Title
Design, Synthesis, and Testing of Synthetic Vectors for siRNA Delivery

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Design, Synthesis, and Testing of Synthetic Vectors for siRNA Delivery

Dissertation

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for the degree of

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in Chemistry

by

Mark Edward Johnson

Dissertation Committee:
Professor Zhibin Guan, Chair
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ABSTRACT OF THE DISSERTATION

Design, Synthesis, and Testing of Synthetic Vectors for siRNA Delivery

By

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Doctor of Philosophy in Chemistry

University of California, Irvine, 2016

Professor Zhibin Guan, Chair

The delivery of nucleic acids, in particular small interfering RNA (siRNA), holds great potential for research and therapeutic applications; however, the design of effective delivery vectors remains a major challenge. In this dissertation, we have pursued a variety of strategies for the synthesis and discovery of vectors for the delivery of siRNA.

Chapter 1 provides a brief introduction to the applications of nucleic acid delivery and the challenges involved in successfully siRNA to cellular targets both in vitro and in vivo. Several previously developed vector systems which heavily influenced the design of the vectors discussed in this dissertation are discussed. Chapter 2 details the design and development of a dendritic peptide bolaamphiphile vector capable of efficient siRNA delivery. Chapter 3 discusses attempts to modify and optimize the original bolaamphiphile system through co-formulation, synthesis of discrete variants, and alteration of the aromatic component. Chapter 4 discusses the design, synthesis, and testing of fluorocarbon modified polyethylenimine for the purpose of siRNA delivery. Chapter 5 explores the use of gold nanoparticles functionalized with dendritic amino acid based ligands, again for the delivery of siRNA.
Chapter 1: Introduction to Synthetic Vectors for Nucleic Acid Delivery

1.1 Applications and Challenges for Nucleic Acid Therapeutics

The delivery of nucleic acids (NA) has long been recognized to hold great therapeutic potential. Despite this promise, however, difficulties involved in safe and effective delivery have prevented the wide spread clinical application of NA therapeutics. When the NA in question is DNA or messenger RNA (mRNA), successful delivery can potentially be used to express any protein of interest. While the successful delivery of DNA requires entry into the nucleus and can lead to permanent genetic modification, properly designed mRNA has recently been demonstrated capable of inducing robust, transient expression of a desired protein target.\(^1\) The delivery of mRNA has recently become an area of great interest due to the high expression levels, cytosolic target and lack of permanent genetic alteration. Alternatively, if the goal is to silence expression of a target protein, delivery of certain types of RNA can be utilized to effectively and transiently knockdown expression with little off-target effects, potentially facilitating the treatment of a wide variety of diseases and congenital disorders.

Since the discovery and elucidation of the RNA interference (RNAi) pathway, the delivery of short interfering RNA (siRNA) has proved invaluable for research applications and promising for the development of novel therapies.\(^1b-f\) In the RNAi pathway, the Dicer protein cleaves endogenous or exogenous RNA to give short, double-stranded RNA fragments approximately 21 nucleotides in length (Figure 1.1).\(^2\) The RNA-
induced silencing complex (RISC) recognizes these fragments and loads the guide strand, with the activated RISC complex proceeding to catalytically degrade mRNAs possessing sequences complementary to the loaded RNA guide strand. Due to the efficacy and specificity of this process, delivery of exogenous siRNA has the potential to efficiently silence the expression of any protein without permanent alteration to the genome. Effective and safe delivery has proven a critical barrier to successful clinical application. Advances in the discovery and understanding of siRNA delivery vehicles, termed “vectors”, has led to exciting developments in the field of RNAi therapeutics. Recent Phase I and II trials have demonstrated up to 98% gene knockdown in liver targets and two Phase III trials are currently underway targeting syndromes caused by mutations in transthyretin.²

The successful delivery of NAs presents a significant challenge due to their physical and chemical properties. Compared to traditional drug molecules, NAs are hydrophilic, high molecular weight and possess a high density of anionic charge. Lacking the proper delivery agent or modifications, naked DNA or RNA has poor cellular uptake and is subject to rapid degradation by endonucleases or clearance from systemic circulation in vivo.³ Delivery vectors are often required to affect sufficient cellular

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Figure 1.1. Mechanism of gene silencing by siRNA. Reprinted from ref. 2.
uptake and induce the desired biological response. While viral vectors have been used due to their high transfection efficiency *in vitro* and in animal models; issues with toxicity, immunogenicity, and negative publicity have greatly hindered their clinical application. In addition, viral vectors are difficult or impossible to modify if one wishes to reduce toxicity/off-target effects, enhance efficiency/specificity, or alter the nature of the cargo. In comparison, given the proper approach simple chemistries and cheap starting material have been shown capable of producing highly modifiable and ultimately effective synthetic vectors. A wide range of synthetic vectors have been studied for the delivery of NAs including lipids, polymers, nanoparticles (NP), peptides, recombinant proteins, and RNA aptamers.

The first requirement for a synthetic vector is that it effectively binds to the desired NA; often this is driven by the electrostatic interaction between a positively charged functionality on the vector, most commonly an amine, and the phosphate backbone of the NA. The binding of NAs by vectors commonly leads to the formation of NPs, termed “complexes” with an appropriate prefix. When used for *in vitro* applications, the next major requirement is that the complex effectively associate with the
cellular membrane and induce sufficient uptake. Due to the overall anionic charge of cellular membranes, cationic functionality is often beneficial for this step as well. Upon initial cellular uptake, most complexes enter the endosomal system and must escape at some point during the subsequent acidification. Failure to do so results in entrapment and lysosomal degradation or exocytosis. One recent imaging study has suggested that siRNA contained in lipid nanoparticle (LNP) therapeutics capable of effective hepatic silencing nonetheless may have as low as a 1-2% endosomal escape efficiency. Once the NA/vector complex has successfully arrived at the target cellular compartment, the cytosol in the case of siRNA and mRNA, the final necessary step is to release the NA cargo.

While the barriers discussed above are the primary concerns for in vitro delivery, if one desires to deliver NAs in vivo additional challenges must be addressed. The complexes must be stable to the presence of serum components, of which negatively charged albumin proteins comprise a large percentage. In addition to purely electrostatic interactions, often vectors contain hydrophobic domains which can promote association with similarly hydrophobic serum components. This can have dramatic effects on efficiency and specificity, with incorporation of the lipoprotein Apolipoprotein E (ApoE) shown to be critical for the efficient hepatic uptake of certain LNP vectors. If the desired application calls for intravenous (i.v.) administration, the circulatory stability of the complexes is of critical importance. The particle size of the complexes is a key parameter, with small particles <20 nM in diameter subject to renal clearance (Figure 1.3). Particles larger than 200 nM in diameter often trigger immune response and larger aggregates can lead to circulatory blockage.
As mentioned previously, the surface charge of the complexes is an important factor for both cell internalization and interaction with serum components. Typically highly cationic complexes interact strongly with negatively charged serum components and cellular membranes, which can lead to toxicity and immune response. If the anionic charge density is too high, unfavorable interactions with negatively charged cell membranes will often prevent effective internalization. For therapeutic applications, the specificity of the complexes to the target tissue must be considered; both to maximize efficiency and minimize off-target effects. Passive targeting of particles with long circulation times via the enhanced permeation and retention (EPR) effect has been shown effective to target NPs to comparatively leaky tumor vasculature. Active targeting of complexes via the incorporation of cell specific ligands is another approach that can enhance specificity for a variety of targets.
1.2 Previous Vectors Studied for the Delivery of siRNA

The research and therapeutic applications of RNAi have led to strong interest in developing vectors capable of effectively delivering siRNA both in vitro and in vivo. A variety of synthetic materials have been explored for this purpose, with polymers and lipids being among the most effective and heavily studied. As discussed previously, endosomal escape of the complexes is a critical step for successful delivery regardless of target and even relatively effective systems may have low efficiency.\textsuperscript{6} For this reason, many effective synthetic siRNA vectors contain functional groups with buffering capacity in the range of endosomal acidification (pH = 5.5-7.4). Polymers such as polyethylenimine (PEI) become more cationically charged as the pH decreases, leading to enhanced membrane lytic activity and subsequent greater endosomal escape.\textsuperscript{11} According to the “proton sponge” theory, this buffering capacity also results in increased osmotic pressure in the endosome, which is also proposed to facilitate release (Figure 1.4).\textsuperscript{12} While increasing the efficiency of endosomal escape, the non-degradable and membrane lytic nature of PEI can often result in toxic effects.

![Figure 1.4. pH responsiveness nature of PEI leads to improved endosomal escape](image-url)
While the first report of successful RNAi therapy in primates made use of a polymeric vector system, lipids have also been heavily utilized for delivery purposes and currently account for a large number of siRNA therapeutics in clinical trials. Early lipid vectors often were highly cationic even at neutral pH, based upon lipids such as DOPE and DOTMA; or in the case of Lipofectamine, perhaps the most common commercial control, the multivalent DOGS (Figure 1.5a). Due to previously discussed reasons, the cationic nature of these early delivery systems often resulted in systemic toxicity and immunogenicity. More recently ionizable lipids which are primarily neutral at pH = 7.4 and only become charged under acidic conditions, such as DLin-KC2-DMA, have proven more efficient and biocompatible for in vivo delivery. Formulation of such lipids with cholesterol, PEG-lipids, and a zwitterionic component such as DOPE/DSPC has been demonstrated to produce highly stable LNPs (Figure 1.5b).

Figure 1.5. Structures of lipids/lipoids used for siRNA delivery. (a) early lipids, (b) components of effective LNP vector, and (c) examples of ionizable lipoids. Adapted from ref. 15, 16, and 17.
Compared to earlier lipids, the low charge density at physiological pH and PEGylation of current LNP delivery systems enhance circulation time and reduce toxic effects. Optimization of the ionizable component through high-throughput screening of synthetically accessible amphiphilic compounds, termed “lipoids”, has led to the discovery of NPs capable of potent hepatic silencing in primates at dosages as low as 0.03 mg / kg (C12-200), with ED$_{50}$ values as low as 0.002 mg / kg (cKK-E12) measured in murine models.$^{16}$ For comparison to commercially available vectors, the most recent in vivo formulation of Lipofectamine, Invivofectamine 3.0, reports an ED$_{50}$ of 0.1 mg / kg for hepatic delivery in mice according to the manufacturers website. Although for a variety of reasons complexes most often tend to accumulate in the liver; recently NPs formed using PEI modified with hydrocarbon epoxides have been reported to specifically target the lungs and endothelium with an ED$_{50}$ of approximately 0.02 mg / kg.$^{17}$ While efficient hepatic uptake of many lipids/LNPs has been demonstrated to be dependent upon incorporation of endogenous ApoE,$^7$ the reasons for the endothelial targeting of the PEI-based NPs are currently not well understood.

Previous work in the Guan lab has focused on the development of synthetic siRNA vectors based upon polymers generated from natural building blocks using simple chemistries. The most successful dendronized polymer, termed “denpol”, was formed from the step-growth polymerization of a diacid and diamine; followed by dendronization using successive couplings of Boc-Lys(Boc)-OH to the amine-functionalized polymer backbone (Figure 1.6).$^{18}$ Compared to PEI, the degradable nature of the polymer backbone and amino acid building blocks of this vector were expected to provide greater biocompatibility. In order to replicate the pH responsive character of PEI, histidine was
functionalized by the carboxylic acid to the outer layer of the amine-terminated denpol, with the imidazole side chain providing buffering capacity in the appropriate range and the primary amine allowing for electrostatic complexation under physiological conditions. Through a focused library screening approach, it was found that additionally an aromatic component, such as Trp/Phe/Tyr, was necessary for effective siRNA delivery with a 3:1 molar ratio of His:Trp functionalized to the periphery proving most efficacious (Figure 1.7). Another critical design component of the denpol system was the integral disulfide bonds of the polymer backbone. Due to relatively high intracellular
concentration of the reducing peptide glutathione, this should allow for efficient polymer degradation and siRNA release once the complexes successfully arrive in the cytosol.

1.3 Summary and Outlook

Although many advances have been made in the development of synthetic NA vectors, the critical challenges of endosomal escape and efficient in vivo delivery to multiple targets have yet to be fully solved. While in vitro transfection is generally considered less challenging than in vivo, certain immortalized and primary cell lines are resistant to most commercially available transfection reagents. New NA vectors and synthetic approaches allowing for increased delivery efficiency or specificity for non-hepatic targets are of great interest for both research and therapeutic applications. While the delivery of siRNA has been a topic of great interest for some time, only recently has the delivery of properly modified mRNA been proven as an effective means to express a protein of interest. The development of vectors for mRNA delivery has thus far been relatively unexplored and may potentially have profound impact.

The following work details efforts to design, optimize, and characterize novel vectors for the delivery of nucleic acids. Using lessons learned from the denpol and other systems, several siRNA delivery systems were designed based upon amphiphilic small molecules, functionalized nanoparticles, or polymers. Amino acid building blocks were commonly utilized due to their biocompatible nature and the wide variety of functionality available. The pH responsive nature of the His side chain and aromatic functionality of Trp were often incorporated to improve cytosolic delivery. In general, focused library approaches were employed to generate series of novel vectors suitable for NA delivery.
Efforts in the Guan lab are ongoing to further optimize the successful systems for specific \textit{in vivo} applications and design new vectors for delivery of both siRNA and mRNA.

\subsection*{1.4 References}


Chapter 2: Design of Dendritic Peptide Bolaamphiphiles for siRNA Delivery

2.1 Introduction

Since its discovery, RNAi has demonstrated tremendous utility in a variety of biological applications, including experimental biology,\textsuperscript{1} functional genomics,\textsuperscript{2} stem cell research,\textsuperscript{3} and disease treatment.\textsuperscript{4-5} The high potency and specificity of gene silencing induced by small interfering RNA (siRNA) makes this technology particularly appealing for medicinal applications; however, safe and efficient delivery of siRNA into targeted cells remains a major challenge.\textsuperscript{5-7} Much effort has been devoted to the development of synthetic delivery vectors\textsuperscript{8} with a variety of systems being investigated, including lipids,\textsuperscript{9-11} peptides,\textsuperscript{12-15} polymers,\textsuperscript{16-19} dendrimers,\textsuperscript{20,21} and gold nanoparticles.\textsuperscript{22,23} Cationic lipids (such as Lipofectamine) and polymers (such as poly(ethylene imine), PEI) have been widely used in a variety of biological studies; however, toxicity and delivery efficiency are often limiting factors for these early vectors.\textsuperscript{24-26} Recently lipid nanoparticles (LNPs)\textsuperscript{8,11,27} and polymeric vectors\textsuperscript{16,28} have demonstrated great promise for therapeutic delivery of siRNA to the liver and solid tumors. Despite major advances, the efficiency of endosomal escape of most vectors is generally low\textsuperscript{29,30} and interaction of conventional cationic lipids and polymers used for gene delivery with the cell membrane can result in membrane disruption, altered cell behavior and cytotoxicity.\textsuperscript{24,26,31-33}

Scheme 2.1. Efficient siRNA delivery using bolaamphiphile vectors
Reproduced with permission from \textit{ACS Central Science}, \textbf{2015}, \textit{1}, 303-312.
Copyright 2015 American Chemical Society.
While combinatorial approaches have proven useful for the discovery of new vectors, a deeper understanding of how structural parameters affect transfection efficiency, circulatory stability, serum resistance, and ultimately in vivo efficacy would be of great value for the further development of synthetic siRNA vectors. Toward this goal, a systematic approach based on direct structure-activity correlation should advance our basic understanding and facilitate the rational design of effective new vectors.

2.2 Results and Discussion

Herein we report the rational design of a dendritic peptide bolaamphiphile for safe and efficient siRNA delivery (Fig. 2.1). Unlike regular lipid-like amphiphiles (termed “mono amphiphile”), which are composed of one or more hydrophobic tails and a single hydrophilic head group, bolaamphiphiles (termed “bola”) are dumbbell-shaped molecules having two hydrophilic head groups connected via a hydrophobic core (Fig. 2.1a-b). In nature, bolaamphiphiles are found in the cell membrane of certain kinds of extremophile archaebacteria with the monolayer assemblies, as opposed to the bi-layer structures of normal lipids, providing improved stability under harsh conditions. The robust self-assembly of bolaamphiphiles makes them attractive for biomaterials applications, with several recent reports of gene delivery using bolaamphiphile-based vectors. We envision that the unique molecular architecture of bolas can be exploited to disfavor insertion into the cell membrane and offer a more biocompatible alternative to conventional lipids. The use of a hydrophobic core shorter than the native phospholipid bilayer should prevent direct membrane insertion due to unfavorable thermodynamics, with the use of relatively large dendritic head groups to disfavor a U-shaped conformation (Figure 2.2a).
The second important design component is the choice of a dendritic peptide head group (Fig. 2.1b,c), for which we chose a lysine-based dendron functionalized with 75 mol% histidine (His) and 25 mol% tryptophan (Trp), the optimal combination for our previous dendronized polymer vector.\textsuperscript{41} The dendritic head group provides multivalent interactions for efficient binding of siRNA, with the size and valency controllable through the use of different dendron generations. Based upon our previous study, Trp improves siRNA binding and cell uptake; while His facilitates endosomal escape due to the pH-responsive nature of the imidazole ring.\textsuperscript{41}

\textbf{Figure 2.1} Design concept and structure of bolaamphiphiles. \textbf{a.} Schematic illustration of mono amphiphile and bolaamphiphile. \textbf{b.} Design of dendritic peptide bolaamphiphiles. Bolaamphiphiles are composed of two dendritic peptide head groups linked to a hydrophobic core by reducible disulfide linkages. The multifunctional dendritic peptide head groups are functionalized with 75 mol% histidine (His) and 25 mol% tryptophan (Trp) on periphery. \textbf{c.} Structures of dendritic peptide bolaamphiphiles. The structure of \textbf{bola-C18-G2} is shown as an example.
The third key component in our bola design is the hydrophobic core for promoting self-assembly in aqueous solution. As shown in Fig. 2.1c, a variety of hydrophobic cores were studied by systematically changing several molecular parameters such as the length, geometry, and chemical nature (hydrocarbon, versus fluorocarbon). Given the unique self-assembly properties of fluorocarbons and previous reports on fluorinated vectors,42-46 we envisioned that the fluorocarbon linker could promote robust self-assembly and also impact the cellular uptake, serum stability, and biodistribution of the complexes. The final design element is the disulfide linkages (Fig. 2.1b,c), connecting the dendritic peptide head groups to the central hydrophobic core to provide stimuli-responsive disassembly of the complexes, facilitating siRNA release.

A typical peptide bola structure (bola-C18-G2) is shown in Fig. 2.1c. The amphiphiles are named as follows: m- for mono amphiphile and bola- for bolaamphiphile, followed by the structure of the hydrophobic core (e.g., C18) and then the generation of the dendron head groups (e.g., G2). With the modular design, all three components could be modified for systematic structure-property studies. Different hydrophobic cores were used, including long chain hydrocarbons (C6-C22) and a fluorocarbon (F10). The length of the hydrophobic cores was chosen to be significantly shorter than the width of a typical phospholipid bilayer membrane so that bolas will not be able to span across the entire bilayer and cause cell membrane disruption. A control molecule containing a hexa(ethylene glycol) core (HEG) was included as the hydrophilic core should not induce self-assembly in aqueous solution. A hydrophobic core containing a 1,2,3-triazole (TZ) was also synthesized to probe the effect of the heterocycle incorporation. The efficient “click” chemistry used in the synthesis of the TZ linker offers opportunity to construct asymmetric bolaamphiphiles in future studies. The size of the dendron head group was varied from first to third generation (G1-G3). For a direct comparison between
mono and bola amphiphiles, three mono amphiphile analogs were synthesized containing either a C11 alkyl tail (m-C11) or one to two oleic acid moieties (m-OA or m-OA2), with the precise structure found in the experimental section. All molecules were synthesized by solution-phase coupling reactions and detailed synthesis and characterization data can be found in the supporting information.

Our hypothesis of low membrane disruption for bolas was first tested by hemolysis assays. Bovine red blood cells (RBC) were treated with the amphiphiles at various concentrations and the release of hemoglobin from RBC was measured as an indicator of membrane lytic activity. Supporting our hypothesis, the mono amphiphiles exhibited much higher hemolysis than bolas (Fig. 2.2b). The hydrophobic tail of mono amphiphiles significantly influenced their hemolytic activity, as the longer OA amphiphile exhibited 4 times higher membrane lysis than the shorter C11 amphiphile. The double tailed OA2 amphiphile induced a relatively high degree of hemolysis at lower concentrations, but plateaued at higher concentration, possibly due to self-assembly at higher concentration. In sharp contrast, the C18 bolas exhibited very low hemolytic activity, with G2 inducing less than 1% hemolysis and G1 ~3% hemolysis at 250 μg/mL. The higher hemolysis induced by G1 bola agrees with our proposed head group size effect, as the small G1 dendron provides less steric hindrance to the U-shaped conformation. The different hemolytic activity of these dendron amphiphiles correlates well with their cytotoxicity. As shown in Fig. 2.2c, all G2 bolaamphiphiles are about two orders of magnitude less cytotoxic to 3T3 fibroblast cells than the analogous mono amphiphiles. The increased cytotoxicity of bola-C18-G3 is presumably due to the higher valency of cationic
The mono and bolaamphiphiles were screened for gene silencing activity in NIH 3T3 cells expressing green fluorescent protein (GFP). Initial transfection and gel electrophoresis experiments suggested that while most vectors fully complexed siRNA by N/P = 10 (the molar ratio of the charged amines of the vector to the phosphates of RNA) the knockdown effect was

charges of the G3 dendron. For comparison, branched poly(ethylene imine) (PEI, $M_n \sim 25$ kDa), a commonly used polymer for gene delivery studies induces much greater toxicity than both mono- and bola-amphiphiles, further confirming the safety of bolas for siRNA delivery.

The mono and bolaamphiphiles were screened for gene silencing activity in NIH 3T3 cells expressing green fluorescent protein (GFP). Initial transfection and gel electrophoresis experiments suggested that while most vectors fully complexed siRNA by N/P = 10 (the molar ratio of the charged amines of the vector to the phosphates of RNA) the knockdown effect was

Figure 2.2. Hemolytic activity and cytotoxicity of bolaamphiphiles. a. Proposed non-membrane disruptive character of dendritic bolaamphiphiles in comparison to monoamphiphiles. Due to the unique molecular architecture, bolaamphiphiles should be more difficult to insert into cell membrane, causing less membrane disruption. In contrast, monoamphiphiles can insert into membrane more easily and cause membrane disruption due to their structural similarity to native phospholipids. b. Hemolytic activity of both mono- and bolaamphiphiles as % hemolysis of bovine red blood cells. c. Cytotoxicity of both mono- and bolaamphiphiles to NIH 3T3 cells as determined by MTT assay.
not saturated until N/P = 30 – 45 and for all further studies a N/P ratio of 45 was utilized (Fig. 2.6). For comparison to the toxicity and hemolysis assays, **bola-F10-G2** complexes formed at N/P = 45 with [siRNA] = 100 nM have a vector concentration of 110 μg/mL, at which no significant toxic effect was observed (Fig. 2.2c). In general, mono amphiphiles showed very little gene silencing with the most efficacious, **m-OA2-G2**, inducing only ~25% knockdown while bolaamphiphiles containing the fluorocarbon (**F10**) or hydrocarbon cores of sufficient length (C18, C22) with G2 or G3 head groups displayed effective gene silencing (Fig. 2.3a,b). The introduction of triazole ring in the hydrophobic core (**TZ**) did not significantly change the transfection efficiency. Bolaamphiphiles based on shorter cores (**bola-C6-G2** and **bola-C12-G2**) or bearing G1 head groups failed to induce a strong knockdown response (Fig. 2.3a). The vector containing a hydrophilic linker (**bola-HEG-G2**) was completely ineffective at gene silencing, presumably due to the lack of hydrophobic self-assembly.

To understand the correlation between molecular structure and delivery efficiency, transmission electron microscopy (TEM) was utilized to examine the morphology of different amphiphile/siRNA complexes. Figure 2.3c-e displays TEM images of different **bola-C18**/siRNA complexes stained with uranyl acetate, demonstrating that the size of the dendritic head group influences the morphology of the assembled nanostructures dramatically. The compound with the smallest head group, **bola-C18-G1**, can pack closely to form twisted nanofibers, similar in morphology to the structures previously reported for well-packed bola assemblies (Fig. 2.3c). As the head group increases in size, both steric effects and charge repulsion disfavor the formation of densely packed nanofibrils, with **bola-C18-G2** and **bola-C18-G3** complexes appearing as circular features consistent with assembly into nanoparticles (Fig. 2.3d,e). The larger head group of G3 bolaamphiphiles further reduces the packing efficiency for the
**Figure 2.3. Transfection, TEM imaging, DLS analysis and gel electrophoresis of different amphiphile/siRNA complexes.**

**a, b.** Initial transfection screening of bola and mono amphiphiles in GFP-expressing NIH 3T3 cells. N/P ratio = 45 and [siRNA] = 100 nM.  
**c-e.** Negative-stain TEM (uranyl acetate) images of vector/siRNA complexes: bola-C18-G1 (c), bola-C18-G2 (d), and bola-C18-G3 (e), scale bars = 200 nm.  
**f, g.** Cryo-TEM imaging of bola-C18-G2 in PBS, scale bar = 200 nm (f) and 100 nm (g).  
**h.** Dynamic light scattering (DLS) particle size and zeta potential analysis of vector/siRNA complexes prepared at N/P = 45 in PBS.  
**i-k.** Dextran sulfate competitive binding assay with different bola complexes (N/P = 45) with the numbers above the wells indicating the S/P ratio (molar ratio of sulfate on dextran sulfate to phosphates on siRNA).  
**l.** Triggered siRNA release by reducing reagent glutathione (GSH). Different bola-G2 complexes (N/P = 45, named by the core) were treated with GSH (+) or PBS (-) for 60 minutes before gel electrophoresis.
hydrophobic core, resulting in larger particles than G2 bolaamphiphiles. Complexes formed using G2 bolaamphiphiles containing fluorocarbon (F10) or 1,2,3-triazole (TZ) cores also displayed nanoparticle morphology (Fig. 2.7a-c), as did both mono-amphiphiles containing a single hydrophobic tail (m-C11-G2, m-OA-G2, Fig. 2.7d,e). The m-OA2-G2 complexes formed fibrillar structures similar to those observed in the bola-C18-G1 images, potentially due to increased hydrophobic interactions from the additional oleate tail enhancing the stability of assembly (Fig. 2.7f). Cryo-TEM was used to observe the morphology of the vector/siRNA complexes in PBS with bola-C18-G2 chosen for initial analysis. The cryo-TEM images of bola-C18-G2 (Fig. 2.3f,g) complexes were characterized by nanoparticles with an average diameter of 88 ± 19 nm which appear to be composed of smaller “granules” approximately 10-20 nm in diameter. Nanoparticles displaying similar “raspberry” morphology have been observed for siRNA complexes of the peptide vector CADY.47

The particle size and zeta potential of the complexes were analyzed using dynamic light scattering (DLS) (Fig. 2.3h). Complexes formed from vectors lacking a hydrophobic core or sufficient length (bola-HEG-G2, bola-C6-G2) did not provide enough signals for analysis, consistent with a lack of assembly. The complexes that formed fibrillar assemblies as observed by TEM (bola-C18-G1, m-OA2-G2) produced scattering signal but the data did not correlate when analyzed via standard methods, possibly due to the formation of irregular aggregates or fibrillar networks. For both bola-C18 and bola-F10 vectors the particle size of G2 was smaller than that of the G3, while the particle size decreased with increasing length of alkyl core from bola-C12-G2, bola-C18-G2, to bola-C22-G2. The trend is consistent with proposed assembly stability, with smaller head group and longer hydrophobic core condensing siRNA into smaller particles and larger head group and shorter core into larger particles. The zeta potential of the
vector/siRNA complexes was also measured, with all samples displaying a positive surface charge between 15-20 mV. Mono amphiphile/siRNA complexes, m-C11-G2 and m-OA-G2, showed similar particle characteristics in DLS, with slightly larger size than the bola analog (bola-C22-G2).

In our bola design, we hypothesized that the more stable nanoparticles formed by bolaamphiphiles should enhance the siRNA binding strength, which was assessed via a competitive binding assay using the anionic polymer dextran sulfate (DS, $M_n \sim 25$ kD). Although m-C11-G2 and bola-C22-G2 have exactly the same chemical composition, the bola complexes exhibited much higher stability with little siRNA release up to S/P = 23 (the molar ratio of sulfate from DS and phosphate from siRNA), while the mono amphiphile complex started to release siRNA at S/P = 8 (Fig. 2.8a,b). Furthermore, the proposed self-assembly model agrees with the stability of different bola complexes. As shown in Fig. 3i-k, despite having head groups with the lowest level of multivalency, bola-C18-G1 exhibited the strongest siRNA binding with no siRNA release up to S/P of 30, presumably due to the stable fibrillar assembly. The vectors bola-C18-G2 and bola-C18-G3, which demonstrated non-fibrillar assembly by TEM, showed siRNA release at the lower S/P ratios of 23 and 15, respectively. The fluorocarbon vector, bola-F10-G2, demonstrated stronger siRNA binding than the hydrocarbon analog bola-C18-G2 with no siRNA release until S/P = 30 (Fig. 2.8c), again presumably due to the more robust self-assembly induced by the fluorocarbon core. The bolaamphiphiles containing either the shortest hydrocarbon (bola-C6-G2) or hydrophilic core (bola-HEG-G2) failed to effectively complex siRNA (Fig. 2.8e,g). Stimuli-responsive siRNA release from bola complexes was demonstrated by incubation of the complexes with glutathione (GSH) followed by subsequent gel electrophoresis, which showed effective decomplexation for the GSH treated samples (Fig. 2.3l).
The different self-assembly behavior was corroborated by infrared (IR) spectroscopy. The methylene (CH$_2$) groups in the alkyl region prefer trans conformation in highly ordered packing structure, resulting in lower frequency for their C-H stretching peaks. Fig. 2.9 shows that from G1 to G3 bola complexes, both the asymmetric (~2930 cm$^{-1}$) and symmetric (~2850 cm$^{-1}$) C-H stretch peaks shifted to higher frequency, indicating less ordered structure in the alkyl region. This agrees with the proposed self-assembly model, with decreasing packing order from nanofibril to nanoparticle, caused by the enhanced electrostatic repulsion and steric associated with increasing valency of the cationic dendron.

The cellular uptake of amphiphile-siRNA complexes was investigated by confocal fluorescence microscopy using Cy3 labeled siRNA (Fig. 2.4a-d). The mono amphiphile (m-C11-G2) complexes showed no cellular uptake with no signal from siRNA detectable (Fig. 2.4a), while bola-C18-G1/siRNA complexes formed aggregates on the cell surface and were not internalized (Fig. 2.4b), in agreement with the observed low gene knockdown for both of these vectors (Fig. 2.3a,b). Complexes formed with both bola-C18-G2 and bola-C18-G3 showed significant cellular uptake of siRNA, with G2 exhibiting higher efficiency (Fig. 2.4c,d). The Cy3-siRNA uptake was further quantified by flow cytometry (Fig. 2.4e-h). Compared to Lipofectamine, Cy3-siRNA complexes with all three mono amphiphiles showed very low cellular uptake (Fig. 2.4e), while bola-C18-G2 and bola-F10-G2 exhibited 20 to 40 times higher uptake than Lipofectamine (Fig. 2.4f). Bolas with either shorter alkane cores (C6 and C12) or a hydrophilic HEG core could not form stable complexes and no cellular uptake of Cy3-siRNA was detected (Fig. 2.4f). Notably, cell uptake efficiency was directly related to the size of head group. Both boa-C18-G2 and bola-F10-G2 showed much higher cell uptake than their G3
analogs (Fig. 2.4g,h), which could be attributed to the higher stability and smaller size of G2 complexes.

To further understand the cell uptake process, we chose **bola-F10-G2** and **bola-F10-G3** as representative vectors to study the cell uptake pathway. Fig. 2.4i-k shows that the cell uptake

**Figure 2.4. Cell uptake of different amphiphile/siRNA complexes.** Cy-3 siRNA was complexed with different amphiphiles and transfected to NIH 3T3 cells. a-d. Confocal fluorescence images of transfected cells (cell nuclei were counter-stained with DAPI, scale bar: 20 µm). e,f. Cell uptake of siRNA complexes with different mono and bola amphiphiles quantified by flow cytometry. g,h. Cell uptake of G2 and G3 bolaamphiphile vectors. i,j. Transfection of Cy3-siRNA complexes carried out at 37 °C or 4 °C. k. Transfection of 3T3 cells pre-treated with sodium azide (NaN₃) and 2-Deoxy-D-glucose (DG). l,m. Endocytic pathway of bola complexes. 3T3 cells were treated with chlorpromazine (30 µM, inhibitor of clathrin-mediated endocytosis), genistein (350 µM, inhibitor of caveolar endocytosis), or ethylisopropylamiloride (EIPA, 30 µM, inhibitor of macropinocytosis) for 1 h before transfection.
of bola/Cy3-siRNA complexes are energy dependent, with low temperature (4 °C) completely shutting down the uptake (Fig. 2.4i,j) and the metabolic inhibitor NaN₃/2-deoxy-D-glucose inhibiting uptake in a concentration dependent manner (Fig. 2.4k). The cell uptake mechanism was further studied by using several small molecule inhibitors to specifically block three common endocytic pathways: clathrin-mediated endocytosis (chlorpromazine), caveolar endocytosis (genistein) and macropinocytosis (EIPA). As shown in Fig. 2.4l and 2.4m, siRNA/bola-F10-G2 complexes were internalized through both clathrin-mediated and caveolar pathways. The bola-F10-G3 complexes entered the cell through caveolae-mediated endocytosis almost exclusively, which can be explained by the larger particle size of the bola-F10-G3 complexes.⁴⁸,⁴⁹ The clear dependence of cell uptake on inhibitor concentration (Fig. 2.10) further confirms the cellular uptake pathway.

To demonstrate the general efficacy of these vectors, transfections were carried out in the following luciferase-expressing cell lines: MDA-MB-231, HEK, INS-1 and 4T1 (Fig. 2.5a). Similar to the results obtained from 3T3 cells, mono amphiphile m-C11-G2 did not achieve any significant gene knockdown and induced substantial toxicity in HEK and 4T1 cells. In contrast, bolaamphiphiles C18-G2 and F10-G2 both displayed robust luciferase knockdown with minimal cytotoxicity. Notably, for the 4T1 cell line in which Lipofectamine failed to induce strong knockdown response, both C18-G2 and F10-G2 effectively silenced luciferase expression. The previously discussed transfection screenings used phosphate buffer (PBS, 10 mM phosphate, 10 mM NaCl, pH = 7.4) for sample preparation (Fig. 2.3a,b and Fig. 2.5a); however, it was observed during optimization that complexes prepared in OptiMEM induced significantly higher knockdown at low concentrations than those prepared in PBS.
Figure 2.5. Transfection study in different cell lines and in the presence of serum. a. Luciferase silencing data for various amphiphiles in different cell lines including MDA-MB-231, HEK 293, β-INS-1, and 4T1. N/P = 45, [siRNA] = 100 nM, viability measured by MTT assay. b,c. Dose/response curves of bola-C18-G2 (b) and bola-F10-G2 (c) vectors in MDA-MB-231 cells. N/P = 45 for all samples, [siRNA] indicated on x-axis. d. Transfection of MDA-MB-231 cells in serum-containing medium. N/P = 45, [siRNA] = 50 nM, final concentration of fetal bovine serum (FBS) in antibiotic-free DMEM transfection media indicated on x-axis.
This optimized protocol was used to further test the transfection efficiency of the vectors in MDA-MB-231 cells, with dose/response curves and IC50 values determined for **bola-C18-G2, bola-C18-G3, bola-F10-G2, and bola-F10-G2**. The fluorocarbon vector **bola-F10-G2** demonstrated highly efficient transfection with an IC50 value of 2.8 nM while the analogous hydrocarbon vector, **bola-C18-G2**, was less effective with an IC50 of 18.7 nM (Fig. 2.5b,c). For both C18 and F10 cores, the G3 variants displayed higher transfection efficiency than the corresponding G2 analogs, with calculated IC50 values of 1.0 nM and 7.4 nM for **bola-F10-G3** and **bola-C18-G2**, respectively (Fig. 2.11). These results suggest that the fluorocarbon linker greatly enhanced the delivery efficiency, with both F10 vectors displaying IC50 values ~7 times lower than the corresponding hydrocarbon analogs. The toxicity of the bolaamphiphile/siRNA complexes prepared using OptiMEM was assessed via MTT assay, with the results corroborating the previously observed trend of G3 inducing greater cytotoxicity than G2 for both C18 and F10 linkers (Fig. 2.12).

Although typical in vitro transfections are performed in reduced serum or serum-free media, successful transfection in the presence of negatively charged serum components is critical for in vivo application. To investigate the serum stability of our vectors, transfections were performed varying the final concentration of fetal bovine serum (FBS) in the transfection media from 0 to 80% (Fig. 2.5d). While Lipofectamine RNAiMAX and **bola-C18-G2** were negatively affected even at low serum content, the gene silencing effects of **bola-F10-G2** were not affected by the presence of up to 80% FBS. The analogous G3 variant, **bola-F10-G3**, showed a slight decrease in efficacy at higher FBS concentrations, which can be attributed to less stable assembly and consequentially weaker siRNA binding due to the larger dendritic head group. The improved serum stability and efficacy of our fluorocarbon bolaamphiphiles compared to those...
containing hydrocarbon cores is in agreement with previous reports on the use of fluorinated lipids for siRNA delivery.\textsuperscript{44} With recent reports of fluorination greatly enhancing DNA transfection efficiency of PAMAM dendrimers,\textsuperscript{45,50} we believe this “fluorocarbon effect” may be generally applicable to other amphiphilic vectors.

2.3 Conclusion

With the high siRNA transfection efficiency in multiple cell lines, excellent serum resistance, and low cytotoxicity and hemolysis, our rationally designed dendritic peptide bolaamphiphiles bode well as promising candidates for gene delivery applications. Compared to the mono amphiphiles studied, all G2 bolaamphiphiles displayed stronger siRNA binding, reduced cytotoxicity, and greatly enhanced transfection efficiency. The size of the cationic dendron was observed to have a major influence on the morphology and biological activity of the complexes, with the fibrillar assemblies formed by \textbf{bola-C18-G1} inducing dramatically less knockdown than the nanoparticle \textbf{bola-C18-G2} and \textbf{bola-C18-G3} complexes. The use of a fluorocarbon, as opposed to hydrocarbon, hydrophobic core greatly enhanced both the transfection efficacy and serum stability of the complexes; suggesting that further investigation into fluorocarbon-based vectors may prove particularly fruitful. The high transfection efficiency of the fluorocarbon vectors and the direct correlation from molecular structure to self-assembly behavior to subsequent biologic activity offers critical insight aiding the rational design of new materials for nucleic acid delivery and other biomaterial applications.
Acknowledgements

I thank Hanxiang Zeng for his contribution to the initial design, testing, and characterization as reported in his thesis. I also thank Tim Tiambeng for assisting with the synthesis. Additionally, I would like to thank Nathan Oldenhuis for synthesizing the triazole variants and performing additional biological testing.

2.4 Supplementary Figures

Figure 2.6. Transfection of NIH-3T3 cells with bolaampiphile vectors. GFP-expressing NIH-3T3 cells were treated with complex solutions at various N/P ratios with [siRNA] = 100 nM. Cells were exposed to transfection media for 4 hours with analysis by flow cytometry after 48 hours. Normalized GFP expression was calculated by comparing mean GFP fluorescence of cells treated with anti-GFP/vector complexes to that of cells treated with complexes formed using non-targeting siRNA.
Figure 2.7. Negative-stain TEM images of amphiphile/siRNA Complexes. Samples prepared at N/P = 45, [siRNA] = 8 μM and stained with uranyl acetate prior to imaging, scale bar = 200 nm.
Figure 2.8. Gel-shift complexation assays. a-c. Dextran sulfate competitive binding assays of amphiphile/siRNA complexes (N/P = 45) with m-C11-G2 (a), bola-C22-G2 (b), and bola-F10-G2 (c). d-h. siRNA complexation assay of different amphiphiles.
Figure 2.9. IR spectra of different bolaamphiphile complexes. All complexes were prepared at N/P = 10 and 20 μM siRNA concentration in PBS buffer and measured using a Thermo Scientific Nicolet iS5 iD5 ATR IR spectrometer.
Figure 2.10. Concentration dependent inhibition of cell uptake by different endocytotic inhibitors. NIH-3T3 cells pretreated with (A) chlorpromazine, (B) geneistein, or (C) EIPA were exposed to Cy-3-siRNA/vector complexes for 4 hours and cellular uptake assessed via FACS.
Figure 2.11. Dose/response curves of G3 vectors in MDA-MB-231 cells. MDA-MB-231 cells were treated with anti-Luc siRNA/vector and non-targeting siRNA/vector complexes at N/P = 45 using the optimized sample preparation protocol and bola-C18-G3 (left) or bola-F10-G3 (right).

Figure 2.12. Cytotoxicity of bolaamphiphile/siRNA complexes. MDA-MB-231 cells were treated with anti-Luc siRNA/vector at N/P = 45 using the optimized sample preparation protocol and after 48 hours the viability assessed via MTT assay.
2.5 Experimental

Materials. Unless otherwise noted, all reagents were used as received from commercial suppliers without further purification. Protected amino acids were purchased from Advanced ChemTech (Louisville, KY) and Aroz Technologies, LLC. (Cincinnati, OH). 1H,1H,12H,12H-perfluoro-1,12-dodecanediol was purchased from Exflour Research Corporation (Round Rock, TX). Coupling reagents were purchased from GL Biochem Ltd. (Shanghai, China). Branched polyethyleneimine (PEI, 25kDa) was purchased from Sigma-Aldrich (St. Louis, MO). Sodium Dextran Sulfate (25kDa) was purchased from TCI America (Portland, OR) and was used as received. GelRed™ siRNA stain was purchased from VWR (Radnor, PA). All siRNA used in this study was purchased from Ambion (Carlsbad, CA) with Silencer® Select negative control siRNA and Silencer®Cy™-3 labeled Negative Control siRNA used for control and cellular uptake studies, respectively. The sequences for the anti-GFP and anti-luc siRNA can be found in below. Lipofectamine RNAiMAX was purchased from Invitrogen (Carlsbad, CA) and used as a positive control following the manufacturer’s protocol.

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequence (sense)</th>
<th>Sequence (anti-sense)</th>
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<tbody>
<tr>
<td>Anti-GFP</td>
<td>5’-CAAGCUAGACCCUGAAGUUCTT-3’</td>
<td>5’-GAACUUCAGGGUCAGCUUGCC-3’</td>
</tr>
<tr>
<td>Anti-Luciferase</td>
<td>5’-AGACUAUAAGAUCAACUUt-3’</td>
<td>5’-AGAUUGAUCUAAUGUCUtg-3’</td>
</tr>
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All reactions were performed using HPLC grade solvents unless otherwise noted. All water used in biological experiments was nanopure water obtained from Barnstead Nanopure Diamond (Waltham, MA). Ultrathin Carbon Type-A, 400 mesh TEM grids were purchased from TED PELLA Inc. (Redding, CA). Unmodified NIH 3T3 cell and engineered NIH 3T3 cell expressing enhanced green fluorescent protein (GFP) were a generous gift from Professor Young Jik Kwon (Department of Chemical Engineering, UC Irvine, CA). Luciferase-expressing HEK,
MDA-MB-231, 4T1 cells were generously provided by Professor Jennifer Prescher (Department of Chemistry, UC Irvine, CA). INS-1 cells were generously donated by Professor Yoko Mullen (City of Hope, Duarte, CA). For hemolysis studies, 100% bovine red blood cells suspension was purchased from Lampire Biological Laboratories (Pipersville, PA). All cell culture media and fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA).

**Instrumentation.** All compounds were characterized by NMR and ES-MS. $^1$H NMR spectra were collected at the UC Irvine NMR Facility and recorded at 500 MHz on Bruker instruments (GN500 or CRYO500). Mass spectral data (ES-MS) was obtained from the UC Irvine Mass Spectrometry Facility and collected with a Micromass LCT spectrometer. $^1$H NMR chemical shifts were reported as values in ppm relative to specified deuterated solvents. The size and zeta potential of bola/siRNA complexes were measured at 633 nm using Zetasizer dynamic light scattering instrument (Malvern Instruments, Malvern, UK) at 25°C with detection angle of 173°. TEM was performed on a FEI Tecnai G2 TF20 high resolution TEM (Electron Imaging Center for NanoMachines, UCLA) operated at an accelerating voltage of 200 kV. The flow cytometry data was obtained on a Becton-Dickinson LSR II flow cytometer (Sue & Bill Gross Stem Cell Research Center, UCI) with an argon ion excitation laser at 488 nm. Confocal fluorescence images were acquired using a Zeiss LSM 510 inverted laser-scanning confocal microscope (Sue & Bill Gross Stem Cell Research Center, UCI). IR spectroscopy was performed using a Thermo Scientific Nicolet iS5 iD5 ATR IR spectrometer.
Synthesis of G2 Bolas with Hydrocarbon Cores

Scheme 2.2. General synthetic route to G2 dendritic peptide bolas having a hydrocarbon core. C18 core is shown as an example in this scheme. The synthesis of bolas with other hydrophobic cores follows the same route.
Synthesis of bola-C18-Cym-NH$_2$ (1): The diacid (121 mg, 0.353 mmol, 1 equiv) and mono-trt protected cystamine (278.8 mg, 0.706 mmol, 2 equiv) were dissolved in 6 mL DCM in a round bottle flask, followed by the addition of DIPEA (135 $\mu$L, 0.777 mmol, 2.2 equiv), EDC•HCl (148.9 mg, 0.777 mmol, 2.2 equiv), and HOBT (105.0 mg, 0.777 mmol, 2.2 equiv). The reaction mixture was left to stir at room temperature overnight. After the reaction, the mixture was diluted with 150 mL DCM and washed with 0.02 N HCl in brine, 1.0 M NaHCO$_3$, and brine three times. The organic layer was dried over Na$_2$SO$_4$ and the solvent was removed in vacuo. The crude product was purified by column chromatography (1~3% TEA in DCM). In a 15 mL round bottom flask, 1 (0.150 mmol) was dissolved in 4 mL DCM and 0.05 mL TIPS, followed by drop-wise addition of 0.5 mL TFA. The reaction was left to stir at rt for 1 h, and all volatiles were removed in vacuum. The crude product was purified by re-dissolving in minimum DCM/MeOH mixture and precipitated in Et$_2$O to give the TFA salt of 2 as a slightly yellow viscous solid (115 mg, 39% yield over 2 steps). $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 2.52 (t, $J = 6.5$ Hz, 2H), 2.30 (t, $J = 6.8$ Hz, 2H), 2.99 (t, $J = 6.7$ Hz, 2H), 2.87 (t, $J = 6.7$ Hz, 2H), 2.21 (t, $J = 7.5$ Hz, 2H), 1.62 (s, 2H), 1.32 (d, $J = 12.5$ Hz, 14H).
**Synthesis of G2-lysine dendron.** In a 100 mL flask equipped with stir-bar, H-Lys-OEt•2HCl (500 mg, 2.02 mmol, 1.0 equiv), and Boc-Lys-Boc-OH•DCHA (2135 mg, 4.04, 2.0 equiv) are added to a solution of DIPEA (0.775 mL, 4.55 mmol, 2.25 equiv) and DMF (35 mL). HOBt (615 mg, 4.55 mmol, 2.25 equiv) and EDC•HCl (872 mg, 4.55 mmol, 2.25 equiv) are added last. The reaction is stirred for 18 h at rt. After 18 h, the mixture was diluted with 70 mL DCM and washed 1 x with 70 mL 0.02 N HCl in brine, 1 x with 70 mL 1.0 M NaHCO₃, and 3 x with 70 mL of brine. The organic layer was dried over Na₂SO₄ and the solvent was removed in vacuum. The product was purified with flash chromatography using a solvent gradient from 70:30 EtOAc:Hexanes to 100:0 EtOAc:Hexanes. After removal of the solvent, 1511 mg of G2-lys dendron ester is obtained as a white solid (90% isolated yield).

The white solid is then dissolved in a solution of THF (11 mL) and H₂O (2.2 mL), and LiOH•H₂O (1518 mg, 36.2 mmol, 20.0 equiv) is added. The reaction is stirred for 3 h at rt. After the reaction is deemed complete the excess THF is removed via vacuum and the remaining mixtures is diluted with 100 mL DCM. 50 mL of brine is added to the mixture and shaken in an extraction funnel yielding an emulsion. 2.0 N HCl is then added drop wise until the reaction separates (~19 mL). The organic layer is then washed with 2 x 50 mL brine. The organic layer was dried over Na₂SO₄ and the solvent was removed in vacuum yielding 1395 mg of the G2-Lysine dendron (white solid, 96% isolated yield).
Synthesis of bola-C18-G2-NH₂ (2). In a two-dram vial, 2 (0.0565 mmol, 1 equiv), boc-protected G2-Lysine-Dendron (90.7 mg, 0.113 mmol, 2 equiv) and DIPEA (21 μL, 0.120 mmol, 2.15 equiv) were dissolved in 2.5 mL DMSO, followed by the addition of EDC•HCl (22.0 mg, 0.120 mmol, 2.15 equiv), and HOBt (16.2 mg, 0.120 mmol, 2.15 equiv). The reaction was left to stir at rt for 24h. After the reaction, the mixture was diluted with 150 mL DCM and washed with 0.02 N HCl in brine, 1.0 M NaHCO₃, and brine three times. The organic layer was dried over Na₂SO₄ and the solvent was removed in vacuum. The crude product was purified by dissolution in MeOH and precipitation in water. If necessary, the product was further purified by column chromatography (20~40% EtOAc in Hexanes). After purification, the Boc was deprotected in a solution of TFA solution (Typically, 1 mL TFA, 1.5 mL anisole, 2.5 mL DCM and 0.1 mL TIPS). The deprotection was done in 4h at rt, followed by solvent removal in vacuo. The product was then purified by dissolution in a minimum amount of MeOH and precipitation in Et₂O. The white precipitate was dissolved in nanopure water, filtered through a 0.22 μm filter and lyophilized to give 3 as a white powder. ¹H NMR (500 MHz, CD₃OD) δ 4.42 – 4.24 (m, 1H), 2.97 (t, J = 6.8 Hz, 1H), 2.85 (m, 1H), 2.52-2.41 (m, J = 14.0, 7.0 Hz, 4H), 2.31 – 2.13 (m, 2H), 2.07 – 2.92 (m, 4H), 2.84 (m, 4H), 2.21 (t, J = 7.5 Hz, 2H), 2.09 – 1.40 (m, 20H), 1.32 (s, 14H). ESI-MS+ (m/z): [M]+H calcd for C₆₄H₁₃₁N₁₆O₈S₄, 1379.91; found, 1379.9.
Synthesis of bola-C18-G2 (3): In a one dram glass vial were added 30 mg of the TFA-salt of the unfunctionalized bola 3 (1 equiv), boc-His(boc)-OH.DCHA (6 equiv) and boc-Trp(boc)-OH (2 equiv). 1.5 mL DMF was added to dissolve the solids, followed by BOP (8.2 equiv) and DIPEA (8.2 equiv). The reaction was left to stir for 24 hours at rt. The protected bola was precipitated in an excess amount of deionized water. After removing water completely, the solid was dissolved in 1 mL TFA, 2 mL DCM, 2 mL Anisole and 0.25 mL TIPS. After stirring overnight, the solvent was removed in vacuo, the resulting solid was redissolved in MeOH and precipitated in Et2O. The white precipitate was dissolved in water and lyophilized to give a white powder and if necessary purified by dialysis (MWCO = 1000) against MeOH. All bolas were characterized by $^1$H NMR. The functionalization ratio was determined by comparison of the characteristic aromatic peaks of imidazole (histidine) and indole (tryptophan) ring.

n=6, bola-C6-G2: $^1$H NMR (500 MHz, CD$_3$OD): δ 8.25-8.14 (6.0 H, histidine, 75 mol%), 7.65 (2.0 H, tryptophan, 25 mol%), 7.38 (2 H, tryptophan, 25 mol%), 7.23-7.06 (10 H), 7.03 (2 H, tryptophan, 25 mol%), 4.36-4.25 (10 H), 4.11 (4 H), 2.67-2.07 (multiple peaks overlapped with solvent peak, integration not accurate), 2.81 (8 H), 2.17 (2 H), 1.83-1.32 (44 H).

n=12, bola-C12-G2: $^1$H NMR (500 MHz, CD$_3$OD): δ 8.25-8.14 (5.8 H, histidine, 72 mol%), 7.65 (2.0 H, tryptophan, 25 mol%), 7.38 (2.0 H, tryptophan, 25 mol%), 7.23-7.06 (10 H), 7.03
(2.0 H, tryptophan, 25 mol%), 4.36-4.25 (10 H), 4.11 (4 H), 2.67-2.07 (multiple peaks overlapped with solvent peak, integration not accurate), 2.81 (8 H), 2.17 (2 H), 1.83-1.32 (56 H).

**n=18, bola-C18-G2 (3):** $^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 8.25-8.14 (5.56 H, histidine, 70 mol%), 7.63 (2.24 H, tryptophan, 28 mol%), 7.39-7.33 (8 H), 7.20-7.01 (2.31 H, tryptophan, 28 mol%), 4.36-4.25 (10 H), 4.11 (4 H), 2.67-2.07 (multiple peaks overlapped with solvent peak, integration not accurate), 2.81 (8 H), 2.17 (2 H), 1.88-1.25 (68 H).

**n=22, bola-C22-G2:** $^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 8.13-8.04 (6.0 H, histidine, 75 mol%), 7.61 (1.98 H, tryptophan, 25 mol%), 7.34 (1.98 H, tryptophan, 25 mol%), 7.19-7.00 (10 H), 6.98 (2.01 H, tryptophan, 25 mol%), 4.36-4.25 (10 H), 4.11 (4 H), 2.67-2.07 (multiple peaks overlapped with solvent peak, integration not accurate), 2.81 (8 H), 2.17 (2 H), 1.88-1.25 (70 H).

2.2 Synthesis of G1 and G3 Bolas

![Scheme](image)

**Scheme 2.3.** Synthetic route for bola-C18-G1.
Synthesis of bola-C18-G1-NH₂ (4). In a two-dram vial, 1 (0.0596 mmol, 1 equiv, 50 mg), Boc-lys(boc)-OH.DCHA (62.8 mg, 0.119 mmol, 2 equiv) and DIPEA (23 µL, 0.131 mmol, 2.2 equiv) were dissolved in 2.5 mL DMSO, followed by the addition of EDC·HCl (25.1 mg, 0.131 mmol, 2.2 equiv), and HOBt (20.1 mg, 0.131 mmol, 2.2 equiv). The reaction was left to stir at rt for 24h. After the reaction, the mixture was diluted with 150 mL DCM and washed with 0.02 N HCl in brine, 1.0 M NaHCO₃, and brine three times. The organic layer was dried over Na₂SO₄ and the solvent was removed in vacuum. The crude product was purified by dissolution in MeOH and precipitation in water. If necessary, the product was further purified by column chromatography (20~40% EtOAc in Hexanes). After purification, the Boc was deprotected in a cocktail of TFA solution (Typically, 1 mL TFA, 1.5 mL anisole, 2.5 mL DCM and 0.1 mL TIPS). The deprotection was done in 4h at rt, followed by solvent removal in vacuo. The product was then purified by dissolution in a minimum amount of MeOH and precipitation in Et₂O. The white precipitate was dissolved in nanopure water, filtered through a 0.22 µm filter and lyophilized to give 4 as a white powder (71.8 mg, 91% yield). ¹H NMR (500 MHz, CD₃OD) δ 2.90 (t, J = 6.3 Hz, 1H), 2.71 – 2.44 (m, 2H), 2.92 (m, 8H), 2.22 (t, J = 7.5 Hz, 2H), 1.94 (d, J = 16.3 Hz, 2H), 1.82 – 1.69 (m, 2H), 1.63 (s, 2H), 1.59 – 1.46 (m, 2H), 1.33 (m, 14H). ESI-MS+ (m/z): [M]+H calcd for C₄₀H₈₂N₈O₄S₄, 867.53; found, 867.5.

Synthesis of bola-C18-G1 (5): In a one drum glass vial were added 30 mg of the TFA-salt of the unfunctionalized bola 4 (0.0227 mmol, 1 equiv), boc-His(boc)-OH.DCHA (36.5 mg, 0.068 mmol, 3 equiv) and boc-Trp(boc)-OH (9.2 mg, 0.0227 mmol, 1 equiv). 1.5 mL DMF was added to dissolve the solids, followed by BOP (42.2 mg, 0.0953 mmol, 4.2 equiv) and DIPEA (17 µL, 0.0953 mmol, 4.2 equiv). The reaction was left to stir for 24 hours at rt. The protected bola was precipitated in an excess amount of deionized water. After removing water completely,
the solid was dissolved in 1 mL TFA, 2 mL DCM, 2 mL Anisole and 0.25 mL TIPS. After stirring overnight, the solvent was removed in vacuo, the resulting solid was redissolved in MeOH and precipitated in Et₂O. The white precipitate was dissolved in water and lyophilized to give 6 as a white powder (40 mg, 81% yield). ¹H NMR (500 MHz, CD₃OD): δ 8.23-8.12 (m, 2.8 H, histidine, 70 mol%), 7.63 (m, 1.07 H, tryptophan, 27 mol%), 7.39-7.33 (m, 2.9 H), 7.20-7.01 (m, 2.14 H, tryptophan, 28 mol%), 4.36-4.11(m, 6H), 2.67-2.07 (multiple peaks overlapped with solvent peak, integration not accurate), 2.81 (8 H), 2.17 (2 H), 1.88-1.25 (48 H).

Scheme 2.4. Synthetic route for bola-C18-G2.
Synthesis of bola-C18-G3-NH₂ (6). In a two-dram vial, 2 (0.0218 mmol, 1 equiv, 50 mg), Boc-lys(boc)-OH.DCHA (92.1 mg, 0.174 mmol, 8 equiv) and DIPEA (31 μL, 0.179 mmol, 8.2 equiv) were dissolved in 2.5 mL DMSO, followed by the addition of BOP (79.2 mg, 0.179 mmol, 8.2 equiv). The reaction was left to stir at rt for 24h. After the reaction, the mixture was diluted with 150 mL DCM and washed with 0.02 N HCl in brine, 1.0 M NaHCO₃, and brine three times. The organic layer was dried over Na₂SO₄ and the solvent was removed in vacuum. The crude product was purified by dissolution in MeOH and precipitation in water. If necessary, the product was further purified by column chromatography (20~40% EtOAc in Hexanes). After purification, the Boc was deprotected in a cocktail of TFA solution (Typically, 1 mL TFA, 1.5 mL anisole, 2.5 mL DCM and 0.1 mL TIPS). The deprotection was done in 4h at rt, followed by solvent removal in vacuo. The product was then purified by dissolution in a minimum amount of MeOH and precipitation in Et₂O. The white precipitate was dissolved in nanopure water, filtered through a 0.22 μm filter and lyophilized to give the TFA salt of 6 as a white powder (79.2 mg, 86% yield). ¹H NMR (500 MHz, CD₃OD) δ 4.42 – 2.9 (m, 12H), 2.85 (m, 2H), 2.52-2.32 (m, 24H), 2.31 – 2.13 (m, 4H), 2.07 – 2.92 (m, 8H), 2.84 (m, 8H), 2.21 (t, J = 7.5 Hz, 4H), 2.09 – 1.40 (m, 90H), 1.32 (s, 28H). ESI-MS+ (m/z): [M] calcd for C₁₁₂H₂₂₆N₃₂O₁₆S₄, 2402.67; found, 1202.8 (M+2H), 802.2 (M+3H).

Synthesis of bola-C18-G3 (7): In a one dram glass vial were added 30 mg of the TFA-salt of the unfunctionalized bola 6 (0.00709 mmol, 1 equiv), boc-His(boc)-OH.DCHA (45.7 mg, 0.0851 mmol, 12 equiv) and boc-Trp(boc)-OH (11.5 mg, 0.0284 mmol, 4 equiv). 1.5 mL DMF was added to dissolve the solids, followed by BOP (50.8 mg, 0.114 mmol, 16.2 equiv) and DIPEA (20 μL, 0.114 mmol, 16.2 equiv). The reaction was left to stir for 24 hours at rt. The protected bola was precipitated in an excess amount of deionized water. After removing water
completely, the solid was dissolved in 1 mL TFA, 2 mL DCM, 2 mL Anisole and 0.25 mL TIPS.

After stirring overnight, the solvent was removed in vacuo, the resulting solid was redissolved in MeOH and precipitated in Et$_2$O. The white precipitate was dissolved in water and lyophilized to give 7 as a white powder (52.7 mg, 93% yield).$^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 8.20 (m, 5.4H), 7.65 – 7.45 (m, 2H), 7.29 (d, $J$ = 7.7 Hz, 2H), 7.15 (t, $J$ = 21.1 Hz, 7H), 7.03 (d, $J$ = 7.6 Hz, 2H), 6.96 (s, 2H), 4.13 (d, $J$ = 75.1 Hz, 15H), 2.39 (d, $J$ = 21.9 Hz, 5H), 2.12 (s, 24H), 2.70 (s, 4H), 2.10 (s, 2H), 1.95 – 0.93 (m, 60H).

2.3 Synthesis of Bolas with Fluorocarbon Core and HEG Core

![Scheme 2.5](image)

**Scheme 2.5.** Synthetic route for bola-F10 core (the subsequent peptide coupling follows the same steps used in the synthesis of bolaamphiphiles having hydrocarbon cores)

**Synthesis of activated carbonate.** 1H,1H,12H,12H-perfluoro-1,12-dodecanediol (827 mg, 1.47 mmol, 1 equiv) was charged to 50 mL RBF and placed under an atmosphere of N$_2$. THF (15 mL) was added along with dry pyridine (0.325 ml, 4.04 mmol, 2.75 equiv) and the mixture stirred until completely dissolved. The reaction mixture was cooled in an ice/water bath
and 4-nitrophenyl chloroformate (815 mg, 4.04 mmol, 2.75 equiv) added. After 1 h of stirring, the ice bath was removed and the reaction was allowed to stir for an addition 8 hours. The reaction mixture was then diluted with DCM (200 ml) and washed sequentially with 0.1 M HCl (2 X 100 mL), and water (2 X 100 mL). The solvent was removed in vacuo and the crude product purified by column chromatography (0-20% EtOAc in hexanes) to give a 609 mg of white solid (46% yield). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.32 (d, $J$ = 9.2 Hz, 2H), 7.43 (d, $J$ = 9.2 Hz, 2H), 4.79 (t, $J$ = 12.0 Hz, 2H).

Synthesis of fluorocarbon core 8. Mono-trityl protected cystamine (130 mg, 0.37 mmol, 2.5 equiv) was weighed into a 25 mL RBF and dry DCM (2 mL) and DIPEA (64 μL, 0.37 mmol, 2.5 eq) added. The activated carbonate (130 mg, 0.145 mmol, 1 eq) was dissolved in dry THF (1 mL) and added drop wise to the solution of cystamine in DCM, with the reaction mixture turning yellow immediately. The reaction was stirred for 8 h at RT then diluted with DCM (50 ml) and transferred to a separatory funnel. The organic layer was washed with sat. NaHCO$_3$ (5 X 50 mL) and then water (2 X 50 mL). After drying with MgSO$_4$ and solvent removal in vacuo, the crude product was purified by column chromatography (10: 90 to 50:50 EtOAc:hexanes) and then deprotected with TFA following the previously reported procedure to the desired product 8 as a clear oil (204 mg, 48% yield). $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 4.70 (t, $J$ = 12.9 Hz, 2H), 2.46 (t, $J$ = 6.5 Hz, 2H), 2.27 (d, $J$ = 6.7 Hz, 2H), 2.96 (t, $J$ = 6.7 Hz, 2H), 2.85 (t, $J$ = 6.7 Hz, 2H). ESI-MS+ (m/z): [M]+H calcd for C$_{22}$H$_{26}$F$_{20}$N$_4$NaO$_4$S$_4$, 941.04; found, 941.0.
Synthesis of bolas with fluorocarbon core. The TFA-salt of the cystamine-functionalized fluorocarbon core (8) was subjected to subsequent coupling reactions of boc-protected lysine as described previously in 2.1 to give **bola-F10-G2** and **bola-F10-G2.**

**Bola-F10-G2-NH2**: $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 4.71 (t, $J$ = 12.6 Hz, 2H), 4.33 (dd, $J$ = 8.6, 5.6 Hz, 1H), 2.96 (t, $J$ = 6.5 Hz, 1H), 2.85 (t, $J$ = 6.6 Hz, 1H), 2.68 – 2.54 (m, 1H), 2.48-2.44 (m, 3H), 2.29-2.18 (m, 2H), 2.06 – 2.93 (m, 4H), 2.91 – 2.77 (m, 4H), 2.08 – 1.34 (m, 18H). ESI-MS+ (m/z): [M]+H calcd for C$_{59}$H$_{98}$F$_{20}$N$_{16}$O$_{10}$S$_{4}$, 1687.62; found, 1687.6.

**Bola-F10-G2**: $^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 8.56-8.47 (m, 5.0 H, histidine, 62 mol%), 7.53-7.04 (14.2 H, tryptophan, 23 mol%), 5.00-4.90 (multiple peaks overlapped with solvent peaks), 4.85-4.10 (14 H), 2.67-2.67 (40 H), 1.71-1.06 (36 H).
Bola-F10-G3: $^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 8.15-7.82 (m, 12.09 H, histidine, 81 mol%), 7.53-7.04 (36.2 H, tryptophan, 26.5 mol%), 4.70-4.50 (4H), 4.41-2.92 (30 H), 2.5-2.9 (multiple peaks overlapped with solvent peaks), 2.76-2.54 (10 H), 1.86-1.12 (88 H).

The synthesis of bola with HEG core was achieved following the same procedure as *bola-F10-G2*, starting with hexa(ethylene glycol) in the first step.

Bola-HEG-G2: $^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 8.77-8.72 (5.91 H, histidine, 74 mol%), 7.63 (2.25 H, tryptophan, 28 mol%), 7.43-7.35 (8 H), 7.21 (2.36 H, tryptophan, 30 mol%), 7.11 (2.30
H, tryptophan, 29 mol%), 7.02 (2.28 H, tryptophan, 28 mol%), 4.33-4.13 (18 H), 2.67-2.07 (multiple peaks overlapped with solvent peak, integration not accurate), 1.80-1.27 (36 H).

2.4 Synthesis of Bola with 1,2,3-Triazole Core

**Scheme 2.6.** Synthesis of 1,2,3-triazole diacid core

**1,2,3-Triazole diacid linker.** In a one-dram vial 11-azidoundecanoic acid (178 mg, 0.74 mmol, 1.0 equiv) and 10-undecynoic acid (135 mg, 0.74, 1.0 equiv) are added to a mixture of tBuOH (1.5 mL) and DI H₂O (1.5 mL) yielding a tan solution. To the solution, Copper (II) sulfate (2.3 mg, 0.015 mmol, 2 mol %) and sodium ascorbate (14.6 mg, 0.074 mmol, 10 mol %) are added. The reaction became a cloudy tan color. The reaction was stirred at rt for 24 h. After 24 h 15 mL DI H₂O is added, and the reaction is stirred for an additional 30 minutes. The mixture is the chilled to -40 °C for 30 minutes and then filtered and washed with 0 °C H₂O. After drying for 1 h, 258 mg of the resulting 1,2,3-triazole diacid linker was obtained (85% isolated yield).

**Synthesis of Bola with Triazole Core.** The 1,2,3-triazole containing diacid was subjected to the same synthetic route as described in Scheme 2.3 to give **bola-TZ-G2**.

**Bola-TZ-G2-NH₂** ¹H NMR (600 MHz, CD₃OD) δ 7.82 (s, 2H), 4.40 (t, \( J = 7.1 \) Hz, 2H), 4.36 (dd, \( J = 8.5, 5.7 \) Hz, 2H), 2.99 (t, \( J = 6.5 \) Hz, 2H), 2.88 (t, \( J = 6.6 \) Hz, 2H), 2.65 – 2.46 (m, 8H), 2.34 (dt, \( J = 2.3, 1.6 \) Hz, 10H), 2.32 – 2.19 (m, 4H), 2.02 – 2.94 (m, 8H), 2.90 – 2.82 (m, \( J = \))
16.1, 10.0, 4.2 Hz, 8H), 2.73 (t, \(J = 7.6\) Hz, 2H), 2.25 – 2.19 (m, \(J = 11.0, 4.0\) Hz, 5H), 2.04 – 1.25 (m, 72H). ESI-MS+ (m/z): [M]+H calcd for \(\text{C}_{67}\text{H}_{133}\text{N}_{19}\text{O}_{8}\text{S}_{4}\), 1459.95; found, 1459.9.

**Bola-TZ-G2:** \(^1\)H NMR (500 MHz, \(\text{D}_2\text{O}\)): \(\delta\) 8.88 – 8.41 (m, 6H 75% Histidine), 7.79 – 6.92 (m, 20H), 4.43 – 2.95 (m, 18H), 2.67 – 2.93 (m, 36H, integration off due to water peaks), 2.93 – 2.42 (m, 10H), 2.32 – 1.96 (m, 4H), 1.92 – 0.70 (m, 72H).

**Scheme 2.7.** Synthesis of monoamphiphile cores
Synthesis of OA2 acid precusor. Oleic acid (457 mg, 1.61 mmol, 2 equiv) and H-Lys-OEt·2 HCl (200 mg, 0.81 mmol, 1 equiv) were dissolved in 7 mL DMF in a 25 mL RBF, followed by the addition of DIPEA (320 µL, 1.82 mmol, 2.25 equiv), EDC.HCl (344 mg, 1.78 mmol, 2.2 equiv), and HOBr (240 mg, 1.78 mmol, 2.2 equiv). The reaction mixture was left to stir at room temperature overnight. After the reaction, the mixture was diluted with 150 mL DCM and washed with 0.02 N HCl in brine, 1.0 M NaHCO₃, and brine three times. The organic layer was dried over Na₂SO₄ and the solvent was removed in vacuo. The crude product was purified by column chromatography (3:1:0.15 hexanes/EtOAc/MeOH) to give the ethyl ester as a clear oil (462.7 mg, 82% yield). ESI-MS+ (m/z): [M]-Na+ calcd for C₄₄H₈₂N₂NaO₄, 725.62; found, 725.6. The purified product was dissolved in THF (6 mL) and LiOH (554 mg, 12.2 mmol, 20 eq.) added along with MeOH (3 mL) and H₂O (3 mL). The reaction mixture was stirred at RT overnight and neutralized with concentrated HCl. DCM (50 mL) was added, the emulsion transferred to a separatory funnel, and washed with 0.02N HCl in brine (3 x 75 mL). After drying with Na₂SO₄ and solvent removal in vacuo the product was isolated as a white powder (392.3 mg, 88% yield). ESI-MS+ (m/z): [M]+Na calcd for C₄₂H₇₈N₂NaO₄, 697.59; found, 697.5.

Synthesis of m-OA2 core 9. In a 25 mL round bottom flask, the acid synthesized in the previous step (392.3 mg, 0.581 mmol. 1 eq.) was dissolved in DCM (5 mL) and EDC (122.5 mg, 0.639 mmol, 1.1 eq.), HOBr (86.3 mg, 0.639 mmol, 1.1 eq.), mono-trityl-cysteamine (240.7 mg, 0.610 mmol, 1.05 eq.), and DIPEA (0.121 mL, 0.697 mmol, 1.2 eq.) added. The reaction mixture was stirred at RT for 24 h followed by dilution with DCM (50 mL) and transfer to a separatory funnel. The organic layer was washed with 0.02N HCl (2 x 50 mL), 1N Na₂SO₃ (2 x 50 mL), and brine (1 x 50 mL). The crude product was purified by column chromatography (1:1
to 0:1 hexanes/EtOAc) to yield the trityl protected product (211 mg, 35%). The purified product was dissolved in DCM (5 mL) then TIPS (0.5 mL) and TFA (0.5 mL) added sequentially. After stirring at RT for 2 h, the solvent was removed in vacuo and the deprotected amine purified by column chromatography (10:1:0.1 DCM/MeOH/TEA) to give the pure product (116 mg, 72%).

ESI-MS+ (m/z): [M]+H calcd for C_{46}H_{89}N_{4}O_{3}S_{2}, 809.63; found, 809.6.

**Synthesis of m-OA core 10.** In a 15 mL round bottom flask, oleic acid (100 mg, 0.354 mmol. 1 eq.) was dissolved in DCM (3 mL) and DMF (1 mL). EDC (74.6 mg, 0.389 mmol, 1.1 eq.), HOBt (52.6 mg, 0.389 mmol, 1.1 eq.), mono-trityl-cysteamine (139.7 mg, 0.354 mmol, 1 eq.), and DIPEA (0.0678 mL, 0.389 mmol, 1.1 eq.) added and the reaction mixture was stirred at RT overnight. The reaction mixture was diluted with DCM (50 mL) and transfer to a separatory funnel. The organic layer was washed with 0.02N HCl (2 x 50 mL), 1N Na_{2}SO_{3} (2 x 50 mL), and brine (1 x 50 mL). The crude product was dissolved in DCM (5 mL) then TIPS (0.5 mL) and TFA (0.5 mL) added sequentially. After stirring at RT for 2 h, the solvent was removed in vacuo and the deprotected amine purified by column chromatography (10:1:0.1 DCM/MeOH/TEA) to give the pure product (102.5 mg, 70% yield in two steps). ESI-MS+ (m/z): [M]+H calcd for C_{22}H_{45}N_{2}O_{4}S_{2}, 417.29; found, 417.2.

**Synthesis of m-C11 core 11.** In a 15 mL round bottom flask, lauric acid (100 mg, 0.500 mmol. 1 eq.) was dissolved in DCM (4 mL). EDC (74.6 mg, 0.389 mmol, 1.1 eq.), HOBt (74.3 mg, 0.55 mmol, 1.1 eq.), mono-trityl-cysteamine (196.8 mg, 0.500 mmol, 1 eq.), and DIPEA (0.096 mL, 0.55 mmol, 1.1 eq.) added and the reaction mixture was stirred at RT overnight. The reaction mixture was diluted with DCM (50 mL) and transfer to a separatory funnel. The organic layer was washed with 0.02N HCl (2 x 50 mL), 1N NaHCO_{3} (2 x 50 mL), and brine (1 x 50 mL). The crude product was dissolved in DCM (5 mL) then TIPS (0.5 mL) and TFA (0.5
mL) added sequentially. After stirring at RT for 2 h, the solvent was removed \textit{in vacuo} and the deprotected amine purified by column chromatography (10:1:0.1 DCM/MeOH/TEA) to give the pure product (130.2 mg, 58% yield in two steps). ESI-MS+ (m/z): [M]+Na calcd for C_{16}H_{34}N_{2}NaOS_{2}, 357.20; found, 357.2.

\textbf{Synthesis of G2 monoamphiphiles.} The His/Trp functionalized G2 monoamphiphiles were synthesized by subjecting 9-11 to the same amino acid coupling reactions previously reported procedure to give \textbf{m-C11-G2, m-OA-G2, and m-OA2-G2.}

\textbf{m-OA2-G2-NH}_{2}: 1H NMR (500 MHz, CDCl3) 5.29 – 5.10 (m, 4H), 4.18 – 4.01 (m, 1H), 2.74 (t, J = 6.5 Hz, 1H), 2.56 – 2.44 (m, 1H), 2.02 (t, J = 6.5 Hz, 2H), 2.85 – 2.60 (m, 8H), 2.12 (dd, J = 8.0, 6.0 Hz, 2H), 2.04 (d, J = 7.5 Hz, 2H), 1.89 (d, J = 5.5 Hz, 10H), 1.82 – 1.71 (m, 2H), 1.41 (dd, J = 47.0, 6.7 Hz, 16H), 1.17 (d, J = 17.5 Hz, 58H), 0.76 (t, J = 6.9 Hz, 6H). ESI-MS+ (m/z): [M]+H calcd for C_{64}H_{125}N_{10}O_{6}S_{2}, 1192.91; found, 1192.91.
**m-OA2-G2:** $^1$H NMR (500 MHz, CD$_3$OD) δ 7.71 (d, 3H), 7.34 (d, $J = 8.1$ Hz, 1H), 7.16 (d, $J = 15.4$ Hz, 1H), 7.09 (t, $J = 7.4$ Hz, 1H), 6.98 (d, $J = 11.4$ Hz, 5H), 5.32 (d, $J = 19.0$ Hz, 4H), 4.49 – 2.79 (m, 7H), 2.59 – 2.35 (m, 3H), 2.23 – 2.93 (m, 9H), 2.77 (dd, $J = 8.0$, 5.0 Hz, 3H), 2.22 (t, $J = 51.9$ Hz, 3H), 1.98 (s, 4H), 1.26 (d, $J = 11.6$ Hz, 38H), 0.86 (t, $J = 6.7$ Hz, 6H).

**m-OA-G2-NHz:** $^1$H NMR (500 MHz, CD$_3$OD) δ 5.51 (s, 1H), 5.37 (t, $J = 4.7$ Hz, 1H), 4.35 (dd, $J = 8.6$, 5.7 Hz, 1H), 2.98 (t, $J = 6.5$ Hz, 1H), 2.87 (t, $J = 6.6$ Hz, 1H), 2.58 (dd, $J = 12.5$, 6.8 Hz, 4H), 2.26 (d, $J = 16.6$ Hz, 3H), 2.97 (dd, $J = 12.5$, 6.1 Hz, 4H), 2.94 – 2.75 (m, 4H), 2.22 (t, $J = 7.5$ Hz, 2H), 2.12 – 1.43 (m, 26H), 1.33 (d, $J = 17.0$ Hz, 21H), 0.92 (t, $J = 7.0$ Hz, 3H). ESI-MS+ (m/z): [M]+H calcd for C$_{40}$H$_{81}$N$_{18}$O$_{4}$S$_{2}$, 801.57; found, 801.57.
m-OA-G2: $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 8.91 (m, 3H), 7.87-7.18 (bm, 8H), 5.36 (m, 2H), 4.34 (m, 7H), 4.28 (s, 9H), 2.26 (m, 10H), 2.82 (m, 5H), 2.20 (m, 3H), 2.04 (m, 27H), 1.31 (m, 41H), 0.92 (m, 5H).

m-C11-G2-NH$_2$: $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 2.68 (t, $J = 5.8$ Hz, 2H), 2.44 (dd, $J = 9.4$, 4.2 Hz, 2H), 2.20 – 2.09 (m, 2H), 2.01 (dd, $J = 9.2$, 4.2 Hz, 2H), 2.37 (dd, $J = 11.0$, 4.1 Hz, 2H), 1.78 (d, $J = 6.4$ Hz, 2H), 1.46 (m, $J = 18.3$ Hz, 15H), 1.05 (t, $J = 7.0$ Hz, 3H). ESI-MS+ (m/z): [M]+H calcd for C$_{34}$H$_{71}$N$_8$O$_4$S$_2$, 719.50; found, 719.5.
m-C11-G2: $^1$H NMR (500 MHz, CD$_3$OD) δ 8.62 (d, $J = 40.8$ Hz, 3 H), 7.73 – 7.49 (m, 2H), 7.41 – 7.22 (m, 3H), 7.05 (m, 3H), 4.39 – 2.86 (m, 7H), 2.56 – 2.31 (m, 5H), 2.20 – 2.86 (m, 7H), 2.86 – 2.58 (m, 4H), 2.11 (d, $J = 7.1$ Hz, 2H), 1.92 – 1.01 (m, 29H), 0.82 (t, $J = 6.6$ Hz, 3H).

**MTT Assay.** NIH 3T3 fibroblast cells were seeded at a density of 5000 cells/ well in 96-well plates 24 h in advance. The culture media was changed from 100 μL DMEM with 10% fetal bovine serum (FBS) to 80 μL plain DMEM immediately before the toxicity assay. 20 μL PBS solution containing different amount of bolas were then added to each well, followed by 4h incubation. The media was then changed back to DMEM with 10% FBS and cultured for another 48h. The media was replaced with 50 μL DMEM solution containing 0.5 mg/mL MTT, followed by 4h incubation at 37 C. 100 μL DMSO was added to the solution to dissolve the formed fomazan and the plate was incubated in a shaker at 37 C for 30 min. MTT reading was obtained by a plate reader (Abs 540 nm). As a positive control, cells were also treated with poly(ethylene imine) (PEI, $M_w = 25$ kDa) at different concentration under the same conditions.

**Hemolysis Assay.** The cell membrane disruption of different dendron amphiphiles were measured by hemolysis assay. The procedure was adapted from literature report with slight modification. $^1$ 100% bovine red blood cell (RBC) suspension was washed with PBS buffer and
collected by centrifugation (10 min, 800G) three times before the assay. 20 µL RBC suspension was then mixed with 80 µL PBS solution containing different amount of amphiphiles, followed by 1 h incubation at 37 °C. RBCs were collected by 10 min centrifugation at 800G and the absorbance of the supernatant was measured at 540 nm. Pure PBS buffer was used as a negative control and 10 mg/mL Triton X-100 solution as the positive control. Percent hemolysis was calculated by comparing the absorbance of the sample with Triton X-100.

**Gel Electrophoresis.** The binding of siRNA to bola was studied by agarose gel electrophoresis. Both siRNA and bola were diluted with 10 mM phosphate buffer pH 7.4. Different amount of bola solutions (5 mg/mL) were added to 5.0 µL 4 µM siRNA solution to achieve different N/P ratio. The same buffer was added to adjust the final volume to 10.0 µL, followed by 30 min incubation at room temperature. 2.5 µL 6X gel loading dye was added to each sample and 10 µL of the mixture was loaded to each well in 1% agarose gel with 1X GelRed dye. The electrophoresis was run in TAE buffer at 60 V for 45 min and the gel was visualized under a UV transilluminator.

**Dextran Sulfate Competitive Binding Assay.** The binding strength of siRNA to bola was studied by competitive binding assay with dextran sulfate (DS). To 5 µL 4 µM siRNA solution was added different bola solution at N/P 40 and the mixtures incubated for 1h at r.t. 1 µL of DS solution at different concentrations was added to the complex to achieve different S/P ratio (the molar ratio of sulfate groups from DS and phosphate groups from siRNA) and the mixture incubated for another 30 min. The samples were then subjected to agarose gel electrophoresis under the aforementioned condition.

**Glutathione Triggered Release of siRNA from Bola Complexes.** To 5 µL 4 µM siRNA solution was added concentrated bola solution to achieve N/P 40 and the final volume was adjusted
to 10 μL by pH 7.4 phosphate buffer. After 1h incubation at room temperature, 1 μL 55 mM glutathione (GSH) was added to the solution to achieve a 5 mM final concentration, followed by 30 min incubation at room temperature. All samples were then subjected to agarose gel electrophoresis under the aforementioned condition.

Negative-stain TEM. siRNA-amphiphile complexes for negative-stain TEM studies were prepared at 2-10 μM siRNA concentration and a N/P ratio of 10 in ddH₂O. In a typical procedure, 10 μL solution containing dendron amphiphiles were added to 10 μL siRNA solution containing 160 pmol negative control siRNA. The solution was briefly vortexed and incubated at rt for 30 m before imaging. TEM grids (Ultrathin Carbon Type-A, 400 mesh) were glow discharged before use. 8 μL samples solution were placed on the grid and let stand for 1 min. The solution was blotted away with a filter paper, while 15 μL 2% Uranyl Acetate was pipetted on to the grid from the other side. After 1 min, the staining process was repeated with another 15 μL 2% Uranyl Acetate solution. All the solution was removed by a filter paper and the grid was left air dry for 10 min before loading into the instrument. Images were obtained on a FEI Tecnai G2 TF20 high resolution TEM operated at an accelerating voltage of 200 kV.

Cryo-TEM. Vector/siRNA complexes for cryo-TEM were prepared at 5 μM siRNA concentration and a N/P ratio of 10 in PBS buffer (10 mM phosphate, 10 mM NaCl, pH = 7.4). In a typical procedure, an appropriate amount of vector was diluted to 12.1 μL using PBS and 1.9 μL of 40 μM siRNA was added. After brief mixing via pipette and 5 minutes incubation, cryo-TEM samples were prepared using a Vitrobot and Quantifoil grids (Cu, 200 mesh, 2 μm holes, glow discharged before use). Samples were placed in a Gatan cryo-holder, transferred to a FEI Tecnai G2 TF20 high resolution TEM, and imaged at 200 kV in low-dose mode.
DLS Measurements. The size and zeta potential of bola/siRNA polyplexes were measured at 633 nm using Zetasizer (NanoZS) dynamic light scattering instrument (Malvern Instruments, Malvern, UK) at 25°C with detection angle of 173°. The stock vector solutions (5 mg/ml) were diluted to 500 μL with PBS (10 mM phosphate, 10 mM NaCl, pH = 7.4) and complexed with 250 μL of 1.5 μM siRNA to give a final [siRNA] of 500 nM with N/P ratio = 45. After 5 minutes incubation, the samples were analyzed for particle size then transferred into a disposable capillary cell for zeta potential analysis.

General Vector/siRNA Complex Preparation (PBS). The TFA-salts of the various amphiphiles were stored at -20° C as 5 mg/ml solutions in ddH2O and prior to complexation the vectors, along with all buffers, were allowed to come to room temperature. Both negative control and targeted siRNA were diluted to 1.5 μM with PBS buffer (10 mM phosphate, 10 mM, NaCl, pH = 7.4). The amount of vector required to give the desired N/P ratio was calculated and the appropriate amount diluted with PBS to a final volume of 12.3 μL per well. The 12.3 μL amphiphile solution was then added to 6.7 μL of 1.5 μM siRNA solution to give final siRNA concentration of 500 nM, followed by brief vortexing. The solution was further agitated on a shaker for 30 min before addition to the transfection media. Generally the complex solutions were prepared at 5X the desired final concentration and for a final volume of 100 μL transfection media, 20 μL of the complex solution with 500 nM [siRNA] was added to each well containing 80 μL of DMEM to give a transfection media with 100 nM [siRNA].

Optimized Vector/siRNA Complex Preparation (OptiMEM). Prior to complexation all vectors and buffers were allowed to equilibrate to room temperature. A 1.5 μM solution of siRNA was prepared using OptiMEM as the dilution buffer and the appropriate amount of vector solution (5 mg/ml) required to give the desired N/P ratio diluted with OptiMEM. The vector solution was
added to the siRNA solution and gently mixed via pipette to give a complex solution with [siRNA] = 500 nM. After 10 minutes incubation without agitation, this concentrated solution was gently mixed via pipette, further diluted to the desired concentrations with OptiMEM, and immediately added to the cell culture media.

**Transfection of 3T3 Cells and Flow Cytometry Analysis.** NIH 3T3 fibroblast cells were seeded at a density of 10,000 cells/well in 48-well plates 24 h in advance. Prior to transfection, the media was replaced with 80 µL plain DMEM solution without serum. Different complex solutions were prepared as described in the general complex procedure (PBS) previously and 20 µL added to each well to make the final siRNA concentration 100 nM. After 4h incubation, the media was changed back to 250 µL DMEM supplemented with 10% fetal bovine serum and cultured for another 48h. Before the analysis, cells were released from each well by Tripsin and harvested by centrifugation (5 min, 500G). Fluorescence of transfected cells was measured on a Becton-Dickinson LSR II flow cytometer with argon ion excitation laser. For each sample, data representing 10,000 objects were collected as a list-mode file and analyzed using FACSDivaTM software (Becton Dickinson, version 6.1.3).

For cell uptake assay, fluorescently labeled negative control siRNA (siRNA-Cy3) was used and the uptake was quantified by the mean Cy3 fluorescence of each cell. For GFP knockdown assay, both targeting siRNA (GFP-siRNA) and non-targeting negative control siRNA (Neg-siRNA) were transfected and the transfection efficiency was calculated by comparing the mean GFP fluorescence of GFP-siRNA treated cells with Neg-siRNA treated cells.

**Transfection in Luciferase Expressing Cell Lines.** Following standard protocols for the handling of the various cell lines, the knockdown effects of the various vectors were assessed in the following luciferase expressing cell lines: MDA-MB-231, INS-1, HEK, and 4T1. After
passaging, the cell were plated in 96-well plates such that they were 30-40% confluent at time of transfection and immediately prior to addition of the complexes the culture media was switched to 80 μL DMEM per well. For initial screening, the vector/siRNA complexes were prepared using the general complex preparation protocol in PBS. The 5X vector/siRNA complexes were prepared as described previously and 20 μL added to each well. After 4 hours the media was replaced with 100 μL of the standard culture media and the cells cultured for 48 hours prior to analysis.

**Transfection Analysis in Luciferase Expressing Cell Lines.** After 48 hours of incubation post-transfection, the culture media was removed and replaced with 100 μL of a 150 μg/mL solution of firefly D-luciferin in PBS buffer. Without any further treatment, the cells were incubated at 37 °C for 5 minutes after which they were imaged using an IVIS lumina II camera. The normalized luciferase knockdown was determined by comparing the overall luminescence of the samples treated with complexes containing anti-luc siRNA to those treated with complexes containing negative control siRNA.

**Transfection of MDA-MB-231 Cells in Presence of Serum.** MDA-MB-231 cells expressing luciferase were seeded at a density of 4,000 cells/ well in a 96-well plates 48 h in advance such that they were ~50% confluent at time of transfection. Vector/siRNA complexes containing either anti-luciferase or control non-targeting siRNA were prepared following the optimized procedure with OptiMEM as the dilution buffer, giving 5X complex solutions with siRNA concentration = 250 nM at an N/P ratio of 45. Immediately before transfection, the culture media was replaced with 80 μL DMEM containing 0%, 12.5%, 25%, 50% or 100% FBS. 20 μL of the 5X complex solutions were then added to each well to make the final siRNA concentration 50 nM and the final FBS composition 0%, 10%, 20%, 40% or 80%. After 4h incubation, the media
was replaced with 100 μL DMEM with 10% FBS. After 48 hours, the luciferase knockdown was assessed as described previously.

**IC50 Determination in MDA-MB-231 Cells.** MDA-MB-231 cells expressing luciferase were seeded at a density of 4,000 cells/well in a 96-well plates 48 h in advance such that they were ~50% confluent at time of transfection. Vector/siRNA complexes containing either anti-luciferase or control non-targeting siRNA were prepared following the optimized procedure with OptiMEM as the dilution buffer, giving 5X complex solutions with the desired siRNA concentrations at a N/P ratio of 45. Immediately before transfection, the culture media was replaced with 80 μL of antibiotic-free OptiMEM. 20 μL of the 5X complex solutions were then added to each well to give final siRNA concentrations of 0.25, 0.5, 1.5, 3, 6.25, 12.5, 16.6, 25, 32.3, 50, 66.6, 75, and 100 nM. After 4h exposure, the media was replaced with 100 μL DMEM containing 10% FBS. After 48 hours, the luciferase knockdown was assessed as described previously. For each vector, the knockdown percentage at half maximal effect was determined and a linear trend between the flanking data points used to calculate the IC50 value.

**Confocal Laser Scanning Microscopy.** Confocal laser scanning microscopy was used to observe the trafficking of labeled siRNA in the transfected cells. Unmodified NIH 3T3 fibroblast cells were seeded at a density of 15000 cells/well on an 8-well chamber slide (Lab-Tek, Rochester, NY) 24h before transfection. Cy3-labeled siRNA was complexed with FITC-labeled amphiphiles at N/P 45 and transfected to the cells under aforementioned conditions. After transfection, the media was changed back to DMEM supplemented with 10% fetal bovine serum. Confocal fluorescence spectroscopy was performed at different time points after the transfection. The nucleus was counter-stained by 25 μg/mL DAPI for 30 min prior to imaging. All confocal images were acquired using a Zeiss LSM 510 inverted laser-scanning confocal microscope. A 40×
numerical aperture of 1.4 oil immersion planapochromat objective was used for all experiments. A 559 nm helium-neon laser, a SMD640 dichroic mirror, and a 575-620 nm band-pass barrier filter were used to obtain the images of Cy3-labeled siRNA. FITC fluorescence of labeled amphiphile was acquired using a 488 nm excitation light, a SDM560 dichroic mirror, and a 505-540 nm band-pass barrier filter. Images of DAPI-stained nuclei were acquired using a 780 nm two-photon excitation light, a 635 nm dichroic mirror, and a 655-755nm band-pass barrier filter. The three fluorescent images were scanned separately and overlaid together with the differential interference contrast image (DIC). The cells were scanned as a z-stack of two-dimensional images (1024×1024 pixels) and an image cutting approximately through the middle of the cellular height was selected to present the intracellular siRNA localization.

**Statistical Analysis.** All quantitative assay were performed in triplicates, data were expressed as mean ± SEM.
Representative NMR Spectra

bola-C6-G2
bola-TZ-G2
2.6 References


Chapter 3: Optimization of Peptide Bolaamphiphile for siRNA Delivery

3.1 Introduction

The preceding chapter detailed the design and testing of a series of dendritic amphiphilic vectors, with the results indicating that those with bolaamphiphile structure and high enough valency induced effective gene silencing. Bolaamphiphiles based upon hydrocarbon cores of appropriate length (C18) produced efficacious vectors; however, the most potent vectors contained a relatively short fluorocarbon domain (F10). Although G3 vectors induced slightly higher knockdown effect at lower concentrations than the G2 analogs, the G3 vectors also induced more cytotoxicity and the siRNA complexes formed had larger particle size and lower colloidal stability. For these reasons the bola-C18-G2 and bola-F10-G2 vectors were selected for further optimization and study.

While effective in vitro, several issues were perceived as hindering further application of the C18 and F10 vectors. Of primary concern for applications requiring systemic administration, it appeared that the colloidal stability of the complexes was poor in buffers containing physiological levels of salt. It was demonstrated by dynamic light scattering (DLS) and microscopy that micron sized aggregates formed rapidly upon addition of bola-C18-G2 or bola-F10-G2 complexes to media containing 100 mM NaCl. Another concern was that the final

![Scheme 3.1 Optimization and study of bolaamphiphile vectors](image-url)
functionalization reaction produced a statistical mixture of products, resulting in active vectors that were not discrete compounds. Aside from increasing the care required to synthesize the vectors, the non-discrete nature complicates characterization, purification, and the study of fine structure/property relationships. The following sections detail further efforts to improve the colloidal stability of the bolaamphiphile vectors through co-formulation,¹⁻³ revise the synthetic approach to produce effective discrete compounds and improve the efficacy and our understanding of this novel class of vectors through variation of the aromatic amino acid component.

**3.2 Co-formulation Studies**

The formulation of cationic or ionizable lipids with cholesterol and PEG has proved incredibly useful for the development of effective LNP delivery systems. Once an effective vector candidate has been identified, this approach allows for customization of surface charge, improved packing efficiency, and ultimately enhanced circulatory stability without any additional synthetic effort.¹⁻³ With the goals of improving the colloidal stability and transfection efficiency, the bola-C18-G2 vector was formulated with cholesterol and a stearic acid modified PEG (C17-PEG) using thin-film hydration from EtOH (Figure 3.1). In this procedure, the hydrophobic components are mixed in a solvent in which they are fully soluble (EtOH), followed by solvent removal in vacuo and resuspension in aqueous buffer or ddH₂O.

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**Figure 3.1** Thin-film hydration for formulation of vectors
The formulations tested consisted of the unmodified bola-C18-G2 vector (C18), a formulation mixed at a 1:1 molar ratio with cholesterol, (C18 / Chol), and one containing the same level of cholesterol with the addition of 5 mol % C17-PEG (M_n = 2,000), referred to as C18 / Chol / 5% PEG. Initially, the colloidal stability of the complexes in low-salt PBS (10 mM phosphate, 10 mM NaCl, pH = 7.4) was investigated using DLS. After addition of siRNA to the formulated vector, the complexes were diluted in low-salt PBS and the particle sized monitored over a period of 24 h (Table 3.1). Both the unmodified sample and the sample formulated only with cholesterol displayed a time-dependent increase in particle size, with the 24 h time point indicating significant aggregation. In contrast, the sample formulated with 5% C17-PEG showed greater colloidal stability with the final time point showing a slight increase in particle size yet still displaying good correlation indicative of well-dispersed, spherical nanoparticles.

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<td>PDI</td>
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<table>
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<td>177</td>
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<td>PDI</td>
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<td>0.15</td>
<td>0.29</td>
<td>0.320</td>
</tr>
</tbody>
</table>

Table 3.1 Colloidal stability of formulated complexes, red values indicate poor correlation.

The stability and cellular uptake of the complexes were next examined with confocal fluorescence microscopy. For this study, Cy3 labeled siRNA was utilized in conjunction with bola-C18-G2 functionalized with 1-2% FITC via the peripheral amines. After exposing NIH-3T3 cells to the complexes for 4 hours, the nuclei were stained with Hoescht 33342 and the samples imaged (Figure 3.2). Compared to the unformulated sample, the two samples formulated with cholesterol and 0 or 5 mol % PEG showed greater co-localization of vector and siRNA signals. While somewhat unexpected, this could suggest that formulation improves the stability of the complexes, possibly due to higher packing efficiency due to the complementarity of the hydrophobic C18 core with cholesterol. The PEGylated sample showed less evidence of
aggregates in the bright-field channel and the internalized complexes appeared to be less aggregated than the formulation containing only cholesterol. These results agree with the previous DLS assay, suggesting that co-formulation with PEG has the potential to improve the colloidal stability of bola-C18-G2 / siRNA complexes.

The transfection efficiency of the formulated sample was next compared to a selection of effective vectors in NIH-3T3-GFP cells. In order to differentiate performance, the knockdown response was assayed at final siRNA concentrations of 10 or 50 nM (Figure 3.3). In general the bola-C18-G2 vectors were ineffective at the lower concentration, with ~40-50% gene knockdown evident at 50 nM for all three formulations tested. While some improvement over the base vector may be evident, all of the formulations were outperformed by the bola-F10-G2 vector and failed to induce greater than 50% silencing at the highest concentration assayed.
Due to the greater transfection efficiency of the bola-F10-G2 compared to the hydrocarbon analogs, formulation of the fluorocarbon vector was pursued, again with the goal of enhancing colloidal stability and efficacy. Compared to the hydrocarbon core of the C18 vectors which should favorably assemble with cholesterol and other aliphatic moieties, the fluorocarbon domain of the F10 vectors is lipophobic in addition to being hydrophobic. Due to this, it was expected that a fluorocarbon-modified PEG would be most suitable for co-assembly and formulation with the bola-F10-G2 vector. Using a simple one-pot synthesis, a mono-functional fluorocarbon domain of similar size to the F10 core was coupled to PEG (Mₘ = 2000) to give the desired compound, referred to as fPEG2k (Scheme 3.2). Initial DLS assays were performed and successfully confirmed that the previously described thin-film hydration of bola-F10-G2 / fPEG2k mixtures produced colloidal stable vectors capable of complexation with siRNA.

![Scheme 3.2 Synthesis of fluorocarbon PEG for formulation with bola-F10-G2](image)

Formulations of bola-F10-G2 with either 5 or 10 mol % fPEG2k were assayed for colloidal stability in low-salt PBS and high-salt PBS (10 mM, phosphate, 100 mM NaCl, pH = 7.4). After preparation of the PEGylated vectors, the samples were complexed with siRNA in low-salt PBS and then diluted 10X with the appropriate buffer. The particle size of the complexes was then monitored over 24 h using DLS (Table 3.2). The complexes showed higher stability under low-salt conditions with the unformulated vector only showing severe aggregation after 24 h; with the same sample aggregating within 4 h when high-salt PBS was used. The
formulated samples showed greatly improved colloidal stability, with the 10 mol % fPEG2k sample essentially completely stable in both buffers up to 24 h.

The transfection efficacy of the formulated vectors was next assayed in MDA-MB-231-Luc cells, utilizing a sample preparation in low-salt PBS designed to ensure colloidal stability. Analysis of the luciferase expression revealed that the PEGylated samples generally showed similar efficacy to the bare vector, with attenuation of knockdown effect when 10 mol % fPEG2k was incorporated. The sample formulated with 5 mol % fPEG2k showed enhanced silencing effect, perhaps due to greater colloidal stability of the complexes allowing for improved cellular uptake and delivery.\textsuperscript{1-3} It was determined that 5 mol % fPEG2k was the desired level of PEGylation given the improved knockdown effect at low siRNA concentration and acceptable stability in high-salt media. It is interesting to note that the attenuation of knockdown begins to

![Table 3.2 Colloidal stability of complexes in low-salt (a) or high-salt PBS (b) determined via DLS.](image-url)
occur when a level of PEGylation (10 mol %) is reached sufficient to fully stabilize the complexes in high-salt media, suggesting aggregation may play a role in effective silencing.

The co-formulation studies were successful in improving the colloidal stability and knockdown efficiency of both bola-C18-G2 and bola-F10-G2 vectors. Given the excellent delivery properties, bola-F10-G2 formulated with 5 mol % fPEG2k was selected for further cellular uptake studies and in vivo delivery application. Thin-film hydration was proved to be an effective means to co-assemble both hydrocarbon and fluorocarbon bolaamphiphile vectors with suitable components, leading to complexes with enhanced stability in physiologically relevant media. Given this success, formulation with cell-specific targeting ligands containing a

Figure 3.4 Transfection MDA-MB-231-Luc cells with of formulated bola-F10-G2 complexes. N/P = 45, [siRNA] indicated, PBS sample prep.
hydrocarbon or fluorocarbon domain to ensure co-assembly seems a promising approach to generate vectors for specific therapeutic or ex vivo applications.

3.3 Synthesis and Study of Discrete Vectors

Although effective for in vitro siRNA delivery, due to the final functionalization step using multiple amino acids all of the previously discussed bolaamphiphile vectors are statistical mixtures of compounds and not discrete in nature. Precise measurement during the final functionalization step and careful characterization are therefore critical to ensure efficacy and reproducibility. It is possible that certain species are more biologically active; however, purification and identification of these via chromatography is impractical due to the complex mixture of components. Aside from necessitating stringent quality control, the non-discrete nature may complicate further application and study due to the multiple species present. If one wishes to systematically modify the bolaamphiphile vectors to determine structure / property relationships, minor batch to batch differences in functionalization ratios could complicate and confound the study of fine differences. Furthermore, any small structural change could require re-optimization of the functionalization ratio to identify the optimal combination; drastically increasing the amount of work required to fully explore the effects of modifications.

Initially, bola-F10 vectors containing the original G2 Lys dendron functionalized with 3 His and 1 Trp residue at defined locations were targeted. Due to the non-equivalency of the four terminal amines, several constitutional isomers are possible and a convergent synthetic approach was designed to allow for the synthesis of the four most synthetically accessible variants. Solid-phase peptide synthesis was employed, making use of the orthogonal Fmoc and Alloc protecting groups to generate discrete dendrons. After synthesis on 2-Cl-Trt resin, the Boc-protected
dendrons were cleaved from the resin using AcOH and solution-phase coupling was attempted to the F10-GO diamine core (Scheme 3.3).

![Scheme 3.3](image)

**Scheme 3.3** Synthesis of discrete peptide dendrons via solid-phase synthesis

Although the solid-phase synthesis successfully generated the protected dendrons, the following coupling step proved difficult. Multiple coupling agents, solvents, and temperatures were investigated; however consistently low yields (<10%) and purification difficulties made this synthetic approach inefficient and impractical. An alternative solution-phase synthesis of similar targets was designed, with the hope that increased scale and the use of simple tripeptides would allow for the synthesis of a variety of discrete vectors (Scheme 3.4). Starting from Lys differentially protected with Fmoc and Boc, this approach was envisioned as a scalable, highly convergent synthesis that would enable synthesis of multiple variants of the C18 and F10 vectors. Unfortunately, while the majority of the synthetic steps proceeded with good efficiency; the final Fmoc deprotection and coupling steps were highly inefficient. As similar difficulties
were encountered in the previous synthesis, it seems likely that the Trt protected His residues interfere with effective coupling.

Due to the success of the co-formulation studies previously discussed, bola-F10-G2 vectors functionalized with 100% His (F10-G2-H) or 100% Trp (F10-G2-W) were synthesized and various mixtures of the two discrete bolaamphiphiles were prepared via thin-film hydration. Due to hydrophobicity of the aromatic side chain, F10-G2-W was insoluble in water and formulations containing >25% of this compound generally showed poor colloidal stability. Formulations of F10-G2-H containing 0-25 mol % of F10-G2-W were screened for transfection activity in MDA-MB-231-Luc cells to determine whether these vectors would display similar efficacy to the previous system (Figure 3.5). Although F10-G2-H was completely ineffective on its own, significant knockdown effect became apparent with incorporation of 20 or 25 mol %

Scheme 3.4 Attempted synthesis of discrete bolaamphiphile vectors through solution-phase synthesis
F10-G2-W. Unfortunately, compared to the previous bola-F10-G2 vector the gene silencing effect was very weak even at high siRNA concentrations.

![Figure 3.5](image) Transfection of MDA-MB-231-Luc cells with discrete formulations. N/P = 45, [siRNA] = 50 or 100 nM, PBS sample prep.

Given the synthetic difficulties in obtaining the 3 His / 1 Trp vectors and the low activity of the discrete formulations, an alternate approach was designed to yield discrete vectors functionalized with dipeptides. By functionalizing the bola-C18-G1 and bola-F10-G1 cores with dipeptides containing two cationic groups, vectors of similar valency but altered functionality were obtained (Figure 3.6). A dipeptide library containing Lys / Arg for additional cationic

![Figure 3.6](image) Structure of dipeptide bolaamphiphile vectors and design of dipeptide library
functionality and His/Trp for effective cellular internalization was designed. This approach proved facile and robust, with no significant synthetic challenges encountered.

The dipeptide vectors were screened for transfection activity in several cell lines, with data from HEK-293-Luc cells displayed in Figure 3.7. Multiple highly efficacious hits were identified with the F10 core again providing more potent gene silencing than the C18. Dipeptide vectors lacking His or Trp were generally ineffective, as were those containing lower cationic valency than the original system (HW and WH). Vectors containing Arg generally were more effective than those with Lys, presumably due to the greater membrane association and cellular activity.

**Figure 3.7** Gene silencing efficiency (left y-axis, columns) and cellular viability (right y-axis, data points) for transfection of HEK-293-Luc cells with dipeptide bolaamphiphile library (N/P 60 and [siRNA] = 20 nM). Cellular viability was assessed by comparing luciferase expression in non-treated cells to cells treated with negative control siRNA-bola complex. OptiMEM sample prep.
uptake provided by the guanidinium side chain. The effective hits promoted potent gene silencing at [siRNA] = 20 nM while inducing little cytotoxicity.

Although synthetic difficulties hindered the study of discrete vectors directly analogous to the previous system, a novel functionalization strategy capable of producing effective, discrete bolaamphiphiles was eventually developed. This approach yielded a surprising number of highly potent vectors and may prove useful for the optimization of other systems. It is important to note that functionalization of the G1 cores with simple dipeptides is incapable of producing compounds of identical valency and functionality to the statistically functionalized vectors. In order to obtain bolaamphiphiles identical in functionality to the initial system, a synthetic approach was designed in which linear peptides containing Lys, His, and Trp are coupled to the G0 diamine cores. By utilizing peptides of various sequences with 3 Lys, 3 His, and 1 Trp residues, it is possible to replicate the functionality of the original system. Efforts are currently ongoing to synthesize and study such linear variants, with initial tests suggesting that bola-F10-G0-HKHKHKW induces comparable gene silencing and without PEGylation has improved colloidal stability over the statistically functionalized bola-F10-G2 vector.

![Scheme 3.5 Synthesis of linear peptide bolaamphiphile variants](image)
3.4 Aromatic bola-F10-G2 Variants

During development of the denpol system, a variety of amino acids and functionalization ratios were screened with ultimately a molar ratio of 3:1 His / Trp proving most efficacious. The work discussed so far has focused on this composition exclusively, although significant discrepancy exists between the bolaamphiphile and denpol systems. After identifying the most effective bolaamphiphile structure, efforts were made to optimize the efficiency and gain further understanding by altering the aromatic component. To this end, a series of statistically functionalized bola-F10-G2 vectors were synthesized in which Trp was replaced with a different natural or unnatural aromatic amino acid (Figure 3.8).

![Chemical structures of alternative aromatic amino acids investigated](image)
One of the benefits of the bolaamphiphile system is the simple chemistry used, which allows for functionalization with a wide variety of either natural or unnatural amino acids. Along with His, alternative Boc-protected aromatic amino acids were functionalized to the bola-F10-G2 periphery in a 3:1 molar ratio, with deprotection affording the desired aromatic variants. For this study, precise control over the functionalization ratio was required and care taken to ensure all vectors contained 25% of the aromatic component, with greater than 1% deviation from this value deemed unacceptable. Although gel-binding assays were performed, transfection experiments were focused on as these provide the best indicator of delivery efficiency.

During previous transfection assays using the bolaamphiphile vectors, it was found that the sample preparation conditions could dramatically affect the observed biological response. When the vector and siRNA were complexed in OptiMEM, a biological media optimized for transfections, greater knockdown effect was observed than when using DMEM or low-salt PBS. Using the optimal sample preparation with OptiMEM, the bola-F10-G2 aromatic variants were

![Figure 3.9](image-url)

**Figure 3.9** Transfection of MDA-MB-231-Luc with variants. N/P = 45, OptiMEM sample prep.
screened for transfection activity in MDA-MB-231-Luc cells (Figure 3.9). The results indicated that multiple aromatic residues other than Trp were capable of promoting efficient cellular internalization and subsequent biological response. Under these conditions, vectors functionalized with Tyr and Phe showed similar efficacy to the Trp vector; although those containing the aliphatic Leu or Val failed to induce significant gene knockdown. The knockdown effect was shown to be independent of the chirality with D-, L-, and a racemic mixtures of Trp all proving equally effective. Methylated analogs of Trp and Tyr showed slightly attenuated silencing at the lower concentration. In general, most aromatic variants induced comparable gene knockdown to Trp except F(NH2), F(CF3), F(Br), and F(Pyr).

In order to explore the dependence of biological response on sample preparation conditions, the vectors were next screened using a complexation protocol designed to minimize the beneficial effects of OptiMEM. For this experiment, the complexes were prepared in low-

![Figure 3.10](image_url) Transfection of MDA-MB-231-Luc with variants. N/P = 45, PBS sample prep.
salt PBS and added to 96-well plates loaded with DMEM. By removing all traces of OptiMEM, it was hoped that any observed differences would be solely the result of the aromatic alterations. The results from this experiment demonstrated the importance of sample preparation, with all of the vectors assayed inducing much less gene silencing than when using the previous conditions (Figure 3.10). Although lower knockdown levels were achieved, some of the same trends previously observed held true. The negative consequences of Trp or Tyr methylation were more pronounced under these conditions. Although Tyr again performed similarly to Trp, the Phe functionalized vector proved completely ineffective. Although several other previously effective variants induced no knockdown, contrary to original expectations the variant containing F(NO2) displayed similar levels of silencing when compared to the Trp vector.

By studying the transfection efficacy of aromatic bola-F10-G2 variants, several interesting observations were made. Although nothing superior to Trp was identified, many unnatural amino acids proved effective for promoting successful transfection when using the optimal sample preparation. In combination with the efficacy of unnatural amino acids, the evidence that chirality plays little role in successful delivery implies that the cellular delivery of siRNA mediated by this system is independent of any receptor or chaperone mediated mechanism. The enhanced knockdown efficacy of the complexes when prepared in OptiMEM may be explained by incorporation of components of OptiMEM into the complexes. An alternative possibility is that this sample preparation encourages aggregation, due to the high salt concentration of OptiMEM, and this is what improves silencing; possibly by sedimentation of the complex agglomerates on top of the cells resulting in an enhanced local concentration of siRNA. The clear dependence on sample preparation and inherent difficulties in drawing comparisons between different cell lines or between in vitro / in vivo experiments make it
possible that certain variants may prove superior in other applications. Due to the difficulty in obtaining meaningful differentiation between these vectors using in vitro assays, further study of these aromatic variants for siRNA delivery was not pursued.

Although for siRNA delivery no amino acid superior to Trp was identified, it is not given that for a different system or NA cargo this would necessarily hold true. After work demonstrating that the original denpol system was highly effective for mRNA delivery, several unnatural aromatic acid denpol variants were synthesized. Although still in the early stages, the first transfection results indicate that Dip functionalization of denpols may greatly enhance successful mRNA delivery compared to the original Trp containing vectors. Study into the delivery of mRNA using unnatural aromatic acid denpol variants is currently ongoing.

Acknowledgements

I would like to deeply thank Alexander Eldredge for contributing to the design, synthesis, characterization, and biological testing of dipeptide functionalized bolaamphiphiles (Chapter 3.3)

3.5 Experimental

Materials. Unless otherwise noted, all reagents were used as received from commercial suppliers without further purification. Protected amino acids were purchased from Advanced Chemtech (Louisville, KY) and Aroz Technologies, LLC (Cincinnati, OH). 1H, 1H, 12H-perfluoro-1,12-dodecanedioil was purchased from Exfluor Research Corporation (Round Rock, TX). Coupling reagents were purchased from GL Biochem Ltd. (Shanghai, China). Sodium Al siRNA used in this study was purchased from Ambion (Carlsbad, CA) with Silencer® Select negative control siRNA and Silencer®Cy™-3 labeled Negative Control siRNA used for control and cellular uptake studies, respectively. The sequences for anti-lucII and anti-GFP siRNA can
be found in Table 3.3. Lipofectamine 2000 and RNAiMAX were purchased from Invitrogen (Carlsbad, CA) and used as a positive control following the manufacturer’s protocol. All reactions were performed using HPLC grade solvents unless otherwise noted. All water used in biological experiments was nanopure water obtained from Barnstead Nanopure Diamond (Waltham, MA). Luciferase-expressing HEK and MDA-MB-231 were provided by Professor Jennifer Prescher (Department of Chemistry, UC Irvine, CA). Unmodified NIH 3T3 cell and engineered NIH 3T3 cell expressing enhanced green fluorescent protein (GFP) were a generous gift from Professor Young Jik Kwon (Department of Chemical Engineering, UC Irvine, CA)

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sequence (sense)</th>
<th>Sequence (anti-sense)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-GFP</td>
<td>5’-CAAGCUGACCCUGAAGUUCTT-3’</td>
<td>5’-GAACUUCAGGGUCAGCUUGGC-3’</td>
</tr>
<tr>
<td>Anti-Luciferase</td>
<td>5’-AGACUAUAAGAUCAUCUtt-3’</td>
<td>5’-AGAUUGAAUCUUAUAGUCCUtg-3’</td>
</tr>
</tbody>
</table>

**Table 3.3.** siRNA sequences used for transfection studies. Lower-case letters denote 2’-O-methyl modified nucleotides.

**Instrumentation.** All compounds were characterized by NMR and ES-MS. $^1$H NMR spectra were collected at the UC Irvine NMR Facility and recorded at 500 or 600 MHz on Bruker instruments (GN600 or CRYO500). Mass spectral data (ES-MS) was obtained from the UC Irvine Mass Spectrometry Facility and collected with a Micromass LCT spectrometer. Matrix assisted laser desorption ionization spectral data (MALDI) was obtained from the UC Irvine Mass Spectrometry Facility and collected with a AB SCIEX TOF/TOF 5800 System. $^1$H NMR chemical shifts were reported as values in ppm relative to specified deuterated solvents. The size and zeta potential of bola/siRNA complexes were measured at 633 nm using Zetasizer dynamic light scattering instrument (Malvern Instruments, Malvern, UK) at 25°C with detection
angle of 173°. The flow cytometry data was obtained on a Becton-Dickinson LSR II flow
cytometer (Sue & Bill Gross Stem Cell Research Center, UCI) with an argon ion excitation laser
at 488 nm. Confocal fluorescence images were acquired using a Zeiss LSM 700 inverted laser-
scanning confocal microscope (Optical Biology Core Facility, UCI). Analytic HPLC analysis
was performed on an Eclipse SDB-C18 reversed phase analytical column (50 mm x 4.6 mm)
using a gradient of 5-95% MeCN in H2O with 0.1 % TFA over 35 minutes and a flowrate of 1.0
mL/min.

Synthesis and Characterization Data

Synthesis of fPEG2k. To a dried 25 mL RBF was added 3 mL of dry THF followed by
the 1,10-H,H-perfluorodecanol (100 mg, 1.0 equiv). The reaction mixture was cooled in an
ice/water bath and DIPEA (2.0 equiv) added, followed by p-nitro-phenylchloroformate (1.0
equiv). The reaction was allowed to gradually warm to RT over 1 hour and the stirred for an
additional 5 hours at rt. The PEG-amine (0.9 equiv), DCM (2 mL) and additional DIPEA were
added and the reaction stirred for 24 h. The reaction mixture was dialyzed against MeOH
(MWCO = 1000) until all yellow color was removed (approximately 6 solvent replacements and
36 h) and the product obtained as a white solid (28% yield). 1H NMR (499 MHz, CDCl3) δ 5.34
(s, 1H), 4.16 (t, J = 13.5 Hz, 2H), 3.21 (s, 15 H), 2.95 (s, 5H).

General Procedure for Functionalization of bola-F10-G2-NH2. The amine-
functionalized F10-G2 core was synthesized as previously described in Chapter 2.5 and utilized
as the TFA salt. To a one dram glass vial were added 5-20 mg of the F10-G2-TFA-salt (1 equiv),
Boc-His(Boc)-OH.DCHA (6 equiv) and the other desired Boc-protected amino acid (2 equiv).
0.25-1.0 mL DMF was added to dissolve the solids, followed by PyBOP (8.5 equiv) and DIPEA
(17 equiv). The reaction was left to stir for 24 hours at rt. The protected vectors were
precipitated in deionized water and collected via centrifugation. After removing water completely in vacuo, the solid was dissolved in 1 mL TFA, 2 mL DCM, 2 mL Anisole and 0.25 mL TIPS. After stirring overnight, the solvent was removed in vacuo, the resulting solid was dissolved in a minimal amount MeOH and precipitated in Et₂O. The white precipitate was purified by dialysis (MWCO = 1000) against MeOH. All bolas were characterized by ¹H NMR with the functionalization ratio determined by comparison of the aromatic peaks of the imidazole (His) to the other aromatic amino acid’s characteristic peaks.

NMR Shifts of bola-F10-G2 Variants

F10-G2-H

¹H NMR (600 MHz, MeOD) δ 8.86 (s, 4H), 7.50 – 7.33 (m, 4H), 4.66 (t, J = 13.7 Hz, 2H), 4.24 (m, 7H), 3.72 – 3.34 (m, 12H), 3.21 (m, 6H), 2.89 – 2.67 (m, 4H), 1.97 – 1.11 (m, 18H).

F10-G2-W

¹H NMR (600 MHz, MeOD) δ 8.00 (s, 4H), 7.69 – 7.49 (m, 4H), 7.40 – 6.93 (m, 12H), 4.63 (t, J = 14.3 Hz, 2H), 4.13 (m, 7H), 3.71 – 3.35 (m, 8H), 3.29-2.95 (m, J = 47.6 Hz, 10H), 2.73 (s, 4H), 1.52 (m, 18H).

F10-G2-H/Y

¹H NMR (600 MHz, MeOD) δ 8.38 (d, J = 73.8 Hz, 3H), 7.26 (d, J = 45.0 Hz, 3H), 7.07 (d, J = 7.8 Hz, 2H), 6.76 (d, J = 7.7 Hz, 2H), 4.68 (t, J = 12.7 Hz, 2H), 4.16 (m, 7H), 3.68 – 3.39 (m, 6H), 3.24 – 2.72 (m, 18H), 2.05 – 1.11 (m, 18H).
F10-G2-H/F

$^1$H NMR (600 MHz, MeOD) $\delta$ 8.46 (m, 3H), 7.43 – 6.94 (m, 8H), 4.69 (s, 2H), 4.29-4.11 (m, 7H), 3.48 (s, 2H), 3.25-2.80 (m, 20H), 1.95-1.09 (m, 18H).

F10-G2-H/V

$^1$H NMR (500 MHz, MeOD) $\delta$ 8.00 (d, $J = 50.6$ Hz, 3H), 7.35 – 6.85 (m, 3H), 4.68 (t, $J = 14.0$ Hz, 2H), 4.14 (m, 7H), 3.42 (s, 4H), 3.26 – 2.71 (m, 12H), 1.94 – 1.06 (m, 19H), 0.99-0.94 (m, 6H).

F10-G2-H/L

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.07-7.96 (m, 3H), 7.31 – 6.98 (m, 3H), 4.67 (t, $J = 13.8$ Hz, 2H), 4.26-4.03 (m, 7H), 3.45 (m, 5H), 3.22 – 2.80 (m, 18H), 1.98 – 1.08 (m, 18H), 0.99-0.94 (m, 6H).

F10-G2-H/W(D)

$^1$H NMR (500 MHz, MeOD) $\delta$ 8.01 – 7.84 (m, 3H), 7.55 (d, $J = 7.7$ Hz, 1H), 7.31 (d, $J = 7.4$ Hz, 1H), 7.13-6.89 (m, 6H), 4.65 (t, $J = 12.8$ Hz, 2H), 4.17 (m, 7H), 3.37 (s, 6H), 3.13 (d, $J = 14.0$ Hz, 18H), 2.08 – 0.97 (m, 18H).

F10-G2-H/W(D/L)

$^1$H NMR (500 MHz, MeOD) $\delta$ 8.01 – 7.84 (m, 3H), 7.55 (d, $J = 7.6$ Hz, 1H), 7.31 (d, $J = 7.5$ Hz, 1H), 7.13-6.89 (m, 6H), 4.65 (t, $J = 12.8$ Hz, 2H), 4.17 (m, 7H), 3.37 (s, 6H), 3.28-2..73 (m, 18H), 2.08 – 0.97 (m, 18H).
F10-G2-H/W(Me)

$^1$H NMR (500 MHz, MeOD) $\delta$ 8.08 – 7.86 (m, 3H), 7.59-7.51 (m, 1H), 7.34-7.28 (m, 1H), 7.15-6.87 (m, 6H), 4.66 (t, $J$ = 13.4 Hz, 2H), 4.45-4.01 (m, 7H), 3.72 (s, 3H), 3.51-3.37 (m, 6H), 3.28-3.01 (m, 16H), 2.01 – 1.05 (m, 18H).

F10-G2-H/Y(Me)

$^1$H NMR (600 MHz, MeOD) $\delta$ 8.45-8.27 (m, 3H), 7.34-7.17 (m 3H), 7.25 (d, $J$ = 7.9 Hz, 2H), 6.72 (d, $J$ = 7.6 Hz, 2H), 4.68 (m, 2H), 4.27-4.02 (m, 7H), 3.95 (s, 3H), 3.60 – 3.38 (m, 6H), 3.24 – 2.72 (m, 18H), 2.05 – 1.11 (m, 18H).

F10-G2-H/F(Me)

$^1$H NMR (600 MHz, MeOD) $\delta$ 8.51-8.32 (m, 3H), 7.45 – 6.91 (m, 7H), 4.66 (m, 2H), 4.32-4.09 (m, 7H), 3.52-3.37 (m, 2H), 3.25-2.88 (m, 20H), 2.25 (s, 3H), 1.92-1.15 (m, 18H).

F10-G2-H/1-Nap

$^1$H NMR (500 MHz, MeOD) $\delta$ 8.09 (s, 1H), 7.86 (d, $J$ = 16.6 Hz, 5H), 7.63 – 7.27 (m, 4H), 7.11-7.02 (m, 3H), 4.41 – 3.90 (m, 7H), 3.40 (s, 6H), 3.25 – 2.60 (m, 20H), 1.91-1.05 (m, 18H).

F10-G2-H/2-Nap

$^1$H NMR (500 MHz, MeOD) $\delta$ 7.83 (m, 8H), 7.48 – 7.29 (m, 3H), 7.06 (d, $J$ = 25.2 Hz, 3H), 4.66 (t, $J$ = 13.5 Hz, 2H), 4.19 (dd, $J$ = 82.3, 43.2 Hz, 8H), 3.39 (s, 8H), 3.22 – 2.89 (m, 10H), 2.78 (s, 4H), 1.98 – 1.03 (m, 19H).
**F10-G2-H/Dip**

$^1$H NMR (500 MHz, MeOH) δ 7.78 (d, $J = 14.1$ Hz, 3H), 7.62 – 7.10 (m, 10H), 7.02 (d, $J = 15.7$ Hz, 3H), 4.67 (t, $J = 14.4$ Hz, 2H), 4.42 – 3.89 (m, 7H), 3.74 – 3.37 (m, 2H), 3.20 – 2.68 (m, 18H), 1.95-1.11 (m, 18H).

**F10-G2-H/Bip**

$^1$H NMR (500 MHz, MeOH) δ 7.99-7.81 (m, 3H), 7.75 – 7.25 (m, 9H), 7.02 (d, $J = 14.9$ Hz, 3H), 4.68 (t, $J = 14.7$ Hz, 2H), 4.40 – 3.95 (m, 7H), 3.75 – 3.36 (m, 6H), 3.25 – 2.68 (m, 16H), 1.98-1.05 (m, 18H).

**F10-G2-H/W(5F)**

$^1$H NMR (500 MHz, MeOD) δ 7.99-7.72 (m, 3H), 7.41 – 7.15 (m, 3H), 6.98 (s, 3H), 6.88 (s, 1H), 4.69 (t, $J = 14.5$ Hz, 2H), 4.45-3.92 (m, 7H), 3.71 – 3.35 (m, 8H), 3.30 – 2.67 (m, 14H), 1.94-1.12 (m, 18H).

**F10-G2-H/W(6F)**

$^1$H NMR (500 MHz, MeOD) δ 7.99-7.72 (m, 3H), 7.50 (s, 1H), 7.14 (s, 1H), 7.13-6.98 (m, 4H), 6.78 (s, 1H), 4.65 (t, $J = 13.8$ Hz, 2H), 4.49 – 3.84 (m, 7H), 3.69 – 3.35 (m, 10H), 3.30 – 2.60 (m, 12H), 1.85-1.11 (m, 18H).
**F10-G2-H/Y(3F)**

$^1$H NMR (600 MHz, MeOD) $\delta$ 8.09-7.90 (m, 3H), 7.50-7.12 (m, 3H), 6.85-6.68 (m, 3H), 4.67 (t, $J = 13.9$ Hz, 2H), 4.60-4.12 (m, 7H), 3.95 – 3.35 (m, 10H), 3.30-3.01 (m, 8H), 2.83 – 2.60 (m, 4H), 1.97 – 1.10 (m, 18H).

**F10-G2-H/F(NH2)**

$^1$H NMR (600 MHz, MeOD) $\delta$ 8.05-7.91 (m, 3H), 7.55-7.19 (m, 3H), 7.02 (d, $J = 7.7$ Hz, 2H), 6.61 (d, $J = 7.7$ Hz, 2H), 4.63 (t, $J = 13.8$ Hz, 2H), 4.62-4.11 (m, 7H), 3.98 – 3.35 (m, 10H), 3.30-3.06 (m, 8H), 2.85 – 2.61 (m, 4H), 1.95 – 1.15 (m, 18H).

**F10-G2-H/F(NO2)**

$^1$H NMR (600 MHz, MeOD) $\delta$ 8.15-7.70 (m, 5H), 7.55 – 7.23 (m, 5H), 4.61 (t, $J = 13.9$ Hz, 2H), 4.13 (m, 7H), 3.72 – 3.35 (m, 8H), 3.29-2.95 (m, 10H), 2.71 (s, 4H), 1.55 (m, 18H).

**F10-G2-H/F(Br)**

$^1$H NMR (600 MHz, MeOD) $\delta$ 8.09-7.93 (m, 3H), 7.81 (d, $J = 7.6$ Hz, 2H), 7.52-7.00 (m, 5H), 4.65 (t, $J = 14.1$ Hz, 2H), 4.52-3.97 (m, 7H), 3.95 – 3.35 (m, 13H), 3.30-3.12 (m, 5H), 2.77 – 2.62 (m, 4H), 1.98 – 1.19 (m, 18H).

**F10-G2-H/F(CF3)**

$^1$H NMR (600 MHz, MeOD) $\delta$ 8.02-7.72 (m, 3H), 7.55-7.28 (m, 5H), 6.96 (d, $J = 7.7$ Hz, 2H), 4.62 (t, $J = 13.9$ Hz, 2H), 4.50-4.05 (m, 7H), 3.97 – 3.31 (m, 9H), 3.30-3.12 (m, 8H), 2.81 – 2.65 (m, 4H), 1.99 – 1.11 (m, 18H).
**F10-G2-H/F(CN)**

\(^1\)H NMR (600 MHz, MeOD) δ 8.11-7.89 (m, 3H), 7.74 (d, J = 7.7 Hz, 2H), 7.62-7.30 (m, 5H), 4.65 (t, J = 13.8 Hz, 2H), 4.52-4.01 (m, 7H), 3.95 – 3.35 (m, 13H), 3.30-3.12 (m, 5H), 2.77 – 2.62 (m, 4H), 1.98 – 1.20 (m, 18H).

**F10-G2-H/Pyr**

\(^1\)H NMR (600 MHz, MeOD) δ 8.02-7.81 (m, 5H), 7.50-7.32 (m, 3H), 7.18 (d, J = 7.6 Hz, 2H), 4.61 (t, J = 14.2 Hz, 2H), 4.49-4.00 (m, 7H), 3.92 – 3.35 (m, 12H), 3.30-3.10 (m, 6H), 2.85 – 2.64 (m, 4H), 1.95 – 1.09 (m, 18H).

**F10-G2-H/Bta**

\(^1\)H NMR (600 MHz, MeOD) δ 8.05-7.95 (m, 3H), 7.91 (s, 1H), 7.78-7.70 (m, 2H), 7.52-7.29 (m, 5H), 4.68 (t, J = 13.9 Hz, 2H), 4.51-3.98 (m, 7H), 3.82 – 3.35 (m, 10H), 3.30-3.11 (m, 8H), 2.91 – 2.65 (m, 4H), 1.98 – 1.10 (m, 18H).

**Synthesis of bola-C18-Cym-NH$_2$ (1):** The diacid (230 mg, 0.671 mmol, 1 equiv) and mono-boc protected cystamine (288.85 mg, 1.679 mmol, 2 equiv) were dissolved in 20 mL DMSO in a round bottle flask, followed by the addition of DIPEA (1075 µL, 6.043 mmol, 9 equiv), and PyBOP (1048 mg, 2.014 mmol, 3 equiv). The reaction mixture was left to stir at room temperature overnight. After the reaction, the mixture was diluted with 150 mL EtOAc and washed with brine three times. The organic layer was dried over Na2SO4 and the solvent was removed in vacuo. The crude product was purified by column chromatography (5% MeOH in DCM). In a 15 mL round bottom flask, 1 (0.150 mmol) was dissolved in 4 mL DCM and 0.05
mL TIPS, followed by drop-wise addition of 0.5 mL TFA. The reaction was left to stir at rt for 1 h, and all volatiles were removed in vacuum. The crude product was purified by re-dissolving in minimum DCM/MeOH mixture and precipitated in Et2O to give the TFA salt of 2 as a slightly yellow viscous solid (260 mg, 48% yield over 2 steps). 1H NMR (500 MHz, MeOD) δ 3.67 – 3.47 (m, 4H), 2.99 (t, J = 6.6 Hz, 4H), 2.87 (t, J = 6.6 Hz, 4H), 2.20 (q, J = 7.8 Hz, 4H), 1.62 (s, 4H), 1.32 (d, J = 12.8 Hz, 28H).

**Synthesis of bola-C18-G1-NH2 (2).** In a two-dram vial, 1 (0.062 mmol, 1 equiv), Boc-Lys-(Boc)-OH (71.5 mg, 0.136 mmol, 2.2 equiv) and DIPEA (53 µL, 0.308 mmol, 5 equiv) were dissolved in 2.5 mL NMP, followed by the addition of PyBOP (77.0 mg, 0.148 mmol, 2.4 equiv). The reaction was left to stir at rt for 24h. After the reaction, the solvent was removed in vacuum. The crude product was purified by dissolution in MeOH and precipitation in water. After purification, the Boc was deprotected in a solution of TFA solution (Typically, 1 mL TFA, 1.5 mL anisole, 2.5 mL DCM and 0.1 mL TIPS). The deprotection was done in 4h at rt, followed by solvent removal in vacuo. The product was then purified by dissolution in a minimum amount of MeOH and precipitation in Et2O.

**Synthesis of bola-C18-G1 Dipeptide Vectors.** In a one-dram glass vial were added 10 mg of the TFA salt of the unfunctionalized 2 (1 equiv) and Boc protected dipeptide (4.5 equiv, specified below). 1.5 mL NMP was added to dissolve the solids, followed by PyBOP (6 equiv) and DIPEA (15 equiv). The reaction was left to stir for 24 hours at rt. The protected bola was precipitated in an excess amount of deionized water. After removing water completely, the solid was dissolved in 1 mL TFA, 2 mL DCM, 2 mL Anisole and 0.25 mL TIPS. After stirring overnight, the solvent was removed in vacuo, the resulting solid was redissolved in MeOH and
precipitated in Et2O. The white precipitate was dissolved in MeOH and purified by dialysis (MWCO = 1000) against MeOH. All bolas were characterized by $^1$H NMR and MALDI.

**Synthesis of bola-F10-G1 Dipeptide Vectors.** The TFA-salt of the cysteamine functionalized fluorocarbon core (F10-G0) was synthesized as described in Chapter 2.5 and subjected to subsequent coupling reactions as described above to give F10-XX.

**NMR Shifts of Dipeptide Vectors**

**C18 HR**

$^1$H NMR (600 MHz, D$_2$O) $\delta$ 8.66 (s, 2H), 7.34 (s, 2H), 4.65 (s, 1H), 4.20 (s, 1H), 4.16 – 3.98 (m, 2H), 3.49 (d, $J = 5.8$ Hz, 4H), 3.03 (d, $J = 218.8$ Hz, 13H), 2.27 – 2.18 (m, 2H), 2.08 – 1.08 (m, 33H).

**C18 RH**

$^1$H NMR (600 MHz, MeOD) $\delta$ 7.75 (d, $J = 6.1$ Hz, 2H), 7.03 (s, 2H), 4.45 (d, $J = 8.2$ Hz, 1H), 4.39 – 4.30 (m, 2H), 4.08 (s, 2H), 3.48 (dd, $J = 17.6$, 14.4 Hz, 4H), 3.27 – 3.17 (m, 9H), 3.11 (s, 3H), 2.90 – 2.80 (m, 5H), 2.20 (t, $J = 7.5$ Hz, 2H), 2.11 – 1.50 (m, 28H), 1.30 (s, 22H).

**C18 HK**

$^1$H NMR (600 MHz, D$_2$O) $\delta$ 8.63 – 8.49 (m, 2H), 7.43 – 7.31 (m, 2H), 4.52 – 4.06 (m, 6H), 3.65 – 3.56 (m, 1H), 3.55 – 3.44 (m, 3H), 3.43 – 3.30 (m, 6H), 3.29 – 3.12 (m, 3H), 3.06 – 2.93 (m, 5H), 2.92 – 2.79 (m, 5H), 2.30 – 2.07 (m, 2H), 1.91 – 1.32 (m, 26H), 1.29 – 1.09 (m, 18H).
C18 KH

$^1$H NMR (500 MHz, MeOH) $\delta$ 7.95 (s, 2H), 7.10 (s, 2H), 4.40 – 4.08 (m, 6H), 3.58 – 3.38 (m, 5H), 3.28 (s, 7H), 3.19 (s, 6H), 2.96 – 2.72 (m, 8H), 2.73 – 2.72 (m, 1H), 2.20 – 2.09 (m, 3H), 1.93 – 1.15 (m, 33H).

C18 KW

$^1$H NMR (600 MHz, D$_2$O) $\delta$ 7.67 – 6.76 (m, 10H), 4.28 (s, 3H), 4.11 (d, $J = 87.5$ Hz, 2H), 3.46 (dd, $J = 97.4$, 17.8 Hz, 4H), 3.27 (d, $J = 55.8$ Hz, 3H), 3.07 – 2.52 (m, 10H), 2.12 (d, $J = 17.0$ Hz, 2H), 1.98 (s, 1H), 1.61 (s, 10H), 1.50 – 0.89 (m, 27H).

C18 WK

$^1$H NMR (600 MHz, MeOD) $\delta$ 7.63 (dd, $J = 21.8$, 7.9 Hz, 2H), 7.37 (t, $J = 8.7$ Hz, 2H), 7.29 – 6.93 (m, 6H), 4.77 (dd, $J = 14.8$, 7.4 Hz, 1H), 4.64 (t, $J = 7.6$ Hz, 1H), 4.19 (dd, $J = 8.6$, 5.5 Hz, 1H), 3.92 – 3.81 (m, 2H), 3.47 (t, $J = 6.3$ Hz, 2H), 3.42 – 3.35 (m, 2H), 3.29 – 2.68 (m, 12H), 2.19 (t, $J = 7.5$ Hz, 2H), 1.94 – 1.10 (m, 32H).

C18 WR

$^1$H NMR (600 MHz, MeOD) $\delta$ 7.66 (dd, $J = 20.9$, 7.9 Hz, 2H), 7.41 (d, $J = 8.2$ Hz, 2H), 7.22 (d, $J = 13.3$ Hz, 2H), 7.16 (t, $J = 7.6$ Hz, 2H), 7.08 (t, $J = 7.4$ Hz, 2H), 4.38 (s, 2H), 4.31 – 4.25 (m, 1H), 4.20 – 4.13 (m, 3H), 4.02 (t, $J = 7.3$ Hz, 1H), 3.55 – 3.40 (m, 4H), 3.36 (dt, $J = 8.4$, 5.7 Hz, 4H), 3.29 – 3.01 (m, 4H), 2.93 (d, $J = 23.4$ Hz, 2H), 2.86 – 2.75 (m, 4H), 2.38 (d, $J = 42.4$ Hz, 3H), 2.19 (t, $J = 7.5$ Hz, 2H), 1.65 (dd, $J = 39.1$, 25.3 Hz, 13H), 1.52 – 1.39 (m, 7H), 1.38 – 1.18 (m, 16H).
C18 RW

$^1$H NMR (500 MHz, MeOH) $\delta$ 7.66 (s, 2H), 7.40 (d, $J = 9.0$ Hz, 2H), 7.30 – 7.00 (m, 6H), 4.29 (d, $J = 48.4$ Hz, 2H), 3.47 (s, 2H), 3.20 (d, $J = 19.5$ Hz, 3H), 2.83 (d, $J = 18.1$ Hz, 6H), 2.26 – 2.13 (m, 3H), 1.35 (dd, $J = 49.0$, 36.5 Hz, 38H).

C18 WH

$^1$H NMR (600 MHz, D$_2$O) $\delta$ 8.52 (d, $J = 11.1$ Hz, 2H), 7.42 (t, $J = 7.0$ Hz, 5H), 7.31 – 6.88 (m, 9H), 4.55 (t, $J = 6.1$ Hz, 1H), 4.44 (t, $J = 6.9$ Hz, 1H), 4.24 (dd, $J = 11.3$, 6.8 Hz, 2H), 4.02 (t, $J = 7.2$ Hz, 1H), 3.56 (s, 1H), 3.50 – 3.21 (m, 9H), 3.19 – 2.72 (m, 13H), 2.14 (t, $J = 6.9$ Hz, 2H), 1.81 (s, 1H), 1.66 (d, $J = 34.5$ Hz, 2H), 1.55 – 1.22 (m, 7H), 1.09 (s, 16H).

C18 HW

$^1$H NMR (499 MHz, MeOH) $\delta$ 7.70 (dd, $J = 27.7$, 19.0 Hz, 3H), 7.40 (d, $J = 7.9$ Hz, 2H), 7.28 – 6.73 (m, 7H), 4.23 (d, $J = 57.8$ Hz, 2H), 3.63 – 3.40 (m, 6H), 3.24 – 2.91 (m, 7H), 2.92 – 2.62 (m, 4H), 2.18 (d, $J = 6.9$ Hz, 2H), 1.60 (s, 4H), 1.49 – 1.04 (m, 20H).

C18 RK

$^1$H NMR (600 MHz, MeOD) $\delta$ 4.48 – 4.27 (m, 3H), 4.21 – 3.95 (m, 4H), 3.68 – 3.43 (m, 6H), 3.24 (s, 6H), 3.01 (dd, $J = 13.5$, 6.9 Hz, 4H), 2.91 – 2.79 (m, 3H), 2.21 (t, $J = 7.5$ Hz, 2H), 2.01 – 1.24 (m, 51H).
C18 KR

$^1$H NMR (600 MHz, MeOD) δ 4.41 – 4.23 (m, 3H), 3.72 (s, 2H), 3.60 – 3.38 (m, 5H), 3.26 – 3.06 (m, 5H), 2.96 (dd, $J = 16.8$, 8.1 Hz, 3H), 2.91 – 2.77 (m, 4H), 2.24 – 2.15 (m, 2H), 2.14 – 1.17 (m, 46H).

F10 RW

$^1$H NMR (600 MHz, MeOD) δ 7.91 – 6.91 (m, 10H), 4.70 (t, $J = 14.0$ Hz, 1H), 4.56 – 4.13 (m, 4H), 3.66 – 3.35 (m, 8H), 3.28 – 3.05 (m, 7H), 2.37 (t, $J = 8.1$ Hz, 1H), 2.10 – 2.00 (m, 2H), 1.96 – 1.57 (m, 9H), 1.54 – 1.23 (m, 7H).

F10 WR

$^1$H NMR (600 MHz, MeOD) δ 7.67 – 7.57 (m, 2H), 7.35 (dt, $J = 12.5$, 6.2 Hz, 2H), 7.21 – 6.96 (m, 6H), 4.68 (dd, $J = 24.9$, 10.5 Hz, 2H), 4.27 – 4.15 (m, 2H), 3.94 (d, $J = 6.1$ Hz, 2H), 3.43 (dd, $J = 12.3$, 5.3 Hz, 2H), 3.29 – 3.10 (m, 7H), 2.97 (d, $J = 10.7$ Hz, 3H), 2.79 (t, $J = 6.6$ Hz, 1H), 2.74 – 2.63 (m, 2H), 2.08 (d, $J = 5.0$ Hz, 4H), 1.88 (s, 4H), 1.67 (d, $J = 54.0$ Hz, 7H), 1.35 – 1.22 (m, 4H).

F10 KH

$^1$H NMR (600 MHz, MeOD) δ 8.79 (d, $J = 6.1$ Hz, 2H), 7.39 (d, $J = 18.0$ Hz, 2H), 4.29 (d, $J = 4.6$ Hz, 1H), 3.98 (s, 2H), 3.69 – 3.56 (m, 1H), 3.55 – 3.42 (m, 5H), 3.28 – 3.10 (m, 7H), 2.98 (d, $J = 6.5$ Hz, 4H), 2.37 (t, $J = 8.1$ Hz, 2H), 2.13 – 1.99 (m, 2H), 1.98 – 1.25 (m, 28H).
**F10 HK**

$^1$H NMR (600 MHz, MeOD) δ 8.15 (d, $J = 6.0$ Hz, 2H), 7.21 (d, $J = 5.7$ Hz, 2H), 4.72 (t, $J = 14.0$ Hz, 2H), 4.42 (d, $J = 8.2$ Hz, 1H), 4.37 – 4.31 (m, 2H), 4.24 (d, $J = 6.2$ Hz, 3H), 3.63 – 3.41 (m, 5H), 3.26 (d, $J = 19.1$ Hz, 8H), 2.96 (dd, $J = 17.1$, 8.4 Hz, 5H), 2.85 (dt, $J = 13.7$, 6.8 Hz, 4H), 1.97 – 1.64 (m, 14H), 1.61 – 1.22 (m, 13H).

**F10 KW**

$^1$H NMR (600 MHz, MeOD) δ 7.81 – 6.98 (m, 10H), 4.56 (s, 1H), 4.44 (d, $J = 6.2$ Hz, 1H), 4.37 – 4.20 (m, 3H), 4.13 (d, $J = 45.1$ Hz, 3H), 3.50 – 3.44 (m, 3H), 3.26 – 2.89 (m, 7H), 2.37 (d, $J = 8.1$ Hz, 1H), 2.07 (dd, $J = 15.2$, 7.5 Hz, 2H), 1.54 (tdd, $J = 61.2$, 47.6, 19.1 Hz, 20H).

**F10 WK**

$^1$H NMR (600 MHz, MeOD) δ 7.63 (dd, $J = 22.0$, 8.0 Hz, 2H), 7.36 (t, $J = 8.0$ Hz, 2H), 7.26 – 6.95 (m, 6H), 4.78 – 4.59 (m, 4H), 4.19 (d, $J = 5.8$ Hz, 1H), 3.90 – 3.77 (m, 2H), 3.44 (s, 2H), 3.29 – 2.62 (m, 14H), 1.92 – 1.09 (m, 23H).

**F10 HR**

$^1$H NMR (600 MHz, MeOD) δ 8.14 (d, $J = 26.3$ Hz, 2H), 7.23 – 7.08 (m, 3H), 4.72 (t, $J = 14.2$ Hz, 3H), 4.67 – 4.58 (m, 1H), 4.29 (dd, $J = 9.1$, 5.0 Hz, 1H), 4.01 (dd, $J = 10.4$, 4.1 Hz, 3H), 3.64 – 3.40 (m, 7H), 3.29 – 3.07 (m, 13H), 2.92 – 2.78 (m, 6H), 2.04 – 1.25 (m, 25H).
F10 RH
$^1$H NMR (500 MHz, MeOH) $\delta$ 7.78 (d, $J = 7.0$ Hz, 4H), 7.06 (s, 5H), 4.77 – 4.67 (m, 5H), 4.54 – 4.31 (m, 11H), 4.17 (d, $J = 5.6$ Hz, 6H), 3.63 – 3.41 (m, 12H), 3.18 (ddd, $J = 22.8$, 14.5, 6.6 Hz, 16H), 3.01 (s, 5H), 2.89 – 2.78 (m, 7H), 2.09 (d, $J = 3.7$ Hz, 6H), 2.02 – 1.50 (m, 29H), 1.43 – 1.27 (m, 9H).

F10 WH
$^1$H NMR (600 MHz, MeOD) $\delta$ 8.07 (dd, $J = 76.5$, 23.6 Hz, 2H), 7.61 (dt, $J = 15.2$, 7.6 Hz, 2H), 7.36 (dd, $J = 13.2$, 7.4 Hz, 2H), 7.27 – 6.92 (m, 9H), 4.74 – 4.43 (m, 3H), 4.29 – 4.03 (m, 3H), 3.22 (dd, $J = 20.2$, 10.0 Hz, 9H), 2.89 – 2.59 (m, 3H), 1.80 – 1.02 (m, 9H).

F10 HW
$^1$H NMR (600 MHz, MeOD) $\delta$ 8.64 (dd, $J = 24.8$, 21.2 Hz, 1H), 7.61 (t, $J = 7.5$ Hz, 1H), 7.43 – 6.97 (m, 4H), 4.77 – 4.60 (m, 1H), 4.24 (s, 1H), 3.66 – 3.37 (m, 2H), 3.19 (ddd, $J = 68.0$, 39.6, 8.3 Hz, 3H), 2.84 (dd, $J = 13.6$, 6.7 Hz, 1H), 1.75 (dd, $J = 60.7$, 6.4 Hz, 1H), 1.56 – 1.17 (m, 2H).

F10 RK
$^1$H NMR (500 MHz, MeOH) $\delta$ 4.78 – 4.68 (m, 3H), 4.50 (ddd, $J = 8.9$, 8.2, 3.7 Hz, 6H), 4.23 (s, 2H), 4.06 – 3.95 (m, 4H), 3.52 – 3.43 (m, 4H), 3.30 – 3.11 (m, 9H), 3.00 – 2.91 (m, 5H), 2.09 – 1.42 (m, 40H).
**F10 KR**

$^1$H NMR (600 MHz, MeOD) δ 4.71 (d, $J = 14.1$ Hz, 2H), 4.46 – 4.23 (m, 5H), 3.63 – 3.43 (m, 6H), 3.22 (d, $J = 40.8$ Hz, 10H), 3.03 – 2.80 (m, 12H), 2.03 – 1.25 (m, 46H).

**Experimental Procedures**

**Formulation of Vectors via Thin-Film Hydration.** To a 1 dram vial were added EtOH (0.25 mL), an appropriate amounts of the vector solution(s) (5-10 mg/ml in ddH$_2$O or MeOH), and any other desired formulation components (typically 2-10 mg/ml stock solutions in EtOH). The solvent was completely removed *in vacuo* and the thin-film rehydrated with an appropriate amount of ddH$_2$O, with mixing via vortex and sonication if necessary to ensure homogenization.

**General Vector/siRNA complex Preparation.** Prior to complexation all vectors and buffers were allowed to equilibrate to room temperature and vortexed. A 1.5 μM solution of siRNA was diluted to the appropriate volume with the desired media (low-salt PBS, OptiMEM or DMEM). The amount of vector solution required to give the desired N/P ratio was added to the diluted siRNA and mixed via pipette to give a 5X complex solution. After 5 minute incubation without agitation, this concentrated solution was gently mixed via pipette and used immediately.

**Transfection of 3T3 Cells and Flow Cytometry Analysis.** NIH 3T3 fibroblast cells were seeded at a density of 10,000 cells/ well in 48-well plates 24 h in advance. Prior to transfection, the media was replaced with 80 μL plain DMEM solution without serum. Different complex solutions were prepared as described in the general complex procedure (PBS) previously and 20 μL added to each well to make the final siRNA concentration 100 nM. After 4h incubation, the media was changed back to 250 μL DMEM supplemented with 10% fetal
bovine serum and cultured for another 48h. Before the analysis, cells were released from each well by Trypsin and harvested by centrifugation (5 min, 500G). Fluorescence of transfected cells was measured on a Becton-Dickinson LSR II flow cytometer with argon ion excitation laser. For each sample, data representing 10,000 objects were collected as a list-mode file and analyzed using FACSDivaTM software (Becton Dickinson, version 6.1.3).

**Transfection and Analysis of Luciferase Expression of HEK-293 and MDA-MB-231 Cells.** Following standard protocols for the handling of the cell lines, the knockdown effects of the various vectors were assayed. After passaging, the cells were plated in 96-well plates at 5,000 cells/well 24 h in advance. Immediately prior to addition of the complexes the culture media was switched to 80 or 90 µL OptiMEM per well. The 5X vector/siRNA complexes were prepared as described previously and 10 or 20 µL added to each well to achieve the desired concentration.

After 48 hours of incubation post-transfection, the culture media was removed and replaced with 100 µL of a 150 µg/mL solution of firefly D-luciferin in OptiMEM buffer. Without any further treatment, the cells were incubated at 37 °C for 5 minutes after which they were imaged using an IVIS lumina II camera. The normalized luciferase knockdown was determined by comparing the overall luminescence of the samples treated with complexes containing anti-luc siRNA to those treated with complexes containing negative control siRNA.

**Confocal Laser Scanning Microscopy.** Confocal laser scanning microscopy was used to observe the trafficking of labeled siRNA in the transfected cells. Unmodified NIH 3T3 fibroblast cells were seeded at a density of 100,000 cells/well on an 8-well chamber slide (Lab-Tek, Rochester, NY) 24h before transfection. Cy3-labeled siRNA was complexed with different PEI at N/P 30 and transfected to the cells under the aforementioned conditions. After transfection, the media was switched back to DMEM with 10% serum. Confocal fluorescence spectroscopy was
performed at 4 h and 24 h post transfection. The nucleus was counter-stained with 0.5 μg/mL solution of Hoechst 33342. All confocal images were acquired using a Zeiss LSM 700 inverted laser-scanning confocal microscope. A 63× plan apochromatic numerical aperture of 1.4 oil immersion DIC III objective or 20× plan apochromatic numerical aperture of 0.8 DIC II objective was used for all experiments. A 555 nm laser and a 546-700 nm band-pass filter were used to obtain the images of Cy3-labeled siRNA. A 405 nm laser and a 400-498 nm band-pass filter were used to obtain the images of the Hoechst 33342 counter-stained nuclei. A 639 nm laser and a 640-700 nm band-pass filter were used to obtain the images of the LysoTracker® Deep Red counter-stained acidic organelles. The fluorescent images were scanned separately and overlaid together with the differential interference contrast image (DIC). The cells were scanned as a z-stack of two-dimensional images (1024×1024 pixels) and an image cutting approximately through the middle of the cellular height was selected to present the intracellular siRNA localization.

**DLS Measurements.** The size and zeta potential of bola/siRNA polyplexes were measured at 633 nm using Zetasizer (NanoZS) dynamic light scattering instrument (Malvern Instruments, Malvern, UK) at 25°C with detection angle of 173°. The stock vector solutions (5 mg/ml) were complexed with 40 μM siRNA diluted in the desired buffer, typically low-salt PBS or OptiMEM to give a final [siRNA] of 2.5 μM, an N/P ratio of 20 and a final volume of 100 μL. After pipette mixing, the samples were transferred into a cuvette and analyzed for particle size.
Representative HPLC Traces of Dipeptide Vectors
Representative $^1\text{H}$ NMR Spectra of Vectors
F10-G2-H
C18-HK
C18-HR
C18-HW
MALDI-TOF Spectra of Dipeptide Vectors
C18-HK: [M]+H calculated 1929.45 [M]+H found 1927.82
C18-HR [M]+H calculated 2040.49 [M]+H found 2039.76

Applied Biosystems MDS Analytical Technologies TOF/TOF™ Series Explorer™ 72039

TOF/TOF™ Reflector Spec #1 [BP = 2040.7, 8071]
C18-HW [M]+H calculated 2160.61 [M]+H found 2159.85

Applied Biosystems MDS Analytical Technologies TOF/TOF™ Series Explorer™ 72039
TOF/TOF™ Reflector Spec #1 [BP = 2182.8, 690]
C18-KH [M]+H calculated 1929.45 [M]+H found 1928.45

Applied Biosystems MDS Analytical Technologies TOF/TOF™ Series Explorer™ 72039

TOF/TOF™ Reflector Spec #1 [BP = 2004.8, 278]
C18-KW [M]+Na calculated 2147.73 [M]+H found 2146.05
C18-RH [M]+H calculated 2040.49 [M]+H found 2039.76
C18-WK [M]+H calculated 2124.73. [M]+H found 2123.95
C18-WR [M]+H calculated 2125.73. [M]+H found 2126.82
F10-HK: [M]+H calculated 2236.4 [M]+H found 2235.5
F10-HR: [M]+H calculated 2348.49. [M]+H found 2347.57
F10-HW [M]+H calculated 2468.61 [M]+H found 2467.57
F10-KH [M]+H calculated 2236.4 [M]+H found 2238.5
F10-KW [M]+H calculated 2432.73 [M]+H found 2341.79
F10-RH [M]+H calculated 2348.49. [M]+H found 2347.61
F10-RW [M]+H calculated 2545.77 [M]+H found 2546.66
F10-WH [M]+H calculated 2467.61 [M]+H found 2467.60
F10-WK [M]+H calculated 2432.73 [M]+H found 2431.49

3.6 References


Chapter 4: Fluorocarbon Modified Polyethylenimine for siRNA Delivery

4.1 Introduction

Since the discovery of the RNA interference pathway, the delivery of small interfering RNA (siRNA) has been of great interest for both research and therapeutic purposes. Many different types of synthetic vectors have been studied for siRNA delivery, including polymers, lipids, and gold nanoparticles. Early delivery systems based upon highly positively charged vectors, such as cationic lipids and polymers, often induce toxicity and immunogenic response. More recently, ionizable lipids have been shown to display improved properties. One synthetic strategy that has proved particularly versatile for the discovery of new vectors is the functionalization of simple polyamines with hydrocarbon (HC) chains, resulting in amphiphilic ionizable compounds termed lipoids. Lipoid vectors have shown great promise for siRNA delivery to the liver and the endothelium; however, the underlying reasons for the tissue specificity are not fully understood. For some lipids and lipoids, efficient hepatic silencing has been shown to be highly dependent upon the incorporation of endogenous Apolipoprotein E (ApoE) into the lipoplexes. While such a “hitchhiking” strategy can be capitalized for some specific applications, strong interactions between the vector/siRNA complexes and serum components are generally undesirable for applications requiring long circulation time or when targeting using a cell specific ligand.

Scheme 4.1. Fluorocarbon PEI promotes efficient siRNA delivery
While attaching hydrophobic moieties (i.e. cholesterol, stearic acid, HC epoxides) to cationic compounds is a common strategy to generate effective siRNA vectors, there are relatively few examples on the use of fluorocarbon (FC) chains. Early studies on hemi-fluorinated lipids have suggested that FC domains can enhance assembly stability and transfection efficiency in the presence of serum. More recent reports have shown that incorporation of FCs either on the periphery or as the hydrophobic core of dendritic vectors can greatly enhance transfection efficacy. FC chains have significantly different properties compared to HC analogs; they are lipophobic in addition to being extremely hydrophobic. Based on these observations, we envisioned that FC functionalized vectors could display strong self-assembly while proving resistant to nonspecific interactions with serum components and aggregation in biological media. This strategy may lead to the development of new vectors having improved delivery efficiency and enhanced tissue specificity induced by any desired targeting moiety.

![Scheme 4.2 Functionalization of PEI with FC epoxides.](image)

**4.2 Results and Discussion**

To test our hypothesis, we selected low molecular weight branched polyethylenimine (PEI, $M_n = 600$) based on prior reports of successful vectors formed by reacting this polymer with HC epoxides. Three commercially available FC epoxides of different lengths were reacted with PEI by heating in EtOH (Scheme 4.2). The resulting FC functionalized polymers are referred to as FX-PEI-Y, where X refers to the total number of carbons atoms in the epoxide and Y indicates the average number of epoxide moieties per PEI molecule, as determined by $^1$H NMR. Two series
of HC analogs (C9 and C18) were synthesized for comparison and are referred to as CX-PEI-Y using similar nomenclature. We initially targeted compounds with lower levels of functionalization (1.0 – 4.0 epoxides per PEI) as we desired vectors with good water solubility. The vectors were fully dissolved in 25 mM sodium acetate buffer at pH 4.5 and these stock solutions used for all subsequent biological testing.

The siRNA complexation properties of the polymers were assayed by preparing complexes at differing N/P ratios (the molar ratio of vector nitrogens to siRNA phosphates) and subjecting them to gel electrophoresis. The results indicated that polymers functionalized with longer FC epoxides complexed siRNA more effectively, with F13-PEI-3.2 showing full complexation at an N/P ratio as low as 5 (Figure 4.1a). The FC PEI samples bound siRNA more strongly than the HC analogs, with C9-PEI-3.2 not fully complexing siRNA until N/P = 40, while F9-PEI-3.2 showed complete binding at an N/P ratio of 30. Competitive binding gel-shift assays with dextran sulfate (\(M_n \sim 25\) kD) further confirmed the binding strengths (Figure 4.1b), with F9-PEI-3.2 showing full release at a S/P ratio (molar ratio of polymer sulfates to siRNA phosphates) of 12 while F13-PEI-3.2 did not fully release until a S/P of 30. The HC vector C9-PEI-3.2 displayed much weaker binding strength with complete release at an S/P of 4.

Figure 4.1. (a) siRNA complexation gel-shift assay of X-PEI-3.2 siRNA at varying N/P ratios. (b) Dextran sulfate competitive binding gel-shift assay for vector/siRNA complexes (N/P = 40) at various S/P ratios.
The synthesized vectors were screened for transfection activity in HEK-293 cells stably expressing firefly luciferase (Luc). Complexes were prepared with either anti-Luc or non-targeting control siRNA at an N/P of 30, the cells transfected, and then luciferase activity quantified via luminescence imaging after 48 hours (Figure 4.2a). In general, samples functionalized with greater than ~2.0 equiv. of any FC epoxide showed potent gene silencing. The C9-PEI samples were generally less effective and had a very narrow effective range with only C9-PE-4.6 inducing greater than 50% silencing effect.

![Graph](image1)

**Figure 4.2.** (a) Transfection of HEK-293 cells with anti-Luc siRNA/X-PEI-Y complexes at N/P = 30. (b) N/P transfection screen of anti-Luc siRNA/X-PEI-3.2 vector complexes in HEK-293 cells.

The cell viability was assessed via lactate dehydrogenase (LDH) assay with the results indicating that the C9-PEI samples were generally more toxic than FC PEI samples, especially at relatively high functionalization levels (Figure 4.5). For example, only 31% of the cells were viable after being treated with siRNA/C9-PEI-4.6 complex (Figure 4.5b). Since C18 functionalized PEI was previously shown more effective for siRNA delivery compared to short
HC functionalized PEI’s, we also compared our data with C18-PEI samples. The transfection efficacy of the C18-PEI samples was similar to previous reports, with the transfection efficiency increasing with increasing levels of functionalization and N/P ratio (Figure 4.6a, c). While the transfection efficacy was comparable to the FC PEI vectors, the effective C18-PEI vectors displayed significantly higher levels of cytotoxicity (Figure 4.6b, d). Presumably, the lower cytotoxicity of FC-PEI’s compared to HC analogs is due to altered complex stabilities or interactions of the FC chains with cellular

Next, several effective samples functionalized with the same level (3.2 eq.) of the various epoxides were selected for further transfection testing at varying N/P ratios (Figure 4.2b). The results indicated that FC vectors began to show effective gene silencing at an N/P of 20, with F9-PEI-3.2 showing increasing knockdown effect up to an N/P of 50. For the F11- and F13-PEI-3.2 vectors, increasing the N/P ratio above 30 did not appear to improve the knockdown response. In contrast, the C9-PEI-3.2 sample only achieved 45% knockdown and did not show any response to increasing N/P ratio.

Confocal fluorescence microscopy was used next to investigate the cell uptake of fluorescently labeled siRNA/ vector complexes. For the microscopy experiments, NIH-3T3 cells plated in chambered slides were exposed to Cy3-siRNA/vector complexes for 24 hrs. The nuclei were stained with Hoechst 33342 and the acidic organelles with Lysotracker (Figure 4.3). The fluorescence images of the samples treated with the FC complexes show significant Cy3 signal both co-localized with the lysosomal stain and successfully delivered to the cytoplasm of the cells (Figure 4.3a-c). In contrast, cells treated with C9-PEI-3.2/siRNA complexes display very weak overall Cy3 signal (Figure 4.3d). The cell uptake of Cy3-siRNA was further quantified by flow cytometry (Figure 4.7). Uptake of the complexes was shown to be heavily dependent upon
both the type and length of the epoxide, with increased length of FC epoxide correlating to increased cellular uptake. Vectors functionalized with the C9 epoxide displayed drastically lower uptake compared to the FC vectors studied. The imaging and flow cytometry results suggest that the FC vectors more effectively deliver siRNA into cells and to the cytoplasm than the HC analogues, in agreement with the transfection data.

![Confocal fluorescence microscopy images](image)

**Figure 4.3.** Confocal fluorescence microscopy images of cells treated with complexes formed between Cy3-siRNA and (a) F9-PEI-3.2, (b) F11-PEI-3.2, (c) F13-PEI-3.2, (d) C9-PEI-3.2. Blue = Hoechst 33342, green = Lysotracker Deep Red, red = Cy3-siRNA. Scale bar = 10 µM.

However, the cell uptake efficiency did not directly correlate with the gene silencing efficiency. While the F13-PEI vectors showed the highest cell uptake, they displayed similar or lower knockdown compared to F9- and F11-PEI vectors (Figure 4.2). These results suggest that siRNA release after cellular entry might be a limiting factor for the strongly binding F13 vectors. Such inverse correlation between binding strength and transfection efficacy has previously been reported for PEI functionalized with HC epoxides.\(^8^b\)

The particle size of the vector/siRNA complexes was analyzed using dynamic light scattering (DLS), with the results showing that the FC vectors condensed siRNA into nanoparticles 100-200 nm in diameter (Table 4.1). In general, FC vectors with higher degrees of functionality tended to condense siRNA into smaller nanoparticles. The morphology of the vector/siRNA complexes (F9-PEI-3.2, F11-PEI-3.2 and F13-PEI-3.2) were examined using graphene oxide...
supported cryogenic transmission electron microscopy (Cryo-TEM) (Figure 4.8). In general, the images for each vector/siRNA complex appeared similar in size and morphology. The images show vesicle-like assemblies with diameters ranging from 20-200 nm, which is consistent with the diameters observed by DLS as well as some smaller rod like particles.

Association with serum components is a critical consideration for in vivo delivery as aggregation or incorporation of endogenous serum components can dramatically affect therapeutic outcome. Upon mixing the vector/siRNA complexes with fetal bovine serum (FBS), both C9- and C18-PEI-3.2 complexes immediately induced turbidity (Figure 4.4a), indicating aggregation. In contrast, FBS mixed with the F9-, F11-, or F13-PEI-3.2 complexes was visually indistinguishable from the control sample diluted with sodium acetate buffer. The association with serum components was further studied by preparing complexes of selected vectors, diluting with 10% fetal bovine serum (FBS) in OptiMEM, and analyzing with DLS. The control sample showed a characteristic peak with size around 5-10 nm which can be attributed to proteins and other serum components. This characteristic peak remained intact upon addition of complexes prepared with the FC vectors (Figure 4.4b). On the contrary, when C9-PEI-3.2/siRNA complexes were added, this peak disappeared completely, with the analysis indicating only nanoparticles in the size regime of the vector/siRNA complexes. These results suggest that the FC
ve

tors interact differently with serum components than the HC analogues, presumably due to the
different chemistry of the hydrophobic regions.

4.3 Conclusion

This study demonstrates that FC functionalization of low molecular weight PEI is an
effective strategy for the discovery of siRNA delivery vectors. Compared to HCs of similar length,
vectors functionalized with FC epoxides showed stronger siRNA binding, lower cytotoxicity,
higher cell uptake and more effective gene silencing. The FC vectors also exhibited knockdown
efficacy over a broader range of functionalization levels. Studies into association of the vectors
with serum components demonstrated that the FC vectors induced less aggregation in the presence
of serum compared to HC analogs. These results suggest that FC functionalization could prove
fruitful for the development of vectors with improved delivery properties and reduced interaction
with serum components.

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performing TEM and cryo-TEM imaging of the complexes.
4.4 Supplementary Figures

**Figure 4.5.** Viability of HEK-293 cells treated with vector/siRNA complexes assessed via LDH assay, N/P = 30.

**Figure 4.6.** Transfection efficacy (a, c) and LDH viability (b, d) of HEK-293 cells treated with C18-PEI/siRNA complexes.
Figure 4.7. Uptake of Cy3-siRNA/vector complexes (N/P=30) by HEK-293 assessed via flow cytometry.

<table>
<thead>
<tr>
<th>z-avg (nm)</th>
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<tr>
<td>f9 PEI 3.2</td>
<td>175 ± 6</td>
</tr>
<tr>
<td>f9 PEI 3.9</td>
<td>155 ± 11</td>
</tr>
<tr>
<td>f11 PEI 2.1</td>
<td>146 ± 9</td>
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</tr>
<tr>
<td>c9 PEI 4.6</td>
<td>172 ± 14</td>
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Table 4.1. Diameter of vector/siRNA complexes analyzed via dynamic light scattering (DLS) by intensity.
Figure 4.8. Cryo-TEM images of X-PEI-3.2 complexes: F9-PEI-3.2 (top), F11-PEI-3.2 (middle) and F13-PEI-3.2 (bottom). Green arrows indicate the vesicle and rod like structures for the complexes and the red arrows indicate contamination from the cryo-TEM preparation.
4.5 Experimental

**Materials.** Unless otherwise noted, all reagents were used as received from commercial suppliers without further purification. Low molecular weight branched polyethylenimine (PEI, $M_n \sim 600$ Da) was purchased from Sigma-Aldrich (St. Louis, MO). The fluorocarbon epoxides were purchased from Oakwood Products Inc. and used without further purification. Sodium dextran sulfate (25kDa) was purchased from TCI America (Portland, OR) and was used as received. GelRed™ siRNA stain was purchased from VWR (Radnor, PA). All siRNA used in this study was purchased from Ambion (Carlsbad, CA) with Silencer® Select negative control siRNA and Silencer® Cy™-3 labeled negative control siRNA used for control and cellular uptake studies, respectively. The sequence for the Silencer® Select anti-Luc siRNA was as follows: 5’-AGACUAUAAGAUUCAAUCUt-3’. Lipofectamine 2000/RNAiMAX were purchased from Invitrogen (Carlsbad, CA) and used as a positive control following the manufacturer’s protocol. All reactions were performed using HPLC grade solvents unless otherwise noted. All water used in biological experiments was nanopure water obtained from Barnstead Nanopure Diamond (Waltham, MA). Ultrathin Carbon Type-A, 400 mesh TEM grids were purchased from TED PELLA Inc. (Redding, CA). HEK-293 cells stably expressing firefly luciferase were donated by Professor Jenifer Prescher (Department of Chemistry, UC Irvine, CA). All cell culture media and fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). Lysotracker Deep Red was used as received from Thermo Fisher (San Jose, CA).

**Instrumentation.** All compounds were characterized by NMR spectroscopy. $^1$H NMR spectra were collected at the UC Irvine NMR Facility and recorded at 500 MHz on Bruker instruments (GN500 or CRYO500). $^{19}$F NMR spectroscopy was performed at 400 MHz on a
Bruker DRX400. $^1$H NMR chemical shifts were reported as values in ppm relative to specified deuterated solvents. The size and zeta potential of vector/siRNA complexes were measured at 633 nm using Zetasizer dynamic light scattering instrument (Malvern Instruments, Malvern, UK) at 25°C with detection angle of 173°. TEM was performed on FEI Sphera microscope operated at 200 keV. The flow cytometry data was obtained using a BD Accuri C6 flow cytometer. Confocal fluorescence images were acquired using a Zeiss LSM 700 inverted laser-scanning confocal microscope (UCI Ayala School of Biological Sciences Optical Biology Core, UCI).

**Functionalization of PEI.** PEI (30 mg, $M_w = 600$) was added into a two dram vial and the desired amount of fluorocarbon or hydrocarbon epoxide was added. A stir bar and ethanol (1 mL) were added and the vial was capped tightly. The reaction mixture was stirred and heated at 90°C for 48 h, allowed to cool to r.t. and the solvent completely removed in vacuo. The average number of epoxides per PEI was determined via $^1$H NMR in CD$_3$OD, by comparing the integration values of the ethyleneimine peaks vs. those of the methine (fluorocarbon samples) or methyl (hydrocarbon samples) of the epoxide. Functionalized PEI samples were dissolved at 2 mg/mL in 25 mM sodium acetate buffer, pH = 4.5 for biological and complexation experiments.

![Scheme 4.3](image)

**Scheme 4.3.** Functionalization of PEI with Alkyl/perfluoroalkyl epoxides
Summary of $^1$H NMR data (all spectra collected at 500 MHz in CD$_3$OD):

**F9-PEI-0.9 (0.93 eq.):** δ 4.31-4.02 (m, 1H), 2.94-2.32 (bm, 60H), 2.32-2.10 (m, 2H).

**F9-PEI-1.4 (1.39 eq.):** δ 4.27-4.05 (m, 1H), 2.97-2.31 (bm, 41H), 2.31-2.09 (m, 2H).

**F9-PEI-1.7 (1.74 eq.):** δ 4.28-4.03 (m, 1H), 2.95-2.35 (bm, 33H), 2.35-2.10 (m, 2H).

**F9-PEI-2.0 (1.98 eq.):** δ 4.35-4.01 (m, 1H), 2.95-2.32 (bm, 29H), 2.32-2.12 (m, 2H).

**F9-PEI-2.2 (2.24 eq.):** δ 4.30-4.00 (m, 1H), 2.96-2.37 (bm, 26H), 2.37-2.08 (m, 2H).

**F9-PEI-2.5 (2.53 eq.):** δ 4.31-4.04 (m, 1H), 2.93-2.34 (bm, 23H), 2.34-2.11 (m, 2H).

**F9-PEI-3.0 (2.97 eq.):** δ 4.26-4.00 (m, 1H), 2.90-2.30 (bm, 20H), 2.30-2.10 (m, 2H).

**F9-PEI-3.2 (3.23 eq.):** δ 4.24-3.98 (m, 1H), 2.90-2.31 (bm, 19H), 2.31-2.04 (m, 2H).

**F9-PEI-3.9 (3.87 eq.):** δ 4.33-4.04 (m, 1H), 2.96-2.34 (bm, 16H), 2.34-2.09 (m, 2H).

**F9-PEI-4.7 (4.70 eq.):** δ 4.31-4.03 (m, 1H), 2.95-2.33 (bm, 13H), 2.33-2.10 (m, 2H).

**F11-PEI-1.0 (1.03 eq.):** δ 4.36-4.07 (m, 1H), 2.98-2.34 (bm, 54H), 2.34-2.14 (m, 2H).

**F11-PEI-1.3 (1.29 eq.):** δ 4.33-4.02 (m, 1H), 2.95-2.34 (bm, 44H), 2.34-2.11 (m, 2H).

**F11-PEI-2.1 (2.12 eq.):** δ 4.28-4.00 (m, 1H), 2.92-2.29 (bm, 27H), 2.29-2.06 (m, 2H).

**F11-PEI-2.3 (2.34 eq.):** δ 4.31-4.02 (m, 1H), 2.92-2.30 (bm, 25H), 2.30-2.09 (m, 2H).

**F11-PEI-3.0 (2.96 eq.):** δ 4.34-4.06 (m, 1H), 2.97-2.35 (bm, 20H), 2.35-2.11 (m, 2H).

**F11-PEI-3.2 (3.18 eq.):** δ 4.27-3.99 (m, 1H), 2.91-2.28 (bm, 19H), 2.28-2.07 (m, 2H).

**F11-PEI-4.3 (4.32 eq.):** δ 4.32-4.01 (m, 1H), 2.96-2.31 (bm, 14H), 2.31-2.10 (m, 2H).

**F11-PEI-4.9 (4.87 eq.):** δ 4.35-4.05 (m, 1H), 2.99-2.36 (bm, 13H), 2.36-2.11 (m, 2H).

**F13-PEI-1.3 (1.32 eq.):** δ 4.36-4.07 (m, 1H), 2.98-2.34 (bm, 43H), 2.34-2.14 (m, 2H).

**F13-PEI-2.1 (2.07 eq.):** δ 4.39-4.11 (m, 1H), 3.03-2.37 (bm, 28H), 2.37-2.10 (m, 2H).

**F13-PEI-2.5 (2.51 eq.):** δ 4.32-4.03 (m, 1H), 2.96-2.30 (bm, 24H), 2.30-2.06 (m, 2H).
F13-PEI-3.0 (3.01 eq.): δ 4.35-4.08 (m, 1H), 2.95-2.35 (bm, 20H), 2.35-2.12 (m, 2H).

F13-PEI-3.2 (3.24 eq.): δ 4.36-4.09 (m, 1H), 2.99-2.30 (bm, 19H), 2.30-2.10 (m, 2H).

F13-PEI-3.7 (3.70 eq.): δ 4.33-4.05 (m, 1H), 2.96-2.34 (bm, 17H), 2.34-2.13 (m, 2H).

F13-PEI-4.2 (4.23 eq.): δ 4.36-4.07 (m, 1H), 2.98-2.34 (bm, 15H), 2.34-2.14 (m, 2H).

F13-PEI-4.4 (4.36 eq.): δ 4.29-4.01 (m, 1H), 2.95-2.26 (bm, 12H), 2.26-2.06 (m, 2H).

C9-PEI-2.4 (2.35 eq.): δ 3.67 (m, 1H), δ 2.92 – 2.24 (bm, 16H), 1.57-1.23 (m, 12H), 0.92 (t, J = 6.5 Hz, 3H).

C9-PEI-2.8 (2.84 eq.): δ 3.68 (m, 1H), δ 2.88 – 2.25 (bm, 21H), 1.55-1.22 (m, 12H), 0.90 (t, J = 6.5 Hz, 3H).

C9-PEI-3.2 (3.20 eq.): δ 3.67 (m, 1H), δ 2.95 – 2.25 (bm, 19H), 1.54-1.22 (m, 12H), 0.92 (t, J = 6.5 Hz, 3H).

C9-PEI-4.0 (4.04 eq.): δ 3.69 (m, 1H), δ 2.91 – 3.28 (bm, 15H), 1.60-1.26 (m, 12H), 0.91 (t, J = 6.5 Hz, 3H).

C9-PEI-4.6 (4.56 eq.): δ 3.66 (m, 1H), δ 2.94 – 2.21 (bm, 14H), 1.55-1.22 (m, 12H), 0.90 (t, J = 6.5 Hz, 3H).

C9-PEI-4.0 (4.02 eq.): δ 3.67 (m, 1H), δ 2.98 – 2.23 (bm, 13H), 1.59-1.23 (m, 12H), 0.91 (t, J = 6.5 Hz, 3H).

C18-PEI-2.1 (2.12 eq.): δ 3.65 (m, 1H), δ 2.83 – 2.30 (bm, 27H), 1.55-1.21 (m, 12H), 0.89 (t, J = 6.5 Hz, 3H).

C18-PEI-2.3 (2.31 eq.): δ 3.67 (m, 1H), δ 2.96 – 2.24 (bm, 25H), 1.57-1.23 (m, 12H), 0.90 (t, J = 6.5 Hz, 3H).

C18-PEI-2.7 (2.73 eq.): δ 3.67 (m, 1H), δ 2.96 – 2.24 (bm, 22H), 1.56-1.22 (m, 12H), 0.89 (t, J = 6.5 Hz, 3H).

C18-PEI-3.0 (3.02 eq.): δ 3.68 (m, 1H), δ 2.97 – 2.21 (bm, 20H), 1.54-1.23 (m, 12H), 0.90 (t, J = 6.5 Hz, 3H).

C18-PEI-3.4 (3.36 eq.): δ 3.66 (m, 1H), δ 2.98 – 2.22 (bm, 18H), 1.57-1.20 (m, 12H), 0.90 (t, J = 6.5 Hz, 3H).
**General Complexation Protocol.** A stock solution of siRNA (40 µM in nuclease-free water) was diluted to the desired concentration with NaAc buffer (25 mM sodium acetate, pH = 5.5). The proper amount of vector solution (2 mg/mL in NaAc buffer) was added to give the desired N/P ratio (molar ratio of amine groups on polymer to phosphate groups from siRNA). After addition of the vector, the sample was mixed via pipette and allowed to incubate at r.t. for 20 minutes prior to use.

**Gel-Shift Complexation Assay.** For each well 4.0 µL of 4 µM siRNA was diluted with an appropriate amount of NaAc buffer. Differing amounts of vector solution (2 mg/mL in NaAc buffer) were added to give the desired N/P ratios and a final volume of 10 µL. After 20 min incubation at r.t., 2 µL of 6X loading dye was added to each sample and loaded into a 1% agarose gel with 1X GelRed dye. The electrophoresis was run in TAE buffer at 60 V for 50 min and the gel was visualized under a UV transilluminator.

**Dextran Sulfate Competitive Binding Assay.** Complexes with N/P ratio = 40 were prepared using 4.0 µL of 4 µM siRNA per well as described previously to give a final volume of 9 µL. After 20 min incubation at r.t., 1 µL of sodium dextran sulfate (DS, Mw = 25 kDa) solution at different concentrations was added to the complexes to achieve different S/P ratios (molar ratio of sulfate groups from DS to phosphate groups from siRNA). The mixture was incubated for another 30 min, then 2 µL of 6X loading dye was added to each sample and mixed via pipetting. The mixture was then immediately loaded into a 1% agarose gel with 1X GelRed dye. The samples were then subjected to agarose gel electrophoresis under the aforementioned condition.

**Transfection in HEK-293-Luc.** Following standard protocols for mammalian cell culture, the gene silencing efficacy of the various vectors were assessed HEK-293 cells engineered to
stably express firefly luciferase. For transfection experiments, cells were seeded in a 96-well plate at a density of 5,000 cells per well and incubated at 37 °C for 24 h. After incubation (when the cells are 30-40% confluent) and immediately prior to addition of the complexes the culture media was switched to 80 μL OptiMEM per well. The 5X vector/siRNA complexes were prepared following the general complexation protocol and 20 μL of the complex solution was added to each well, giving a final siRNA concentration of 40 nM. The plates were gently agitated and returned to the incubator. 48 hours post-transfection, the transfection media was removed and replaced with 100 μL of a 150 μg/mL solution of firefly D-luciferin in OptiMEM. Without any further treatment, the cells were incubated at 37 °C for 5 minutes after which they were imaged using an IVIS lumina II camera. The normalized luciferase knockdown was determined by comparing the overall luminescence of the samples treated with complexes containing anti-Luc siRNA to those treated with complexes containing negative control siRNA.

**Transfection of Cy3-siRNA and Analysis with Flow Cytometry.** HEK-293 were seeded in a 48-well plate at a density of 10,000 cells per well and incubated at 37 °C for 24 h. After incubation (when the cells are 30-40% confluent) and immediately prior to addition of the complexes the culture media was switched to 200 μL OptiMEM per well. Vector/siRNA complexes were prepared using Cy3 labeled siRNA following the general complexation protocol and 50 μL of the complex solutions added to each well. After 24 hours of incubation, the cells were released by treatment with trypsin (150 μL per well), transferred into 1.5 mL Eppendorf tubes and analyzed using flow cytometry.

**Cell Viability Assays.** HEK-293 cells were plated and treated with vector/siRNA complexes as described in the transfection protocol. For the LDH assay, immediately prior to luciferase analysis, 50 μL of the culture media was removed and assayed for LDH activity.
following the manufacturer’s instructions for the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher).

**Confocal Microscopy.** Confocal laser microscopy was used to track cyanine-3 (Cy-3) labeled siRNA in the transfected cells. Unmodified NIH 3T3 fibroblast cells were seeded at a density of 15000 cells/well on an 8-well chamber slide (Lab-Tek, Rochester, NY) 24h before transfection. Cy3-labeled siRNA was complexed with different PEI at N/P = 30 and transfected to the cells under the aforementioned conditions. After transfection, the media was switched back to DMEM with 10% serum. Confocal fluorescence spectroscopy was performed at 4 h and 24 h post transfection. The nucleus was counter-stained with 0.5 μg/mL solution of Hoechst 33342. Acidic organelles were counterstained with LysoTracker® Deep Red following the product manual at a concentration of 75 nM. All confocal images were acquired using a Zeiss LSM 700 inverted laser-scanning confocal microscope. A 63× plan apochromatic numerical aperture of 1.4 oil immersion DIC III objective or 20× plan apochromatic numerical aperture of 0.8 DIC II objective was used for all experiments. A 555 nm laser and a 546-700 nm band-pass filter were used to obtain the images of Cy3-labeled siRNA. A 405 nm laser and a 400-498 nm band-pass filter were used to obtain the images of the Hoechst 33342 counter-stained nuclei. A 639 nm laser and a 640-700 nm band-pass filter were used to obtain the images of the LysoTracker® Deep Red counter-stained acidic organelles. The fluorescent images were scanned separately and overlaid together with the differential interference contrast image (DIC). The cells were scanned as a z-stack of two-dimensional images (1024×1024 pixels) and an image cutting approximately through the middle of the cellular height was selected to present the intracellular siRNA localization.
**Statistical Analysis.** All biological assays were performed in triplicate, with data expressed as the mean ± STDEV.

**DLS Measurements.** The size and zeta potential of the vector/siRNA complexes were measured at 633 nm using Zetasizer (NanoZS) dynamic light scattering instrument (Malvern Instruments, Malvern, UK) at 25°C with detection angle of 173°. For particle size measurements, complexes were prepared at N/P = 30 following the general complexation protocol to give solutions in NaAc buffer with [siRNA] = 5 μM. After 20 minutes incubation, the samples were diluted with the desired buffer and the particle size and zeta potential measured.

**FBS Turbidity Assay.** Fetal bovine serum (FBS) was thawed and 50 μL added to 0.5 mL Eppendorf tubes. Following the standard complexation protocol, siRNA/vector complexes (N/P = 30, [siRNA] = 1 μM) were prepared using F9-PEI-3.2, F11-PEI-3.2, F13-PEI-3.2, C9-PEI-3.2, or C18-PEI-3.0 vectors. 50 μL of the prepared complexes were added to the thawed FBS and mixed via pipette. For the control sample, 50 μL of FBS was diluted with 50 μL of NaAc buffer. After 5 minutes, a camera was used to acquire an image of the diluted FBS.

**FBS Turbidity Assay -DLS.** Complexes were prepared using the X-PEI-3.2 series of vectors following the previously reported protocol at N/P = 30 to give a siRNA concentration of 1 μM. After mixing, 20 μL of the complexes were diluted into 80 μL of 10% FBS in OptiMEM and analyzed via DLS. For the control sample, 20 μL of NaAc buffer was added to 80 μL of 10% FBS in OptiMEM and analyzed.

**Graphene Oxide Supported Cryo-TEM.** Graphene oxide TEM grids were prepared as previously described using Quantifoil R2/2 TEM grids. For standard cryo-TEM, Quantifoil R2/2 TEM grids were plasma cleaned prior to use using an EMITECH K950X for 3 minutes at 20 mAmp and 0.2 mbar. Then either using the graphene oxide TEM grids or the
plasma cleaned Quantifoil grids, 7 μL of sample was added to the TEM grids and left for 10 minutes at high humidity before being blotted with a piece of filter paper and immediately plunged into liquid ethane. The grids were then transferred to liquid nitrogen for storage and transferred to the microscope where they were imaged at < -170 °C using a precooled Gatan 626 cryo-transfer holder.

Representative NMR Spectra

$^1$H Spectra

![Representative NMR Spectra](image)
$^{19}$F Spectra

F9-PEI-3.0

F11 PEI 3.2
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Chapter 5: Design of Ligands for Gold Nanoparticle Mediated siRNA Delivery

5.1 Introduction

The delivery of siRNA has the potential to treat a wide range of conditions including viral infection, neurodegenerative disease, and cancer.\(^1\) Due to the poor cellular uptake and rapid degradation of naked siRNA, the design of effective delivery agents is a critical step if the therapeutic potential of this technology is to be realized.\(^2\) A variety of synthetic vectors have been studied for this purpose, including polymers,\(^3\) lipids,\(^4\) dendrimers,\(^5\) peptides,\(^6\) and functionalized inorganic nanoparticles.\(^7\) Gold nanoparticles (GNPs) in particular present an attractive platform for delivery applications due to their biocompatibility, optical properties, well-defined size/shape, and ease of functionalization via the gold-thiolate bond.\(^8\) Several GNP-based approaches to siRNA delivery have been demonstrated, including covalent attachment of thiol-bearing RNA,\(^9\) electrostatic “wrapping” of GNPs with various charged polymers,\(^10\) and functionalization of the gold surface with cationic ligands.\(^11\)

Although studies have revealed that particles functionalized with a sufficient density of thiolated siRNA can enter cells through a process mediated by class A scavenger receptors,\(^12\) the successful delivery of siRNA by nanoplexes formed through complexation with cationic GNPs is often dependent upon incorporation of functionality allowing for endosomal escape to avoid entrapment and subsequent degradation.\(^{11a,b,d}\) GNPs functionalized with ligands terminated in

![Scheme 5.1 Functionalization of GNPs for siRNA delivery](image)
multiple copies of an oligo-(ethyleneimine) motif have been demonstrated to promote successful intracellular delivery of siRNA.\textsuperscript{11a} The incorporation of pH-responsive moieties, in the case of oligo-(ethyleneimine) secondary or tertiary amines, in the range of endosomal acidification (pH 5-7.4) is a strategy often used to improve the endosomal escape of delivery vectors. Following our previous strategy making use of natural building blocks, we sought to design amino acid bearing ligands to functionalize GNPs for promoting successful siRNA delivery.

5.2 Results and Discussion

Initially, we sought to optimize GNPs for siRNA delivery through co-functionalization with mixtures of simple ligands displaying a single copy of His, Trp, or Lys (Figure 5.1). It was thought that GNPs would provide an ideal platform for siRNA vectors due to the accessibility of various sizes / shapes and the ease of functionalization. Our first design consisted of an alkylthiol anchor coupled to a short oligo-ethylene glycol segment and terminated in a single amino acid. It was envisioned that the alkylthiol region would enable strong coordination of the ligand to the nanoparticle surface, while the flexible linker would give the cationic head group the conformational freedom necessary for effective siRNA binding. The positive charge required for complexation and membrane association is provided by the N-terminal α-amino groups or ε-amines on Lys side chains. Oleylamide-capped GNPs (d = 6.0 ± 1.1 nm) were chosen for this study due to the weakly coordinating nature of the stabilizing ligand and ease of purification/synthesis. All of the ligands studied make use of the same alkylthiol anchor and are referred to beginning with any modifications to the linker region, followed by the degree of headgroup dendronization, and finally the single amino acid codes for the terminal amino acid or peptide. For example, the ligand G1-K consists of the standard linker dendronized by the coupling of a single Lys, and terminated with Lys residues for a total of four primary amines per ligand.
The series of single amino acid ligands (G0-H, G0-W, G0-K, and G1-K), was synthesized using standard solution phase techniques and olelylamide-GNPs were co-functionalized with various combinations using standard ligand displacement techniques. After purification via centrifugation, the functionalized nanoparticles were readily dispersable in water with successful replacement of oleylamine evident from their water solubility and a slight red-shift of the UV/Vis absorbance maxima from 520 nm to 523-525 nm (Figure 2.2A). The functionalized nanoparticles were screened for colloidal stability and subjected to gel-shift assays to determine siRNA complexation. Nanoparticles functionalized with mixtures containing ≥50% of G0-K or G1-K were typically stable in ddH2O for several weeks, except for those containing >25% of G0-W.
which showed a red-shift in the $\lambda_{\text{max}}$ and immediate aggregation. The initial gel-shift assays revealed that GNPs functionalized with only G0-H and G0-W failed to effectively retard the migration of siRNA. In contrast, GNPs functionalized with mixtures containing G0-K or G1-K showed complete complexation beginning at molar ratios of approximately 15-20 siRNA per GNP (Figure 5.2B), suggesting that the positive charge provided by the $\alpha$-amino group of His alone is insufficient to effectively complex siRNA. TEM imaging was used to confirm the particle size and morphology of the GNPs (Figure 5.2C,D). Subsequent screening for transfection activity revealed moderate knockdown of GFP expression at siRNA to GNP ratio of 5 to 1; however the efficiency of these vectors compared unfavorably to that of Lipofectamine (Figure 5.3).
Results from the initial study suggested that GNPs functionalized solely with His were incapable of siRNA complexation and failed to induce efficient gene silencing when used in combination with Lys and Trp containing ligands. It was thought that further optimization would be wasted effort and a second series of ligands of higher valency was designed, incorporating more Lys residues to enhance the siRNA binding and colloidal stability of the functionalized GNPs. Additionally, the length of the region linking the alkylthiol anchor to the headgroup was increased to allow for greater flexibility. The artificial amino acid Stp was chosen for this purpose due to the presence of the oligo-ethyleneimine motif, which has been shown to promote endosomal escape. An oligo-ethylene glycol linker was also utilized in order to demonstrate the importance of the secondary amines to successful transfection.
The second generation of ligands was synthesized using solid-phase techniques and Fmoc-chemistry, assembling the alkylthiol anchor on 2-Cl Trt resin. GNPs functionalized with second generation ligands displayed stronger siRNA binding, with complete complexation occurring at 40-50 siRNA per GNP. The binding strength of the second generation ligands was further investigated via competitive gel-shift assays using dextran sulfate, with results suggesting that both increased length of the linking region and presence of secondary amines resulted in stronger siRNA binding (Figure 5.4). DLS measurements of Stp-G2-KKW functionalized GNPs complexed with siRNA at a 5:1 molar ratio showed a mono-disperse population with a hydrodynamic radius of 26 ± 5 nm in ddH2O, with the formation of aggregates of approximately 1 μm when prepared in either DMEM or OptiMEM.

The transfection efficiency of GNPs functionalized with the second generation ligands was initially assessed at a siRNA concentration of 66 nM and experiments confirmed that nanoplexes formed at molar ratio of 5:1 siRNA to GNPs were typically more effective than those complexed at higher ratios (Figure 5.5). Incorporation of terminal Trp residues appeared to enhance the gene silencing ability of the functionalized GNPs as shown by the comparison of Stp-G2-KK, Stp-G2-KKL, and Stp-G2-KKW. Similarly, increased valency of the head group also led to a corresponding increase in transfection efficiency with Stp-G2-KKW showing

**Figure 5.4.** Dextran sulfate competitive binding assays of functionalized GNPs.
greater knockdown than Stp-G1-KKW. The importance of the linker region was evident within the series G2-KKW, EG8-G2-KKW, Stp-G2-KKW, and Stp4-G2-KKW. While the two ligands containing the unmodified linker or oligo-ethyleneglycol segment had similar transfection efficiencies, the incorporation of a single Stp unit greatly enhanced the GFP knockdown; with four repeats of Stp proving even more effective. Further transfection experiments were performed probing the concentration dependence of the vectors, including GNPs functionalized with 5 mol % of a PEG-SH (M_w = 5000) with respect to the cationic ligand. In agreement with previous experiments, the GNPs functionalized with Stp4-G2-KKW showing the most effective knockdown, attaining >70% gene silencing at a siRNA concentration of 16 nM.

Figure 5.5. Transfection screening of functionalized GNPs in NIH-3T3-GFP cells. Non-targeting (-) or GFP (+) siRNA used, with siRNA: GNP molar ratio indicated on x-axis, [siRNA] = 66 nM.
5.3 Conclusion

The results from this study show that GNPs functionalized with simple amino acid-based ligands failed to affect efficient gene silencing. Incorporation of the artificial amino acid Stp in the linking region and increasing the valency of the peptide headgroup led to significant enhancement of transfection efficiency. The tri-peptide KKW proved to be more effective than KKL or KK, suggesting that the aromatic side-chain of Trp capable of promoting successful delivery. Although the optimized ligands displayed efficient silencing of GFP expression, assays revealed that functionalized GNPs even with 5 mol % PEG rapidly aggregated in typical cell culture media. Further investigation of this system was not pursued due to the lengthy ligand synthesis, poor yield of functionalized GNPs after purification, and lack of colloidal stability.
5.4 Experimental

Materials and Methods. Reactions were performed using standard Schlenk techniques under a nitrogen atmosphere unless otherwise noted. Flash chromatography was performed with applied air pressure and the indicated solvent systems over Fisher silica gel (230 - 400 mesh). Reagents for organic syntheses were obtained from Sigma-Aldrich or Acros and used without any further purification. Coupling reagents, Fmoc-protected amino acids, and 2-chlorotrityl chloride resin (100-200 mesh, 1.1 mmol/g) were purchased from AAPPTec. The synthesis of N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-11-(tritylthio)undecanamide was performed according to the reported procedure\textsuperscript{14}. The synthesis of Stp was carried out according to the procedure described by Wagner \textit{et al.}\textsuperscript{15} while the on-resin synthesis of the alkylthiol linker was adapted from the procedure reported by Albericio \textit{et al.}\textsuperscript{16} The synthesis of the EG\textsubscript{8} amino acid building block was adapted from the approach used by Davis \textit{et al.}\textsuperscript{17} with the two tetraethylene glycol starting materials (TrtO-EG\textsubscript{4}-OH and BnO-EG\textsubscript{4}-OTs) synthesized following standard procedures.

Nanopure water (ddH\textsubscript{2}O) was used for all biological experiments and obtained using a Barnstead Nanopure Diamond D91101. Sodium Dextran Sulfate (25 kDa) was purchased from TCI America, diluted to the desired concentration with ddH\textsubscript{2}O, and stored at -20 °C. Silencer anti-GFP siRNA, Silencer Select negative control siRNA, and Lipofectamine RNAiMAX were purchased from Invitrogen. GelRed\textsuperscript{TM} siRNA stain was purchased from VWR. Unmodified NIH 3T3 cell and engineered NIH 3T3 cell expressing enhanced green fluorescent protein (GFP) were gifted by Professor Young Jik Kwon (Department of Chemical Engineering, UC Irvine, CA). Cell culture media and fetal bovine serum (FBS) were purchased from Invitrogen.

Instrumentation. \textsuperscript{1}H and \textsuperscript{13}C NMR spectroscopy was performed at 500 MHz and 125 MHZ, respectively, using a Bruker GN500 or CYRO500. Unless otherwise noted peaks reported
are referenced to TMS (CDCl$_3$) or internal solvent standard (d4-MeOH/d6-DMSO). Mass
spectral data (ESI / MS, MALDI-TOF, and HRMS) was obtained at the UC Irvine Mass
Spectrometry Facility and collected with a Micromass LCT spectrometer. Analytic HPLC
analysis was performed on an Eclipse SDB-C18 reversed phase analytical column (50 mm x 4.6
mm) using a gradient of 5-95% MeCN in H$_2$O with 0.1 % TFA over 35 min and a flowrate of 1.0
mL/min. Preparative HPLC was performed on a Millipore Waters 600E system using an Agilent
Prep C18 column (250 mm x 21.2 mm) with a flowrate of 10.0 mL/min.

**General Procedure for Synthesis of Single Amino Acid Ligands.** To a stirred solution
of N-(2-(2-((aminoethoxy)ethoxy)ethyl)-11-(tritylthio)undecanamide (300 mg, 0.400 mmol),
DIPEA (129 mg, 2.5 equiv) and protected amino acid (1 eq. Boc-Lys(Boc)-OH, Boc-His(Boc)-
OH, or Boc-Trp(Boc)-OH) in DMF (3 mL) was added HOBT (256 mg, 0.500 mmol). Upon
dissolution of the HOBT, EDC HCl (215 mg, 1.05 eq) was added and the mixture purged with
nitrogen. The reaction was allowed to stir overnight and the solvent removed *in vacuo* at 55°C.
The crude product was dissolved in DCM (50 mL) and washed with DI water (3 x 50 mL) prior
to purification via CC (2-4% MeOH/DCM) to give the protected amino acid ligand. The
protected amino acid ligand (0.2 mmol) was dissolved in DCM (4 ml) in a 15 ml RBF and the
solution purged with nitrogen. Anisole (2 mL) was added followed by the dropwise addition of
TFA (4 ml). The reaction was stirred at RT until complete deprotection was accomplished (2-6
h), monitoring reaction completion with MS and TLC. The solvent was then reduced *in vacuo*
and the deprotected ligands precipitated in cold Et$_2$O.
**Scheme 5.2** Synthesis of EG₈ Building Block. (i) NaH, THF, 73%; (ii) TFA, TES, DCM, 79%; (iii) TsCl, NaOH, THF/H₂O; (iv) NaN₃, EtOH, reflux, 80% (2 steps); (v) H₂, Pd/C, TFA, EtOH, 96%; (vi) Fmoc-OSu, DIPEA, Succinic Anhydride, DMAP, THF/MeCN; 69%.

Octaethylene glycol benzyl trityl ether (2a). TrtO-EG₈-OH (11.6 g, 26.6 mmol) and BnO-EG₈-OTs (10.9 g, 25.0 mmol) were weighed into an oven-dried 250 mL RBF equipped with a stirbar. The flask was sealed, purged with N₂, and dry THF (100 mL) was added. The mixture was stirred in order to dissolve the starting materials and 60% NaH dispersion in mineral oil (1.2 g, 30 mmol) added. After stirring for 12 h, the THF was removed *in vacuo* and the residue dissolved in DCM (200 mL). The solution was transferred to a separation funnel and washed with sat. ammonium chloride (200 mL), water (200 mL), and brine (200 mL). The organic layer was dried with MgSO₄, filtered, and concentrated *in vacuo* until all solvent was gone. The crude product was purified by flash chromatography (10% EtOAc/hexanes) to yield a
clear oil (12.8 g, 73%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.47 (d, $J$ = 7.3 Hz, 6H), 7.28 (dd, $J_1$ = 7.5 Hz, $J_2$ = 7.5 Hz, 6H), 7.24-7.37 (m, 5H), 7.22 (t, $J$ = 7.1 Hz, 3H), 4.57 (s, 2H), 3.59-3.73 (m, 30H), 3.24 (t, $J$ = 5.2 Hz, 2H). ESI / MS (ES+/MeOH) $m/z$ calculated for C$_{42}$H$_{54}$O$_9$ (M + Na): 725.37, found 725.3.

Octaethylene glycol monobenzyl ether (2b). Compound 2a (10.5 g, 15.0 mmol) was weighed into a 100 mL RBF and dissolved in DCM (45 mL). The flask was sealed, purged with N$_2$, and a stirbar added prior to addition of TES (5.8 g, 50 mmol). Next, TFA (5 mL) was added dropwise and the reaction mixture was stirred for 2 h. The solvent was removed in vacuo, the residue redissolved in methanol, (50 mL) and sodium methoxide added (1.35 g, 25.0 mmol). The resulting mixture was heated to 60 °C and stirred for 12 h in order to completely cleave the undesired trifluoro-acetate side product. The reaction progress was monitored via TLC (30% EtOAc/hexanes). After removal of solvent in vacuo, the residue was partitioned between EtOAc (75 mL) and 0.5M HCl (75 mL). The aqueous layer was extracted with EtOAc (2 x 25 mL) and the combined organic fractions washed with water (100 mL) and brine (100 mL). The organic phase was dried (MgSO$_4$), filtered, the solvent removed in vacuo, and the residue purified by flash chromatography (30% EtOAc/hexanes) to give a clear oil (5.45 g, 79%). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.24-7.37 (m, 5H), 4.57 (s, 2H), 3.59-3.75 (m, 32H). ESI / MS (ES+/MeOH) $m/z$ calculated for C$_{23}$H$_{40}$O$_9$ (M + Na): 483.26, found 483.2.
**Azido-octaethylene glycol benzyl ether (2c).** Compound 2b (5.00 g, 10.8 mmol) was weighed into a 100 mL RBF and dissolved in THF (25 mL). The solution was cooled in a water/ice bath and a solution of NaOH (1.0 g, 25 mmol) in water (10 mL) was added. Tosyl chloride (2.48 g, 13.0 mmol) was added and the reaction mixture stirred for 1 h after which the cooling bath was removed and the reaction stirred for an additional 8 h at RT. The organic solvent was removed *in vacuo* and water/DCM (50/50 mL) added to the flask. The mixture was transferred to a separatory funnel and the organic phase isolated. The aqueous layer was extracted with DCM (2 x 25 mL) and the combined organic fractions washed with water (50 mL) and brine (50 mL) prior to drying (MgSO₄), filtration, and removal of solvent *in vacuo*. The resultant clear oil was then dissolved in EtOH (50 mL) and NaN₃ (1.05 g, 16.2 mmol) added. The reaction mixture was equipped with a reflux condenser and heated to reflux in an oil bath. After stirring for 12 h, the reaction was allowed to cool to room temperature and the EtOH removed *in vacuo*. The residue was partitioned between water/DCM (50 mL/50 mL), transferred to a separatory funnel and the organic layer isolated. The aqueous phase was extracted with DCM (2 x 25 mL) and the combined organic fractions washed with water (2 x 100 mL). After drying with MgSO₄, filtration, and removal of solvent *in vacuo* the crude product was purified by flash chromatography (10% EtOAc/hexanes) to give a clear oil (4.2 g, 80%). ¹H NMR (500 MHz, CDCl₃) δ 7.25-7.39 (m, 5H), 4.59 (s, 2H), 3.60-3.79 (m, 32H). ESI / MS (ES+/MeOH) m/z calculated for C₂₃H₃₉N₃O₈ (M + Na): 508.26, found 508.3.
**Mono-deoxy-amino-octaethylene glycol (2d).** Compound 2c (2.00 g, 4.10 mmol) was weighed into a dry 100 mL RBF equipped with stirbar and dissolved in EtOH (40 mL). The flask was sealed and the solution degassed for 15 min with N₂ prior to addition of 5% Pd/C (220 mg, 0.20 mmol). TFA (0.4 mL) was added to the reaction mixture and a H₂ balloon was connected to the flask. A vent needle was inserted into the septa and H₂ was allowed to bubble through the reaction mixture for 1 h, refilling the balloon with H₂ as necessary. The reaction was allowed to stir for an additional 12 h under a H₂ atmosphere, at which point the flask was purged with N₂ and the catalyst removed by vacuum filtration through celite. The solvent was removed *in vacuo* to give the TFA salt 2d as an off-white oil (1.90 g, 96%). ¹H NMR (500 MHz, D₂O) 3.55-3.85 (m, 32H). ESI / MS (ES+/MeOH) m/z calculated for C₁₆H₃₅N₈ (M + H): 370.24, found 370.3.

**1-(9H-fluoren-9-yl)-3,29-dioxo-2,7,10,13,16,19,22,25,28-nonaaxa-4-azadotriacontan-32-oic acid (2e).** Compound 2d (1.90 g, 3.93 mmol) was added to an oven-dried 50 mL RBF and dissolved in dry THF (20 mL). DIPEA (1.00 g, 7.95 mmol) was added and the reaction mixture was cooled in a water/ice-bath. A solution of Fmoc-Osu (1.41 g, 4.20 mmol) in THF:MeCN (5 mL : 10 mL) was added dropwise over a period of 1 h at which point the ice-bath was removed and the reaction mixture allowed to warm to RT. After stirring for an additional 10 h, succinic anhydride (500 mg, 5.00 mmol) was added, followed by DIPEA (1.3 g, 10 mmol) and DMAP (25 mg, 0.20 mmol). The reaction was stirred at RT for 12 h followed by solvent
removal in vacuo. The resultant residue was partitioned between 0.2 M HCl (100 mL) and DCM (75 mL), transferred to a separatory funnel, and the organic layer isolated. The aqueous phase was extracted with DCM (2 x 25 mL) and the combined organic fractions washed with 0.2 M HCl (100 mL), water (100 mL), and brine (100 mL). After drying (MgSO₄), filtration, and solvent removal in vacuo; the crude product was purified by flash chromatography (2-5% MeOH in DCM) to give 2e as a viscous oil (2.01 g, 69%). ¹H NMR (500 MHz, CDCl₃) δ 7.86 (d, J = 7.5 Hz, 2H), 7.55 (d, J = 7.6 Hz, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.29 (t, J = 7.4 Hz, 2H), 4.58-4.71 (m, 2H), 4.37-4.51 (m, 1H), 4.19 (t, J = 5.3 Hz, 2H), 3.50-3.79 (m, 28H), 3.25 (t, J = 5.2 Hz, 2H), 2.72-2.83 (m, 4H). ESI/MS (ES+/MeOH) m/z calculated for C₃₅H₄₉NO₁₃ (M + Na): 714.31, found 714.3.

**Synthesis and Characterization of GNPs.** The synthesis of oleylamine-stabilized GNPs was performed following the protocol reported by Gao et al.¹⁸. Glassware used for nanoparticle synthesis was cleaned with aqua regia, washed thoroughly with Nanopure water, and oven dried prior to use. Hydrogen tetrachloroaurate (III) trihydrate (ACS reagent) and oleylamine (70% tech.) were obtained from Sigma-Aldrich and used without any further purification. The GNPs were purified by precipitation/centrifugation as reported and stored as a solution in DCM at 4°C. The nanoparticle stock solution was dropcast onto Lacey Formvar / Carbon 200 mesh grids (Ted Pella, Inc.) and imaged using bright field transmission electron microscopy (TEM) using a FEI Tecnai TF-12 microscope operating at 120 keV. The particle size distribution was determined through analysis using ImageJ software (Cambridge, MA) and the extinction coefficient at 506 nm calculated according to the procedure reported by Liu et al.¹⁹. The stock solution was diluted in DCM and the UV/Vis spectra recorded using a Genesys 6 spectrophotometer (Thermo Scientific); with the concentration calculated using the absorbance at
506 nm and the calculated extinction coefficient ($\epsilon_{506} = 1.89 \times 10^7 \text{ M}^{-1}\text{cm}^{-1}$ for 6 nm diameter GNPs).

**Functionalization of OA GNPs.** OA GNP stock solution (0.5 mL, 0.5 nmol) was added to a 20 mL scintillation vial and diluted to 8 mL with DCM. The vial was placed on a VWR Mini Shaker set to 350 rpm and a solution of Stp-G2-KKW in MeOH 10 mg/ml (0.501 mL, 1.13 µmol) was added dropwise. The reaction was allowed to shake for 10 min at which point additional MeOH was added in order to increase the ratio of DCM:MeOH to approximately 2:1. After an additional h of agitation the vial was visually inspected for precipitation, and if necessary, additional MeOH was added to completely dissolve the NPs. After allowing 24 h for the ligand exchange to take place, the solvent was removed *in vacuo* and the red residue washed with 5% MeOH in DCM (3 x 10 mL). The remaining traces of organic solvent were removed *in vacuo* and the residue dissolved in 100 mM HCl (12 mL) to give a red solution. The solution was transferred to 1.5 mL Eppendorf tubes and centrifuged at 15k rpm for 2 h at 4° C. The majority of the supernatant was removed via vacuum aspiration and the nanoparticles redispersed in a total of 12 mL ddH$_2$O. The precipitation / dispersion procedure was repeated three times and the purified GNPs were resuspended in ddH$_2$O to give a final volume of approximately 150 µL. The dark red solution was sonicated briefly to ensure complete dispersion, the concentration calculated via UV/Vis spectroscopy, and the functionalized NPs stored at 4° C.
**General Solid-Phase Synthesis Information.** Ligands were synthesized using solid-phase techniques with standard Fmoc chemistry on 2-chlorotrityl resin. Cleavage of the Fmoc protecting group was accomplished by treatment with 20% piperidine in DMF (3 x 7 min). For coupling of standard amino acids, 4 equivalents of the protected amino acid with respect to the number of amines on the resin was used with DMF as the solvent, HCTU as the coupling agent and DIPEA as the base in a 4:4:8 ratio (amino acid : HCTU : DIPEA). The amino acids were pre-activated with HCTU/DIPEA for 1-2 min and the resin, then exposed to the coupling solution for 30-45 min. Monitoring reaction completion via the Kaiser test with double coupling was performed if necessary. Coupling of Fmoc-Stp(boc)$_3$-OH and Fmoc-EG$_8$-OH was accomplished using 2 equiv. of amino acid and HCTU per amine with 20% collidine in DMF as solvent/base. Reaction time was increased to 8-12 h in order to ensure complete coupling with

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**Scheme 5.3.** On-resin Synthesis of Alkylthiol Linker. (i) 11-mercaptoundecanoic acid, DCM; (ii) EDC, HOAt, DIPEA, DMF.
confirmation via the Kaiser assay. Between coupling and deprotection steps, the resin was washed with DMF (3 x 10 mL) and DCM (3 x 10 mL). Cleavage and deprotection were achieved simultaneously by exposing the resin to a solution containing 90:5:3:2 (TFA : thioanisole : EDT : anisole) for 2.5 h, followed by concentration in vacuo and precipitation in cold Et₂O. Unless otherwise noted ligands were re-precipitated in ether to remove excess scavengers and purified by preparative HPLC using an Agilent Prep C18 column (250 mm x 21.2 mm) with 0.1% TFA/H₂O and 0.1% TFA/MeCN as the solvents. After purification, the ligands were lyophilized and stored at -30° C.

**Procedure for Preparation of Resin 1.** 2-Chlorotrityl chloride resin (910 mg, 0.100 mmol) and 11-mercaptoundecanoic acid (438 mg, 0.200 mmol) were weighed into a dry 20 mL scintillation vial. Dry DCM (12 mL) was added to the vial, after which the vial was purged with N₂ then capped and sealed with parafilm. The vial was placed on a VWR Mini Shaker and agitated for 15 h at 350 rpm. The reaction mixture was transferred to a peptide synthesis vessel and the resin isolated via filtration. The resin was washed with DCM (3 x 15 mL) and then was reacted with a solution contain DCM, MeOH, DIPEA in a 8:1:1 ratio for 15 min with N₂ agitation. After the capping step, the resin was washed with DCM (2 x 15 mL) and DMF (2 x 15 mL) prior to addition of a solution of Fmoc-1-amino-4,7,10-trioxa-13-tridecanamine hydrochloride (2.44 g, 0.600 mmol), DIPEA (155 mg, 1.2 mmol) and HOAt (816 mg, 0.600 mmol) in DMF (10 mL) to the reaction vessel. EDC (1.15g, 0.600 mmol) was added and the mixture agitated using N₂ for 24 h. The vessel was drained and the resin washed with DMF (3 x 15 mL) and DCM (3 x 15 mL). After drying in vacuo, the resin loading was determined by treating a small quantity of resin (3-5 mg) with 20% piperidine and quantifying the amount of
Fmoc-piperidine adduct in solution by measuring the absorbance at 301 nm (1.05 g, 0.64 ± 0.02 mmol/g).

**General Peptide Synthesis (Stp-G2-KKW).** Resin 1 (154 mg, 0.100 mmol) was added to an empty 25 mL peptide synthesis vessel and swelled in DMF (10 mL) with N₂ agitation for 20 min. The initial Fmoc group was removed by treatment with 20% piperidine in DMF (3 x 7 min) and the resin washed with DMF (3 x 10 mL) and DCM (3 x 10 mL). Fmoc-Stp(boc)₃-OH (1.63 g, 0.200 mmol) and HCTU (827 mg, 0.200 mmol) were weighed into a scintillation vial and dissolved in 20% collidine in DMF (6 mL). After mixing for 1 minute, the coupling solution was added to the reaction vessel and the mixture allowed to react with the resin for 8 h under N₂ agitation. The resin was washed and the completion of the reaction was confirmed via the Kaiser test prior to deprotection of the terminal Fmoc group. Next, Fmoc-Lys(Fmoc)-OH (236 mg, 0.400 mmol) was coupled following the standard amino acid coupling procedure; followed by Fmoc-deprotection and coupling of another Fmoc-Lys(Fmoc)-OH (472 mg, 0.800 mmol). The Fmoc groups were removed and the following amino acids coupled sequentially: Fmoc-Lys(Boc)-OH (750 mg, 1.60 mmol), Fmoc-Lys(Boc)-OH (750 mg, 1.60 mmol), and Boc-Trp(Boc)-OH (648 mg, 1.60 mmol). After washing, the resin was transferred to a dry 25 mL RBF along with a stirbar and placed under a N₂ atmosphere. A solution containing a 90:5:3:2 mixture of TFA, thioanisole, EDT, anisole was freshly prepared and 10 mL added to the resin-containing RBF for cleavage. The mixture was stirred for 2.5 h, at which point the resin was filtered off and the solution concentrated in vacuo to ~1 mL. The deprotected peptide was precipitated in ice-cold Et₂O (50 mL) and isolated by centrifugation at 4100 rpm for 5 min. The ether was decanted, and the pellet washed with cold Et₂O (2 x 20 mL) and the white solid redissolved in methanol. The crude peptide was precipitated again in ice-cold Et₂O (50 mL) and
isolated via centrifugation again. The peptide was purified by preparative HPLC (5% MeCN to 50% MeCN over 60 min, monitoring at 285 nm). The pure fractions were combined and lyophilized to give a fluffy white solid (148 mg, 33%)

**Gel-shift Complexation Assay.** The siRNA stock solution (40 µM) and functionalized GNP stock solutions (1-2 µM) were diluted to appropriate concentrations with ddH2O. Differing amounts of GNPs were added to 5.0 µL of 4 µM siRNA to achieve the desired siRNA : GNP molar ratios. The final volume was adjusted to 10.0 µL with ddH2O and the samples incubated for 30 min at RT on a VWR Mini Shaker set to 350 rpm. A 1% agarose gel with 1X GelRed dye was prepared and 2.0 µL 6X gel loading dye was added to each sample prior to loading. The gel was electrophoresed in 1X TBE buffer (pH 7.9) for one hour at 60 V and the gel visualized under a UV transilluminator.

**Dextran Sulfate Competitive Binding Assay.** Stock solutions of sodium dextran sulfate (DS), 4 µM siRNA solution and various functionalized GNPs in ddH2O were prepared. The GNPs were added to 5.0 µL of 4 µM siRNA to give a siRNA : GNP molar ratio of 5 : 1 and the final volume adjusted to 10.0 µL with ddH2O. After incubating for 30 minutes, 1 µL DS solution of different concentration was added to the nanoplexes to give the desired S/P ratios (molar ratio of the sulfate groups on DS to the phosphate groups on siRNA). After an additional 30 min of incubation, the samples were subjected to agarose gel electrophoresis as described previously.

**MTT Assay.** 24 h prior to the experiment, NIH 3T3 fibroblast cells were seeded at 5000 cells / well in 96-well plates. The normal cell-culture media (10% FBS in DMEM) was replaced with 80 µL of pure DMEM. Stock solutions (1-2 µM) of the functionalized GNPs were added to 4 µM siRNA at a molar ratio of 5:1. After 30 min incubation, the nanoplexes were diluted as
necessary with pure DMEM and 20 μL added to each well to give the desired siRNA concentration. After 4 h incubation, the media was replaced with 10% FBS in DMEM and the cells cultured for an additional 48 h. The media was removed and 50 μL of DMEM containing 0.5 mg/mL MTT added, followed by 4h incubation at 37 °C. The formazan was dissolved by addition of 100 μL DMSO and the cell viability determined by measuring absorbance at 540 nm with a plate reader.

**Transfection Assay.** GFP producing NIH 3T3 fibroblast cells and unmodified NIH 3T3 cells were seeded at a density of 1000 cells/ well in 48-well plates 24 h prior to the experiment. GNP / siRNA nanoplexes at various molar ratios were prepared as described previously and diluted in pure DMEM to the appropriate concentration. The culture media was replaced with 200 μL of pure DMEM and 50 μL of the nanoplex solutions added to give the desired siRNA concentration. After incubating at 37 °C for 4 h, the media was changed back to 10% FBS in DMEM. The cells were cultured for an additional 48 h at which point they were Tripsinized and collected via centrifugation. The GFP fluorescence of the released cells was analyzed using a Becton-Dickinson LSRII flow cytometer with argon ion excitation laser at 488 nm (Becton-Dickinson, Franklin Lakes, NJ). For each sample, the GFP fluorescence of 10,000 objects was determined using FACSDivaTM software (Becton Dickinson, version 6.1.3).
MALDI-TOF and Structures of Ligands

**Stp-G2-KKW**

Chemical Formula: $C_{136}H_{233}N_{37}O_{20}S$

Exact Mass: 2772.81
Stp-G2-KKL
Chemical Formula: C_{119}H_{237}N_{33}O_{20}S
Exact Mass: 2480.83
Stp-G2-KK
Chemical Formula: C_{29}H_{193}N_{29}O_{10}S
Exact Mass: 2028.49
Stp-G1-KKW
Chemical Formula: C_{81}H_{141}N_{21}O_{12}S
Exact Mass: 1632.08
G2-KKW
Chemical Formula: C_{127}H_{268}N_{32}O_{18}S
Exact Mass: 2501.61

M + H
$\text{EG}_{2}\text{-G2-KKW}$

Chemical Formula: $C_{14}\,H_{24}\,N_{3}\,O_{2}\,S$

Exact Mass: 2952.85

$-\text{M} + \text{Na} - \text{H}_2\text{O}$
\[ ^1H \text{NMR Spectra of Stp-G2-KKW} \]
$^1$H NMR Spectra of Stp-G2-KKW
$^1$H NMR Spectra of EGs-G2-KKW
$^1$H NMR Spectra of Stp-G1-KKW
$^1$H NMR Spectra of G0-W
$^1$H NMR Spectra of G0-H
5.5 References


