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DIRECT MEASUREMENT OF RUPTURE FORCE OF SINGLE TRIAZOLE MOLECULE BY ATOMIC FORCE MICROSCOPE AND SOLID PHASE SYNTHESIS OF MONODISPERSE POLYETHYLENE GLYCOLS

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DIRECT MEASUREMENT OF RUPTURE FORCE OF SINGLE TRIAZOLE
MOLECULE BY ATOMIC FORCE MICROSCOPE
AND
SOLID PHASE SYNTHESIS OF MONODISPERSE POLYETHYLENE GLYCOLS

By
Ashok Khanal

A DISSERTATION
Submitted in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY
In Chemistry

MICHIGAN TECHNOLOGICAL UNIVERSITY

2018

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This dissertation has been approved in partial fulfillment of the requirements for the Degree of DOCTOR OF PHILOSOPHY in Chemistry.

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Preface

The initial ideas of all research projects in this dissertation involves study of rupture force measurement of single triazole molecule and the solid phase synthesis of monodispersed PEGs, were conceived by Dr. Shiyue Fang. The research described in this dissertation was conducted under his supervision.

All writing of this dissertation (Chapter 1, Chapter 2 and Chapter 3) is carried out by Mr. Ashok Khanal and revised by Dr. Shiyue Fang.

All research work in Chapter 2 was conducted and written by Mr. Ashok Khanal, with the exceptions of (1) collecting the AFM data, which was performed by Dr. Fei Long under the mentoring of Dr. Shiyue Fang and Dr. Reza Shahbazian Yassar; (2) the portions of early chemistry studies utilized in this project was assisted by Dr. Bin Cao. The content in Chapter 2 was previously published in *Chem. Eur. J.* **2016**, 22, 9760-9767.

All experiments in Chapter 3 were conducted by Mr. Ashok Khanal. The content in Chapter 3 was previously published in *Chem. Eur. J.* **2017**, 23, 15133-15142.

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List of Abbreviations

AAC	alkyne-azide cycloaddition
Ac	acetyl
AFM	atomic force microscope
APTES	3-aminopropyltriethoxysilane
AuNP	gold nanoparticle
Bn	benzyl
CuAAC	copper catalyzed alkyne-azide cycloaddition
DMF	<i>N,N</i> -dimethylformamide
DMTr	4,4'-dimethoxytrityl
DNA	deoxyribonucleic acid
DVP	divinylbenzene
EDCI	1-ethyl-3-(3- dimethylaminopropyl)carbodiimide
Eq	equivalent
ESI	electrospray ionization
Et	ethyl

FDA	Food and Drug Administration
Fmoc	9-fluorenylmethoxycarbonyl
g	gram
h	hour(s)
HOMO	highest occupied molecular orbital
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectroscopy
Hz	hertz
<i>i</i> Pr	isopropyl
<i>J</i>	coupling constant
K	kilo
Da	dalton
LRMS	low resolution mass spectroscopy
LUMO	lowest un-occupied molecular orbital
M	molar
MALDI	matrix-assisted laser desorption/ionization
Me	methyl

mg	milligram
MHz	megahertz
mL	millilitre
mM	milimolar
mmol	millimole
MS	mass spectroscopy
<i>n</i> Bu	butyl
NHS	<i>N</i> -hydroxysuccinimide
nm	nanometer
NMR	nuclear magnetic resonance
nN	nanonewton
ODN	oligodeoxynucleotide
OEG	oligoethyleneglycol
PEG	polyethylene glycol(s)
Ph	phenyl
pN	piconewton
PNA	peptide nucleic acid
ppm	part per million

RNA	ribonucleic acid
<i>t</i> Bu	<i>tert</i> -butyl
THF	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TOF	time of flight
Ts	tosyl
μL	microliter
μmol	micromol

Abstract

This dissertation includes two topics, which are related to each other: (1) Rupture force measurement of single triazole molecule by atomic force microscope (AFM) and (2) Solid phase stepwise synthesis of polyethylene glycols (PEGs).

The reaction of an alkyne and an azide to form a 1,2,3- triazole has found wide applications. However, the stability of triazole under mechanical stress is unclear. We investigated the cycloreversion at the single molecular level using atomic force microscopy. A mica surface functionalized with a molecule containing a 1,2,3-triazole moiety in the middle and an activated ester at the end was prepared. An AFM tip with amino group was ramped over the surface, which temporarily linked the tip to the surface through amide bond formation. During retraction, the triazole broke and the forces were recorded. Most of the forces were below 860 pN. The resulting alkyne were labeled with gold nanoparticles (AuNPs). AFM imaging revealed AuNPs at the predefined locations, proving that 1,2,3-triazole could rupture with force below 860 pN.

We used commercially available functionalized polyethylene glycol (PEGs) for functionalizing of mica surface and AFM tip. These PEGs were polydispersed admixtures with different size of tags and not ideal for many applications. There is a need to develop a new method to synthesize a monodispersed PEGs. We believe that if monodisperse PEGs were used, our data would be much better. In addition, monodispersed PEGs can form single molecular conjugates during chemical reactions

which allow precise characterization of all PEGs conjugates. This lead us to conceive an idea to prepare monodisperse PEGs.

Significant efforts have been made in the area, to achieve monodisperse PEGs using solution phase but the drawbacks include the need of multiple column chromatography, low overall yield, and contamination by depolymerized products. To overcome all shortcomings, we have developed a new technology to synthesize monodispersed PEGs on solid support. The Wang resin, which contains the 4-benzyloxy benzyl alcohol function, was used as the support. On this solid support, tetraethylene glycol monomer was added stepwise. Polyethylene glycol (PEG) with eight and twelve glycol units and its derivatives were synthesized. The synthetic cycle consists of deprotonation, Williamson ether formation (coupling), and detritylation. Cleavage of the PEG from solid support was achieved with trifluoroacetic acid. From this method, we prepared highly pure products having different functionalities at the two ends without any chromatographic purification in the entire synthesis.

Chapter 1

Introduction

This dissertation is based on two peer reviewed articles. This chapter includes an introduction of all subsequent chapters that are included in this dissertation. It consists of two parts. The first part describes the direct measurement of rupture force of single triazole molecule by Atomic Force Microscope (AFM). Because the alkyne-azide cycloaddition (AAC) reaction is widely used, and how stable the 1,2,3-triazole product is under mechanical stress could be an important consideration for some applications, we were interested in directly measuring the force for the rupture of a single 1,2,3-triazole molecule using AFM and gaining strong evidence that the rupture products are an alkyne and an azide.

The second part describes the possibility of using solid phase stepwise synthesis to prepare monodisperse PEGs. This is to address the problems faced by using polydispersed PEGs such as inconsistent composition, difficult characterization and loss of biological activities. People are using solution phase to synthesize monodispersed PEGs. Drawbacks of this method include the need of multiple column chromatography to purify intermediate PEGs and the final PEG product, low overall yield, contamination by depolymerized products due to the harsh conditions needed for the key ether formation. Unlike solution phase, solid phase synthesis is simple, less expensive and all chromatography purifications can be avoided. In addition, solid phase synthesis allows for the possibility for automation.

1.1 Measurement of Rupture Force of Single Triazole Molecule

1.1.1 Atomic Force Microscope

Atomic Force Microscope is a very powerful analytical tool and has been applied to wide range of scientific and technological fields like materials science, chemistry, physics, nanotechnology, nanoscience, biology, biophysics and biochemistry.

The key components of AFM are shown in Figure 1.1. It has a cantilever, as shown in Figure 1.1 (a), fixed at one end which possess a sharp tip near its free end. It is the main sensing part of the instrument. Silicon and the silicon nitride are the most common materials for the preparation of the cantilever and its geometry is either rectangular or triangular. The cantilever can be moved across the surface to scan the sample. A piezo-electric crystal, as shown in Figure 1.1 (b), is used for bending and movement of the cantilever. The expansion and the contraction of the piezo-electric crystal is proportional with applied voltages. The laser is used to detect the deflection of the cantilever, as shown in Figure 1.1 (c). When the cantilever approaches the surface, the attractive force between the tip and the surface makes deflection of it. The deflection of the cantilever towards or away from the surface is monitored by a laser beam. All cantilever deflections can cause slight changes in the direction of reflected beam. The laser is reflected from the top of the cantilever onto a mirror, as shown in Figure 1.1 (d), and then into a photodiode detector as in Figure 1.1 (e). A position sensitive photodiode is used to track the changes of reflected beam. The topography of the sample surface influences the deflection of the cantilever, and is monitored by the instrument. Direct

force measurement between AFM tip and sample surface is the function of the gap between tip and the surface.

Figure 1. 1. A schematic representation of the AFM instrument. (a) Cantilever; (b) Piezo-electric crystal; (c) Laser; (d) Mirror; (e) Position sensitive photodiode detector; (f) Data processor; (g) Image; (h) Scanner.

1.1.2 Force Curves

AFM has been used to measure surface interactions by means of force distance curves since 1989.¹ AFM is widely used for its high resolution imaging capabilities, but

it is also a powerful tool to calculate the bond rupture forces between the AFM tip and the surface. It is a huge milestone to relate theoretical and experimental calculations. When the tip approaches the surface, initially the interactions are too small to measure and the tip remains in its undisturbed position. If the tip is kept going towards the surface, at some point the attractive forces between the tip and the sample surface overcome and the tip jumps into contact with the surface (Figure 1.2). In these cases the attractive forces are usually generated from Van der Waals and capillary forces.

Figure 1.2. AFM tip pulled towards the sample surface

As the separation between the tip and the sample is decreased further, there is experienced a strong repulsive force between the tip and sample atoms. The tip and sample are considered to be in contact, when the repulsive force is predominate. This repulsive force causes the cantilever to bend. Which is shown in the Figure1.3.

Figure 1.3. AFM tip is in contact with sample surface

Finally, when the tip approaches away from the surface a large distance separates the tip and the sample. There is no interaction between them and again the tip remains undisturbed position (Figure 1.4).

Figure 1.4. AFM tip far from the sample surface

So, the atomic force microscope can measure and record the different forces encountered during the approach, contact and retraction of the cantilever from a surface. The data is displayed as a X-Y curve. The X axis represents the distance for the approach and retract of the tip. Similarly the Y axis represents the deflections of the tip. These deflections are recorded and converted to force. This force-versus-distance curve, is simply known as “force curves” (Figure 1.5).²

Figure 1.5. Schematic representation of force distance curve

I, as shown in Figure 1.5, represents the cantilever is not in contact with or far from the surface, as a result, no force is measured there. **II**, as shown in Figure 1.5, corresponds that the cantilever is in contact with the sample surface because of attractive force. **III**, as shown in Figure 1.5, represents the cantilever is in hard contact with the

sample surface that makes tip starts to bend because of repulsive force. **IV**, as shown in Figure 1.5, represents the cantilever is withdrawn upwards from the surface. In our experiment we broke the 1,2,3-triazole moiety in to an alkyne and an azide during this step.

1.1.3 1,3-Dipolar Cycloaddition

1,3-Dipolar cycloaddition is the concerted reaction between a 1,3-dipole and a dipolarophile. 1,3-Dipole is a dipolar compound having positive and negative charge, which is stabilized by resonance within three atoms. Dipolarophile is generally unsaturated compound that can undergo 1,3-dipolar cycloaddition. Triazole (**1.3**) is a five membered ring heterocyclic compound. 1,2,3- triazole was first synthesized by Rolf Huisgen using an azide (**1.1**) and an alkyne (**1.2**) via 1,3-dipolar cycloaddition reaction,³ as shown in Scheme 1.1.

Scheme 1.1. Formation of 1,2,3-triazole from 1,3-dipolar cycloaddition

The reaction was commonly known as the alkyne-azide cycloaddition (AAC). Later on K. Barry Sharpless and Morten Meldel developed the copper catalyzed version of alkyne-azide cycloaddition which is known as copper catalyzed alkyne-azide

cycloaddition (CuAAC).⁴ The reaction is compatible with polar functional groups and polar solvents including aqueous buffer, and is completely orthogonal to functionalities naturally occurring in biomolecules. It has good kinetics under mild conditions, and its product is highly thermally and photochemically stable⁵. Due to these characteristics, the reaction is given the name click reaction. In most cases, a terminal alkyne is used, and the reaction needs copper (I) for catalysis. To avoid copper, strained alkynes have been employed, and copper-free AAC reactions have emerged^{6,11}. The cycloaddition reaction of an alkyne and an azide (AAC) to form a 1,2,3-triazole has been widely used in areas such as chemical biology,⁶ bioconjugate chemistry,⁷ supramolecular chemistry,⁸ surface chemistry,⁹ polymer science,¹⁰ and drug delivery.¹¹ In addition, the beauty of this reaction is leading to high yield, selective, quick, quantitative and simple to operate.

1.1.4 Triazole Moiety Under Mechanical Stress

Despite all these compatibilities, stabilities and wide applications of the AAC reactions, the stability of the 1,2,3-triazole product under mechanical stress has received little attention,¹²⁻¹⁴ and the external force that a 1,2,3-triazole linkage can tolerate is unknown.

The idea of cycloreversion is supported by a number of recent experimental studies^{12-13,15} and theoretical calculations.^{13-14,16} These studies showed that under suitable mechanochemical conditions cycloreversion reactions may have lower energy barriers than homolytic σ -bond scissions.

1.1.4.1 Sonication for Cycloreversion

Sonication is the process of making physical vibration by using an electrical current. The powerful vibration can be used to break the molecule. Sonication is used for a wide variety of applications including: solubilize compounds, cleaning, crystallization, extraction, emulsification, protein purification, sonochemistry and mechanochemistry. Nowadays, sonication is also used to break mechanophore by applying force via polymer chain segments. Among them cycloreversion¹²⁻¹⁶ reaction of an alkyne and an azide is pretty common.

The experimental studies were carried out under ultrasonic conditions on bulk scales, and the cycloreversion reactions of adducts of furan-maleimide, anthracene-maleimide, and alkyne-azide were investigated. In the studies, the adducts were embedded in the center of linear polymers, and the solution of the conjugates were subjected to sonication. Due to rapid microbubble growth and collapse, different segments of the polymers in different microenvironments move with different speeds and in different directions, and stretching forces are exerted on the cycloaddition adducts as well as other portions of polymer. Analyses following sonication indicated that the adducts were split into their cycloaddition reactants. Rupture of σ -bonds in other portions of the polymer chains was negligible. Besides these [2+4] and [2+3] adducts, 1,2-dioxetane, which belongs to a [2+2] adduct, was also studied under similar conditions, and was split into two ketones.¹⁷ These experiments could not give force

information. However, product analyses indicated that cycloreversions were favored over rupture of typical covalent bonds in the polymer chains.

Despite of all the new discoveries in cycloreversion from sonication, the deep understanding of such phenomena is still in progress. One probable challenge in unravelling the mechanisms maybe due to the lack of quantitative force control. Since in most of the mechanochemistry studies, mechanical force was introduced by ultrasound, and the interplay between dissolved polymer molecules and ultrasound is complex and rather indirect. Single molecule force spectroscopy provide a new option to study cycloreversion more directly and quantitatively. By anchoring one polymer between an AFM probe and substrate, the rupture forces of covalent bond could be measured by pulling the polymer until chain rupture.

1.1.5 Mechanical Force for Triazole Cycloreversion

The 1,2,3-triazole moiety was prepared with catalyst-free cycloaddition of strain-promoted cyclooctyne and azide, by chemical modification on mica surface. Then the terminal end was modified to NHS ester, as shown in Figure 1.2 (1.4). AFM probe with terminal amine functional group was prepared, as shown in Figure 1.2 (1.9). Polyethylene glycol (PEG) was used during the chemical modification of the tip and the mica surface. The purpose of using PEG is to increase the length of the linker. Ramping the probe into the surface can form amide bond, as shown in figure 1.2 (1.5). The amide bond was proven to be robust in polymer chain and could be formed rapidly upon

contact of AFM probe and surface, therefore was chosen in our experiment to provide strong anchoring, as shown in Scheme 1.2.

By retracting the AFM probe away from surface, mechanical stress was introduced to stretch the polyethylene glycol molecules until the weakest bond was broken. Since 1,2,3-triazole was assumed to be the weakest, cycloreversion reaction would happen. We found that the linkage between the tip and surface could even not withstand a 900 pN retraction force.

We were not sure about the force that it resulted from the splitting of 1,2,3-triazole or not. If the 1,2,3-triazole moiety is ruptured that leaves alkyne groups on the mica surface, as shown in Figure 1.2 (1.6). To prove this assumption, the mica surface (1.6) was chemically modified to (1.7), which possess NHS terminal group. Then the NHS terminal groups of (1.7) can react with amino-functionalized AuNPs (5 nm in diameter) through amide bond again as shown in Figure 1.1.(1.8).

To further confirm that the forces detected by AFM were attributed to the splitting of the 1,2,3-triazole molecule within the polymer chain, we carried out the AFM experiments at various locations on mica surface in a patterned fashion. The lined pattern was 200 nm in interval, and the squared pattern was 500 nm for side length. At each location, at least 5 bond dissociation events were observed before moving to the next. This ensures enough number of alkyne residuals at each location for the AuNPs attachment. In this way, non-specific adsorption of AuNPs on mica surfaces could be excluded due to the stochastic nature.

Scheme 1.2. Schematic of AFM single molecule force spectroscopy measurement on cycloreversion of 1,2,3-triazole. (1.4) Mica surface modified with PEG molecule containing 1,2,3-triazole and terminated with NHS groups. (1.9) AFM probe modified with PEG molecule terminated with amino groups (1.5) Upon contact of the probe and surface, strong amide bond is formed. (1.6) By retracting the probe away from surface, weakest triazole moiety breaks leaving alkyne residue on the surface. (1.7) The alkyne on the surface were converted to NHS terminal groups. (1.8) Amino-functionalized AuNPs are introduced.

Therefore, if AuNPs were observed at the same location as the force spectroscopy experiments, then the mechanically activated cycloreversion could be confirmed. As a result, patterned AuNPs were observed directly through AFM imaging which excluded the possibility of non-specific adsorption of AuNPs on mica surface (Figure 1.6).

Therefore, the measured rupture forces with a value of below 860 pN were attributed to the ring opening of the cycloreversion.

Figure 1.6. (A) Line pattern with 200 nm intervals after AuNPs attachment.
(B) Square pattern with 500 nm after AuNPs attachment.

1.1.6 Importance of Pure PEGs

Before rupture force measurement of triazole ring by AFM, we chemically modified mica surface and AFM probe. We used commercially available polyethylene glycols (Fmoc-NH-PEG-O-C₃H₆-CO-NHS) for chemical modification of surface and tip as shown in figure 1.7. There is no doubt that the chemical modifications of surface and tip were accurate. Otherwise, the AuNP patterning experiment would not give expected results. However, if we synthesize the part I of surface A and part II of tip B before attaching to the surface and the tip, as shown in Figure 1.7, then the characterization would be easy and precise. In this case, we believe that our data would be much better. Majority of commercially available PEGs are typically made by polymerization of ethylene oxide. Due to the randomness of the process, the products are polydispersed admixtures of many different PEGs

Figure 1.7. (A) Chemically modified surface using PEGs
(B) Chemically modified tip using PEGs

So, the commercially available PEGs used in this experiment may create difficulties in characterization of part I of surface A and part II of tip B after the synthesis. Although admixtures are currently used for almost all experiments, they are not ideal for reasons such as difficulty to achieve consistent composition for products from different batches, difficulty to characterize the products, due to heterogeneity of physical properties caused by the different sizes of the PEG tags. These disadvantages of commercially available PEGs used in the experiment, envision us to think about synthesis of monodisperse/pure PEGs. The idea is not limited to improve our experimental data only

but has a variety of other applications, which are discussed later. Monodispersed PEG is made up of a single oligomer, which forms single molecular conjugates during chemical reactions. It allows complete and more precise characterization of all the PEG conjugates. In conclusion, monodisperse PEGs can reduce or eliminate the problems associated with polydispersed PEGs.

1.2 Solid Phase Synthesis of Monodisperse PEGs

1.2.1 Applications

Polyethylene glycols (PEGs) are versatile, chemically stable, amphiphilic oligomers with repeating units of ethylene oxide. They have several unique physical and biophysical properties compared to other synthetic polymers. They are nontoxic, biocompatible, water-soluble, nonimmunogenic and nonatigenic polymers with diverse applications. Covalent conjugation of PEGs with biomolecules and drugs is known as PEGylation. PEGylation improves the water solubility and biocompatibility of drugs. It is the most effective method to improve therapeutic properties of drugs by less frequent dosing.²⁸ The PEG used for such applications requires a size of 4K Da to have the desired biophysical effects, and the most commonly used size is 40K Da.²⁹ PEGs can be applied to surface science. Chemical modification of the surface can be achieved by using PEGs. In mechanical force measurement project, we also used PEGs for modification of tips and surfaces.³⁰ PEGylated nanoparticles in drug delivery systems make a significant contribution. PEGylation improves the dispersion of nanoparticles in vivo.³¹ PEG modified carbon nanotubes³² have various biomedical applications. They

have been successfully tested in the field of neurology, vaccination and oncology. They are also used to make bioactive organic-inorganic hybrid nanocomposites³³ materials emended in inorganic matrices. They are widely used in bioconjugation³⁴ because of their flexibility and hydrophilicity. In some of the applications, the size of the PEG used was lower than 1K Da. So it is better to name it oligoethylene glycol (OEG).

1.2.2 Synthesis and Complications

The classical method for PEG synthesis was from ethylene oxide in which purification and separation of pure oligomers is impossible. The size distribution (molecular weight) can be characterized statistically by its weight average molecular weight and its number average molecular weight, so they have varying length and molecular weight.³⁵ If this admixture of PEG is used for PEGylation to prepare drugs then it is difficult to achieve consistent composition. In addition, it can cause difficulty with characterization, loss of biological activities and formation of different size molecules which results in different physical properties. These shortcomings are the major challenges for polydispersed PEGs to get FDA approval.³⁶ Different research groups across the globe have tried to get monodispersed PEGs by solution phase stepwise organic synthesis.³⁷ The longest monodispersed PEGs successfully prepared from this method is about 2K Da.³⁸ but there are a lot of problems in this method. First, depolymerization is a major problem during ether formation processes.³⁹ In depolymerization the deprotonated PEG reacts intramolecularly (Scheme 1.3). During coupling process, base can deprotonate the growing linker to form an anion (**1.12**). Some

of the anions, instead of coupling with monomer, depolymerization involves the elimination of oxirane molecule to form (**1.13**). The depolymerization initiated by the anion is also known as anionic depolymerization. After anionic depolymerization it is impossible to separate n-1 mer PEG.⁴⁰ In addition, this method requires a lot of chromatographic separation of the product, intermediate and monomer.

Scheme 1.3. Representation of depolymerization process

Anionic depolymerization is a serious problem for large scale synthesis of monodisperse PEGs from solution phase,⁴¹⁻⁴² so we start the innovative synthesis of monodisperse PEGs by using solid phase synthesis.

1.2.3 Solid Phase Synthesis

The concept of solid phase synthesis was first introduced by Robert Bruce Merrifield and honored by the Nobel Prize in Chemistry in 1984. In solid phase synthesis, the chemical reactions of the substrate attachment to the solid support occur in a stepwise fashion and there are methods for attachment and detachment from the

supports. Desired product is assembled by stepwise addition of monomers. The desired intermediates of the products are anchored to the solid support, so purification of compounds bound to the solid support is achieved by simple washing. This allows the use of a large excess of reagents, improving the efficiency of chemical transformations. As a result solid phase synthesis is rapid and efficient.

Solid phase synthesis has a lot of advantages over solution phase synthesis. First, solid phase synthesis does not require any chromatographic purification. The impurities and the excess reagent are removed by washing the solid support. The overall process is very fast as it does not require set up of the reaction, extraction and purification. An excess of reagent can be used to complete the reaction quickly. The main beauty of this synthesis is that it can be automated. In addition, the desired product is cleaved selectively at the end of the synthesis and, most of the time, the solid support is reusable. Our lab is using this technology for the synthesis of oligonucleotide (ODN), deoxyribonucleic acid (DNA), peptides and peptide nucleic acid (PNA).¹⁸⁻²⁷ Recently we are able to synthesize monodisperse PEGs by using this solid phase technology for the first time.

We executed the synthesis of PEGs on solid support by using an ABI-394 DNA/RNA synthesizer on a small scale and using peptide synthesis vessel on a large scale. In both cases Wang resin was used for solid support (Figure 1.8) which has polystyrene as a solid support and 4-benzyloxy benzyl alcohol function as a growing chain.

Figure 1.8. Solid support (Wang resin)

We started PEG synthesis using an ABI-394 DNA/RNA synthesizer, as shown in Figure 1.9. A homemade column with 10 ml capacity was prepared (Figure 1.9 A). Then the column was connected to the synthesizer with long lines. It was placed on an orbital shaker for agitation. (Figure 1.9 B). The reagents were transferred from the synthesizer manually, and excess monomer was recovered in an Erlenmeyer flask (Figure 1.9 C).

In this way we were able to collect the excess monomer that can be used for another coupling. The recovered monomer was almost pure with slight contamination with the side product. The side product was from the β -elimination of the monomer. So, the recovered monomer can be used in the next cycle as the side product cannot react with the linker.

Figure 1.9. (A) Large homemade column; (B) The setup of homemade column with an ABI-394 DNA/RNA synthesizer. The column is shaken using an orbital shaker. (C) An ABI-394 DNA/RNA synthesizer with TCA solution is in bottle 14, the monomer solution is in bottle 15, the excess monomer is delivered to the Erlenmeyer flask for recovery. The bottle positions are numbered as those in the operational manual of the synthesizer.

In addition, we also executed the large scale synthesis of PEGs by using a peptide synthesis vessel. Agitation was achieved by clamping the vessel in the modified rotary evaporator prepared in the lab (Figure 1.10). The reagents were removed by applying a vacuum or positive nitrogen pressure.

Figure 1.10. The setup of larger scale solid phase PEG synthesis using a peptide synthesis vessel.

1.2.4 PEG Synthetic Cycle

During solid phase synthesis of PEGs, the monomers are added sequentially one at a time. Each addition requires three steps which is called a synthetic cycle. The three steps are deprotonation, coupling and detritylation. At the end of the synthesis, the PEGs are cleaved from the solid support. The brief descriptions of the three steps are given in the following paragraphs and Scheme 1.4.

1.2.4.1 Deprotonation

In the synthesis cycle of PEGs, deprotonation is the first step. As shown in the Scheme 1.4, it converts **1.15** to **1.16**. The Wang resin, which contains the 4-benzyloxy benzyl alcohol function, was used as the support. Thus the growing chain of Wang resin contains a hydroxyl group at the end. The proton from the hydroxyl group needs to be removed before the coupling step. Deprotonation was achieved using *t*BuOK THF solution at room temperature. Once the proton is removed the resin is ready for the coupling step in the cycle.

1.2.4.2 Coupling

In the coupling step, the alkoxide **1.16** is coupled with the monomer **1.17** via the Williamson ether formation reaction to give **1.18**. The monomer contains a tosyl group at one end and a dimethoxytrityl group at the other. The tosyl group is kicked out by the alkoxide ion leaving DMTr group at the end.

1.2.4.3 Detritylation

The growing chain of PEGs contains 4,4'-dimethoxytrityl (DMTr) at the end which needs to be removed before the deprotonation step. In this step, the DMTr protecting group of **1.18** is removed with a dilute acid to give **1.19**. Desired PEG is prepared by repeating the above three steps.

1.2.4.4 Cleavage

The previous three steps are repeated as one cycle for adding each monomer until the PEG synthesis is complete. The growing linker is stable to all the reagents used. As the desired length of PEG is reached, the linker is cleaved with trifluoroacetic acid (TFA) at room temperature.

Scheme 1.4. Polyethylene glycol synthesis cycle

1.2.5 Synthesis

In this work, we demonstrate our results for using solid phase technology for stepwise synthesis of monodisperse PEGs and their derivatives. The synthetic design of all PEGs and their derivatives are shown in the Scheme 1.5. The growing linker of **1.19** after coupling with monomer **1.17**, produces **1.24**. The compound **1.24** upon detritylation generates compound **1.25** from which the (PEG)₈ is achieved by cleaving (**1.26**). Similarly, (PEG)₈ derivatives (**1.28** and **1.29**) can be prepared from **1.25** utilizing functionalization and cleavage. Treatment of **1.25** with different monomers (**1.17** and **1.37**), form the compounds **1.30** and **1.33**. The protecting group of **1.30** is removed and then cleaved to get (PEG)₁₂ i.e. **1.32**. The (PEG)₁₂ derivatives (**1.34** and **1.35**) can be prepared from **1.33** utilizing functionalization and cleavage.

We can use excess reagents due to the ease of removing them after the reactions (solution phase methods generally cannot do this), the Williamson ether formation reaction can be performed with high efficiency. Also, excess reactants at room temperature and in shorter time, avoids the highly undesirable PEG depolymerization reaction. Depolymerization produces the shorter PEGs that are impossible to remove from the product.³⁹ This was completely suppressed.

Scheme 1.5. PEGs and their synthesis design

Significantly, all chromatography purifications can be avoided in this method. In addition, the monomers used for the synthesis did not require chromatographic purification and impure ones could be used directly. The synthesis of the monomers and the impurities are shown in the Scheme 1.6. The details of monomer synthesis is explained in Chapter 3. Both of the monomers have tosyl group on one end that can be kicked out by the alkoxide group of the growing linker. During this coupling step of synthetic cycle, the byproducts, as shown in Scheme 1.6, **1.38** and **1.39** do not participate. So, they are removed by washing the resin.

Scheme 1.6. Synthesis of monomers

All intermediates in the solid phase synthesis were purified by washing, and pure final products were obtained without chromatography. Using the technology, we were able to synthesize close-to-monodisperse (PEG)₈ (compound **1.26** in the Scheme 1.5), (PEG)₁₂, (compound **1.32** in the Scheme 1.5) and their derivatives (compounds **1.28**, **1.29**, **1.34** and **1.35** in the Scheme 1.5) with quantitative yields.

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Chapter 2

Evidence of Splitting 1,2,3-Triazole into an Alkyne and Azide by Low Mechanical Force in the Presence of Other Covalent Bonds.

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Abstract

The cycloaddition reaction of an alkyne and azide to form a 1,2,3-triazole is widely used in many areas. However, the stability of the triazole moiety under mechanical stress is unclear. To see if a triazole could be selectively split into an alkyne and azide in the presence of other typical covalent bonds, a mica surface functionalized with a molecule containing a triazole moiety in the middle and an activated ester at the end was prepared. An atomic force microscope (AFM) tip with amino groups on its surface was ramped over the mica surface at predefined locations, which could temporarily link the tip to the surface through amide bond formation. During retraction, the triazole or another bond in the linkage broke, and a force was recorded. The forces varied widely at different ramps from close to 0 pN to 860 pN due to non-specific adhesions and to the inherent inconsistency of single bond rupture. If some of the forces were from triazole cycloreversion, there would be alkynes at the predefined ramping locations. The surface was reacted with an azide carboxylic acid followed by labeling with amino Au nanoparticles (AuNPs). AFM imaging revealed AuNPs at the predicted locations, which provided evidence that under certain conditions triazole could be split selectively in the presence of other bonds at forces below 860 pN.

2.1 Introduction

The alkyne-azide cycloaddition (AAC) reaction to form a 1,2,3-triazole has been widely used in areas such as chemical biology, bioconjugate chemistry, supramolecular chemistry, surface chemistry, polymer science, and drug delivery.¹⁻⁶ The reaction is compatible with polar functional groups and polar solvents and is orthogonal to functionalities in biomolecules. It has good kinetics, and its product is thermally and photochemically stable.⁷⁻⁸ However, despite all the studies and applications, the external force that a 1,2,3-triazole can tolerate is unclear.⁹⁻¹⁰ An recent paper aimed to determine if a triazole could be split selectively in the presence of typical covalent bonds using ultrasound polymer mechanochemistry had been retracted.⁹ A follow-up paper by a different research group using an atomic force microscope (AFM) single molecule force spectroscopy technique suggested that the chance for selective triazole rupture in the presence of C-Si bond is low. In several thousands of events, only three showed potential triazole rupture, but there was no suitable method to differentiate triazole rupture from other bond rupture.¹⁰ Earlier density functional theory calculation indicated force-induced selective triazole rupture is unlikely.¹² A more recent calculation considered the effect of pulling directions, and concluded that cycloreversion of certain triazoles is in principle possible.¹³ Recently, our laboratory needed to covalently anchor an enzyme between an AFM tip and a surface. One approach was to embed the enzyme in a molecule with one end attached to an AFM tip and the other terminated with a strained alkyne. Approaching the tip to a surface with covalently attached azides would link the tip to the surface through an AAC reaction

and accomplish the anchoring task. In model studies, we observed that the linkage between the tip and surface could not even withstand an 900-pN retraction force. In addition to other potential causes such as failure to functionalize the tip or the surface, one bold hypothesis was that the newly formed 1,2,3-triazole was broken with low mechanical force. In this Paper, we provide evidences suggesting that the possibility of selective 1,2,3-triazole rupture in the presence of other covalent bonds should not be excluded based on our current knowledge, and mechanical cycloreversion may be easier than currently predicted by theoretical calculations.

2.2 Results and Discussions

2.2.1 Potential Approaches for Mechanical Triazole Cycloreversion Studies

One approach for the study of 1,2,3-triazole cycloreversion would be to ramp an AFM tip covalently functionalized with azides over a surface functionalized with a monolayer of strained-alkynes. Because strained alkynes can react with azides in the absence of a catalyst, approaching the tip to the surface would form a triazole. When the tip is retracted, if selective cycloreversion is possible, the triazole would be split back into the alkyne and azide. The forces for the rupture would be easily recorded by the AFM. Repeated ramping would generate sufficient data for statistical analysis, which would give the ranges of mechanical forces for splitting a triazole under various conditions such as different force loading rates. Unfortunately, this approach did not work well in our hands. The problem was that we did not know whether the forces

observed in the force-separation curves were from covalent bond ruptures including triazole cycloreversion or from non-specific interactions. Ramping AFM probes without azides on the tip over alkyne surface also gave similar force-separation curves. Non-specific adhesion forces could be reduced by incorporating PEG spacers.¹⁴ but the results were not significantly better. Distinguishing the non-specific forces from those from covalent bond rupture was impossible. In addition, we had no means to prove that any triazole was indeed split into an alkyne and azide using this approach.

To solve these problems, we decided to use an approach involving splitting pre-formed triazoles. Specifically, a triazole is embed into the middle of a linear molecule. One terminus of the molecule is covalently linked to a mica surface, which is atomically flat. The other terminus has an activated carboxylic acid ester, which can react with a primary amine at room temperature. An AFM tip functionalized with a monolayer of amino groups is then ramped over the mica surface. The ramping locations are not random. Instead, they are selected at predefined locations such as on a line with defined intervals. While the tip is in close proximity to the surface during the ramp, there will be a chance to form one or more amide bonds between the amino groups on the tip and the activated carboxylic acid ester groups on the surface. This will temporarily link the tip to the surface covalently. During retraction, the triazole or another covalent bond in the linkage will break, and the rupture force is recorded. If the breakage occurs at the triazole and the products are an alkyne and azide, the one on the surface can be detected by AFM imaging after labeling with gold nanoparticles (AuNP) using the AAC reaction. Non-specific adhesion of AuNPs to surface is expected to be unavoidable, but they must

be at random locations. In contrast, the AuNPs anchored to the locations of alkynes or azides resulted from triazole cycloreversion should show the predefined pattern. If imaging finds the anticipated AuNP deposition patterns, we can draw a conclusion that 1,2,3-triazole cycloreversion in the presence of other covalent bonds is possible, and the forces for the rupture are equal to or lower than the largest ones observed during the ramps.

2.2.2 Preparation of Surface and AFM Tip

To implement the plan, we prepared the mica surface **2.1** and the AFM tip **2.2**. As shown in Scheme 2.1, freshly pilled mica was reacted with 3-aminopropyltriethoxysilane (APTES) to give amino mica **2.3**. Reaction of **2.3** with the commercially available Fmoc-NH-PEG-(CH₂)₃-C(=O)-NHS (**2.4**; Fmoc, 9-fluorenylmethoxycarbonyl; NHS, *N*-hydroxysuccinimide) gave surface **2.5**. The Fmoc group in **2.5** was removed with piperidine to give **2.6**, which was reacted with compound **2.7** to give the alkynylated surface **2.8**. Formation of a 1,2,3-triazole between the alkyne on **2.8** and the azide in **2.9** gave surface **2.10**. The carboxylic acid groups on **2.10** were activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and reacted with *N*-hydroxysuccinimide (NHS) to give the target surface **2.1**, which had a covalently anchored monolayer of molecules with a 1,2,3-triazole in the middle and an activated carboxylic acid ester at the terminus.

The AFM tip **2.2** was prepared from a silicon nitride probe with a silicon tip (Scheme 2.2). Aminization of the tip with APTES gave **2.11**,¹⁵ which was reacted with

compound **2.4** to give tip **2.12**. The Fmoc group was removed with piperidine to give the target amino tip **2.2**. Incorporating PEG into the linkages on the surface and the tip was expected to reduce non-specific adhesions¹⁴ and to increase the mobility of the NHS ester and amino groups to facilitate the amide formation reaction.

Scheme 2.1. Preparation of surface **2.1**: a) 1% APTES in PhMe, rt, 20 min, then 100 °C, 1 h; b) **2.4**, Et₃N, CHCl₃, rt, 5 h; c) 20% piperidine in DMF, rt, 30 min; d) **2.7**, Et₃N, DMF, rt, 12 h; then Ac₂O, pyridine, CHCl₃, rt, 3 h; e) **2.9**, iPrOH, rt, 12 h; f) EDCI, NHS, DMF, 50 °C, 5 h.

Scheme 2.2. Preparation of AFM tip **2.2**: a) 1% APTES in PhMe, rt, 12 h, then 95 °C, 10 min; b) **2.4**, Et₃N, CHCl₃, rt, 5 h; c) 20% piperidine in DMF, rt, 30 min.

2.2.3 Splitting Triazole with AFM

The probe **2.2** was ramped over surface **2.1** in isopropanol at room temperature with a force loading rate of about 5 nN/s at retraction (Scheme 2.3). For each ramp, a force-separation curve was recorded. A typical curve that showed adhesion force at non-zero probe-surface separation is presented in Figure 2.1. While approaching tip **2.2** to surface **2.1** and before retracting to a certain distance, an amino group in **2.2** and an NHS ester in **2.1** was expected to have chances to react, and an amide bond could be formed. In the cases that an amide bond was formed, the tip was covalently linked to the surface to give **2.13** (Scheme 2.3). During retraction, the PEG was first stretched, and the adhesion force increased. When the force reached approximately 500 pN, the value then abruptly returned to zero, indicating that a bond in the linkage was broken.

Scheme 2.3. Splitting 1,2,3-triazole and labelling alkyne on surface with AuNP: a) Approach tip **2.2** to surface **2.1** on AFM in *i*PrOH; b) retract tip from surface; c) *n*BuNH₂, CHCl₃, rt, 3 h; d) **2.9**, *i*PrOH, rt, 12 h; e) EDCI, NHS, DMF, 50 °C, 5 h; f) **2.17**, DMF, rt, 12 h

It should be noted that this curve might be from covalent bond rupture including triazole rupture. It might also be from disruption of a non-specific interaction, in which case no amide was formed during the ramp. With multiple ramps, it was hypothesized that some would cause amide bond formation and covalent bond rupture. It was further

hypothesized that the bond rupture may occur at the 1,2,3-triazole in the linkage **2.13**, splitting it into the strained alkyne in **2.14** and the azide in **2.15** (Scheme 2.3).

Figure 2.1. A typical single 1,2,3-triazole molecule rupture force curve. AFM tip **2.2** was ramped over surface **2.1**, and the two were temporarily linked together covalently through amide bond formation (see Scheme 2.3). During retraction following the course of the ramp, the PEG chains in **2.1** and **2.2** were first stretched, which resulted in an increase in the adhesion force. This portion of the force curve fits well with the worm-like chain model (the green dotted line). When the pulling force was increased to ~500 pN, the 1,2,3-triazole broke, and the force abruptly returned to zero.

To find evidence that supports the hypotheses, probe **2.2**, which had multiple amino groups, was ramped over surface **2.1** at pre-defined locations (Scheme 2.3). Each ramp was expected to consume zero, one or more amino groups. Six ramping locations were selected, which were on one line at intervals of 200 nm. At each location, ramping was performed multiple times until at least five curves showing adhesion forces at non-zero probe-surface separation were obtained. Some of the curves did not have any force peak,

and some had a peak at zero probe-surface separation with forces less than 100 pN. In these cases, the amide bond formation reaction probably did not occur. Other curves had one or more peaks at non-zero probe-surface separation. These peaks might originate from the rupture of 1,2,3-triazole. It is also possible that the peaks were from non-specific interactions between the molecules on the probe and surface. The force values are listed in Table 2.1. After the ramps, each spot was expected to have zero, one or more strained alkynes (**2.14**). Other areas of the surface were still covered with the long molecule with the NHS carboxylic acid ester groups at the end and the triazole in the middle intact (not shown in Scheme 2.3).

Table 2.1. Forces potentially split 1,2,3-triazole at predefined locations on a mica surface giving AuNPs in Figure 2.2.

Location	Force (pN)
1	200, 170, 330, 190, 110, 160, 140, 190, 450, 460
2	170, 200, 860, 190, 250, 250, 150, 250, 200, 220
3	240, 310, 160, 250, 100, 220, 350, 270, 340, 340, 310, 150, 250, 230, 200, 370, 340
4	160, 150, 250, 200, 330, 180
5	230, 230, 270, 260, 210, 250, 270
6	220, 500, 250, 260, 200, 200, 240, 260, 330

2.2.4 Labeling Alkynes and Imaging Specifically Deposited AuNPs

The surface was unmounted from AFM, and the NHS ester groups were inactivated with excess *n*-butylamine. To label the alkynes resulted from triazole cycloreversion on **2.14**, the surface was incubated in a solution of azide carboxylic acid **2.9**. This converted the alkynes on the six spots to carboxylic acid groups through the AAC reaction (Scheme 2.3). The carboxylic acid groups were then activated to give **2.16**, which was reacted with excess amino functionalized 5 nm AuNP (**2.17**) to give **2.18**. The surface was washed and dried and then imaged with AFM in air. As observed in Figure 2.2 (see insert), AuNPs were detected as the reverse image of the AFM tip at five of the six locations. The height of the nanoparticles ranged from 3.9 to 8.6 nm (Figure 2.2), which was in good agreement with 5 nm AuNP. The width at the base of the height graphs was about 35 nm, which was consistent with the diameter of tip curvature. As expected, the five nanoparticles were roughly on one line with intervals close to 200 nm. The direction of the line also agreed well with the deposition direction, which was from top to bottom. The line was not perfect, which could be caused by factors such as the different locations of amino groups on tip **2.2** that participated in the amide bond formation reaction, the long PEG spacer (~25 nm), and thermal drift of AFM.

Figure 2.2. AFM image of patterned AuNPs on a mica surface (upper right insert) and the height graph of the five AuNPs. The patterned AuNP image was generated by ramping AFM probe **2.2** over surface **2.1** at predefined locations. The six locations were on one line at intervals of 200 nm. Due to 1,2,3-triazole cycloreversion, the six locations contained strained alkynes, which were converted into activated carboxylic acid esters and labelled with amino AuNP (see Scheme 2.3). The nanoparticles were imaged with AFM in air and were observed as the reverse image of AFM tip at the expected locations with only one missing. The height graph of the five spots agreed well with 5 nm AuNPs.

2.2.5 Possible Force Values for Triazole Splitting

The forces responsible for generating the alkyne in **2.14**, which made the attachment of AuNPs to the pre-defined locations possible, must be among those in Table 2.1. The largest ones measured at locations 1-6 were 460, 860, 370, 330, 270, and 500 pN, respectively. It is impossible to tell which force was responsible for deposition

of the AuNP at each spot. However, it is apparent that the forces must be equal to or lower than the largest values if the NPs were deposited through triazole cycloreversion. This would lead us to draw a conclusion that 1,2,3-triazole could be split into an alkyne and azide with mechanical forces as low as 330 pN assuming that the absence of AuNP at spot 5 was due to the absence of an alkyne. However, this number is much lower than those predicted by density functional theory calculations, which suggested that mechanical triazole rupture should occur with forces in the nanonewton range.^{12,13} If we assume that one or more of the five NPs at the locations with lowest forces were from nonspecific deposition, we would be able to reach a conclusion with higher than 330 pN rupture forces, which would bring the experimental values closer to calculated ones. This is reasonable because chances exist that one or more of the five NPs being deposited nonspecifically even though the chance for nonspecific deposition of all the five NPs at the pre-defined locations is extremely low as analyzed in the next paragraph. With these considerations, we would conservatively state that triazole rupture can be caused by forces below 860 pN instead of the more bold number of 330 pN under the conditions the experiment was carried out.

2.2.6 The Chance for Random AuNP Deposition on a Line is Low

The chance for accidental deposition of the AuNPs on all the five of the six pre-defined spots is low. To locate the particles, we scanned an area of $60 \times 60 \mu\text{m}^2$. There were a total of about 3.6×10^4 NPs in the area, most of which of course are deposited non-specifically. For specific deposition, if depositing a NP to an area of $10 \times 10 \text{ nm}^2$

is considered accurate, the scanned area can be divided into 3.6×10^7 positions. For filling one chosen position with a NP, there are 3.6×10^4 possibilities. Because the orientation of the surface during the ramping and imaging experiments was kept consistent, for filling the second position with a NP, there were only two choices, one above and the other below the first NP at a distance of 200 nm. Therefore the possibility to deposit the second NP to the right positions is 2×10^{-3} . For the same reason, the possibility to deposit subsequent NPs onto the line with 200 nm interval is also 2×10^{-3} . As a result, the possibility for five NPs to line up accidentally as shown in Figure 2 is as low as 5.76×10^{-7} . This analysis tells us that the possibility of splitting 1,2,3-triazole into an alkyne and azide selectively by mechanical force is indeed extremely high.

2.2.7 More Triazole Splitting, Labeling and Imaging Experiments

To obtain more evidences, we repeated the single molecule triazole cycloreversion, labeling and imaging experiments two more times. In one of them, six ramping locations on a line with 200 nm interval were chosen (Figure 2.3, see insert). In another, four locations on the corners of a square with sides of 500 nm were chosen (Figure 2.4, see insert). AuNPs were found at all anticipated positions. In these two repeating experiments, multiple NPs at each locations were found. These results may seem less ideal than the parent experiments where only one NP was found at each location. However, multiple NPs at each location are more plausible because at each location, ramping was carried out multiple times and for each ramp more than one triazole could be split. The difference in the results between parent and repeating experiments is not

difficult to explain. In the parent experiments, the density of surface and tip functionalization might be lower and we might be lucky that we were able to deposit one particle at each of the five out of the six locations. In the repeating experiments, the density of surface and tip functionalization might be higher. As a result, multiple NPs at each location were found. The height graphs of selected NPs at each location (Figures 2.3-2.4) also agreed well with 5 nm AuNP. The force values of the ramps are in Table 2.2. As can be seen, none of the numbers reached nanonewton range.

Table 2.2. Forces potentially split 1,2,3-triazole at predefined locations on a mica surface giving AuNPs in Figures 2.3-2.4.

Location	Force for Figure 2.3 (pN)	Force for Figure 2.4 (pN)
1	124, 272, 144, 71	350, 500, 410
2	255, 297, 226, 232, 95	620, 130, 210, 160, 260
3	145, 191, 118, 422	220, 560, 130
4	259, 206, 119, 257	165, 235, 175, 230
5	292, 307, 417, 339	-
6	541, 472, 376, 449, 386	-

Figure 2.3. AFM image of patterned AuNPs on a mica surface (upper right insert) and the height graph of selected AuNPs. The patterned AuNP image was generated similarly as described in the caption of Figure 2.2. The height graphs agreed well with 5 nm AuNPs.

Figure 2.4. AFM image of patterned AuNPs on a mica surface (upper right insert) and the height graph of the AuNPs. The patterned AuNP image was generated similarly as described in the caption of Figure 2 by ramping probe 2.2 over surface 2.1 at the corners of a square with sides of 500 nm. The height graphs agreed well with 5 nm AuNPs.

2.2.8 Selective Cycloreversion and Lower Than Calculated Forces

Several research groups have used density functional theory to evaluate the possibility of selective triazole cycloreversion and the forces needed for the process.¹¹⁻¹³ Uggerud and co-worker have shown that both C-C bond homolytic scission and 1,2,3-triazole rupture can be facilitated by external mechanical forces.¹² However, in order to achieve selective cycloreversion of a 1,4-substituted 1,2,3-triazole over C-C bond scission, the external stretching forces have to be lower than 1 nN at ambient conditions. With higher forces, C-C bond homolytic scission is favored. The energy needed for cycloreversion to occur at 1 nN is as high as 70 kcal/mol, which is in agreement with a separate calculation.¹¹ This amount of energy is difficult for molecules to acquire at room temperature. The calculations further predicted that for the triazole to undergo cycloreversion at ambient conditions, forces of 13.5 nN are needed, which exceeds values for typical covalent bond rupture.¹² More recently, Blank and co-workers carried out calculations using 1,5-substituted 1,2,3-triazoles and triazoles formed from strained alkynes as substrates.¹³ They found that the forces for cycloreversion can be as low as 4 nN when a force loading rate of 10^8 nN/s is used. They concluded when the linkers to the triazoles are polytetrahydrofuran or polymethacrylate, selective cycloreversion is possible under ultrasonic conditions.

Our experimental data only partially agree with results from the calculations.¹¹⁻¹³ We indeed observed potential selective cycloreversion of a 1,2,3-triazole that is formed from a strained alkyne over typical covalent bond scissions, and the rupture forces were

consistently lower than 1 nN, which is needed for the selectivity according to Uggerud and co-worker.¹² However, we could not provide an explanation on how the triazole acquires the calculated activation energy of about 70 kcal/mol at room temperature. In addition, our observed rupture forces (less than 860 pN) are much lower than the calculated values (13.5 nN¹² and 4 nN¹³). It is noted that our triazole substrate is different from those used in the calculations and further we used a much lower force loading rate than one of the calculations (~ 5 nN/s vs 10^8 nN/s).^{3e} The loading rate should be even lower if the long flexible linkages connecting the triazole to the tip and surface are considered. As a result, the rupture events may actually occurred under close to equilibrium conditions. In order to see if our lower than expected force values were a result of erroneous probe calibration, control experiments using a version of the surface **2.1** without a triazole in the linkage were carried out. Among the force curves obtained, 20% showed rupture forces in the nanonewton range, which indicates that the low forces in the parent experiments were probably not a result of systematic error.

Uggerud and co-worker mentioned several potential reasons for observed selective triazole cycloreversion under ultrasonic conditions, which they predicted unlikely by calculation.^{9,12} Solvation is one of them, but their calculation showed an increase of activation energy in a polar solvent instead. Others include rapid heating, involvement of excited electronic states, solvent molecule assisted ring opening, and radical process. In our AFM experiment, rapid heating can be ruled out. Involvement of excited electronic states and radical process are highly unlikely due to the mild conditions although we cannot exclude the possibility of excitation caused by a sharply reduced

HOMO and LUMO gap of a structure made possible by the stretching forces. Solvent assisted ring opening is as likely as under ultrasonic conditions.

Besides the explanations suggested by Uggerud and co-worker, it might be worthwhile to pay attention to one major difference between mechanical triazole cycloreversion and mechanical homolytic covalent bond scission, which exists in their energy profiles. For cycloreversion, the transition state energy, which is mainly from bending the alkyne and azide, is much higher than the product (~ 20 kcal/mol). In contrast, for homolytic covalent bond rupture, the transition state energy is close or equal to the products, which are radicals. It is noted that the difference of rupture forces for triazole cycloreversion between calculated (13.5 nN¹² and 4 nN¹³) and observed (less than 860 pN) values is big; while the difference of rupture forces for covalent bond scission between calculated (e.g. 4.78 nN for C-Si) and observed (2.0 nN) values is considered acceptable.¹⁶ However, if this difference in transition state energies relative to products is a cause of the much lower than calculated forces for triazole cycloreversion, a rationale remains to be proposed.

2.3 Conclusions

In conclusion, we have provided strong evidences that selective mechanical cycloreversion of 1,2,3-triazole is possible in the presence of other typical covalent bonds. The measured force (below 860 pN) is however significantly lower than calculated values (4 nN or higher) in the literature.^{12,13} The finding is expected to be important for research projects in areas such as surface chemistry, material science, and

nanotechnology. For example, for anchoring molecules to a surface or microspheres, it would be beneficial to avoid using AAC reaction with certain alkyne substrates if significant mechanical stress is expected. The same caution should be taken for conjugating biomolecules to various vehicles for applications in biology and medicine. When the AAC reaction is used for the synthesis of polymers, the mechanical reversibility of the reaction may provide benefits to self-healing.^{13,18} In addition, the triazole function could be an excellent tool in studies involving transporting single molecules to pre-determined locations on the surface.¹⁹ The method itself used in the study is also remarkable. Currently, for studies on mechanochemical reactions at the single-molecule level, one challenge is the validation of the reaction products, which may be different from those of thermal and photochemical reactions.²⁰ Even in studying mechanochemical reactions on a bulk scale with ultrasound, chemical analysis remains challenging because of the high mass ratio of polymer to functional groups of interest. Therefore, most efforts in mechanochemistry have been directed toward theory and simulations.²⁰ The method used in the present work for product identification provides new opportunities in the research area. We expect it to be adopted for studying other mechanochemical reactions.

2.4 Experimental Section

2.4.1 Materials

AFM probes (silicon nitride probe with silicon tip; cantilever back side coated with 45 nm Ti/Au; f_0 15 kHz; k 30 pN/nm) were purchased from Bruker (Santa Barbara, CA; model, MSNL). Mica (muscovite mica, V-5 quality, 50 × 75 mm, 0.15 – 0.21 mm thickness) was from Electron Microscopy Science (Hatfield, PA; Cat# 71855-01). Fmoc-NH-PEG-O-C₃H₆-CO-NHS (**2.4**, PEG-MW 3,000; PEG = polyethylene glycol) was from Rapp Polymere GmbH (Tuebingen, Germany; Cat# 133000-22-35). Amine (Au-DHLA-PEG400-NH₂) gold nanoparticle (AuNP **2.17**; 5 nm diameter; OD 1, 0.4 mM Au in water) was from AC Diagnostics (Fayetteville, AR; Cat# AMG-0500-P-1). CHCl₃ (J. T. Baker, HPLC grade), Et₃N (Aldrich, freshly distilled), DMF (Aldrich, anhydrous, 99.8%), APTES (Aldrich, reagent grade, ≥ 98.0%), Ac₂O (EMD, ACS grade, 99%), MeOH (Aldrich, semiconductor grade), *i*PrOH (Aldrich, HPLC grade) and pyridine (Aldrich, ACS reagent grade, ≥ 99%) were used as received. Compounds **2.7**²¹ (*exo* isomer was used in all cases) and **2.9**²² were synthesized following literature procedures. All AFM experiments were carried out on a Nanoscope V, Dimension Icon Atomic Force Microscope (Bruker, Santa Barbara, CA) at room temperature. The spring constants of probes were calibrated using the thermal noise method,²³ and were in the range of 16.7-23.9 pN/nm.

2.4.2 Preparation of Mica Surface 2.5

Freshly pillared mica surface was washed with CHCl_3 (3 times) and dried by blowing a gentle flow of argon over the surface for about 2 min. The mica was then incubated in a solution of APTES (1%) in toluene (freshly distilled) for 20 min (Scheme 2.1). The solution was removed and the surface was washed with toluene for 5 times. The surface was cured at 100 °C in an oven for 1 h, and it was stored in a desiccator until use.²⁴ This gave amino mica surface **2.3**. The surface was incubated in a solution of compound **2.4** (2.5 mM) and Et_3N (0.5%) in CHCl_3 at rt for 5 h under inert atmosphere with occasional gentle shaking. The mica was taken out from the solution and washed with CHCl_3 (3 times) and MeOH (3 times) sequentially, and then dried by blowing a gentle flow of argon over the surface for about 2 min. Unreacted amino groups were capped by incubating the surface in a solution of Ac_2O (10 mM) and pyridine (10 mM) in CHCl_3 at rt for 3 h. The surface was washed and dried as described above giving surface **2.5**.

2.4.3 Preparation of Mica Surface 2.1

Surface **2.5** was incubated in a 20% solution of piperidine in DMF (v/v) at rt for 30 min. It was then washed with DMF (3 times), CHCl_3 (3 times), and MeOH (3 times), and dried by blowing a gentle flow of argon over the surface for about 2 min. This gave surface **2.6** (Scheme 2.1). The surface was incubated in a solution of compound **2.7** (10 mM) and Et_3N (30 mM) in DMF at rt for 12 h under inert atmosphere. It was then washed with DMF (3 times), CHCl_3 (3 times), and MeOH (3 times), and dried as described above. Unreacted amino groups were capped by incubating the surface in a

solution of Ac₂O (10 mM) and pyridine (10 mM) in CHCl₃ at rt for 3 h. The surface was washed and dried as described above giving surface **2.8**. The surface was incubated in a solution of compound **2.9** (10 mM) in *i*PrOH at rt for 12 h. It was then washed with *i*PrOH (3 times), CHCl₃ (3 times), and MeOH (3 times), and dried as described above giving surface **2.10**. The surface was incubated in a solution of *N*-hydroxy-succinimide (NHS, 10 mM) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, 10 mM) in DMF at 50 °C for 5 h under inert atmosphere. It was then washed with dry DMF (3 times), and CHCl₃ (3 times), and dried as described above. The reaction was repeated for one more time to ensure completion. This gave surface **2.1**.

2.4.4 Aminization of AFM Probe

Commercially available AFM probe was washed with CHCl₃ (2 times) and dried by blowing a gentle flow of argon over the surface for about 2 min (Scheme 2.2). The probe was then incubated in a piranha solution (H₂O₂-H₂SO₄, v/v 1/3) at rt for 30 min, and washed with water (6 times), MeOH (3 times), and CHCl₃ (3 times), and dried by blowing a gentle flow of argon over the surface for about 2 min. A desiccator was flushed with argon. A solution of APTES (8% v/v) in freshly distilled toluene and the AFM probe were placed in the desiccator quickly.

The desiccator was sealed, and allowed to stand at rt for 12 h. The probe was taken out and placed in a beaker. The beaker was heated open to air on an oil bath with a pre-heated temperature of 95 °C for 10 min. After cooling to rt, the probe was washed with

CHCl₃ (3 times) and MeOH (3 times) sequentially, and dried by blowing a gentle flow of argon over the surface for about 2 min.¹⁵ This gave probe **2.11**.

2.4.5 Preparation of AFM Probe 2.2

Probe **2.11** was incubated in a solution of compound **2.4** (2.5 mM) and Et₃N (0.5%) in CHCl₃ at rt for 5 h under inert atmosphere with occasional gentle shaking (Scheme 2.2). The probe was taken out from the solution and washed with CHCl₃ (3 times) and MeOH (3 times) sequentially, and then dried by blowing a gentle flow of argon over the surface for about 2 min. This gave probe **2.12**. The probe was incubated in a solution of piperidine (20%, v/v) in DMF at rt for 30 min. It was then washed with DMF (3 times), CHCl₃ (3 times), and MeOH (3 times), and dried as described above giving probe **2.2**.

2.4.6 Splitting Single 1,2,3-Triazole Molecule

To the mica surface **2.1**, which had scratches at a location useful as reference for locating patterned AuNPs during imaging (see supporting information for details), in a shallow petri dish was added a few drops of *i*PrOH via a pipette. AFM tip **2.2** was moved to an area devoid of contamination close to a recognizable scratch under guidance of the AFM's optical camera, and a picture was taken with the camera. The probe was then engaged to the surface using 0 nm scan size with approximately 1 nN setpoint (Scheme 2.3). The instrument is then changed into a ramp mode. Ramping was carried out at six different locations on a line with 200 nm intervals. Each ramp has a chance to form **2.13** temporarily, which is then split into **2.14** and **2.15** during tip retraction. On each position, about 10 ramps were carried out. The ramp parameters were: ramp distance,

250 nm; ramp rate, 0.5 Hz; surface delay time 20 s; contact force 500 pN. The force curves were recorded. A typical one is shown in Figure 2.1. The force values are in Table 2.1. The resulting alkynes on the mica surface **2.14** were labeled with AuNPs as described below to give the image shown in Figure 2.2 (see insert).

2.4.7 Attaching AuNP to Locations on Mica That Have Alkynes Produced Via Splitting 1,2,3-Triazole

The surface **2.14** from the AFM experiment – splitting single 1,2,3-triazole molecule – was first incubated in a solution of *n*-BuNH₂ (10 mM) in CHCl₃ at rt for 3 h to inactivate the NHS ester groups on surface. The surface was then washed with CHCl₃ (3 times), and MeOH (3 times), and dried by blowing a gentle flow of argon over the surface for about 2 min. The procedure was repeated to ensure complete inactivation. The surface was then incubated in a solution of **2.9** (10 mM) in *i*PrOH at rt for 12 h, and washed with *i*PrOH (3 times), CHCl₃ (3 times), and MeOH (3 times), and dried as described above. The carboxylic acid groups were then activated by incubating in a solution of EDCI (10 mM) and NHS (10 mM) in DMF at 50 °C for 5 h under inert atmosphere, and the surface was washed with DMF (3 times), and CHCl₃ (3 times), and dried as described above. The activation was repeated for one more time. This gave **2.16**. The surface was then incubated in a solution of amino AuNP (**2.17**, 0.04 mM, 5 nm diameter) in DMF at rt for 12 h, and washed with DMF (8 times), water (8 times) and MeOH (5 times), and dried as described above. This gave surface **2.18**, which was used for imaging described below.

2.4.8 Imaging Mica Surface with Patterned AuNPs

The surface **2.18** and a Scanasyst-Air probe (f_0 70 kHz; k 0.4 N/m) from Bruker were mounted onto AFM in air. Using the optical camera of AFM as a guidance, the probe was moved to the same location of triazole rupture experiment on the surface using the scratches as reference by comparing to the picture taken during triazole rupture experiments (see supporting information for details). After engaging the tip to surface, an area of $60 \times 60 \mu\text{m}^2$ was scanned in air using the Scanasyst mode. AuNPs were detected as the reverse image of AFM tip at five of the six locations. The image is shown in Figure 2.2 (see insert). The height graphs of the five AuNPs are shown in Figure 2.2. To further ensure that the nanoparticles were not a result of non-specific adsorption and were lined up accidentally, two other surfaces were prepared and imaged under the same conditions; one was the same with six locations in a line at which probe **2.1** was ramped and the other with four locations at the corners of a square with sides of 500 nm at which probe **2.1** was ramped. The force values are in Table 2.2. AuNPs were detected as the reverse image of AFM tip at all of the expected locations. The images and the height graphs of AuNPs are shown in Figures 2.3-2.4.

2.5 Additional Information on Locating the Area of Patterned AuNPs on Surface for Imaging

The mica surface was scratched for searching the area of patterned AuNPs for imaging.

Before mounting the surface onto AFM for performing triazole rupture experiments, the surface was scratched at a location. After mounting the probe and surface onto AFM, an area devoid of contamination near the scratches was chosen for ramping, and a picture was taken using the optical camera of the AFM (left picture), which is a little bit blurred because the surface was in isopropanol. The probe was then engaged to the surface, and ramping was carried out in predefined locations in the area according to the experimental procedure in the paper, which split triazoles and generated alkynes on the surface. The surface was unmounted and processed to attach AuNPs to the locations of alkynes as described in the paper. It was then remounted onto AFM in air for imaging. With the guidance of optical camera and the picture taken during the triazole rupture experiment, the probe was moved to the location of alkynes generated by splitting triazole using the scratches as reference. A picture was taken using optical camera (right picture; probe dimension, length 115 μm , width 25 μm), which is more clear because the surface was in air. A relatively large area ($60 \times 60 \mu\text{m}^2$) was scanned to ensure that the patterned AuNPs were covered.

Figure 2.5. After mounting the probe and surface onto AFM

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Chapter 3

Solid Phase Stepwise Synthesis of Polyethylene Glycol

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Abstract

Polyethylene glycol (PEG) and derivatives with eight and twelve ethylene glycol units were synthesized by stepwise addition of tetraethylene glycol monomers on a polystyrene solid support. The monomer contains a tosyl group at one end and a dimethoxytrityl group at the other. The Wang resin, which contains the 4-benzyloxy benzyl alcohol function, was used as the support. The synthetic cycle consists of deprotonation, Williamson ether formation (coupling), and detritylation. Cleavage of PEGs from solid support was achieved with trifluoroacetic acid. The synthesis including monomer synthesis was entirely chromatography-free. PEG products including those with different functionalities at the two terminals were obtained in high yields. The products were analyzed with ESI and MALDI-TOF MS and were found close to monodispersity.

3.1 Introduction

Polyethylene glycol (PEG) has several attracting structural, physical and biophysical properties, which include stable, flexible and neutral backbone, and good solubility in water and many other solvents. It is biocompatible, nonimmunogenic and nonantigenic. For these reasons, it has been widely used in the pharmaceutical industry and biomedical research for PEGylating biomacromolecules to improve drug solubility and stability, and reduce immunogenicity and dosing frequency.¹⁻⁹ The PEG used for such applications requires a size of 4K Da to have the desired biophysical effects, and the most commonly used size is 40K Da.¹⁰⁻¹³ Besides applications as PEGylating agents in pharmaceuticals, PEG and its derivatives are also used frequently in other areas including surface science,¹⁴⁻¹⁵ nanotechnology,¹⁶⁻¹⁸ carbon nanotube functionalization,¹⁹⁻²¹ organic-inorganic hybrid materials²² and bioconjugation.²³⁻²⁶ In some of these cases, the sizes can be smaller than 1K Da and the compounds are more suitable to be called oligoethylene glycol (OEG), even though in some articles including this one they are still called PEG for convenience.

PEGs are typically made by polymerization of ethylene oxide. Due to the randomness of the process, the products are polydisperse admixtures of many different molecules of varying length and molecular weight.²⁷⁻²⁹ Although admixtures are currently used for PEGylation of pharmaceuticals, they are not ideal for reasons such as difficulty to achieve consistent composition for products from different batches, difficulty to characterize the products, losses of intended biological activities of

pharmaceutical ingredient due to heterogeneity of physical properties caused by the different sizes of the PEG tags, and challenges to obtain FDA approval.^{12,13,29,30-31} As a result, significant efforts have been made to synthesize monodisperse PEGs via stepwise organic synthesis.^{11-13,27-31,32-40} Currently, the longest PEGs that can be synthesized using stepwise organic synthesis are those with about 45 ethylene glycol units, which corresponds to molecular weights of about 2K Da.^{12,28,29} Challenges for the synthesis of longer PEGs include low efficiency of Williamson ether formation reaction when applied to long PEG substrates, depolymerization of PEG during the ether formation process,^{27,40} and the lack of technology to separate PEGs of different lengths.^{11,12,28,29} For short PEG (e.g. 8 – 40 ethylene glycol units) synthesis, one of the major problems is the need of multiple column chromatography to purify monomers, intermediates and the final product. This problem has severely impeded the practical use of stepwise synthesis for inexpensive large scale monodisperse PEG production.^{12,28,33,34,36}

In this paper, we report our results on using solid phase technology for stepwise synthesis of monodisperse PEGs and their derivatives. We envisioned that using solid phase technology, we would be able to avoid all chromatographic purifications. In addition, due to the ease of purification of intermediates and product by washing, we could use excess reactants to overcome the low efficiency of the key Williamson ether formation reaction. Further, because excess reactants could be used and the ether formation reaction could be performed at lower temperature and in a shorter time, the anionic PEG depolymerization reaction, which generates shorter PEGs that are impossible to remove from product,^{27,40} could be completely suppressed. Our results are

encouraging. Significantly, the monomers used for the synthesis did not require chromatographic purification and impure ones could be used directly, the Williamson ether formation reaction could proceed at room temperature with useful rates, the anionic PEG depolymerization might be completely suppressed, all intermediates in the solid phase synthesis were purified by washing, and pure final products were obtained without chromatography. Using the technology, we were able to synthesize close-to-monodisperse (PEG)₈ and (PEG)₁₂ and their derivatives with high yields.

3.2 Results and Discussion

3.2.1 The Solid Phase PEG Synthesis Design

The solid phase synthesis route is illustrated in Scheme 3.1. The (PEG)₄ (tetraethylene glycol) derivative **3.1**, which can be synthesized from the inexpensive (PEG)₄, was chosen as the monomer for the stepwise synthesis.

The Wang resin (**3.2**), which is 1% divinylbenzene cross-linked polystyrene dotted with 4-benzyloxy benzyl alcohols, was chosen as the solid support. The loading of the resin can be as high as 1.0 mmol/g and the resin can be purchased at remarkably low prices. A typical synthesis cycle consists of three steps, which are deprotonation, coupling and detritylation. In the first step, the resin is deprotonated with a base such as *t*BuOK to convert **3.2** to **3.3**. In the second step, the alkoxide **3.3** is coupled with monomer **3.1** via the Williamson ether formation reaction to give **3.4**. In the third step, the 4,4'-dimethoxytrityl (DMTr) protecting group in **3.4** is removed with a dilute acid to give **3.5**.

Scheme 3.1. The solid phase stepwise PEG synthesis design.

The cycle is then repeated to give **3.6**. At this stage, the PEG is asymmetric, which is highly desirable in most applications but more challenging to make using other methods.^{12,28,34} However, if it were cleaved from the solid support, it would become symmetric. As a result, before cleavage, **3.6** should be functionalized to give **3.7**, in which the R group must be stable under the acidic conditions needed for the cleavage.

After functionalization, the product is cleaved from the resin with a strong acid such as TFA to give asymmetric PEG **3.8** (Scheme 3.1).

3.2.2 Analytical Methods for Monitoring the Completeness of Solid Phase PEG Synthesis Reactions

To successfully implement the solid phase PEG synthesis design to make monodisperse PEGs without any chromatographic purifications, each of the three steps in all synthesis cycles must be 100% complete and devoid of any side reactions that alter the length of the growing PEG. Therefore, analytical methods that could accurately monitor the progress of the reactions were needed. During the course of our studies, we successfully evolved such methods. For steps 1-2, in the first cycle (converting **3.2** to **3.4**, Scheme 3.1), because the reactions were found easy to complete, we did not develop any method to check their completeness. For subsequent cycles, since the Williamson coupling reaction is not highly efficient and the efficiency of the reaction drops with increasing length of PEG substrates as demonstrated in solution phase synthesis,^{11,28} monitoring the progress of the reaction to ensure complete reaction before carrying out step 3 was crucial. Otherwise, the final PEG product would not be monodisperse. Therefore, before next detritylation, a small portion of resin (e.g. 4 mg) was treated with TFA, and the cleaved PEG was analyzed with ESI-MS (Method A). If only peaks corresponding to expected PEG product appeared, the reaction was complete. If peaks corresponding to PEG that did not undergo the coupling reaction appeared, the reaction was incomplete. If the latter happens, the resin was subjected to deprotonation and

coupling again. This was repeated until un-coupled PEG peaks completely disappear in MS.

For step 3 (e.g. converting **3.4** to **3.5**, Scheme 3.1), complete detritylation before going to the next deprotonation and coupling reactions was also required for obtaining monodisperse PEG product. Otherwise, the un-detritylated PEG would fail to couple in the current cycle and gave PEGs shorter than the final PEG product at the end of the solid phase synthesis. Since the trityl cation side product of the detritylation reaction had an intense red to light yellow color depending on concentration, it was possible to visualize the progress of the reaction. However, this was not enough to ensure complete detritylation to the degree necessary for obtaining monodisperse PEG at the end of the synthesis. For this reason, we evolved two methods to monitor detritylation more accurately. One was to analyze the last batch of detritylation solution with ESI-MS (Method B). Specifically, a portion of the last batch of detritylation solution was concentrated to dryness and dissolved in methanol (e.g. 2 ml), and the methanol solution was analyzed with ESI-MS. If DMTr cation was observed, the detritylation reaction was incomplete. In this case, detritylation was repeated. In our studies, we tested the sensitivity of ESI-MS to detect DMTr cation using standard DMTrCl solutions, and found that the cation could be reliably observed when the concentration of DMTrCl solution was above 0.001 ng/ μ l. If DMTr cation was not observable in ESI-MS, to further ensure complete detritylation, we intended to analyze the resin using an additional method (Method C). This method involved treating a small portion of the resin (e.g. 6 mg) with a mixture of excess *t*BuOK and BnBr, which alkylated the

detritylated PEGs but not un-detritylated ones. After cleaving with TFA, the products were analyzed with ESI-MS. If MS showed that all PEGs were benzylated, the detritylation reaction was complete. If un-benzylated PEG appeared and treating the resin with *t*BuOK and BnBr further did not reduce un-benzylated PEG, detritylation was incomplete (Method C). In the latter case, detritylation should be executed more times.

3.2.3 Synthesis of (PEG)₁₂ on a DNA/RNA Synthesizer

To find out the feasibility of the solid phase technology and quickly identify the conditions for each step of the synthesis cycle, we started our studies using an ABI-394 DNA/RNA synthesizer on a small scale (275 mg resin, 0.9 mmol/g loading, 0.25 mmol PEG product) using a similar procedure we reported previously for the synthesis of peptides and peptide nucleic acids using the same synthesizer.⁴¹ A homemade synthesis column was used as the reaction vessel, which was similar to the commercially available 10 μ mol DNA synthesis column for the synthesizer except that it had a larger capacity (10 ml, Figure 3.8). To agitate the reaction mixture, the column was connected to the synthesizer with relatively long lines so that it could be placed on an orbital shaker. Using the setup, the reagents and solvents including 0.1 M *t*BuOK THF solution, 0.31 M THF solution of monomer **3.1**, TCA solutions, DCM and THF could be delivered to the synthesis column conveniently from the bottles on the synthesizer.

The required monomer **3.1** is a known compound, and was conveniently synthesized from (PEG)₄ in two steps by tritylation with DMTrCl and tosylation with tosyl chloride (TsCl).^{28,42-43} The products were purified with flash column

chromatography. Using the procedure, we were able to prepare **3.1** at scales of 10 grams with reasonable efforts. The solid phase synthesis started with swelling the resin in THF for about 20 minutes, which was then removed using the synthesizer's reverse flush function. Deprotonation was achieved using 0.1 M *t*BuOK THF solution at room temperature for 30 minutes. This converted **3.2** to **3.3** (Scheme 3.1). The excess base and solvent were removed by reverse flush and rinsing the resin with dry THF. Coupling was achieved by delivering a 0.31 M solution of monomer **3.1** in THF to the column and shaking the column at room temperature for seven hours. This converted **3.3** to **3.4**. After coupling, the excess monomer **3.1** was conveniently recovered by delivering to a bottle on the synthesizer, which contains a basic solution (e.g. 5% Na₂CO₃). Under these conditions, the recovered **3.1** had good purity with only slight contamination by the side product resulted from β -elimination of **3.1** as indicated by TLC (Figure 3.9). As stated earlier, we did not design a procedure for monitoring the completeness of the deprotonation and coupling steps for the first synthesis cycle. However, to obtain monodisperse PEG at the end of solid phase synthesis, complete conversion of **3.2** to **3.4** must be achieved. Otherwise, the remaining hydroxyl group in **3.2** would participate in the coupling reaction in the subsequent cycles, and gave shorter PEGs. With these considerations, we repeated the deprotonation and coupling steps two more times under similar conditions. The resin was then washed thoroughly and subjected to detritylation (converting **3.4** to **3.5**), which was achieved using 3% trichloroacetic acid (TCA) in DCM and 3% TCA in toluene. The acid solutions were delivered to the column and then removed by reverse flush. We monitored the progress of the reaction with ESI-MS using

Methods B and C, and found that complete detritylation could be achieved with 3% TCA in DCM for 10 times followed by 3% TCA in toluene for five times. This concluded the first synthesis cycle and the resin-(PEG)₄ (**3.5**) was obtained.

In the second synthesis cycle for converting **3.5** to **3.10** (Scheme 3.2), the deprotonation and coupling steps were carried out under the same conditions used in the first cycle.

Scheme 3.2. Solid phase stepwise synthesis of (PEG)₁₂ (**3.13a**).

After coupling four times, ESI-MS analysis using Method A indicated that the reaction was complete. Detritylation of **3.9** to give **3.10** was achieved under similar conditions used in the first cycle except that the resin was further treated with a cocktail

containing 5% TCA, 1% triisopropylsilane (TIPS), 5% MeOH, 5% thioanisole, and 5% phenol in DCM (Cocktail A) five times. ESI-MS analyses using Methods B and C indicated that the additional treatments were needed for 100% conversion of **3.9** to **3.10**. In the third cycle for converting **3.10** to **3.12** (Scheme 3.2), the deprotonation and coupling steps were conducted five times. ESI-MS analysis using Method A indicated complete reaction. Complete detritylation was achieved using 5% TCA in toluene (10 times), Cocktail A (10 times) and Cocktail B (20% TCA, 1% TIPS, 5% MeOH, 5% thioanisole, and 5% phenol in DCM; 10 times). We were aware that at this stage the detritylation conditions were quite harsh, and the PEG could be prematurely cleaved from the resin. We carefully analyzed the detritylation solutions with ESI-MS, and found that no detectable amount of PEG fell off from the resin, which indicated that the 4-benzyloxy benzyl alkyl ether linkage was completely stable under these conditions.

Cleaving the PEG **3.13a** from the resin **3.12** was found fairly straightforward even though we were concerned that the 4-benzyloxy benzyl alkyl ether linkage might be shielded by the PEG and the cleavage reaction might be difficult.²⁸ A portion of **3.12** (128 mg out of 384 mg) was treated with pure TFA at room temperature for two hours (Scheme 3.2). The resin was washed with THF, and the washes and TFA were combined. The volatiles were evaporated, and the residue was co-evaporated with water. Pure PEG **3.13a** was obtained as a light yellow oil. ESI-MS (Figure 3.1) and ¹H and ¹³C NMR (Figures A.1-A.2) analyses indicated that the synthesis was successful.

Figure 3.1. ESI-MS of (PEG)₁₂ (3.13a) from small scale synthesis.

In ESI-MS, we did not see any (PEG)₄ and (PEG)₈, which indicated that all the reactions from 3.2 to 3.12 were 100% complete. One impurity was (PEG)₁₁, which was most likely from the starting (PEG)₄ used for making monomer 3.1 because ESI-MS analysis of (PEG)₄ also showed small amount of (PEG)₃ (Figure A.3). This observation was consistent with previous reports, which also noted contamination of commercial (PEG)₄ with (PEG)₃.²⁸ Another possibility for the appearance of (PEG)₁₁ was depolymerization of the deprotonated PEG intermediates^{27,40} but this possibility was

low given the mild conditions we used in the coupling step.²⁷ Other than containing minute amount of (PEG)₁₁, PEG **3.13a** was highly pure. We also analyzed the sample with MALDI-TOF-MS (image in ESI, A.23). The (PEG)₁₁ impurity was less obvious. The amount of **3.13a** obtained was 36 mg, which corresponds to an 81% overall yield based on 33% (128 mg out of 384 mg) of 275 mg resin and 0.9 mmol/g loading of resin **3.2**. However, in theory, since all reactions from **3.2** to **3.12** were 100%, the overall yield should be quantitative (45 mg). The discrepancy between theoretical and experimental yields could be attributed to incomplete cleavage of **3.13a** from **3.12**, but we subjected the remaining resin to additional cleavage and no more PEG was obtained. Other reasons for the lower than theoretical yield could be loss of resin during the synthesis, the consumption of portions of resin for ESI-MS analysis, loss of PEG after cleavage and lower than stated loading of the resin.

3.2.4 Synthesis of Longer PEGs and PEG Derivatives

After successful synthesis of (PEG)₁₂, we tested to couple **3.12** with **3.1** to synthesize longer PEGs under similar conditions used in the first three cycles. For the synthesis of (PEG)₁₆, after five rounds of coupling, ESI-MS analysis using Method A showed 86% (PEG)₁₆ and 14% (PEG)₁₂ (Figure A.4). For (PEG)₂₀, the resin carrying (PEG)₁₆ and (PEG)₁₂ was coupled with **3.1** five times. ESI-MS analysis using Method A showed 77% (PEG)₂₀ and 23% (PEG)₁₆ (Figure A.5). Although these results were significant because the longer PEGs could be easily separated from shorter ones using techniques involving tagging followed by chromatography, polymerization or

extraction^{34,36,44-47} such studies had not been pursued. Instead, it came to our attention that the synthesis of pure (PEG)₁₂ and its asymmetric derivatives using a convenient method without any chromatography is highly significant. The compound and some of its derivatives are known^{26,28,36,48} and commercially available, but they could have been made using procedures involving multiple column chromatography purifications and therefore are highly expensive. With this consideration, we decided to pursue the synthesis of derivatives of **3.13a** and the synthesis of one of them on a multiple gram scale.

A significant advantage of the solid phase method is that the difficult-to-access asymmetric PEG derivatives can be easily synthesized.¹² To demonstrate this, we prepared compounds **3.13b-e** (Scheme 3.3).

The asymmetric BnO(PEG)₈ (**3.13b**) was synthesized by simply soaking **3.10** in a THF solution of BnBr and *t*BuOK, which gave **3.14**. Treating **3.14** with TFA gave the product **3.13b**. The product was purified by precipitation from THF with Et₂O. Derivatization of **3.13b** to **3.13d** was achieved in solution using the excellent reaction conditions for tosylation of alcohols first reported by Ouchi and co-workers.⁴⁹ Thus, treating **3.13b** with slightly excess TsCl in the presence of NaOH in a solvent mixture of THF and water at room temperature quantitatively converted the starting material to **3.13d**. The product was purified by partition between water and ether, passing through a Celite pad and precipitation from diethyl ether with hexanes.

Scheme 3.3. Synthesis of (PEG)₈ and (PEG)₁₂ derivatives **3.13b-e**.

For the synthesis of **3.13c**, we coupled **3.10** with the monomer **3.15** to give **3.16** directly (Scheme 3.3) instead of using the route involving benzylation of **3.12**. The required additional monomer **3.15** was prepared on small scale according to literature procedure.⁵⁰⁻⁶⁰ Cleavage of **3.13c** from **3.16** and tosylation of **3.13c** to give **3.13e** were achieved as described for **3.13b** and **3.13d**. As described below, using the solid phase method, we actually did not need to use pure monomers **3.1** and **3.15**, and the monomers did not need any chromatographic purification. If this were considered, preparing **3.13b-**

e were all entirely chromatography-free. The products were analyzed with ESI-MS and ^1H and ^{13}C NMR (Figures A.6-A.13). As shown in ESI-MS (Figure 3.2), compounds **3.13c** and **3.13e** were devoid of derivatives of $(\text{PEG})_4$ and $(\text{PEG})_8$, which could be formed from incomplete reactions during solid phase synthesis, were not observable. Derivatives of $(\text{PEG})_{11}$, which could be from the $(\text{PEG})_3$ in the starting $(\text{PEG})_4$ or less likely from depolymerization of PEG under basic conditions,^{27,40} could be observed, but the amounts were minimal. Similar results were observed for **3.13b** and **3.13d** (Figures A.14-A.15). We also analyzed **3.13b-e** with MALDI-TOF-MS (images in ESI, A.24-A.27). The impurities arisen from PEG depolymerization were less obvious.

Figure 3.2. ESI-MS of $\text{BnO}(\text{PEG})_{12}$ (**3.13c**, left) and $\text{BnO}(\text{PEG})_{12}\text{OTs}$ (**3.13e**, right) from small scale synthesis.

3.2.5 Synthesis of BnO(PEG)₁₂ Manually on a Larger Scale in a Peptide Synthesis Vessel

After establishing feasibility of the solid phase PEG synthesis method and successful identification of reaction conditions using small scale synthesis on an automated synthesizer, to demonstrate the practical usefulness of the method, we decided to prepare the asymmetric BnO(PEG)₁₂ (**3.13c**) on a significantly larger scale (theoretically 6.3 mmol, 4.02 grams of product). The synthetic route involving coupling **3.10** with **3.15** was used (Scheme 3.3). Relatively large amount of monomers **3.1**,^{27,28,42-43,55} and **3.15**⁵⁰⁻⁶⁰ was required for the synthesis and they were prepared using procedures similar to reported ones with slight but important modifications. Importantly, we did not purify any of the compounds with column chromatography, and impure monomers were used directly for the solid phase synthesis.

Scheme 3.4. Synthesis of monomers DMTrO(PEG)₄OTs (**3.1**) and BnO(PEG)₄OTs (**3.15**) at multigram scale without chromatography.

For the synthesis of **3.1** (Scheme 3.4), the scale was at the level of 147 mmol of DMTrCl, which was the limiting starting material for the first step of the synthesis. With this scale, theoretically 96 grams of **3.1** could be produced at the end of monomer synthesis. Slow addition of a DMTrCl (1 equivalent) solution in pyridine to the solution of (PEG)₄ (5 equivalent) in the same solvent gave the desired DMTrO(PEG)₄ and the symmetric ditritylated (PEG)₄ side product **3.17**. Other materials in the crude reaction mixture include pyridine, excess (PEG)₄, and pyridinium chloride. These impurities were readily removed by evaporation and partition. After these simple manipulations, TLC (Figure 3.6) and ¹H NMR (Figure A.16) indicated that the desired product DMTrO(PEG)₄ was only contaminated with small quantities of **3.17**. Compound **3.17** could be removed with flash column chromatography, but at this scale, it was inconvenient and therefore not pursued. Because the next tosylation reaction and later the coupling reaction on solid phase using **3.1** did not require accurate amount of materials, we did not determine the molar ratio of **3.1** and **3.17**, and simply treated the mixture as pure **3.1** for calculation purpose even though it was possible that about 5 mol% **3.17** were present. Using the procedure involving TsCl and NaOH,¹⁵ DMTrO(PEG)₄ was converted to monomer **3.1** with quantitative conversion according to TLC. After several simple manipulations including partition and precipitation, monomer **3.1** was only contaminated with **3.17** according to TLC (Figure 3.3) and ¹H NMR (Figure A.17).

Figure 3.3. TLC of DMTrO(PEG)₄OTs (**3.1**) synthesized at large scale without chromatography purification. Eluent: hexanes/EtOAc/Et₃N 7:3:0.5. Left lane, DMTrO(PEG)₄ contaminated with **3.17**; middle lane, co-spot of left and right lane samples; right lane, product from the tosylation reaction without chromatography purification

Again, **3.1** and **3.17** were not separated and the mixture was used directly for solid phase PEG synthesis. For the synthesis of **3.1**, it is notable that due to the use of the slow addition technique, we were able to keep the ditritylated side product **3.17** at a minimum while using only five equivalents (PEG)₄. In contrast, in some previous reports concerning the synthesis of DMTrO(PEG)₄ and similar compounds, 10 equivalents (PEG)₄ were used and the amount of symmetric side product could be higher.^{28,33,34}

The synthesis of monomer **3.15** was carried out at the scale of 208 mmol NaH, which was the limiting starting material of the first step of the synthesis (Scheme 3.4).

With this scale, theoretically 73 grams of **3.15** could be produced at the end of monomer synthesis. Although synthesis of the compound has been reported previously,⁵⁰⁻⁶⁰ to minimize the formation of di-benzylated and depolymerized products [e.g. BnO(PEG)₃]⁴⁰ and avoid chromatography purification, we modified the conditions, which mainly featured the use of a different ratio of (PEG)₄, NaH and BnBr, carrying out alkylation at lower temperature and timely quenching the reaction. We treated four equivalents (PEG)₄ in THF with one equivalent NaH at 0 °C. The resulting alkoxide was alkylated with 0.8 equivalents BnBr at 40 °C. This gave the mono-benzylated BnO(PEG)₄ and small quantities of di-benzylated **3.18** according to TLC (Figure 3.7) and ESI-MS (Figure A.18). After removing the remaining (PEG)₄, NaBr, and THF by simple manipulations such as evaporation and partition, The product BnO(PEG)₄ and **3.18** were not separated, and the mixture were subjected to tosylation as described for the synthesis of **3.1** (Scheme 3.4). Without chromatography, monomer **3.15**, which was contaminated with **3.18** (TLC, Figure 3.4; ¹H NMR, Figure A.19), was used directly for the solid phase PEG synthesis. For the synthesis of BnO(PEG)₄, in reported procedures, more equivalents NaH or other bases were generally used. Under those conditions, the yields of mono-benzylated product were usually around 70% or lower⁵⁰⁻⁵⁹ although a few papers reported higher yields.⁶⁰

Figure 3.4. TLC of BnO(PEG)₄OTs (**3.15**) synthesized at large scale without chromatography purification. Eluent: hexanes/EtOAc 1:1. Lane 1 (from left), TsCl; lane 2, co-spot of lanes 1 and 3 samples; lane 3, BnO(PEG)₄ contaminated with **3.18**; lane 4, co-spot of lanes 3 and 5 samples; lane 5, product of the tosylation reaction without chromatography. The amount of **3.18** is minute and cannot be easily seen but can be seen in ESI-MS (Figure A.18).

The product was mostly purified with chromatography to remove the di-benzylated **3.18** except that in one report, a two-step procedure was used, which enabled chromatography-free production of pure BnO(PEG)₄.⁶¹ Compared to known methods, we achieved higher yields of mono-benzylated product and potentially lower percentage of di-benzylated product. In addition, our conditions could have also reduced the depolymerized BnO(PEG)₃, which is highly important for stepwise monodisperse long PEG synthesis because a small percentage of shorter monomers will rapidly accumulate in the growing PEG in a repetitive synthesis and render the entire synthesis useless.^{27,40} The above advantages of the modified conditions can be explained using Scheme 3.5.

a higher molar ratio of (PEG)₄ over NaH or other bases than in previous reports, in the course of the reaction, the molar ratio of **3.22** over **3.19** (**3.20** as well) was minimized according to the equation $[3.22]/[3.19] = K[3.21]/[(\text{PEG})_4]$ where K is the equilibrium constant, and therefore the chance for the formation of **3.18** was reduced. The use of less equivalents BnBr than NaH was intended to minimize the formation of the depolymerized product BnO(PEG)₃.⁴⁰ With less BnBr, at the stage close to the end of the reaction, the last few molecules of BnBr were expected to be easier to find their reaction partners compared with the case in which equal moles of NaH and BnBr were used. This minimized the life time of **3.19** -**3.20** that were converted to **3.21**, and therefore reduced their chance to depolymerize to **3.23**, which could be benzylated to **3.24** including the highly undesired BnO(PEG)₃. Under our conditions, we believe that depolymerization to give BnO(PEG)₃ was minimal. The small amount of BnO(PEG)₃ in ESI-MS (Figure A.18) was likely from the minute (PEG)₃ in (PEG)₄ starting material (Figure A.3). It is important to note that with less equivalents BnBr than NaH, even after the reaction was complete, if it had not been quenched timely, the product **3.21** would have had more chances to equilibrate to **3.22**, which could be depolymerized to BnO(PEG)₃.⁴⁰ Therefore, the reaction should be closely monitored and quenched once BnBr was consumed. An alternative is to use more equivalents BnBr than NaH, in which case at the end of the reaction, no anionic species remain, and therefore there is no need to quench the reaction timely. However, a drawback is that the remaining BnBr is difficult to be completely removed from product without chromatography.

With procedures for easy access of large quantities of **3.1** and **3.15** in hand, the solid phase PEG synthesis virtually has no limitations in terms of scalability. To execute our plan on synthesizing BnO(PEG)₁₂ (**3.13c**) at 6.3 mmol scale, seven grams of Wang resin (0.9 mmol/g loading) was swelled in a simple 100 ml peptide synthesis vessel (Figure 3.10) in THF. The volume of the swelled resin was about 50 ml. THF was removed, and the resin was treated with 0.2 M *t*BuOK (1.1 equivalents) for 20 minutes at room temperature. After removing the liquids, the resin was rinsed with dry THF, and the solution of monomer **3.1** (0.31 M, 2 equivalents), which was contaminated with **3.17** (at calculation, **3.1** was assumed pure, so the actual concentration and equivalents were slightly lower), was added to the synthesis vessel. The vessel was rotated gently on a rotatory evaporator at room temperature for 24 hours (Figure 3.10). This gave **3.4**. The ditritylated **3.17** was inert during the coupling reaction, and was conveniently removed with the excess **3.1** by filtration. The recovered **3.1** (TLC, Figure 3.11; ¹H NMR, Figure A.20), which was in the filtrate and contaminated by **3.17** and the side product resulted from β-elimination of **3.1**, could be reused, but since we had synthesized large quantities of **3.1**, this had not been tested. The resin was washed sequentially with THF, THF/H₂O (v/v 1:1) and dry THF, and dried under vacuum. The deprotonation and coupling steps were repeated two more times using 0.8 equivalents *t*BuOK and 1.5 equivalents **3.1** to ensure complete alkylation of **3.3**. As in our small scale studies, the completeness of the reaction was not assessed.

For removing the DMTr group on **3.4** to give **3.5**, conditions for the small scale studies as described earlier with slight modifications were used (see details in ESI).

Deprotonation of **3.5** and alkylating with **3.1** to give **3.9** in the second synthetic cycle were performed under similar conditions in the first cycle. However, after finishing the first two couplings (first coupling, 1.1 equivalents *t*BuOK, 2 equivalents **3.1**; second coupling, 0.8 equivalents *t*BuOK, 1.5 equivalents **3.1**; room temperature, 24 hours for each coupling), we carried out ESI-MS analysis using Method A. The result indicated that the reaction had already reached 100% completion. Therefore, the third coupling was not executed. This informed us that the coupling step in the first cycle could be simplified as well. Detritylation of **3.9** to give **3.10** was achieved under the similar conditions described for converting **3.4** to **3.5** (see details in ESI). The completeness of the detritylation was determined with ESI-MS using Methods B and C. Coupling of **3.10** with **3.15** (Scheme 3.3), which was contaminated with **3.18**, was carried out using the same procedure described for converting **3.5** to **3.9** in this larger scale synthesis. ESI-MS analysis using Method A after two couplings also indicated that the reaction reached completion. It is noted that the recovered excess **3.15** after the couplings was also of good quality according to TLC (Figure 3.12) and could be reused. Cleaving **3.13c** from the resin (**3.16**) was achieved using TFA at room temperature as described for small scale synthesis. The product (**3.13c**) appeared as a light yellow thick oil. The amount was 3.22 grams, which corresponds to an 80% overall yield based on a 0.9 mmol/g loading of resin **3.2**. The lower than 100% yield could be caused by factors described in small scale synthesis of **3.13a**.

PEG **3.13c** was analyzed with ESI-MS (Figure 3.5), and ^1H and ^{13}C NMR (Figures A.21-A.22), which indicated that the synthesis was successful. Like the small scale

synthesis, we did not see any (PEG)₄ and (PEG)₈ in ESI-MS. However, small amount of BnO(PEG)₁₁ was observable. Again, this impurity may come from (PEG)₃ in the (PEG)₄ starting material or less likely depolymerization of the deprotonated PEG in the coupling step.^{27,40} Other than that minute impurity, the product was highly pure.

Figure 3.5. ESI-MS of BnO(PEG)₁₂ (3.13c) from larger scale synthesis using a peptide synthesis vessel.

2.3.6 Additional Discussions

Overall, our study has demonstrated that synthesis of monodisperse PEGs using stepwise solid phase technology is feasible. In addition, we have found that the Williamson ether formation reaction can proceed at room temperature with acceptable rates when one reactant is in excess. Carrying out this reaction at room temperature has several significant advantages, which include less likely to form the difficult-to-remove shorter PEGs resulted from depolymerization of deprotonated PEG reactants (refer to **3.23** in Scheme 3.5),^{27,40} less likely for the tosylate reactant to undergo β -elimination, which consumes reagent and potentially cause problems for product purification,²⁸ less capital cost for large scale synthesis and automated synthesis, and as it is almost always true that milder conditions give cleaner products. Other significant findings include that the 4-benzyloxy benzyl alkyl ether linker and the DMTr protecting group of the monomer are compatible for the synthesis even though both are acid-sensitive and the linker has to stay intact completely when the DMTr group is removed under acidic conditions repeatedly. The polar *t*BuOK can penetrate into the relatively hydrophobic polystyrene matrix to efficiently deprotonate hydroxyl groups. Deprotonation of PEGs on the solid support with *t*BuOK prior to adding the tosylate monomers does not cause depolymerization of PEGs at all or to any noticeable degree. With a procedure that separates deprotonation and alkylation (i.e. do these sequentially instead mixing the alcohol and tosylate with the base), the tosylate β -elimination side reaction is minimal. The linker can be readily cleaved using TFA even though it might be wrapped by PEGs, which may slow down the reaction.²⁸ In addition, we have developed reliable analytical

methods for monitoring the progress of the solid phase reactions (i.e. Methods A, B and C). Developing these methods is highly important because using the methods, we can ensure that every step is 100% complete, which is required for making monodisperse PEG.

The solid phase PEG synthesis technology has several advantages compared with typical solution phase technologies: (1) There is no need to use column chromatography to purify monomers. This has been demonstrated by our larger scale synthesis in a peptide synthesis vessel. The impurities in the monomers such as **3.17** and **3.18** were inert during the coupling reaction and were conveniently removed by simple washings after coupling. In contrast, using solution phase synthesis, the monomers have to be purified with chromatography and impurities such as **3.17** and **3.18** have to be removed, because even though they are inert during coupling, they will contaminate the product and may become more difficult to remove. One exception is the method reported by Kinbara group.^{34,36} The authors elegantly demonstrated the synthesis of monodisperse PEGs without chromatography. However their method is still not ideal because it suffers from drawbacks including limited length of PEGs that can be synthesized, multiple extractions, loss of precious product in each coupling step as symmetric dimers, decreasing purity of product with increasing PEG length, and potential complexity caused by the β -elimination side reaction.²⁸ (2) There is no need to handle the highly hydrophilic PEG intermediates and purifying them with column chromatography. Highly polar and water soluble organic compounds are usually difficult to handle and purify with column chromatography by synthetic chemists. Using solution phase

methods, after every step, the PEG product has to be isolated and purified with column, which is expensive and imposes significant challenges. The challenge becomes more serious as the length of PEG increases. Using the solid phase technology, these problems do not exist at all. (3) The difficult-to-remove shorter PEGs resulted from depolymerization of deprotonated PEG intermediates^{27,40} can be avoided or kept minimum. Due to the simplicity to clean up the PEG product anchored to solid support by washing, excess monomers can be used for the coupling reaction to increase reaction rate and lower reaction temperature. Both decrease the chance of PEG depolymerization. In contrast, in solution phase synthesis, if one reactant is used in excess, the product purification process usually becomes complicated. As a result, close to equal molar reactants are usually used and the reaction is typically carried out at elevated temperatures.^{27,28,33,34,39} (4) The vinyl ether side product from β -elimination of tosylate has no chance to affect PEG product purity. During the coupling reaction, slight β -elimination of tosylate is unavoidable²⁸ although this is largely not discussed in previous reports. In solution phase synthesis, if it were not removed by chromatography, it could be hydrolyzed, incorporated into growing PEGs and generate shorter PEGs. Using the solid phase technology, the vinyl ethers were conveniently washed away. (5) The solid phase method does not involve using any transition metal catalyst or high dilution technique. Some of the solution methods require these and therefore may be less ideal for large scale monodisperse PEG production.^{12,29} (6) Finally, one important advantage that solid phase synthesis always enjoys is the possibility for automation. Since excess reagents can be removed by simple washing and all the reactions on solid

support can be carried out at room temperature, it is easy to envision that the PEG synthesis could be readily achieved on commercially available peptide synthesizers without any modification of the instruments.

To evaluate the practical utility of the solid phase technology for large scale PEG production, a rough estimation of the cost structure for the synthesis of BnO(PEG)₁₂ (**3.13c**) at the scale of theoretical 1 mol (637 g) final PEG product is given. If the synthesis is carried out in one batch, a reaction vessel of 15 L is needed. The required chemicals mainly include resin **3.2**, (PEG)₄, DMTrCl, TsCl, BnBr, NaH, *t*BuOK, TCA, TFA, NaOH and solvents. The amount of resin **3.2** is 1.1 kg, which is roughly two times the mass of the product. We were able to purchase **3.2** at a price of \$1.8/g for 100 g. With this price, which could be lower if more were purchased, 1.1 kg costs \$1,980. The cost of other chemicals including solvents is estimated to be around \$5,000 if the procedure described for the synthesis of **3.13c** in the peptide synthesis vessel is scaled up proportionally. So, the total material cost is around \$7,000. Potentially, the resin can be reused, and the excess monomers in the coupling step and the TCA solution in the detritylation step as well as the TFA for cleavage could be recovered in a cost efficient fashion. If these were considered, the material cost could be lower. For labor, one chemist should be able to finish the synthesis in two weeks without having to work intensely. Considering that compounds similar to **3.13c** are currently highly expensive (e.g. Ph₃CO(PEG)₁₂OCPh₃ \$850-1,300/g, Ph₃CO(PEG)₁₀ \$1,800/g) and the high prices may be a result of the need of multiple column chromatography purifications during their production, there is a high chance for the solid phase PEG synthesis method to find

practical use. Another way to evaluate the practical utility of the solid phase method is to compare its cost structure with that of typical solution phase methods. The solid phase method needs a resin and excess monomers, but has the advantage of avoiding purification of monomers and intermediates with column chromatography. The overall yield of the final product is high or quantitative. The product is more likely to be devoid of impurities resulted from depolymerization. In contrast, typical solution phase methods do not need a resin and use close to equal molar reactants. These reduce material costs. However, they typically need multiple tedious and expensive chromatographies to purify PEG starting materials, intermediates and product, and when the PEG compounds are long, purification may not always be feasible. In addition, the overall yield of the PEG product will not be quantitative and can be quite low.¹² Overall, we believe that the additional costs from resin and excess monomers in our solid phase method can be easily offset by the costs of column chromatography purifications in solution phase methods, and there is a high chance for the solid phase method to be widely adopted for PEG synthesis.

2.4 Conclusions

In conclusion, we have demonstrated that the stepwise solid phase technology is suitable for the synthesis of monodisperse or close to monodisperse PEGs and their derivatives. Advantages of the method include rendering the entire synthesis chromatography-free, milder conditions for the key Williamson ether formation reaction to minimize anionic depolymerization of PEG intermediates and increase

monodispersity of products, and high or quantitative overall yield. We also developed analytical methods for monitoring the completeness of the solid phase reactions, which is required for the synthesis of monodisperse PEGs. Using the technology, we successfully synthesized PEG derivatives with eight and twelve ethylene glycol units with close to monodispersity. Currently we are tuning conditions to achieve long PEG synthesis with little purification efforts and searching solutions to further increase monodispersity of PEG products.

2.5 Experimental Section

2.5.1 General Experimental Information

All reagents including (PEG)₄ (tetraethylene glycol, 99%, Aldrich), DMTrCl (4,4'-dimethoxytrityl chloride, 99.7%, Chem-Impex), TsCl (tosyl chloride, 98%, Aldrich), NaH (60% in mineral oil, Aldrich), BnBr (benzyl bromide, 99%, Bean Town Chemical), Wang resin (polystyrene functionalized with 4-benzyloxy benzyl alcohol, 1% DVB, 0.9 mmol/g loading, 100-200 mesh, AAPPTec LLC), *t*BuOK (1 M solution in THF, Aldrich), TCA (trichloroacetic acid, 99%, Aldrich) and TFA (trifluoroacetic acid, 99%, Aldrich), and solvents from commercial sources were used as received unless noted otherwise. (PEG)₄ was co-evaporated with toluene under vacuum. THF was dried using an Innovative Technology's PureSolv™ system. Pyridine was distilled over CaH₂ under nitrogen. All reactions were carried out in oven-dried glassware under nitrogen. Thin layer chromatography (TLC) was all performed using Sigma-Aldrich TLC plates, silica gel 60F-254 over glass support, 250 μm thickness. ¹H and ¹³C NMR spectra were

measured on a Varian UNITY INOVA spectrometer at 400 and 100 MHz, respectively. Chemical shifts (δ) were reported in reference to solvent peaks (residue CHCl_3 at δ 7.24 ppm for ^1H and CDCl_3 at δ 77.00 ppm for ^{13}C). HRMS was obtained on a Thermo HR-Orbitrap Elite Mass Spectrometer. LRMS was obtained on a Thermo Finnigan LCQ Advantage Ion Trap Mass Spectrometer. MALDI-TOF MS of compounds **3.13a-e**, which do not show impurities including depolymerized PEG products well, are included at the end of the supporting information. They were obtained on Bruker's microflexTM LRF MALDI-TOF System.

2.5.2 Large Scale Synthesis of Monomers 3.1 and 3.15

DMTrO(PEG)₄: The procedure is similar to reported ones^{28,42} with slight but important modifications. To a solution of (PEG)₄ (143 g, 127 ml, 738 mmol, 5 equiv.) in dry pyridine (150 ml) in a round-bottomed flask was added a solution of DMTrCl (50 g, 147.6 mmol, 1 equiv.) in dry pyridine (200 ml) via cannula dropwise under a nitrogen atmosphere at rt over ~8 h. After addition, stirring was continued at rt for ~5 h. Most pyridine was removed under reduced pressure with a small amount left to keep the mixture basic. The residue was partitioned between EtOAc (500 ml) and 5% Na_2CO_3 (300 ml). The organic phase was washed with 5% Na_2CO_3 (300 ml $\times$ 4), dried over anhydrous Na_2SO_4 , and filtered. The filtrate was evaporated to dryness, co-evaporated with toluene (50 ml $\times$ 2) and further dried under vacuum giving the product DMTrO(PEG)₄, which was contaminated with small amount of DMTrO(PEG)₄ODMTr (**3.17**), as a thick light yellow oil (total 70.2 g; TLC, Figure 3.6; R_f for DMTrO(PEG)₄,

0.10; for **3.17**, 0.80; SiO₂, hexanes/EtOAc/Et₃N 1:2:0.15; ¹H NMR, Figure A.16). The mixture was not separated and was used directly for the next tosylation reaction. The reaction was also performed at smaller scales and the product was purified with flash column chromatography and pure product was used for the next tosylation reaction.

Figure 3.6. TLC of DMTrO(PEG)₄ from large scale synthesis without chromatography. Eluent: hexanes/EtOAc/Et₃N 1:2:0.15. Left lane, DMTrOH; middle lane, co-spot of left and right lane samples; right lane, DMTr(PEG)₄ contaminated with **3.17**.

DMTrO(PEG)₄OTs (3.1): The solutions of DMTrO(PEG)₄ (contaminated with small amount of **3.17**; total 69 g; assumed 139 mmol as if it were pure, 1.0 equiv.) in THF (125 ml) and NaOH powder (22 g, 556 mmol, 4.0 equiv.) in water (75 ml) were combined and stirred at 0 °C for 30 min. The solution of TsCl (39.8 g, 208 mmol, 1.5 equiv.) in THF (100 ml, note that it is important to keep the ratio of total THF and water at around 3:1 v/v) was added dropwise over 10 min while the reaction mixture was stirred vigorously at 0 °C. After addition, stirring was continued while the temperature was raised to rt gradually. The progress of the reaction was monitored with TLC, and

complete reaction was observed within 24 h. The mixture was partitioned between 5% Na_2CO_3 (300 ml) and EtOAc (500 ml). The aqueous phase was extracted with EtOAc (200 ml $\times$ 2). The combined organic phase was dried over anhydrous Na_2SO_4 and filtered. Volatiles were removed under reduced pressure. The residue was dissolved in toluene and volatiles were removed under vacuum from an oil pump. Compound **3.1**, which was contaminated with **3.17**, was obtained as a thick light yellow oil (total 88.2 g; TLC, Figure 3; R_f for **3.1**, 0.25; for **3.17**, 0.50; SiO_2 , hexanes/EtOAc/ Et_3N 7:3:0.5; ^1H NMR, Figure A.17). The product,²⁸ which was contaminated with **3.17**, was dried over fresh Drierite under high vacuum overnight, and was used directly for the solid phase PEG synthesis. The reaction was also performed at smaller scales and the product was purified with flash column chromatography and pure product was used for solid phase PEG synthesis.

BnO(PEG)₄: The procedure is similar to reported ones⁴⁰⁻⁵⁰ with slight but important modifications. A suspension of NaH (60% in mineral oil, 8.3 g, 208 mmol, 1 equiv.) in dry THF (30 ml) under nitrogen was cooled on an ice bath. The solution of (PEG)₄ (144 ml, 833 mmol, 4 equiv.) in dry THF (100 ml) was added dropwise over ~1.5 h. After addition, the ice bath was removed. BnBr (20 ml, 167 mmol, 0.8 equiv.) was added via a syringe. The reaction mixture was stirred vigorously at 40 °C. The progress of the reaction was monitored closely with TLC. Once BnBr was consumed, which took 24 h in this case, the reaction was quenched with water timely. THF was removed under reduced pressure and the residue was partitioned between EtOAc (600 ml) and water (500 ml). The aqueous layer was extracted with EtOAc (200 ml $\times$ 2). The combined

organic phase was washed with water (400 ml $\times$ 4) and brine (300 ml $\times$ 2), dried over anhydrous Na₂SO₄, and filtered. The filtrate was evaporated to dryness and further dried under vacuum giving the product BnO(PEG)₄,⁵² which was contaminated with small amount of BnO(PEG)₄OBn (**3.18**), as a thick light yellow oil (total 42 g; TLC, Figure 3.7; *R_f* for BnO(PEG)₄, 0.40; for **3.18**, 0.80; SiO₂, EtOAc/MeOH 20:1).

Figure 3.7. TLC of BnO(PEG)₄ from large scale synthesis without chromatography. Eluent: EtOAc/MeOH 20:1. Left lane, (PEG)₄; middle lane, co-spot of left and right lane samples; right lane, product of the benzylation reaction without chromatography. In the right lane, **3.18** is much less than it appears to be because sample in the lane was heavily spotted. The middle lane was less spotted and relative amounts of **3.18** and BnO(PEG)₄ are better indicated. The TLC was stained with I₂ and then visualized under UV. If not stained with I₂, **3.18** could be hardly seen under UV due to small quantity. The small quantity of **3.18** can also be seen in ESI-MS (Figure A.18).

ESI-MS of the mixture showed small amount of BnO(PEG)_3 (Figure A.18), which was most likely from $(\text{PEG})_3$ in the $(\text{PEG})_4$ starting material according to ESI-MS (Figure A.3). BnO(PEG)_4 and **3.16** were not separated and the mixture was used directly for the next tosylation reaction. The reaction was also performed at smaller scales and the product was purified with flash column chromatography and pure product was used for the next tosylation reaction.

BnO(PEG)₄OTs (3.15): A similar procedure for the tosylation of DMTrO(PEG)_4 to give **3.1** was used. Thus reaction of BnO(PEG)_4 (contaminated **3.18**; total 40 g; assumed 284 mmol as if it were pure, 1 equiv.) in THF (125 ml), NaOH powder (22.5 g, 563 mmol, 4.0 equiv.) in water (75 ml) and TsCl (40 g, 211 mmol, 1.5 equiv.) in THF (100 ml) gave product **3.15**,⁵⁵ which was contaminated with **3.18**, as a thick light yellow oil (total 59.1 g, TLC, Figure 4; R_f for **3.15**, 0.30; for **3.18**, 0.32; SiO_2 , hexanes/EtOAc 1:1; ^1H NMR, Figure A.19). The product, which was contaminated with **3.18**, was dried over fresh Drierite under high vacuum overnight, and was used directly for the solid phase PEG synthesis. The reaction was also performed at smaller scales and the product was purified with flash column chromatography and pure product was used for solid phase PEG synthesis.

2.5.3 Small Scale Solid Phase PEG Synthesis on a DNA/RNA Synthesizer

(PEG)₁₂: The Wang resin (**3.2**, 275 mg, 0.9 mmol/g loading, 0.25 mmol) was loaded into a homemade 10 ml synthesis column (Figure 3.8). The column was attached to one of the synthesizer's usual synthesis column positions via two relatively long tubes and

placed on an orbital shaker (Figure 3.8). Dry THF (9 ml) was delivered to the column and the resin was allowed to swell at rt for 20 min. The THF was removed using the reverse flush function of the synthesizer. *t*BuOK solution (0.1 M, 3 ml, 0.3 mmol, 1.2 equiv.) was delivered to the column, and the column was shaken at rt for 15 min. The solution was removed and the deprotonation was repeated. After the base solution was removed, monomer **3.1** (0.31 M in THF, 4 ml, 1.24 mmol, 5 equiv.) was delivered. The column was shaken at rt for 7 h. The liquid was flushed to a flask containing 5% Na₂CO₃.

Figure 3.8. The setup of solid phase PEG synthesis using an ABI-394 DNA/RNA synthesizer. The plastic column is the reaction vessel, which is placed on an orbital shaker. THF is in bottle 18. TCA solution is in bottle 14. *t*BuOK is in bottle 11. The solution of monomer DMTrO(PEG)₄OTs (**3.1**) is in bottle 15. Excess monomer **3.1** is delivered to bottle 10 (the Erlenmeyer flask) for recovery. The bottle positions are numbered as those in the operation manual of the synthesizer.

TLC analysis of the recovered **3.1** after extraction with EtOAc showed that the compound was contaminated with about 2% β -elimination product (Figure 3.9). The resin was washed with THF (10 ml $\times$ 3), and the ether formation process was repeated two times. After the last coupling, the resin was washed extensively (THF/H₂O, v/v, 1:1, 10 ml $\times$ 3; THF, 10 ml $\times$ 5). This converted **3.2** to **3.4**.

Figure 3.9. TLC of excess monomer DMTrO(PEG)₄OTs (**3.1**) recovered after the coupling step in the small scale PEG synthesis using an ABI-394 DNA/RNA synthesizer. Eluent: hexanes/EtOAc/Et₃N 7:3:0.5. Left lane, pure **3.1**; middle lane, co-spot of left and right lane samples; right lane, recovered **3.1**.

The solutions of 3% TCA in DCM (10 ml $\times$ 10) and in PhMe (10 ml $\times$ 5) were delivered to the column and then removed by reverse flush as many times as needed until red or orange color no longer appeared. Completeness of detritylation was determined using Methods B and C. After detritylation, the resin was washed with DCM

(10 ml) and THF (10 ml $\times$ 5), and dried under vacuum over Drierite. This converted **3.4** to **3.5**.

For converting **3.5** to **3.9**, the deprotonation and coupling steps were carried out as described above for a total of four times. ESI-MS analysis using Method A indicated complete reaction. For converting **3.9** to **3.10**, the resin was treated with 3% TCA in DCM (10 ml $\times$ 10), 3% TCA in PhMe (10 ml $\times$ 5) and a cocktail containing 5% TCA, 1% triisopropylsilane (TIPS), 5% MeOH, 5% thioanisole, and 5% phenol in DCM (Cocktail A, 10 ml $\times$ 5). ESI-MS analysis using Methods B and C indicated complete reaction. For converting **3.10** to **3.12**, the deprotonation and coupling steps were conducted five times and detritylation was achieved using 5% TCA in toluene (10 ml $\times$ 10), Cocktail A (10 ml $\times$ 10) and Cocktail B (20% TCA, 1% TIPS, 5% MeOH, 5% thioanisole, and 5% phenol in DCM; 10 ml $\times$ 10). ESI-MS using Methods A, B and C indicated that the reactions were complete.

To cleave PEG **3.13a** from resin **3.12**, a portion of the resin (128 mg of 384 mg), which had been washed extensively using conditions described earlier, was placed in a 1.5 ml centrifuge tube. Pure TFA (300 μ l) was added and the mixture was shaken at rt for 2 h. The tube was spun shortly and the supernatant was removed to another 1.5 ml centrifuge tube with a pipette. The resin was washed with TFA (100 μ l $\times$ 2) and THF (100 μ l $\times$ 3). The supernatant and the washes were combined. Volatiles were evaporated by blowing nitrogen over the surface of the solution. The residue was dissolved in water, vortexed and centrifuged. The supernatant was transferred to another centrifuge tube

and volatiles were removed under vacuum. The residue was dissolved in THF (100 μ l) and precipitated with diethyl ether 200 μ l giving the product **3.13a**²⁷ as a light yellow oil: 36 mg, 66 μ mol, 81% yield based on 0.9 mmol/g loading of resin **3.2**; ¹H and ¹³C NMR images are in Figures A.1-A.2; ESI-MS (Figure 1) m/z : [M+NH₄]⁺ calcd for C₂₄H₅₀O₁₃NH₄, 564.4; found, 564.4.

2.5.4 Synthesis of PEG Derivatives

BnO(PEG)₈ (3.13b): To resin **3.10** (100 mg of 355 mg resin **3.10** from 275 mg resin **3.2**, 0.069 mmol, 1 equiv.) in a 1.5 ml centrifuge tube was added THF (0.2 ml), BnBr (0.3 ml, 2.5 mmol, 36 equiv.) and *t*BuOK (1 M in THF, 0.3 ml, 0.3 mmol, 4.3 equiv.). The tube was rotated on rotary evaporator at rt for 24 hours. After a short spin, the supernatant was removed. The resin was washed extensively (THF/H₂O, v/v, 1:1, 0.5 ml $\times$ 3; THF, 0.5 ml $\times$ 5; DCM, 0.5 ml $\times$ 5), and **3.13b** was cleaved from the resin using a similar procedure described for **3.13a**. Product **3.13b**^{55,35} appeared as a light yellow oil: 26 mg, 0.056 mmol, 82% yield based on 0.9 mmol/g loading of resin **3.2**; ¹H and ¹³C NMR images are in Figures A.6-A.7. ESI-MS (Figure A.14) m/z : [M+NH₄]⁺ calcd for C₁₃H₄₀O₉Na, 483.3; found 483.4.

BnO(PEG)₁₂ (3.13c): Resin **3.10** (120 mg of 355 mg resin **3.10** from 275 mg resin **3.2**, 0.083 mmol, 1 equiv.) in a 1.5 ml centrifuge tube was washed with dry THF (300 μ l $\times$ 5) by suspending the resin in the solvent, shaking for 1 min, centrifuging, and removing supernatant. The tube was flushed briefly with nitrogen. Monomer **3.15** (0.3 M, 0.6 ml, 0.18 mmol, 2.1 equiv.) and *t*BuOK (1 M in THF, 0.3 ml, 0.3 mmol, 3.6

equiv.) were added. The tube was capped and sealed with Parafilm. Then it was rotated on rotary evaporator at rt for 10 h. After a short spin and centrifugation, the supernatant was removed. The resin was washed with a mixture of THF and water (1:1, v/v; ~0.5 ml $\times$ 2), and dry THF (~0.5 ml $\times$ 5), and dried under vacuum over fresh Drierite overnight. The coupling was repeated two more times. The tube was centrifuged, and the supernatant was removed with a pipette. The resin was washed extensively (THF/H₂O, v/v, 1:1, 0.5 ml $\times$ 3; THF, 0.5 ml $\times$ 5; DCM, 0.5 ml $\times$ 5), and **3.13c** was cleaved from the resin using a similar procedure described for **3.13a**. The product appeared as a light yellow oil: 42 mg, 0.066 mmol, 79% yield based on 0.9 mmol/g loading of resin **3.2**; ¹H NMR (400 MHz, CDCl₃; Figure A.8): δ 3.58-3.70 (m, OCH₂CH₂O, 48H), 4.55 (s, ArCH₂O, 2H), 7.25-7.33 (m, ArH, 5H); ¹³C NMR (100 MHz, CDCl₃; Figure A.9): δ 61.88, 69.64, 70.53, 70.76, 70.80, 72.76, 73.43, 127.77, 127.92, 128.54, 138.47; ESI-MS (Figure 2) m/z : [M+H]⁺ calcd for C₃₁H₅₆O₁₃H, 637.3799; found, 637.3781.

BnO(PEG)₈OTs (3.13d): Prepared at a small scale using the procedure described for large scale tosylation of DMTrO(PEG)₄OH to give DMTrO(PEG)₄OTs (**3.1**). Thus, the reaction of **3.13b** (20 mg, 0.043 mmol, 1 equiv.) in THF (0.5 ml), TsCl (10 mg, 0.052 mmol, 1.2 equiv.) in THF (0.25 ml) and NaOH (8 mg, 0.19 mmol, 4.5 equiv.) in water (0.2 ml) gave **3.13d**⁵⁵ as light yellow oil after purification by passing its solution through a Celite pad and precipitation from Et₂O with hexanes: 21 mg, 0.034 mmol, 81% yield; ¹H and ¹³C NMR are in Figures A.10-A.11. ESI-MS (Figure A.15) m/z : [M+Na]⁺ calcd for C₃₀H₄₆O₁₁SNa, 637.3; found, 637.4.

BnO(PEG)₁₂OTs (3.13e): Prepared as described for **3.13d**. Thus, the reaction of **3.13c** (25 mg, 0.039 mmol, 1 equiv.) in THF (0.5 ml), TsCl (9 mg, 0.047 mmol, 1.2 equiv.) in THF (0.25 ml) and NaOH (7 mg, 0.17 mmol, 4.5 equiv.) in water (0.25 ml) gave **3.13e** as light yellow oil after purification by passing its solution through a Celite pad: 24 mg, 0.03 mmol, 77% yield; ¹H NMR (400 MHz, CDCl₃; Figure A.12): δ 2.42 (s, CH₃Ar, 3H) 3.56-3.67 (m, OCH₂CH₂O, 46H), 4.13 (t, *J* = 4.8 Hz, ArOCH₂, 2H), 4.54 (s, ArCH₂O, 2H), 7.24-7.32 (m, ArH, 7H), 7.77 (d, *J* = 8.4 Hz, ArH, 2H); ¹³C NMR (100 MHz, CDCl₃; Figure A.13): δ 21.85, 68.89, 69.44, 69.65, 70.77, 70.85, 70.95, 73.44, 127.77, 127.93, 128.18, 128.55, 130.01, 133.25, 138.49, 144.96; ESI-MS (Figure 3.2) *m/z*: [M+H]⁺ calcd for C₃₈H₆₂O₁₅SH, 791.3888; found, 791.3859.

2.5.5 Larger Scale Solid Phase Synthesis of BnO(PEG)₁₂ (3.13c) in a Peptide Synthesis Vessel

First cycle (converting 3.2 to 3.5): The Wang resin (**3.2**, 0.9 mmol/g, 7 g, 6.3 mmol) was loaded in a 100 ml peptide synthesis vessel with a coarse porosity fritted glass resin support (Figure 3.10).

Figure 3.10. The setup of larger scale solid phase PEG synthesis using a peptide synthesis vessel

The vessel was capped with a rubber septa and flushed briefly with a nitrogen flow. Dry THF (80 ml) was added via a syringe. The rubber septa was replaced with the plastic cap coming with the vessel (the cap is less likely to swell and provides better sealing), and the vessel was gently shaken on a rotary evaporator (~0.5 rounds per second) at rt for 20 min. The swelled resin had a volume of ~50 ml. The cap was replaced with a septa, and the vessel was placed under nitrogen provided via a needle from a gas line. The bottom of the vessel was attached to a round-bottomed flask, and THF was flushed to the flask with a positive nitrogen pressure. After all THF came down, the bottom of the vessel was closed with the stopcock.

*t*BuOK (0.2 M in THF, 35 ml, 7.0 mmol, 1.1 equiv.) was added to the synthesis vessel via a syringe. The septa was replaced with the cap, and the vessel was gently shaken by rotating on a rotatory evaporator at rt for 20 min. The vessel was placed under

nitrogen and the liquid was removed under positive nitrogen pressure using the procedure described earlier for removing THF from the vessel. To the resin was added dry THF (30 ml) via a syringe under nitrogen. The vessel was gently shaken manually for ~5 sec, and the THF was removed with nitrogen pressure as described earlier. To the resin under nitrogen, monomer **3.1** (0.31 M in THF, 41 ml, 12.7 mmol, 2 equiv.; contaminated with **3.17**; **3.1** was assumed pure at calculation, so the actual concentration and equiv. were lower) was added via a syringe. The septa was replaced with the cap and the vessel was shaken gently on a rotatory evaporator at rt for 24 h. The vessel was taken off, and the cap was removed. Any resin on the cap was washed to the vessel with THF. The liquid was removed from the vessel to a round-bottomed flask via vacuum filtration in air or by applying positive nitrogen or air pressure. The resin was washed with THF (30 ml $\times$ 2). The liquid and THF washes were combined, evaporated and partitioned between EtOAc and 5% Na₂CO₃. The organic layer was washed with brine and analyzed with TLC (Figure 3.11), which showed spots corresponding to **3.1**, **3.17** and the vinyl ether side product resulted from β -elimination of **3.1**.

Figure 3.11. TLC of excess monomer DMTrO(PEG)₄OTs (**3.1**) recovered after the coupling step in the larger scale PEG synthesis using peptide synthesis vessel. Eluent: hexanes/EtOAc/Et₃N 7:3:0.5. Left lane, **3.1** contaminated with **3.17**; middle lane, co-spot of left and right lane samples; right lane, recovered unused **3.1** contaminated with **3.17** and β -elimination product.

The organic phase was evaporated to dryness under reduced pressure and a small portion was analyzed with ¹H NMR (Figure A.20). The resin was washed with a mixture of THF and water (1:1, v/v; ~30 ml $\times$ 2), and dry THF (~30 ml $\times$ 5); and then dried under vacuum over fresh Drierite overnight. The resin was swelled with THF as described earlier. The *t*BuOK (0.2 M in THF, 25 ml, 5.0 mmol, 0.8 equiv.) deprotonation, THF wash and alkylation with **3.1** (0.31 M in THF, 30 ml, 9.3 mmol, 1.5 equiv.; contaminated with **3.17**; **3.1** was assumed pure at calculation) were repeated two times with slightly different molar equivalents of reagents and volumes of solutions. After the monomer was removed and recovered, the resin was washed with a mixture of THF and water (1:1, v/v; ~30 ml $\times$ 2), and then THF (~30 ml $\times$ 2). ESI-MS analysis

to check the completeness of the coupling using Method A was not performed in this first cycle. This completed the synthesis of **3.4**.

Detritylation of **3.4** to give **3.5** was achieved under similar conditions for small scale synthesis. The experiments were conducted in air. Specifically, to the resin in the synthesis vessel was added 3% TCA in DCM (40 ml). The vessel was shaken shortly and the liquid was removed by vacuum filtration. This acid treatment was conducted for a total of 12 times. The resin was washed with DCM (40 ml) and THF (40 ml $\times$ 5), and dried under vacuum over Drierite. ESI-MS analysis using Methods B-C showed that the detritylation was complete. This gave **3.5**.

Second cycle (converting 3.5 to 3.10): Deprotonation and alkylation of **3.5** to give **3.9** was conducted similarly as described for converting **3.2** to **3.4** in the first cycle except that the process was only repeated one time instead of two times (first coupling: *t*BuOK, 1.1 equiv.; monomer **3.1**, 2 equiv.; second coupling: : *t*BuOK, 0.8 equiv.; monomer **3.1**, 1.5 equiv.). ESI-MS analysis indicated that the reaction was 100% complete. Detritylation of **3.9** to give **3.10** was carried out under similar conditions for converting **3.4** to **3.5** except that 5% instead of 3% TCA in DCM was used (40 ml, 5 min, 10 times) and additional treatments were conducted using the cocktail 5% TCA, 1% triisopropylsilane (TIPS), 5% MeOH, 5% thioanisole, and 5% phenol in DCM (Cocktail A; 40 ml, rt, 30 min, 2 times), followed by 5% TCA in DCM (40 ml, 5 min, 4 times). The resin was washed with DCM (40 ml) and THF (40 ml $\times$ 5), and dried under vacuum over Drierite. ESI-MS analysis using Methods B-C showed that the detritylation was complete. This gave **3.10**.

Third cycle (converting 3.10 to 3.16): Deprotonation and alkylation of **3.10** to give **3.16** was conducted under the same conditions as described for converting **3.5** to **3.9** in the second cycle in this larger scale synthesis using **3.15**, which contained small amount of **3.18**, as the monomer. The recovered excess **3.15** was analyzed with TLC (Figure 3.12). The resin was washed with a mixture of THF and water (1:1, v/v; ~30 ml $\times$ 3), and then THF (~30 ml $\times$ 2); and was dried under vacuum over fresh Drierite overnight (10.53 g). This gave **3.16**.

Figure 3.12. TLC of excess monomer BnO(PEG)₄OTs (**3.15**) recovered after the coupling step in the larger scale PEG synthesis using peptide synthesis vessel. Eluent: hexanes/ethyl acetate 1:1. Left image, visualized under UV. Right image, the same TLC visualized under UV after I₂ stain. Left lane: **3.15** contaminated with **3.18** before coupling; middle lane: co-spot of left and right lane samples; right lane: **3.15** recovered after coupling. The minute quantities of **3.18** and elimination product could only be seen after I₂ stain under UV.

Cleavage and analysis (3.13c): Cleaving **3.13c** from **3.16** was accomplished similarly as described for small scale synthesis. To the resin (10.53 g) that had been swelled with in the synthesis vessel was added TFA (20 ml), and the mixture was shaken

gently on a rotary evaporator at rt for 2 h. The TFA solution was filtered to a round-bottomed flask by applying a positive pressure, and the resin was washed with TFA (5 ml $\times$ 1), DCM (20 ml $\times$ 1) and THF (20 ml $\times$ 5). The TFA solution and the washes were combined, and evaporated on a rotatory evaporator under reduced pressure (to prevent TFA vapor to go to water, a NaOH trap was connected between water aspirator and the evaporator). The residue was dissolve in water (20 ml) and volatiles were removed under vacuum from an oil pump. The product **3.13c** was purified by precipitation from THF (~5 ml) with Et₂O (~20 ml) and appeared as a light yellow oil; 3.22 g; 80% based on 0.9 mmol/g loading of resin **3.2**; ¹H and ¹³C NMR images are in Figures A.21-A.22. ESI-MS image is in Figure 3.5.

2.5.6 Analytical Methods for Monitoring Completeness of Solid Phase Reactions

Method for monitoring completeness of the Williamson ether formation reaction with ESI-MS (Method A): In order to obtain monodisperse PEG at the end of the solid phase synthesis, all ether formation reactions must be 100% complete. To monitor the completeness of the reaction, ~4 mg of resin (**3.9**, **3.11** or **3.16**), which had been extensively washed with THF, THF/water and THF as described earlier after the coupling reactions, was placed in a 1.5 ml centrifuge tube. The resin was further washed with DCM (100 μ l $\times$ 2). TFA (100 μ l) was added and the mixture was gently shaken by rotating on a rotary evaporator at rt for 2 h. This cleaved the PEG from resin, and in cases of **3.9** and **3.11** the DMTr group was also detached. The tube was centrifuged

shortly, and the supernatant was transferred to another 1.5 ml centrifuge tube. The resin was washed with TFA ($100\ \mu\text{l} \times 2$) and then THF ($100\ \mu\text{l} \times 3$). The TFA and THF solutions were combined and evaporated by blowing nitrogen over the surface of the solution. The residue was dissolved in water ($300\ \mu\text{l}$) and in cases of **3.9** and **3.11**, the solution was extracted with hexanes ($200\ \mu\text{l} \times 5$) to remove DMTrOH. In the case of **3.16**, no extraction was conducted. Alternatively, in the cases of **3.9** and **3.11**, the DMTr group was removed before cleavage with 3% TCA solution in DCM. The water solution or water layer was evaporated to dryness. The residue was placed under a vacuum from an oil pump for 10 h, and then dissolved in 2 ml MeOH. The theoretical concentration of the cleaved PEG was 1.7 mM. A portion of the solution was further diluted 100 times and analyzed with ESI-MS. If only peaks corresponding to the desired product [(PEG)₈ for **3.9**, (PEG)₁₂ for **3.11**, BnO(PEG)₁₂ for **3.16**] were observed, the Williamson ether formation reaction was complete. If peaks corresponding to PEG that failed to undergo the ether formation reaction [(PEG)₄ for **3.9**, (PEG)₈ for **3.11**, (PEG)₈ for **3.16**] were observed along with peaks from the desired product, the ether formation reaction was incomplete, and had to be repeated.

Method for monitoring completeness of the detritylation reaction by detecting DMTr cation with ESI-MS (Method B): In order to obtain monodisperse PEG at the end of the solid phase synthesis, the detritylation reactions must be 100% complete. The resin to be detritylated (**3.4**, **3.9** or **3.11**) was first treated with TCA solutions or cocktails as described earlier in the synthesis column or the peptide synthesis vessel until the colors of the solutions before and after the treatment were about the same. The resin

was washed with TCA two times if a TCA cocktail was used in the previous detritylation, and then treated with 3% TCA for an additional time. The volume of TCA solution was minimal but adequate to cover the resin. The mixture was agitated at rt for 10 min, and filtered. A portion of the filtrate (500 μ l) was concentrated to dryness. The residue was mixed with water and volatiles were removed. The residue was then dissolved in MeOH (500 μ l) and the suitability of the concentration for ESI-MS was estimated by spotting on a TLC plate using a capillary tube and developing with hexanes. If TLC showed orange or red color of DMTr cation upon contacting with acid (5% H₂SO₄), the analysis could be stopped and detritylation of the resin was incomplete and needed to be repeated. If no color, the solution was analyzed with ESI-MS. If DMTr cation was observed in MS, the detritylation reaction was incomplete and needed to be repeated. If DMTr cation was not observable, we intended to further confirm the completeness of detritylation using Method C below.

Method for monitoring completeness of the detritylation reaction by benzylation followed by ESI-MS analysis (Method C): If Method B did not detect any DMTr cation with ESI-MS, ~6 mg of resin (**3.4**, **3.9** or **3.11** after detritylation) was placed in a 1.5 ml centrifuge tube, and washed with toluene (200 μ l) and dry THF (200 μ l $\times$ 5) by suspending the resin in the solvents, shaking for 1 min, centrifuging, and removing supernatant with a pipette. The tube was then flushed briefly with nitrogen. BnBr (100 μ l, 0.85 mmol, 200 equiv.), *t*BuOK (1 M solution in THF, 100 μ l, 0.1 mmol, 25 equiv.) and dry THF (100 μ l) were added. The tube was capped and sealed with Parafilm. The mixture was agitated by rotating the tube on a rotary evaporator at rt for 24 h. The tube

was centrifuged, and the supernatant was removed with a pipette. The resin was washed with a mixture of THF and water (1:1, v/v; $\sim 200\ \mu\text{l} \times 2$), THF ($\sim 200\ \mu\text{l} \times 2$) and then DCM ($\sim 200\ \mu\text{l} \times 2$), and about 2 mg was treated with TFA (100 μl) by rotating the tube on a rotary evaporator at rt for 2 h. The tube was centrifuged and supernatant was transferred to another 1.5 ml centrifuge tube. The resin was washed with TFA (100 $\mu\text{l} \times 2$) and then THF (100 $\mu\text{l} \times 3$). The TFA and THF solutions were combined and evaporated by blowing nitrogen over the surface of the solution. To the residue was added 200 μl water. After a short vortex and centrifuge, the volatiles were evaporated under vacuum, which helped to remove residue TFA. The residue was dissolved in MeOH (1 ml). The theoretical concentration of the cleaved PEG derivative was 1.8 mM. The solution was diluted 100 times and analyzed with ESI-MS. If only peaks corresponding to the benzylated product [BnO(PEG)_4 for **3.4**, BnO(PEG)_8 for **3.9**, BnO(PEG)_{12} for **3.11**] was observed, the detritylation reaction was complete. If peaks corresponding to DMTr cation (which should not happen if Method B did not detect it), or un-benzylated PEG [$(\text{PEG})_4$ for **3.4**, $(\text{PEG})_8$ for **3.9**, $(\text{PEG})_{12}$ for **3.11**] were observed along with peaks from the benzylated product, the detritylation reaction was incomplete, and had to be repeated. In some cases in which un-benzylated PEG was detected, we subjected the remaining half amount of resin to an additional benzylation under the same conditions. After cleavage, we observed that the ratio of un-benzylated and benzylated PEGs did not change. This confirmed that the appearance of un-benzylated PEG was not a result of incomplete benzylation, and instead was due to incomplete detritylation.

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Appendix A

Supporting Information for Chapter 3

Solid Phase Stepwise Synthesis of Polyethylene Glycol

Figure A.1. ^1H NMR of (PEG)₁₂ (**3.13a**) from small scale synthesis.

Figure A.2. ^{13}C NMR of $(\text{PEG})_{12}$ (**3.13a**) from small scale synthesis.

tetraethyleneglycol194 #333 RT: 6.48 AV: 1 NL: 2.60E6
T: + c ESI Full ms [50.00-2000.00]

Figure A.3. ESI-MS of commercial (PEG)₄ showing that it was contaminated with minute (PEG)₃.

4 20% 10 hr 4 days #1 RT: 0.00 AV: 1 NL: 2.59E7
T: + c ESI Full ms [150.00-2000.00]

Figure A.4. ESI-MS of $(\text{PEG})_{16}$ with $(\text{PEG})_{12}$ contamination.

peg 2+3_160210151808 #39 RT: 0.89 AV: 1 NL: 2.25E7
T: + c ESI Full ms [150.00-2000.00]

Figure A.5. ESI-MS of $(\text{PEG})_{20}$ with $(\text{PEG})_{16}$ contamination.

Figure A.6. ¹H NMR of BnO(PEG)₈ (**3.13b**) from small scale synthesis.

Figure A.8. ¹H NMR of BnO(PEG)₁₂ (**3.13c**) from small scale synthesis.

Figure A.10. ¹H NMR of BnO(PEG)₈OTs (**3.13d**) from small scale synthesis.

Figure A.11. ¹³C NMR of BnO(PEG)₁₂OTs (**3.13d**) from small scale synthesis.

Figure A.12. ¹H NMR of BnO(PEG)₁₂OTs (**3.13e**) from small scale synthesis.

Figure A.13. ¹³C NMR of BnO(PEG)₁₂OTs (**3.13e**) from small scale synthesis.

benzyl2 #77 RT: 1.93 AV: 1 NL: 1.82E7
T: + c ESI Full ms [150.00-2000.00]

Figure A.14. ESI-MS of BnO(PEG)₈ (**3.13b**) from small scale synthesis. The peak at m/z 439.5 may not all be from [M-44+Na]⁺ because no corresponding [M-44+NH₄]⁺ and [M-44+H]⁺ could be observed. Typically, these associated peaks should be observable if the [M-44+Na]⁺ peak is as strong as indicated and the [M+Na]⁺ peak is accompanied with significantly strong [M+NH₄]⁺ and [M+H]⁺ peaks.

ots2pegbenz #117 RT: 2.79 AV: 1 NL: 1.01E8
T: + c ESI Full ms [200.00-1100.00]

Figure A.15. ESI-MS of BnO(PEG)₈OTs (**3.13d**) from small scale synthesis.

Figure A.16. ¹H NMR of DMTrO(PEG)₄ from large scale synthesis without chromatography. The compound was contaminated with small amount of DMTrO(PEG)₄ODMTr (**3.17**).

Figure A.17. ¹H NMR of DMTrO(PEG)₄OTs (**3.1**) synthesized at large scale without chromatography purification. The compound was contaminated with small amount of DMTrO(PEG)₄ODMTr (**3.17**).

impurebezpegots3 #5 RT: 0.11 AV: 1 NL: 7.28E6
T: + c ESI Full ms [150.00-2000.00]

Figure A.18. ESI-MS of $\text{BnO}(\text{PEG})_4$ contaminated with small amount of $\text{BnO}(\text{PEG})_4\text{OBn}$ (**3.18**) synthesized at large scale without chromatography purification. The amount of $\text{BnO}(\text{PEG})_3$ is minute and it is most likely from $(\text{PEG})_3$ in the starting $(\text{PEG})_4$. See Figure 3.S5.

Figure A.19. ¹H NMR of BnO(PEG)₄OTs (**3.15**) synthesized at large scale without chromatography purification. The compound was contaminated with small amount of BnO(PEG)₄OBn (**3.18**).

Figure A.20. ¹H NMR of DMTrO(PEG)₄OTs (**3.1**) recovered after the coupling step in the larger scale PEG synthesis using peptide synthesis vessel. The compound was contaminated with DMTrO(PEG)₄ODMTTr (**17**) and the vinyl ether product resulted from β-elimination of **3.1** under basic conditions.

Figure A.21. ¹H NMR of BnO(PEG)₁₂ (**3.13c**) from larger scale synthesis in a peptide synthesis vessel

Figure A.22. ¹³C NMR of BnO(PEG)₁₂ (**3.13c**) from larger scale synthesis in a peptide synthesis vessel.

Figure A.23. MALDI-TOF-MS of (PEG)₁₂ (**3.13a**).

Figure A.24. MALDI-TOF-MS of BnO(PEG)₈ (3.13b).

Figure A.25. MALDI-TOF-MS of BnO(PEG)₁₂ (3.13c).

Figure A.26. MALDI-TOF-MS of BnO(PEG)₈OTs (**3.13d**).

Figure A.27. MALDI-TOF-MS of BnO(PEG)₁₂OTs (**3.13e**).

Appendix B

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