Title
Rational Design of Synthetic Vectors for siRNA Delivery

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Author
Zeng, Hanxiang

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Rational Design of Synthetic Vectors for siRNA Delivery

DISSEPTION

submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Chemistry

by

Hanxiang Zeng

Dissertation Committee:
Professor Zhibin Guan, Chair
Professor Shiou-Chuan Tsai
Professor Elizabeth R. Jarvo

2014
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<th>Description</th>
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<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>BOP</td>
<td>(Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>CD</td>
<td>cyclodextrin</td>
</tr>
<tr>
<td>CQ</td>
<td>chloroquine</td>
</tr>
<tr>
<td>DIPEA</td>
<td>diisopropylethylamine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMS</td>
<td>dimethyl suberimidate•2HCl</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>EDC</td>
<td>3-(Ethyliminomethyleneamino)-N,N-dimethylpropan-1-amine</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>LNP</td>
<td>lipid nanoparticle</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>$M_n$</td>
<td>number average molecular weight</td>
</tr>
<tr>
<td>MTBE</td>
<td>methyl tert-butyl ether</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>$M_w$</td>
<td>weight average molecular weight</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>OEI</td>
<td>oligoethylenimine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>--------------------------------</td>
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<tr>
<td>PAMAM</td>
<td>poly(amido amine)</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethylenimine</td>
</tr>
<tr>
<td>PG</td>
<td>polyglycerol</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-Induced Silencing Complex</td>
</tr>
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<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscope</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TIPS</td>
<td>triisopropylsilane</td>
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Chapter 2 and chapter 6 of this dissertation are a reprint of the materials as they appear in the Journal of American Chemical Society. I would like to thank the American Chemical Society for the permission to include them in the dissertation. The co-authors listed in these publications are also acknowledged for their collaboration work.
Curriculum Vitae

Education

PhD 2008-2014
Chemistry University of California, Irvine Advisor: Zhibin Guan

BS 2004-2008
Chemistry Tsinghua University

Honors and Awards

- UC Irvine Michael Zach Award May 2013
- Selected Attendee for ACS Organic Chemistry Graduate Research Symposium July 2012
- Allergan Fellowship 2012
- Poster Award for the 6th Fluorescence Dynamics Workshop October 2011
- Tsinghua University Qirui Fellowship 2007
- Tsinghua University Changxing Fellowship 2006
- China National Scholarship 2005

Work Experience

Graduate Research Assistant Guan Group, UC Irvine 2008 to 2014

- Successfully designed several synthetic vectors for siRNA delivery, including nanogels, dendronized polymers, dendritic polyglycerols, gold nanoparticles and bolaamphiphiles.
- Developed a novel catalytic system for dehydrogenative polyamide synthesis.
- Assisted the development of peptide-saccharide copolymer for tissue engineering.
- Assisted the development of clicked fluoropolymer for microfluidic device fabrication.

Visiting Researcher Haag Group, Freie Universität Berlin, Germany Oct to Dec 2012

Collaborated with the group members on developing amino acid functionalized dendritic polyglycerol for siRNA delivery.
Safety Coordinator  
Guan Group, UC Irvine  
2011 to 2014

Coordinated with the Department of Environment, Health and Safety to ensure the safety of laboratory work and environment.

Teaching Assistant  
Chemistry Department, UC Irvine  
2009 to 2014

Instructed various laboratory courses of General Chemistry, Organic Chemistry and lecture course of Analytical Chemistry.

Undergraduate Research Assistant  
Hua Group, Tsinghua University  
2006 to 2008

Developed several environmentally friendly catalytic systems for alkyne functionalization.

Professional Affiliation

Member of American Chemical Society  
March 2011 to present

Publications


**Patent**


**Conference Presentations and Posters**

1. Edwards Lifesciences Center Heart to Heart Training Club, Irvine, CA June 11, 2013


4. UC Irvine Pfizer Symposium June 2, 2012

5. American Chemical Society 243rd National Meeting, San Diego, CA March 26, 2012

6. UC Irvine Chemistry Graduate Student and Postdoc Colloquium February 24, 2012

7. 6th LFD Workshop in Advanced Fluorescence Imaging and Dynamics, Irvine, CA October 21, 2011


9. UC Irvine Pfizer Symposium December 11, 2010

10. UC Irvine Chemistry Graduate Student and Postdoc Colloquium November 19, 2010
ABSTRACT OF THE DISSERTATION

Rational Design of Synthetic Vectors for siRNA Delivery

By

Hanxiang Zeng

Doctor of Philosophy in Chemistry

University of California, Irvine, 2014

Professor Zhibin Guan, Chair

Despite promising potentials for disease treatment, clinical application of RNAi is greatly limited by the lack of safe and effective delivery vectors. In this dissertation, we aim to achieve efficient siRNA delivery by developing novel synthetic biomaterials through rational molecular design and in-depth structure-property study.

Chapter 1 introduces the background of siRNA delivery. Major challenges in synthetic vector design are discussed, followed by current approaches to overcome these obstacles. Several successful synthetic vectors are surveyed with an emphasis on materials with dendritic architectures.

Chapter 2 describes dendronized polymer (denpol) for efficient siRNA delivery. The denpol architecture combines multivalency of dendrons and flexibility of polymer backbone to achieve strong siRNA binding. Through in vitro screening of a focused denpol library, we identified that histidine and aromatic amino acid functionalized denpols achieved high delivery efficiency with minimum cytotoxicity. Fluorescence trafficking study revealed that aromatic groups enhanced cell uptake and histidine helped endosomal escape. Such combination could also be applied to other systems. Chapter 3 describes tryptophan and histidine functionalized
dendritic polyglycerol for siRNA delivery. And chapter 4 investigates the application of tryptophan-histidine combination on a novel bolaamphiphile structure. Compared to normal amphiphiles, bolaamphiphiles do not disrupt cell membrane and thus greatly reduces toxicity. The molecular structure of bolaamphiphiles determines their self-assembly behavior with siRNA and subsequent biological activity. The optimal dendron bolaamphiphile achieved effective silencing at low siRNA concentration.

Chapter 5 highlights the importance of formulation process in delivery. In a stimuli-responsive nanogel system, we found that only in situ cross-linking could effectively encapsulate siRNA and achieve functional delivery.

Chapter 6 describes a novel Ru catalytic system for polyamide synthesis. All the biomaterials we developed in previous chapters are based on polyamides, as the amide bonds provide good chemical stability while maintain biodegradability. Current syntheses of polyamides involve harsh conditions and/or produce stoichiometric amount of toxic waste. We developed the first catalytic polyamidation by dehydrogenation of diols and diamines to achieve high atomic economy with no waste generation. The high catalytic selectivity also offers the opportunity to efficiently incorporate polyamines into the polymer backbone without tedious protection/deprotection steps.
Chapter 1: Synthetic Vectors for siRNA Delivery

Small interfering RNA (siRNA) has shown great potentials in a variety of biological applications, including functional genomics, tissue regeneration, bioengineering and disease treatment. The sequence specific gene silencing and broad protein target spectrums make siRNA especially appealing for medicinal application. However, the lack of safe and effective delivery vectors remain a major challenge in this field. Virus has been extensively used as vectors for siRNA delivery, but the intrinsic immunogenicity and infectious nature limited their clinic application. Synthetic vectors, on the hand, offer improved biocompatibility and more control over the material properties, but often suffer from low transfection efficiency. In this chapter, challenges for synthetic vector design will be discussed, followed by a short survey of successful synthetic vectors, with a focus on dendrimer and dendron based materials.

1.1 RNA interference (RNAi)

RNA interference is a naturally occurring process to regulate gene expression by RNA molecules. Two most common pathways involved in RNAi are microRNA (miRNA) and siRNA (Figure 1.1). In miRNA pathway, endogenously produced double stranded pre-miRNA is transported from the nucleus into the cytoplasm. Dicer protein then cuts the double stranded RNA (dsRNA) into 21-nucleotides (nt) fragments and loads them into RNA-induced silencing complex (RISC). RISC unwinds the double stranded RNA, cleaves the passenger strand, and finds specific messenger RNA (mRNA) through base pairing with the guide strand. RISC binding then inhibits protein expression of the corresponding mRNA. siRNA pathway utilize the same machinery. Unlike miRNA which requires several steps of processing prior to RISC loading, the 21-nt siRNA can be directly introduced exogenously or obtained by Dicer cleavage of long dsRNA. siRNA are directly loaded into RISC and follow the same pathway for gene
silencing. In general, the guide strand of siRNA binds to mRNA with perfect base pairing, leading to mRNA cleavage and degradation, while most miRNA guide strands have imperfect match to mRNA, which only suppresses mRNA translation. Although both miRNA and long dsRNA have been used for gene silencing, 21-nt siRNA is most commonly used in therapeutic application, thus will be the focus of our discussion.

Figure 1.1 RNA interference pathways. (Reprinted from reference 1)

Ever since its discovery, siRNA has been extensively studied for medicinal application. Successful activation of siRNA pathway could effectively silence gene expression with high specificity, and virtually any protein targets could be regulated. Large efforts and capitals have been devoted to develop RNAi-based therapeutics. Over 20 RNAi drugs are currently
undergoing clinical trial (Table 1.1), and FDA has recently approved the first antisense drug Kynamro® for the treatment of homozygous familial hypercholesterolemia.

### Table 1.1 RNAi based drugs in clinical trial. (As of October 2013, reprinted from reference[^3])

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>Delivery system</th>
<th>Disease</th>
<th>Phase</th>
<th>Status</th>
<th>Company</th>
<th>ClinicalTrials.gov identifier</th>
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<td>ALN-VSP02</td>
<td>KSP and VEGF</td>
<td>LNP</td>
<td>Solid tumours</td>
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[^3]: DPC, dynamic polyconjugate; LNP, lipid nanoparticle; NP, nanoparticle; shRNA, short hairpin RNA.
1.2 Critical Challenges in siRNA Delivery

In order to successfully deliver siRNA, there are multiple barriers to overcome at different stages,\(^5,6\) including toxicity and immunogenicity of both siRNA and delivery materials, serum stability and tissue targeting and penetration for systemic delivery, and effective cell uptake, endosomal escape and siRNA dissociation in the cytoplasm at cellular level. Several important lessons have been learned in previous studies to overcome these barriers, and Figure 1.2 summarizes some of the current approaches.\(^7\)

![Figure 1.2 Challenges in siRNA delivery and current approaches to overcome these barriers. (Reprinted from reference\(^7\))](image-url)
Biocompatibility is the first concern in siRNA delivery. Although siRNA generally does not exhibit cytotoxicity, it can be recognized by the innate immune system and induce pro-inflammatory response.\(^8\) The immune activation is highly dependent on the siRNA sequence and RNA molecular structure, therefore rational choice of target sequence and chemical modification of RNA molecules could help minimize the immunogenicity.\(^9\) On the other hand, delivery materials could induce various cytotoxicity and immune responses. When designing synthetic vectors, biocompatibility should always be a priority consideration. And it has been shown that the use of biodegradable materials could prevent long term accumulation and lower the adverse impact to biological systems.\(^10\)

Systemic delivery of siRNA poses several important challenges: RNase degradation and renal clearance can quickly diminish the amount of available siRNA in circulation, and non-specific uptake and accumulation prevents siRNA from reaching the target site.\(^11\) Chemical modification of siRNA have been used to increase RNase resistance, such as fluorination and methylation at the 2’-position of the nucleotides, and conjugation with cholesterol.\(^12\) Vectors with strong siRNA binding could also prevent enzymes from approaching and increase the half-life of siRNA in serum. Furthermore, conjugation of protein resistant polymers, such as poly(ethylene glycol) (PEG)\(^13\) and polysaccharide\(^14,15\) not only increases the serum stability but also reduces toxicity and prolongs circulation time.

The delivery to specific tissue site could be achieved through either passive or active targeting. Nanoparticles tend to accumulate in liver and kidney for clearance, thus making them popular targets for siRNA therapy.\(^16\) Similarly, due to leaky vessels and poor lymphatic drainage, nanoparticles could accumulate in cancer tissues through enhanced permeation and retention (EPR) effect.\(^17\) However, passive targeting is only limited to a few tissue types, active targeting
is required for broader applications. Modification of delivery vectors with targeting ligand can help localize the siRNA complexes to specific cell type through ligand-receptor interaction. Antibodies,\textsuperscript{18} aptamers,\textsuperscript{19} proteins,\textsuperscript{20} peptides\textsuperscript{21} and small molecule ligands\textsuperscript{22} have been explored for targeted siRNA delivery, although higher specificity is still desired for clinical applications.

\textbf{Figure 1.3} Uptake and intracellular trafficking of targeted siRNA delivery (Reprinted from reference \textsuperscript{23})
Upon reaching the target tissue site, delivery vectors need to carry siRNA across the cell membrane and transport siRNA into the cytoplasm. Generally cationic charge\textsuperscript{23} and amphiphilic structure\textsuperscript{24} could enhance cell membrane interaction and facilitate endocytosis. Cell penetrating peptide, such as TAT and octaarginine are also used to enhance cell uptake,\textsuperscript{10} and \textit{de novo} designed CADY peptide have been shown to delivery siRNA through direct membrane penetration.\textsuperscript{25} Most vectors enter the cell through endocytosis. If trapped in endosome, siRNA and vectors will be transported to lysosome, where enzyme degradation and clearance will remove siRNA from the cell. Therefore, endosomal escape is a critical step for functional delivery (Figure 1.3).\textsuperscript{26} During the transportation and fusion of early endosome into lysosome, the protons are pumped into endosome to decrease the pH from 7.4 to 5.5. The pH change has been extensively used for endosomal escape. Protonable vectors could disrupt endosome membrane through “proton sponge” effect\textsuperscript{27} or enhanced amphiphilicity\textsuperscript{28} during the acidification process. And upon reaching the cytoplasm, reducible vectors could facilitate the release of siRNA and subsequent RISC complex loading.\textsuperscript{29}

\textbf{1.3 Synthetic Vectors for siRNA Delivery}

Synthetic vectors for siRNA delivery can be categorized into two classes: cationic polymers and lipids. Cationic polymers are generally large macromolecules containing multiple amine groups for siRNA condensation. The overall polymer can be either hydrophilic or amphiphilic for better membrane interaction. Most successful polymeric vectors contain functional groups with good buffering capacity from pH 5.5-7.4, which could help endosomal escape through proton sponge effect. Cationic lipids are small molecules with a hydrophilic headgroup and a hydrophobic tail. These lipids can self-assemble into vesicles in water and
condense with siRNA to form multi-lamellar liposome structure. Most liposomes were formulated with different lipids to achieve multiple functions in the same particle: cationic lipids were used for siRNA binding, cholesterol for increased stability, PEG lipid for serum stability and protonable lipids for endosomal escape. In this section, two representative vectors, cyclodextrin polymer and lipid nanoparticle will be discussed. As the thesis focus on a specific type of polymeric vector, dendrimer, examples of dendrimer-based vectors will be covered afterwards.

1.3.1 Cyclodextrin Polymer Nanoparticle for siRNA Delivery

![Diagram of cyclodextrin polymer nanoparticle for siRNA delivery](image)

Figure 1.4 Cyclodextrin polymer nanoparticle for targeted siRNA delivery. (Modified from reference 30)
Cycloextrin polymer (CDP) based nanoparticle is the first delivery system that entered clinical trial for cancer therapy and demonstrated specific gene silencing in humans through targeted systemic delivery.\textsuperscript{20} CDP is a short oligomer (x=5, Figure 1.4) composed of cyclodextrin (CD) and diamidine. The strong basicity of the amidine groups mediates effective siRNA condensation, and the guest-host interaction with CD provides orthogonal motif for co-formulation with different components. Both ends of the polymer are capped with imidazole groups, which helps endosomal escape with their good buffering capacity.\textsuperscript{31} Although the CDP alone exhibited efficient transfection \textit{in vitro},\textsuperscript{32} systemic delivery requires co-formulation with PEG and targeting ligands. Adamatane terminated PEG (PEG-AD) and PEG modified transferin (Tf-PEG-AD) can be loaded into the nanoparticle through the inclusion of the hydrophobic adamantane in the CD ring. PEG prevents nanoparticle aggregation in serum and prolongs circulation time,\textsuperscript{33} while transferrin binds to CD71 receptor on cancer cell surface, improving the specific cell uptake.\textsuperscript{34} The targeted CDP nanoparticle has demonstrated successful systemic siRNA delivery in several animal models, and phase-I clinical trial results indicated RNAi-specific gene inhibition in human melanoma patients.\textsuperscript{30}

\textbf{1.3.2 Lipid Nanoparticle for siRNA Delivery}

Before the use in siRNA delivery, lipids have been extensively studied for the delivery of other therapeutic reagents, including DNA and hydrophic drugs. Their clinic potentials have been strengthened as several liposome-based anti-cancer drug formulations approved by FDA.\textsuperscript{35} Therefore, a great amount of efforts have been put into the optimization of lipid-based system for siRNA delivery.
The cationic lipid structure contains three parts: head group, linker and hydrophobic tails [Figure 1.5(A)]. The head group can be either permanently charged ammonium groups or ionizable amines. The ammonium mediates stronger siRNA binding, while ionizable amine groups induce lower toxicity and help endosomal escape. The linker group can be tuned for different degradability, from stable ether and amide bonds to hydrolysable ester, phosphoester groups. Both the length and unsaturation degree of the hydrophobic tails can be varied, resulting in different self-assembly behavior and stability of the resulting lipid nanoparticles. The structure of each component significantly changes the lipid transfection efficiency, although direct structure-property relationship is hard to establish. Instead, screening of large cationic lipid library was used to find the optimal vectors.37-39

Besides lipid structure, formulation is also critical to generate efficient lipid nanoparticle (LNP) for siRNA delivery. One single lipid generally cannot meet all the requirements for successful delivery; therefore multiple components are formulated into one LNP to achieve different functions. Most common formulation of LNP includes cationic lipids, neutral lipids,
lip-PEG and cholesterol [Figure 1.5 (B)]. Cationic lipids help siRNA binding, neutral lipids dilute the charge and lowers toxicity, lipid-PEG increases the serum stability, and cholesterol enhances the overall self-assembly through strong hydrophobic interactions. The process for formulation is also important, and it has been found that microfluidic mixing generates stable LNP with high potency both in vitro and in vivo.

Successful silencing by LNP delivery in non-human primates was first reported by the Langer group in 2006, and several LNP systems are in clinic trials for the treatment of hypercholesterolaemia, transthyretin-mediated amyloidosis and cancer. (Table 1.1)

1.3.3 Dendrimer-Based Delivery Vectors

![Figure 1.6](image1.png)

**Figure 1.6** Structure of (A) 2nd generation poly(amido amine) dendrimer (G2-PAMAM) and (B) amine terminated dendritic polyglycerol (PG-NH₂)

Dendrimer is a repetitively hyperbranched molecule with well-defined structure. The high density of surface functional groups could mediate multivalent biological interactions, and the single molecular weight and well-defined structure make dendrimers more preferable than polydispersed polymers for biological studies. Several cationic dendrimers have been studied for siRNA delivery, with poly(amido amine) dendrimer (PAMAM) most commonly used due to its straightforward synthesis. Higher generation PAMAM have been successfully used for siRNA
delivery both in vitro\textsuperscript{41} and in vivo,\textsuperscript{42} and transfection efficiency could be further improved by chemical modification with PEG,\textsuperscript{43} amino acids,\textsuperscript{44} peptides,\textsuperscript{21} saccharides,\textsuperscript{45} hydrocarbon\textsuperscript{46} and fluorocarbon.\textsuperscript{47}

Dendritic polymers are an alternative to perfect dendrimers, with similar hyperbranched structure but much simpler synthesis. (often achieved in one step) And with careful control, the polydispersity could be maintained relatively low (PDI < 1.5). As an example, hyperbranched polyglycerol (PG) has been widely used for biomaterial applications, showing high biocompatibility.\textsuperscript{48} And amine-terminated PG (PG-NH\textsubscript{2}) was also developed for successful siRNA delivery.\textsuperscript{49,50}  

![Figure 1.7 Structure of different dendron amphiphiles for siRNA delivery. (A) PAMAM amphiphile (B) PAMAM lipid (C) PG-NH\textsubscript{2} amphiphile (D) PG-DAPMA amphiphile (Adapted from reference \textsuperscript{51-54})]
As a combination of dendrimer and lipid, dendron amphiphiles have attracted attention for siRNA delivery in recent years. In a typical dendron amphiphile structure, hydrophobic tail is attached to the focal point of the dendron. (Figure 1.7) The hydrophobic interaction promotes dendron self-assembly, therefore only low generation dendrons are needed for strong siRNA binding, and strenuous synthesis of high generation dendron can be simplified. Furthermore, the amphiphilic structure enhances cell membrane interaction, results in improved cell uptake. Both PAMAM$^{51,52}$ and PG$^{53,54}$ based dendron amphiphiles have been developed to deliver siRNA in cell culture and mouse models.

![Figure 1.7](image)

**Figure 1.7** Peptide amphiphiles for siRNA delivery. (A) Amphiphilic peptide tweezer (B) Lipopetide (C) Dynamic peptide amphiphile (Adapted from reference$^{38,39,55}$)

Dendritic peptide headgroups are advantageous in biocompatibility and degradability. The unique structure of the peptide headgroup could also be used for specific biologic
interactions. An amphiphile peptide tweezer has been designed with the headgroup for specific siRNA binding through intercalation and the hydrophobic tail for self-assembly. [Figure 1.8 (A)] Combinatorial approaches were also used to identify optimal peptide amphiphiles, and structures with multiple hydrophobic tails were found to be generally more potent for siRNA delivery. [Figure 1.8 (B)-(C)]

1.4 Rational Design of Synthetic Vectors

From previous studies, several important lessons have been learned for synthetic vector design. Firstly, due to the short and rigid structure of siRNA, highly branched architecture and chain flexibility are important for stable complex formation. Secondly, amphiphilic molecules can help both cellular uptake and endosomal escape through enhanced membrane permeability. Furthermore, pH responsive moiety can facilitate endosomal membrane rupture through either “proton sponge” effect or increased amphiphilicity at lower pH. Lastly, by taking advantages of the reducing environment in the cytoplasm, reducible bonds can be introduced into the vector for efficient release of siRNA.

With these considerations, we have rationally designed several synthetic vectors. These molecules are all composed of naturally occurring building blocks to ensure biocompatibility and degradability, and dendritic structures were used for effective siRNA binding. Vectors were functionalized with aromatic amino acids for membrane anchoring and histidine for endosomal escape, and disulfide bonds were introduced to facilitate siRNA release in the cytoplasm. Four different classes of molecules, dendronized peptide, dendritic polyglycerol, peptide bolaamphiphile and peptide nanogels will be discussed in the following chapters.
Reference


Chapter 2: Multifunctional Dendronized Peptide Polymer Platform for Safe and Effective siRNA Delivery

2.1 Introduction

Since its discovery, small interference RNA (siRNA) has been investigated both \textit{in vitro} and \textit{in vivo} for the treatment of several diseases including cancer, inflammation, diabetes and neurodegenerative diseases.\textsuperscript{1,2} Despite its potential, therapeutic application of siRNA is greatly hindered by the lack of safe and effective delivery vectors.\textsuperscript{3} Both viral and non-viral vectors have been studied intensively in the last decades. Viral vectors, although with higher efficiency in general, have safety concerns with their infectious nature and immunogenicity.\textsuperscript{4} On the other hand, synthetic non-viral vectors offer versatile and precise structure control and present as promising candidates for siRNA delivery.\textsuperscript{5-6} Among different synthetic vectors, cationic lipids,\textsuperscript{7} polymers,\textsuperscript{8-12} dendrimers,\textsuperscript{13-15} peptides\textsuperscript{17,18} and nanoparticles\textsuperscript{19,20} are the most common systems and have been shown to be effective for siRNA delivery \textit{in vitro}. However, many suffered from low efficiency and toxicity or immunogenicity \textit{in vivo}, few have progressed into clinical trials and there have been no FDA-approved siRNA delivery vectors so far.\textsuperscript{3}

In order to successfully deliver siRNA, there are multiple barriers to overcome at different stages,\textsuperscript{21,22} including strong siRNA binding for complex formation, biocompatibility, serum stability and tissue penetration for systemic delivery, and effective cell uptake, endosomal escape and siRNA dissociation in the cytoplasm at cellular level.\textsuperscript{23} Several important lessons have been learned in previous studies to overcome these barriers. Firstly, due to the short and rigid structure of siRNA,\textsuperscript{24} highly branched architecture\textsuperscript{25-27} and chain flexibility\textsuperscript{27} are important
for stable complex formation. Secondly, amphiphilic molecules can help both cellular uptake and endosomal escape through enhanced membrane permeability.\textsuperscript{28-32} Furthermore, pH responsive moiety can facilitate endosomal membrane rupture\textsuperscript{33,34} through either “proton sponge” effect\textsuperscript{35} or increased amphiphilicity at lower pH.\textsuperscript{28} Lastly, by taking advantages of the reducing environment in the cytoplasm, disulfide bonds have been introduced into the polymer for efficient unpacking of siRNA.\textsuperscript{36,37}

On the basis of these lessons, herein we describe a novel amino-acid based biodegradable dendronized polymer (denpol) platform for effective siRNA delivery (Fig. 2.1). Although both individual dendrons\textsuperscript{27} and dendrimers\textsuperscript{16,38,39} have been investigated for siRNA delivery, the denpol architecture represents a novel design with several advantages. Several studies have indicated that the rigidity of high generation dendrimers makes them non-ideal for siRNA binding and delivery,\textsuperscript{27,40} and introduction of flexibility into dendrimers can significantly improve their transfection efficiency.\textsuperscript{41-43} Lower generation dendrimers or single dendrons, on the other hand, lack the multivalency for strong siRNA binding.\textsuperscript{44,45} In contrast, \textit{denpol architecture combines the multivalency of dendrimers and conformational flexibility of linear polymers}, representing a conceptual advancement in siRNA carrier design. Furthermore, the facile synthesis of lower generation denpols allows for easy structural permutation and optimization, and the designed denpols are fully composed of natural amino acids to ensure biodegradability and low toxicity.
2.2 Results and Discussion

To demonstrate our concept, we constructed a small focused library of amino acid based denpol by a “graft-from” approach (Scheme 1). First, the polymer backbone was synthesized by step-growth polymerization of dicysteine (1) and L-lysine (2) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as the coupling reagent (polymer $M_n \sim 15$ kD, PDI $\sim 1.8$ by GPC). Dicysteine was introduced into the polymer backbone as the environmentally responsive motif for facilitated siRNA release in the cytoplasm.36,37 Onto the linear polymer backbone (3), L-lysine-based dendron was then grown generation by generation through solution-phase peptide coupling. Finally, after reaching the desired generation, the outer layer of the dendron was functionalized by a combination of one hydrophilic and one hydrophobic amino acid at various ratios (5). In our combinatorial synthesis, three hydrophilic amino acids (lysine: K, serine: S, and histidine: H) and four hydrophobic amino acids (tryptophan: W, phenylalanine: F, tyrosine: Y, and leucine: L) were included in our library. Enabled by the highly efficient

Figure 2.1 Concept of multifunctional amphiphilic dendronized polypeptide vectors for siRNA delivery.
peptide coupling reaction, a small focused library of G1-G2 amphiphilic denpols were quickly generated (Scheme 1). For simplicity, the denpols were named after the generation of the dendron and the compositions of the amphiphilic amino acids on the outer layer using one-letter amino acid codes. For example, G2 75H-25W represents a denpol carrying multivalent second-generation denrons having 75 mol% histidine and 25 mol% tryptophan incorporated on the outer layer. Details of denpol synthesis and characterization can be found in the Supporting Information (SI).

Scheme 2.1 Synthetic Route for the Denpol Library
The binding capability of the denpols with siRNA was initially assayed by gel electrophoresis, and most denpols could completely complex with siRNA at N/P ratio from 10~30. The binding strength was further evaluated by competitive binding assay using an anionic polymer, dextran sulfate (DS, MW=25 kD) as the challenger (selected examples are shown in Figure 2.2A-C). For this purpose, polyplexes prepared at N/P 40 were incubated with different amount of DS to compete with siRNA. A number of trends were observed in the competitive binding assay. Firstly, the generation of dendron is important for binding strength to siRNA. Second generation denpol has much stronger binding strength than first generation (Fig. 2.2A & 2.2B), due to the increased multivalent binding sites from the dendrons. Secondly, the composition of amino acids on the outer layer also has a significant impact on the binding capability to siRNA. In our small library of denpols, tryptophan (W) incorporated denpols show the strongest binding to siRNA. For example, no appreciable siRNA release could be observed for G2 75H-25W at S/P up to 30 (Fig. 2.2C). It has been reported that indole ring on tryptophan could intercalate into nucleotide base pairs, which could contribute to the increased binding affinity of tryptophan functionalized denpol to siRNA. Lastly, due to the dicysteine building block on polymer backbone, the denpols are responsive to reducing reagent for releasing complexed siRNA. For this assay, different denpol/siRNA polyplexes were treated with glutathione (GSH) at a concentration close to physiological conditions (5 mM). Gel assay shows that complete siRNA release was observed for most denpols after the GSH treatment (Fig. 2.2D). For the tryptophan containing G2 denpol, which has the strongest binding affinity to siRNA, the binding strength was significantly reduced. Most likely, the reduced binding affinity to siRNA after GSH treatment is due to the reduction-triggered degradation of the denpol, which
decreases the multivalency for binding. We envision that such GSH triggered release of siRNA should be beneficial for intracellular siRNA delivery.

Figure 2.2 Gel electrophoresis study of denpol siRNA complexation. (A)-(C) Dextran sulfate competition with different siRNA/denpol polyplexes prepared at N/P 40: (A) G1 75H-25F, (B) G2 75H-25F, (C) G2 75H-25W. (D) siRNA release from polyplexes prepared at N/P 40 after the treatment of GSH (5 mM) at r.t. for 30 min (“+”: treated with GSH; “-”: without GSH treatment).

The size of the polyplexes was investigated by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The DLS results showed that most denpols were able to condense siRNA into particles smaller than 100 nm in diameter and the polyplexes has moderate positive charge (zeta-potential ~ 15 mV) (in SI, Figure 2.8). Figure 2.3C shows a representative DLS curve of polyplexes prepared from G2 75H-25W at N/P 40 (z-avg = 80 nm, PDI = 0.317). TEM provides direct visualization of the polyplexes, which shows spherical nanoparticles with diameters ~ 20-80 nm (Figure 2.3A and Figure 2.9). After GSH treatment,
however, no discreet nanoparticle could be observed under TEM (Fig. 2.3B), which confirmed the particle responsiveness to reducing agent as observed by gel assay (Fig. 2.2D).

Figure 2.3 Basic characterizations of the denpol/siRNA polyplexes. TEM images of siRNA and G2 75H-25W polyplexes at N/P 10 before (A) and after (B) glutathione treatment. (C) Size distribution measured by DLS for G2 75H-25W/siRNA polyplexes at N/P 40. (D) MTT assay of selected denpols using NIH 3T3 cell line.

The efficacy of siRNA transfection was first screened using an engineered NIH 3T3 cell line expressing enhanced green fluorescence protein (GFP). siRNA against GFP was complexed
with different denpols at N/P 20–120 and transfected to 3T3 cells cultured in a 96-well plates for 4h. After 48 h incubation, the GFP fluorescence of each well was measured by a plate reader and the cell viability was determined by MTT assay. GFP fluorescence was then normalized by percent viability to eliminate toxicity-related GFP reduction. Two benchmark vectors, branched poly(ethylene imine) (PEI, MW ~ 25 kD) and Lipofectamine, were used as the positive control. The screening results of transfection efficiency for G2 denpols at optimal N/P ratio are summarized in Figure 2.11. (Complete screening shown in Figure 2.12-13). An important trend can be generalized from the data: both aromatic groups and histidine are critical for effective silencing. Denpols carrying both histidine and aromatic amino acids show very high transfection efficiency and low cytotoxicity (75H-25F, 88H-12W, 75H-25Y). Without aromatic groups (75H-25L), no transfection could be observed. And without histidine, substantial silencing only occurred at a very high ratio of hydrophobic amino acid (25K-75F), which caused significant cytotoxicity.
Figure 2.4 Flow cytometry analysis of transfected NIH 3T3 cells. (A) Transfection summary of selected G2 denpols at optimal N/P ratio in serum free media. (G2 75H-25W and 75K-25F were transfected at N/P 80 and the rest at N/P 120) (B) Comparison of *in vitro* transfection efficacy between Lipofectamine and G2 75H-25W at different serum concentration.

On the basis of the initial screening results, several denpols were selected for more detailed investigation. The dose-dependent toxicity was first determined by MTT assay. As shown in Figure 2.3D, all the denpols are two orders of magnitude less toxic than PEI, suggesting the current denpol as a very safe platform for siRNA delivery. The transfection of
selected denpols was then repeated and analyzed by flow cytometry for more accurate measurement. The results agreed well with the initial screening result and confirmed that both histidine and aromatic groups are critical for successful GFP silencing (Fig. 2.4A). Denpols with either K-F (lacking H) or H-L (lacking aromatic residue) combinations didn’t show any significant gene knockdown. Scrambled siRNA/G2 75H-25W was also transfected and showed minimum effect to GFP expression, indicating high specificity and low off-target effect (Fig. 2.4A).

For a successful *in vivo* delivery system, the polyplexes must be able to protect siRNA from the RNase in the serum, and should also have minimum aggregation with negatively charged proteins in serum. In order to study the serum compatibility of the current system, transfection of denpol/siRNA was carried out in DMEM solution containing 10% to 75% fetal bovine serum. Denpol G2 75H-25W was chosen because it showed the highest siRNA binding affinity and high transfection efficiency in serum-free condition. At all serum concentrations tested, this denpol showed significantly higher transfection efficiency over Lipofectamine (Fig. 2.4B). Even at 75% serum concentration, higher than 50% knockdown could still be observed for this denpol.
In order to gain insights into the role of different functional groups, we conducted intracellular fluorescence trafficking study using a Cy3-labeled siRNA. Three amphiphilic analogues of G2 denpols were chosen for comparative studies: one having both H and an aromatic residue (G2 75H-25F), one having an aromatic residue but no H (G2 75K-25F), and the
last one having H but no aromatic residue (G2 75H-25L). Various Cy3-labeled polyplexes were exposed to 3T3 cells for 4 h in serum free media and then replaced with normal media with 10% serum. Confocal fluorescence images were taken at different time points after the transfection. As shown in Figure 2.5, the aromatic residue (F) is critical for cellular uptake. While no siRNA internalization was observed with H-L functionalized denpol (G2 75H-25L), both H-F and K-F combinations show very effective cell uptake (G2 75H-25F, G2 75K-25F). On the other hand, the buffering capacity of histidine is also critical for successful delivery. In G2 75K-25F transfected cells, the siRNA fluorescence greatly diminished after 6 h, and no siRNA could be observed 24 h after transfection. In contrast, siRNA remained present in G2 75H-25F transfected cells up to 24 h. It is hypothesized that the buffering capacity of histidine could aid endosomal membrane disruption through either “proton sponge” mechanism or increased amphiphilicity. Without the pH responsive groups (G2 75K-25F), endocytosed siRNA would likely be transported to lysosome, followed by enzymatic degradation and fast clearance.

On the basis of the transfection and fluorescence trafficking results, we hypothesized that amphiphilicity of aromatic amino acids and the buffering capacity of histidine work synergistically for effective siRNA delivery in the current denpol system. Amphiphilicity has been shown to be important for cellular membrane interaction in several peptide and polymer vectors. More specifically, Liu and coworkers has recently reported that aromatic amino acid (Trp, Phe, Tyr) functionalized gold nanoparticle has increased cellular uptake over nanoparticles with alkyl hydrophobic amino acids (Val, Leu, Ile), which was related to better membrane anchoring and permeation of the aromatic groups. We have observed similar results in our study: denpols with aromatic amino acids (F, W, Y) showed effective cellular uptake and transfection while no cellular uptake or silencing was observed with Leucine functionalized
denpol (G2 75H-25L). Presumably, the relatively large aromatic hydrophobic groups enhance cellular membrane interaction for the denpol complexes.

Efficient endosomal escape is also critical for successful siRNA delivery as most vectors were internalized by endocytosis. The buffering capacity of histidine is known to aid endosomal membrane rupture through either “proton sponge” mechanism or increased amphiphilicity. Therefore, denpols containing histidine only require a relatively low ratio of aromatic groups for effective delivery, while denpols without histidine functionalization need a high ratio of aromatic amino acids incorporation, which also induces cytotoxicity by disrupting cell membrane. The pH responsiveness ensures the biocompatibility of denpols at neutral pH and increased membrane lysis at acidic pH in endosome to facilitate endosomal escape.

2.3 Conclusion

In summary, we have introduced novel denpol architecture for the vector design of siRNA delivery. We synthesized a focused library of multifunctional amphiphilic dendronized peptide polymer for siRNA delivery. MTT assays indicated that the denpols generally have very low cytotoxicity. Through 96-well fluorescence screening, we were able to quickly identify several candidates that can effectively transfec siRNA to NIH 3T3 cells. More quantitative cell flow cytometry studies have confirmed the screening data and showed significantly improved transfection efficacy of the best-performing denpol over lipofectamine in serum-containing media. Further detailed intracellular fluorescence trafficking study revealed that the combination of histidine and aromatic residues is critical for both cell uptake and endosomal escape: while aromatic residues enhance cell uptake, the buffering capacity of histidine facilitates endosomal membrane rupture and therefore enhances the transfection efficacy. The high delivery efficiency
and low toxicity suggest denpol as a promising platform for siRNA delivery. The basic structure-property information obtained in this study should also be applicable to the design of other types of synthetic vectors for gene delivery.

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2.4 Experimental and supporting information

2.4.1 General

**Materials.** Unless otherwise noticed, 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). Coupling reagents were purchased from GL Biochem Ltd. (Shanghai, China). Branched polyethyleneimine (PEI, 25kDa) was purchased from Sigam-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). Silencer anti-GFP siRNA, Silencer Select negative control siRNA, Silencer Cy™-3 labeled Negative Control siRNA and Lipofectamine RNAiMAX were purchased from Invitrogen (Carlsbad, CA). All reactions were performed in HPLC grade unless otherwise noted. All water used in biological experiments was nanopure water obtained from Barnstead Nanopure Diamond (Waltham, MA). 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). Cell culture media, Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA).

**Instruments.** All the denpols were characterized by NMR and the molecular weight and molecular weight distribution of denpol backbone was measured by gel permeation chromatography (GPC). \(^1\)H NMR spectra were recorded at 500 MHz on Bruker instruments. \(^1\)H NMR chemical shifts were reported as values in ppm relative to deuterated solvents D\(_2\)O (4.80). GPC was performed on an Agilent 1100 SEC system using a OHpak SB-803 HQ column from
Shodex, and the molecular weight was determined with respect to poly(ethylene glycol) (PEG) standards purchased from Aldrich. DMF with 0.1% LiBr (wt/v) was used as the eluent at a flow rate of 1.0 mL/min with column temperature at 45°C. The size and zeta potential of denpol/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 nanoparticle formed by denpol/siRNA complexes was visualized on a FEI/Philips CM-20 conventional TEM operated at an accelerating voltage of 200 kV. The flow cytometry data was obtained on a Becton-Dickinson LSR II flow cytometer with argon ion excitation laser at 488 nm (Becton-Dickinson, Franklin Lakes, NJ). And confocal fluorescence images were acquired using a Zeiss LSM 510 inverted laser-scanning confocal microscope.
2.4.2 Denpol synthesis and characterization

**Synthesis of polymer backbone 1:** H-Lys-OEt·2HCl (2.471 g, 10.0 mmol), (Boc-Cys-OH)₂ (4.405 g, 10.0 mmol) were dissolved in DMSO (15.0 mL) in a wide, shallow glass jar. Once the amino acids were dissolved after vigorous stirring, EDC.HCl (11.502 g, 60.0 mmol), HOBt (2.973 g, 22 mmol), and DIPEA (4.355 mL, 25 mmol) were added to the reaction. The reaction vessel was capped and the mixture was left to stir at room temperature for 48 hours. One drop of the crude reaction mixture was diluted with DMF and subjected to GPC analysis. Crude polymer
was precipitated by adding the reaction mixture to water and then separated by centrifugation. After completely removing water, the polymer was dissolved in 50 mL trifluoroacetic acid (TFA) and 10 mL DCM and left to stir for 24 hours at rt. After deprotection, the solvent was removed with rotary evaporation and the solid was redissolved in methanol. Deprotected polymer was then obtained by precipitation of the methanol solution in diethyl ether. The polymer was further purified by dialysis against MeOH for 2 days with MWCO 3,500. After removing MeOH in vacuo, the polymer was lyophilized to give a white powder. (3.30g, 56% yield) $^1$H NMR (500 MHz, D$_2$O): $\delta$ 4.52-4.34 (2H), 4.34-4.18 (3H), 3.47-3.12 (6H), 2.0-1.86 (1H), 1.86-1.68 (1H), 1.68-1.52 (2H), 1.52-1.37 (2H), 1.34-1.23 (t, 3H). The protected polymer was characterized by GPC with 0.1% LiBr DMF as the eluent, poly(ethylene glycol) (PEG) standards were used as the reference: $M_n = 15.0$ kDa, $M_w/M_n = 1.76$.

**Figure 2.6** GPC trace of denpol backbone before (A) and after (B) purification.

**Synthesis of G1 Denpol 2.** In a 25 mL round bottom flask, 2.20 g 1 (3.72 mmol, 1 equiv) and 3.93 g boc-lys(boc)-OH.DCHA (7.44 mmol, 2 equiv) was dissolved with 10 mL DMF, followed by 1.42 mL DIPEA (8.18 mmol, 2.2 equiv) and 3.62 g BOP (8.18 mmol, 2.2 equiv). The reaction
was left to stir at rt overnight. The protected denpol was then precipitated by added the solution into water. After completely removing water, the polymer was dissolved in 20 mL TFA/DCM solution (3:1) and stirred overnight at rt. Excess TFA and DCM was removed in vacuo, the resulting polymer was redissoved in MeOH and precipitated in ether. The precipitate was dissolved in water and lyophilized to give a white powder. (3.29g, 81 % yield) $^1$H NMR (500 MHz, D$_2$O): $\delta$ 4.74-4.55 (2H), 4.38-4.24 (1H), 4.24-4.13 (2H), 4.03 (2H), 3.28-2.88 (10H), 1.97-1.26 (18H), 1.23 (3H).

**Synthesis of G2 Denpol 3.** In a 25 mL round bottom flask, 2.00 g 2 (1.81 mmol, 1 equiv) and 3.81 g boc-lys(boc)-OH.DCHA (7.22 mmol, 4 equiv) was dissolved with 10 mL DMF, then added 1.30 mL DIPEA (7.60 mmol, 4.2 equiv) and 3.36 g BOP (7.60 mmol, 4.2 equiv). The reaction was left to stir at rt overnight. The protected denpol was then precipitated by added the solution into water. After completely removing water, the polymer was dissolved in 20 mL TFA/DCM solution (3:1) and stirred overnight at rt. Excess TFA and DCM was removed in vacuo, the resulting polymer was redissoved in MeOH and precipitated in ether. The precipitate was dissolved in water and lyophilized to give a white powder. (3.20g, 85 % yield). $^1$H NMR (500 MHz, D$_2$O): $\delta$ 4.74-4.63 (1H), 4.63-4.49 (1H), 4.42-4.13 (2H), 4.03 (2H), 3.28-2.88 (10H), 1.97-1.26 (18H), 1.23 (3H).

**Typical procedure for denpol functionalization:** In a one drum glass vial were added 30 mg of unfunctionalized denpol at desired generation, and two different boc-protected amino acids with specified ratio. 1 mL DMF was added to dissolve the solids, followed by adding BOP (1.05 equiv to the primary amines) and DIPEA (1.05 equiv to the primary amines). The reaction was
left to stir for 24 hours at rt. Protected denpol was precipitated in an excess amount of deionized water. After removing water completely, the solid was dissolved in 3 mL TFA, 1mL DCM and 0.1 mL triisopropylsilane as the scavenger. After stirring overnight, excess TFA and DCM was removed in vacuo, the resulting polymer was redissovd in MeOH and precipitated in ether. The precipitate was dissolved in water and lyophilized to give a white powder. All denpols were characterized by $^1$H NMR. The functionalization ratio in NMR was calculated by comparing the characteristic side chain peak with the aliphatic region in lysine. (see Figure 2.6 for an example)
Figure 2.7 Representative $^1$H NMR assignment of functionalized denpol (G2 7S:25F)
**G1 DENPOL:**

![Diagram of G1 DENPOL molecule]

**G1 75K-25F:** white powder, 92% yield, $^1$H NMR (D$_2$O): $\delta$ 7.37 (3H), 7.24 (2H), 4.73-3.84 (11H), 3.33-2.84 (18H), 2.01-1.12 (39H). Percent functionalization by $^1$H NMR: 75%K-23%F.

**G1 75K-25W:** white powder, 71% yield, $^1$H NMR (D$_2$O): $\delta$ 7.84-6.93 (5H), 4.48-3.83 (11H), 3.50-2.66 (18H), 2.13-0.77 (39H). Percent functionalization by $^1$H NMR: 75%K-19%W.

**G1 75H-25F:** white powder, 87% yield, $^1$H NMR (D$_2$O): $\delta$ 8.66 (3H), 7.67-6.92 (8H), 4.64-3.90 (11H), 3.49-2.77 (18H), 2.06-1.04 (21H). Percent functionalization by $^1$H NMR: 68%H-26%F.

**G1 75H-25W:** white powder, 91% yield, $^1$H NMR (D$_2$O): $\delta$ 8.79-8.32 (3H), 7.73-6.84 (8H), 4.66-3.88 (11H), 3.46-2.87 (18H), 1.98-1.06 (21H). Percent functionalization by $^1$H NMR: 75%H-25%W.

**G1 75H-25Y:** white powder, 87% yield, $^1$H NMR (D$_2$O): $\delta$ 8.64 (3H), 7.48-7.24 (3H), 7.17-6.93 (2H), 6.91-6.67 (2H), 4.67-3.96 (11H), 3.45-2.92 (18H), 1.95-1.06 (21H). Percent functionalization by $^1$H NMR: 70%H-24%Y.
G2 DENPOL:

G2 25K-75F: white powder, 67% yield, $^1$H NMR (D$_2$O): δ 7.35 (18H), 7.24 (12H) 4.66-3.83 (19H), 3.33-2.80 (34H), 2.01-0.93 (57H). Percent functionalization by $^1$H NMR: 25%K-62%F.

G2 50K-50F: white powder, 63% yield, $^1$H NMR (D$_2$O): δ 7.38 (12H), 7.27 (8H), 4.69-3.88 (19H), 3.40-2.86 (34H), 2.05-0.98 (69H). Percent functionalization by $^1$H NMR: 50%K-45%F.

G2 50H-50F: white powder, 79% yield, $^1$H NMR (D$_2$O): δ 8.48 (4H), 7.28-6.93 (24H), 4.67-3.77 (19H), 3.33-2.60 (34H), 1.86-0.76 (45H). Percent functionalization by $^1$H NMR: 41%H-41%F.

G2 75K-25F: white powder, 84% yield, $^1$H NMR (D$_2$O): δ 7.33 (6H), 7.23 (4H), 4.73-3.87 (19H), 3.36-2.80 (34H), 2.07-0.92 (81H). Percent functionalization by $^1$H NMR: 75%K-23%F.

G2 75K-25W: white powder, 92% yield, $^1$H NMR (D$_2$O): δ 7.63-6.91 (10H), 4.62-3.79 (19H), 3.53-2.61 (34H), 2.09-0.68 (81H). Percent functionalization by $^1$H NMR: 75%K-23%W.

G2 75S-25F: white powder, 93% yield, $^1$H NMR (D$_2$O): δ 7.34 (6H), 7.23 (4H), 4.73-4.02 (19H), 4.02-3.81 (12H), 3.31-2.85 (22H), 1.93-0.98 (45H). Percent functionalization by $^1$H NMR: 73%S-22%F.
G2 75H-25F: white powder, 69% yield, $^1$H NMR (D$_2$O): $\delta$ 8.50 (6H), 7.31 (16H), 4.87-3.99 (19H), 3.44-2.83 (34H), 2.00-1.11 (45H). Percent functionalization by $^1$H NMR: 75%H-25%F.

G2 75H-25W: white powder, 78% yield, $^1$H NMR (D$_2$O): $\delta$ 8.64 (6H), 7.74-6.78 (16H), 4.46-3.86 (19H), 3.47-2.63 (34H), 2.04-0.80 (45H). Percent functionalization by $^1$H NMR: 75%H-21%W.

G2 75H-25Y: white powder, 76% yield, $^1$H NMR (D$_2$O): $\delta$ 8.66 (6H), 7.38 (6H), 7.05 (4H), 6.76 (4H), 4.63-3.95 (19H), 3.48-2.77 (34H), 1.97-1.05 (45H). Percent functionalization by $^1$H NMR: 75%H-20%Y.

G2 75H-25L: white powder, 79% yield, $^1$H NMR (D$_2$O): $\delta$ 8.68 (6H), 7.39 (6H), 4.48-3.84 (19H), 3.48-2.87 (30H), 1.99-1.10 (51H), 0.90 (12H). Percent functionalization by $^1$H NMR: 75%H-22%L.

G2 88H-12F: white powder, 69% yield, $^1$H NMR (D$_2$O): $\delta$ 8.77-8.25 (7H), 7.31 (10H), 7.20 (2H), 4.72-4.01 (19H), 3.42-2.83 (34H), 1.92-1.09 (45H). Percent functionalization by $^1$H NMR: 83%H-15%F.

G2 88H-12W: white powder, 84% yield, $^1$H NMR (D$_2$O): $\delta$ 8.72-8.26 (7H), 7.63-6.88 (12H), 4.74-3.96 (19H), 3.43-2.70 (34H), 1.94-1.06 (45H). Percent functionalization by $^1$H NMR: 88%H-12%W.

G2 88H-12Y: white powder, 76% yield, $^1$H NMR (D$_2$O): $\delta$ 8.80-8.50 (7H), 7.41 (7H), 7.10 (2H), 6.95-6.72 (2H), 4.66-4.00 (19H), 3.51-2.82 (34H), 1.89-1.18 (45H). Percent functionalization by $^1$H NMR: 86%H-11%Y.
2.4.3 Denpol/siRNA binding study

**Gel electrophoresis.** The binding of siRNA to denpol was studied by agarose gel electrophoresis. Both siRNA and denpol were diluted with 10 mM pH 7.4 phosphate buffer. Different amount of denpol solutions (5 mg/mL) were added to 5.0 μL 4 μM siRNA solution to achieve different N/P ratio (the molar ratio of primary amine groups from denpol and phosphate groups from siRNA). 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 (pH 7.9) 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 denpol was studied by competitive binding assay with dextran sulfate (DS). To 5 μL 4 μM siRNA solution was added different denpol solution at N/P 40 and incubated for 1h at room temperature. 1 μL DS solution of different concentration 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 incubated for another 30 min. The samples were then subjected to agarose gel electrophoresis under the aforementioned condition.

**Glutathione triggered release of siRNA from denpol complexes.** To 5 μL 4 μM siRNA solution was added concentrated denpol 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 polyplex 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.
**DLS measurements.** The size and zeta potential of denpol/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°. Both denpol and siRNA were diluted in nanopure water, and 50 μL denpol solution was added to 50 μL 1.5 μM siRNA solution (N/P 40), followed by brief vortexing. After 30 min incubation at rt, DLS measurement was taken. The solution was then diluted with 600 μL PBS, and subjected to zeta-potential measurement. At least three measurements were taken for each sample and the mean values were reported.

![Figure 2.8 DLS measurement of different denpol/siRNA complexes](image)

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<td>13.5</td>
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*Figure 2.8 DLS measurement of different denpol/siRNA complexes*
Figure 2.9 DLS measurement of G2-75H25W/siRNA complexes at different ratio. (Note: Due to the presence of excess free polymer, samples at N/P 80 and 120 are very polydispersed and the measurement is less accurate.)

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<td>Z-Avg (d.nm)</td>
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<tr>
<td>Zeta potential (mV)</td>
<td>14.9</td>
<td>15.9</td>
<td>16.5</td>
</tr>
</tbody>
</table>

**TEM Characterization.** The nanoparticle formed by denpol/siRNA complexes was visualized on a FEI/Philips CM-20 conventional TEM operated at an accelerating voltage of 200 kV. Samples were prepared by placing a drop of siRNA/denpol complex solution in DI water (1 mg/mL) on a TEM grid (Ted Pella, Silicon Monoxide Type-A, 300 Mesh). The excess solvent was removed by placing the sample on a filter paper. The siRNA/denpol complexes were stained by placing a drop of 1.0 wt % aqueous solution of uranyl acetate for 1 minute, followed by removal of excess solvent.
2.4.4 Biological Studies

**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 before the toxicity assay. 20 μL PBS solution containing different amount of denpols were 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. MTT reading was obtained by a plate reader (Abs 540 nm). As a positive control, cells were also treated with poly(ethylene imine) (PEI) at different concentration under the same conditions.

**Transfection Screening.** NIH 3T3 fibroblast cells expressing GFP were seeded at a density of 5000 cells/well in 96-well plates 24 h in advance. Prior to transfection, the media was replaced with 80 μL DMEM solution without FBS. 20 μL different polyplex solutions at variant N/P ratios were added to each well to make a final siRNA concentration of 100 nM, followed by
4h incubation at 37 °C. The media was changed back to DMEM with 10% FBS. After 48h, the fluorescence of each well was measured by a plate reader (Ex. 460 nm, EM. 525 nm) and the background signal was removed by subtracting the fluorescence of non GFP-expressing NIH 3T3 cells. After fluorescence reading, the viability was measured by MTT assay and the fluorescence was normalized by percent viability to eliminate toxicity-induced GFP reduction. As positive controls, two bench-mark vectors, PEI and lipofectamine RNAiMAX were also included in the study, and the transfection was carried out following the manufacturer’s protocol (100 nM siRNA concentration). Figure 2.10 summarizes the transfection results of G2 denpols at the optimal N/P ratio, and Figure 2.11 and 2.12 are the complete screening data of G1 and G2 denpols at various N/P ratios.
Figure 2.11 Screening results of G2 denpols at optimal N/P ratio.
Figure 2.12 Transfection screening of G1 Denpols
Figure 2.13 Transfection screening of G2 Denpols
Flow cytometry analysis of transfected cells. NIH 3T3 fibroblast cells expressing GFP 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 200 µL DMEM solution without serum. 50 µL different siRNA/denpol complex solutions were added to each well to make the final siRNA concentration 100 nM. After 4h incubation, the media was changed back to DMEM with 10% serum and cultured for another 48h. Before the analysis, cells were released from each well by Tripsin and harvested by centrifugation. The GFP fluorescence of transfected cells was measured on a Becton-Dickinson LSR II flow cytometer with argon ion excitation laser at 488 nm (Becton-Dickinson, Franklin Lakes, NJ). 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). The transfection efficiency was presented by compare the fluorescence of transfected cells with nontreated cells.

Confocal Laser Scanning Microscopy. Confocal laser scanning microscopy was used to track 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 denpols at N/P 80 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 different time point after the transfection. The nucleus was counter-stained with DAPI. 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. 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 two 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.

Figure 2.14 Intracellular fluorescence trafficking of transfected NIH 3T3 cells at higher magnification. Cells were incubated with Cy3-labeled siRNA (red) complexed with different dendrons for 4h. The media was changed back to fresh DMEM with 10% serum. Fluorescence images were taken at 0 h, 6 h or 24 h after the transfection. Cell nucleus was counter-stained with DAPI (blue).

**Statistical Analysis.** All quantitative assay were performed in triplicates, data were expressed as mean ± SEM.
2.4.5 NMR Spectra
$^1$H Spectrum of Denpol Backbone 1
$^1$H Spectrum
G1 75H-25Y
$^1$H Spectrum
G2 25k/75F
$^1$H Spectrum
G2 75K-25F
\(^1\)H Spectrum
G2 75S:25F
$^1$H Spectrum

G2 75H-25L
$^1H$ Spectrum

G2 88H-12Y
Chapter 3: Amino Acid Functionalized Dendritic Polyglycerol for Safe and Effective siRNA Delivery

3.1 Introduction

RNA interference (RNAi) is a naturally occurring process which regulates protein expression using RNA molecules. Since its discovery, RNAi has been widely used in different biological fields including functional genomics, stem cell differentiation, and bioengineering. Due to the broad spectrum of protein targets and high silencing specificity, small interfering RNA (siRNA) is also a popular target for medicinal application. Many RNA-based drugs have undergone clinical trials for a variety of diseases, and FDA recently approved the first antisense therapeutic, Kynamro®, for the treatment of homozygous familial hypercholesterolemia.

Despite its promising potential, medicinal application of siRNA is greatly limited by the lack of safe and effective delivery vectors. siRNAs are negatively charged macromolecules that do not pass across the cell membrane readily, and can be easily degraded by RNase in biological fluids. Successful synthetic vectors could protect siRNA from degradation and deliver it across the cell membrane in vitro, however, many vectors suffer from low transfection efficiency and/or high toxicity in vivo. Along with a variety of classes of compounds, dendrimers and dendritic polymers have received a lot of attention due to their highly branched architecture, well-controlled nanostructure and multivalent display of binding moieties allowing for effective siRNA interaction. Dendritic polyglycerol (PG) offers further advantages in its straightforward synthesis, controllable molecular weight and branching, chemical stability and excellent biocompatibility. As a major thrust of our research, we have developed several PG...
based materials for a variety of biomedical applications, including bio-antifouling,\textsuperscript{13} inflammation suppression,\textsuperscript{14,15} virus inhibition,\textsuperscript{16,17} drug delivery\textsuperscript{18} and tissue engineering\textsuperscript{19}. The chemical versatility of PGs has also enabled us to generate single amine and oligamines functionalized PG materials for siRNA delivery.\textsuperscript{20,21} Although these PG-amines have shown good transfection efficiency both \textit{in vitro} and \textit{in vivo}, the toxicity escalates at higher concentration resulting in a relatively narrow therapeutic window. In this study, we aimed to improve the PG-based siRNA delivery vectors by the conjugation of amino acids. Through systematically tuning the amino acid combination, functionalization ratio, ligand density and the size of PG core, we have identified several amino acid functionalized PGs (PG-AAs) which showed greatly reduced cytotoxicity and improved transfection efficiency even in the presence of high serum concentration.

3.2 Experimental Section

Materials. Unless otherwise noticed, all reagents were used as received from commercial suppliers without further purification. Protected amino acids were purchased from abcr GmbH (Karlsruhe, Germany). Coupling reagents were purchased from Fisher (Schwerte, Germany). Sodium Dextran Sulfate (25kDa) was purchased from TCI America (Portland, OR) and was used as received. GelRed\textsuperscript{TM} siRNA stain was purchased from VWR (Radnor, PA). Silencer Select anti-GFP siRNA, Silencer Select negative control siRNA, and Lipofectamine RNAiMAX were purchased from Invitrogen (Carlsbad, CA). All reactions were performed in HPLC grade solvents unless otherwise noted. All water used in biological experiments was nanopure water obtained from Barnstead Nanopure Diamond (Waltham, MA). 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).
Cell culture media, Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA).

**Functionalization of PG with amino acids.** Amine terminated polyglycerol (PG-NH$_2$) with different molecular weight and different degree of amine functionalization were prepared following literature procedures. A combination of His with one of three aromatic aminos (Phe, Tyr, Trp) was then conjugated to the PG-NH$_2$ by BOP coupling. In a typical reaction, a MeOH solution containing 30 mg PG-NH$_2$ was concentrated by removing most of the MeOH *in vacuo*. 1.5 mL DMSO was added to dilute the solution and the mixture was left under vacuum for another 30 min to remove the remaining MeOH. Two different Boc-protected amino acids with specified molar ratio were added to the DMSO solution, followed by BOP (1.2 equiv. to the amount of free amine groups) and DIPEA (1.2 equiv. to the amount of free amine groups). The reaction mixture was left to stir at room temperature for 24h, transferred into dialysis tubing (MWCO 1000), and dialyzed against MeOH for 2~3 days to remove all small molecules. After dialysis, the solvent was removed *in vacuo* and the obtained solid re-dissolved in a mixture of 5 mL TFA, 1 mL DCM and 0.2 mL trisopropylsilane (TIPS). The deprotection was allowed to proceed overnight and the reaction mixture was dialyzed against an aqueous solution of 0.1 N HCl for 2 days. The samples were lyophilized to give the PG-AAs as light yellow solids. The functionalization ratio of the two different amino acids was determined by comparing the aromatic peaks with the peaks from the PG backbone (Figure 3.8). A list of all PG-AAs used in this study and their NMR functionalization ratio are summarized in Table 3.1. For use in this study, PG-AAs were dissolved in nanopure water at 5 mg/mL concentration and stored at -20 °C.

**Gel electrophoresis.** The binding of siRNA to PG-AAs was studied by agarose gel electrophoresis. siRNA and the various PG-AAs were diluted with 10 mM pH 7.4 phosphate
buffer. Different amount of PG solutions were added to 5.0 μL of 4 μM siRNA solution to achieve desired N/P ratios (molar ratio of amino groups from PG to the phosphate groups from siRNA). The same buffer was added to adjust the final volume to 10.0 μL, followed by 30 min agitation on a shaker 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 a 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 different PG-AAs was studied by competitive binding assay with dextran sulfate (DS). To a 5 μL of 4 μM siRNA solution was added 5 μL of PG-AA solution to give a N/P ratio of 40 and the mixture was agitated on a shaker for 30 min at room temperature. 1 μL DS solution at different concentration was then added to the complex to achieve different S/P ratios (the molar ratio of sulfate groups from DS to phosphate groups from siRNA) and the mixture was agitated for another 30 min. The samples were then subjected to agarose gel electrophoresis under the aforementioned condition.

**DLS measurements.** The size and zeta potential of PG/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°. Both PG-AA and siRNA were diluted by nanopure water, and 50 μL PG-AA solution was added to 50 μL of 3.0 μM siRNA solution to achieve N/P ratio of 5, followed by brief vortexing. After 30 min agitation on a shaker, the samples were subjected to DLS measurement. Afterwards, the 100 μL solution was diluted with 500 μL PBS solution, and subjected to ζ-potential measurement. At least three measurements were taken for each sample and the mean values were reported.
**TEM Characterization.** The nanoparticles formed by PG-AA/siRNA complexes were visualized on a FEI/Philips CM-20 conventional TEM operated at an accelerating voltage of 200 kV. Samples were prepared by placing a drop of siRNA/PG-AA complex solution (N/P = 5, 1 mg/mL) in DI water on a TEM grid (Ted Pella, Silicon Monoxide Type-A, 300 Mesh). The excess solvent was blotted away by a filter paper. The siRNA/denpol complexes were stained by placing a drop of 1.0 wt % aqueous solution of phosphotungstic acid (PTA), followed by removal of excess solvent.

**Transfection.** NIH 3T3 fibroblast cells expressing enhanced green fluorescence protein (GFP) 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 200 μL plain DMEM without serum. To prepare the complex solution, siRNA was diluted from stock solution to 1.5 μM by phosphate buffer (10 mM phosphate, pH 7.4). Different PG-AA solutions were diluted by the same phosphate buffer to a volume of 41.7 μL and then added to 8.3 μL siRNA solution (12.5 pmole). After brief vortexing, the complex solution was further agitated on a shaker for 30 min. The 50 μL complex solution was then added to each well to make the final siRNA concentration 50 nM. (For transfection carried out at different siRNA concentration, the amount of siRNA and polymers were adjusted accordingly.) After 4h incubation, the media was changed back to 250 μL DMEM with 10% fetal bovine serum and cultured for another 48h. Before flow cytometry analysis, cells were released from each well by Tripsin and harvested by centrifugation. The GFP fluorescence of transfected cells was measured on a Becton-Dickinson LSR II flow cytometer with argon ion excitation laser at 488 nm (Becton-Dickinson, Franklin Lakes, NJ). 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). The transfection efficiency was calculated by
comparing the mean fluorescence of transfected cells with cells that received no treatment. As a positive control, Lipofectamine RNAiMAX was used to transfect 3T3 at 100 nM siRNA concentration following the manufacturer’s manual.

**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 to 80 μL plain DMEM without serum before the toxicity assay. 20 μL buffered solution containing different amount of PG/siRNA (N/P=30) complexes were added to each well, followed by 4h incubation. Immediately after the incubation, the media was replaced with 50 μL DMEM containing 1.0 mg/mL MTT, followed by 4h incubation at 37 °C. Afterwards, 100 μL DMSO was added to each well, and the plate left in a shaker at 37 °C for 15 min to dissolve the formed formazan. MTT reading was obtained by a plate reader (Abs 540 nm), and the percent viability was calculated by comparing the absorbance with untreated cells.

**Statistical Analysis.** All transfection were performed in triplicates and MTT assays in quadruplicates, data were expressed as mean ± SEM.
3.3 Results and Discussion

Scheme 3.1 Functionalization of PG-NH$_2$ with Different Amino Acids.

**Functionalization of PG with Amino Acids.** In our previous studies on dendronized peptide polymers, we have found that polymers functionalized with histidine and an aromatic amino acid exhibited high siRNA transfection efficiency and low cytotoxicity.\textsuperscript{22} It was proposed that aromatic amino acids could enhance cell uptake through membrane anchoring and the buffering capacity of histidine could help the polyplexes escape endosome through the “proton sponge” effect or increased amphiphilicity under acidic conditions. Therefore, we chose to conjugate PG with similar amino acid combinations. Primary amine terminated PG was synthesized following previously reported procedures.\textsuperscript{21} The amine density of PG-NH$_2$ was controlled during the synthetic process, and all the amines were consumed in the following conjugation reaction. PG with two different molecular weight, PG-14kD ($M_n = 14.4$ kDa, PDI = 1.6) and PG-100kD ($M_p = 99.1$ kDa, $M_n = 54.7$ kDa, PDI = 2.75) were synthesized and the amine density was varied from 35% to 70%. Two Boc-protected amino acids were then conjugated to PG-NH$_2$ by BOP.
coupling, followed by TFA deprotection. Three different aromatic amino acids, phenylalanine (F), tyrosine (Y) and tryptophan (W) were used in combination with histidine (H), and the functionalization ratio was controlled by the loading ratio during the conjugation reaction. The exact incorporation ratio was calculated by comparing the aromatic proton peaks with the peaks from PG backbone in $^1$H NMR (Figure 3.8), and the obtained ratio agrees well with the loading ratio. These PG-AAs were named by the molecular weight of the PG core, the amine density, the one letter amino acid code and its corresponding functionalization ratio. For example, 14kD-50%-75H25W refers to a PG molecule with a 14 kD PG core and 50% amine functionalization, with 75 mol% of the amine conjugated to histidine and 25 mol% conjugated to tryptophan. A list of PG-AAs synthesized in this study and their functionalization ratios are summarized in table 3.1.

**siRNA Binding.** The binding of siRNA to PG-AA was studied by agarose gel electrophoresis. As shown in Figure 3.9(A) and 3.9(B), most PG-AAs could effectively complex siRNA at N/P ratio of 5~8. In order to compare the binding strength, competitive binding assays were carried out using an anionic polymer, dextran sulfate (DS). Different amounts of DS were added to PG-AA/siRNA complexes solution. The more amounts of competitors (indicated by S/P ratio) needed to release siRNA from the complexes, the stronger the binding strength of the polymer is. The results are summarized in Figure 3.1, and indicate that all of the PG-AAs show strong siRNA binding with siRNA release only at high S/P ratios. The tryptophan functionalized PG exhibited the highest binding strength, with 14kD-70%-75H25W showing little siRNA release at S/P = 30 (Figure 3.1B); while 14kD-70%-75H25Y had significantly more siRNA released at S/P = 30 (Figure 3.1C) and 14kD-70%-75H25F started to release siRNA at S/P = 18 (Figure 3.1A). A similar trend could be observed in PGs with different ratios of tryptophan incorporation [Figure
3.9(C): 14kD-70%-90H10W exhibited significant siRNA release starting from S/P = 23, while 14kD-70%-60H40W showed no siRNA release up to S/P = 30. The increased binding strength is proposed to result from the intercalation of tryptophan indole ring into the siRNA base pairs.23

**Figure 3.1** Dextran sulfate competitive binding assay. PG/siRNA complexes were prepared at N/P = 40 and different amounts of dextran sulfate were added to compete with siRNA.

In addition to functional group effects, multivalency contributes greatly to the strong siRNA binding of cationic dendrimers and dendritic polymers. By recruiting multiple binding motifs on the same molecule, dendritic polymers can reduce the entropic penalty of
complexation and increase the overall binding strength.\textsuperscript{11} In our system, the multivalency was controlled by ligand density of grafted amino acids and molecular weight of the PG core. As all the amines on the PG were fully conjugated, the ligand density of grafted amino acids was directly determined by the amine density of PG core. Figure 3.1B, 1D and 1E shows that PG-AAs with higher ratios of amino acid incorporation bind to siRNA stronger, due to the increased degree of multivalency. Similarly, increasing the molecular weight of PG core also results in more binding motifs on the same molecule. As shown in figure 3.1F, even at a lower functionalization ratio, 100kD PG samples exhibited the strongest siRNA binding, with no release observed up to 30 equivalence of competitor present.

\textbf{Characterization of PG/siRNA Complexes.} The size and zeta potential of PG/siRNA complexes were measured by dynamic light scattering (DLS). Figure 3.2A and 3.2B shows the intensity curve of a few representative PG-AA complexes. In general, PG-AAs formed smaller nanoparticles with a broader size distribution compared to the amine terminated PGs. In figure 3.2B, a minor peak with smaller size was also observed in the 100kD-35%-75H25W complex solution, which was possibly due to the presence of unbound polymers. Besides smaller size, all the amino acids functionalized PG showed significantly lower surface charge compared to the PG-NH$_2$ counterpart.\textsuperscript{(Figure 3.2C)} As excessive positive charge on the nanoparticle could disrupt cell membranes and cause toxicity,\textsuperscript{24} we expect that the amino acid functionalization would reduce the cytotoxicity of PG-based delivery vectors. The morphology of the complexes was further studied by TEM imaging. Figure 3.2D shows a representative image of 100kD-35%-75H25W/siRNA complexes with phosphotungstic acid staining. Spherical nanoparticles with diameter ranging from 20 to 60 nm could be observed.
Figure 3.2 Characterization of different PG/siRNA complexes. (A)-(B) Intensity distribution of 14 kD (A) and 100 kD (B) PG complexes. (C) Summary of DLS measurement. (D) TEM image of PG-100kD-75H25W/siRNA complexes. All complexes prepared in water at N/P = 5.

Transfection Conditions. The siRNA transfection efficiency of PG-AAs was tested in vitro on a GFP-expressing NIH 3T3 fibroblast cell line. Anti-GFP siRNA was complexed with different polymers and added to the cell culture media, the GFP fluorescence was observed by a fluorescence microscope and the mean fluorescence per cell quantified by flow cytometry. An initial transfection condition screening was carried out with two PG samples derived from a 14kD PG-NH2 core, 14kD-70%-75H25W and 14kD-70%-60H40W. The results in figure 3.10 show that both samples could effectively silence GFP expression at 100 nM siRNA.
concentration, with N/P ratio from 10 to 30. A dose-response curve of these two complexes was then generated to find the optimal transfection concentration. As shown in figure 3.3A, effective silencing could be observed at siRNA concentrations higher than 25 nM, and the transfection saturated at 50 nM with approximately 80% knockdown. As a comparison, Lipofectamine only achieved a similar level of knockdown at 100 nM siRNA concentration. The low effective siRNA concentration indicates a high transfection efficiency of the current PG systems. As the silencing effect appeared to saturate at 50 nM, all of the following transfection studies to systematically compare different PG samples were carried out at this concentration.

Figure 3.3 (A) Dose-Response curve of siRNA silencing efficiency in NIH 3T3 cells. 14kD-70%-75H25W and 14kD-70%-60H40W were complexed with anti-GFP siRNA at N/P ratio of 30 and 15 respectively and transfected to GFP expressing 3T3 cells. The fluorescence of transfected cells were analyzed by flow cytometry. (B) Images of 3T3 cells transfected by 14kD-70%-75H25W under the aforementioned condition. Top: fluorescence images; Bottom: phase contrast images. Scale bar: 100 μm.
Figure 3.4 (A) Transfection summary of PG samples with different amino acid combination and functionalization ratio. All PG complexes were prepared at N/P = 30 and transfection carried out at 50 nM siRNA concentration. Lipofectamine was transfected at 100 nM siRNA concentration following the manufacturer’s instruction. (B)-(C) MTT assay of 3T3 cells treated by PG/siRNA complexes (N/P = 30) at different concentrations under the same condition.

In addition, the flow cytometry data was confirmed by fluorescence imaging (Figure 3.3B). As the siRNA concentration was increased from 25 nM, the GFP signal greatly diminished with almost no fluorescence detected at 50 nM and above. The cell density remained the same in the phase contrast images, indicating high cell viability.
Comparison of Different Amino Acid Combination and Functionalization Ratio. In order to find the optimal amino acid combination, 14kD-70%-NH₂ was functionalized with histidine and three different aromatic amino acids, phenylalanine (F), tyrosine (Y) and Tryptophan (W) at various molar ratios. The transfection efficiency and toxicity profile of these PG samples are summarized in Figure 3.4. While Phe and Tyr functionalized PGs showed minimal to low transfection, 14kD-70%-75H25W showed effective gene silencing. (Figure 3.4A) The efficiency was much improved over unfunctionalized 14kD-70%-NH₂ and similar level of knockdown could only be achieved by Lipofectamine at higher siRNA concentration. It was also shown that the functionalization ratio of Trp is important for effective transfection, as 14kD-70%-90H10W was not effective for siRNA delivery, yet both samples with higher Trp incorporation, 14kD-70%-75H25W and 14kD-70%-60H40W, showed more than 70% GFP knockdown. (Figure 3.4A) Previous gel electrophoresis studies have shown that Trp incorporation enhances siRNA binding of PGs, which may contribute to the critical role of Trp in transfection. Furthermore, the larger indole ring in Trp increases cell membrane interaction and therefore helps complex internalization.²⁵,²⁶

The cytotoxicity of the polyplexes was quantified via MTT assay with Figure 3.4B and 4C showing that all PG-AAs exhibited less toxicity to 3T3 cells than unfunctionalized 14kD-70%-NH₂. 3T3 cells treated with PG-AA complexes all remained greater than 50% viability up to 200 nM siRNA concentration, while those treated with 14kD-70%-NH₂ polyplexes showed only 20% viability at 100 nM siRNA concentration. The results indicate that amino acid functionalization could significantly improve the safety profile of PG-based vectors. On the other hand, Trp incorporation while enhancing transfection efficiency, also induced more cytotoxicity. The most effective vectors in this series of PG-AAs, 14kD-70%-75H25W and 14kD-70%-
60H40W, displayed greater toxicity than the other samples. Therefore, we continued to optimize the His-Trp functionalized PGs, aiming to further reduce the toxicity while maintain the high transfection efficiency.

**Effect of Ligand Density.** In polymeric delivery systems, the density of amine groups, which is usually the siRNA binding motif, has a profound effect for its biological properties. Higher amine density generally increases siRNA binding strength as well as cell membrane interaction, which subsequently enhances the transfection efficiency. However, high cationic charge can lead to cell membrane disruption, induce severe toxicity, and too strong siRNA binding strength can also hinder release in the cytoplasm. By tuning the ligand density we hoped to identify optimal vectors which give a good balance between transfection efficiency and cytotoxicity. Four more His/Trp functionalized PGs were synthesized from 50% and 35% PG-NH₂. The functionalization ratio of H-W was chosen to be 75H-25W and 60H-40W as these ratios were previously shown to be most effective. The transfection efficiency and toxicity profile of these PG-AAs are summarized in Figure 3.5 in comparison to PG-AAs with 70% functionalization ratio. For PG with 50% ligand density, both 75H-25W and 60H-40W functionalized PGs showed improved silencing effect over the 50% PG-NH₂ counterpart. For the PGs with 35% ligand density, however, the transfection efficiency was significantly reduced, possibly due to the decreased degree of multivalency. The toxicity assay showed the expected trend with lower ligand density exhibiting less cytotoxicity to 3T3 cells (Figure 3.5B). Among all of the PG vectors in this series, we found that 14kD-50%-75H25W to be optimal with a high level of knockdown at 50 nM siRNA concentration and no significant cytotoxicity up to 200 nM siRNA concentration. The increased transfection efficiency and reduced toxicity results in a wider therapeutic window than the previously developed single amine or oligoamines terminated PGs.
Figure 3.5 (A) Transfection summary of H-W functionalized PGs with different ligand density. All PG complexes were prepared at N/P = 30 and transfection carried out at 50 nM siRNA concentration. Lipofectamine was transfected at 100 nM siRNA concentration following manufacturer’s instruction. (B) MTT assay of the cells treated by different PG/siRNA complexes under the same condition.
Figure 3.6 (A) Transfection summary of high molecular weight PG samples. All PG complexes were prepared at N/P = 30 and transfection carried out at 50 nM siRNA concentration. Lipofectamine was transfected at 100 nM siRNA concentration following manufacturer’s instruction. (B) MTT assay of the cells treated by different PG/siRNA complexes under the same condition.

High Molecular Weight PG. Besides alterations to the ligand density, the degree of multivalency can also be controlled by varying the molecular weight of the PG core. Higher molecular weight PG presents more functional groups on the surface and increases the number of available binding sites per polymer. By using higher molecular weight PG, we hoped to achieve effective siRNA binding and transfection while allowing for lower ligand density and greater biocompatibility. To test our hypothesis, we synthesized 100kD-35%-75H25W and 100kD-35%-60H40W from a high molecular weight PG core. The transfection efficiency and toxicity were assessed as previously described with the results summarized in Figure 3.6. Similar to the other PG samples, the H-W functionalized PG showed greatly reduced toxicity over unfunctionalized 100kD-35%-NH2. More importantly the increased multivalency of the higher molecular PG
reduced the ligand density required for efficient transfection. 100kD-35%-75H25W showed high silencing effect at 50 nM siRNA concentration (Figure 3.6A) while maintaining a low toxicity profile up to 200 nM concentration (Figure 3.6B).

**Transfection of Negative Control siRNA.** In order to confirm that the effective protein knockdown observed in our study was indeed the result of sequence specific RNA interference, several selected vectors were complexed with non-targeting negative control siRNA and transfected to 3T3 cells under the aforementioned conditions. As shown in figure 3.7A, all the samples showed minimal effect on the GFP expression, confirming the sequence specific silencing and indicating that the current PG systems have low off target effects.

**Transfection in Serum Containing Media.** In order to develop a successful vector for *in vivo* applications, the complexes must be stable in biological fluid. High concentration of negatively charged proteins in serum can compete with siRNA in binding and cause nonspecific aggregation. Furthermore the vector also needs to effectively protect siRNA from degradation by the RNase found in serum. To test the potential of PG-AAs for systemic delivery, we carried out *in vitro* transfection in culture media containing different concentrations of fetal bovine serum (FBS). The two optimal vectors identified from the previous studies, 14kD-50%-75H25W and 100kD-35%-75H25W, were used and the results are shown in figure 3.7B. Both vectors displayed effective knockdown in 10% serum media. The transfection efficiency of low MW PG and Lipofectamine decreased at higher serum concentration, while the silencing from higher MW PG at N/P=60 showed little dependence on serum concentration, with greater than 70% knockdown achieved at 75% serum concentration. The strong siRNA binding from high MW PG-AA (Figure 3.1F) as well as good protein resistant properties of the PG-core presumably
contribute to the high serum stability of the corresponding siRNA complexes and the results indicate that the high MW PG is a potential candidate for in vivo delivery applications.

Figure 3.7 (A) Transfection summary of different PGs with negative control siRNA. All PG complexes were prepared at N/P = 30 and transfection carried out at 50 nM siRNA concentration. (B) Transfection summary of PG-AAs in serum containing media. All samples were transfected at 100 nM siRNA concentration with specified N/P ratio.
3.4 Conclusion

In summary, we have developed an amino acid functionalized dendritic polyglycerol system for safe and effective siRNA delivery. Compared to amine-terminated PGs, PG-AAs showed improved transfection efficiency and a broader therapeutic window. The amino acids combination, functionalization ratio, ligand density and molecular weight of the PG cores were systematically varied, leading to two optimized PG-AAs, 14kD-50%-75H25W and 100kD-35%-75H25W. Furthermore, vectors based upon higher molecular weight PG remained effective even in media containing 75% serum. The low toxicity profile, high transfection efficiency and high serum stability make the current PG-AA system a promising candidate for clinical applications.

References


Biopharm.* **2013**, *85*, 756.


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(20) Fischer, W.; Calderon, M.; Schulz, A.; Andreou, I.; Weber, M.; Haag, R. *Bioconjugate


2013*, *135*, 4962.


3.5 Supporting Information

3.5.1 List of Amino Acid Functionalized PG in this study.

Table 3.1 PG samples and their functionalization ratio.

<table>
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<th>Sample Name</th>
<th>Ligand Density</th>
<th>Functionalization Ratio</th>
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<tr>
<td></td>
<td></td>
<td>Histidine</td>
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<tr>
<td>14kD-70%-75H25F</td>
<td>68%</td>
<td>68%</td>
</tr>
<tr>
<td>14kD-70%-75H25Y</td>
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</tr>
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<td>60%</td>
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3.5.2 $^1$H NMR Assignment and Calculation of Functionalization Ratio

The NMR spectrum of 14kD-70%-60H40W was used as an example for the assignment. The NMR spectra of all the other PG-AAs were very similar, differing only in the integration values of the various peaks. The calculation of the actual functionalization ratio was carried out as follows:

Peak ~8.5 contains 1 H from His; integration set to 1.00

Peak from 7.0~7.5 contain 1 H from histidine and 5 Hs from tryptophan, therefore

Molar amount of Trp = (4.60-1.00)/5 = 0.72.

Both His and Trp contribute 3 Hs in the region 2.5~4.5, therefore

Amount of Hs from PG core = 2.27+16.30-1*3-0.72*3 = 13.41

And each repeating unit of PG has 5 Hs, therefore

Molar amount of PG repeating units = 13.41/5 = 2.68

Only 68% of PG repeating units were converted to amines, therefore

Molar amount of amines = 2.68*0.68 = 1.82

His% = 1/1.82 = 55%

Trp% = 0.72/1.82 = 40%
Figure 3.8 Representative $^1$H NMR assignment of functionalized PG (14kD-70%-60H40W)
3.5.3 Additional Gel Electrophoresis Data of PG-AA/siRNA Complexation

Figure 3.9 (A) – (B) Complexation of different PG samples with siRNA. (C) Dextran Sulfate competitive binding assay of PGs with different Trp incorporation.
3.5.4 Initial Transfection Screening Results.

Figure 3.10 Transfection results of PG-AAs at different N/P ratios. Transfection was tested in NIH 3T3 cell line with 100 nM siRNA concentration. The GFP fluorescence was quantified by flow cytometry.
Chapter 4: Peptide Bolaamphiphiles as Functional Biomaterials

4.1 Introduction

Recent years have seen rapid development in self-assembling peptide based biomaterials. These designer peptides can form various nano to macro-scaled architectures through supramolecular interactions, and the material properties could be precisely controlled by the molecular structure. As peptides play an essential role in biological system, self-assembling peptides also offer great opportunity to incorporate bioactive ligands and provide additional benefits of biocompatibility and biodegradability. Recent research has found broad applications of these materials in the delivery of therapeutic reagents, tissue engineering, diagnostics, antimicrobial materials and stabilization of membrane proteins.

Figure 4.1 Structures of mono amphiphile (A) and bolaamphiphile (B).

The biomaterial application of self-assembling peptides can be exemplified by the peptide amphiphile (PA) developed in the Stupp group. These PAs contain a peptide headgroup covalently attached to a hydrophobic alkyl tail. They can self-assemble into well-defined nanofibers and form hydrogel under physiological conditions. The resulting materials exhibit high bioactivity and have been extensively studied in regenerative medicine. However, PAs have a high propensity to form fibers and their applications in vesicular form have been very limited. Therefore it is highly desirable to design novel peptide structures that can form a wider range of morphologies.
In search of new molecular architectures, bolaamphiphilies have drawn our attention. Unlike lipid-type amphiphiles (termed as “mono amphiphile” in this paper), bolaamphiphilies contain two hydrophilic headgroups connected by a hydrophobic chain in the middle. (Figure 1B) Such structure is inspired by the unusual cell membrane found in archaebacteria. The monolayer membranes formed by bolaamphiphilies provide superior stability over lipid bilayer membranes, and help archaebacteria survive in extreme conditions.\textsuperscript{13} Synthetic analogs and their self-assembly have been studied\textsuperscript{14,15}, and some simple peptide bolaamphiphyles have been shown to form various self-assembly structures.\textsuperscript{16,17} However, their functional application has yet to be explored. Herein, we describe a new class of bolaamphiphilies with peptide dendron headgroups. Compared to their mono amphiphile counterpart, peptide bolaamphiphilies showed low membrane lytic activity and low cytotoxicity. And depending on the molecular structure, peptide bolaamphiphilies can self-assemble with oligonucleotides into various morphologies, including nanofiber, monolayered vesicle and micellar spheres. We also report here our studies of the peptide bolaamphiphilies in a specific biomaterial application, siRNA delivery.

### 4.2 Design and Synthesis of Peptide Bolaamphiphiles

The peptide bolaamphiphile structure (Figure 4.2 bola-C\textsubscript{18}-G2) was designed with the following considerations: 1. A L-lysine-based dendron provides a charged hydrophilic headgroup. The amines on the outer layer of the dendron can be readily conjugated with bioactive ligands and the multivalency enhances the corresponding ligand-receptor interactions. 2. A long chain alkyl diacids as the core promotes self-assembly through hydrophobic interactions. 3. The hydrophobic core and the headgroups are linked through dicystamine, which can be cleaved under reducing environment\textsuperscript{18} and provide stimuli-responsive disassembly of the peptide bolaamphiphile.
Figure 4.2 Structure of different peptide bolaamphiphiles

The synthesis of peptide bolaamphiphiles was achieved through solution phase coupling reaction (Scheme 4.1). Because of the modular design, each part of the bolaamphiphiles could be conveniently modified, giving us opportunities for detailed structure-property analysis and fine tuning of the desired bioactivity. In this study, the length of the alkyl chain was varied from 6 carbons to 22 carbons, resulting in both increased hydrophobicity and more space for alkyl chain packing. Fluorinated diols were used for stronger hydrophobic interactions and hexaethylene glycol as a hydrophilic control with no intramolecular attraction in aqueous solution. A triazole ring was introduced to provide different geometry in the core, where 1,4-triazole gives a more linear alkyl chain and 1,5-triazole a kinked analog. In the linker region, a triethylene glycol was
used as a non-reducible control in our study, and we envision that other stimuli-responsive motifs could also be introduced for different applications. The generation of L-lysine dendron was varied from 1	extsuperscript{st} generation to 3	extsuperscript{rd} generation, giving different headgroup size as well as degree of multivalency. For direct comparison of the bolaamphiphile with conventional mono amphiphile structure, we also synthesized three mono amphiphile analogs. (Figure 4.3) The headgroup and the linker remained the same, with hydrophobic tailed varied from dodecanoic acid (C11) to single and double oleic acid.

![Figure 4.3 Structure of different mono peptide amphiphiles](image)

For biomaterial application of peptide bolaamphiphiles, we focus on siRNA delivery in the current study. We have previously found that dendronized polymer with histidine and tryptophan functionalization exhibited high siRNA transfection efficiency with minimum
It was proposed that tryptophan enhances cell uptake by membrane anchoring and histidine helps siRNA escape from the endosome through “proton sponge” effect or increased amphiphilicity under acidic condition. Therefore, we chose to conjugate histidine and tryptophan to the peptide bolaamphiphiles for siRNA delivery applications. Boc-protected amino acids were coupled to the outer layer of the dendron through BOP coupling, followed by TFA deprotection. (Scheme 4.1) The functionalization ratio of histidine was 75 mol% and tryptophan 25 mol%, which were the optimal ratios found in previous studies. The same conjugation reaction was carried out for all bolaamphiphiles and mono amphiphiles. And their potential for biomaterial application was investigated in the following study.

**4.3 Cytotoxicity of Peptide Bolaamphiphiles**

Cell membranes are composed of amphiphilic phospholipids, therefore amphiphiles can insert into the lipid bilayer. In some cases, such amphiphilicity could be used to enhance material-cell membrane interaction; however, it could also cause membrane disruption and induce cytotoxicity. To investigate the potential of peptide bolaamphiphiles for biomaterial application, we first tested their toxicity in a model cell line, NIH 3T3 fibroblasts. The cells were treated with various bolaamphiphiles for 4h, and the cell viability was determined via MTT assay 48h after the treatment. One very interesting result was found in this study: bolaamphiphiles exhibited much lower cytotoxicity than mono amphiphiles. As shown in figure 4.4(A), m-C11-G2-75H25W and bola-C22-G2-75H25W have identical functional group composition, yet the mono amphiphile were 10 times more toxic than the bolaamphiphile. The longer hydrophobic tail in m-OA-G2-75H25W induced even higher toxicity, yet the hydrophobic chain length showed little effect in the cytotoxicity of bolaamphiphiles bola-C18-G2-75H25W and bola-C22-G2-75H25W. Also shown in figure 4.4 (B), varying the headgroup or the use of fluorinated...
core did not significantly change the toxicity profile, and cells remained higher than 50% viable up to 1 mg/mL concentration. Two commonly used transfection reagents, branched polymer poly(ethylene imine) (PEI) and poly(amido amine) dendrimer (PAMAM) were included as a control, with both showing significant higher cytotoxicity to 3T3 cells. The low cytotoxicity profile of peptide bolaamphiphiles, especially compared with their mono amphiphile analogs, indicate that they are a good candidate for biomaterial application.

Figure 4.4 MTT assay of different peptide bolaamphiphiles.

4.4 Hemolytic Acitivity of Peptide Bolaamphiphiles

Several studies have shown that amphiphilic molecules cause toxicity through membrane disruption, and therefore we propose that the vastly different toxicity profile of mono amphiphiles and bolaamphiphiles are due to their different membrane lytic activities. To test the
hypothesis, hemolysis studies were carried out using bovine red blood cells (RBC), and the release of hemoglobin from RBC were measured as an indicator of membrane lytic activity. As we expected, figure 4.5 (A) shows that mono amphiphiles exhibit much higher hemolysis than bolaamphiphiles. The hydrophobic tail of mono amphiphiles significantly changes their hemolytic activity, as the longer OA amphiphile exhibited 4 times higher membrane lysis than the shorter C11 amphiphile. Double tailed OA₂ amphiphile shows higher hemolysis at lower concentration, but plateaued at higher concentration, possibly due to self-assembly at high concentration. On the other hand, all bolaamphiphiles exhibited very low hemolytic activity, with G2 and G3 inducing below 1% hemolysis and G1 ~3% hemolysis at 250 μg/mL. It should be noted that the different hemolysis was not caused by the assembly behavior, as all the concentration tested are below the critical micelle concentration (CMC) for both mono amphiphiles (Except for m-OA₂-G2-75H25W) and bolaamphiphiles.

Figure 4.5 Hemolysis assay of different peptide mono amphiphiles and bolaamphiphiles.
Although the exact membrane interaction at molecular level is beyond the scope of the current study, we hypothesize that the different hemolytic activity is directly related to their ability of membrane insertion. As shown in figure 4.6, mono amphiphile can readily insert into the lipid bilayer, burying the alkyl tail into the hydrophobic layer and exposing the charged headgroup to the hydrophilic environment. However, because of its unique structure, the membrane interaction of bolaamphiphile is very limited. The steric hindrance and charge repulsion of the two dendron headgroups prevent a U-shaped morphology for membrane insertion. On the other hand, because the hydrophobic core is significantly shorter than the width of lipid bilayer, direct insertion of the bolaamphiphile into the cell membrane will cause unfavorable interaction between the hydrophilic head group and hydrophobic layer in the membrane. Therefore, although being an amphiphilic molecule, peptide bolaamphiphiles cannot insert the hydrophobic core into the lipid bilayer and exhibit less membrane disruption.

**Figure 4.6** Proposed cell membrane interaction of mono amphiphile and bolaamphiphile
4.5 Self-Assembly of Peptide Bolaamphiphiles with siRNA

The positively charged amine groups on dendron surface prevent peptide bolaamphiphiles to self-assemble at physiological conditions. Addition of anionic polymers neutralizes the charge and promotes self-assembly. In this study, we used siRNA as the anionic polymer, and different amphiphile/siRNA complexes were visualized by TEM. (Figure 4.7)

Figure 4.7 TEM images of different bolaamphiphile-siRNA complexes. (A) bola-C18-G1-75H25W, (B) bola-C18-G2-75H25W, (C) bola-C18-G3-75H25W, (D) m-C11-G2-75H25W, (E) bola-1,4-TZ-G2-75H25W, (F) bola-1,5-TZ-G2-75H25W. All samples were prepared at 2.0 mg/mL concentration with N/P ratio of 10. Samples were stained with 2% uranyl acetate before TEM imaging. Scale bar: 100 nm.
Figure 4.8 Proposed different self-assembly structures between peptide bolaamphiphiles and siRNA. (A) Twisted fiber. (B) Single-layered vesicle. (C) Micellar aggregate.

Figure 4.7 (A)-(C) shows that the size of the headgroup significantly changes the self-assembly behavior. With smaller headgroup, C18-G1 bolaamphiphiles can closely pack with each other and form nanofibers. The chirality of the headgroup also induces the twists into the fiber [Figure 4.8 (A)] and the pitches could be observed in TEM [indicated by red arrows in Figure 4.7 (A)]. As the headgroup size increases, both steric and charge repulsion prevent highly ordered packing in nanofibers. C18-G2 forms single-layered vesicle with siRNA [Figure 4.7 (B) and Figure 4.8 (B)] and C18-G3 could not form any ordered packing, with only micellar aggregates observed. [Figure 4.7 (C) and Figure 4.8 (C)] A similar trend was also observed in bolaamphiphiles with fluorinated core. (Figure 4.15) Vesicular structures with dark edge could be observed with bola-F10-G2-75H25W complexes and dark spherical aggregates observed with bola-F10-G3-75H25W. Fibril structure was not observed in F10-G1 bolaamphiphiles, [Figure S4.2 (D)] possibly because the shorter hydrophobic core could not form highly ordered
fiber structure. On the other hand, geometry of the hydrophobic core also influences the self-assembly. Bolaamphiphile with a more linear core containing 1,4-triazeole ring could form vesicles with siRNA, [Figure 4.7 (E)] yet the kinked 1,5-triazeole analog prevents effective packing and only micellar aggregates were observed.[Figure 4.7 (F)] For the assembly of mono amphiphiphiles, both m-C11-G2-75H25W [Figure 4.15 (A)] and m-OA-G2-75H25W [Figure 4.7 (D)] complexes forms vesicles, possibly with a bilayer membrane, and fibril network was observed with the double tailed m-OA2-G2-75H25W complex. [Figure 4.15 (B)]

The different self-assembly behavior could be confirmed by IR spectrum. Methylene CH2 groups in the alkyl region prefers trans conformation in highly ordered packing structure, resulting in lower wavenumber of C-H stretch peaks. Figure 4.16 shows that from G1 to G3 bolaamphiphile complexes, both the asymmetric (~2930 cm⁻¹) and symmetric (~2850 cm⁻¹) C-H stretch peaks shifted to higher wavenumber, indicating less ordered structure in the alkyl region. The decreased packing order agrees with the corresponding fiber, vesicle and micelle assembly structure.

Furthermore, the different assembly structure also influences siRNA binding strength. In the competitive binding assays shown in figure 4.9, G1 bolaamphiphile exhibited the strongest binding, with siRNA release only at the highest S/P ratio (molar ratio of sulfate from DS and phosphate from siRNA). As the packing order decreases, the assembly becomes less stable and siRNA binding weakens. G2 bolaamphiphile shows siRNA release from S/P = 18 and G3 bolaamphiphile from S/P =11. A dicystamine linker was introduced in the bolaamphiphile structure so that environmental stimuli could trigger disassembly. Figure 4.9 (E) confirms the responsiveness to reducing reagent: upon GSH treatment, the self-assembled complex was disrupted and siRNA released from different bolaamphiphile complexes. The size of spherical
complexes observed in TEM is also measured by dynamic light scattering (DLS). The intensity curve in Figure 4.9 (D) shows that average diameter of the complexes ranges from 100~250 nm, which agree with the TEM images.

**Figure 4.9** (A)-(C) Dextran sulfate competitive binding assay. Different bolaamphiphile-siRNA complexes were prepared at N/P=40 and treated with different amount of DS. (D) DLS measurement. Peptide bolaamphiphile complexes were prepared in PBS buffer with N/P =45 and siRNA concentration of 2 μM. (E) Glutathione (GSH) triggered siRNA release. All the samples are G2 bolaamphiphiles with 75H25W functionalization, only the hydrophobic core are indicated in the image. Complexes were prepared at N/P=40 and treated with 10 mM GSH (+) or PBS (-) for 4h at 37 °C.
4.6 Peptide Bolaamphiphiles for siRNA Delivery

For the biomaterial application of peptide bolaamphiphiles, we focused on siRNA delivery in this study. Sequence specific gene silencing from siRNA has great potential for disease treatment, yet safe and effective delivery vectors remain a major challenge in the field. Several amphiphilic dendron structures have been explored for siRNA delivery, including PAMAM, poly(glycerol) and peptide dendrons conjugated to alkyl tail, as well as PAMAM dendrimer with long chain alkane core. However, most systems need co-formulation with cationic lipids, which may cause membrane disruption and induces cytotoxicity. In our study, peptide bolaamphiphiles showed significantly lower hemolysis and toxicity than the corresponding mono amphiphiles, and both vesicular and micellar complexes form spherical nanoparticle with diameters <100 nm, making them very appealing for siRNA delivery application.

4.6.1 Cell uptake of bolaamphiphile complexes

![Fluorescence trafficking of siRNA uptake](image)

**Figure 4.10** Fluorescence trafficking of siRNA uptake. Cy3-siRNA (red) was complexed with different amphiphiles at N/P = 30 and transfected to NIH 3T3 cells. Fluorescence images were taken 4h after the transfection. Nucleus was counter stained with DAPI (blue)
The transfection of peptide bolaamphiphiles was first studied by cellular uptake of siRNA. Figure 4.10 shows the fluorescence images of NIH 3T3 cells transfected with Cy3 labeled siRNA. Mono amphiphile was not able to delivery siRNA into the cells and no Cy3 fluorescence could be observed. G1 bolaamphiphiles forms aggregates attached to the cell surface, however, there large particles could not be internalized and no siRNA was observed in the cytoplasm. Both G2 and G3 bolaamphiphiles could delivery siRNA into the cells, with G2 bolaamphiphile exhibiting significantly higher efficiency.

The Cy3-siRNA uptake was then quantified by flow cytometry, with results summarized in Figure 4.11. Compared with Lipofectamine, all three mono amphiphiles treated cells showed no siRNA uptake [Figure 4.11 (A)], while bolaamphiphiles bola-C18-G2-75H25W and bola-F10-G2-75H25W exhibited 20 to 40 times higher uptake than the control. [Figure 4.11 (B)] Bolaamphiphiles with either shorter alkane core or hydrophilic HEG core could not form stable complexes, and did not delivery Cy3-siRNA into the cells. [Figure 4.11 (B)] It is very interesting to notice that cell uptake was directly related to the morphologies of self-assembled complexes. Vesicular complexes formed by C18 and F10 G2 bolamaphiphiles showed much higher cell uptake than their G3 micellar complexes. Similarly, bolaamphphiles containing 1,4-triazole core shows higher uptake than the 1,5-triazole analog. The three graphs in Figure 4.11 (C) are plotted in different scale for better visualization, and among them, F10-G2-75H25W transfected cells exhibited the highest siRNA uptake.
Figure 4.11 Quantification of siRNA uptake by flow cytometry. NIH 3T3 cells were exposed to different amphiphile/Cy3-siRNA complexes with N/P = 30 and siRNA concentration of 100 nM. After 4h transfection, cells were washed with PBS, released from culture plate and subjected to flow cytometry assay. (A)-(B) Cell uptake of mono amphiphile and bolaamphiphile. (C) Comparison between vesicular and micellar complexes.

4.6.2 Gene silencing of peptide bolaamphiphiles

The gene silencing of peptide bolaamphiphiles was studied in an engineered NIH 3T3 cell line with green fluorescence protein (GFP) expression. Transfection efficiency was
calculated by comparing the anti-GFP siRNA complexes treated cells with scrambled siRNA treated ones. Figure 4.12 summarizes the transfection results. Similarly to cell uptake studies, mono amphiphiles showed very low transfection efficiency, with only m-OA2-G2-75H25W inducing ~25% knockdown. Neither bolaamphiphiles with hydrophilic HEG core or fibril complexes formed by G1 bolaamphiphiles was able to silence GFP expression, while vesicular (C18-G2 and F10-G2) and micellar (C18-G3 and F10-G3) complexes both exhibited very high protein knockdown. [>80%, Figure 4.12 (B)]

We also found that the 100 nM siRNA used in the transfection was well-saturated concentration. Although micellar complexes showed much lower uptake than vesicular complexes [Figure 4.11 (C)], both were able to induce high level of GFP silencing under the 100 nM condition. However, upon reducing siRNA concentration, the difference in transfection efficiency became obvious. Bola-F10-G2-75H25W remained effective as low as 25 nM siRNA concentration, while bola-F10-G3-75H25W showed almost linear dependency on siRNA concentration from 25 to 75 nM transfection. [Figure 4.12 (C)] Lipofectamine also exhibited similar trend, with better silencing only achieved at higher siRNA concentration. The low effective dosage of bola-F10-G2-75H25W makes it a promising candidate for siRNA delivery application. Furthermore, the direct relationship from molecular structure to self-assembly behavior and subsequent biologic activity offers great opportunity to rationally design peptide bolaamphiphiles for different biomaterial application.
Figure 4.12 Transfection summary of (A) mono amphiphiles and (B) peptide bolaamphiphiles. (C) Dose-response relationship. siRNA were complexed with different bolaamphiphiles at N/P = 45 and transfection carried out at 100 nM siRNA concentration in (A) and (B). Average cell GFP fluorescence was determined by flow cytometry 48h after the transfection, and % GFP expression calculated by comparing anti-GFP siRNA treated cells with scrambled siRNA treated ones.
4.6.3 Uptake pathway and intracellular trafficking of peptide bola amphiphiles

Figure 4.13 Cell uptake pathway of peptide bola amphiphiles. (A)-(B) NIH 3T3 cells were treated with 30 μM chlorpromazine, 350 μM genistein, or 30 μM EIPA to specifically inhibit different uptake pathway. (C)-(D) Cells were transfected at low temperature. (E) NaN₃ and 2-deoxy-D-glucose (DG) were used to inhibit ATP synthesis and deplete energy in treated 3T3 cells.

For better understanding the bioactivity of peptide bola amphiphiles, **bola-F10-G2-75H25W** and **bola-F10-G3-75H25W** were chosen as representative vesicles and micelle aggregates to study the cell uptake pathway and subsequent intracellular trafficking. Figure 4.13
(C)-(E) shows that the cell uptake of the peptide bolaamphiphile/Cy3-siRNA complexes are energy dependent, with low temperature completely blocking the uptake and NaN3/2-deoxy-D-glucose inhibiting uptake in a concentration dependent manner. Several pathways are involved for energy intensive active uptake, and we used small molecule inhibitors to specifically block three most common pathways: clathrin-mediated endocytosis (chlorpromazine), caveolar endocytosis (Genistein) and macropinocytosis (EIPA)\(^{31,32}\). As shown in figure 4.13 (A) and (B), vesicular complexes (F10-G2) were internalized through both clathrin mediated and caveolar pathways, while micellar complexes exclusively enters the cell through caveolar endocytosis. Dependency of the inhibitor concentration was also observed (Figure 4.17), confirming the cellular uptake pathway.

![Graph showing %GFP Expression](image)

**Figure 4.14** Effect of endosomal acidification on peptide bolaamphiphile transfection. NIH 3T3 cells were treated with 100 nM bafilomycin for 1h before the transfection.
After endocytosis, cargoes trapped in the endosome are transported to lysosome and degraded by the hydrolytic enzymes.\textsuperscript{31,33} To achieve functional siRNA delivery, the complexes need to escape from endosome during the process. In our previous studies on dendronized polymers, we have found that histidine functionalization could help endosomal escape through “proton sponge” effect or enhanced amphiphilicity under acidic conditions. Bafilomycin inhibits vacuolar-type H\textsuperscript{+}-ATPase and prevents acidification of endosome. Under neutral condition, imidazole ring in the histidine could not be protonated, and fails to facilitate endosomal escape. As shown in Figure 4.14, a vacuolar proton pump inhibitor, bafilomycin, significantly reduces the transfection efficiency of peptide bolaamphiphiles, confirming the critical role of histidine in siRNA delivery.

4.7 Conclusion

We have successfully developed a novel class of bolaamphiphile with peptide dendron headgroup. Compared with conventional mono amphiphile structure, peptide bolaamphiphiles showed no membrane disruption and low cytotoxicity. Bolaamphiphiles could self-assemble with siRNA to form various nano-objects, and several peptide bolaamphiphiles exhibited high siRNA transfection efficiency \textit{in vitro}. Furthermore, we were able to directly link the molecular structure of bolaamphiphiles to their self-assembly behavior and biological activity. We envision that such control could enable rational design of new peptide bolaamphiphiles for a wider range of biomaterial application.

Reference


4.8 Experimental and Supporting Information

4.8.1 General Information

Materials. Unless otherwise noticed, 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). Coupling reagents were purchased from GL Biochem Ltd. (Shanghai, China). Branched polyethyleneimine (PEI, 25kDa) was purchased from Sigam-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). Silencer anti-GFP siRNA, Silencer Select negative control siRNA, Silencer Cy™-3 labeled Negative Control siRNA and Lipofectamine RNAiMAX were purchased from Invitrogen (Carlsbad, CA). All reactions were performed in 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). 100% bovine red blood cells suspension was purchased from Lampire Biological Laboratories (Pipersville, PA). Cell culture media, Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA).

Instruments. All compounds were characterized by NMR and MS. ¹H NMR spectra were recorded at 500 MHz on Bruker instruments. ¹H NMR chemical shifts were reported as values in ppm relative to specified deuterated solvents. The size and zeta potential of
denpol/siRNA polyplexes 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).
4.8.2 Peptide bolaamphiphile synthesis and characterization

Scheme 4.1 General synthetic route of peptide bolaamphiphiles

**Synthesis of cystamine-terminated linker 1:** Dissolve diacid and mono-trt protected cystamine in DCM in a round bottle flask, followed by the addition of DIPEA, EDC.HCl, and HOBt. The reaction mixture was left to stir at room temperature overnight. After the reaction, the mixture was diluted with DCM and washed with HCl, NaHCO₃, and brine. The organic layer was dried over Na₂SO₄ and the solvent was removed.
removed in vacuo. The crude product was purified by column chromatography (1–3% TEA in DCM).

**Deprotection of Trt-protected linker.** 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 precipitate in Et₂O.

**Synthesis of Lysine-based bolaamphiphile 3.** 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 (23.0 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 dissolving in MeOH and precipitate 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 in a minimum amount of MeOH and precipitation in Et₂O. The white precipitate was dissolved in nanopure water, filtered over a 0.22 µm filter and lyophilized to give 3 as a white powder.
Synthesis of His-Trp Functionalized DA 4: In a one drum glass vial were added 30 mg of unfunctionalized DA 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. Protected DA 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 redissoved in MeOH and precipitated in Et₂O. The white precipitate was dissolved in water and lyophilized to give a white powder. All DA were characterized by ¹H NMR. The functionalization ratio was determined by comparison of the characteristic aromatic peaks of imidazole (histidine) and indole (tryptophan) ring.

¹H NMR Analysis of Different DAs

n=12, C12-G2-75H25W: $^1$H NMR ($d^4$-MeOH): 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), 3.67-3.07 (multiple peaks overlapped with solvent peak, integration not accurate), 2.81 (8 H), 2.17 (2 H), 1.83-1.32 (56 H).


n=22, C22-G2-75H25W: $^1$H NMR ($d^4$-MeOH): δ 8.13-8.04 (6.40 H, histidine, 80 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), 3.67-3.07 (multiple peaks overlapped with solvent peak, integration not accurate), 2.81 (8 H), 2.17 (2 H), 1.88-1.25 (70 H).

F10-G2-75H25W: $^1$H NMR (D$_2$O): δ 8.56-8.47 (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), 3.67-2.67 (40 H), 1.71-1.06 (36 H).
HEG-G2-75H25W: $^1$H NMR ($d^4$-MeOH): $\delta$ 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), 3.67-3.07 (multiple peaks overlapped with solvent peak, integration not accurate), 1.80-1.27 (36 H).

4.8.3 Cytotoxicity and hemolysis studies

**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 DAs 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) 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. 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.

4.8.4 TEM studies

Figure 4.15 Additional TEM images of different amphiphile complexes.
Unless otherwise specified, all siRNA-amphiphile complexes for TEM studies were prepared at 8.0 μM siRNA concentration and N/P ratio of 10. 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 1~2 h 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, in the meanwhile, 15 μL 2% Uranyl Acetate was pipetted on to the grid from the other side. After 1 min, repeat the staining process 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 putting into the TEM machine. Images were obtained on a FEI Tecnai G2 TF20 high resolution TEM operated at an accelerating voltage of 200 kV.

**Figure 4.16** IR spectrum of different bolaamphiphile complexes. All complexes were prepared at N/P = 10 and 20 μM siRNA concentration.
4.8.5 siRNA binding study

**Gel electrophoresis.** The binding of siRNA to DA was studied by agarose gel electrophoresis. Both siRNA and DA were diluted with 10 mM phosphate buffer pH 7.4. Different amount of DA 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 DA was studied by competitive binding assay with dextran sulfate (DS). To 5 µL 4 µM siRNA solution was added different denpol 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 denpol complexes.** To 5 µL 4 µM siRNA solution was added concentrated DA 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.
DLS measurements. The size and zeta potential of DA/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°. Both denpol and siRNA were diluted in nanopure water, and 50 µL DA solution was added to 50 µL 1.5 µM siRNA solution (N/P 40), followed by brief vortexing. After 30 min incubation at rt, DLS measurement was taken. The solution was then diluted with 600 µL PBS, and subjected to zeta-potential measurement. At least three measurements were taken for each sample and the mean values were reported.

4.8.6 Cell uptake and protein knockdown

Sample preparation. The complex solution for transfection was prepared by simple mixing of the amphiphile solution and siRNA solution. In a typical procedure, 1.5 µM siRNA solution was prepared by diluting the stock solution with PBS buffer. Different amphiphile solution was also diluted by PBS buffer to a final volume of 12.3 µL. The 12.3 µL amphiphile solution was then added to 6.7 µL 1.5 µM siRNA solution, followed by brief vortexing. The solution was further agitated on a shaker for 30 min before transfection. As a positive control, Lipofectamine RNAI MAX was complexed with the same amount of siRNA following the vendor’s manual.

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.

**Transfection and flow cytometry.** 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. 20 μL different complex solutions were 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.

**Statistical Analysis.** All quantitative assay were performed in triplicates, data were expressed as mean ± SEM.
Figure 4.17 Concentration dependent inhibition of cell uptake.

4.8.7 $^1$H NMR Spectra
Chapter 5: *In situ* Cross-linked Biodegradable and Stimuli-Responsive Nanogel for Efficient siRNA Delivery

5.1 Introduction

Gene silencing effects introduced by small interfering RNA (siRNA) have shown great potency in specific regulation of protein expression and consequently, have been considered as a promising therapeutic approach for various diseases.\(^1\)\(^-\)\(^4\) Despite its potential, the clinical application of siRNA is still limited due to the lack of safe and efficient delivery systems.\(^5\)\(^-\)\(^7\) Both viral and non-viral vectors have been intensively studied for siRNA carrier. Although non-viral vectors are often found to be less efficient than viral vectors, non-viral systems have the advantage of being less immunogenic and offering greater synthetic versatility.\(^8\)\(^,\)\(^9\)

Among different non-viral delivery vectors, nanogels made by cross-linked hydrophilic polymers have gained much attention recently due to their high loading capacity, stability and responsiveness to environmental stimuli such as pH, temperature, ionic strength, and light.\(^10\)\(^-\)\(^14\) The cross-linked network allows efficient encapsulation of cargos and the responsiveness to pH change or reducing reagent has been shown to facilitate the release of siRNA upon entering the cytoplasm while maintaining the stability in extracellular environment.\(^15\)\(^-\)\(^19\) Biocompatibility of the vectors is another concern for therapeutic applications of siRNA. Polycationic materials could condense siRNA via electrostatic interaction, but often induce cytotoxicity by interrupting cellular membrane integrity.\(^20\) As an ongoing effort in our group, we have been utilizing natural building blocks such as amino acids and saccharides to construct synthetic biomaterials that exhibit excellent biodegradability and minimum toxicity.\(^21\)\(^-\)\(^23\) Herein, we designed a natural
amino acid based polymer precursor and cross-linked it into a stimuli-responsive nanogel, in order to generate a siRNA delivery vector which was both biocompatible and efficient.

In one of our recent studies, we have successfully developed a surfactant-free method for the preparation of biocompatible and biodegradable nanogels.\textsuperscript{24} Several peptide-based nanogels were synthesized and biocompatibility assay showed that these nanogels exhibited minimal cytotoxicity to NIH 3T3 fibroblast cells \textit{in vitro}.\textsuperscript{24} Whereas our previous study was focused on method development for surfactant-free synthesis of nanogels and investigation of their basic biomaterial properties, the current study is investigating the application of our biocompatible nanogel system for siRNA delivery. In the previous study, we have designed a tetra-lysine (\textbf{TetK})-based pre-polymer, which contains a tetra-L-lysine motif for siRNA binding and a dicysteine motif for triggered degradation and release under reducing environment (Figure 5.1). \textbf{TetK} nanogel was selected for the current siRNA delivery study because it showed good biocompatibility and polylysine has been widely used in gene delivery.\textsuperscript{25-27} The stability of polypplex formed between cationic vectors and siRNA, known to be sensitive to many factors such as concentration, ratio, solvent and order of mixing etc.,\textsuperscript{28} is critical for efficient delivery. Recent studies have shown that \textit{in situ} cross-linking either the core or the shell of siRNA/polymer complexes could increase the stability and transfection efficiency.\textsuperscript{29-32} In this study, we investigated the \textit{in situ} cross-linking of \textbf{TetK}/siRNA complexes by a poly(ethylene glycol) cross-linker and compared its efficiency with complexes prepared by either pre-polymer or pre-formed nanogel.
5.2 Materials and Methods

5.2.1 Materials. Unless otherwise noticed, 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). Coupling reagents were purchased from GL Biochem Ltd. (Shanghai, China). Branched polyethyleneimine (PEI, 25kDa) was purchased from Sigam-Aldrich (St. Louis, MO). Sodium Dextran Sulfate (25kDa) was purchased from TCI America (Portland, OR). GelRed™ siRNA stain was purchased from VWR (Radnor, PA). Orange gel loading dye (6X) was purchased from New England Biolabs (Ipswich, MA). Alexa Fluor 488 carboxylic acid, succinimidyl ester was purchased from Invitrogen (Carlsbad, CA). Silencer GFP siRNA, Silencer Select negative control siRNA and Silencer Cy™-3 labeled Negative Control siRNA were purchased from Ambion (Austin, TX). RNase ONE™ Ribonuclease was purchased from Promega (Madison, WI). All reactions were
performed in HPLC grade solvents that were further purified by passing through an alumina column, and reagent grade solvents were used for aqueous workup and flash chromatography. 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). Dulbecco’s modified Eagle’s medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Invitrogen (Carlsbad, CA).

5.2.2 Nanogel Synthesis

**Synthesis of TetK pre-polymer and PEG crosslinker.** The synthesis of TetK pre-polymer and PEG cross-linker were reported in another manuscript.\(^{24}\) Briefly, the tetralysine monomer was synthesized by conventional liquid phase coupling reactions and the dicysteine diacid was activated by pentafluorephenol (PFP). The two monomers were reacted in dry DMF overnight to give the protected copolymer with number average molecular weight ranging from 15~20 kDa and polydispersity (PDI) around 1.5. Boc protecting groups were removed by 30% trifluoroacetic acid (TFA) in dichloromethane, resulting a cationic polymer with five free amines each repeating unit. The activated PEG cross-linker was obtained from carbonyl diimidazole (CDI) and poly(ethylene glycol) with number molecular weight of 2000. All small molecules were characterized by mass spectrometer and nuclear magnetic resonance (NMR) spectroscopy, and polymers by gel permeation chromatography (GPC) and NMR. The detailed synthesis was reported in another manuscript.\(^{24}\)

**Preparation of pre-formed TetK nanogel.** The pre-formed TetK nanogel was synthesized by previously reported surfactant-free method with slight modification.\(^{24}\) To 10 mg of TetK pre-polymer dissolved in 0.50 mL DMF was added 0.45 mL methyl tert-butyl ether
(MTBE). Then 5.7 mg PEG\textsubscript{2000} (15 mol\% to the amount of free amines from the pre-polymer) was dissolved in 46 μL DMF (125 mg/mL) and added into the polymer solution, followed by 3 μL pyridine. The reaction was stirred for ~24 h at room temperature. Afterwards, the reaction mixture was diluted with MeOH/H\textsubscript{2}O (1:1) solution and then dialyzed against MeOH/H\textsubscript{2}O (MWCO 50,000) for 24 h, followed by nanopure water for 48 h and was lyophilized afterwards. Nanogel in 35~50\% yield could be obtained in white powder form after purification.

**Preparation of *in situ* crosslinked TetK/siRNA nanogel.** *In situ* cross-linked TetK/siRNA nanogel was synthesized by the following scheme (Figure 5.2). A stock solution of 40 μM siRNA was prepared using RNase free water. 100 μL of the stock siRNA solution was diluted in 0.25 mL DMF solution. With respect to different N/P ratios, different amount of TetK pre-polymer solution (10 mg/mL in DMF) was added drop wise to the siRNA solution under vigorous stirring. More DMF was added to adjust the final polymer concentration to 4 mg/mL. The mixture was allowed to stir at room temperature for 1h before 15 mol\% PEG\textsubscript{2000} (75 mg/mL in DMF) and 2.5 μL pyridine were added. The mixture was reacted overnight and then diluted with RNase free water. The free polymer, siRNA and small organic molecules were removed by centrifuge filtration with a 30,000 MWCO filter for three times. The final aqueous solution was lyophilized for two days to give the TetK/siRNA nanogel in 45~60\% yield. The nanogels were resuspended in 1 mL RNase free water and stored at -80 °C before use.
Figure 5.2 *In situ* crosslinking for preparation of TetK/siRNA nanogel

5.2.3 Nanogel Characterization

**DLS measurements.** The size and zeta potential of both the pre-formed TetK nanogel and *in situ* cross-linked TetK/siRNA nanogel were measured at 633 nm using Zetasizer (NanoZS) dynamic light scattering instrument (Malvern Instruments, Malvern, UK) at 25°C with detection angle of 173°. Both nanogels were lyophilized and resuspended in 10 mM pH 7.4 phosphate buffer before measurement. At least three measurements were taken for each sample. Mean values are reported.

**Complexation of siRNA with TetK pre-polymer and pre-formed TetK nanogel.** Polycomplexes of siRNA with Tetk pre-polymer and pre-formed TetK nanogel were analyzed by agarose gel electrophoresis. Both siRNA and TetK pre-polymer or nanogel were diluted with 10 mM pH 7.4 phosphate buffer. Different amount of pre-polymer or pre-formed nanogel solutions (5 mg/mL) were added to 7.5 μL 4 μM siRNA solution to achieve different N/P ratios (From N/P 1 to 20). The same phosphate buffer was added to adjust the final volume to 12.5 μL.
After 1h 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 TBE 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 the nanogel was investigated by dextran sulfate (DS) challenge. To 7.5 μL 4 μM siRNA solution was added either pre-polymer or pre-formed nanogel at N/P 40 and incubated for 1h at room temperature. 1 μL DS solution of different concentration was added to the complex to achieve different S/P ratio (charge ratio between sulfate from DS and phosphate from siRNA) and incubated for another 30 min. Similarly, *in situ* cross-linked nanogel containing 30 pmol siRNA was diluted in 10 mM phosphate buffer and treated with 1 μL different DS solution for 30 min. The samples were then subjected to agarose gel electrophoresis under the aforementioned condition.

**Glutathione triggered release of siRNA from the nanogel.** To 7.5 μL 4 μM siRNA solution was added different amount of pre-formed TetK nanogel solution at different N/P ratio and phosphate buffer to adjust the final volume to 12 μL. After incubation for 1h at room temperature, 1 μL 65 mM glutathione (GSH) was added to the polyplex solution to achieve a 5 mM final concentration, followed by another 30 min incubation at room temperature. Similarly, *in situ* crosslinked TetK/siRNA nanogels prepared at N/P ratio 20, 40 and 80 were also treated with 5 mM GSH solution for 30 min. All samples were then subjected to agarose gel electrophoresis.
siRNA quantification for *in situ* cross-linked TetK/siRNA nanogel. The amount of siRNA encapsulated in the *in situ* nanogel was quantified by first releasing the siRNA and then staining with a fluorescent dye GelRed.\textsuperscript{35} In 96 well plates, 1 – 5 \( \mu \)L stock nanogel solutions were diluted separately in 70 \( \mu \)L 1X GelRed solution with 5 mM glutathione and then incubated at room temperature overnight. The fluorescence was measured by a 96-well plate reader (Excitation wavelength 290 nm, Emission wavelength 600 nm). A standard curve was generated in parallel by treating a 4 \( \mu \)M siRNA stock solution under the same condition. The siRNA concentration was calculated by comparing the fluorescence of the nanogel and standard. Sample calculation could be found in the supporting information (SI, Part 3).

RNase digestion. The ability of the nanogel to protect siRNA from enzymatic degradation was studied by RNase digestion. Different amounts of either TetK pre-polymer or pre-formed TetK nanogel (5 mg/mL) was complexed with 3 \( \mu \)L siRNA solution (4 \( \mu \)M) to achieve N/P ratio 20, 40 and 80. 10 mM pH 7.4 phosphate buffer was added to adjust the final volume to 10 \( \mu \)L. After incubation at room temperature for 1 h, 1 \( \mu \)L of RNase One\textsuperscript{TM} solution (550 U/mL) was added to each solution to achieve a final RNase concentration of 50 U/mL. The mixture was then incubated for 4 h at 37 °C. The *in situ* cross-linked nanogel was also treated with 50 U/mL RNase under the same condition. After digestion, all the samples were diluted in a 96-well plate with 70 \( \mu \)L 1X GelRed solution with 5 mM GSH and the fluorescence immediately measured by a plate reader. As a control, polyplexes made from either pre-polymer, pre-formed or *in situ* cross-linked nanogels were treated with 1 \( \mu \)L RNase-free phosphate buffer under the same condition and compared with the samples treated with RNase.
5.2.4 Biological Assay

**Transfection.** NIH 3T3 fibroblast cells expressing enhanced green fluorescence protein (GFP) were seeded at a density of 5000 cells/well in 48-well plates in 250 μL DMEM with 10% FBS and cultured for 24 h in 37°C/5% CO₂ incubator. Prior to transfection, the media was replaced with 180 μL fresh DMEM solution with or without 10% FBS. 20 μL 1 mM chloroquine (CQ) solution was added to each well to make a final concentration of 100 μM, followed by 2h incubation at 37 °C. 50 μL polyplex solution containing 25 pmol siRNA made from either pre-polymer, pre-formed or *in situ* cross-linked nanogel were added to each well and incubated for another 4h. The media was changed back to 250 μL DMEM with 10% FBS and cultured in an incubator for 72h before the analysis. Qualitative assay of the GFP level in 3T3 cells was first obtained by fluorescence microscopy (Nikon Eclipse Ti, Melville, NY) with digital imaging (Nikon DS-Qi1, NIS-Elements Advance Research Software, Melville, NY). Cells in each well were directly imaged under the microscope. Then the GFP expressing level was quantified by flow cytometry. Briefly, 150 μL Tripsin was added to each well to release the cells. After washing two times with PBS, the GFP fluorescence of transfected cells was measured on a Becton-Dickinson LSR II flow cytometer (Stem Cell Research Center, UC Irvine) with argon ion excitation laser at 488 nm (Becton-Dickinson, Franklin Lakes, NJ). For each sample, data representing 10,000 objects were collected as a list-mode file and analyzed using FACSDiva™ software (Becton Dickinson, version 6.1.3). The transfection efficiency was determined by comparing the fluorescence of transfected cells with control GFP expressing cells which received no treatment (negative control). Branched poly(ethylene imine) (PEI) was chosen as a positive control because it is one of the most commonly used cationic polymer vector for gene delivery. PEI was complexed with siRNA at N/P 10 and transfected to NIH 3T3 cells under similar
conditions without CQ treatment. Different complexes with scrambled siRNA were prepared at N/P 80 and also transfected in parallel with CQ treatment to serve as a negative control. All experiments were carried out in triplicate and data are expressed as mean ± SEM.

**Fluorescence labeling of TetK pre-polymer and in situ cross-linked TetK/siRNA nanogel.** A 10 mg/mL stock solution of Alexa Fluor 488 NHS ester was prepared by dissolving 1 mg dye in 100 µL anhydrous DMSO. 7.5 µL of the dye solution was added to a 1.5 mL anhydrous DMF solution containing 7.5 mg TetK pre-polymer and the mixture was stirred at room temperature overnight. The unreacted dye and organic solvent was removed by diluting in water and centrifuging through a 3000 MWCO filter for three times. The aqueous solution was lyophilized to give 5.4 mg labeled polymer in 71% yield. The amount of Alexa Fluor 488 dye coupled to the polymer was quantified by comparing the fluorescence of the labeled polymer with free dye in a 96 well plate reader (Excitation wavelength 488 nm, Emission wavelength 525 nm). A dual labeled TetK/siRNA nanogel was then prepared by Alexa Fluor 488 labeled TetK pre-polymer and Cy3 labeled negative control siRNA at N/P 20 under aforementioned in situ cross-linking condition.

**Confocal Laser Scanning Microscopy.** Confocal laser scanning microscopy was used to track the internalization of in situ cross-linked TetK/siRNA nanogel. Unmodified NIH 3T3 fibroblast cells were seeded at a density of 5000 cells/well on an 8-well chamber slide (Lab-Tek, Rochester, NY) 24h before transfection. The cells were then treated in 200 µL fresh DMEM solution with or without 100 µM CQ for 4h. 50 µL dual labeled TetK/siRNA nanogel solution containing 25 pmol siRNA was then added to each well and incubated at 37 °C for 4h. After transfection, the media was switched back to DMEM with 10% serum. Confocal fluorescence spectroscopy was performed at different time point after the transfection. 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. Green fluorescence of Alexa 488 labeled TetK nanogel was acquired using a 488 nm excitation light from a multiple argon laser, a SDM560 dichroic mirror, and a 505-540 nm band-pass barrier filter. 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. The two 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 and an image cutting horizontally through approximately the middle of the cellular height was selected to differentiate internalized nanogel from those adsorbed on cellular surface.

5.3 Results and Discussion

Nanogel Synthesis and Characterization

We initially investigated the applicability of pre-formed TetK nanogel for siRNA delivery. For this purpose, the TetK nanogel was synthesized by the surfactant-free method developed in our laboratory. After re-suspending in Ph 7.4 phosphate buffer, the nanogel has a diameter ranging from 150 to 200 nm and zeta potential 30~40 mV. However, complexation of siRNA with the pre-formed nanogel led to large aggregates as observed by DLS measurement (z-average > 500 nm). We propose that this be due to poor permeability of siRNA into the nanogel, which results in siRNA binding preferentially on the surface of the nanogel. Neutralization of the positive surface charge leads to decreased electrostatic repulsion between different nanogels and causes aggregation. To circumvent this problem, we envision that it will ensure more efficient encapsulation by first complexing siRNA with excess amount of TetK pre-polymer and then
cross-linking in situ to form a stable TetK/siRNA nanogel. Three different TetK/siRNA nanogels at different N/P ratio were prepared by the in situ cross-linking approach (Table 1), and stable nanogel could be obtained in 45% - 60% yield after centrifugal filtration, regardless of N/P ratio. The amounts of siRNA in the nanogels were quantified by GelRed staining, and 45-50% of loaded siRNA could be encapsulated in the nanogel after purification. The size of nanogel was measured by DLS in pH 7.4 phosphate buffer. The diameter ranged from 250 to 300 nm at relatively low N/P ratio, and decreased to less than 200 nm at N/P 80.

### Table 5.1 Synthesis of in situ cross-linked TetK/siRNA nanogels

<table>
<thead>
<tr>
<th>N/P</th>
<th>20</th>
<th>40</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (nm)</td>
<td>275±20</td>
<td>282±60</td>
<td>184±45</td>
</tr>
<tr>
<td>Yield</td>
<td>48%</td>
<td>58%</td>
<td>59%</td>
</tr>
<tr>
<td>siRNA recovery</td>
<td>46%</td>
<td>45%</td>
<td>48%</td>
</tr>
</tbody>
</table>

1. siRNA recovery is calculated by the amount of encapsulated siRNA divided by the amount of loaded siRNA in the nanogel preparation.

### Gel Electrophoresis

The binding strength of siRNA to TetK pre-polymer, pre-formed and in situ cross-linked nanogel was evaluated by competitive binding study with a polyanionic polymer, dextran sulfate (DS) (Figure 5.3 A-C). Different amount of DS was added to the polyplexes to achieve different molar ratio of sulfate from DS and phosphate from siRNA (S/P ratio). After incubating DS and TetK/siRNA complexes in phosphate buffer for 30 min, all samples were subjected to agarose gel eletrophoresis. While the pre-formed TetK nanogel showed some improvement in siRNA binding strength compared to pre-polymer (Figure 5.3A&B), in situ cross-linked TetK/siRNA nanogel showed much stronger binding to siRNA, where no release of siRNA was observed up
to S/P 30 (Figure 5.3C). Dextran sulfate competition studies suggest that the cross-linked nanogel network binds stronger to siRNA than pre-polymer, and *in situ* cross-linking further improves the binding strength by efficient encapsulation of siRNA inside the nanogel.

![Figure 5.3 Gel Electrophoresis Assay](image)

**Figure 5.3** Gel Electrophoresis Assay. siRNA complexes were prepared at N/P 40 with TetK pre-polymer (A), pre-formed TetK nanogel (B) and *in situ* cross-linked TetK/siRNA nanogel (C) and then treated with different concentration of dextran sulfate for 30 min before loading to the agarose gel. (D) Glutathione triggered release of siRNA from *in situ* cross-linked TetK/siRNA nanogels. (E) Release of siRNA from pre-formed nanogel/siRNA complexes.

One important property for successful delivery is the facile release of the cargo after internalization into the cell. Because of the reducing environment in the cytoplasm, disulfide bond has been included in a variety of systems for reduction triggered release. In the TetK
nanogel, a dicysteine component was incorporated in the polymer backbone, which can be cleaved by reducing reagent glutathione (GSH). The GSH triggered release of siRNA was examined by agarose gel electrophoresis (Figure 5.3, D & E). Both *in situ* cross-linked TetK/siRNA nanogel and complexes of pre-formed nanogel with siRNA were treated with GSH (5 or 10 mM) for 30 min. In both scenarios, siRNA release was observed at all N/P ratios after incubating with GSH.

**RNase Digestion**

Systemic administration of siRNA for disease treatment requires enough circulation time so siRNA can be delivered to target tissue or cells. An effective delivery vector should protect siRNA from enzymatic degradation during vascular transport. In order to access the resistance of siRNA complexed by TetK nanogel toward RNase degradation, digestion test was carried out with three different siRNA complexes (Figure 5.4). After incubation with 50 U/mL RNase One™ at 37°C for 4h, free siRNA was completely digested with less than 5% recovery. In contrast, complexes made from pre-polymer or *in situ* cross-linked nanogel showed very good protection of siRNA from degradation with higher than 80% recovered at all N/P ratios after the RNase treatment. Interestingly, the complexes made from pre-formed TetK nanogel showed minimum protection, only ~10% siRNA could be recovered after the digestion. The inability of protection further supports our hypothesis that siRNA is preferentially adsorbed onto the surface of pre-formed nanogel instead of being encapsulated. The surface adsorbed siRNA on the pre-formed nanogel are more exposed to the outer environment and therefore more susceptible to degradation. Furthermore, electrostatic repulsion of positive charged vectors is known to prevent cationic RNase from approaching RNA.\(^{37}\) Surfacted bound siRNA neutralized the surface charge and therefore decreased the protective effect of positively charged nanogel.
Figure 5.4 RNase Digestion Assay. siRNA complexes were prepared at different N/P ratio with linear TetK polymer, pre-formed and in situ crosslinked nanogel. The complexes were incubated with 50 U/mL RNase One at 37°C for 4h. Remaining siRNA was released by GSH and quantified by GelRed staining.

Tranfection to NIH 3T3 cells

The transfection study was carried out using engineered NIH 3T3 cells expressing enhanced green fluorescence protein (GFP). Anti-GFP siRNA complexes with different N/P ratios were prepared by either TetK pre-polymer, pre-formed nanogel or in situ cross-linking. The transfection studies were carried out in both serum and serum-free conditions. As shown in Figure 5.5, TetK pre-polymer showed moderate transfection in serum-free media with increased efficiency at higher N/P ratio. However, upon addition of serum into the cell culture media, the efficacy quickly diminished. Pre-formed nanogel, despite stronger affinity with siRNA compared to pre-polymer, showed minimum transfection at all N/P ratio in either media. Presumably, this was caused by the large aggregation after complexation as revealed by DLS measurement. In addition, neutralization of positive charge on nanogel surface by siRNA adsorption may reduce the interaction with cell membrane, further decreasing the transfection efficiency. In situ cross-
linked TetK/siRNA nanogel showed the highest silencing effect. In serum-free media, higher than 85% silencing effect could be achieved, and no significant difference was observed between different N/P ratios. In transfection media containing 10% serum, the efficiency was slightly hampered, but still 60-80% knockdown was observed. The efficiency was also positively related to the N/P ratio, with higher N/P nanogel achieving more efficient gene silencing. Presumably, the improved transfection efficacy of in situ cross-linked nanogel in serum containing media results from stronger siRNA binding affinity, better protection by the encapsulation, and lack of aggregation of polyplexes. The use of PEG cross-linker may also contribute to serum stability for the TetK/siRNA nanogel system. PEG is widely used in biomaterial applications for its excellent biocompatibility, and have also shown to increase the stability of cationic vectors in serum by the shielding effect from anionic proteins. The high transfection efficacy of PEG cross-linked nanogel indicated that the current system could be applicable for in vivo systems.

As negative controls, scrambled siRNA complexes at N/P 80 were also prepared and transfected under the same conditions. Compared with untransfected cells, there is no significant silencing effect of the scrambled siRNA complexes in all three conditions, indicating minimum non-specific silencing effect of our system. Polyethyleneimine (PEI) has been widely used in DNA/siRNA delivery because of its high tranfection efficacy. Branched PEI (MW = 25 kDa) complexed with siRNA at N/P 10 was also included in the transfection study as a positive control. Similar to reported results, while relatively high silencing effect was observed in serum-free conditions for PEI, the efficiency is greatly reduced in serum-containing media due to aggregation.
Figure 5.5 Summary of transfection results. NIH 3T3 cells expressing GFP were treated with different samples and the GFP fluorescence of transfected cells was measured by flow cytometer. The transfection efficiency was presented by comparing the fluorescence of transfected cells with untransfected samples.

Qualitative analysis of transfection of 3T3 cells by fluorescence microscopy agreed with the quantitative flow cytometry data. Figure 5.6 shows one set of images from three different siRNA complexes prepared at N/P 80. Cells treated with pre-formed nanogel showed almost the same level of green fluorescence as the blank control. While cells transfected with linear TetK pre-polymer showed some reduction in fluorescence, cells transfected with in situ cross-linked nanogel showed almost no fluorescence. The diminished green fluorescence confirmed the silencing effect from nanogel transfection. (Other fluorescence images is summarized in the supporting information, Figure 5.10)
Figure 5.6 Fluorescence and phase images of cells transfected with different complexes at N/P 80. NIH 3T3 cells were transfected under aforementioned conditions. 72h after the transfection, cells were imaged under a Nikon Eclipse Ti fluorescence microscope. Scale bar: 100 μm.

The delivery efficiency of the in situ cross-linked TetK/siRNA nanogel was further investigated by dose-response relationship. 3T3 cells pretreated with 100 μM CQ was transfected with different amount of in situ nanogel at N/P 80 in serum-free media (Figure 5.7). The concentration of the nanogel was presented by the amount of siRNA encapsulated. The gene silencing effect was shown to be concentration dependent. While 10 nM siRNA showed only
minimum silencing effect, increasing siRNA concentration to 25 nM led to a dramatic increase of the silencing efficiency to 60%. The transfection efficacy gradually saturated at higher concentration, with the silencing increased to 80% at 100 nM siRNA concentration. The same effect could be observed from the peak shift in the histogram (Figure 5.7B) and also supported by qualitative fluorescence images with diminished green fluorescence from cell treated by higher concentration nanogel solution (Figure 5.7C). For the siRNA delivery applications, it is preferred to use less amount of siRNA because of the relatively high cost of siRNA and also the possibility of non-specific effect at higher concentration. Our system showed that effective silencing could be obtained with as low as 25 nM siRNA.

**Intracellular trafficking of in situ cross-linked nanogel vector**

The intracellular localization of siRNA and nanogel was visualized by confocal fluorescence microscopy. For this purpose, the TetK pre-polymer was labeled with a green fluorescent dye, Alexa 488 and siRNA with a red dye, Cy3. *In situ* cross-linked nanogel with dual labels were then prepared following aforementioned condition. After merging the two fluorescence images obtained by the microscope, co-localization of siRNA and nanogel was indicated by yellow colored spots.
Figure 5.7 Dose-response relationship of *in situ* cross-linked nanogel. NIH 3T3 cells was transfected with *in situ* nanogel with N/P ratio 80 at different concentration adjusted to siRNA amount. (A) Summary of flow cytometry data. (B) Histogram of a representative sets of transfection data at different concentrations. (C) fluorescence images (Top) and phase contrast images (bottom) of cells treated with different concentrations of nanogel.

As the TetK nanogel has little buffering capacity in pH 5–7, we added chloroquine (CQ) in our transfection studies to facilitate endosomal escape. In order to further elucidate the effect of CQ, NIH 3T3 cells were treated with or without CQ and then transfected with labeled nanogel. As shown in Figure 5.8, efficient internalization could be observed both with and without CQ treatment right after the transfection. However, cells treated without CQ quickly cleared out
Figure 5.8 Confocal fluorescence images of NIH 3T3 cells transfected with dual labeled TetK/siRNA nanogel. TetK pre-polymer was labeled with Alexa Fluor 488 dye (green), and siRNA were labeled with Cy3 (Red). In situ cross-linked nanogel were synthesized with the two labeled component and PEG cross-linker. The cells were treated with or without chloroquine for 4h and then exposed to the complexes for another 4h before changing culture media back to DMEM with 10% serum. Confocal fluorescence images were taken at 0, 8, 16, and 48 hours post-transfection.

siRNA with only minimal amount of siRNA (red) observed 8 h after transfection and almost no siRNA after longer durations. In contrast, both free siRNA (red) and siRNA complexed in the nanogel (yellow) were present in cells treated with CQ for up to 48h. CQ has been widely used in gene delivery to assist endosomal escape.\textsuperscript{44,45} It is hypothesized that the accumulation of CQ in endosome increases the pH and therefore inhibits the fusion of endosome to lysosome and also lowers the activity of hydrolytic enzymes. In addition, the increased pH causes more protons and counterion chlorides to flush into endosome and increase the osmotic pressure, which may eventually lead to endosome burst and release of the cargo into cytoplasm. Such effect was observed in our study, nanogels in cells with no CQ treatment were transported to lysosome and
siRNA were degraded and quickly removed from the cells. Furthermore, carefully scrutinizing the images at 0 and 8h reveals that the fluorescence is less localized in cells treated with CQ as indicated by larger and more diffusive spots, while smaller and more localized fluorescence spots were observed in cells without CQ treatment. This also suggests that CQ facilitate more efficient escape of the TetK/siRNA nanogel from the endosomes into the cytoplasm.

5.4 Conclusion

In this study, we investigated the applicability of a biodegradable and stimuli-responsive nanogel made from natural amino acids for siRNA delivery. The siRNA complexes were prepared with pre-polymer, pre-formed nanogel or in situ cross-linking. Although pre-formed nanogel showed improved binding affinity of siRNA over pre-polymer, the poor permeability of siRNA into the nanogel resulted in large aggregates, inefficient protection from RNase, and minimum transfection efficacy. In sharp contrast, in situ cross-linking ensures efficient encapsulation of siRNA and achieves not only the strongest binding affinity but also improved protection of siRNA from enzymatic degradation. Transfection in NIH 3T3 cells pretreated with chloroquine shows that the in situ cross-linked TetK/siRNA nanogel has high gene silencing effect both in serum-free and 10% serum media with minimum nonspecific interaction. Fluorescence trafficking study confirmed efficient cellular uptake of the nanogel and also indicated that chloroquine helps endosomal escape of internalized nanogel and siRNA. The biocompatibility and high transfection efficacy suggests the in situ cross-linked system a potent candidate for siRNA delivery application. Another advantage of this system is the modular
design and synthetic versatility of the pre-polymer, and currently chemical modification of pre-polymer to avoid the use of chloroquine is under investigation.

Reference


(3) Davidson, B. L.; McCray, P. B. Nat. Rev. Genet. 2011, 12, 329.


(18) Blackburn, W. H.; Dickerson, E. B.; Smith, M. H.; McDonald, J. F.; Lyon, L. A.


5.5 Supporting Information

5.5.1 siRNA quantification in *in situ* cross-linked TetK/siRNA nanogel

The amount of siRNA encapsulated in the *in situ* nanogel was quantified by first releasing the siRNA and then staining with a fluorescent dye GelRed. In 96 well plates, 1 – 5 µL stock nanogel solution was diluted in 70 µL 1X GelRed solution with 5 mM glutathione and then incubated at room temperature overnight. The fluorescence was measured by a 96-well plate reader (Excitation wavelength 290 nm, Emission wavelength 600 nm). A standard curve was generated in parallel by treating 4 µM siRNA solutions under the same condition. The sample siRNA concentration could be directly calculated by plugging the fluorescence into the standard curve, however, we found this method has a higher calculation error due to different background fluorescence in different samples. Therefore, the siRNA concentration was calculated by linear aggregation.

\[
F = a \cdot C + b
\]

\[
F = a \cdot \frac{VC_1}{70+V} + b
\]

\[
F = aC_1\left[\frac{V}{70+V}\right] + b
\]

\[
F = aC_1V_{cal} + b
\]

\[
V_{cal} = \frac{V}{70+V}
\]

The above equations assume that the fluorescence intensity (F) is in linear relationship with final siRNA concentration (C) in each sample. The concentration C could then be expressed by the stock concentration C₁ and the dilution factor. After transformation, we can see that F is in
linear relationship with $V_{\text{cal}}$. The $F/V_{\text{cal}}$ plot of a sample solution was compared with standard siRNA solution, and the ratio of the slope is proportional to the ratio of siRNA concentration. Figure 5.9 is the plot of one sample (HZ176) with the standard siRNA solution, the siRNA concentration of HZ176 could be calculated as following:

$$C (HZ176) = \frac{11087}{14442} \times 4 \ \mu\text{M} = 3.07 \ \mu\text{M}$$

![Sample plot of siRNA quantification.](image)

**Figure 5.9** Sample plot of siRNA quantification.

### 5.5.2 Fluorescence imaging of transfected NIH 3T3 cells

Fluorescence images of transfected NIH 3T3 cells was obtained by fluorescence microscope (Nikon Eclipse Ti, Melville, NY) with digital imaging (Nikon DS-Qi1, NIS-Elements Advance Research Software, Melville, NY). A 10× objective was used for all experiments and GFP
fluorescence was acquired using a 488 nm excitation light from a multiple argon laser, a SDM560 dichroic mirror, and a 505-540 nm band-pass barrier filter. Figure 5.10 shows the images of cells transfected with different samples.

**Figure 5.10 (A)** Fluorescence and phase images of cells treated by complexes prepared with anti-GFP siRNA at N/P 20. Scale bar: 100 µM.
Figure 5.10 (B) Fluorescence and phase images of cells treated by complexes prepared with anti-GFP siRNA at N/P 40. Scale bar: 100 μM.
**Figure 5.10 (C)** Fluorescence and phase images of cells treated by complexes prepared with scrambled siRNA at N/P 80. Scale bar: 100 μM.
Chapter 6: Direct Synthesis of Polyamides via Catalytic Dehydrogenation of Diols and Diamines

6.1 Introduction

Natural and synthetic polyamides are among the most important families of polymers. Natural polyamides, including proteins and peptides, are essential macromolecules in living organisms. Synthetic polyamides exhibit high strength, toughness, and stability, therefore have received numerous applications in fiber products and engineering plastics,\(^1\) and recently found great potentials for many functional applications.\(^2\) In biomedical studies, polyamides have also attracted much attention because they have shown good biocompatibility and degradability.\(^3\) For example, polylysine (PLL) and poly(amido amine) dendrimer (PAMAM) as well as their derivatives have been widely used in biomaterial applications.\(^4,5\) Our lab has previously developed functional polyamides as biomaterials for gene delivery,\(^6\) tissue engineering,\(^7\) and protein-resistant applications.\(^8\) The growing interest in polyamide-based functional materials and biomaterials has motivated us to develop more efficient direct synthesis of functional polyamides.

![Scheme 6.1 Concept of direct polyamidation via dehydrogenation](image)

*Scheme 6.1* Concept of direct polyamidation via dehydrogenation
Polyamides are conventionally synthesized by condensation between amines and carboxylic acids or their derivatives. In such reactions, the acids are usually pre-activated to form acyl chlorides\(^2\) or active esters,\(^9\) or \textit{in situ} activated by stoichiometric amounts of coupling reagents,\(^10\) the later being used extensively in peptide synthesis.\(^11\) High temperature melt condensation is used for commercial synthesis of aliphatic polyamides (Nylon’s).\(^1\) All these syntheses either involve harsh conditions and/or produce stoichiometric amount of toxic wastes. Alternatively, polyamides can also be produced by ring-opening polymerizations of lactams\(^12\) or N-carboxy-amino acid anhy-dride.\(^13\) However, the preparation of the cyclic monomers still encounters the same limitations aforementioned for amidation reaction. While several mild amidation methods have been developed for peptide synthesis,\(^14-15\) their efficiency and substrate scope have hindered their application for polyamide synthesis. Given the broad importance of polyamides for both technological and biomedical applications, an atom economic synthesis that avoids toxic waste generation and/or harsh conditions is highly desirable.

Milstein and coworkers reported recently a direct dehydrogenative amidation from alcohols and amines using a PNN pincer Ru catalyst.\(^16\) Following this seminal work, several other groups reported similar amidation systems, which feature Ru catalysts with pincer ligands\(^17\) and NHC ligands,\(^18-20\) rhodium catalysts\(^21\) and supported silver catalysts.\(^22\) Given the high efficiency of the catalytic process and minimal side product (H\(_2\)) produced, we were inspired to apply this catalytic reaction to polyamide synthesis (Scheme 6.1). Herein, we report the first successful direct polyamidation from diols and diamines using the Milstein Ru catalyst.
6.2 Results and Discussion

In Milstein’s original paper, while both mono- and diamines were shown suitable for the catalytic amidation, only the reactions of mono-alcohols have been reported. Therefore, we first investigated the reactivity of diols by running model reactions between a series of alkyl diols and benzylamine (Scheme 6.2 in the SI). Surprisingly, the results show that a minimum spacer of six carbons between the two hydroxyl groups is necessary for an efficient conversion. The exact reason for the inactivity of diols with shorter spacers is unclear at this moment, which could be due to chelation of the two hydroxyl groups to the active catalytic center. It is worth noting that this effect is only for free hydroxyl groups, because oxygen atoms in oligo(ethylene glycol)s used in later studies do not affect the catalytic activity.

Next, a model polyamidation between tri(ethylene glycol) (1b) and m-xylylene diamine (2b) was chosen to optimize polymerization conditions (Table 6.1). Oligo(ethylene glycol)s (OEGs) were chosen in our study because they offer sufficient distance between the two –OH groups, have good solubility, and, most importantly, they are FDA approved biomaterials having minimal cytotoxicity and immunogenicity. Initially, we followed the same conditions reported in Milstein’s original paper for dehydrogenation. Heating a mixture of 1b and 2b with 0.2 mol% Milstein catalyst for 28 h, however, only resulted in mostly dimers and trimers (entry 1). Increasing catalyst loading and reaction time has moderate positive effect on the polymerization (entries 2-4); but the molecular weight of the polymer was still relatively low (number-averaged molecular weight, \( M_n < 3 \) kD). One possible factor is that, due to extensive hydrogen bonding between amide linkages, the resulting polyamide has limited solubility in toluene, which may prematurely stop chain growth. To address this issue, we reasoned that more polar solvents should break the inter-chain hydrogen bonds and increase the solubility. Therefore, several polar
solvents were screened (See Table 6.3 in the SI) and anisole was found to be a good solvent in which the catalyst remained highly active and the $M_n$ of the polyamide was dramatically increased (entry 5). Too polar solvents such as DMSO seemed to suppress the activity of the dehydrogenation reaction (entry 6); however, addition of a relatively small amount of DMSO to anisole could increase the polarity without significantly lowering catalyst activity, thus the $M_n$ of the polymer could be further improved to more than 22 kDa (entry 7). For even less soluble polymers, the ratio of DMSO could be further increased with higher catalyst loading to maintain both the polyamide solubility and catalyst activity (Table 6.2, entries 1-3).

Table 6.1 Condition screening for a model catalytic polyamidation

<table>
<thead>
<tr>
<th>Entry</th>
<th>Cat. Loading (mol%)</th>
<th>Reaction Time (h)</th>
<th>Solvent</th>
<th>Conversion (%)</th>
<th>$M_n (10^3)$</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>28</td>
<td>toluene</td>
<td>56</td>
<td>&lt; 1.0</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>28</td>
<td>toluene</td>
<td>77</td>
<td>1.1</td>
<td>1.14</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>48</td>
<td>toluene</td>
<td>82</td>
<td>1.6</td>
<td>1.92</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>48</td>
<td>toluene</td>
<td>89</td>
<td>3.2</td>
<td>1.59</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>48</td>
<td>anisole</td>
<td>&gt;99</td>
<td>13.8</td>
<td>1.67</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>48</td>
<td>DMSO</td>
<td>79</td>
<td>1.4</td>
<td>1.60</td>
</tr>
<tr>
<td>7</td>
<td>1.0</td>
<td>48</td>
<td>anisole/DMSO (6:1)</td>
<td>&gt;99</td>
<td>22.6</td>
<td>1.51</td>
</tr>
</tbody>
</table>

$^a$ Reaction condition: 1.0 mmol triglycol, 1.0 mmol xylylenediamine and 0.01 mmol catalyst are premixed in 1.5 mL solvent in glovebox, then heated under N$_2$ flow. $^b$ Determined by NMR of the crude reaction mixture. $^c$ Number average molecular weight, determined by Gel Permeation chromatography (GPC). $^d$ Polydispersity index, determined by GPC. $^e$ No polymer peak in GPC, only oligomers observed by NMR.
Using our optimized condition, we then synthesized a series of polyamides using various diols and diamines (Table 6.2). Because the ultimate molecular weight of each polymer is greatly affected by its solubility in the polymerization solution, either anisole or anisole/DMSO mixed solvent was used for better result. We observed that while anisole/DMSO mixed solvent worked better for less soluble polyamides (entries 1-3), anisole gave larger polymers for more soluble systems (entries 4-17), because the addition of DMSO increased the polyamide solubility while decreased the catalyst activity. With oligo(ethylene glycol)s as diol monomers, a variety of diamines, having aliphatic or aromatic, linear or cyclic spacers, were effective for the polyamidation, resulting in polymers with $M_n$ ranging from 10 to 30 kD (entries 1-7). For diamines having longer alkyl chain between two amino groups, the polymer become less polar and more soluble in anisole, therefore dodecanediamine ($2e$) gives much larger polymer than propanediamine ($2d$). Tri-functional monomer, tris(2-aminoethyl)amine ($2h$), could also be incorporated into the polyamides, resulting in a branched polymer (entry 8).

On the other hand, different diols could also polyermize with 2,2'-\(\text{O}\)-bis(ethyamine) ($2g$) under the same conditions (entries 9-13). Similarly, both linear and cyclic, aromatic and alkyl spacers were tested and shown to be well tolerated in this catalytic system. Because of solubility issues, alkyl diols alone only give low molecular weight oligomers. However, polyamides larger than 20 kDa could be achieved by copolymerizing them with tetra(ethylene) glycol to interrupt the crystallinity of the alkyl chain and thus improve solubility (entries 12-13). It should be noted that due to limited solubility and phase separation during polymerization, a few systems gave bi-modal molecular weight distribution in GPC measurement (entries 1, 2, 8, 15 and 17).
The dehydrogenative polyamidation should follow a similar catalytic process as proposed by Milstein et al.\textsuperscript{16} Presumably, after a catalytic cycle for dehydrogenation of an alcohol to the corresponding aldehyde, reaction with an amine will form the hemiaminal, which is subsequently converted to an amide linkage by a second catalytic cycle of dehydrogenation. One interesting feature of the Milstein catalyst is the selectivity for primary amines in presence of an unprotected secondary amine.\textsuperscript{16} This offers an exciting opportunity for direct synthesis of functional polyamides containing secondary amino groups circumventing tedious protection and deprotection steps.\textsuperscript{24} To test this selectivity in polyamidation, diethylene triamine (2i), and naturally occurring spermidine (2j) and spermine (2k) were directly used in polyamidation without any protection. Based on \textsuperscript{1}H NMR analysis, the secondary amines in polymers 3o-3q remain intact during the direct polyamidation (Figure 6.1-6.5 in the SI). With natural polyamines and biocompatible OEGs linked by degradable amide bond, these cationic polymers are promising candidates for biomedical applications. In the same manner, piperazine-diethanol (1e) could also react with polyamines, giving a linear polyamide with high cationic charge density (entry 17).
### Table 6.2 Catalytic dehydrogenative polyamidation

<table>
<thead>
<tr>
<th>Entry</th>
<th>Diol</th>
<th>Diamine</th>
<th>Conversion° (yield%)</th>
<th>$M_n$ (10^3)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1°</td>
<td>1a</td>
<td>2c</td>
<td>99 (89)</td>
<td>11.9°</td>
<td>3.09</td>
</tr>
<tr>
<td>2°</td>
<td>1b</td>
<td>2c</td>
<td>&gt;99 (87)</td>
<td>12.7°</td>
<td>2.80</td>
</tr>
<tr>
<td>3°</td>
<td>1b</td>
<td>2d</td>
<td>&gt;99 (84)</td>
<td>19.8</td>
<td>1.86</td>
</tr>
<tr>
<td>4°</td>
<td>1b</td>
<td>2e</td>
<td>&gt;99 (88)</td>
<td>28.4</td>
<td>1.75</td>
</tr>
<tr>
<td>5°</td>
<td>1b</td>
<td>2f</td>
<td>98 (77)</td>
<td>13.6°</td>
<td>-</td>
</tr>
<tr>
<td>6°</td>
<td>1a</td>
<td>2g</td>
<td>&gt;99 (73)</td>
<td>19.4</td>
<td>1.59</td>
</tr>
<tr>
<td>7°</td>
<td>1b</td>
<td>2g</td>
<td>&gt;99 (78)</td>
<td>22.1</td>
<td>1.56</td>
</tr>
<tr>
<td>8°</td>
<td>b</td>
<td>2h</td>
<td>&gt;99 (71)</td>
<td>21.2°</td>
<td>2.12</td>
</tr>
<tr>
<td>9°</td>
<td>1c</td>
<td>2g</td>
<td>99 (85)</td>
<td>15.0</td>
<td>1.59</td>
</tr>
<tr>
<td>10°</td>
<td>1d</td>
<td>2g</td>
<td>&gt;99 (79)</td>
<td>16.5</td>
<td>1.69</td>
</tr>
<tr>
<td>11°</td>
<td>1e</td>
<td>2g</td>
<td>&gt;99 (76)</td>
<td>19.5</td>
<td>1.65</td>
</tr>
<tr>
<td>12°/</td>
<td>1f</td>
<td>1c (1:1)</td>
<td>&gt;99 (88)</td>
<td>24.2</td>
<td>1.65</td>
</tr>
<tr>
<td>13°/</td>
<td>1g</td>
<td>1c (1:1)</td>
<td>&gt;99 (90)</td>
<td>21.5</td>
<td>1.63</td>
</tr>
<tr>
<td>14°/</td>
<td>1a</td>
<td>2i</td>
<td>99 (91)</td>
<td>8.2°</td>
<td>2.89</td>
</tr>
<tr>
<td>15°</td>
<td>1b</td>
<td>2j</td>
<td>97 (65)</td>
<td>6.8°</td>
<td>2.56</td>
</tr>
<tr>
<td>16°</td>
<td>1b</td>
<td>2k</td>
<td>99 (70)</td>
<td>9.6°</td>
<td>1.65</td>
</tr>
<tr>
<td>17°</td>
<td>1e</td>
<td>2l</td>
<td>99 (72)</td>
<td>11.3°</td>
<td>3.06</td>
</tr>
</tbody>
</table>

° Reaction conditions: 1.0 mmol diol, 1.0 mmol diamine and 0.01 mmol catalyst are premixed in 1.5 mL solvent in glovebox, then heated under N2 flow for 48 h. Determined by 1HNMR of the crude reaction mixture. Isolated yield after precipitation in toluene. In anisole/DMSO (4:1), 2 mol% catalyst loading. In anisole, 1 mol% catalyst loading. Bi-modal GPC distribution. Not soluble in GPC solvent, calculated from 1HNMR. Molecular weight measured by Multi-Angle Laser Light Scattering (MALLS). GPC taken after acylation by Ac2O to avoid strong interaction with the GPC column gel material. Reaction carried out at 2.5 mmol scale.
6.3 Conclusion

In conclusion, we report here the first successful direct polyamidation by catalytic dehydrogenation of diols and diamines. This method avoids the requirement of stoichiometric pre-activation or in situ activation reagents required for conventional polycondensation method and provides a much cleaner process with high atomic economy. The high catalytic selectivity of this method also offers the opportunity of efficient synthesis of functional polyamides. Given the broad importance of functional polyamides, this atom economic polyamidation may find widespread utility in many technological and biomedical applications. Our current focus is on investigation of different dehydrogenation catalytic systems for further broadening the scope of this method as well as on application of this method to the synthesis of highly functional polyamides as new biomaterials.

References


6.4 Supporting information

6.4.1 General information.

All reagents were used as received from commercial suppliers without further purification unless otherwise specified. Milstein catalyst was purchased from STREM and other reagents were purchased from Sigma-Aldrich and Alfa-Aesar. Toluene and DMF were purified through alumina filtration system, other solvents were in HPLC grade and used as received without further purification. All reactions were set up in a Vacuum Atmosphere glove box charged with nitrogen and carried out in flame-dried glassware under constant N$_2$ flow. $^1$H NMR spectra were recorded at 500 MHz and $^{13}$C NMR spectra were recorded at 125 on Bruker instruments. $^1$H NMR chemical shifts were reported as values in ppm relative to deuterated solvents DMSO-$d_6$ (2.50). Data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet) and integration. Multiplets were reported over the range (in ppm) it appeared. $^{13}$C NMR data were recorded relative to the solvent signals of DMSO-$d_6$ (39.51). Mass spectrometry data (MS/ESI) were acquired with a Micromass autospec mass spectrometer to determine the molecular weight of small molecules. Gel permeation chromatography (GPC) was performed on an Agilent 1100 SEC system using a OHpak SB-803 HQ column from Shodex to determine molecular weights and molecular weight distributions, $M_w/M_n$, of linear polymer samples with respect to poly(ethylene glycol) (PEG) standards purchased from Aldrich. DMF with 0.1% LiBr (wt/v) was used as the eluent at a flow rate of 1.0 mL/min with column temperature at 45°C. Molecular weight and molecular weight distribution of branched polymer is determined by static light scattering method: $dn/dc$ of the sample is
measured by plotting RI detector area vs. sample concentration at fixed injection volume and comparing the slope with PEG standards (dn/dc = 0.135) in the same aforementioned GPC system. Light scattering data was acquired from a Dawn DSP 18-angle laser light scattering detector (MALLS, laser wavelength $\lambda = 632$ nm, Wyatt Technology, Santa Babara, CA) coupled to the GPC system.
6.4.2 Solvent screening of Milstein catalyst for direct amidation

Two sample reactions were used for screening the solvent effect on the catalyst (Table S6.1). For low boiling point solvents (entries 1-7), the reaction of 1,2-ethylenediamine and 2-methoxyethanol was used. For high boiling point solvent (entries 8-13), substrates with higher boiling point, benzylamine and 1,12-dodecanediol, were used to avoid the loss of substrate during heating. Among all solvents screened, anisole gives the highest reaction activity. Furthermore, addition of a small amount of DMSO in anisole could increase the polarity of the solvent without significantly decreasing the catalyst activity.

Table 6.3 Solvent screening for direct amidation using the Milstein catalyst

<table>
<thead>
<tr>
<th>Entry</th>
<th>Solvent</th>
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<td>DMSO</td>
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<tr>
<td>6</td>
<td>t-Butanol</td>
<td>15% conv. c</td>
<td>13</td>
<td>Anisole/DMSO (3:1)</td>
<td>96% conv. d</td>
</tr>
</tbody>
</table>

* Reaction conditions: 2.5 mmol dodecane-1,12-diol, 5 mmol benzylamine and 0.01 mmol Milstein catalyst in 2-3 mL solvent. *b* Oligo(ethylene ammonium) chloride salt was formed. *c* NMR conversion based on alcohol. *d* NMR conversion based on benzyl amine.
6.4.3 Effect of spacer between two hydroxyl groups of diols on direct amidation

In Milstein’s original paper (Science 2007, 317, 790), only the reactions of mono-alcohols have been reported. This prompted us to test the reactivity of diols by running model reactions between benzyl amine and alkyl diols with different spacer lengths (Scheme S6.1). The results show that a minimum spacer of six carbons between the two hydroxyl groups is necessary for an efficient conversion. It is worth noting that this effect is only for free hydroxyl groups, because oxygen atoms in oligo(ethylene glycol) used in later studies do not affect the catalytic activity.

Scheme 6.2 Model reaction for catalytic amidation of diols with benzylamine
6.4.4 Procedure for the catalytic dehydrogenative amidation of diols and benzylamine: The Milstein Ru catalyst (4.5 mg, 0.010 mmol), a diol (2.523 mmol), benzylamine (540.7 mg, 5.046 mmol), and toluene (3.0 mL) were mixed in a 25 mL round-bottom flask under an atmosphere of purified nitrogen in a glove box. The flask was equipped with a condenser and the solution was heated to reflux for 24 h under equilibrium with constant nitrogen flow. At the end of the reaction, an aliquot of the reaction solution was withdrawn for NMR analysis of the reaction conversion. The rest of the reaction mixture was allowed to stand at room temperature and the bis-amides crystallized from the solution upon cooling. The liquid was removed by decantation and the solids were washed with toluene. The resulting bis-amides were dried under vacuum overnight and isolated yield was calculated.

\[
\text{N}^4,\text{N}^6\text{-dibenzylhexanediamide}: \text{Colorless solid, 94\% isolated yield, } \text{H NMR (DMSO-}d_6\text{): } \delta 8.26 (t, 2H, J = 5.9 \text{ Hz}), 7.30-7.18 (m, 10H), 4.23 (d, 4H, J = 5.9 \text{ Hz}), 2.26 (m, 4H), 1.51 (m, 4H). \]

\[\text{C NMR (DMSO-}d_6\text{): } \delta 171.7, 139.5, 127.9, 126.9, 126.4, 41.9, 35.0, 24.9. \text{ MS (ESI), } m/z \text{ calcd for (C}_{22}\text{H}_{28}\text{N}_{2}\text{O}_{2} + \text{H}}^+: 325.2; \text{ found: 325.2.} \]

\[
\text{N}^4,\text{N}^8\text{-dibenzyloctanediamide: Colorless solid, 92\% isolated yield, } \text{H NMR (DMSO-}d_6\text{): } \delta 7.96 (bs, 2H), 7.32-7.21 (m, 10H), 4.29 (d, 4H, J = 5.8 \text{ Hz}), 2.16 (m, 4H), 1.56 (m, 4H), 1.31(m, 4H). \]
$^{13}$C NMR (DMSO-$d_6$): $\delta$ 171.8, 139.5, 127.9, 126.9, 126.4, 41.8, 35.2, 28.2, 25.0. HRMS (ESI), $m/z$ calcd for (C$_{22}$H$_{28}$N$_2$O$_2$ + Na)$^+$ : 375.2048; found: 375.2049.

$N^1,N^{10}$-dibenzyldodecanediamide: Colorless solid, 96% isolated yield, $^1$H NMR (DMSO-$d_6$): $\delta$ 8.09 (bs, 2H), 7.32-7.21 (m, 10H), 4.26 (d, 4H, $J$ = 5.8 Hz), 2.13 (t, 4H, $J$ = 7.4 Hz), 1.55 (m, 4H), 1.25 (m, 12H). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 171.8, 139.5, 127.9, 126.9, 126.4, 41.8, 35.2, 28.6, 28.5, 28.4, 25.0. HRMS (ESI), $m/z$ calcd for (C$_{26}$H$_{36}$N$_2$O$_2$ + Na)$^+$ : 431.2675; found: 431.2661.
6.4.5 Procedure for the catalytic polymerization of diamines with diols: The Milstein catalyst (4.5 mg, 0.010 mmol), a diamine (1.0 mmol), a diol (1.0 mmol), and anisole/DMSO (6:1) (1.5 mL) were mixed in a 10 mL round-bottom flask under an atmosphere of purified nitrogen in a glove box. The flask was equipped with a condenser and the solution was heated at 120 °C for 48 h under equilibrium with constant nitrogen flow. Anisole was removed in vacuo and the resulted brown solid was dissolved in hot DMF. Upon complete dissolution, an aliquot of the crude reaction mixture were taken for NMR characterizations after evaporating the solvent. The polymers were precipitated by pouring the DMF solution into an excess amount of toluene. The polymer solids were isolated by centrifugation, dried under vacuum overnight, and weighed for yield calculation. A portion of the solids were redissolved in DMF (0.1% LiBr) and subjected to GPC characterization. The polymer yields are reported in the Table 1 & 2 in the main text, and their ¹H NMR and GPC characterization data are summarized below.

![Poly(m-xylylene triglycolylamide) 3a](image)

**Poly(m-xylylene triglycolylamide) 3a:** ¹H NMR (DMSO-d₆): δ 8.27 (t, 2H), 7.11-7.22 (m, 4H), 4.25 (d, 4H), 3.93 (s, 1H), 3.64 (s, 1H). GPC (0.1% LiBr in DMF): $M_n = 22.6\times10^3$ g/mol, $M_w = 34.1\times10^3$ g/mol.
Poly(p-xylylene triglycolylamide) 3b: $^1$H NMR (DMSO-$d_6$): $\delta$ 8.23 (t, 2H), 7.17 (s, 4H), 4.25 (d, 4H), 3.92 (s, 4H), 3.62 (s, 4H). GPC (0.1% LiBr in DMF): $M_n = 11.9 \times 10^3$ g/mol, $M_w = 36.8 \times 10^3$ g/mol; bi-modal distribution: $M_{p1} = 67.1 \times 10^3$ g/mol, $M_{p2} = 9.3 \times 10^3$ g/mol ($M_p$ stands for peak molecular weight).

Poly(p-xylylene tetruglycolylamide) 3c: $^1$H NMR (DMSO-$d_6$): $\delta$ 8.17 (t, 2H), 7.18 (s, 4H), 4.26 (d, 4H), 3.93 (s, 4H), 3.58 (m, 8H). GPC (0.1% LiBr in DMF): $M_n = 12.7 \times 10^3$ g/mol, $M_w = 35.6 \times 10^3$ g/mol; bi-modal distribution: $M_{p1} = 66.4 \times 10^3$ g/mol, $M_{p2} = 13.5 \times 10^3$ g/mol.

Poly(trimethylene tetruglycolylamide) 3d: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.75 (t, 2H), 3.87 (s, 4H), 3.63 (s, 8H), 3.11 (q, 4H), 1.55 (quint, 2H). GPC (0.1% LiBr in DMF): $M_n = 19.8 \times 10^3$ g/mol, $M_w = 36.8 \times 10^3$ g/mol.

Poly(dodecamethylene tetruglycolylamide) 3e: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.22 (br, 2H), 3.87 (s, 4H), 3.63 (s, 8H), 3.14 (q, 4H), 1.28-1.47 (m, 20H). GPC (0.1% LiBr in DMF): $M_n = 28.4 \times 10^3$ g/mol, $M_w = 49.7 \times 10^3$ g/mol.
Poly(1,4-trans-cyclohexyl triglycolylamide) 3f: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.10 (m, 2H), 3.86 (s, 4H), 3.60 (m, 10H), 1.83 (d, 4H), 1.35 (m, 4H). Conversion = 98%, $M_n = 13.6 \times 10^3$ g/mol.

Poly(3,6-dioxo-1,8-octylene triglycolylamide) 3g: $^1$H NMR (DMSO-$d_6$): two sets of peaks because of different conformations at r.t. set 1: $\delta$ 7.68 (t, 2H), 3.90 (s, 4H), 3.63 (s, 4H), 3.54 (s, 4H), 3.45 (t, 4H), 3.33 (q, 4H), set 2: 7.23 (t, 2H), 3.89 (s, 4H), 3.62 (s, 4H), 3.53 (s, 4H), 3.43 (t, 4H), 3.26 (q, 4H). GPC (0.1% LiBr in DMF): $M_n = 19.0 \times 10^3$ g/mol, $M_w = 29.3 \times 10^3$ g/mol.

Poly(3,6-dioxo-1,8-octylene tetraglycolylamide) 3h: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.64 (t, 2H), 3.89 (s, 4H), 3.59 (b, 8H), 3.50 (s, 4H), 3.43 (t, 4H), 3.27 (q, 4H). GPC (0.1% LiBr in DMF): $M_n = 22.1 \times 10^3$ g/mol, $M_w = 34.5 \times 10^3$ g/mol.

Poly(3,6-dioxo-1,8-octylene $p$-diphenoxymethyleneamide) 3j: $^1$H NMR (DMSO-$d_6$): $\delta$ 8.02 (t, 2H), 6.92 (s, 4H), 4.43 (s, 4H), 3.48 (b, 4H), 3.43 (t, 4H), 3.28 (q, 4H). GPC (0.1% LiBr in DMF): $M_n = 15.0 \times 10^3$ g/mol, $M_w = 23.8 \times 10^3$ g/mol.
Poly(3,6-dioxa-1,8-octylene-co-triethylamino tetracyglycolylamide) 3i: $^1$H NMR (DMSO-$d_6$): δ 7.64 (t), 7.59 (t), 7.50 (t), 3.88 (s), 3.58 (br), 3.50 (s), 3.47 (t), 3.43 (t), 3.27 (q), 3.13-3.20 (m), 2.56-2.52 (m). Molar composition: tris(2-aminoethyl)amine 20%, 3,6-dioxa-1,8-octylenediamine 80%. MALLS (0.1% LiBr in DMF): $dn/dc = 0.176$ mL/g; $M_n = 21.2 \times 10^3$ g/mol, $M_w = 44.9 \times 10^3$ g/mol.

Poly(3,6-dioxa-1,8-octylene propane-2,2-diyl-bis($p$-phenoxlmethylene)amide) 3k: $^1$H NMR (DMSO-$d_6$): δ 8.02 (t, 2H), 6.92 (s, 4H), 4.43 (s, 4H), 3.48 (b, 4H), 3.43 (t, 4H), 3.28 (q, 4H). GPC (0.1% LiBr in DMF): $M_n = 16.5 \times 10^3$ g/mol, $M_w = 27.9 \times 10^3$ g/mol.
Poly(3,6-dioxa-1,8-octylene N,N-methylenepiperazineamide) 3l: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.65 (t, 2H), 3.50 (s, 4H) 3.43 (t, 4H), 3.25 (q, 4H), 2.90 (s, 4H), 2.45 (br, 8H). GPC (0.1% LiBr in DMF): $M_n = 19.5 \times 10^3$ g/mol, $M_w = 32.2 \times 10^3$ g/mol.

Poly(3,6-dioxa-1,8-octylene tetraglycolamide)-co-poly(3,6-dioxa-1,8-octylene hexamethyleneamide) 3m: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.50-7.81 (m, 4H), 3.91 (s, 4H), 3.58 (s, 8H), 3.49 (s, 8H), 3.44 (t, 4H), 3.38 (t, 4H), 3.27 (q, 4H), 3.18 (q, 4H), 2.05 (br, 4H), 1.44 (br, 4H). Percent composition: 51% tetra(ethylene)glycol, 49% hexanediol. GPC (0.1% LiBr in DMF): $M_n = 24.2 \times 10^3$ g/mol, $M_w = 39.9 \times 10^3$ g/mol.

Poly(3,6-dioxa-1,8-octylene tetraglycolamide)-co-poly(3,6-dioxa-1,8-octylene hexadecamethyleneamide) 3n: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.50-7.81 (m, 4H), 3.90 (s, 4H), 3.58 (m, 8H), 3.49 (m, 8H), 3.44 (t, 4H), 3.38 (t, 4H), 3.27 (q, 4H), 3.18 (q, 4H), 2.09 (t, 4H), 1.53 (t, 4H), 1.30 (br, 20H). Percent composition: 52% tetra(ethylene) glycol, 48% hexanediol. GPC (0.1% LiBr in DMF): $M_n = 21.5 \times 10^3$ g/mol, $M_w = 35.0 \times 10^3$ g/mol.
Poly(2-aminodiethylene triglycolylamide) 3o: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.69-7.46 (t, 2H), 3.89 (s, 4H), 3.64-3.63 (s, 4H), 3.16 (q, 4H), 2.64-2.50 (t, 4H). GPC (0.1% LiBr in DMF): $M_n = 8.2 \times 10^3$ g/mol, $M_w = 23.7 \times 10^3$ g/mol; bi-modal distribution: $M_{p1} = 62.0 \times 10^3$ g/mol, $M_{p2} = 14.3 \times 10^3$ g/mol.

Poly(3-aminooctamethylene triglycolylamide) 3p: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.69-7.46 (t, 2H), 3.85 (s, 4H), 3.60 (s, 8H), 3.16-3.07 (m, 4H), 2.50-2.43 (m, 4H), 1.57-1.34 (m, 6H). Due to interactions of the polymer with the GPC column, the secondary amino group of this polymer was acetylated for GPC analysis (see next section).

Poly(3,8-diaminododecamethylene tetrarglycolylamide) 3q: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.69-7.46 (t, 2H), 3.85 (s, 4H), 3.59 (s, 8H), 3.18 (q, 4H), 2.54-2.43 (m, 8H), 1.56-1.34 (m, 8H). Due to interactions of the polymer with the GPC column, the secondary amino group of this polymer was acetylated for GPC analysis (see next section).
Poly(2-aminodiethylene triglycolylamide) 3r: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.64 (t, 2H), 3.14 (q, 4H), 2.90 (s, 4H), 2.54 (t, 4H), 2.36 (br, 8H). Due to interactions of the polymer with the GPC column, the secondary amino group of this polymer was acetylated for GPC analysis (see next section).

6.4.6 Procedure for the acetylation of secondary amine: To avoid interaction of secondary amine with the GPC column, polymer 3k and 3l were acetylated after polyamidation. After the polymerization, the reaction mixture was dissolved in 5 mL hot DMF. Half of the resulting solution was taken into a 15 mL round bottom flask into which 0.5 mL acetic anhydride (~10 equiv) was added. After being heated at 60 °C overnight, all volatiles were removed under vacuum. The remaining solid was dissolved in DMF and then subjected to GPC and NMR for characterization.

Acetylated 3p: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.69-7.46 (t, 2H), 3.88 (m, 4H), 3.60 (m, 8H), 3.21-3.05 (m, 8H), 1.96 (m, 3H), 1.69-1.14 (m, 6H). GPC (0.1\% LiBr in DMF): $M_n = 6.8 \times 10^3$ g/mol, $M_w = 17.5 \times 10^3$ g/mol; bimodal distribution: $M_p^1 = 5.2 \times 10^3$ g/mol, $M_p^2 = 57.7 \times 10^3$ g/mol.
Acetylated 3q: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.69-7.46 (t, 2H), 3.88 (m, 4H), 3.58 (m, 8H), 3.22-3.05 (m, 12H), 1.96 (m, 6H), 1.69-1.14 (m, 8H). GPC (0.1% LiBr in DMF): $M_n = 9.6 \times 10^3$ g/mol, $M_w = 15.8 \times 10^3$ g/mol.

Poly(2-aminodiethylene triglycolylamide) 3o: $^1$H NMR (DMSO-$d_6$): $\delta$ 7.64 (t, 2H), 3.32 (m, 4H), 3.20-3.25 (m, 4H), 2.90 (s, 4H), 2.43 (br, 8H), 1.99 (s, 3H). GPC (0.1% LiBr in DMF): $M_n = 11.3 \times 10^3$ g/mol, $M_w = 34.5 \times 10^3$ g/mol; bi-modal distribution: $M_p^1 = 62.2 \times 10^3$ g/mol, $M_p^2 = 7.6 \times 10^3$ g/mol.
6.4.7 $^1$H NMR confirmation of selectivity of primary amine over secondary amine for polyamidation

The Milstein catalyst’s selectivity of primary amine over secondary amine for polyamidation was confirmed by $^1$H NMR of the synthesized polyamides 3o, 3p and 3q (See Part 8 for the NMR spectra). In polymer 3o (Fig. 6.1), the integration of the protons on the adjacent carbons of the amide (-CH$_2$-NH-CO-) is 3.95, and that of the secondary amine (-CH$_2$-NH-CH$_2$-) is 3.99. The ratio of the two integrations agrees with the theoretical value of 1:1 within experimental error.

In polyamide 3p and 3q (Fig. 6.2 & 6.3), the ratio of integrations of (-CH$_2$-NH-CO-) and (-CH$_2$-NH-CH$_2$-) also matches the expected structure. Furthermore, the free secondary amines in polyamide 3p and 3q were acetylated by acetic anhydride. The $^1$H NMR spectra of the acetylated polymers (Fig. 6.4 & 6.5) confirm that an exactly stoichiometric amount of acetyl groups (one acetyl group/repeat unit in 3p and two in 3q) was incorporated into the polymers, unambiguously proving that the secondary amino groups remain intact during the catalytic polyamidation process.

It should be noted that the $^1$H NMR spectra of polyamides 3o-q display some complicated patterns and multiplets are observed for many resonances. Presumably, this may result from a diverse array of different polymer conformations associated with different hydrogen-bonding interactions.
Figure 6.1 $^1$H NMR spectrum of polymer 3o
Figure 6.2 $^1$H NMR spectrum of polymer $3p$
Figure 6.3 $^1$H NMR spectrum of polymer 3q
Figure 6.4: $^1$H NMR spectrum of acetylated polymer 3p
Figure S6.5 $^1$H NMR spectrum of acetylated polymer 3q
6.4.8 NMR Spectrum of polyamides (All NMR data are recorded from crude reaction mixture after evaporating solvent.)
6.4.9 Representative GPC trace of polyamides
6.4.10 Molecular Weight measurement of branched polyamides 3i

Molecular weight and molecular weight distribution of branched polymer 3i is determined by static light scattering method. dn/dc of the sample is measured by plotting RI detector area vs. sample concentration at fixed injection volume and comparing the slope with PEG standards ($M_n = 12140$, $dn/dc = 0.135$) in the same aforementioned GPC system (Figure 6.6). Light scattering data was acquired from a Dawn DSP 18-angle laser light scattering detector (MALLS, laser wavelength $\lambda = 632$ nm, Wyatt Technology, Santa Babara, CA) coupled to the GPC system (Figure 6.7). The molecular weight and molecular weight distribution is calculated from software ASTRA 4.7 from Wyatt Technology using Zimm plotting treatment.

![Figure 6.6](image1.png)  
**Figure 6.6** dn/dc of 3i and PEG standard  

![Figure 6.7](image2.png)  
**Figure 6.7** MALLS and RI signal of 3i