAGE gels and could be detected with Coomassie-based stain (Figure 2B). As the ratio of PEG₂₄:G3 was increased, an apparent higher shift in the molecular weight of each band was observed suggesting increased functionalization of the dendrimers with saturation at higher PEG₂₄ concentrations (Figure 2B, left panel). We confirmed that this molecular weight increase was due to the addition of PEG₂₄ crosslinker by staining a duplicate SDS-PAGE gel with PEG-specific iodine stain (Figure 2B, right panel).

MALDI-TOF analysis of PEG modified dendrimers

While the SDS-PAGE analysis strongly indicated successful PEG₂₄ **2** modification of the G3 PAMAM dendrimers **1**, we wanted to confirm this by MALDI-TOF analysis. This was a more accurate technique for analyzing the mass of G3 PAMAM dendrimers **1**, PEG₂₄ crosslinker **2**, and the products of each PEG₂₄:G3 dendrimer reaction ratio. MALDI-TOF analysis suggested that the average mass of the G3 PAMAM dendrimers **1** and PEG₂₄ **2** crosslinker alone was 6937 Da and 1280 Da respectively which closely agreed with the mass reported by the manufacturer (Table 1). Each of the PEG₂₄:G3 reactions showed increasing mass as the ratio of PEG₂₄ to dendrimer was increased, confirming the SDS-PAGE results. The MALDI-TOF spectra showed a distribution of individual mass peaks. Figure 3A is a representative spectrum from the 5:1 PEG:G3 dendrimer reaction ratio. In addition to a broad peak that corresponds to the mass of the G3 dendrimer **1** alone, distribution of individual peaks were observed that iteratively increased by 1280 Da, corresponding to the theoretical mass addition of each PEG₂₄ **2** crosslinker.

As the PEG₂₄:G3 stoichiometric ratio increased, we observed an upward shift in the overall mass, but reduced resolution of the individual mass peaks (data not shown). Thus, for the calculation of the average number of PEG₂₄ **2** additions to the G3 dendrimer **1**, we selected the mass of the highest peak, or group of peaks. We are reporting the highest signal peak in Table 1 assuming that each reaction resulted in a Gaussian distribution of PEG₂₄ molecules incorporated around this central peak.

The mass increase for the 5:1, 11:1, 16:1, and 32:1 PEG₂₄:G3 dendrimer reaction ratios corresponded to 5, 16, 20, and 20 PEG **2** additions, respectively (Figure 3B and Table 1). Surprisingly, we observed over 100% addition of the calculated PEG₂₄ **2** to the dendrimers **1** for 5:1 and 11:1 reaction ratios. One explanation for this could be the difference in the reported and actual concentration of PEG₂₄ **2** or G3 dendrimer **1** lots. Similar to the SDS-PAGE analysis, saturation between 16:1 to 32:1 PEG₂₄ to G3 dendrimer reaction ratio was observed with a maximum addition of 20 PEG₂₄ units per dendrimer. This suggested that after crosslinking, PEG₂₄ occupied up to 63% of the available amines on the dendrimer (Figure 3B). We did not aim to saturate the dendrimers with PEG₂₄ since excess unreacted PEG can be difficult to remove from the products. Therefore, we chose the 11:1 PEG₂₄:G3

dendrimer reaction ratio to synthesize the PEG modified dendrimer scaffold for dendribody production.

Crosslinking optimization of scFv6H4Cys to PEG modified dendrimers

After optimizing the PEG₂₄:G3 dendrimer reaction ratio, we began a series of experiments aimed at optimizing the addition of multiple scFv6H4Cys **4** molecules to the PEG₂₄ modified dendrimer scaffold. These optimization experiments included varying numerous parameters known to affect maleimide-thiol reaction efficiency including: scFv to dendrimer stoichiometric ratio, ionic strength, EDTA concentration, temperature, and pH.²¹

The endpoint of the reaction efficiency was the number of scFv6H4Cys **4** molecules that could be linked to the PEG modified G3 dendrimer **3** core. We could determine the number of scFv6H4Cys **4** on each dendrimer since the reaction products formed distinct and well-resolved bands in an SDS-PAGE gel. We carefully examined both the maximum number of higher-order conjugates as well as the relative amounts of each species. To determine if temperature influenced the reaction efficiency, the dendribody conjugation was allowed to proceed at 10° 25° and 37°C for 2 hrs, and analyzed by SDS-PAGE (Figure 4A). The reaction temperature had a moderate effect on the efficiency of the reaction, with the 25°C condition having a slightly higher efficiency in the number of scFv6H4Cys **4** molecules conjugated to PEG modified dendrimers. In our studies, NaCl and EDTA concentrations had negligible effects on the efficiency of the reaction (data not shown).

To determine how pH affected the crosslinking efficiency, we conducted six reactions ranging from pH 6.2 to 7.2. This range was selected based on manufacturer's instructions and previous reports.²¹ The optimal reaction was found to be pH 6.4 (Figure 4B). A Western blot (Figure 5B) probed with an anti-FLAG antibody further confirmed that the discrete banding pattern observed for the dendribodies **5** in the SDS-PAGE analysis was indeed from the addition of increasing numbers of scFv6H4Cys **4** to the PEG modified dendrimers **3**.

Purification of dendribodies by immobilized metal affinity chromatography

For the functional assay, it was first necessary to separate the prototype dendribodies **5** from the unconjugated PEG modified dendrimers **3** and scFv6H4Cys **4**. We attempted several chromatographic purification techniques, including ion exchange (IEX, data not shown) and size exclusion chromatography (SEC). These two approaches were not suitable for dendribody purification due to either incomplete separation (IEX), or poor recovery of the product (SEC). However, SEC was a useful analytical tool for determining the native size of the dendribodies. Based on a standard curve of known molecular weight proteins, the calculated molecular weights of the predominant peaks of the dendribodies and unreacted scFv6H4Cys were determined to be 162 and 27 kDa respectively (Figure 5A). The dendribodies eluted as a wide peak with molecular weight ranging from 90 to 305 kDa.

We were ultimately able to utilize the hexa-histidine (His6) tag of scFv6H4Cys **4** for purification of the dendribodies. The dendribody reaction mixture was applied to a Ni(II) affinity column and eluted with an imidazole gradient over 20 column volumes. The multiple dendribodies and reactants eluted at different concentrations in the imidazole gradient, presumably due to their relative strength of interaction with the immobilized nickel groups on the chromatography resin. Unreacted PEG modified dendrimer did not bind to the IMAC column and was detected in the flow through fractions (Figure 6A and B). Dendribodies with lower number of scFv6H4Cys were eluted in the early fractions whereas the higher-order dendribodies eluted later with increasing imidazole concentration. Unreacted scFv6H4Cys eluted from the column predominantly in the later fractions with a peak around 50% (235 mM) imidazole. The early elution fractions (Figure 6B, lanes 4

through 6) were pooled and concentrated (Figure 6C). The Western blot confirmed that this pooled fraction was enriched in lower order dendribodies. This confirmation was important since the lower order dendribodies migrate to a similar position on a gel as PEG₂₄ modified dendrimers **3**.

Determination of *in vitro* activity of dendribodies

To determine if the prototype dendribodies retained METH-binding function, a saturation binding equilibrium dialysis experiment was performed using a scFv6H4Cys and dendribody concentration capable of binding 20% of a 50,000 dpm ³H-METH solution. An initial titration was performed to find a protein concentration that would provide a good signal-to-noise ³H-METH binding ratio at low concentrations, while minimizing the effects of ligand depletion.²² We compared the absolute affinities of the unreacted scFv6H4Cys **4** and the purified lower-order dendribodies **5** to ³H-METH. The dissociation constant (K_D) of the dendribodies **5** and scFv6H4Cys **4** were 1.2 and 1.1 nM, respectively (Figure 7). The PEG modified dendrimers showed negligible nonspecific binding to ³H-METH.

DISCUSSION

The therapeutic application of the synthesis and testing of a novel anti-METH antibody fragment-dendrimer conjugate (dendribody) is to treat METH abuse and associated medical conditions. Site-specific sulfhydryl conjugation is becoming a common conjugation strategy to create bioconjugates. This strategy has several advantages over less selective conjugation strategies. First, site-specific conjugation distal to the METH binding site of the scFv6H4Cys **4** can lessen the likelihood of steric interference during METH interaction. Second, since the cysteine residue is located at the C-terminus of the protein, potential polymerization and unwanted side reactions could be avoided. Third, the heterobifunctional PEG-based linkers we used are an important alternative to carbon-based spacers for protein-dendrimer conjugation because of their increased flexibility, solubility, decreased chance of aggregation, and reduced immunogenicity potential.^{23–27} Thus, this elegant conjugation strategy could produce dendribodies with higher multivalency, solubility, and reduced risk of antigenicity.

It was critical to analyze each reaction product during the dendribody development to allow optimal control of the stoichiometry of PEG₂₄ **2** and scFv6H4Cys **4** additions to the dendrimer **1**. We found the PEG₂₄ modified dendrimers **3** separated well on a 4–12% gradient polyacrylamide gel and were detectable with Coomassie-based stain (Figure 2B). This was interesting, since neither G3 PAMAM dendrimers nor PEG₂₄ alone take up this stain well. Additionally, we found that the intensity of Coomassie-based staining in gels decreased as the number of PEG₂₄ additions to the G3 dendrimer increased. The reason for this is not clear, but could be due to steric factors influencing entrapment of the dye, or net charge changes to the molecule. The apparent size of the PEG₂₄ modified dendrimers in an SDS-PAGE gel was larger than the size determined by MALDI-TOF. This could be attributed to an inherent property of PEG to increase the hydrodynamic volume of its conjugates.²⁸ We also observed high molecular weight bands in the PEG₂₄ modified dendrimer lanes (Figure 2B), which were most likely due to their dimerization. These bands were not seen in the final dendribody reaction products.

The DTT treatment of scFv6H4Cys allowed the PEG₂₄ maleimide group **2** to covalently link it to the G3 dendrimer. Since scFv6H4Cys **4** contains four internal cysteines that form two stabilizing disulfide bonds,²⁹ we were concerned that DTT-treatment of the scFv6H4Cys would reduce these internal disulfide bridges and lead to loss of function, however this was not the case. SDS-PAGE analysis confirmed that the maleimide-sulfhydryl reactions routinely produce dendribodies with a denatured size ranging from 50 to ~200 kDa (Figure

4). However, SEC indicated a native size range for the dendribody peak of 90 to 305 kDa. This native size shift in the SEC analysis is most likely due to hydrodynamic volume changes induced by the PEG moiety, as the scFv6H4Cys peak was 27 kDa, agreeing with SDS-PAGE analysis. This change in apparent size might have important effects on *in vivo* PCKN parameters such as volume of distribution and clearance.

Despite the inclusion of excess molar amounts of PEG₂₄ modified dendrimer **3** and scFv6H4Cys **4** in the reaction mixture in an attempt to achieve even higher order dendribodies, we rarely observed higher than six scFv per dendrimer. This was not entirely unexpected, since our computer simulations based on the dimensions of each component (Figure 1) indicated that scFv6H4Cys molecules could cause steric inhibition at higher-order incorporations. It might be possible to create even higher order (higher multivalency) dendribodies by using a generation 4 or generation 5 dendrimer with 64 and 128 reactive amine groups, respectively. However, the potential for toxicity increases as the generation of PAMAM dendrimers increases.³⁰ The choice of a G3 PAMAM dendrimer **1** represented a compromised balance between the number of functional groups and safety.

The dendribody elution profile from the IMAC column was the result of two predominant factors; accessibility of the His6 tag to the Ni(II) groups, and/or multivalency. The lower order dendribody products with one scFv6H4Cys per dendrimer contain a single His6 affinity tag. The potential for His6-Ni(II) interaction could be hindered by the PEG modified dendrimer **3**, and most importantly, direct hindrance by the high PEG:His6 ratio in the lower order dendribodies. Higher order dendribodies with three to five scFv6H4Cys **4** groups (lower PEG:His6 ratio) eluted later, possibly due to the multiple His6 tags available to bind to the Ni(II) in an additive or synergistic fashion, similar to the cooperative avidity found with IgG or IgM interactions with multiple antigenic epitopes. Interestingly, we found that none of the dendribodies could be detected in Western blots by probing with an anti-His6 antibody (data not shown), suggesting that any degree of PEG modification was sufficient to block IgG access to the 6His tag, but not the much smaller bead-chelated Ni(II). The His6 moiety of the unreacted scFv6H4Cys **4** was unhindered and eluted in the later, higher concentration imidazole fractions.

The affinity of scFv6H4Cys for METH ($K_D = 1.1$ nM) was determined by saturation binding equilibrium dialysis, however a previously reported affinity for parent scFv6H4 ($K_D = 10$ nM) was established using a bead-based radioimmunoassay,²¹ which given the inherent discrepancies in the assays, could account for the differences in their affinities. Since the X-ray crystal structure of scFv6H4 indicated that METH binding occurs via a very deep pocket,²⁹ we were concerned that conjugation could affect METH interactions. Significant charge or steric changes even distal to the binding site of the antibody fragment have been shown to reduce affinity.²⁷ However, the scFv6H4Cys retained its affinity for METH in the conjugated form, indicating that the conjugation process had little or no effect on the binding pocket of the protein.

CONCLUSIONS

These studies describe initial steps in the development of a novel anti-METH scFv-based nanomedicine for METH abuse. We successfully conjugated multiple anti-METH scFvs to a dendrimer nanoparticle with no loss of affinity for METH. This is, to the best of our knowledge, the first description of conjugating an anti-drug of abuse antibody fragment to a nanoparticle with detailed characterization of reaction intermediates. We think that the dendribody design is a promising platform to customize PCKN parameters (size dependent extension of *in vivo* half-life), improving efficacy (multivalency) and safety of biotherapeutics.

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ABBREVIATIONS

scFv	single-chain variable fragment
V_H	variable heavy region
V_L	variable light region
PCKN	pharmacokinetic
METH	(+)-methamphetamine
[³H]METH	(+)-2',6'- ³ H(n) methamphetamine
SEC	size exclusion chromatography
PAGE	polyacrylamide gel electrophoresis
MALDI-TOF	matrix assisted laser desorption ionization – time of flight
BMGY	buffered minimal glycerol-complex medium
G3	generation 3
PAMAM	polyamidoamine
PEG	polyethylene glycol

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Figure 1.

A. Schematic of polyamidoamine (PAMAM) dendrimer consisting of a core, three generations of synthesis, and terminal groups. **B.** Structure representative of heterobifunctional polyethylene glycol (PEG) linker with N-hydroxysuccinimide (NHS) ester and maleimide groups with 24 ethylene oxide repeat units {succinimidyl-[(N-maleimidopropionamido)-tetracosaehtyleneglycol] ester}. **C.** Design schematic of scFv6H4Cys. V_H, variable heavy region; V_L, variable light region; Linker, a 15 amino acid linking region; His6, 6-histidine tag for aiding purification; Cys, engineered cysteine residue for site-specific conjugation; FLAG, epitope for antibody recognition. Approximate dimensions of each dendribody component indicated to illustrate scale.

Figure 2.

A. UV spectroscopy monitoring of NHS crosslinking reaction. Representative UV wavelength scans from 220–325 nm of increasing reaction ratios of PEG₂₄ to G3 PAMAM dendrimers before and after desalting. The solid and dotted lines represent the PEG₂₄ modified dendrimers before and after desalting respectively. **B.** SDS-PAGE reducing gels loaded with reaction products of increasing ratios of PEG₂₄ to G3 dendrimer: (lane 1) purified scFv6H4Cys, (lanes 2 and 6) 5:1, (lanes 3 and 7) 11:1, (lanes 4 and 8) 16:1, and (lanes 5 and 9) 32:1. The left gel panel is stained with Coomassie based GelCode Blue stain and the right is stained with PEG-specific iodine stain.

Figure 3.

A. Representative MALDI-TOF spectrum showing the peaks resolved from a 5:1 PEG₂₄:G3 dendrimer reaction. **B.** Plot of the average number of PEG₂₄ incorporated per dendrimer versus each reaction ratio as calculated from MALDI-TOF analysis.

Figure 4.

A. SDS-PAGE reducing gel showing temperature optimization of the PEG₂₄ modified dendrimer crosslinking to scFv6H4Cys (dendribody) reaction: (Lane 1) PEG₂₄: G3 dendrimer (reaction ratio 11:1), (Lane 2) purified scFv6H4Cys, (Lane 3) reaction incubated at 10°C, (Lane 4) 25°C and (lane 5) 37°C. **B.** SDS-PAGE reducing gel from pH optimization study: (Lane 1) purified scFv6H4Cys, (Lane 2) PEG₂₄:G3 dendrimer (reaction ratio 11:1), dendribodies synthesized in conjugation buffer adjusted at pH 6.2 (Lane 3), 6.4 (Lane 4), 6.6 (Lane 5), 6.8 (Lane 6), 7.0 (Lane 7) and 7.2 (Lane 8).

Figure 5.

A. SEC analysis of dendribodies before purification and DTT treated scFv6H4Cys. The solid and dotted lines represent the dendribodies and scFv6H4Cys respectively. DTT treated scFv6H4Cys exists *in vitro* mainly as a monomer shown by prominent peak at 27 kDa. There is also trace quantity of dimeric species eluting at approximately 15 ml. The dendribodies elute as a single peak of 162 kDa followed by the peak of unreacted scFv6H4Cys. **B.** Western blot analysis showing (Lane 1) purified scFv6H4Cys, (Lane 2/Pre) dendribodies before purification and (Lanes 3–13) eluting fractions 1–11.

Figure 6.

Purification of the dendribodies by immobilized metal affinity chromatography: **A.** Separation profile of dendribodies from unreacted PEG₂₄ modified dendrimers and scFv6H4Cys. Peak assignments: (1–3) unreacted PEG₂₄ modified dendrimers, (4–6) dendribodies and (7–15) mixture of dendribodies and unreacted scFv6H4Cys. **B.** SDS-PAGE analysis of the fractions eluted with imidazole gradient **C.** SDS-PAGE analysis of pooled and concentrated elution fractions 4–6.

Figure 7.

In vitro saturation binding assay: Saturation data for specific binding of ^3H -METH to scFv6H4Cys, dendribodies and PEG₂₄ modified dendrimers with corresponding Scatchard plots (inset). ScFv6H4Cys retained affinity for ^3H -METH after conjugation to the dendrimers. Data points are the mean $\pm$ S.E.M of triplicate determinations.

Scheme 1.

Synthesis of anti-METH scFv6H4Cys-dendrimer conjugates (dendribodies). G3 PAMAM dendrimer **1** is reacted with a heterobifunctional PEG₂₄ crosslinker **2** to produce PEG modified dendrimer **3** along with the loss of NHS group. **3** is then conjugated to reduced scFv6H4Cys **4**, via a thioether bond to produce the dendribody **5**. Reagents and conditions: (a) Phosphate buffered saline, pH 7.4, 2 mM EDTA, room temperature (RT), dark, shaking, 2 hr; (b) 50 mM Sodium phosphate buffer, pH 6.4, 150 mM NaCl, 2 mM EDTA, RT, dark, shaking, 2 hr.

Table 1

The theoretical and experimental mass additions of PEG₂₄ to G3 PAMAM dendrimers corresponding to each reaction ratio of PEG₂₄:G3 dendrimer. The apparent molecular weights of PEG₂₄ and dendrimer are obtained from the manufacturers and calculated from MALDI-TOF spectra.

PEG:dendrimer reaction ratio	Theoretical MW ^a Da	MALDI-TOF ^b <i>m/z</i>	PEG/dendrimer ratio
5:1	13324	13144	5
11:1	20998	29259	16
16:1	27393	34126	20
32:1	47857	34913	20

^aTheoretical mass addition by dendrimer and PEG is 6929 and 1279 Da respectively

^bExperimental mass addition by dendrimer and PEG is 6937 and 1280 Da respectively