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NON-CHROMATOGRAPHIC OLIGONUCLEOTIDE PURIFICATION AND AUTOMATED POLYETHYLENEGLYCOL SYNTHESIS

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AND

AUTOMATED POLYETHYLENEGLYCOL SYNTHESIS

By

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Author Contribution Statement

All the initial ideas of all research projects reported in chapter 1, chapter 2, chapter 3, chapter 4, and chapter 5 in this dissertation were conceived by Dr. Shiyue Fang.

All the experiments in chapter 2, 4, and 5 and data analysis in this dissertation was carried out by Ms. Dhananjani Eriyagama under the supervision of Dr. Shiyue Fang. All writing of this dissertation is carried out by Dhananjani Eriyagama and revised by Dr. Shiyue Fang.

Chapter 2 of this document is previously published in Organic process research and development (OPRD) journal in 2018. All the experiments for the chapter 2 were done by Dhananjani Eriyagama under the supervision of Dr. Shiyue Fang.

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List of Abbreviations

ODN	Oligodeoxynucleotide
LC-MS	Liquid Chromatography mass spectrometry
PEG	Polyethylene glycol
ESI-MS	Electrospray ionization mass spectrometry
DNA	Deoxyribonucleic acid
dA	Deoxyadenosine
dC	Deoxycytidine
dG	Deoxyguanosine
dT	Deoxythymidine
PCR	Polymerase chain reaction
FDA	Food and Drug Administration
CPG	Controlled pore glass
DMTr	Dimethoxy trityl
RP-HPLC	Reversed Phase High Performance Liquid Chromatography
PAGE	Polyacrylamide Gel Electrophoresis
RP	Reversed Phase
TMEDA	Tetramethyl ethylenediamine
CaH_2	Calcium hydride
TLC	Thin layer Chromatography
DCM	Dichloromethane
THF	Tetrahydro furan
EtOAc	Ethyl acetate
$NaHCO_3$	Sodium bicarbonate
Na_2SO_4	Sodium sulfate

Et_3N	Triethyl amine
NaCl	Sodium chloride
Rf	Retardation factor
MeOH	Methanol
MeCN	Acetonitrile
DIEA	Diisopropylethylamine
MALDI TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
OD_{260}	Optical density
RNA	Ribonucleic acid
UV_{260}	Ultraviolet (nm 260)
HFIP	Hexafluoro-2-propanol
Bz-dA	5'-Dimethoxytrityl-N-benzoyl-deoxyAdenosine
Ac-dC	5'-Dimethoxytrityl-N-acetyl-2'-deoxyCytidine
*i*Bu-dG	5'-Dimethoxytrityl-N-isobutyryl-deoxyGuanosine
Ac_2O	Acetic anhydride
H_2O	Water
NH_4OH	Ammonium hydroxide
$(NH_4)_2S_2O_4$	Ammonium persulfate
NaOAc	Sodium acetate
AcOH	Acetic acid
*n*BuOH	*n* butanol
AFM	Atomic force microscope
CNT	Carbon nanotube
PG	Protecting group

t-BuOK	Potassium *tert*-butoxide
DMF	Dimethyl formamide
TFA	Trifluoro acetic acid
KHMDS	Potassium Hexamethyl disilyl amide
DMTrCl	Dimethoxy trityl chloride
DMSO	Dimethyl sulfoxide
NMP	N-Methyl-2-pyrrolidone
Ts	Tosyl
Me	Methyl
TsCl	Tosylchloride
SiO_2	Silicon dioxide
MVA	Measuring vessel A
RV	Reaction Vessel
TCA	Trichloro acetic acid
MOM	Methoxy methyl
LDA	Lithium diisopropyl amine
NaH	Sodium hydride
LAH	Lithium aluminium hydride

Abstract

Synthetic oligodeoxynucleotides (ODN) have various applications in many areas such as, synthetic biology, chemical biology, antisense drug development, and data storage. As a result, there is a high demand for synthetic ODNs. Many advances have been made with ODN synthesis the purification still remains a bottleneck. The non-chromatographic purification method is developed to address this problem. Similarly, polyethylene glycols (PEGs) have been used in many areas including pharmaceutical applications, surface science, and nanomedicine due to its unique properties. However, monodispersed PEG synthesis is expensive with existing methods. A novel automated solid phase synthesis method is developed to obtain monodispersed PEGs.

The first two chapters are an introduction to synthetic oligodeoxynucleotide purification. These chapters describe oligonucleotides and applications, chemical synthesis of oligodeoxynucleotides, impurities in synthetic oligonucleotides, and current purification methods. Even though many chromatographic purification methods are available for synthetic ODN purification, there is no method that is capable of high throughput purification, large scale ODN purification, and long ODN purification. To address this problem, we developed two non-chromatographic purification methods which are based on polymerization, to purify synthetic ODNs. One is catching failure sequences by polymerization, and the second method is catching full-length sequences by polymerization. The second method is less expensive for large-scale and long ODN purification compared to the first method. It is demonstrated that this new non-chromatographic method is suitable for high throughput, large scale, and long ODN purification.

The final chapters describe applications of polyethylene glycol and current PEG synthesis methods. For many applications monodispersed PEGs are preferred. However, PEGs synthesized using current synthesis methods are polydispersed and excessive purification is required to obtain monodispersed PEGs. Therefore, it is expensive to obtain monodispersed PEGs using these existing methods. An automated solid-phase stepwise synthesis method is developed for monodispersed PEG synthesis. This solid-phase

synthesis method is cost effective because the purification is not required to obtain monodispersed PEGs using this method. Monodispersed PEGs with eight, twelve, and sixteen ethylene glycol units and their derivatives were synthesized using automated stepwise addition of tetra ethylene glycol monomer without chromatographic purification. The monomer consists of a 4,4′ dimethoxy trityl group at one end and the tosyl group at the other hydroxyl group. Wang resin was used as the solid support for the automated synthesis. The automated synthesis cycle consists of three steps, deprotonation, coupling, and detritylation. PEGs were cleaved off from the solid support and analyzed with ESI-MS. After PEG_{12} synthesis, the coupling step with DMTr monomer had some complications and therefore a new monomer with a smaller protecting group was designed. Using this new monomer monodispersed PEGs with ten, fifteen, and twenty ethylene glycol units were synthesized. The synthesis was done using automated solid-phase stepwise addition of penta ethylene glycol units without chromatographic purification. The penta ethylene glycol monomer consists of a 2-phenethyl group at one end, and the tosyl group at the other hydroxyl group was used for the synthesis. The first step is deprotection and deprotonation, and the second step is coupling. These two steps were performed alternatively until the PEG with the desired length was obtained. After the synthesis, PEGs were cleaved from the solid support and analyzed using ESI-MS.

1. Introduction to Oligodeoxynucleotides

1.2 Oligodeoxynucleotides

Oligodeoxynucleotides (ODNs) (Figure 1.1) are short, single-stranded deoxynucleic acid (DNA) sequences. They consist of a 2'-deoxyribose phosphate backbone and four types of nucleobases. These nucleoside bases can be doxy-adenosine (dA), deoxy-guanosine (dG), deoxy-cytidine (dC), and deoxy-thymidine (dT). A nucleotide consists of a deoxyribose phosphate group and a nucleoside. These ODN sequences can be chemically synthesized in an automated manner using the phosphate diester method[1]. The length of ODN sequences can vary from 15-200 nucleotides. The structure of an ODN sequence is shown in Figure 1.1.

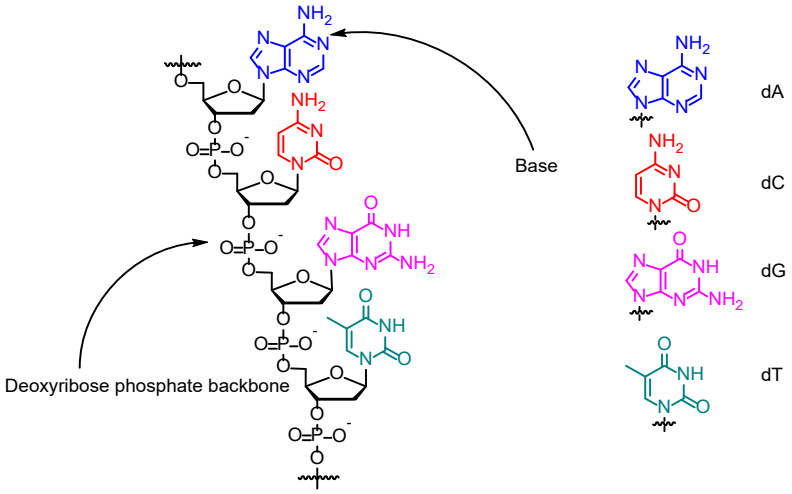


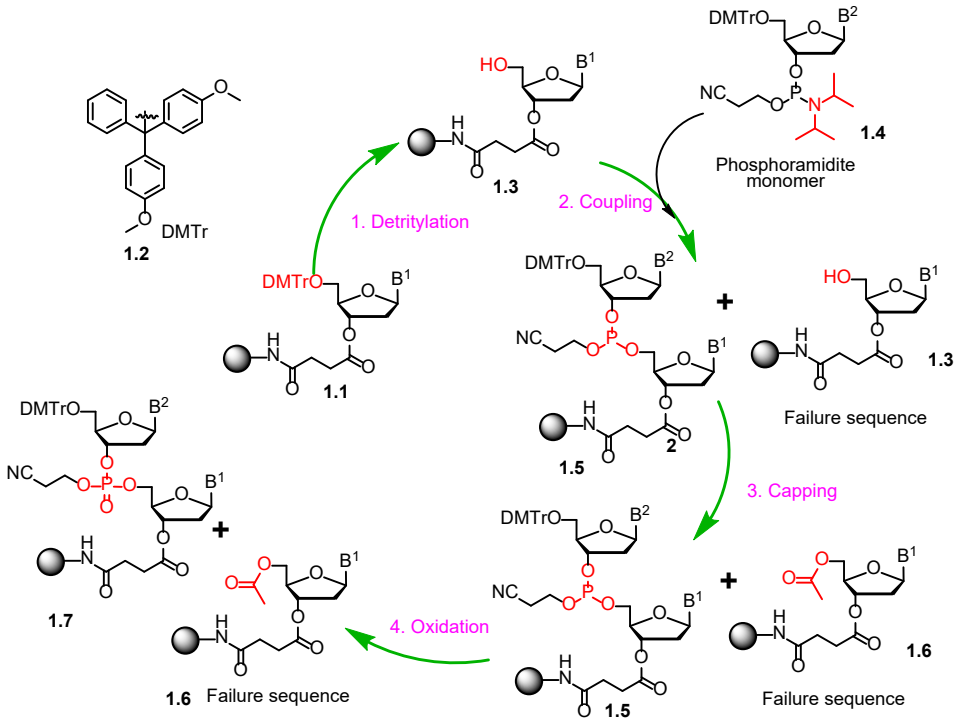
Figure 1.1:Structure of ODN

1.3 Applications of synthetic ODNs

Apart from using as primers for PCR and DNA sequencing[2], synthetic oligodeoxynucleotides found applications in different areas such as synthetic biology[3, 4], antisense drug development[5], long-term data storage[6], and chemical biology[7, 8]. As of 2020, there are 10 FDA-approved oligonucleotide-based drugs[9] in the market and about 60 oligonucleotide-based drugs in clinical trials[9]. On the other hand, synthetic biology is expanding drastically, and much research involved uses synthetic ODNs modified or unmodified. The annual market for synthetic ODNs in academia is about $800 million.

1.4 ODN synthesis

The phosphate triester method or the phosphoramidite method is the most used method for ODN synthesis. Usually, the exocyclic amino group, 5′ hydroxyl group, and the phosphate group are protected and deprotected during the synthesis as needed. The ODN sequence is synthesized on a solid support, known as controlled pore glass (CPG). Usually, the first nucleotide is pre-attached to the solid support via a base cleavable linker. The phosphoramidites are added sequentially one at a time to the 5′ end of the existing oligodeoxynucleotide chain. The ODN synthesis cycle consists of four steps shown in Scheme 1.1 and is further described in the following paragraphs. The four steps are detritylation, coupling, capping, and oxidation. After repeating these four steps, one phosphoramidite is added to the growing ODN chain. After the solid phase synthesis is done, the ODN is cleaved from the solid support and obtained crude ODNs.



Scheme 1.1:Synthesis cycle of ODNs

1.4.1 Detritylation

The first step of the synthesis cycle is known as detritylation. The 5′ hydroxyl group of the nucleotide is usually protected with the 4,4′ dimethoxy trityl (DMTr) (**1.2**, Scheme 1.1) group to prevent side reactions. During this step the DMTr group on the 5′ hydroxy group of the existing base on the solid support (**1.1**, Scheme 1.1) is removed using a 3% trichloroacetic acid in dichloromethane solution. After the detritylation reaction the resulting compound (**1.3**, Scheme 1.1) has a free hydroxyl group that can react during the next step of the synthesis cycle.

1.4.2 Coupling

The second step of the synthesis cycle is coupling. In this step, the next phosphoramidite (**1.4**, Scheme 1.1) is attached to the existing nucleotide (**1.3**, Scheme 1.1) on the solid support. The phosphoramidite solution in acetonitrile and the activator solution are delivered simultaneously to increase the rate of the coupling reaction. Usually, one of the solutions of 1*H*-tetrazole, 5-ethylthio-1*H*-tetrazole, 2-benzylthiotetrazole, or 4,5-dicyanoimidazole is used as the activator solution. The activator reacts with the diisopropyl amino group of the phosphoramidite monomer to make that a better leaving group. Then the 5′ hydroxyl group of the nucleotide (**1.3**, Scheme 1.1) on the solid support reacts with the new phosphoramidite monomer (**1.4**, Scheme 1.1) and generates the growing ODN chain (**1.5**, Scheme 1.1).

1.4.3 Capping

The next step of the synthesis cycle is known as capping. Even though the coupling step is efficient, some of the 5′ hydroxyl groups can remain unreacted. These groups can undergo coupling reactions during the successive synthesis cycles and generate deletion sequences. Therefore, the unreacted 5′ hydroxyl groups (**1.3**, Scheme 1.1) are reacted with acetic anhydride and protected with an acetyl group (**1.6**, Scheme 1.1). As a result, they cannot undergo further reactions during the next synthesis steps. These capped ODN sequences are known as failure sequences.

1.4.4 Oxidation

The fourth or final step of the ODN synthesis cycle is oxidation. The phosphite triester bond (**1.6**, Scheme 1.1) is unstable under acidic conditions. Therefore, during this step, the unstable phosphite triester is oxidized to the stable phosphate triester (**1.7**, Scheme 1.1) using iodine solution in water and pyridine.

1.4.5 Cleavage

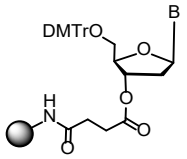
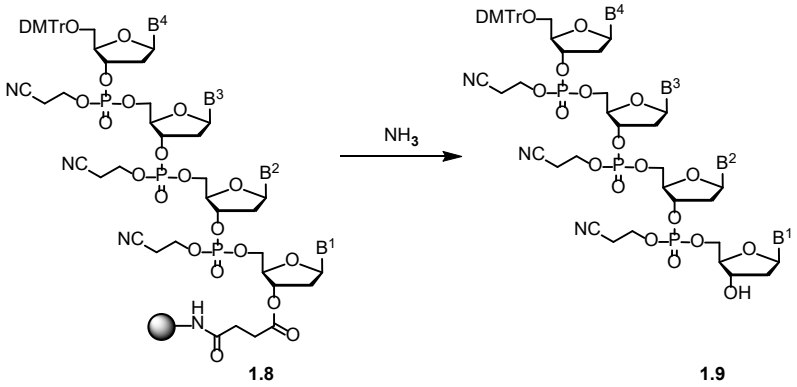


Figure 1.2:Nucleoside is pre-attached to the base via a linker



Scheme 1.2:ODN cleavage form the solid support

During the entire solid-phase oligonucleotide synthesis, the 3′ hydroxyl group of the ODN sequence is attached to the solid support via a succinyl linker (Figure 1.2). This linkage (**1.8**, Scheme 1.2) can be cleaved and obtain cleaved ODN sample (**1.9**, Scheme 1.2) using 28% aqueous ammonium hydroxide solution at room temperature. This process can be done on the synthesizer or manually after the ODN synthesis.

1.4.6 Deprotection

Exocyclic amino groups of nucleosides dA, dC, and dG are nucleophilic and must be protected throughout the ODN synthesis. dT is not required to protect. Phosphite groups are also protected during the synthesis, typically with the 2-cyanoethyl group. 5′ hydroxyl

group of the deoxyribose sugar moiety is temporarily protected with the DMTr group, and this group is removed as needed during the ODN synthesis. Protected dA, dC, dG, and dT phosphoramidites (**1.10**, **1.11**, **1.12**, and **1.13** Figure 1.3) are usually used for ODN synthesis and are commercially available.

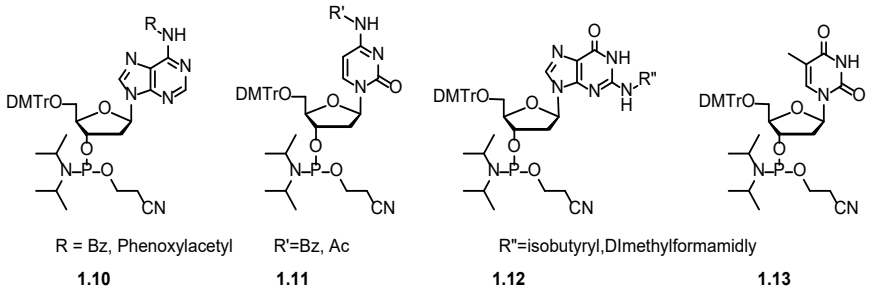
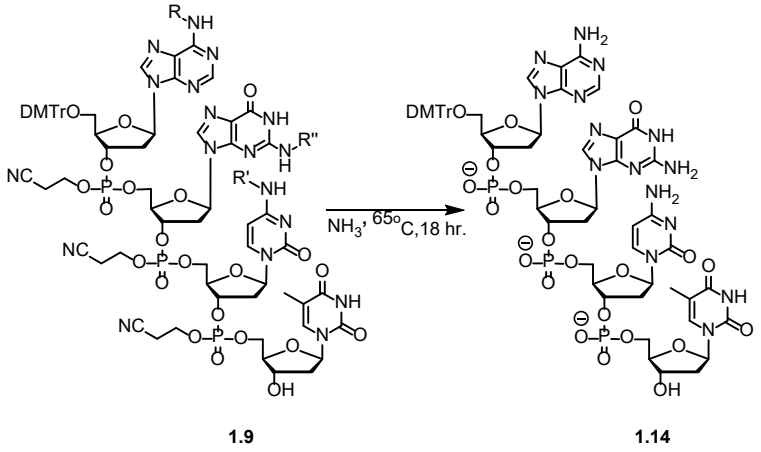


Figure 1.3:Protected phosphoramidites.

After the ODN synthesis, synthesized ODNs (**1.9**, Scheme 1.3) can be fully deprotected (**1.14**, Scheme 1.3) by treating that with 28% ammonia solution in water, at 65ºC for 18 hours. It is noteworthy some protecting groups that can be removed with milder conditions have been developed.



Scheme 1.3:Deprotection of synthetic ODNs with ammonia.

1.5 Impurities in synthetic ODNs

Synthetic ODNs contain three types of impurities. For most applications, ODNs needed to be purified. The first type of impurities is small organic molecules. These are from protecting groups of exocyclic amino groups or phosphate protecting groups of ODN

sequences. These small organic molecules can be easily removed by precipitation. Deprotected ODN sequences are not soluble in organic solvents such as *n*-butanol, and the small organic molecules are soluble in organic solvents. As a result, we can use *n*-butanol or ethanol to remove small organic molecules while the deprotected ODN sequences remain in the precipitate.

The second impurity types are deletion sequences, addition sequences, and de-purinated sequences. Deletion sequences occurred due to incomplete detritylation or incomplete capping reactions. Addition sequences result from pre-mature detritylation during the coupling step. These are hard to remove. However, the formation of these sequences can be easily avoided by adjusting synthesis conditions during the ODN synthesis.

The third type of impurities is known as failure sequences. These are harder to remove due to similar physical and chemical properties. Truncated sequences are generated due to an incomplete coupling reaction during each cycle when the phosphoramidite is added to the ODN sequence. As a result, these are harder to avoid. The content of failure sequences could be varied from 30% to 60% of the crude ODN sample.

1.6 Current ODN purification methods

Current purification methods mainly involved Reverse-Phase (RP) High-Performance Liquid Chromatography (HPLC)[10, 11], Polyacrylamide gel electrophoresis (PAGE)[12], affinity purification[13, 14], cartridge purification[15, 16], and solid-phase purification[17]. These methods will be further described in following the paragraphs.

1.6.1 Reverse-phase (RP) high performance liquid chromatography (HPLC)

This method is based on hydrophobic and hydrophilic interactions of a molecule of interest between the stationary and mobile phase[11, 18]. ODNs are hydrophilic compared to organic molecules and less hydrophilic than inorganic salts. The stationary phase in the column contains hydrophobic C-18 chains. As a result, ODN will bind strongly to the stationary phase than inorganic salts and elute later with a more non-polar solution

containing a solvent like acetonitrile. More hydrophobic molecules will bind strongly to the solid phase and elute later than the ODNs. Depending on the length of ODNs, the time it takes to elute will vary, and it is suitable for ODNs up to 60mers easily. However, this method is unsuitable for longer oligonucleotides due to the similar retention time. The instrumentation is also expensive. This method is adopted for large-scale purification, but it is costly and labor-intensive.

1.6.2 Polyacrylamide gel electrophoresis (PAGE)

This method separates ODNs based on their mass to charge ratio[12]. An electric field is applied to the gel, and ODNs with different lengths travel at different speeds during the process. So ODNs with different lengths can be separated using this method. Even though this method is suitable for obtaining high pure ODNs (up to 98%), it is not suitable for large-scale purification. The purification method is time-consuming and labor-intensive. It is not suitable for high throughput purification as well.

1.6.3 RP-cartridge purification

Hydrophobicity of ODNs is used to separate ODNs in this method[15, 16]. During the ODN synthesis, the 5' hydroxyl group is usually tagged with the DMTr group, and as a result, the full-length ODN sequences are hydrophobic compared to failure sequences. The solid support of the cartridge is hydrophobic, and full-length sequences with the DMTr group are retained in the cartridge longer than failure sequences without the DMTr group. As a result, the ODNs can be separated using this method. This method is inexpensive compared to other methods. However, this method is not suitable for long ODN purification. The separation solely depends on hydrophobicity introduced by the DMTr group, which diminishes when the ODN chain gets longer.

1.6.4 Fluorous affinity purification

This method is also an affinity-based purification method[13, 14]. The solid support is fluorinated, and the full-length ODN sequence is tagged with a fluorous moiety. The full-length ODN sequences will elute slower than the failure sequences because they will bind to the fluorous solid support more tightly than the failure sequences without the fluorous

moiety. This method is suitable for obtaining ODNs with high purity. However, this method is expensive.

1.6.5 Biotin-streptavidin affinity purification

Biotin and avidin have strong interaction, and in this purification method, that interaction is used to retain full-length ODN sequences from the crude ODN sample[17]. The full-length ODN sequence is tagged with a biotin molecule during the ODN synthesis. Then streptavidin is used to pull full-length sequences from the crude ODN sample. The full-length sequences with the biotin tag will interact with streptavidin, while failure sequences without the biotin tag can be washed away. After the isolation, the ODN is cleaved from the streptavidin to obtain pure ODNs. This method is suitable for quantities of ODN purification. However, this method is expensive.

1.7 Limitations of existing purification methods

Current ODN purification methods mainly involve chromatographic methods, and as a result, methods are not suitable for high throughput purification, long ODN purification or large-scale purification. Most of the methods are time-consuming, expensive, and labor-intensive.

1.8 Catching by polymerization methods

We recently developed two non-chromatographic methods based on polymerization to overcome these problems. In these methods, we tagged either failure sequences or full-length sequences with a polymerizable function and caught failure sequences or full-length sequences, respectively. Both the methods are described in further detail in the next paragraphs.

1.8.1 Catching failure sequences by polymerization

The first method is called catching failure sequence by polymerization[19, 20]. In this method, the failure sequences were capped with a capping agent, which has a polymerizable function. Capping agents (**1.15**, **1.16**, **1.17**, and **1.18**; Figure 1.4) were used for the ODN synthesis. Here acrylamide group is used as the polymerizable group. As a

result, failure sequences contain a polymerizable function while the uncapped full-length sequences do not contain a polymerizable function.

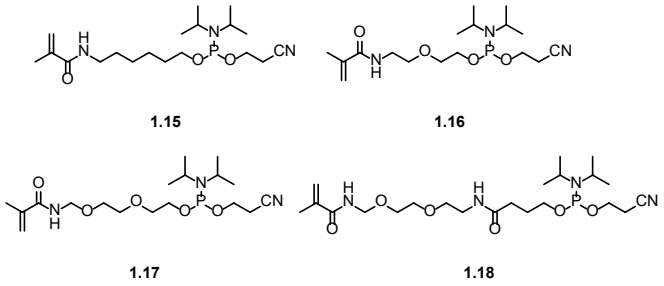
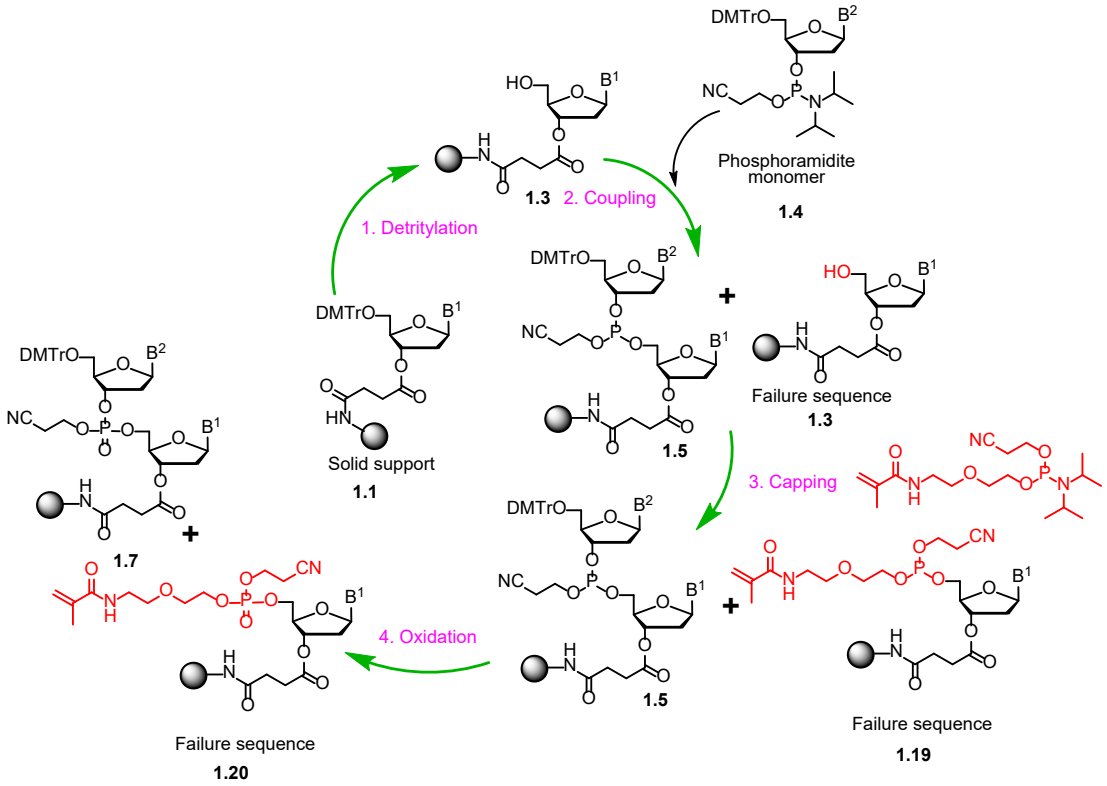


Figure 1.4:Polymerizable capping agents used during the synthesis

So far four polymerizable capping agents (**1.15**, **1.16**, **1.17**, and **1.18**, Figure 1.4) have been tested by our research group. The polymerizable capping agent **1.16** (Figure 1.4) has been used because it is easier to synthesize.

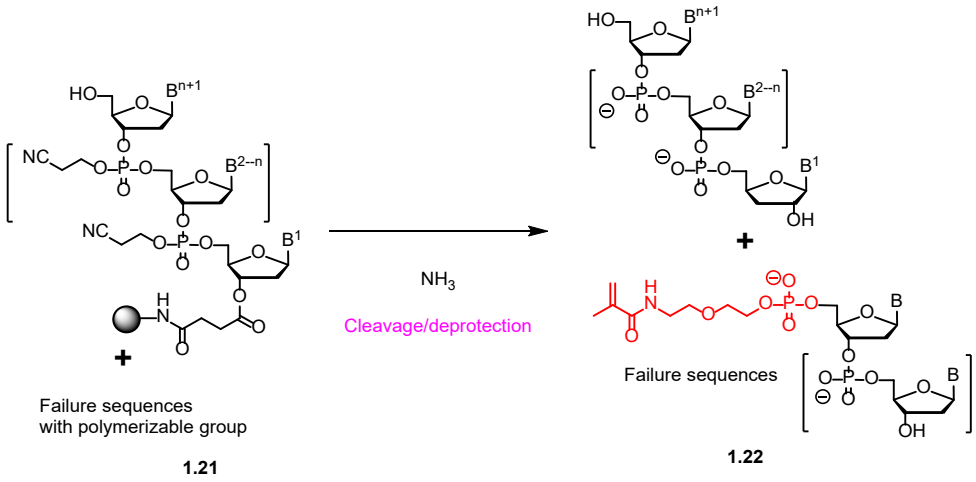
The script of the ODN synthesis was slightly modified to cap failure sequences with the polymerizable capping agent. Instead of using acetic anhydride for capping during the ODN synthesis, the polymerizable capping agent was used. As a result, the failure sequences will have a polymerizable function, and the full-length sequence will not have a polymerizable function.



Scheme 1.4:Modified automated ODN synthesis with polymerizable tagging agent

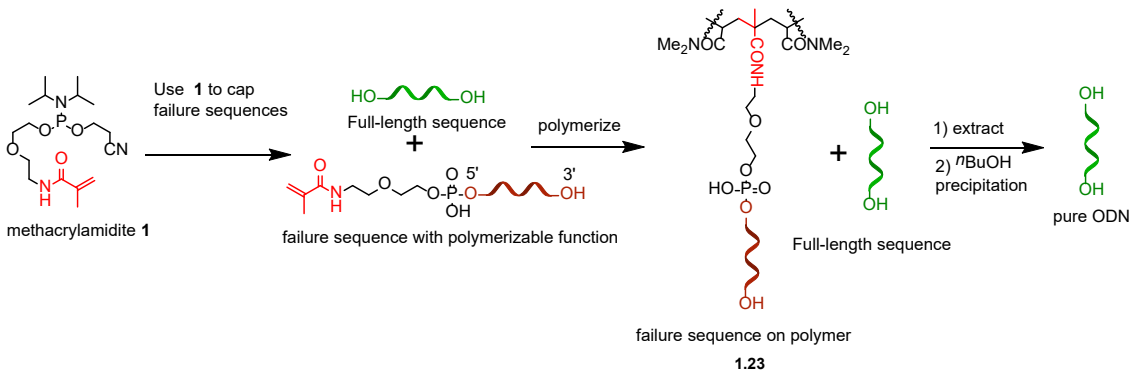
The first step, detritylation, and the second step, coupling, is performed similarly to a typical ODN synthesis. The next capping step was modified slightly. A polymerizable phosphoramidite molecule was used instead of acetic anhydride as a capping agent. The polymerizable phosphoramidite capping agent reacts similarly to a phosphoramidite monomer during the coupling step. Reactivity of the capping agent is assumed higher than that of the phosphoramidite monomer because of the smaller size. After that, the oxidation step was performed to oxidize trivalent phosphite to pentavalent phosphate.

After the synthesis was done, the ODN sample was treated with 28% ammonium hydroxide at 65° C for 18 h to cleave and deprotect ODNs (**1.22**, Scheme 1.5).



Scheme 1.5:ODN deprotection and cleavage

The crude ODN sample can be copolymerized with polymerization mix. The polymerization mix contains *N,N*-dimethylacrylamide, *N,N′*-Methylenebisacrylamide, *N,N,N′,N′* -tetramethylethylenediamine (TMEDA), and ammonium persulfate. *N,N*-dimethylacrylamide is the monomer, *N,N′*-Methylenebisacrylamide acts as a cross-linker during the polymerization process. TMEDA and ammonium persulfate act as initiators during the radical polymerization reaction. When the polymer is formed the failure sequences with the polymerizable function are going to attached to the polymer (**1.23**, Scheme 1.6). The full-length sequences are going to remain in the solution can be extracted. The *n*-butanol precipitation is done to remove any small organic impurities, and the pure ODNs can be obtained.



Scheme 1.6:ODN purification using catching failure sequences by polymerization

The polymerization procedure is shown in Figure 1.5. First, the crude ODN sample was mixed with the polymerization mix. Then the mixture was quickly transferred into a spin tube. After that, the mixture was allowed to polymerize at room temperature for about ten minutes. Then full-length sequences can be extracted from the polymer while failure sequences are bound to the polymer. The full-length sequences still contain small organic molecules from the protecting groups. Small organic molecules can be removed by *n*-butanol precipitation. After the precipitation, pure ODN can be obtained using this method.

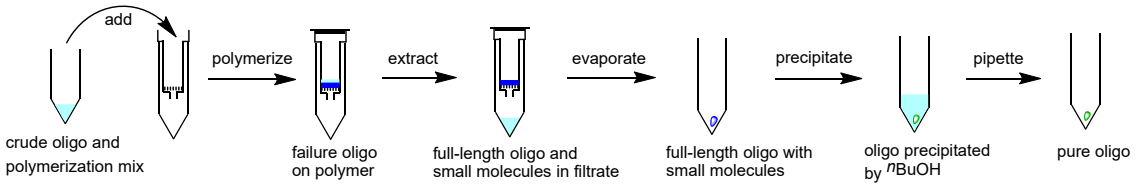


Figure 1.5:ODN purification using catching failure sequence by polymerization

This method is suitable for shorter ODN purification. The polymerizable capping agent is used in each step to cap failure sequences. When the length of the ODN sample gets longer, the amount of capping agent needed increases. Therefore, this method can be costly for long ODN purification, which is one of the method's limitations.

1.8.2 Catching full-length sequence by polymerization

The other method developed is catching full-length sequence by polymerization technique. Here we attached a polymerizable tagging agent with a cleavable linker to the full-length ODN sequence during the ODN synthesis. The other failure sequences are capped with acetic anhydride as usual after each coupling reaction. As a result, the full-length ODN sequence has a polymerizable function while other failure sequences do not have the polymerizable group.

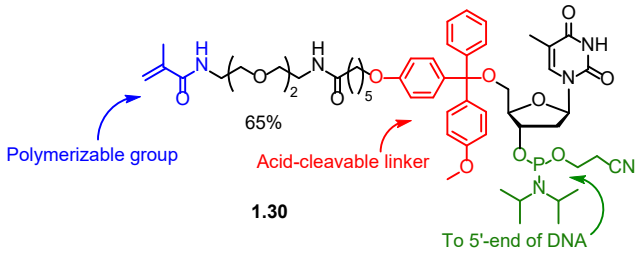
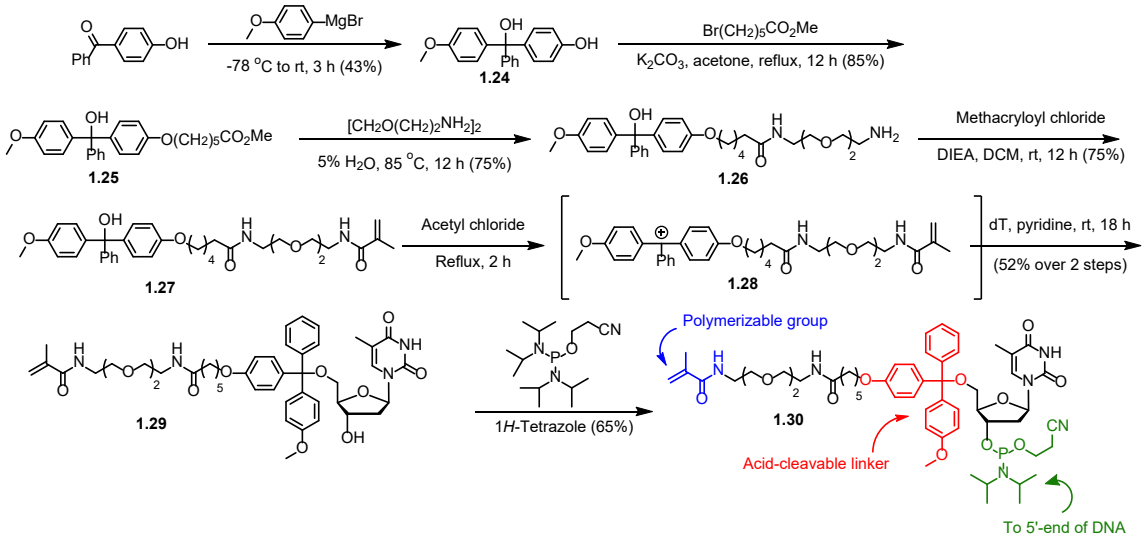


Figure 1.6:Polymerizable dT phosphoramidite

The polymerizable dT phosphoramidite (**1.30**, Figure 1.6) has a polymerizable methacrylamide function attached to the 5' hydroxyl group via a cleavable linker. The phosphoramidite is attached to the ODN sequence as the last monomer during the ODN synthesis (Scheme 1.8). As a result, full-length ODN sequences will have a polymerizable function. Final detritylation is not performed after the ODN synthesis.

1.9 Synthesis of the polymerizable phosphoramidite



Scheme 1.7:Synthesis of the polymerizable dT phosphoramidite

All reactions were performed in oven-dried glassware under a nitrogen atmosphere using standard Schlenk techniques. Reagents and solvents available from commercial sources were used as received unless otherwise noted. CH_2Cl_2 (DCM), pyridine, and acetone were distilled over CaH_2. THF was distilled over Na/benzophenone. Thin-layer chromatography (TLC) was performed using Sigma-Aldrich TLC plates, silica gel 60F-

254 over glass support, 0.25 µm thickness. Flash column chromatography was performed using SiliCycle silica gel, particle size 40–63 µm.

Compound 1.24: To a solution of 4-hydroxy benzophenone (2.00 g, 10 mmol, 1.0 equiv.) in dry THF (15 mL) was added 4-methoxyphenylmagnesium bromide (0.5 M, 40.0 mL, 24 mmol, 2.0 equiv.) dropwise at 0 °C under nitrogen. After 8 h, the mixture was quenched with saturated $NaHCO_3$ (50 mL) and extracted with EtOAc (40 mL × 3). The combined organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated. The crude was purified via precipitation from DCM by hexanes. The compound **1.24** was obtained as a bright orange solid with 93% yield.

Compound 1.25: To a solution of compound **1.24** (5.00 g, 16.33 mmol, 1.0 equiv.) in dry acetone (80 mL) were added methyl-6-bromohexanoate (2.60 mL, 16.33 mmol, 1.0 equiv.) and K_2CO_3 (9.00 g, 65.32 mmol, 4.0 equiv.) at room temperature under nitrogen from a Schlenk line. After refluxing overnight, the volatiles were removed under reduced pressure and the remaining reaction mixture was quenched with saturated NaCl (50mL) and EtOAc (50 mL × 3). The combined organic layer was dried over anhydrous Na_2SO_4 filtered and concentrated. The crude was purified by flash chromatography. R_f = 0.4 (hexanes/EtOAc/Et_3N, 3:1:0.4). The compound **1.25** is obtained as a yellow oil with 85% yield.

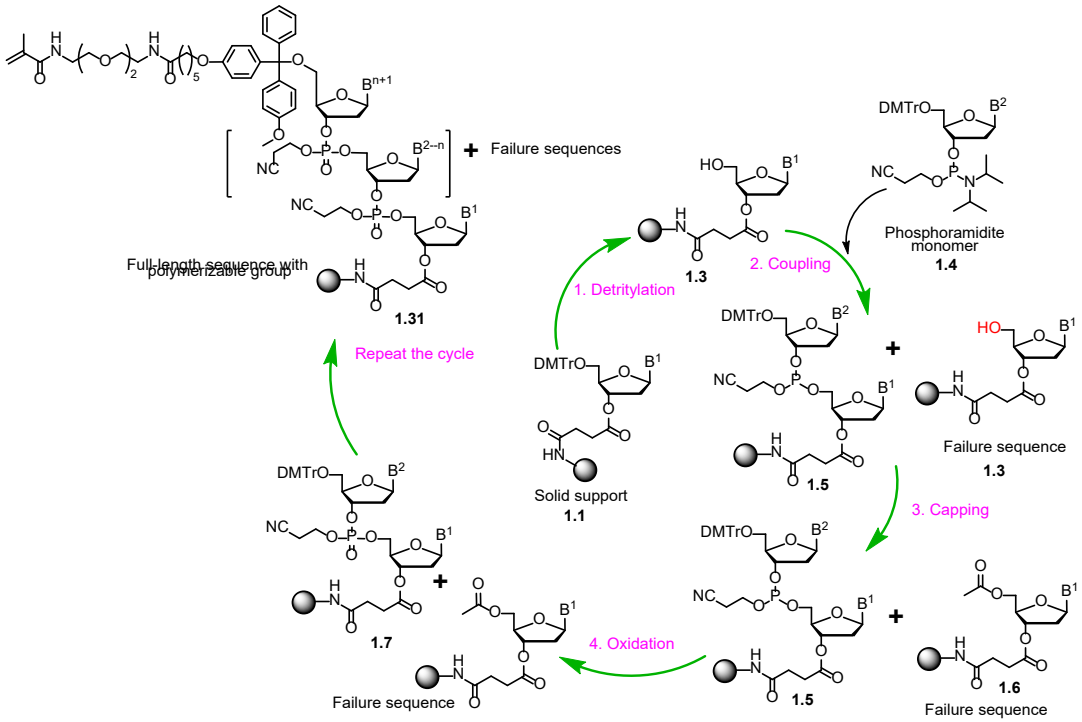
Compound 1.26: To compound **1.25** (3.00 g, 6.91 mmol, 1.0 equiv.), 2,2ˈ-(ethylenedioxy) bis(ethylamine) (3.10 mL, 20.73 mmol, 3.0 equiv.) and water (0.700 mL) were added at room temperature under nitrogen from a Schlenk line. After stirring at 85 °C for 8 h the reaction mixture was concentrated and purified by flash chromatography. R_f = 0.5 (Et_2O/MeCN/MeOH/Et_3N, 5:2:2:1). The compound **1.26** was obtained as a pale-yellow oil with 75% yield.

Compound 1.27: To a solution if compound **1.26** (2.70 g, 4.90 mmol, 1.0 equiv.) in dry DCM was added DIEA (2.60 mL, 14.7 mmol, 3.0 equiv.) at room temperature under nitrogen from Schlenk line. Methacrolyl chloride (479 µL, 4.9 mmol, 1.0 equiv.) in dry DCM (10 mL) was added to the reaction mixture at 0° C slowly via a cannula. After 8 h

the reaction mixture was quenched with 10% Na_2CO_3 (10 mL) and extracted with DCM (15 mL × 3). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated. The crude product was purified using flash chromatography. R_f = 0.3 (hexanes/EtOAc/Et_3N, 1:5:0.6). The compound **1.27** was obtained as a yellow oil with 75% yield.

Compound 1.29: To compound **1.27** (2.08 g, 3.36 mmol, 1.0 equiv.) was added acetyl chloride (4.00 mL, mmol, equiv.) at room temperature under nitrogen from Schlenk line. After stirring the reaction mixture at room temperature for two hours, excess acyl chloride was evaporated, and the contents were washed with dry hexanes to remove any traces of acetyl chloride. The content was dried under vacuum for one hour. Then to a solution of thymidine (1.22 g, 5.06 mmol, 1.5 equiv.) in freshly distilled pyridine (10 mL) at room temperature under nitrogen from Schlenk line was added the dried intermediate crude in pyridine (10 mL) via cannula. After stirring at room temperature for 8 h, the reaction mixture was quenched with 10% Na_2CO_3 (40 mL × 3) and extracted EtOAc (100 mL). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated. The crude was purified using flash chromatography. R_f = 0.4 (EtOAc/MeOH/Et_3N, 9:1:0.1). The compound **1.29** was obtained as a pale-yellow oil with % yield.

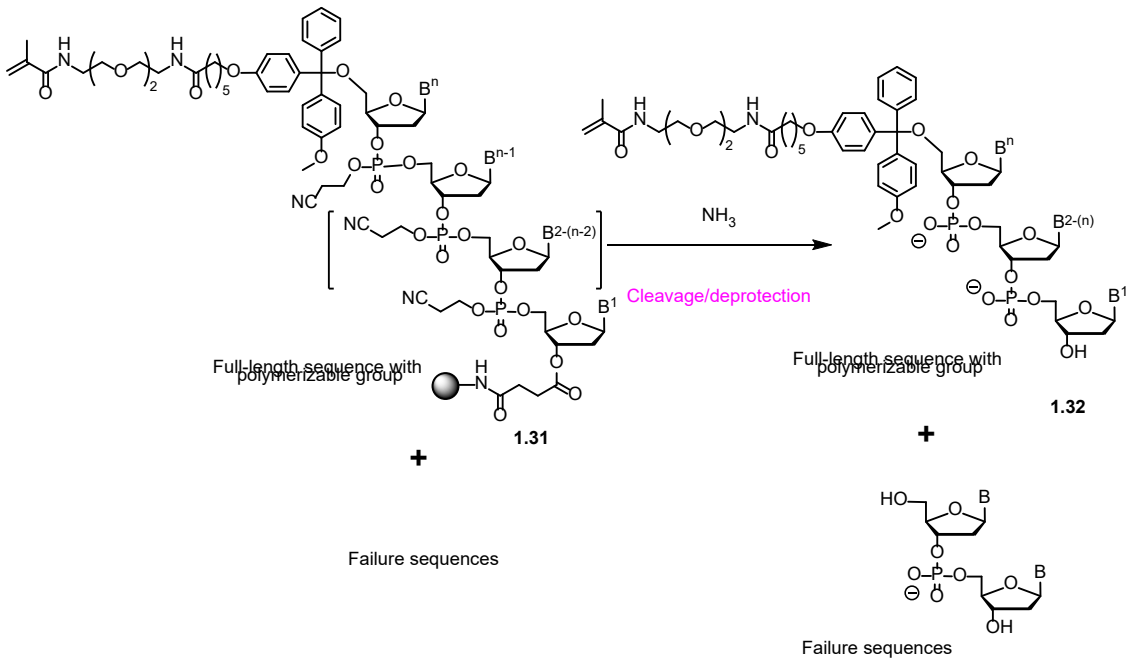
Compound 1.30: To a solution of compound **1.29** (0.408 mg, 0.48 mmol, 1.0 equiv.) in dry DCM (10 mL) under nitrogen from a Schlenk line were added 2 cyanoethyl *N, N,N′,N′* tetraisopropyl phosphoramadite (0.231 mL, 0.73 mmol, 1.5 equiv.) and diisopropyl ammonium tetrazolide (0.125 g, 0.73 mmol, 1.5 equiv.) .After stirring the reaction mixture for 5 h at room temperature the reaction mixture was concentrated and purified using flash chromatography. R_f = 0.5 (EtOAc/acetone/Et_3N, 7:3:0.5). The compound **1.30** was obtained as a pale-yellow oil with 65% yield.



Scheme 1.8:Attaching polymerizable tagging agent during the ODN synthesis.

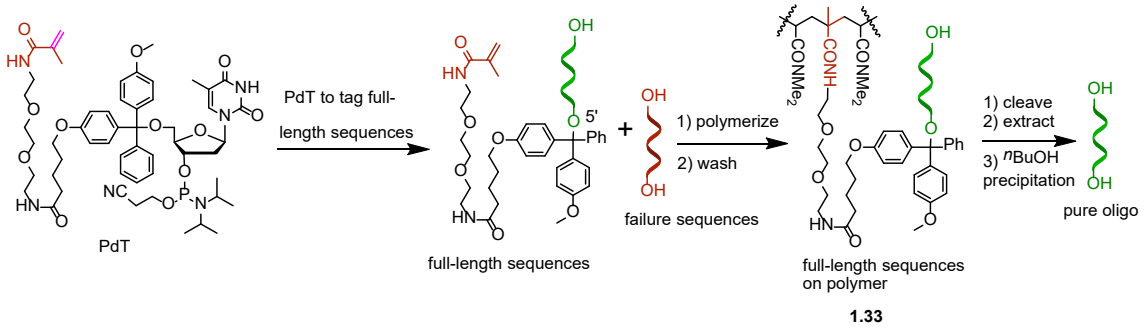
ODN synthesis was done using the same conditions described above. Here acetic anhydride was used as the capping agent using the ODN synthesis. The phosphoramidite (**1.30**, Figure 1.6) was attached to the ODN sequence as the last monomer during the ODN synthesis (Scheme 1.8). As a result, full-length ODN sequences will have a polymerizable function. Final detritylation is not performed after the ODN synthesis.

After the synthesis, the CPG was treated with 28% ammonia solution at 65 °C for 18 h. The crude ODN sample can be obtained after the cleavage and deprotection (**1.32**, Scheme 1.8). This crude ODN sample contains full-length sequences tagged with the polymerizable group and failure sequences without a polymerizable function.



Scheme 1.9:Cleavage and deprotection of ODN tagged with polymerizable group

The crude ODN sample was mixed with the polymerization mix, which was described earlier. The full-length ODN sequences were attached into the polymer via the cleavable linker. Here radical polymerization conditions were used for the polymerization reaction. The full-length sequences were incorporated into the polyacrylamide gel (Scheme 1.9). The failure sequences and other impurities remain in the solution. The failure sequences and other impurities can be washed away while the full-length sequences are still attached to the polymer. The full-length ODN can be cleaved with 80% acetic acid in water. Pure ODN can be obtained after *n*-butanol precipitation which was done to remove any small organic molecules present in the sample.



Scheme 1.10:Purification using catching full-length sequence by polymerization

A schematic diagram of the purification procedure is shown in Figure 1.7. First, the crude ODN sample was mixed with polymerization mix and quickly transferred into a spin tube. After the polymerization was done, the failure sequences could be washed away with water while the full-length sequences were still attached to the polymer. Then the full-length sequences were cleaved from the linker using 80% acetic acid solution. The gel was further extracted with water to collect any left-over ODN sequences. After evaporating the acetic acid and water, the pure ODNs were precipitated with *n*-butanol to remove any small organic molecules from the sample. After that, pure ODNs can be obtained.

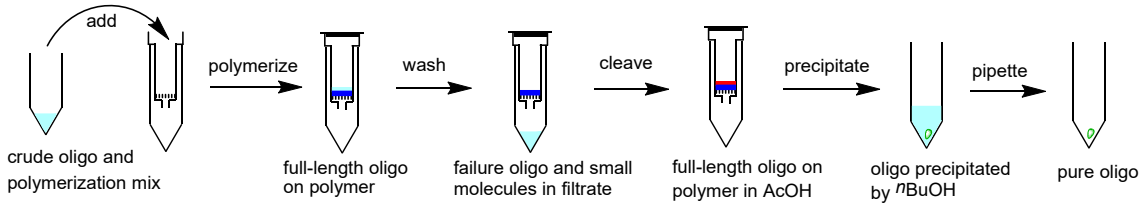


Figure 1.7:ODN purification using catching full-length sequence by polymerization

This method is convenient because the polymerizable phosphoramidite was only used once, as the last monomer, during the ODN synthesis to tag the full-length ODN sequence. As a result, this method can be used to purify ODNs as long as 303-mer, high throughput purification, and large scale (60 μmole scale) purification, are further described in the next chapter.

1.10 Advantages of non-chromatographic purification methods

Most purification methods are chromatographic methods, while our methods are not. As a result, the method is capable of large-scale ODN purification, Long ODN purification, and high-throughput ODN purification. The method involves simple mixing and filtering steps and is hence less labor-intensive. The method also does not require expensive instrumentation or expensive reagents. As a result, it is cost-effective. It uses fewer solvents than other purification methods, and it is more environmentally friendly than most other methods.

2 Parallel, Large Scale and Long Synthetic Oligodeoxynucleotide Purification Using the Catching Full-Length Sequence by Polymerization Technique

Keyword: oligonucleotide, purification, synthesis, polymerization, large scale, high throughput

2.1 Abstract

The catching by polymerization synthetic oligodeoxynucleotide (ODN) purification technique was shown to be potentially suitable for high throughput purification by purifying 12 ODNs simultaneously, to be convenient for large-scale purification by purifying at 60 μmol synthesis scale, and to be highly powerful for long ODN purification by purifying ODNs as long as 303-mer. LC-MS analysis indicated that the ODNs purified with the technique have excellent purity.

2.2 Introduction

In recent years, several emerging research areas including synthetic biology,[3, 4, 21-23]CRISPR genome editing,[24-28] antisense therapeutics[5, 29] and DNA data storage[6, 30-32] created a high demand for synthetic oligodeoxynucleotides (ODNs). To meet this demand, high throughput and large-scale ODN production are required. Although significant progress has been made on high throughput and large scale ODN synthesis in the last few years, limited progress has been made on high throughput and large-scale purification.[7, 15, 17, 33-36] Currently ODN purification technologies mainly include HPLC and gel electrophoresis. Although HPLC has been adapted for large-scale purification, it is highly expensive, requires expensive instrument and large volumes of harmful organic solvents, and is not suitable for high throughput purification. Gel electrophoresis is tedious, cannot be scaled up and is not suitable for high throughput purification either. Other methods for ODN purification include cartridge purification,[15, 16, 37] fluorous affinity purification[13, 14] and biotin-streptavidin affinity purification.[38-40] To our knowledge, these methods have limited success on large-scale and high throughput purification although cartridge purification has been widely used for small scale purification. We recently reported a new

technique for synthetic ODN purification, which we call catching full-length sequence by polymerization.[19, 41] The method involves capping the failure sequences in each synthetic cycle with acetic anhydride or other reagents as in typical ODN synthesis and tagging the desired full-length sequence with a polymerizable methacrylamide group at the end of synthesis. After cleavage and deprotection, the full-length sequence is co-polymerized into an insoluble cross-linked polyacrylamide polymer, while the failure sequences and other impurities remain in solution. After washing away the impurities with water, pure full-length ODN is cleaved from the polymer. In this paper, we report experimental procedures that prove the suitability of the technique for high throughput and large-scale ODN purification, and the unlimited power of the technology for isolation of long ODNs (up to 303-mer) from the complex mixtures of crude ODNs. In addition, to further confirm the high purity of the ODNs purified with the technique, several samples were thoroughly analyzed with LC-MS. This method represents a new dimension of analysis of the ODNs purified with the catching by polymerization techniques in the context of our previous analysis efforts.

Table 2.1:ODN numbering and length [a]

ODN	length	ODN	length
1a	15-mer	**1n**	43-mer
1b	21-mer	**1o**	64-mer
1c	23-mer	**1p**	80-mer
1d	19-mer	**1q**	90-mer
1e	20-mer	**1r**	110-mer
1f	20-mer	**1s**	151-mer

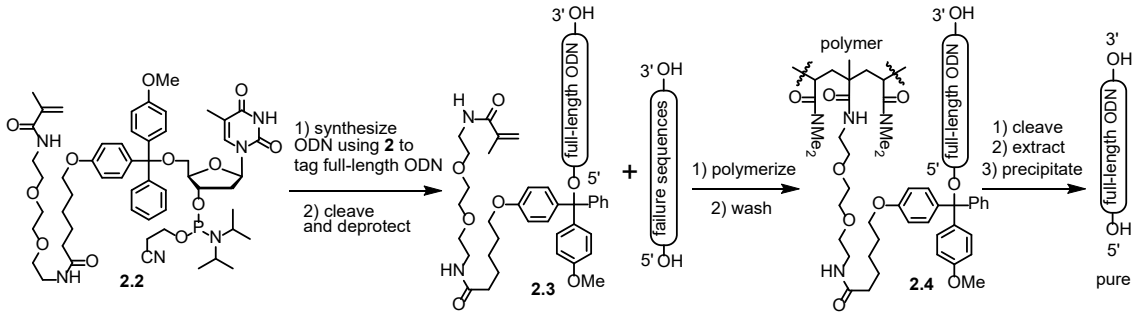
1g	20-mer	**1t**	197-mer
1h	21-mer	**1u**	203-mer
1i	21-mer	**1v**	225-mer
1j	22-mer	**1w**	251-mer
1k	26-mer	**1x**	275-mer
1l	28-mer	**1y**	303-mer
1m	32-mer		

[a] Sequence information is provided in Appendix.

2.3 Results and discussions

2.3.1 Parallel purification

To provide evidence that the technique is suitable for high throughput ODN purification, an experiment was set up to purify 12 different ODNs simultaneously. The ODNs (**1a-l**, Table 1) were selected from the Φ29 DNA polymerase gene. The equipment needed for the parallel purification were a multichannel pipette and a 96-well micro plate centrifuge. The ODNs were synthesized on an automated MerMade-6 synthesizer by standard DNA synthesis. Acetic anhydride was used to cap failure sequences during the synthesis. In the last synthetic cycle, the tagging reagent (**2.2**, Scheme 2.1) instead of a standard nucleoside phosphoramidite was used for coupling. Capping and oxidation were then carried out under normal conditions, but detritylation was omitted, which otherwise would remove the polymerizable methacrylamide tag introduce with (**2.2**, Scheme 2.1). After the synthesis, the ODNs were cleaved from the solid support (CPG) and fully deprotected under typical conditions. The fully deprotected ODNs (**2.3**, Scheme 2.1) had a polymerizable methacrylamide tag at their 5′-end (Scheme 2.1). The crude ODNs contained (**2.3**, Scheme 2.1) and impurities including failure sequences and small molecules such as benzamide.



Scheme 2.1:The catching full-length sequence by polymerization ODN purification technique

The parallel purification began after the solution of the 12 crude ODNs in concentrated ammonium hydroxide in 12 centrifuge tubes were evaporated to dryness in a vacuum centrifugal evaporator. Suitable volumes of water were added to the 12 tubes using the 12-channel pipette. The tubes were shaken gently to dissolve the ODNs. The polymerization solution containing *N*,*N*-dimethylacrylamide and *N*,*N*,*N*′,*N*′-methylenebis(acrylamide), and the polymerization initiation solutions of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) and ammonium persulfate were then added sequentially. The tubes were gently shaken, and the solutions were quickly transferred to the top compartment of 12 centrifugal filter units. The mixtures were allowed to polymerize over the filter in the units at room temperature for about one hour. The gels represented by (**2.4**, Scheme 2.1) were broken into several pieces. Water was added, and then removed by a short spin in a centrifuge. Several rounds of washing removed impurities including the failure sequences. To the gel was added 80% acetic acid, which cleaved the trityl ether bond in (**2.4**, Scheme 2.1) and cleaved the full-length ODN from the polyacrylamide polymer. A short spin in a centrifuge separated the solutions from the gels. The gels were washed with water using the procedure involving adding water and spin for several times. The top compartment of the centrifugal filter units along with the gels in them were discarded and the solutions in the tubes were evaporated to dryness. The residue was dissolved in a small amount of ammonium hydroxide solution, and then *n*-butanol was added. The mixtures were then agitated and centrifuged. The full-length ODNs were

precipitated. The supernatants were removed with the 12-channel pipettes. The precipitates were pure ODNs (Scheme 2.1). The steps involving precipitation with *n*-butanol from ammonium hydroxide solution may be omitted, and the purity of ODNs is not compromised according to HPLC analysis. However, we still suggest carrying out the precipitation procedure because it could remove any residue acetic acid from the ODNs, which could damage ODN over time.

The 12 purified ODNs were analyzed with RP HPLC. The critical sections of the HPLC profiles are shown in Figure 2.1. The full profiles of crude and pure ODNs are provided in Supporting Information. All ODNs were highly pure (> 99%) except for one of them. In the HPLC profile of **1i**, an additional small peak eluting before the major ODN peak was observed. The identity of this peak is unknown. We also noticed that in the profile of 1k, there were some impurities before the ODN peak, which could be failure sequences. We believe that this small amount of impurities could be easily removed by washing the polyacrylamide gel **2.4** more thoroughly. For all the 12 ODNs, MALDI-TOF MS analyses gave predicted molecular peaks (Appendix). It is remarkable that in all the cases, the spectra were very clean. Except for a few minor peaks, all appreciable peaks were accountable based on the ODN structures (Appendix).

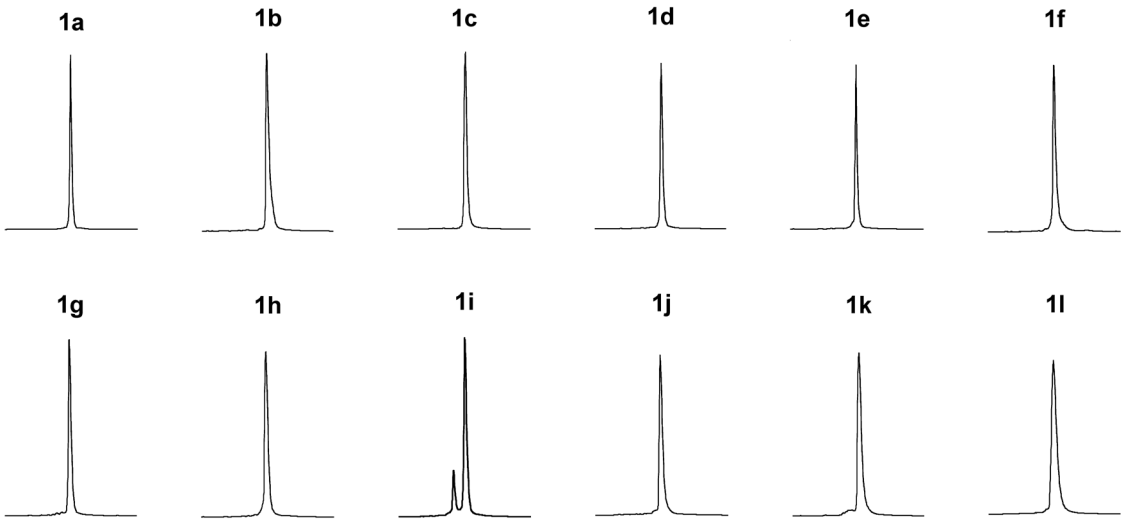


Figure 2.1:Critical sections of the HPLC profiles of ODNs **1a-l**. The ODNs were purified in a high throughput fashion using the catching full-length sequence by polymerization technique.

The protocol described above for the parallel purification of 12 ODNs does not contain any complicated manipulations, and all the steps could be performed in a parallel fashion. Therefore, in principle, the purification technique can potentially be scaled up to purify 96 or more ODNs simultaneously without significantly increasing the working load. Because high throughput ODN synthesis technologies have been developed in the past few years, we believe that our catching by polymerization technique could bring about transformative changes in the industry of high throughput ODN production.

2.3.2 Large-scale purification

To prove that the catching by polymerization technique is suitable for large-scale ODN purification, we synthesized the 32-mer ODN **1m** at 60 μmol scale. The synthesis conditions were similar to those for the synthesis of the ODNs for parallel purification but with some modifications including carrying out the synthesis using two 30 μmol columns. Experimental details are disclosed in the experimental section. The catching by polymerization procedure was also similar to the parallel purification except that the polymerization was performed in a round bottom flask and the polyacrylamide gel (**2.4**, Scheme 2.1) was transferred into a Büchner funnel with glass sintered filtration disc for

washing away impurities and cleaving ODN from the gel. The former was achieved with trimethylamine solution and water. The latter was conducted with 80% acetic acid. In the processes, removing liquids from the funnel was achieved by applying vacuum from a water aspirator. The filtrate containing the full-length ODN was evaporated to dryness, which gave a light-yellow sticky oil. Upon precipitation from concentrated ammonium hydroxide by *n*-butanol, the ODN appeared as a white solid (photo in Appendix). HPLC analysis indicated that the ODN was highly pure (Appendix). The OD_{260} was determined to be 13,075, which corresponds to a 65% yield for the synthesis and purification. The yield was significantly higher than what we obtained earlier (15%).[31] The improved yield was likely a result of more efficient coupling of (**2.2**, Scheme 2.1) to the 5'-end of ODN during synthesis and more complete extraction of the full-length ODN from the gel during purification.

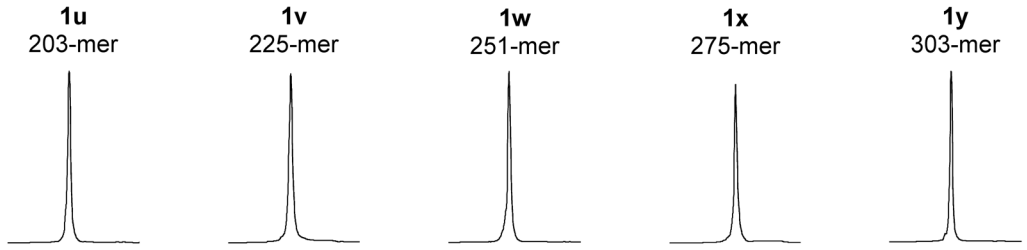


Figure 2.2:Critical sections of the HPLC profiles of the long ODNs **1u-y**. The ODNs were purified using the catching full-length sequence by polymerization technique.

2.3.3 Long ODN synthesis and purification

A highly impressive feature of the catching full-length sequence by polymerization technique is its power to purify long ODNs. We believe that as long as an ODN can be synthesized, no matter how long it is and how complex the crude mixture is, using the technique, the full-length ODN could potentially be isolated. We previously demonstrated the isolation of ODNs as long as 197-mer from complex crude mixture.[41] In the current studies, we successfully synthesized and purified ODNs **1n-1y**, which ranged from 43-mer to 303-mer (Table 1). The ODN synthesis conditions were similar as described for the synthesis of ODNs for parallel purification with some modifications. The syntheses were conducted on CPGs with larger pore sizes (1400 Å and 2000 Å) and at smaller scales (0.2

μmol). Before synthesis, the CPG was subjected to the capping conditions to block any free hydroxyl and amino groups. During synthesis, coupling was conducted three times instead of two. It is noted that during the long ODN syntheses, when the ODNs reached the length of 140-nucleotide, no red or orange color of the trityl cation could be observed in the synthesis column during detritylation. The synthesizer could not detect any trityl signal either. However, we assumed that a small portion of the nucleotide chains were still not capped and could continue to grow in subsequent synthetic cycles. We therefore allowed the synthesis to continue.

We were not certain if we could isolate ODNs longer than 200-mers and detect them with HPLC. Therefore, we gradually increased the length from 203-mer (**1u**), 225-mer (**1v**), 251-mer (**1w**), 275-mer (**1x**), to 303-mer (**1y**). After successful synthesis and purification of shorter ones in the series, we went further to the next longer ones. The purification procedure was similar as described for parallel purification except that the ODNs were purified one at a time. In addition, urea was added to the polymerization solution to destroy any possible secondary structures of the long ODNs, or hybrids formed between the long ODNs and failure sequences. Destroying these structures is important for washing away impurities efficiently. Overall, in all the purification cases, single sharp peaks of ODNs were observed in the HPLC profiles. The critical sections of the HPLC profiles of **1u-y** are shown in Figure 2.2. The full profiles of crude and pure **1n-y** are provided in Appendix.

We believe that the success of synthesizing ODNs with lengths up to 303-nucleotide and using the catching full-length sequence by polymerization technique to isolate them from the complex crude mixture is noteworthy. To our best knowledge, so far, no other technique has the capability to isolate target ODNs from the complex mixtures generated from the many steps of chemical synthesis needed for assembling the long ODNs. In principle, gel electrophoresis could purify 303-mer ODNs because it could resolve a 303-mer from a 302-mer. However, when the mixture is highly complex and the mass percentage of the desired full-length ODN in the mixture is low, gel electrophoresis is impractical for the application. The solid phase extraction approach, which relies on the

formation of a covalent bond between a functionalized solid support and the full-length ODN, could be an option. However, reactions between large molecules are inherently slower than the same reactions between smaller molecules, and it is difficult for large ODNs to diffuse into the matrix of solid phase to form the covalent bond, this approach is unlikely to achieved satisfactory results for long ODN purification either.[33] The purification approaches based on the interactions between antigen and antibody such as biotin and streptavidin could also suffer the problem of difficulty for large molecules to diffuse into solid matrix.[38-40] The fluorous affinity purification method has been demonstrated for long ODN purification, but when the length of the ODNs reaches 100-mer, differentiation between the full-length sequence and failure ones becomes difficult.[13, 14] Other methods such as HPLC and hydrophobic cartridge purification are clearly unsuitable for the application. When considering technologies for long ODN purification, it is important to note that resolution of ODNs of different lengths is only one of the challenges. Another challenge, which is to isolate the desired sequence that is of low mass percentage from a complex mixture, is even more difficult to overcome. The catching full-length sequence by polymerization technique is well suited to overcome both challenges, and therefore it is a promising tool for accomplishing this difficult task.

2.3.4 LC-MS analysis of the ODNs purified with the catching-by-polymerization technique

One concern about the catching by polymerization purification technique is the damage of ODNs in the acrylamide radical polymerization step. So far, we have used RP HPLC, anion-exchange HPLC and MALDI-TOF MS to characterize the ODNs. No ODNs that were damaged by radicals were detected using these analytical methods.[20, 41-44] In addition, we subjected the four nucleosides – adenosine, cytidine, guanosine and thymidine – to the acrylamide radical polymerization conditions; HPLC analyses of the recovered nucleosides showed that they were identical with authentic nucleosides, and the recovered guanosine was different from 8-oxo-guanosine – the commonly observed radical damaged guanosine.[42] We had synthesized ODNs containing 8-oxo-guanosine, and compared them with the ODNs synthesized with unmodified nucleosides and purified with the catching by polymerization technique, no damage in the purified ODNs was found either.[19] We also

conducted the experiments involving enzymatic digestion of the purified ODNs into nucleotides, converting the nucleotides to nucleosides, and HPLC analysis of the nucleosides; HPLC analysis indicated that the nucleosides were intact.[19] However, ODNs are relatively large molecules, their characterization, especially determining their purity, is highly challenging. Therefore, more dimensions of characterization of the ODNs purified by the catching by polymerization technique is always desirable. For this reason, we selected three sequences (**1a-c**) from the ODNs purified using the catching by polymerization technique, and analyzed them with LC-MS.

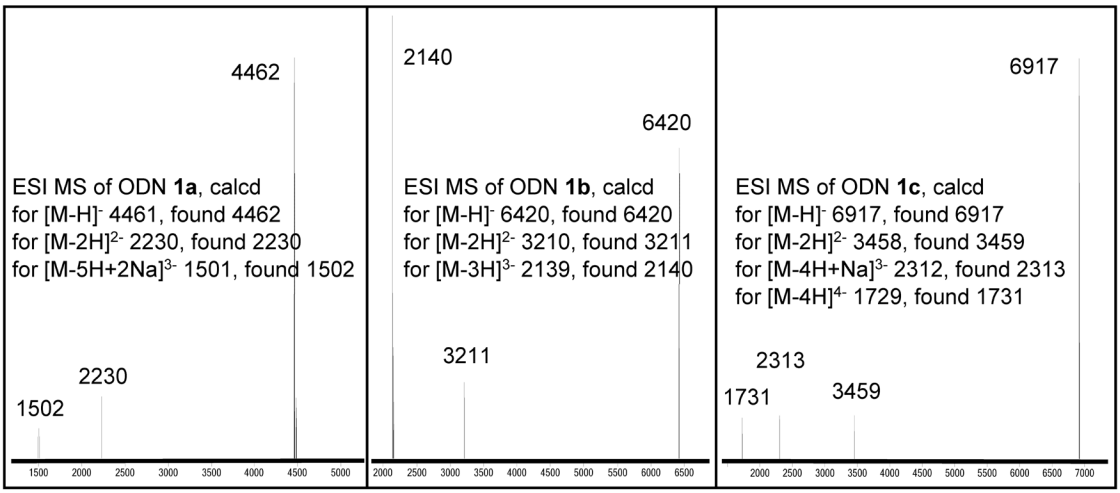


Figure 2.3:Critical sections of the MS spectra of ODNs **1a-c**. The sections are from full MS spectra obtained from the full elution peak range in LC-MS experiments. More detailed LC-MS data are provided in Supporting Information.

The critical sections of the MS spectra of the three ODNs (**1a-c**) obtained from the full elution peak range are shown in Figure 2.3. The full MS spectra from the full elution peak range as well as selected sliced LC peak ranges are provided in Supporting Information. Overall, all the spectra were very clean. Except for peaks from double or triple charged full-length ODNs, additional peaks with masses lower than the predicted molecular masses were not observed or very insignificant. Additional peaks with masses

higher than the predicted molecular weight other than common metal adducts of the ODNs were not detected in all the three cases. These observations indicated that all failure sequences and other impurities in the crude ODNs were washed away during purification. In addition, the n-1 deletion sequences and n+1 addition sequences, which could be resulted from incomplete capping of failure sequences and double coupling due to premature detritylation, respectively, were not formed in any appreciable amounts during ODN synthesis. Importantly, the results once again prove that the conditions for the acrylamide radical polymerization reaction during purification did not cause ODN damages.

2.4 Conclusion

To advance the catching full-length sequence by polymerization technique to the level of practical use for ODN purification, we successfully adapted the technique for parallel purification by purifying 12 ODNs simultaneously. In addition, we further demonstrated the suitability of the technique for large-scale purification and the power of the technique for long ODN purification. It is remarkable that the technique can readily isolate ODNs up to 303 nucleotides in length with low mass percentages from highly complex crude mixtures. Further, we used LC-MS to analyze three ODNs purified with the catching by polymerization technique. This represents an additional dimension of characterization of the ODNs purified with the technique. The results further proved that the ODNs were highly pure. Currently we are making efforts to identify a niche market for the catching by polymerization technique to enter the field of ODN production. We believe that the technique will be equally suitable for purification of RNA and other biooligomers including peptides,[45] and we are also making efforts in those areas.

2.5 Experimental section

2.5.1 General Information

Reagents and CPG for ODN synthesis were purchased from Glen Research and Bioautomation and were used as received. The polymerizable tagging agent **2.2** was synthesized according to reported procedure.[31] Chemicals used in the catching by polymerization procedure were purchased from Aldrich and were used directly. The

centrifugal filtering unit was purchased from Aldrich. Analytical RP HPLC was performed on a JASCO LC-2000Plus System with PU-2089Plus Quaternary Gradient pump and UV-2075Plus detector. Column: C-18, 5 μm, 100 Å, 250 × 3.20 mm. Detection: UV at 260 nm. Eluents and gradient for pure and crude **1a-m** and crude **1n-y**: solvent A, 0.1 M triethylammonium acetate and 5% acetonitrile; solvent B, 90% acetonitrile; time, 0-60-80 min, B%, 0-45-100; flow rate 1.0 mL/min. Eluents and gradient for pure **1n-y**: solvent A, 200 mM HFIP and 8.1 mM Et_3N; solvent B, methanol; time, 0-5-25-80 min, B%, 5-5-70-70; flow rate 1 mL/min. MALDI-TOF MS were obtained on Bruker's microflex™ LRF MALDI-TOF System.

2.5.2 ODN Synthesis

All ODNs were synthesized on a MerMade 6 solid phase synthesizer. The synthesizer manufacturer recommended synthesis scripts were used with slight modifications. Concisely, long chain amino alkyl CPG (pore size, 1000 Å for ODNs **1a-m**, 1400 Å for **1n-r**, 2000 Å for **1s-y**) was used as solid support. Detritylation: 2% DCA in DCM, 90 sec × 2 (50 sec × 4 for 1m). Coupling: 0.1 M commercial 2-cyanoethyl 5′-DMTr Bz-dA,Ac-dC, *i*Bu-dG and dT in acetonitrile, 100 sec × 2 (100 sec × 3 for **1m-y**). Capping: cap A, THF/pyridine/Ac_2O (8:1:1), cap B, 16% methylimidazole in THF, 50 sec × 2 (50 sec × 4 for 1m). Oxidation: 0.02 M I_2 in THF/pyridine/H_2O, 60 sec (90 and then 25 sec, and repeated 1 time for 1m). In the last synthetic cycle, the tagging phosphoramidite 2 instead of a standard nucleoside phosphoramidite was used in the coupling step (100 sec × 5 for 1m, 100 sec × 3 for others). At the end of the synthesis, detritylation was not performed. Special for **1n-y**, before synthesis, the CPG was subjected to capping (50 sec × 2) to block any free hydroxyl and amino groups.

2.5.3 Parallel Purification

The ODNs **1a-l** were synthesized on a 1 μmol scale under conditions described in ODN Synthesis. The CPGs were divided into 5 equal portions. One portion was suspended in conc. NH_4OH (300 μL) in sealed 1.5 mL centrifuge tubes and heated at 55 °C for 12 h. After cooling to rt and removing supernatants, the CPGs were washed with conc. NH_4OH (100 μL). The combined supernatants and washes for each ODN were evaporated to dryness in a vacuum centrifugal evaporator. To the ODNs in 12 centrifuge tubes were

added 50 μL water using a 12-channel pipette. The tubes on a 96-well plate were shaking gently to dissolve the ODNs. A small portion of the solution (1 μL) was injected into HPLC to generate the crude profiles. A polymerization solution (12 μL) containing *N*,*N*-dimethylacrylamide (6.6 M) and *N*,*N*′-methylenebis(acrylamide) (0.33 M) was added to the remaining solution. After shaking the plate gently, the polymerization initiator solutions *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (0.66 M, 5 μL) and $(NH_4)_2S_2O_4$ (0.22 M, 5 μL) were added sequentially. The 12 solutions were transferred to the top compartment of 12 centrifugal filter units using the 12-channel pipette. The tubes were closed, and the mixtures were allowed to polymerize at rt for ~1 h. The polyacrylamide gels were loosened or broken into pieces, and NaOAc solution (20%, 250 μL or more) was added, which was removed with a spin in a centrifuge after standing for 3 min. The washing was repeated 8 times. Then the gels were washed with a Et_3N solution (5%, 250 μL or more, 3 min) for 6 times, and finally with water (250 μL or more, 0 min standing time) for 6 times. All the filtrates were discarded. To the gel was added minimum volumes of 80% AcOH that could cover the gel (~100 μL). The cleaving reaction was allowed to proceed at rt for 3-5 min. The cleaving solutions were collected by spinning in a centrifuge. The cleavage procedure was repeated 3 times. The gels were washed with water (~100 μL × 2, 3-5 min standing for each), and the combined cleaving solutions and washes were evaporated to dryness in a vacuum centrifugal evaporator. To the ODNs were added conc. NH_4OH (100 μL). After warming at 65 °C for 10 min and cooling to rt, *n*BuOH (900 μL) was added. The tubes were vortexed (~20 sec), and then centrifuged at ~14K rpm for ~3 min. The supernatants were removed with the 12-channel pipette, and the residues were the purified ODNs. For HPLC analysis, to the ODNs were added 50 μL water, and 20 μL was inject into HPLC to obtain the profiles of pure ODNs. The OD_{260} values of the ODNs were obtained as described in Supporting Information.

2.5.4 Large-Scale Purification

The ODN **1m** was synthesized using two 30 μmol synthesis columns under conditions described in ODN Synthesis. Cleavage and deprotection were carried out in a sealed pressure tube with conc. NH_4OH (25 mL, 65 °C, 12 h). The CPG and ODN solution

were separated by filtration, and the CPG was washed with NH_4OH (5 mL × 5). The solution and washes were combined in a round bottom flask. A small portion (1 μL) was used to generate the crude HPLC profile. After adding ~1 mL DIEA to the remaining solution, the mixture was concentrated to close to dryness on a rotary evaporator under high vacuum. To the ODN was added water (3.4 mL), *N*,*N*-dimethylacrylamide (2.27 mL), and *N*,*N*′-methylenebis(acrylamide) (70 mg), and the flask was sealed with a rubber septa and placed under nitrogen from a gas line via a needle. The flask was shaken gently for the contents to form a homogenous solution. The nitrogen line was shifted to vacuum from a water aspirator to degas the solution briefly, and the line was shifted back to nitrogen. The initiator solutions *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (0.66 M, 10 μL) and $(NH_4)_2S_2O_4$ (0.22 M, 10 μL) were added via syringes. The flask was shaken shortly to mix the contents, and then allowed to stand at rt under nitrogen. After 1 h, a gel was formed, which was broken into small pieces and transferred into a 60 mL Büchner funnel with a glass sintered filtration disc. The gel was washed with 5% Et_3N (15 mL × 12, 30 min incubating for each wash), and then water (20 mL × 12, 0 min incubating). The ODN was then cleaved from the gel with 80% AcOH (10 mL × 5, rt, 10 min incubating each time). The gel was washed with water (10 mL× 8, rt, 15 min incubating). The gel was further soaked in water overnight (80 mL, rt), from which significant amount of ODN was recovered. Additional extraction of the gel with water did not give any ODN. All the solutions containing ODN including the AcOH solution, the water washes and soaking solution were combined and evaporated to dryness. The residue was dissolved in conc. NH_4OH (5 mL), *n*BuOH (45 mL) was added. Upon mixing, a white solid was formed. The supernatant was removed. The solid was dissolved in water and transferred into a centrifuge tube. Volatiles were removed under vacuum in a centrifugal evaporator giving the ODN as a white solid. The ODN was dissolved into water (5 mL). A small portion was injected into HPLC to generate the pure profile. The OD_{260} value of 1m was obtained as detailed in Supporting Information.

2.5.5 Long ODN Purification

The long ODNs **1n-y** were synthesized at 0.2 μmol under the conditions described in ODN Synthesis. Cleavage and deprotection were achieved using conc. NH_4OH at 55 °C

for 12 h. The catching by polymerization purification procedure was similar as described for parallel purification with the following modifications. The ODNs were purified one by one instead of simultaneously. Before adding the polymerization solution, the ODNs were dissolved in a urea solution (7 M, 50 μL) instead of water. After precipitation with *n*BuOH, the ODNs were dissolved in water (100 μL), heated to 60 °C, and cooled to 0 °C rapidly. Immediately, 20 μL of the solution was injected into HPLC to obtain the pure HPLC profiles. The OD_{260} values of the ODNs were obtained as detailed in Supporting Information.

2.5.6 LC-MS Analysis

All analyses were performed on an Agilent 1200 HPLC System, which was coupled to an Agilent 6224 Time of Flight LC/MS system with Electrospray Ionization source. Column: Agilent Extended C-18, 1.8 μm, 80 Å, 50 × 2.1 mm. Detection: negative total ion content. Eluents and gradient: solvent A, 200 mM HFIP and 8.1 mM TEA; solvent B, methanol; time, 0-1-11-11.5-14.5-15 min; B%, 10-10-70-90-90-10; flow rate 0.2 mL/min. Column temperature: 40 °C. Critical sections of MS of full elution peak ranges of ODNs **1a-c** are in Figure 3. Full profiles of LC, and full MS spectra of full elution peak and selected sliced peak ranges are in Appendix.

3 Introduction to Polyethylene Glycol

3.1 Polyethylene glycol

Polyethylene glycol (Figure 3.1) (PEG) is a polymer consisting of ethylene glycol units. Due to interesting physical and chemical properties such as high solubility in water and other organic solvents, flexible, neutral, and stable structure, non-immunogenicity, non-antigenicity, and biocompatibility[46-50] PEGs have found many applications in various fields.

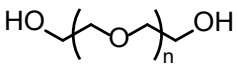


Figure 3.1:Structure of polyethylene glycol

3.2 Applications of polyethylene glycol

PEGs are biocompatible, and as a result they are used in the pharmaceutical industry as an ingredient that can be attached to therapeutics to increase the solubility, stability, and reduce the therapeutics immunogenicity. Attachment of the PEG chain to therapeutics which is known as PEGylation. PEGs are also used in drug delivery.[51] Apart from that, PEGs are used in other areas such as surface science[52], nanotechnology[53],cross-linkers[54], bioconjugation[55, 56], organic inorganic hybrid materials[57].

3.2.1 Pharmaceutical applications

3.2.1.1 PEGylation of therapeutics

PEG or PEG derivatives can be attached covalently to therapeutics, known as PEGylation. This process increases the stability, solubility, controlled release, extended half-life, enhanced pk/pk (pharmacokinetic/pharmacodynamic) profile, and bioactivity[51, 58, 59]. Bioactivity of the PEGylated drugs can be influenced by the length of the PEG chain, the PEGylation site, the linker chemistry, and the PEGylation reaction temperature[54, 59].

3.2.1.2 PEGs as drug delivery vehicles

PEGs can be used as drug delivery systems such as liposome[60], dendrimers[61], micelles[62], and carbon nanotubes[63, 64]. Use of liposomes and dendrimers as drug vehicles

can increase the stability and circulation time of the drug. Surface modification of nanoparticles used for drug delivery can enhance the dispersion of nanoparticles in the aqueous environment and have a higher drug loading capacity. PEGs can also be used to form micelles to deliver drugs. These will increase the colloidal stability, has high drug loading, enhanced encapsulation efficiency, and long circulation time[54, 65].

3.2.2 PEG linkers

The PEG linkers are extensively applied in surface modification[66]. In one application, PEGs are attached between the surface and peptide chains, and these PEGs linkers can enhance the specificity of peptide binding[58]. Another application is modifying the silicon atomic force microscope (AFM) cantilever tips to enhance the resolution of the images, by reducing the cantilever tip surface size[67].

3.2.3 Organic-inorganic hybrid materials

Organic-inorganic hybrid materials have changed the Chemical, physical, biomedical, optical, and chromatographic properties of molecules of interest. PEGs are hydrophilic, and water-soluble can be used to obtain hybrid PEG-Silica particles that are biocompatible.

3.2.4 Carbon nanotube functionalization

Carbon nanotubes (CNT) are used in many applications. High toxicity and poor water solubility of CNTs limits their applications. Recent studies have found that by attaching PEGs the cytotoxicity of CNTs can be reduced, and as a result they can be used for biomedical applications. Functionalizing CNT surfaces by PEGs will increase the water solubility of CNTs, hence can be used for many applications such as studies with live cells[68].

3.3 Current PEG synthesis methods

Synthesis of PEG is mainly employed by polymerization of ethylene oxide solution-phase synthesis. Even though these two methods are widely used, some limitations are associated with these methods. These two methods will be discussed in detail in the following sections.

3.3.1 Polymerization of ethylene oxide

PEGs can be synthesized by polymerization of ethylene oxide[69]. Both acid-catalyzed polymerization and base-catalyzed polymerization can be used to polymerize ethylene oxide. However, acid-catalyzed polymerization generates polymers with low molecular weight. Cationic catalysts also can be used to polymerize ethylene oxide[70]. In general, anionic catalysts are preferred. Using anionic catalysts, high molecular weight polymers can be obtained. The PEGs synthesized using these methods are polydispersed, which means they are mixtures of molecules with different lengths due to the randomness of the synthesis method. Purification of these admixtures of PEGs is challenging and requires multiple chromatographic purifications. This increases the cost of monodispersed or closer to monodispersed PEGs. As a result, admixtures of PEGs are used for many applications, including nanomedicine and pharmaceutical applications.

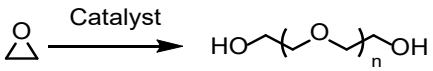


Figure 3.2:A typical polymerization of ethylene oxide

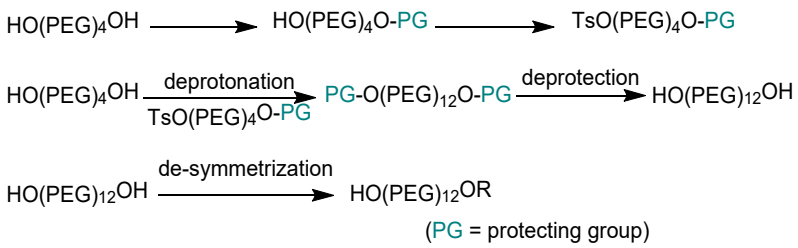
Therapeutics which are PEGylated with polydispersed admixtures of PEGs are difficult to characterize. Their consistency varies from batch to batch, their physical, chemical, pharmacokinetics, and biological activities also vary, and it is harder to get FDA approval due to these reasons. Therefore, efforts have been made to obtain monodispersed PEGs for decades.

3.3.2 Solution phase synthesis

Solution-phase stepwise PEG synthesis has been developed to synthesize monodispersed PEGs. Usually, Williamson ether formation reaction is used for the PEG elongation. PEG elongation could be bidirectional, meaning both hydroxyl groups converted to alkoxide will undergo Williamson ether formation reaction with the monomer at the same time, or unidirectional, which means only one end reacts at a time. Typically, deprotonated PEG starting material generated an alkoxide, which is reacted with PEG moiety with a leaving group. Alkyl sulfonates or primary halides are used as a leaving

group during the synthesis. After that, the protecting groups are removed to obtain the hydroxyl groups. Then these reactions can be repeated until the desired length is obtained.

The schematic diagram of bidirectional PEG synthesis is shown in Scheme 3.1. Both ends of the PEG_4 starting material are deprotonated and reacted with the PEG_4 monomer via Williamson ether formation reaction. Then protecting groups at both ends of the PEG molecule are removed to obtain hydroxyl groups at both ends. Then deprotonation, coupling, and deprotection steps are repeated until the PEGs with desired length is obtained.

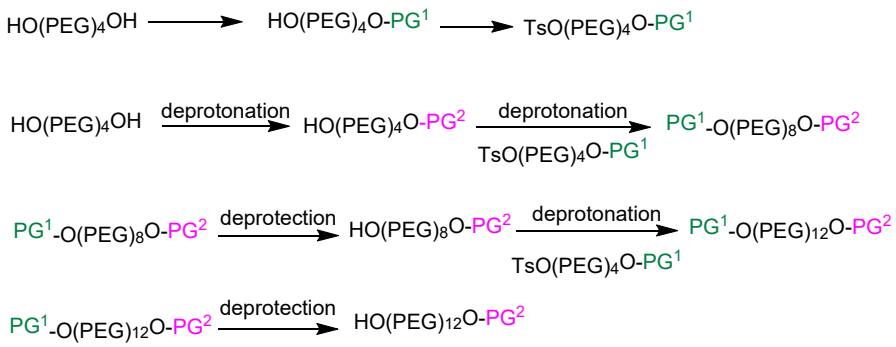


Scheme 3.1:Solution-phase bidirectional monodisperse PEG synthesis

The schematic diagram of unidirectional PEG synthesis is shown in Scheme 3.2. In unidirectional elongation, only one end undergoes ether formation reaction. Typically, in this method, two types of PEG monomers are used. One hydroxyl group of the one tetra ethylene glycol starting material is temporarily protected with a protecting group (**PG_1**) which can be readily removed after the coupling reaction. The other hydroxyl group of the same PEG molecule is converted to a group that acts as the leaving group during the Williamson ether formation reaction. The other PEG unit has a protecting group (**PG_2**) compatible with the **PG_1**.

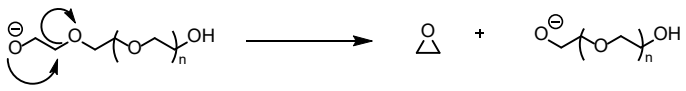
First, the mono protected PEG moiety is deprotonated and reacted with the PEG moiety, which has the temporary protecting group, **PG_1**. After the elongated PEG is obtained, the **PG_1** is removed in the presence of **PG_2**. Then the hydroxyl group can further

react with the monomer to obtain longer PEGs. This process can be repeated till the PEGs with the desired length are obtained.



Scheme 3.2:Solution-phase unidirectional monodisperse PEG synthesis

Even though stepwise synthesis is employed for monodispersed PEG synthesis, the method has some limitations. When PEGs get longer, the efficiency of the ether formation reaction decreases. Therefore, it is not easy to obtain PEGs with longer lengths. Elevated temperatures are needed to be used for longer times for Williamson ether formation reaction, and as a result, the depolymerization side reaction occurs. The anionic depolymerization reaction is shown in Scheme 3.3. This process generates PEGs with shorter lengths. Moreover, multiple chromatographic purifications are needed to purify monomers, intermediates, and the final product to obtain monodispersed PEGs with solution-phase synthesis.



Scheme 3.3:Depolymerization of polyethylene glycol

3.4 Solid phase PEG synthesis

Solid-phase PEG synthesis has several advantages over solution-phase PEG synthesis. With solid-phase synthesis, PEG intermediates can be synthesized without chromatographic purification, excess reactants can be used to drive the reactions to the completion, excess reagents can be removed from the reaction mixture simply by washing

with solvents, Williamson ether formation reaction can be carried out at room temperature, and as a result, the depolymerization reaction is suppressed. The final product can be obtained without column chromatographic purification. Most importantly, the synthesis can be automated.

3.5 Automated PEG synthesis

Solid-phase PEG synthesis is automated using a peptide synthesizer, solid support, and protected monomer. Automated synthesis has several advantages over manual synthesis. The reaction conditions can be controlled well without human errors. The synthesis is less time-consuming and reduces operational costs. Less laborious, increase reproducibility, and hence decrease the final cost of the product. Scaling up the synthesis is not laborious as well due to the automation.

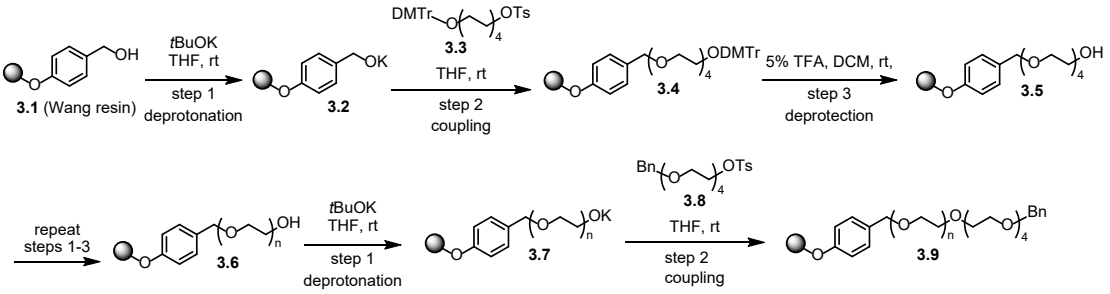
Figure 3.3:Peptide synthesizer that was used for automated PEG synthesis

Our research group developed a solid-phase synthesis method for monodispersed PEG synthesis. These two methods are briefly discussed in the following paragraphs and will be discussed in detail in the next two chapters.

3.5.1 PEG synthesis with a monomer containing an acid labile protecting group

Wang resin (**3.1**, Scheme 3.4) is used as the solid support for the automated solid-phase synthesis. Wang resin is a 1% divinylbenzene cross-linked polystyrene resin with

0.9 mmol/ gram loading and functionalized with 4-benzyloxy benzyl alcohol. The typical synthesis cycle consists of three steps, which are deprotonation, coupling, and detritylation. The deprotonation of the resin (**3.1**, Scheme 3.4) was done by treating the hydroxyl group with a base such as *t*-BuOK for 10 minutes to obtain the deprotonated product (**3.2**, Scheme 3.4). Then the excess base was removed from the resin by simply draining it from the reaction vessel. Then excess base left in the reaction vessel was removed by washing with dry DMF. After deprotonation, the alkoxide (**3.2**, Scheme 3.4) can undergo the coupling reaction with the monomer (**3.3**, Scheme 3.4) via ether formation reaction to give the intermediate (**3.4**, Scheme 3.4). The coupling reaction was done for 6 hours at room temperature. This reaction can be repeated for complete conversion. During the third step, the DMTr protecting group on the PEG chain was removed by treating the resin with 5% TFA in DCM, a mild acidic condition to obtain PEG intermediate (**3.5**, Scheme 3.4). The DMTr cation has a red-orange color depending on the concentration. The deprotection step was performed until the red-orange color of the filtrate disappeared. These three steps can be repeated to give PEGs (**3.6**, Scheme 3.4) at any length. Asymmetric functionalization of the PEGs can be accomplished by simply coupling with a different monomer (**3.8**, Scheme 3.4), where the R group is stable under cleavage conditions. The PEG (**3.6**, Scheme **3.4**) is deprotonated with *t*BuOK again to obtain the alkoxide (**3.7**, Scheme 3.4), which can undergo an ether formation reaction with the monomer (**3.8**, Scheme 3.4). This can generate asymmetric PEGs (**3.9**, Scheme 3.4). After cleaving PEGs from the solid support, we can obtain PEGs with one hydroxyl group protected with the R group.



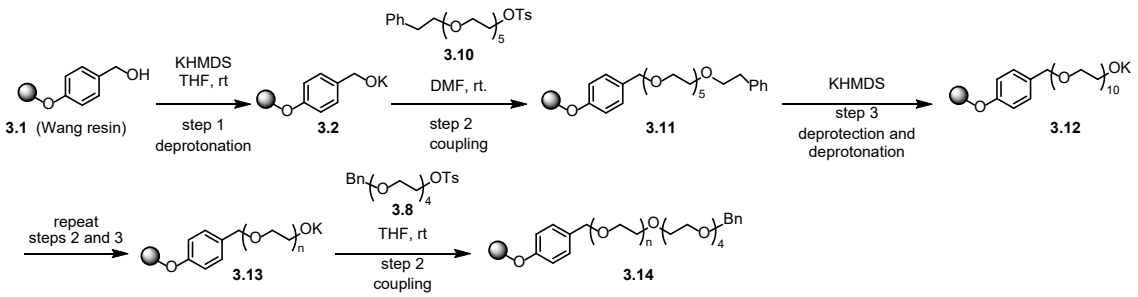
Scheme 3.4:Solid-phase synthesis scheme using monomer with acid labile protecting group

We were able to use this method to synthesize PEG_{12}. However, when the PEG grows longer, the coupling reaction rate decreases, which is the major limitation of this method. The deprotection and deprotonation reactions were performed in two steps, which increased the solvent consumption for the synthesis.

Therefore, we designed a monomer protected with a base-labile group. When the protecting group is removed using a base, via β elimination, it generates the alkoxide. As a result, deprotection and deprotonation can be performed in one step instead of two steps. This saves time and solvents. Therefore, this monomer is more economical than the other monomer that we used previously.

3.5.2 PEG synthesis with a monomer containing a base labile protecting group

The automated PEG synthesis using a monomer with a base labile protecting group is shown in Scheme 3.5. Wang resin was used as the solid support. The typical synthesis cycle consists of three steps: deprotonation, coupling, and deprotection. The deprotonation of the resin (**3.1**, Scheme 3.5) was done by treating the hydroxyl group with a base such as KHMDS for 10 minutes to obtain the alkoxide (**3.2**, Scheme 3.5). Then the excess base was removed from the resin by simply washing with dry DMF. After deprotonation, the alkoxide (**3.2**, Scheme 3.5) can undergo the coupling reaction with the monomer (**3.10**, Scheme 3.5) via Williamson ether formation reaction to give the PEG intermediate (**3.11**, Scheme 3.5). The coupling reaction was done for 6 hours at room temperature. During the third step, the 2-phenethyl protecting group on the PEG chain was removed by treating the resin with 0.25 M KHMDS solution in THF, a strong basic condition to obtain alkoxide (**3.12**, Scheme 3.5). The protecting group removal is irreversible. By repeating the deprotection conditions two times, we obtained a fully deprotected and deprotonated PEG chain (**3.12**, Scheme 3.5) on the solid support. After this coupling step and deprotection step (and deprotonation) can be repeated to give PEGs (**3.13**, Scheme 3.5) with any length. Asymmetric functionalization of the PEGs can be accomplished by simply coupling with a different monomer (**3.8**, Scheme 3.5), where the R group is stable under cleavage conditions.



Scheme 3.5:Solid-phase synthesis scheme using monomer with base labile protecting group

Results obtained using these two methods are described in next two chapters.

4 Automated PEG synthesis with PEG_4 monomer containing an acid labile protecting group

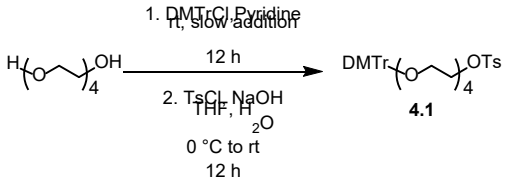
4.1 Abstract

PEGs with eight, twelve, and sixteen ethylene glycol units and their derivatives were synthesized by automated stepwise addition of tetra ethylene glycol monomer without chromatographic purification. The monomer consists with 4, 4′ dimethoxy trityl group at one end and the tosyl group at the other hydroxyl group. Wang resin which is a cross-linked polystyrene resin with 4 benzyloxy benzyl alcohol function is used as the solid support for the automated synthesis. The automated synthesis cycle consists of three steps, deprotonation, coupling and detritylation. PEGs were cleaved off from the solid support by treating the solid support with pure trifluoro acetic acid. The cleaved PEGs were analyzed with ESI-MS. The PEGs were found to be monodispersed.

4.2 Introduction

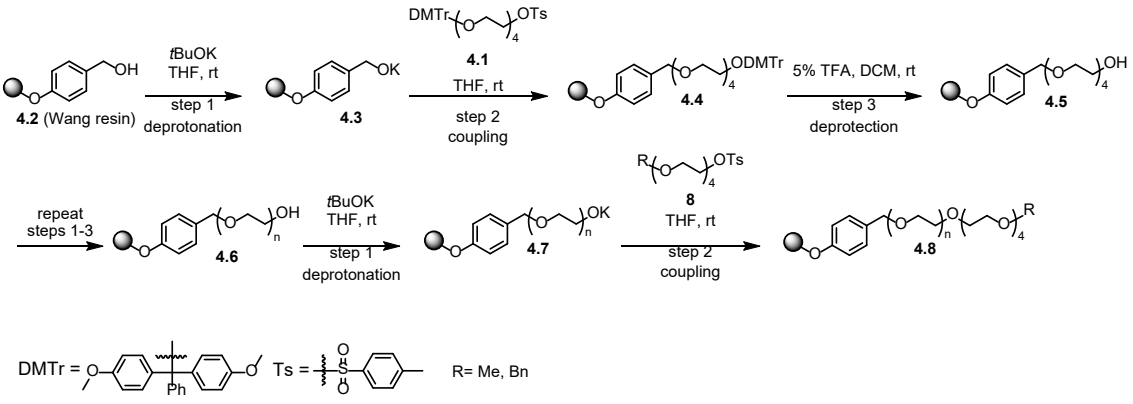
Due to its unique physical and chemical properties such as high solubility in water and other solvents, stable neutral and flexible structure, and biocompatibility, polyethylene glycol (PEG) is found a variety of applications in various fields, including the pharmaceutical industry, nanomedicine, surface science, carbon nanotube functionalization, and organic-inorganic hybrid materials. Due to these applications there is a high demand for PEGs. Even though the PEG synthesis has been employed for decades, most methods that are used to synthesize PEGs give polydisperse PEG mixtures. As a result, purification of those PEG mixtures is needed, and intensive purification increases the cost of monodispersed PEG products. Our research group designed a novel method called the automated solid phase stepwise synthesis of polyethylene glycol to overcome these problems.

4.3 Results and discussion



Scheme 4.1:Synthesis of the monomer with DMTr acid labile group

Readily available tetra ethylene glycol is used as the starting material for monomer synthesis. One hydroxyl group is protected with a 4,4' dimethoxy trityl (DMTr) group. The mono-protection is promoted by slowly adding the 4,4' dimethoxy trityl chloride (DMTrCl) at room temperature and using an excess amount of tetra ethylene glycol. Then the product was isolated via extraction. The starting material, PEG_4, is more soluble in water, while the product is readily soluble in the organic phase. Then the mono protected $DMTrOPEG_4OH$ is tosylated to obtain the monomer (**4.1**, Scheme 4.1) used for the coupling reaction.



Scheme 4.2:Solid phase synthesis of PEG using monomer with acid labile protecting group

Automated solid-phase synthesis is illustrated in Scheme 4.2. The automated synthesis was done using the CSBio peptide synthesizer and the Wang resin as the solid support. Wang resin is a 1% divinylbenzene cross-linked polystyrene resin with 0.9 mmol/ gram loading and functionalized with 4-benzyloxy benzyl alcohol. The typical synthesis cycle consists of three steps, which are deprotonation, coupling, and detritylation. The deprotonation of resin (**4.2**, Scheme 4.2) was done by treating the hydroxyl group with a base such as *t*-BuOK for 10 minutes to obtain the alkoxide (**4.3**, Scheme 4.2). Then the

excess base was removed from the resin by simply washing with dry DMF. After deprotonation, the alkoxide (**4.3**, Scheme 4.2) can undergo a coupling reaction with the monomer (**4.1**, Scheme 4.2) via Williamson ether formation reaction to give PEG intermediate (**4.4**, Scheme 4.2). The coupling reaction was done for six hours at room temperature. The DMTr protecting group on the PEG chain was removed during the third step by treating the resin with 5% TFA in DCM, a mild acidic condition to obtain deprotected PEG chain (**4.5**, Scheme 4.2). The DMTr cation has a red-orange color depending on the concentration. The deprotection step was performed until the red-orange color of the filtrate disappeared. These three steps can be repeated to give elongated PEGs (**4.6**, Scheme 4.2) with any length. Asymmetric functionalization of the PEGs can be accomplished by simply coupling with a different monomer (**4.8**, Scheme 4.2), where the R group is stable under cleavage conditions.

In order to find the best conditions for the automated synthesis, we tested THF, DMF, DMSO, and NMP for deprotonation and coupling steps during the PEG_0 to PEG_8 synthesis. Obtained ESI-MS data is shown in the results and discussion section. Of all the solvents, DMF gave the best results, and we used DMF as the solvent for deprotonation and coupling steps. The DCM is chosen during the detritylation deprotection step.

Figure 4.1 shows the ESI-MS obtained from a PEG_8 synthesis with THF as the solvent during the deprotonation and coupling step. The synthesis was done using the same conditions described in the previous paragraph. The deprotonation and the coupling steps were performed with THF as the solvent. *t*BuOK was dissolved in THF, and the monomer was dissolved in THF. The washings after the deprotonation were also done using THF. The MS data shows complete conversion, and no depolymerization side reaction occurs during the coupling reaction.

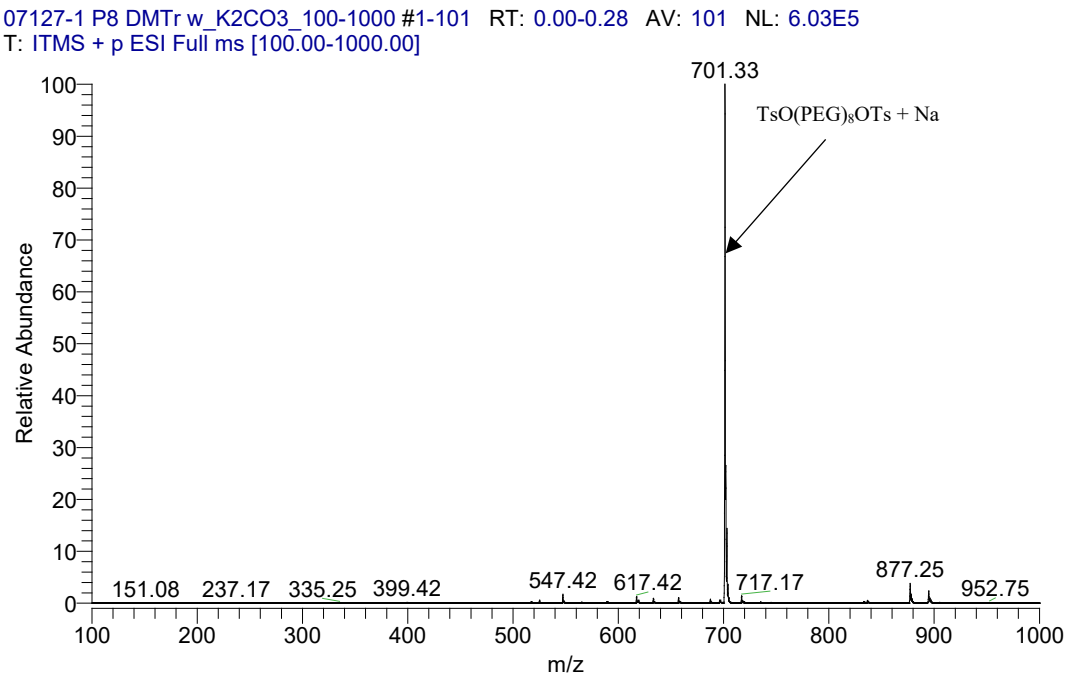


Figure 4.1:ESI-MS of PEG_8 synthesis with THF as solvent

Figure 4.2 shows the ESI-MS obtained from a PEG_8 synthesis with DMF as the solvent during the deprotonation and coupling step. The synthesis was done using the same conditions described in previous paragraph. *t*BuOK and the monomer were dissolved in DMF. The washings after the deprotonation were also done using DMF. The MS data shows complete conversion and there is no depolymerization side reaction occurs during the coupling reaction.

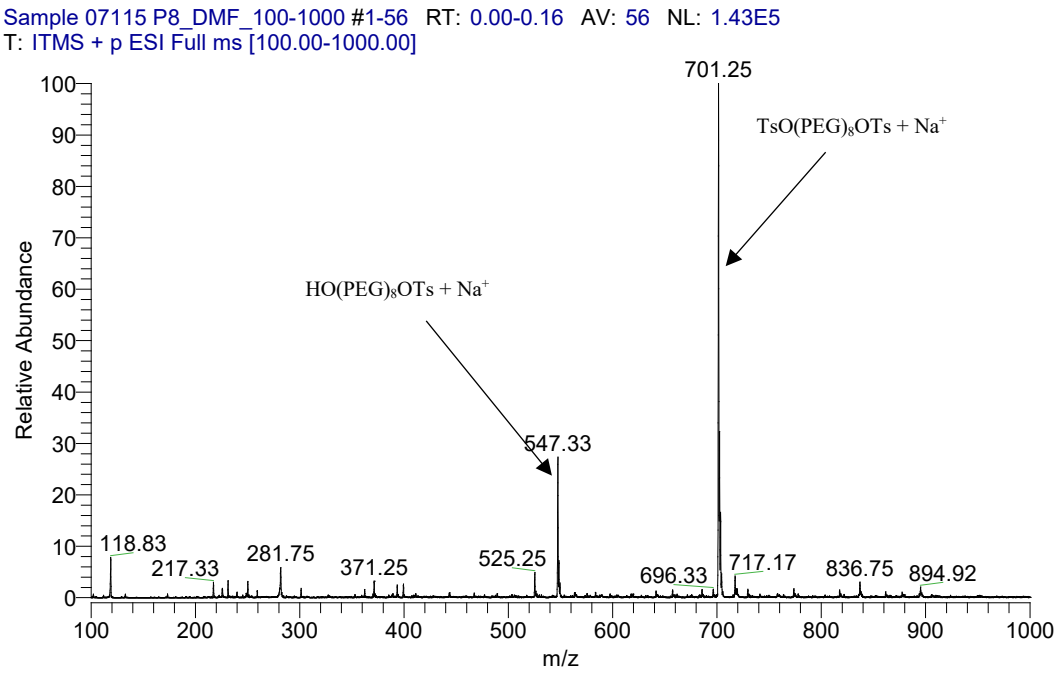


Figure 4.2:ESI-MS of PEG_8 synthesis with DMF as solvent

Figure 4.3 shows the ESI-MS obtained from a PEG_8 synthesis with NMP as the solvent during the deprotonation and coupling step. The synthesis was done using the same conditions described in previous paragraph. *t*BuOK was dissolved in NMP and the monomer is also dissolved in NMP. The washings after the deprotonation were also done using NMP. The MS data shows complete conversion and there is no depolymerization side reaction occurs during the coupling reaction.

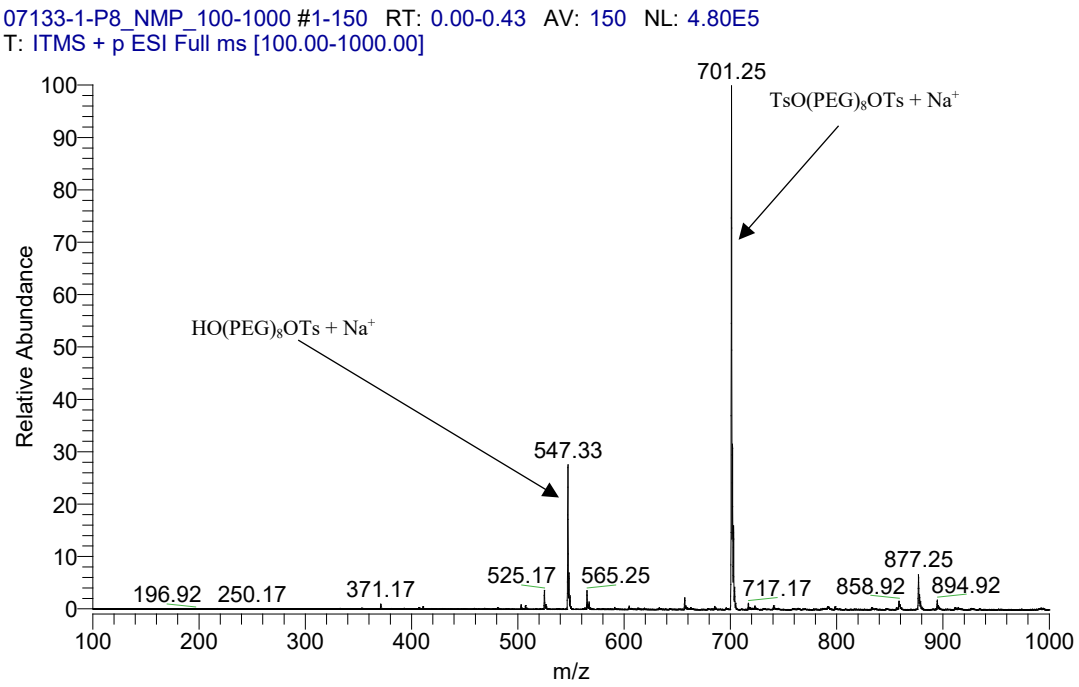


Figure 4.3:ESI-MS of PEG_8 synthesis with NMP as solvent

Figure 4.4 shows the ESI-MS obtained from a PEG_8 synthesis with DMSO as the solvent during the deprotonation and coupling step. The synthesis was done using the same conditions described in previous paragraph. *t*BuOK was dissolved in DMSO and the monomer is also dissolved in DMSO. The washings after the deprotonation were also done using DMSO. The MS data shows complete conversion and there is no depolymerization side reaction occurs during the coupling reaction.

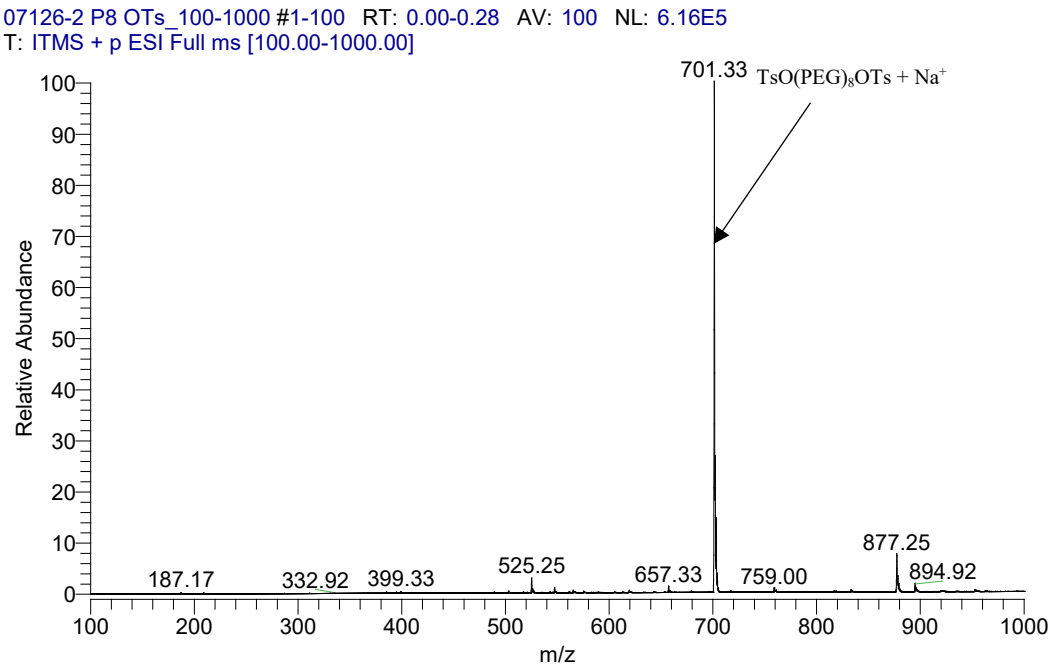
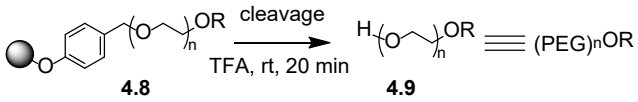


Figure 4.4:ESI-MS of PEG_8 synthesis with DMSO as solvent

After careful consideration, DMF was chosen as the solvent for deprotonation and coupling steps because it is less expensive compared to DMSO and NMP, and it suppresses the depolymerization side reaction during the coupling step with longer PEGs.



TFA = trifluoroacetic acid; R = Bn, Me, Ac or other acid-stable groups

Scheme 4.3:Cleavage of PEG from the solid support

After the PEG with desired length is obtained, the PEG is cleaved off from the solid support by treating the Wang resin with PEGs with pure trifluoro acetic acid for 20 minutes. Then the resin was washed with THF/water (1:1) solution three times to extract any PEG left in the sample. Extracts were combined and evaporated TFA, THF and water under reduced pressure to obtain pure PEG sample.

For analytical purposes, cleaved PEG is reacted with Tosyl chloride in the presence of NaOH in water and THF solution and extracted with Ethyl acetate. Tosylation was done

to makes the ESI-MS analysis easier. Solvents were removed by evaporation under vacuum. The tosylated PEG samples were analyzed with ESI-MS.

ESI-MS of PEG_8 sample is shown in Figure 4.5. A TLC of the PEG_8 sample was also compared with an authentic sample. The analyses indicated the synthesis was complete. In ESI-MS, there is no PEG_4 (M=307) present, which indicates that the reaction from **4.2** to **4.6**, where n=8, is complete. The depolymerization side reaction is also not present in the sample. This means the conditions that we used during the deprotonation and coupling reactions indeed can suppress the depolymerization side reaction.

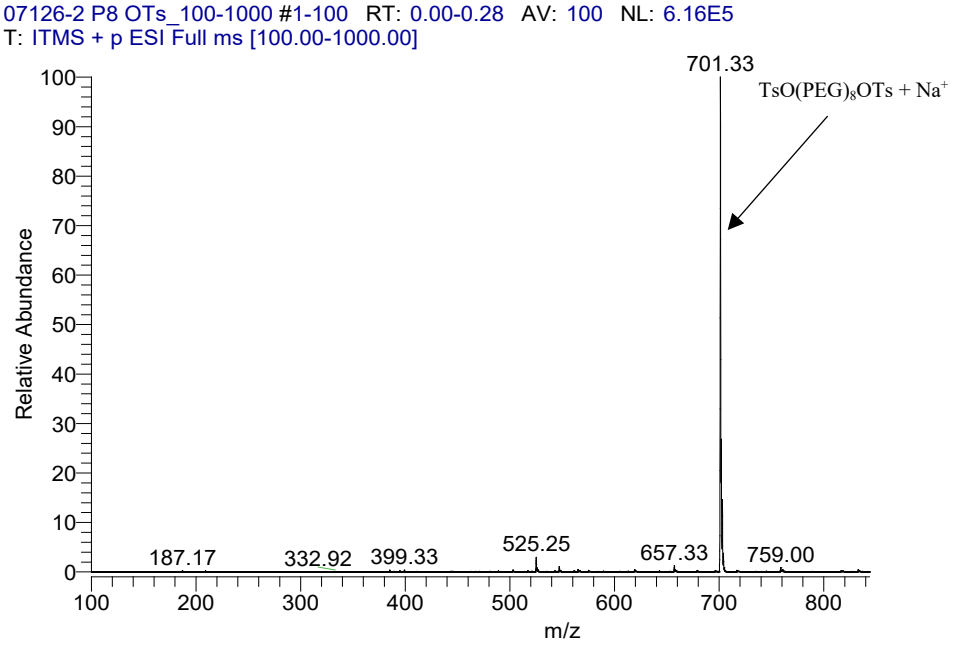


Figure 4.5:ESI-MS of $(PEG)_8$ synthesis

After successful synthesis of PEG_8, the PEG_{12} synthesis was done. We used the same reaction conditions that described above to converter PEG_8 to PEG_{12}. The ESI-MS of PEG_{12} sample is shown in Figure 4.6. A TLC of the PEG_{12} sample was also compared with an authentic sample of PEG_8. The analyses indicated the synthesis was complete. In ESI-MS, there is no PEG_8 (M=701) present, which indicates that the reaction from **4.2** to **4.6**, where n=12, is complete. The depolymerization side reaction is also not present in the

sample. This means the conditions that we used during the deprotonation and coupling reactions can suppress the depolymerization side reaction.

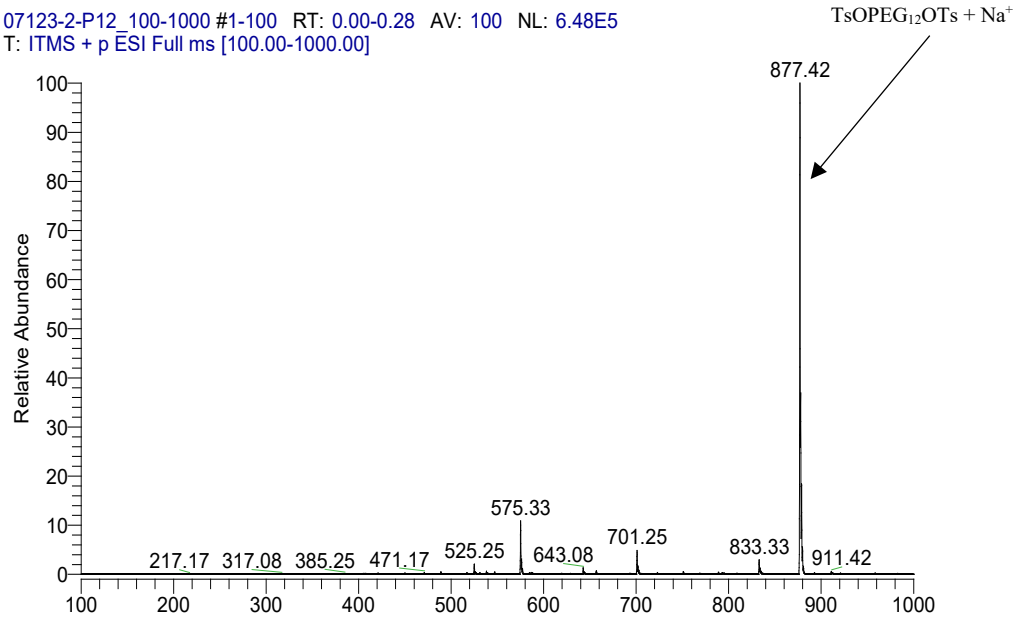


Figure 4.6:ESI-MS of $(PEG)_{12}$

We were able to synthesize PEG_{12} consecutively without stopping at PEG_8 and analyzing the sample. The ESI-MS of PEG_{12} sample is shown in Figure 4.7. The analysis indicated the synthesis was complete. In ESI-MS, there is no PEG_8 (M=701) present, which indicates that the reaction from **4.2** to **4.6**, where n=12, is complete.

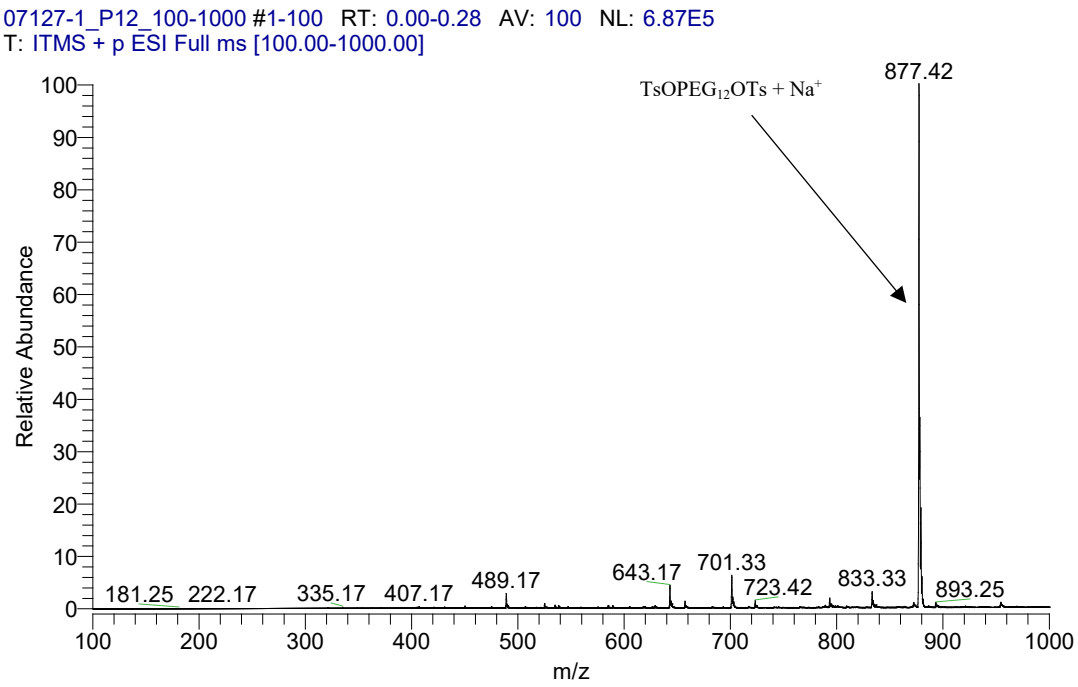


Figure 4.7:ESI-MS data for consecutive PEG_{12} synthesis

After obtaining PEG_{12}, we used the same reaction conditions that described above to converter PEG_{12} to PEG_{16}. The MS data for the PEG_{16} sample is shown in Figure 4.8. The sample contains ~20% PEG_{12}. The analytical data implied that the conversion from PEG_{12} to PEG_{16} is incomplete. It also indicated that there is some depolymerized PEG_{16} present in the sample. This means the coupling reaction is slower compared to the coupling reaction of PEG_{12} or PEG_8. As a result, the depolymerization side reaction occurs in the reaction mixture. We repeated the synthesis of PEG_{16} several times and tried multiple couplings on PEG_{12} to PEG_{16}. We also modified the reaction conditions and still the reaction was incomplete. All the results were consistent. Therefore, human errors were excluded.

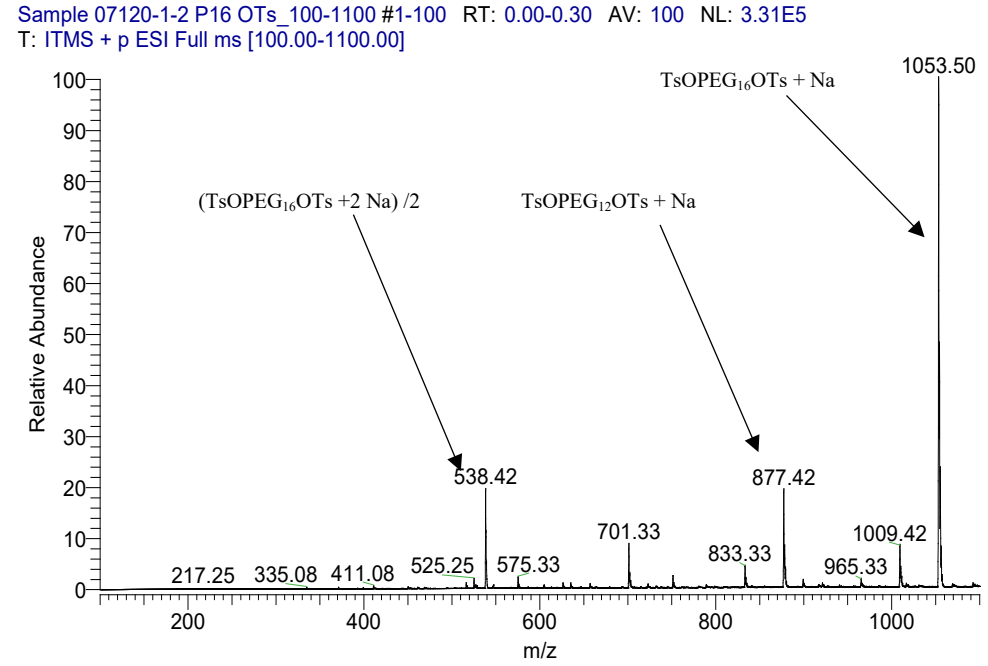


Figure 4.8:ESI-MS of $TsOPEG_{16}OTs$

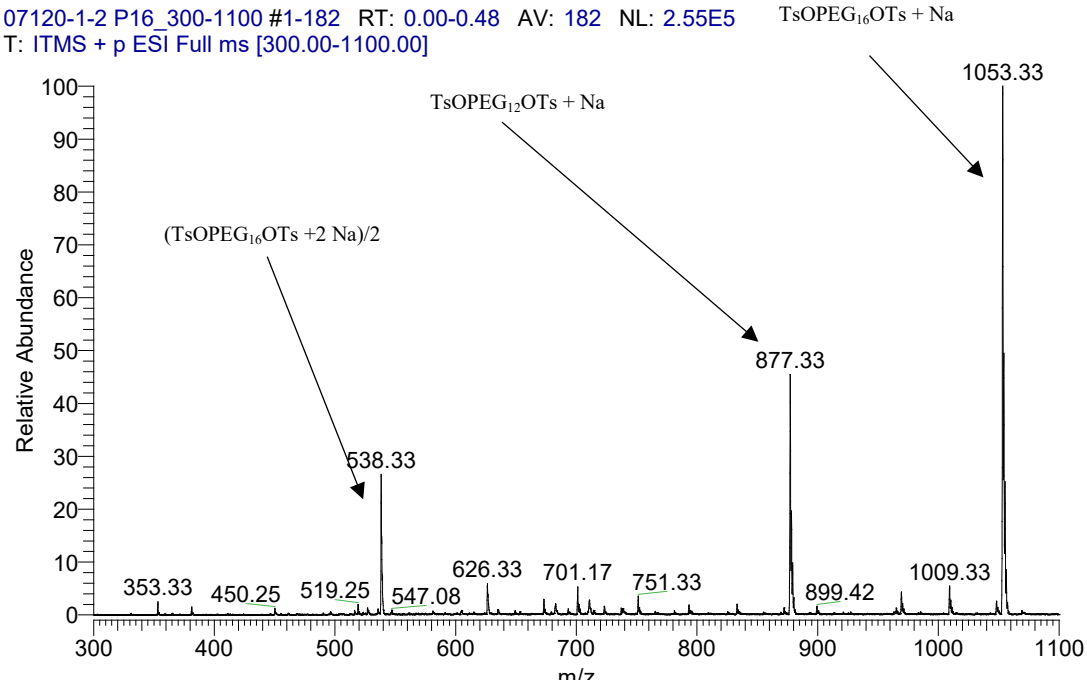
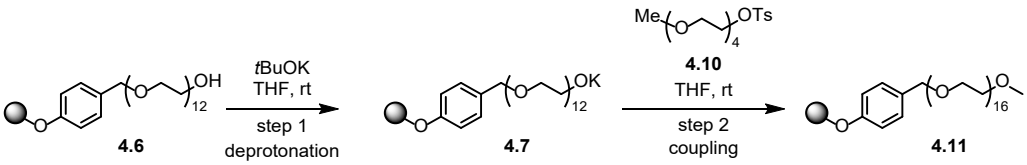


Figure 4.9:PEG_{16} synthesis with five times coupling from PEG_{12} to PEG_{16}

There are two possibilities for the incomplete conversion. 1). It could be due to incomplete deprotection during the detritylation step. DMTr removal is a reversible reaction, and as a result some deprotected hydroxyl groups can react with DMTr cation. 2). It could also be resulted from incomplete coupling reaction. To find out the reason for incomplete conversion of PEG_{16}, we used tosylated methoxy PEG_4 monomer (**4.10**) instead of DMTr PEG_4 OTs monomer (**4.1**). The synthesis scheme is shown in Scheme 4.4. After the synthesis is done, we cleaved the PEG_{16} sample from the solid support. Here tosylation reaction was not executed.



Scheme 4.4:The synthesis scheme for PEG_{16} derivative synthesis

ESI-MS data (Figure 4.10) shows that there is no PEG_{12} left in the sample. This implies that the detritylation step is complete. However, the data shows that there is PEG_{12} OMe present in the sample, apart from the expected mass which is PEG_{16} OMe. This implies that there is PEG_8 left in original PEG_{12} sample. So, our PEG_{12} coupling was also not complete. However, results shows that the coupling reaction with the new monomer was complete. There is no PEG_8 or PEG_{12} left in the sample. This suggests that the DMTr monomer is not reacting completely during the coupling step, with longer PEGs.

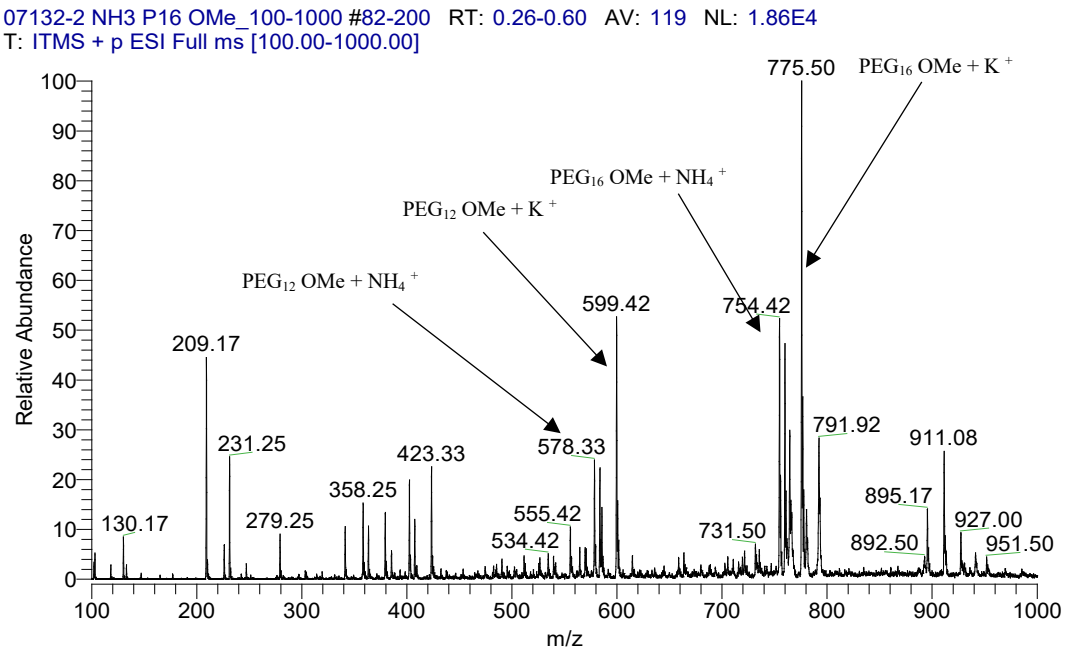
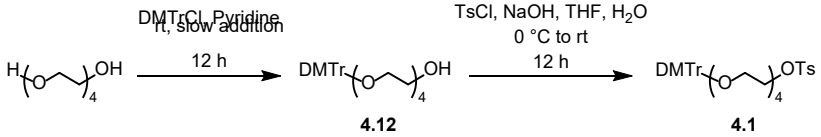


Figure 4.10:ESI-MS of Methoxy PEG_{16} synthesis

4.4 Conclusions

In conclusion, we have demonstrated that the automated stepwise solid phase technology is suitable for the synthesis of monodisperse PEGs and their derivatives. Advantages of the method include chromatography- free synthesis, minimize human errors, reduce time and solvents required for the synthesis hence bring down the overall cost of the PEG synthesis, milder conditions for the Williamson ether formation reaction to minimize depolymerization of PEGs to increase the monodispersity of PEGs, and the overall yield. Using the technology, we successfully synthesized PEG derivatives with eight and twelve ethylene glycol units with close to monodispersity. Even though the PEG_8, PEG_{12} samples are pure, the PEG $_{16}$ sample had some unreacted PEG_{12} left in the sample. According to our MS data, we hypothesized that, the DMTr protecting group is too hydrophobic and may stick to the hydrophobic polystyrene solid support. Therefore, the DMTr monomer is not suitable for monodispersed PEG synthesis longer than PEG_{12}.

4.5 Experimental



Scheme 4.5:Monomer synthesis

***DMTrO(PEG)$_4$ OH* (4.12)**: To a solution of (PEG)$_4$ (142 g, 127.0 ml, 737 mmol, 10 equiv.) in dry pyridine (150 ml) in a round-bottomed flask was added a solution of DMTrCl (25 g, 73.7 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 reaction 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 (150 ml × 4), dried over anhydrous $MgSO_4$, and filtered. The filtrate was evaporated to dryness, co-evaporated with toluene (50 ml × 2) and further dried under vacuum giving the product DMTrO(PEG)$_4$ (**4.12**, Scheme 4.5) which was contaminated with small amount of DMTrO(PEG)$_4$ODMTr , as a thick light yellow oil (total 34.7 g; R_f for DMTrO(PEG)$_4$, 0.10; SiO_2, hexanes/EtOAc/Et_3N 1:2:0.15). The mixture was not separated and was used directly for the next tosylation reaction.

DMTrO(PEG)$_4$OTs (**4.1**) :The solutions of **DMTrO(PEG)$_4$OH** (**4.12**, Scheme 4.5) (contaminated with small amount of DMTrO(PEG)$_4$ODMTr ; total 34.7 g; assumed 69.9 mmol as if it were pure, 1.0 equiv.) in THF (100 ml) and NaOH pearls (33.5 g, 839.4mmol, 12.0 equiv.) in water (100 ml) were combined and stirred at 0 °C for 1h. The solution of TsCl (14.98 g, 78.7 mmol, 1.125 equiv.) in THF (200 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 1h, 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 (150 ml × 3). The combined organic phase was dried over anhydrous $MgSO_4$ and filtered. Volatiles were removed under reduced pressure. The residue was co-evaporated with toluene (50 ml×2) and further dried under vacuum giving the product DMTrO(PEG)$_4$OTs. (compound **4.1**, Scheme 4.5) which was contaminated with DMTrO(PEG)$_4$ODMTr, was obtained as a thick light yellow oil (total 43.2 g; TLC; R_f, 0.25; SiO_2, hexanes/EtOAc/Et_3N 7:3:0.5). The product, which was contaminated with DMTrO(PEG)$_4$ODMTr, was dried over P_2O_5 under high vacuum overnight, and was used directly for the automated solid-phase PEG synthesis.

(PEG)$_8$: The Wang resin (Scheme 4.2) **4.2** (1.0 g, 0.9 mmol/g loading, 0.9 mmol) was loaded into a 20 ml reaction vessel. Then the reaction vessel was attached to the peptide synthesizer. Dry THF (15 ml) was delivered to the reaction vessel using transfer MVA to RV function of the synthesizer, and the resin was allowed to swell at rt for 10 min while mixing. Then the THF was removed using the RV to waste function of the synthesizer. The resin was washed with anhydrous THF for 5 times. Dry DMF (15 ml) was delivered to the reaction vessel using transfer MVA to RV function of the synthesizer, and the resin was allowed to mix at rt for 10 min. Then the DMF was removed using the RV to waste function of the synthesizer. Then *t*BuOK solution in DMF (0.25 M, 15 ml, 3.75 mmol, 4.1 equiv.) was delivered to the reaction vessel, and was mixed at rt for 5 min. The solution was removed, and the deprotonation was repeated one more time. This converted Wang resin **4.2** to deprotonated intermediate **4.3**. After the base solution was removed, the resin was washed with 15 ml of anhydrous DMF for 3 minutes for two more times to remove excess *t*BuOK left in the reaction vessel. After that, the monomer **4.1** (0.5 M in DMF, 15 ml, 7.5 mmol, 8.33 equiv.) was delivered into the reaction vessel. The column was mixed at rt. for 6 hours. The monomer solution was flushed to waste. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 3); DMF (10 ml ×3); DMSO (10 ml × 3). The deprotonation reaction and the ether formation coupling reaction were repeated for two more times, to ensure the complete conversion of **4.2** to **4.4**. The solutions of 5% TFA in DCM (10 ml × 10) were delivered to the column, mixed for ten minutes, and then removed by RV to waste function as many times as needed until red or orange color

no longer appeared. After detritylation, the resin was washed with DCM (10 ml × 3) and THF (10 ml × 7). This converted **4.4** to **4.5**.

For converting **4.5** to **4.6**, where n=8, the deprotonation, coupling and deprotection steps were carried out as described above for one more time. ESI-MS analysis indicated complete reaction.

(PEG)$_{12}$: The Wang resin (Scheme 4.2) **4.2** (1.0 g, 0.9 mmol/g loading, 0.9 mmol) was loaded into a 20 ml reaction vessel. Then the reaction vessel was attached to the peptide synthesizer. Dry THF (15 ml) was delivered to the reaction vessel using transfer MVA to RV function of the synthesizer, and the resin was allowed to swell at rt for 10 min while mixing. Then the THF was removed using the RV to waste function of the synthesizer. The resin was washed with anhydrous THF for 5 times. Dry DMF (15 ml) was delivered to the reaction vessel using transfer MVA to RV function of the synthesizer, and the resin was allowed to mix at rt for 10 min. Then the DMF was removed using the RV to waste function of the synthesizer. Then *t*BuOK solution in DMF (0.25 M, 15 ml, 3.75 mmol, 4.1 equiv.) was delivered to the reaction vessel, and was mixed at rt for 5 min. The solution was removed, and the deprotonation was repeated one more time. This converted Wang resin **4.2** to deprotonated intermediate **4.3**. After the base solution was removed, the resin was washed with 15 ml of anhydrous DMF for 3 minutes for two more times to remove excess *t*BuOK left in the reaction vessel. After that, the monomer **4.1** (0.5 M in DMF, 15 ml, 7.5 mmol, 8.33 equiv.) was delivered into the reaction vessel. The column was mixed at rt. for 6 hours. The monomer solution was flushed to waste. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 3); DMF (10 ml ×3); DMSO (10 ml × 3). The deprotonation reaction and the ether formation coupling reaction were repeated for two more times, to ensure the complete conversion of **4.2** to **4.4**. The solutions of 5% TFA in DCM (10 ml × 10) were delivered to the column, mixed for ten minutes, and then removed by RV to waste function as many times as needed until red or orange color no longer appeared. After detritylation, the resin was washed with DCM (10 ml × 3) and THF (10 ml × 7). This converted **4.4** to **4.5**. Then for converting **4.5** to **4.6**, where n=8, the deprotonation, coupling and deprotection steps were carried out as described above for one

more time. For converting **4.5** to **4.6**, where n=8, the deprotonation, coupling steps were conducted three times and detritylation was achieved using 3% TCA in DCM (10 ml × 10). After this an ESI-MS analysis was performed on a portion of the PEG_8 sample, to ensure the reaction was complete. Then for converting **4.6** (n= 8) to **4.6**, where n=12, the deprotonation, coupling steps were conducted three times and detritylation was achieved using 5% TFA in DCM (10 ml × 10). After this an ESI-MS analysis was performed on a portion of the PEG_{12} sample.

(PEG)$_{16}$: The Wang resin (Scheme 4.2) **4.2** (1.0 g, 0.9 mmol/g loading, 0.9 mmol) was loaded into a 20 ml reaction vessel. Then the reaction vessel was attached to the peptide synthesizer. Dry THF (15 ml) was delivered to the reaction vessel using transfer MVA to RV function of the synthesizer, and the resin was allowed to swell at rt for 10 min while mixing. Then the THF was removed using the RV to waste function of the synthesizer. The resin was washed with anhydrous THF for 5 times. Dry DMF (15 ml) was delivered to the reaction vessel using transfer MVA to RV function of the synthesizer, and the resin was allowed to mix at rt for 10 min. Then the DMF was removed using the RV to waste function of the synthesizer. Then *t*BuOK solution in DMF (0.25 M, 15 ml, 3.75 mmol, 4.1 equiv.) was delivered to the reaction vessel, and was mixed at rt for 5 min. The solution was removed, and the deprotonation was repeated one more time. This converted Wang resin **4.2** to deprotonated intermediate **4.3**. After the base solution was removed, the resin was washed with 15 ml of anhydrous DMF for 3 minutes for two more times to remove excess *t*BuOK left in the reaction vessel. After that, the monomer **4.1** (0.5 M in DMF, 15 ml, 7.5 mmol, 8.33 equiv.) was delivered into the reaction vessel. The column was mixed at rt. for 6 hours. The monomer solution was flushed to waste. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 3); DMF (10 ml ×3); DMSO (10 ml × 3). The deprotonation reaction and the ether formation coupling reaction were repeated for two more times, to ensure the complete conversion of **4.2** to **4.4**. The solutions of 5% TFA in DCM (10 ml × 10) were delivered to the column, mixed for ten minutes, and then removed by RV to waste function as many times as needed until red or orange color no longer appeared. After detritylation, the resin was washed with DCM (10 ml × 3) and THF (10 ml × 7). This converted **4.4** to **4.5**. Then for converting **4.5** to **4.6**, where n=8, the

deprotonation, coupling and deprotection steps were carried out as described above for one more time. For converting **4.5** to **4.6**, where n=8, the deprotonation, coupling steps were conducted three times and detritylation was achieved using 5% TFA in DCM (10 ml × 10). After this an ESI-MS analysis was performed on a portion of the PEG_8 sample, to ensure the reaction was complete. Then for converting **4.6** (n= 8) to **4.6**, where n=12, the deprotonation, coupling steps were conducted three times and detritylation was achieved using 5% TFA in DCM (10 ml × 10). After this an ESI-MS analysis was performed on a portion of the PEG_{12} sample, to ensure the reaction was complete. Then for converting **4.6** (n= 12) to **4.6**, where n=16, the deprotonation, coupling steps were conducted three times and detritylation was achieved using 5% TFA in DCM (10 ml × 10). ESI-MS analysis indicated in-complete conversion of PEG_{12} to PEG $_{16}$.

MeO(PEG)$_{16}$: The Wang resin (Scheme 4.2) **4.2** (1.0 g, 0.9 mmol/g loading, 0.9 mmol) was loaded into a 20 ml reaction vessel. Then the reaction vessel was attached to the peptide synthesizer. Dry THF (15 ml) was delivered to the reaction vessel using transfer MVA to RV function of the synthesizer, and the resin was allowed to swell at rt for 10 min while mixing. Then the THF was removed using the RV to waste function of the synthesizer. The resin was washed with anhydrous THF for 5 times. Dry DMF (15 ml) was delivered to the reaction vessel using transfer MVA to RV function of the synthesizer, and the resin was allowed to mix at rt for 10 min. Then the DMF was removed using the RV to waste function of the synthesizer. Then *t*BuOK solution in DMF (0.25 M, 15 ml, 3.75 mmol, 4.1 equiv.) was delivered to the reaction vessel, and was mixed at rt for 5 min. The solution was removed, and the deprotonation was repeated one more time. This converted Wang resin **4.2** to deprotonated intermediate **4.3**. After the base solution was removed, the resin was washed with 15 ml of anhydrous DMF for 3 minutes for two more times to remove excess *t*BuOK left in the reaction vessel. After that, the monomer **4.1** (0.5 M in DMF, 15 ml, 7.5 mmol, 8.33 equiv.) was delivered into the reaction vessel. The column was mixed at rt. for 6 hours. The monomer solution was flushed to waste. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 3); DMF (10 ml ×3); DMSO (10 ml × 3). The deprotonation reaction and the ether formation coupling reaction were repeated for two more times, to ensure the complete conversion of **4.2** to **4.4**. The solutions

of 5% TFA in DCM (10 ml × 10) were delivered to the column, mixed for ten minutes, and then removed by RV to waste function as many times as needed until red or orange color no longer appeared. After detritylation, the resin was washed with DCM (10 ml × 3) and THF (10 ml × 7). This converted **4.4** to **4.5**. Then for converting **4.5** to **4.6**, where n=8, the deprotonation, coupling and deprotection steps were carried out as described above for one more time. For converting **4.5** to **4.6**, where n=8, the deprotonation, coupling steps were conducted three times and detritylation was achieved using 5% TFA in DCM (10 ml × 10). After this an ESI-MS analysis was performed on a portion of the PEG_8 sample, to ensure the reaction was complete. Then for converting **4.6** (n= 8) to **4.6**, where n=12, the deprotonation, coupling steps were conducted three times and detritylation was achieved using 5% TFA in DCM (10 ml × 10). After this an ESI-MS analysis was performed on a portion of the PEG_{12} sample, to ensure the reaction was complete. Then for converting **4.6** (n= 12) to **4.11**, the deprotonation, coupling steps were conducted three times. ESI-MS analysis indicated complete conversion of PEG_{12} to PEG $_{16}$OMe.

Cleavage of PEG from the resin: To cleave PEG from the resin, a portion of the dry resin (50 mg), which had been washed extensively using conditions described earlier, was placed in a 1.5 ml centrifuge tube. Pure TFA (300 µl) was added, and the mixture was shaken at rt for 2 hrs. 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 (50 µl × 2) and THF (50 µl × 3). The supernatant and the washes were combined. Volatiles were evaporated under vacuum. 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 µl) and precipitated with diethyl ether 200 µl giving the product as a light-yellow oil: ~6 mg.

Tosylation of PEG after cleaving it off from the solid support: After the PEG was obtained, PEG was dissolved in 50 µL of THF. Then NaOH (10 equiv) dissolved in 50 µL of H_2O was added into the reaction mixture. Then it was mixed for about 2 minutes. After that TsCl (3equiv) dissolved in 100 µL of THF was added into the reaction mixture and allowed

to react for 18 h. The mixing was done by shaking the Eppendorf tube. Then the reaction mixture was extracted with EtOAc and analyzed using ESI-MS.

5 Automated PEG synthesis with PEG_5 monomer containing a base labile protecting group

5.1 Abstract

Monodispersed PEGs with ten, fifteen, and twenty ethylene glycol units were synthesized using automated solid-phase stepwise addition of tetra ethylene glycol units without chromatographic purification. The Penta ethylene glycol monomer consists of a 2-phenyl ethyl group at one end, and the tosyl group at the other hydroxyl group was used for the synthesis. Wang resin which is a cross-linked polystyrene resin with 4 benzyloxy benzyl alcohol function, is used as the solid support for the automated synthesis. 2 phenyl ethyl protecting group can be removed using a stronger base like KHMDS via β-elimination. Therefore, deprotection and deprotonation steps were performed as one step. The automated synthesis cycle consists of only two steps. The first step is deprotection and deprotonation, and the second step is coupling. These two steps were performed alternatively until the PEG with the desired length was obtained. After the synthesis, PEGs were cleaved off from the solid support using pure trifluoroacetic acid. Volatiles were evaporated with reduced pressure, and the samples were analyzed using ESI-MS.

5.2 Introduction

Automated stepwise solid-phase synthesis is employed for monodispersed PEG synthesis using Wang resin, a polystyrene resin as the solid support, and $DMTrPEG_4OTs$ monomer. Experiments and results obtained in our previous studies were described in detail in Chapter 4. When the PEG length increases, hydrophilic PEG chains are directed outwards from the hydrophobic Wang resin. However, the hydrophobic DMTr group of the monomer interacts with the polystyrene Wang resin. This scenario decreases the rate of the Williamson ether formation reaction during the coupling step. As a result of the incomplete coupling reaction, obtaining PEGs longer than PEG_{12} synthesis using this monomer is not feasible. Therefore, we designed a different monomer with a smaller protecting group.

5.3 Results and discussions

First, a monomer protected with Methoxymethyl (MOM) group was used. MOM is also an acid labile protecting group instead of the DMTr protecting group. The coupling reaction from PEG $_8$ to PEG $_{12}$ was done using OTsPEG$_4$OMOM instead of OTsPEG$_4$ODMTr monomer. The ESI-MS data is shown in figure 5.1. The reaction conditions were the same and DMTr coupling reaction. After the synthesis the PEG was cleaved from the resin using pure TFA for 20 minutes. This cleaved off the PEG from the resin and removed MOM protecting group on PEG moiety. After the cleavage and deprotection PEG is reacted with tosyl chloride for the analytical purposes. The reaction conditions were further described in experimental section.

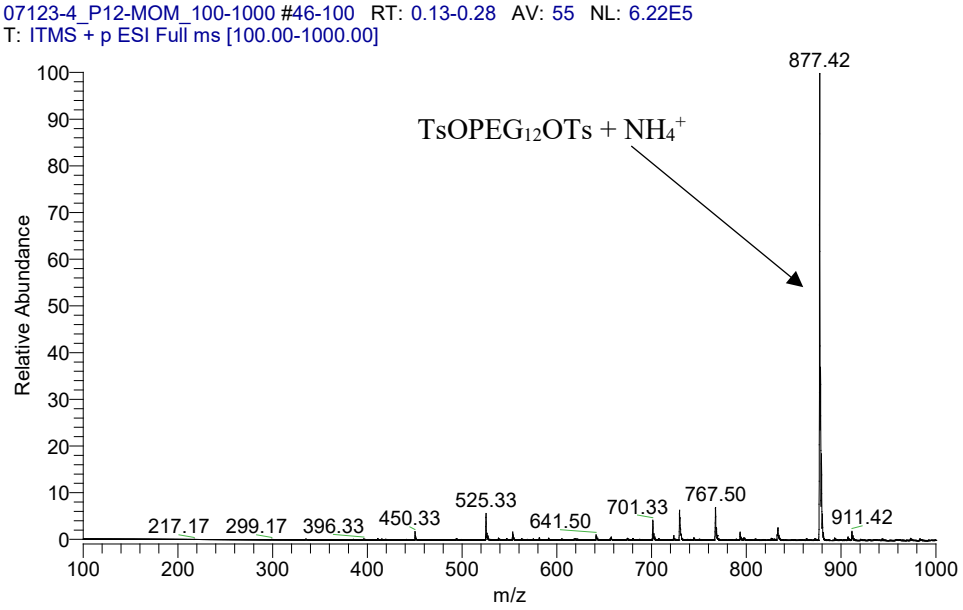


Figure 5.1:ESI-MS for PEG12 synthesized with MOM monomer

According to the ESI analysis the coupling reaction was complete. The depolymerization side reaction was suppressed during the deprotonation and coupling reactions.

However, the PEG synthesis using this monomer is not practical due to several reasons. 1.Unlike DMTr monomer, the cation obtained during the deprotection of MOM group does not have a color, and it is difficult to monitor the completion of the deprotection reaction. 2. The MOM protecting group is less labile than the DMTr protecting group and

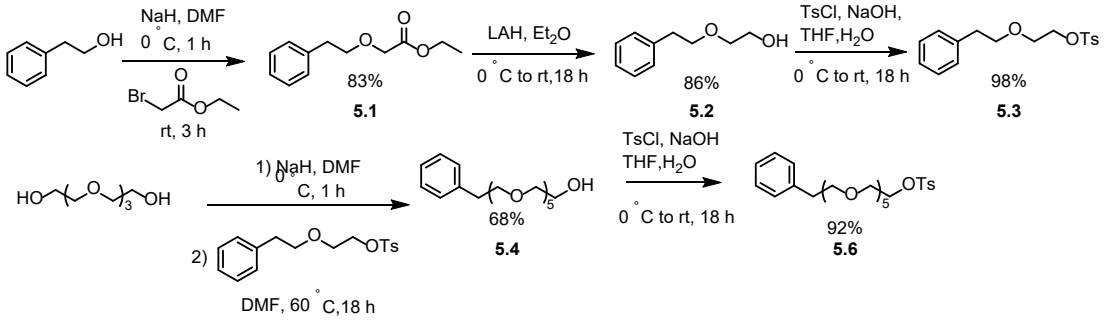
stronger conditions are required for the deprotection. These conditions also can cleave of the linker, which is going to affect the yield and the purity of the PEG that obtained using this method. 3. The cleavage of the PEGs synthesized using this method did not have high yields. The yield for the PEG obtained after the cleavage is very low compared to the other PEG synthesis even if the same conditions were used. We hypothesized that the cation generated from the deprotected MOM group reacts with the phenyl groups in the Wang resin. As a result, it is harder to cleave off the PEG from the rein. This is one of the major reasons we did not proceed with this MOM monomer.

Therefore, base labile protecting group was designed for automated solid-phase PEG synthesis. This new protecting group is smaller than the DMTr protecting group. Therefore, we assumed that the hydrophobic interactions of the protecting group and the Wang resin will not affect the rate of the coupling reaction.

To use a base labile protecting group in solid phase synthesis, the protecting group should be able to be removed using a base which is stable at room temperature. It is not practical to lower the temperature of the reaction vessel during the synthesis and therefore, the base also should be less reactive. The protecting group should also be stable under basic Williamson ether formation reaction. I.e.: the deprotonated PEG moiety should not be strong enough to deprotect the base labile protecting group during the Williamson ether formation reaction. Several protecting groups were tested (Appendix A.3) for those two criteria. The phenyl ethyl protecting group that gave the best results so far, and therefore, the phenyl ethyl group was selected as the base labile protecting group for the solid phase PEG synthesis.

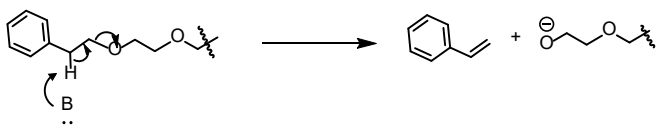
The monomer synthesis is shown in Scheme 5.1. Deprotonated 2-Phenylethanol starting material was reacted with ethyl bromoacetate to obtain compound **5.1**. Then the resulting ester (compound **5.1**, Scheme 5.1) was reduced to its alcohol **5.2** using lithium aluminium hydride (LAH). After that the alcohol group of compound **5.2** was reacted with *p*-toluene sulfonyl chloride to obtain compound **5.3**. Deprotonated PEG_4 moiety was reacted with tosylated compound **5.3** to obtain compound **5.4**. Finally, the compound **5.4**

was reacted with *p*-toluene sulfonyl chloride to obtain the new base labile PEG monomer **5.5**.



Scheme 5.1:Monomer synthesis with the base labile group

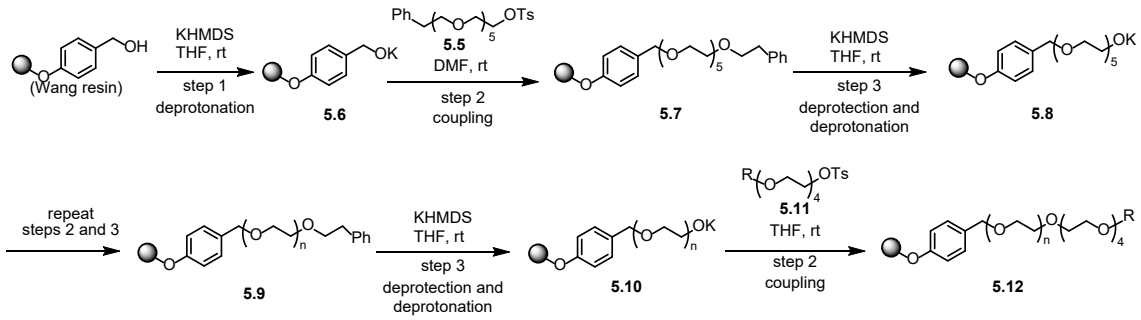
The deprotection and deprotonation mechanism of the base labile protecting group is shown in Scheme 5.2. The electron-withdrawing phenyl group decreases the pKa of the α hydrogen atom and makes it more acidic. As a result, a stronger base can abstract the H, promoting β elimination. When the protecting group is removed, it generates the alkoxide and styrene by product. We have several options for the base. We could use Potassium hexamethyl diamide (KHMDS) or Lithium diisopropyl amine (LDA). However, LDA is not compatible with the solid phase automated synthesis because the base is not stable at room temperature. It is very reactive and needs to generate freshly before each application. Therefore, we used KHMDS, a base that is stable at room temperature and less reactive than LDA.



Scheme 5.2:Deprotection mechanism of base-labile protecting group

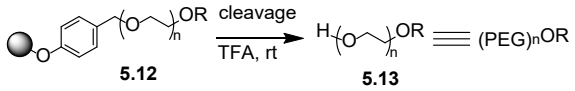
Automated stepwise solid-phase PEG synthesis scheme is shown in Scheme 5.3. Wang resin was used as the solid support and the CSBio peptide synthesizer was used for automation. Wang resin is a cross-linked polystyrene resin with 0.9 mmol/ gram loading. The synthesis consists of three steps, initial deprotonation of the hydroxyl group on the Wang resin, coupling and the deprotection of the protecting group. The deprotection of the ethyl phenyl group generates styrene and the alkoxide, which can undergo Williamson

ether formation reaction. The hydroxyl group of the Wang resin was treated with KHMDS to obtain deprotonated resin **5.6**. The excess base was washed away using anhydrous DMF. Then deprotonated resin **5.6** can undergo coupling reaction with the monomer **5.5**, via Williamson ether formation reaction to give **5.7**. The excess monomer is washed away using DMF. The resin is also washed with water/ THF (1:1) solution to remove any inorganic substances present in the sample. Then the resin **5.7** is treated with Potassiumhexamethyldisilylamide (KHMDS) to promote β elimination to obtain deprotected and deprotonated compound **5.8**. Excess base was washed away using anhydrous DMF. This cycle can repeat to give **5.9** with any length. Asymmetric functionalization of the PEGs can be accomplished by simply coupling with a different monomer **5.11**, where R group is stable under acidic conditions.



Scheme 5.3:Automated solid phase PEG synthesis

After obtaining the PEGs with desired length, the Wang resin with the PEG is dried under vacuum for 30 minutes and treated it with pure TFA for 30 minutes. Then the cleaved PEG was extracted using THF (300 µL ×3) the extracts were combined and 300 µL of deionized water is added to the extract and evaporated under high vacuum. The PEG was co-evaporated with toluene (500 µL ×3), to remove any residual TFA., and treated with aqueous 28% NH_4OH solution to remove TFA adducts from the PEG chain. Then the cleaved PEG was analyzed using ESI-MS.



TFA = trifluoroacetic acid; R = Bn, Me, Ac or other acid-stable groups

Scheme 5.4:PEG cleavage from the solid support using TFA

ESI-MS of PEG_{10} phenethyl sample is shown in Figure 5.2. The analysis indicated the synthesis was complete. In ESI-MS, there is no PEG_5 phenethyl or PEG_5OH present, which indicates that the reaction from **5.7** to **5.9**, where n=10, is complete. There is no evidence for depolymerization side reaction. There is no M-44 (536) peak is in the MS data. This means the conditions that we used during the deprotection and coupling reactions indeed can suppress the depolymerization side reaction.

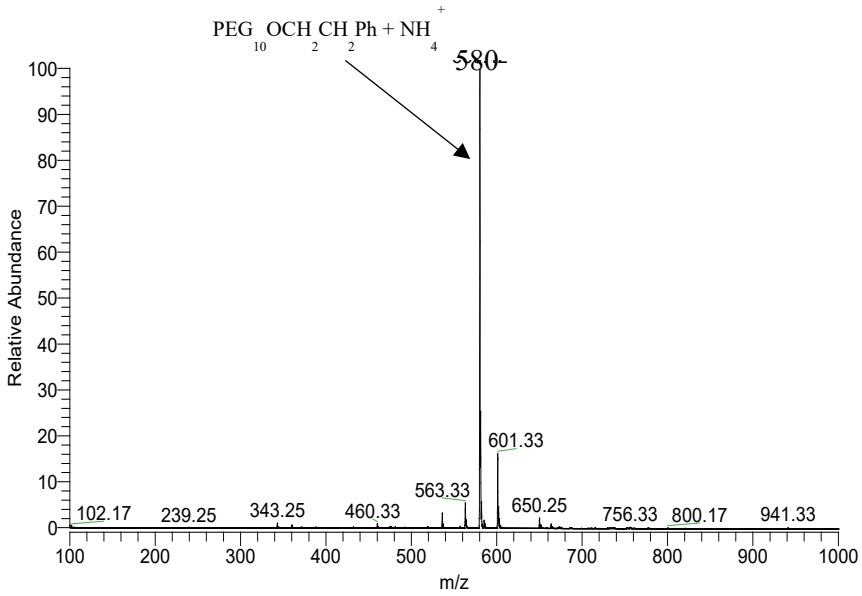


Figure 5.2:ESI-MS of PEG_{10}

ESI-MS of PEG_{15} phenethyl sample is shown in Figure 5.3. The analysis indicated the synthesis was complete. In ESI-MS, there is no PEG_{10} phenethyl or PEG_{10} OH present, which indicates that the reaction from **5.9** (n=10) to **5.9** (n=15), is complete. There is no evidence for depolymerization side reaction. There is very little M-44 peak (756) is in the MS data. This means the conditions that we used during the deprotection and coupling reactions indeed can suppress the depolymerization side reaction. The monomer is less

hydrophobic that the DMTr monomer that we used in previous studies, and the rate of coupling reaction is not affected due to the hydrophobicity of the protecting group.

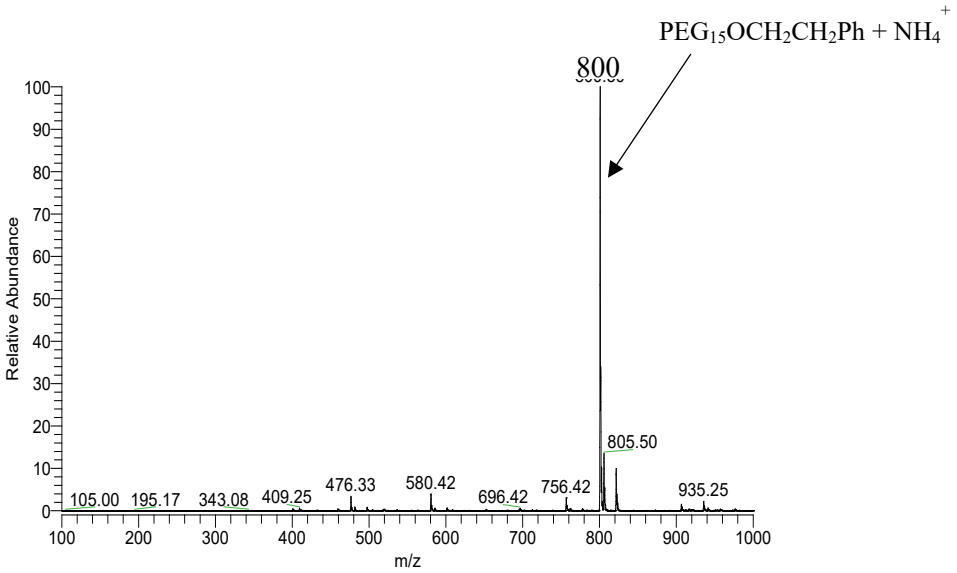


Figure 5.3:ESI-MS of PEG_{15}

ESI-MS of PEG_{20} phenethyl sample is shown in Figure 5.4. The analysis indicated the synthesis was near completion. In ESI-MS, there is very little PEG_{15} phenethyl or PEG_{15} OH present, which indicates that the reaction from **5.9** (n=15) to **5.9**, where n=20, is near completion. There is no evidence for depolymerization side reaction. There is a little bit of M-44 (976) peak is in the MS data. This means the conditions that we used during the deprotection and coupling reactions can suppress the depolymerization side reaction.

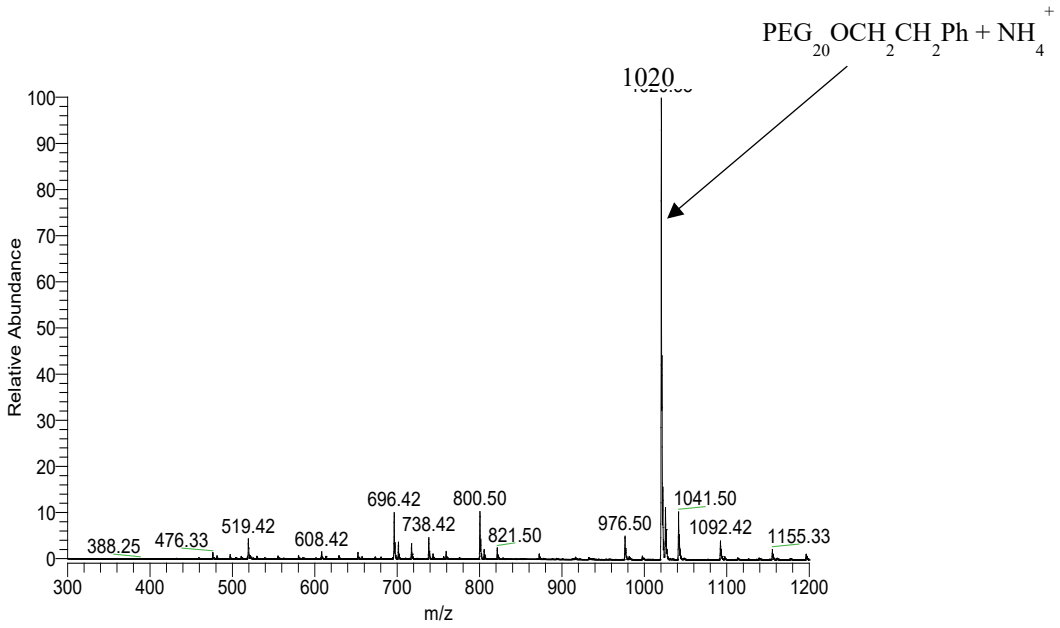


Figure 5.4:ESI-MS of PEG_{20}

Currently we are testing conditions to obtain purer PEG_{20} using this method. We are also testing conditions for consecutive synthesis using this method.

5.4 Conclusions

In conclusion, we have demonstrated that the automated stepwise solid phase technology is suitable for the synthesis of monodisperse PEGs and their derivatives. Advantages of the method include chromatography- free synthesis, minimize human errors, deprotection and deprotonation steps can be performed at the same time, reduce time and solvents required for the synthesis hence bring down the overall cost of the PEG synthesis, milder conditions for the Williamson ether formation reaction to minimize depolymerization of PEGs to increase the monodispersity of PEGs, and the overall yield. Using the technology, we successfully synthesized PEG derivatives with ten and fifteen ethylene glycol units with close to monodispersity. Even though the PEG_{10}, PEG_{15} samples are pure, the PEG_{20} sample had some unreacted PEG_{15} left in the sample. We were able to obtain $PEG_{20,}$ closer to monodispersity, using this method.

5.5 Experimental

General information: All compounds from commercial sources were used as received unless noted otherwise. Anhydrous DMF was dried using molecular sieves. Compounds **5.1-5.5** were synthesized following reported procedure. All reactions were carried out under nitrogen using oven-dried glassware. Thin layer chromatography (TLC) was performed using Sigma-Aldrich TLC plates, silica gel 60F-254 over glass support, 250 μm thickness. ^{1}H and ^{13}C NMR spectra were obtained on a Varian spectrometer at 400 MHz,100 MHz respectively. Chemical shifts (δ) were reported in reference to solvent peaks (residue $CHCl_3$ at δ 7.24 ppm for ^{1}H and $CDCl_3$ at δ 77.00 ppm for ^{13}C). LRMS was obtained on a Thermo Finnigan LCQ Advantage Ion Trap Mass Spectrometer.

Ethyl 2-phenthoxyacetate (**5.1**): The suspension of NaH (60% in mineral oil, 3.64 g, 82.8 mmol, 1.0 equiv.) in anhydrous DMF (150 mL) in a 1 L 2-neck round bottom flask under nitrogen was cooled on an ice bath. The solution of $Ph(CH_2)_2OH$ (10.0 mL, 82.8 mmol, 1.0 equiv) in anhydrous DMF (250 mL) was added dropwise via a cannula over ~1 h. After addition, the reaction mixture was stirred at 0 °C for ~1 h. This gave the clear solution of $NaO(CH_2)_2Ph$. Ethyl bromo acetate (13.8 g; 82.8 mmol, 1.0 equiv), was dissolved in anhydrous DMF (100 mL). The solution of $NaO(CH_2)_2Ph$ was added to the solution of ethyl bromo acetate dropwise via a cannula. After addition, the mixture was stirred at 0 °C for 4 h. After that, the reaction was quenched with EtOH. DMF was removed on a rotary evaporator under high vacuum. The residue was partitioned between EtOAc (700 mL) and saturated NaCl (150 mL). The organic phase was washed with saturated NaCl (150 mL × 3), dried over anhydrous $MgSO_4$, and filtered. The filtrate was evaporated to dryness under reduced pressure and further dried under high vacuum. The residue was purified with flash chromatography (SiO_2, EtOAc/hexanes 1:4) to give compound **5.1** (14.4 g, 83%) as a clear oil: TLC Rf = 0.6 (SiO_2, hexanes/EtOAc 4:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27-7.17 (m, 5H), 4.20-4.15 (q, 2H), 4.04 (s, 2H), 3.74-3.71 (t, 2H), 2.94-2.90 (t, 2H), 1.26-1.22 (t, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.48, 138.58, 129.03, 128.53, 126.45, 72.83, 68.71, 61.03, 36.45, 14.53.

2-Phenethoxyethan-1-ol (**5.2**): Lithium aluminium hydride (LAH) (1.98 g, 51.8 mmol, 3.0 equiv.) was placed in a 1 L two neck round bottomed flask and flush with nitrogen for 10 minutes and cooled down to 0 °C with an ice bath. Then anhydrous Et_2O (75 mL) in another flask under nitrogen was slowly added into the reaction flask with LAH. Then a solution of Ethyl 2-Phenthoxyacetate (14.4 g, 69.1 mmol, 1.0 equiv) in anhydrous Et_2O (300 mL) was added dropwise via a cannula over ~1 h. The ice bath was replaced with a water bath to make sure the reaction occurs while addition of ester. After addition, the reaction mixture was stirred at rt for 8 h. After that, the reaction mixture was cooled down to 0 °C using an ice water bath. Then 1.98 ml of water was added very slowly to quench excess LAH in the reaction mixture. After that 1.98 ml of 15% NaOH aqueous solution was added to the reaction mixture. Then 5.94 ml of water was added slowly. The LAH in the reaction mixture is turned to a white suspension after the last aliquot of water was added. Then the reaction mixture was filtered and dried over anhydrous $MgSO_4$ and filtered again. The filtrate was evaporated to dryness under reduced pressure and further dried under high vacuum. The residue was purified with flash chromatography (SiO_2, EtOAc/hexanes 1:5) to give compound **5.2** (9.96 g, 86%) as a clear oil. : TLC Rf = 0.3 (SiO_2, hexanes/EtOAc 4:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28-7.18 (m, 5H), 3.69-3.66 (t, 4H), 3.53-3.51 (t, 2H), 2.90-2.86 (t, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.91, 128.55, 128.50, 126.44, 72.18, 61.96, 36.53.

2-Phenethoxyethyl 4-methylbenzenesulfonate (**5.3**): Compound **5.2** (5.7 g, 31.1 mmol, 1.0equiv.) was dissolved in THF (70 ml) and added it into a round bottomed flask and was cooled down to 0 °C with an ice bath. Then NaOH (12.45 g, 311 mmol, 10 equiv.) was dissolved in water (70 ml) and added into the reaction mixture. Then the mixture was allowed to stir vigorously at 0 °C for 1h. After that *p*-toluene sulfonyl chloride (8.86 g, 46.6 mmol, 1.5 equiv.) in THF (140 mL) was added dropwise via a cannula over ~1 h. After addition, the reaction mixture was stirred at 0 °C – rt. for ~18 h. Then the reaction mixture was partitioned between EtOAc (500 mL) and saturated NaCl (50 mL). The organic phase was washed with saturated NaCl (50 mL × 3), dried over anhydrous $MgSO_4$, and filtered. The filtrate was evaporated to dryness under reduced pressure and further dried under high vacuum. The residue was purified with flash chromatography (SiO_2,

EtOAc/hexanes 1:4) to give compound **5.3** (7.06 g, 98%) as a clear oil: TLC Rf = 0.6 (SiO_2, hexanes/EtOAc 4:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76-7.74 (d, 2H) 7.30-7.12 (m, 8H), 4.12-4.10 (t, 2H), 3.60-3.55 (m, 4H), 2.79-2.76 (t, 2H), 2.40 (s, 3H; ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.89, 138.74, 133.19, 129.94, 128.11, 126.41, 72.47, 69.49, 68.46, 36.41, 21.94.

17-Phenyl-3,6,9,12,15-pentaoxaheptadecan-1-ol (**5.4**): The suspension of NaH (60% in mineral oil, 0.98 g, 24.5 mmol, 1.2 equiv.) in anhydrous DMF (50 mL) in a 2-neck round bottom flask under nitrogen was cooled on an ice bath. The solution of tetra ethylene glycol (19.7 g, 17.5 mL, 204 mmol, 5.0 equiv) in anhydrous DMF (150 mL) was added dropwise via a cannula over ~1 h. After addition, the reaction mixture was stirred at 0 °C for ~1 h. This gave the clear solution of $NaOPEG_4OH$. Compound **5.3** (4.7g; 20.4 mmol, 1.0 equiv), was dissolved in anhydrous DMF (50 mL) and the solution was added to the reaction mixture at 60 °C dropwise via a cannula over ~3h. After addition, the mixture was stirred at 60 °C for 8 h. After that, the reaction was quenched with EtOH. DMF was removed on a rotary evaporator under high vacuum. The residue was partitioned between EtOAc (400 mL) and saturated NaCl (50 mL). The organic phase was washed with saturated NaCl (50 mL × 3), dried over anhydrous $MgSO_4$, and filtered. The filtrate was evaporated to dryness under reduced pressure and further dried under high vacuum. The residue was purified with flash chromatography (SiO_2, EtOAc/hexanes 2:1) to give compound **5.4** (4.73 g, 68%) as a clear oil: TLC Rf = 0.3 (SiO_2, hexanes/EtOAc 1:2); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17-7.09 (m, 5H), 3.61-3.54 (m, 22H), 2.81-2.78 (t, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.96, 129.02, 128.39, 126.23, 72.45, 70.82, 70.75, 70.43, 69.30, 36.47.

17-Phenyl-3,6,9,12,15-pentaoxaheptadecyl 4-methylbenzenesulfonate (**5.5**): Compound **5.4** (4.3 g, 12.5 mmol, 1.0equiv.) was dissolved in THF (30 ml) and added it into a round bottomed flask and was cooled down to 0 °C with an ice bath. Then NaOH (5.0 g, 125 mmol, 10 equiv.) was dissolved in water (30 ml) and added into the reaction mixture. Then the mixture was allowed to stir vigorously at 0 °C for 1h. After that *p*-toluene sulfonyl chloride (3.5 g, 18.8 mmol, 1.5 equiv.) in THF (60 mL) was added dropwise via a cannula

over ~1 h. After addition, the reaction mixture was stirred at 0 °C – rt. for ~18 h. Then the reaction mixture was partitioned between EtOAc (200 mL) and saturated NaCl (25 mL). The organic phase was washed with saturated NaCl (25 mL × 3), dried over anhydrous $MgSO_4$, and filtered. The filtrate was evaporated to dryness under reduced pressure and further dried under high vacuum. The residue was purified with flash chromatography (SiO_2, EtOAc/hexanes 1:1) to give compound **5.5** (5.23 g, 92%) as a clear oil: TLC Rf = 0.4 (SiO_2, hexanes/EtOAc 1:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74-7.72 (d, 2H) 7.29-7.15 (m, 7H), 4.10-4.08 (t, 2H), 3.62-3.51 (m, 20H), 2.86-2.82 (t, 2H), 2.38 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.88, 139.02, 133.15, 129.95, 129.03, 128.08, 126.28, 72.50, 70.94, 70.48, 69.51, 68.88, 36.53, 21.93.

(PEG)$_{10}$: The Wang resin (1.0 g, 0.9 mmol/g loading, 0.9 mmol) (Scheme 5.2) was loaded into a 20 ml peptide synthesis vessel. The reaction vessel was attached to the peptide synthesizer. Dry THF (15 ml) was delivered to the reaction vessel using transfer MVA to RV function of the synthesizer, and the resin was allowed to swell at rt for 10 min while mixing. Then the THF was removed using the RV to waste function of the synthesizer. The resin was washed with anhydrous THF for five times, DMF for five times, DMSO for five times and NMP for five times alternatively for 10 minutes each time. Then KHMDS solution in THF (0.25 M, 15 ml, 3.75 mmol, 4.1 equiv.) was delivered to the reaction vessel, and was mixed at rt for 5 min. The solution was removed, and the deprotonation was repeated one more time. After the base solution was removed, the resin was washed with anhydrous DMF for 3 minutes for two more times to remove excess KHMDS left in the reaction vessel. This converted Wang resin to **5.6**. After that, the monomer **5.5** (0.5 M in DMF, 15 ml, 7.5 mmol, 8.33 equiv.) was delivered into the reaction vessel. The column was mixed at rt. for six hours. The monomer solution was flushed to waste. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 3); DMF (10 ml ×3); DMSO (10 ml × 3). This converted **5.6** to **5.7**. To obtain **5.8**, KHMDS solution in THF (0.25 M, 15 ml, 3.75 mmol, 4.1 equiv.) was delivered to the reaction vessel, and was mixed at rt for 5 min. The solution was removed, and the deprotonation was repeated one more time. After the base solution was removed, the resin was washed with anhydrous DMF for 3 minutes for two times to remove excess KHMDS left in the reaction

vessel. After that, the monomer **5.5** (0.5 M in DMF, 15 ml, 7.5 mmol, 8.33 equiv.) was delivered into the reaction vessel. The column was mixed at rt. for six hours. This converted **5.8** to **5.9** (n=10). Then the monomer solution was flushed to waste. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 3); DMF (10 ml ×3); DMSO (10 ml × 3). ESI-MS analysis indicated complete conversion.

(PEG)$_{15}$: The Wang resin (1.0 g, 0.9 mmol/g loading, 0.9 mmol) was loaded into a 20 ml peptide synthesis vessel (Scheme 5.2). The reaction vessel was attached to the peptide synthesizer. Dry THF (15 ml) was delivered to the reaction vessel using transfer MVA to RV function of the synthesizer, and the resin was allowed to swell at rt for 10 min while mixing. Then the THF was removed using the RV to waste function of the synthesizer. The resin was washed with anhydrous THF for five times, DMF for five times, DMSO for five times and NMP for five times alternatively for 10 minutes each time. Then KHMDS solution in THF (0.25 M, 15 ml, 3.75 mmol, 4.1 equiv.) was delivered to the reaction vessel, and was mixed at rt for 5 min. The solution was removed, and the deprotonation was repeated one more time. After the base solution was removed, the resin was washed with anhydrous DMF for 3 minutes for two more times to remove excess KHMDS left in the reaction vessel. This converted Wang resin to **5.6**. After that, the monomer **5.5** (0.5 M in DMF, 15 ml, 7.5 mmol, 8.33 equiv.) was delivered into the reaction vessel. The reaction vessel was mixed at rt. for six hours. The remaining monomer solution was flushed to waste. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 5); DMF (10 ml ×5); DMSO (10 ml × 5); NMP (10 ml × 5) alternatively. This converted **5.6** to **5.7**. To obtain **5.8**, KHMDS solution in THF (0.25 M, 15 ml, 3.75 mmol, 4.1 equiv.) was delivered to the reaction vessel, and was mixed at rt. for 5 min. Then the solution was removed, and the deprotonation was repeated one more time. After the base solution was removed, the resin was washed with anhydrous DMF for 3 minutes for two more times to remove excess KHMDS left in the reaction vessel. After that, the monomer **5.5** (0.5 M in DMF, 15 ml, 7.5 mmol, 8.33 equiv.) was delivered into the reaction vessel. The reaction vessel was mixed at rt. for six hours. This converted **5.8** to **5.9** (n=10). The monomer solution was then flushed to waste. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 5); DMF (10 ml ×5); DMSO

(10 ml × 5); NMP (10 ml × 5). To obtain **5.10**, where n= 10, KHMDS solution in THF (0.25 M, 15 ml, 3.75 mmol, 4.1 equiv.) was delivered to the reaction vessel, and was mixed at rt for 5 min. The solution was removed, and the deprotonation was repeated one more time. After the base solution was removed, the resin was washed with anhydrous DMF for 3 minutes for two more times to remove excess KHMDS left in the reaction vessel. After that, the monomer **5.5** (0.5 M in DMF, 15 ml, 7.5 mmol, 8.33 equiv.) was delivered into the reaction vessel. The reaction vessel was mixed at rt. for six hours. This converted **5.10** (n=10) to **5.9** (n=15). The monomer solution was flushed to waste. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 3); DMF (10 ml ×3); DMSO (10 ml × 3). ESI-MS analysis indicated complete reaction.

(PEG)$_{20}$: The Wang resin (1.0 g, 0.9 mmol/g loading, 0.9 mmol) was loaded into a 20 ml peptide synthesis vessel (Scheme 5.2). The reaction vessel was attached to the peptide synthesizer. Dry THF (15 ml) was delivered to the reaction vessel using transfer MVA to RV function of the synthesizer, and the resin was allowed to swell at rt for 10 min while mixing. Then the THF was removed using the RV to waste function of the synthesizer. The resin was washed with anhydrous THF for five times, DMF for five times, DMSO for five times and NMP for five times alternatively for 10 minutes each time. Then KHMDS solution in THF (0.25 M, 15 ml, 3.75 mmol, 4.1 equiv.) was delivered to the reaction vessel, and was mixed at rt for 5 min. The solution was removed, and the deprotonation was repeated one more time. After the base solution was removed, the resin was washed with anhydrous DMF for 3 minutes for two more times to remove excess KHMDS left in the reaction vessel. This converted Wang resin to **5.6**. After that, the monomer **5.5** (0.5 M in DMF, 15 ml, 7.5 mmol, 8.33 equiv.) was delivered into the reaction vessel. The reaction vessel was mixed at rt. for six hours. The remaining monomer solution was flushed to waste. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 5); DMF (10 ml ×5); DMSO (10 ml × 5); NMP (10 ml × 5) alternatively. This converted **5.6** to **5.7**. To obtain **5.8**, KHMDS solution in THF (0.25 M, 15 ml, 3.75 mmol, 4.1 equiv.) was delivered to the reaction vessel, and was mixed at rt. for 5 min. Then the solution was removed, and the deprotonation was repeated one more time. After the base solution was removed, the resin was washed with anhydrous DMF for 3 minutes for

two more times to remove excess KHMDS left in the reaction vessel. After that, the monomer **5.5** (0.5 M in DMF, 15 ml, 7.5 mmol, 8.33 equiv.) was delivered into the reaction vessel. The reaction vessel was mixed at rt. for six hours. This converted **5.8** to **5.9** (n=10). The monomer solution was then flushed to waste. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 5); DMF (10 ml ×5); DMSO (10 ml × 5); NMP (10 ml × 5). To obtain **5.9**, where n= 15, KHMDS solution in THF (0.25 M, 15 ml, 3.75 mmol, 4.1 equiv.) was delivered to the reaction vessel, and was mixed at rt for 5 min. The solution was removed, and the deprotonation was repeated one more time. After the base solution was removed, the resin was washed with anhydrous DMF for 3 minutes for two more times to remove excess KHMDS left in the reaction vessel. After that, the monomer **5.5** (0.5 M in DMF, 15 ml, 7.5 mmol, 8.33 equiv.) was delivered into the reaction vessel. The column was mixed at rt. for six hours. This converted **5.10** (n=10) to **5.9** (n=15). The monomer solution was flushed to waste. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 5); DMF (10 ml ×5); DMSO (10 ml × 5); NMP (10 ml × 5). ESI-MS analysis indicated complete reaction. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 3); DMF (10 ml ×3); DMSO (10 ml × 3). To obtain **5.9**, where n=20, KHMDS solution in THF (0.25 M, 15 ml, 3.75 mmol, 4.1 equiv.) was delivered to the reaction vessel, and was mixed at rt for 5 min. The solution was removed, and the deprotonation was repeated one more time. After the base solution was removed, the resin was washed with anhydrous DMF for 3 minutes for two more times to remove excess KHMDS left in the reaction vessel. After that, the monomer **5.5** (0.5 M in DMF, 15 ml, 7.5 mmol, 8.33 equiv.) was delivered into the reaction vessel. The column was mixed at rt. for six hours. The monomer solution was flushed to waste. Then the resin was washed with THF (10 ml × 2), THF/H_2O, v/v, 1:1, (15 ml × 5); THF, (10 ml × 3); DMF (10 ml ×3); DMSO (10 ml × 3). ESI-MS analysis indicated complete reaction.

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A Appendix A: supporting information for Parallel, Large Scale and Long Synthetic Oligodeoxynucleotide Purification Using the Catching Full-Length Sequence by Polymerization Technique

A.1 ODN sequences

Table 1:ODN sequences

ODN	length	sequence
1a	15-mer	TTT ATT CCC CAA CAT
1b	21-mer	CAA AGT AGC GTG CAC TTT TGC
1c	23-mer	TCA CAT GAT ACC ATT CTC CTA AT
1d	19-mer	TCG GCA TAC CAT GAT TAC T
1e	20-mer	TAC TTG CTG CTT AGA CCG CT
1f	20-mer	CCC ACG TTT TAG CGC TTC GT
1g	20-mer	CTG TGT CGC ATG TAA AAG GT
1h	21-mer	CAC GCT TCA TGA TAT AAC CCT
1i	21-mer	TTG CCA TGA TTG ACA ACC AAT
1j	22-mer	CAA TGG AAG TAC CAT TGA TAC T
1k	26-mer	TAG ACC TAC TAG ATA GGT TCC CAC GT

1l	28-mer	TAA TGG AAG TAC CAT TGA TAC TAC CAT T
1m	32-mer	TAG TTT TAT AAT TTC ATC AGC AGT GTT ACC GT
1n	43-mer	TCG GCT CAA CTC AAA CCTATC AAA CTT GTA ACC CCT CGG CGC T
1o	64-mer	TAT TAT AGC ATA CTC TGT AAT AGT TGT CAA ACA TTA ATA GTT AAT TCT CCC ATT CTA AAA CGA T
1p	80-mer	TAC TCA TAA TAC TGT TTA CCG TCA TCA TCT TGA AGC AAC ATT GTC ACA TCG TAT GAG TCA ACA AAA TCA TTT TGC ACC AT
1q	90-mer	TAT TAT AGC ATA CTC TGT AAT AGT TGT CAA ACA TTA ATA GTT AAT TCT CCC ATT CTA AAA CGA TTT GAT CGT TTA TTT CTA CAA TTA GAT
1r	110-mer	CAT TAT AGC ATA CTC TGT AAT AGT TGT CAA ACA TTA ATA GTT AAT TCT CCC ATT CTA AAA CGA TTT GAT CGT TTA TTT CTA CAA TTA GAT GGA CTC CAT TTG TAC CGA AT
1s	151-mer	TCA ACA AAA TCA TTT TGC ACC ATG TGG AGC ACC TCC AAA TAA CAC CTT TAT AAC CCA TGT GGC GTA ATC ATT GTT TTC CAT CCT AGA AAG CTC ATA CAA TGC GTT TTT CAT GAG TTT ATT TTC ATG CTC TAG TTT AGT CAT CTT CTT TTC T
1t	197-mer	CCC CAA CAT ACA CAT GAC AAT GGA AGT ACC GTA CCA TTG ATA CTA CCA TTA TAG CAT ACT CTG TAA TAG TTG TCA AAC ATT AAT AGT TAA TTC TCC CAT TCT AAA ACG ATT TGA TCG TTT ATT TCT ACA ATT AGA TGG ACT CCA TTT GTA CCG AAT GGA TGG ACT TGT AAC TTT ATC GTA CCA TCT TTA AAC ATA TT
1u	203-mer	TTT AAT CCC CAA CAT ACA CAT GAC AAT GGA AGT ACC GTA CCA TTG ATA CTA CCA TTA TAG CAT ACT CTG TAA TAG TTG TCA AAC ATT AAT AGT TAA TTC TCC CAT TCT AAA ACG ATT TGA TCG TTT ATT TCT ACA ATT AGA TGG ACT CCA TTT GTA CCG AAT GGA

		TGG ACT TGT AAC TTT ATC GTA CCA TCT TTA AAC ATA TT
1v	225-mer	TTT AAT CCC CAA CAT ACA CAT GAC AAT GGA AGT ACC GTA CCA TTG ATA CTA CCA TTA TAG CAT ACT CTG TAA TAG TTG TCA AAC ATT AAT AGT TAA TTC TCC CAT TCT AAA ACG ATT TGA TCG TTT ATT TCT ACA ATT AGA TGG ACT CCA TTT GTA CCG AAT GGA TGG ACT TGT AAC TTT ATC GTA CCA TCT TTA AAC ATA TTC GCA ATA TGT TCT TCT AAC TCT
1w	251-mer	TTT AAT CCC CAA CAT ACA CAT GAC AAT GGA AGT ACC GTA CCA TTG ATA CTA CCA TTA TAG CAT ACT CTG TAA TAG TTG TCA AAC ATT AAT AGT TAA TTC TCC CAT TCT AAA ACG ATT TGA TCG TTT ATT TCT ACA ATT AGA TGG ACT CCA TTT GTA CCG AAT GGA TGG ACT TGT AAC TTT ATC GTA CCA TCT TTA AAC ATA TTC GCA ATA TGT TCT TCT AAC TCT GCA CGC TTC ATG ATA TAA CCC TCC TT
1x	275-mer	TTT AAT CCC CAA CAT ACA CAT GAC AAT GGA AGT ACC GTA CCA TTG ATA CTA CCA TTA TAG CAT ACT CTG TAA TAG TTG TCA AAC ATT AAT AGT TAA TTC TCC CAT TCT AAA ACG ATT TGA TCG TTT ATT TCT ACA ATT AGA TGG ACT CCA TTT GTA CCG AAT GGA TGG ACT TGT AAC TTT ATC GTA CCA TCT TTA AAC ATA TTC GCA ATA TGT TCT TCT AAC TCT GCA CGC TTC ATG ATA TAA CCC TCC TTA TCA GAT GTC AAA TAT AGT TTT CT
1y	303-mer	TTT AAT CCC CAA CAT ACA CAT GAC AAT GGA AGT ACC GTA CCA TTG ATA CTA CCA TTA TAG CAT ACT CTG TAA TAG TTG TCA AAC ATT AAT AGT TAA TTC TCC CAT TCT AAA ACG ATT TGA TCG TTT ATT TCT ACA ATT AGA TGG ACT CCA TTT GTA CCG AAT GGA TGG ACT TGT AAC TTT ATC GTA CCA TCT TTA AAC ATA TTC GCA ATA TGT TCT TCT AAC TCT GCA CGC TTC ATG ATA TAA CCC TCC TTA TCA GAT GTC AAA TAT AGT TTT CTC ACG GCT CAA CTC AAA CCT ATC AAA CTT

RP HPLC of ODNs

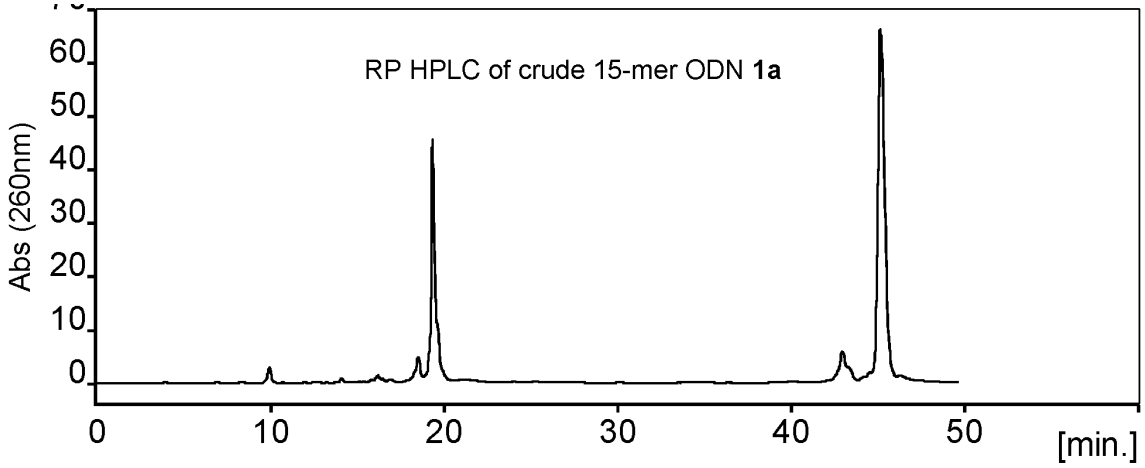


Figure A.1:RP HPLC profile of crude 15-mer **1a**

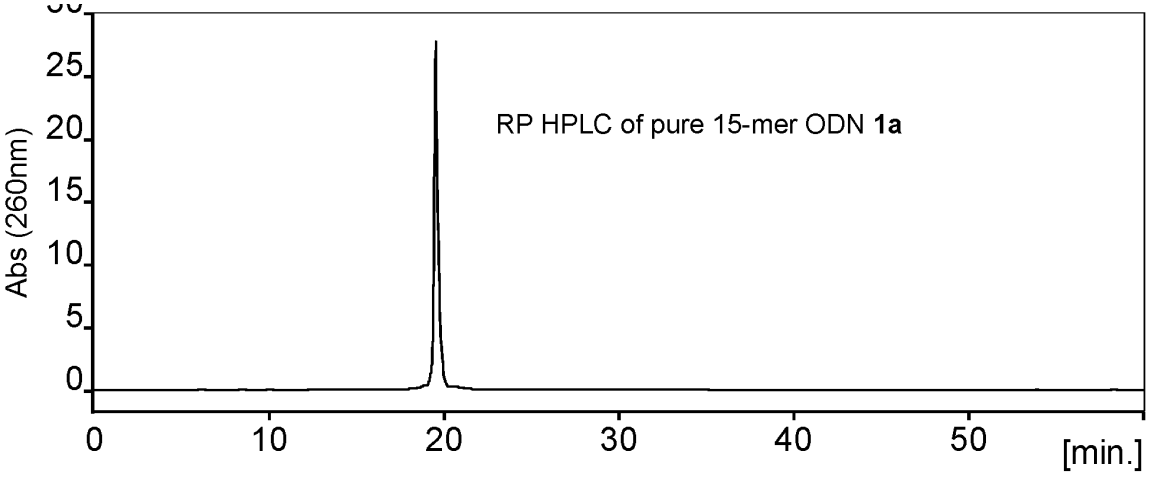


Figure A.2:RP HPLC profile of pure 15-mer **1a**

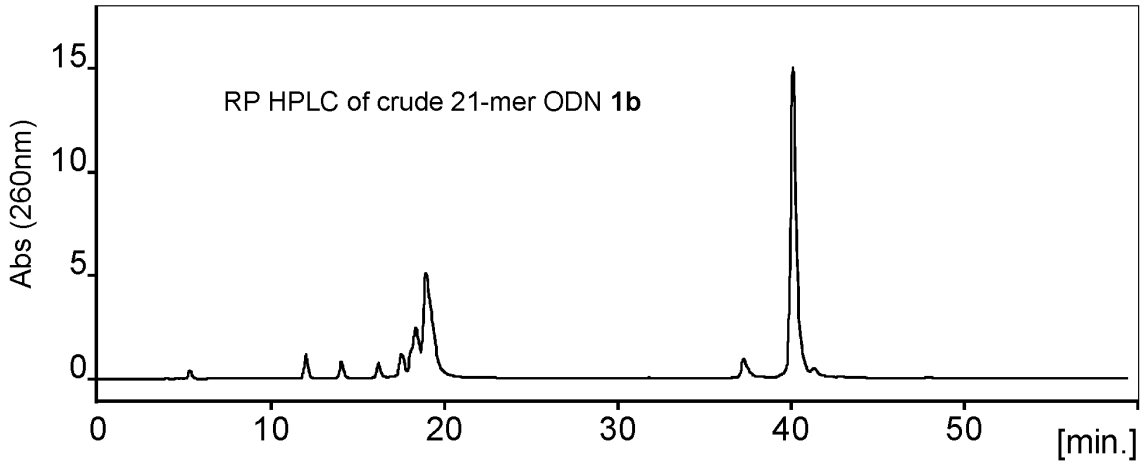


Figure A.3:RP HPLC profile of crude 21-mer **1b**

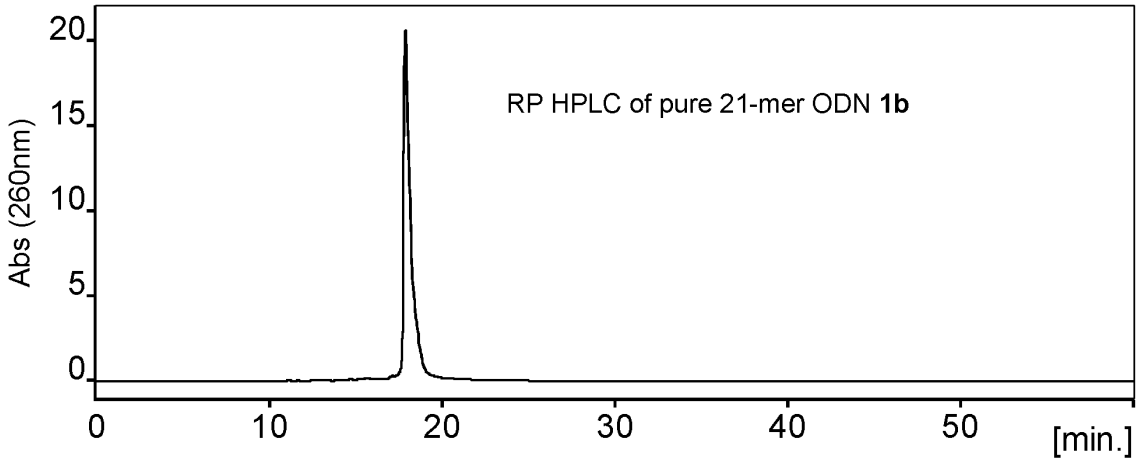


Figure A.4:RP HPLC profile of pure 21-mer **1b**

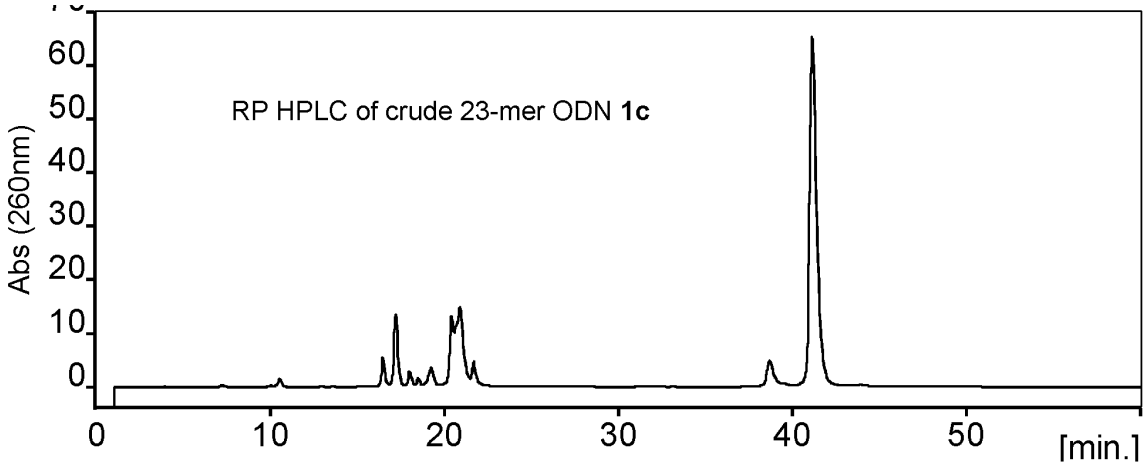


Figure A.5:RP HPLC profile of crude 23-mer **1c**

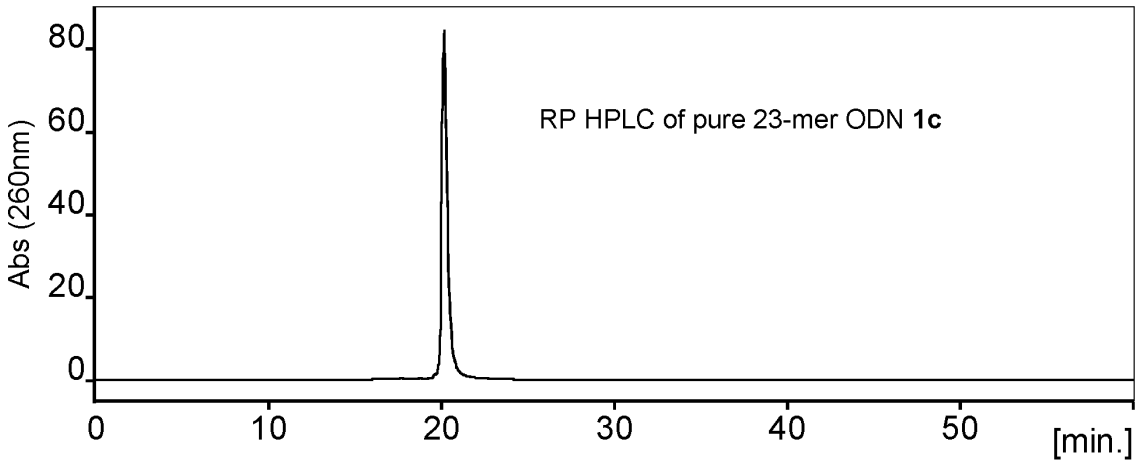


Figure A.6:RP HPLC profile of pure 23-mer **1c**

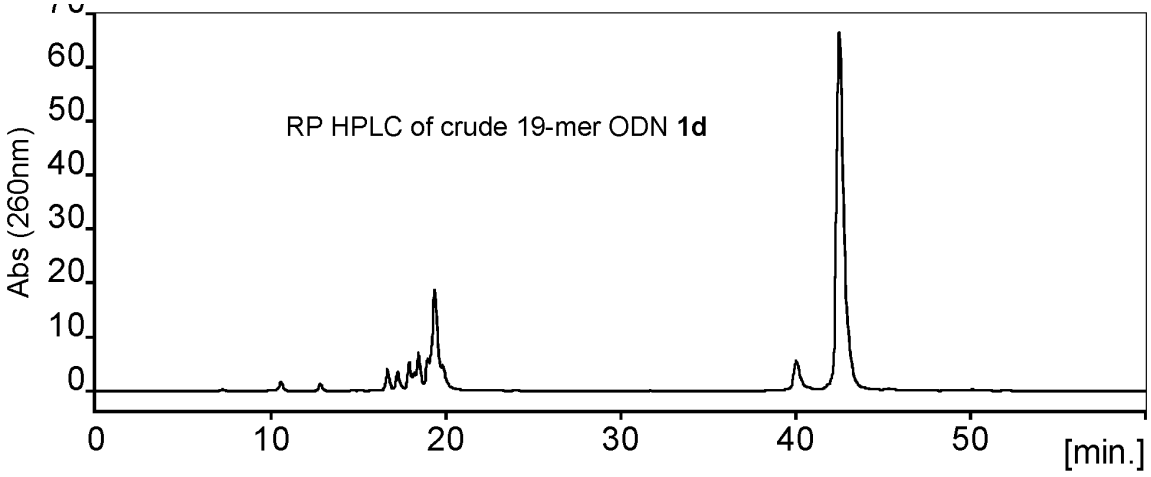


Figure A.7:RP HPLC profile of crude 19-mer **1d**

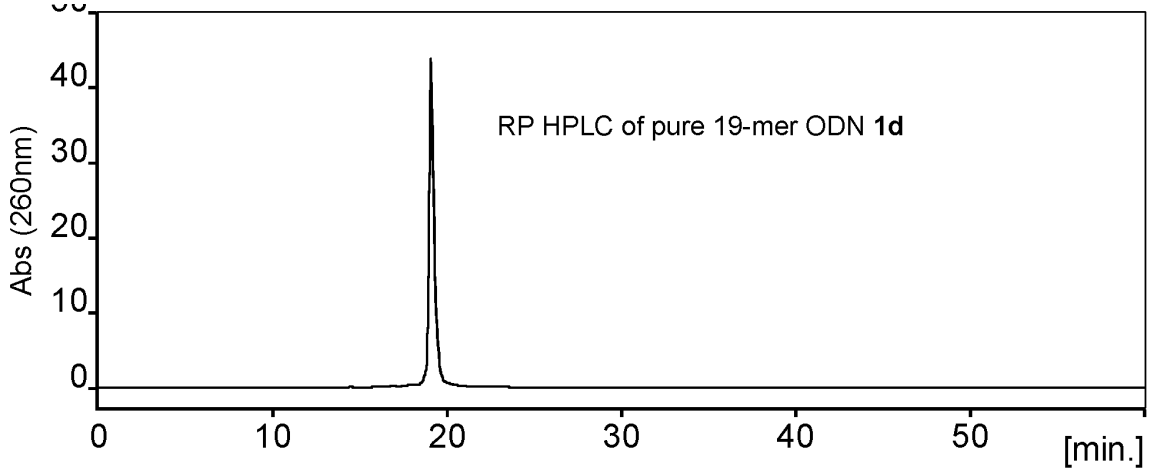


Figure A.8:RP HPLC profile of pure 19-mer **1d**

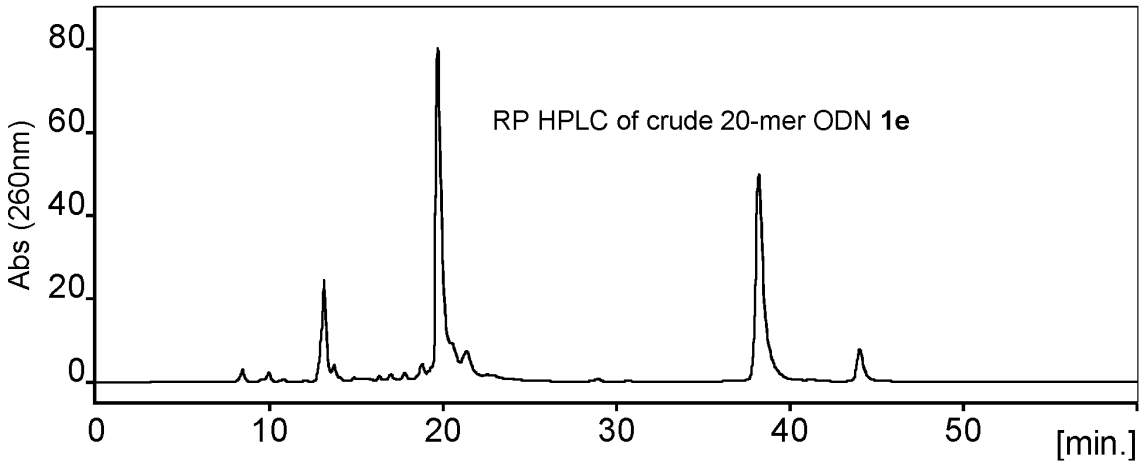


Figure A.9:RP HPLC profile of crude 20-mer **1e**

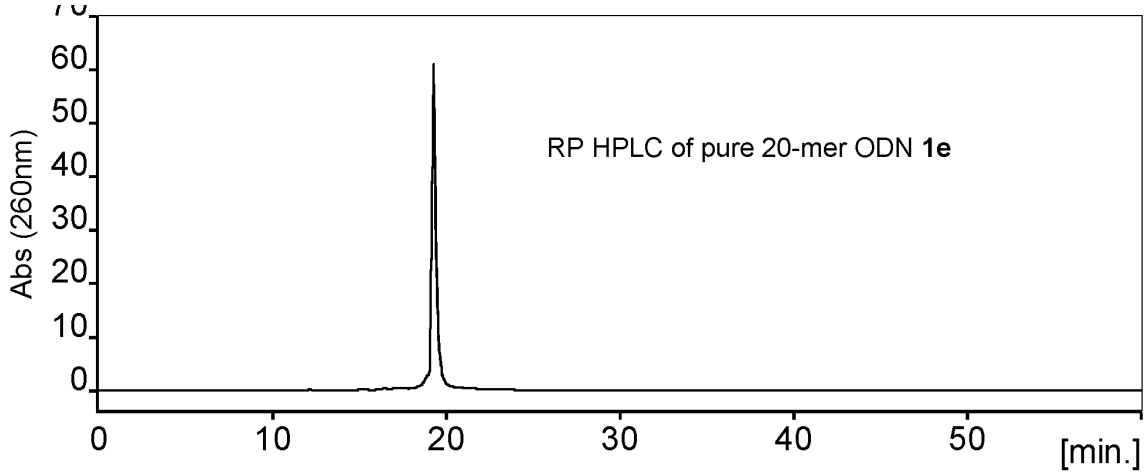


Figure A.10:RP HPLC profile of crude 20-mer **1e**

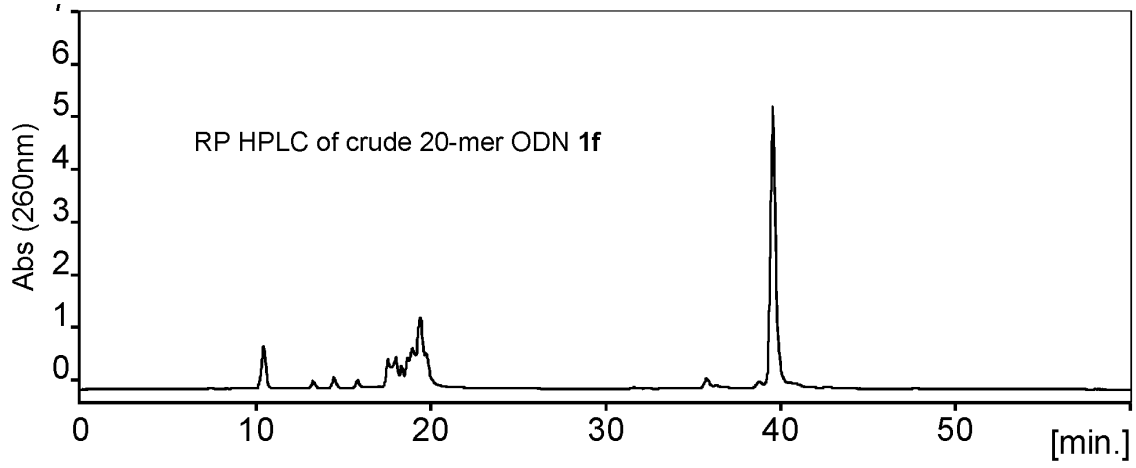


Figure A.11:RP HPLC profile of crude 20-mer **1f**

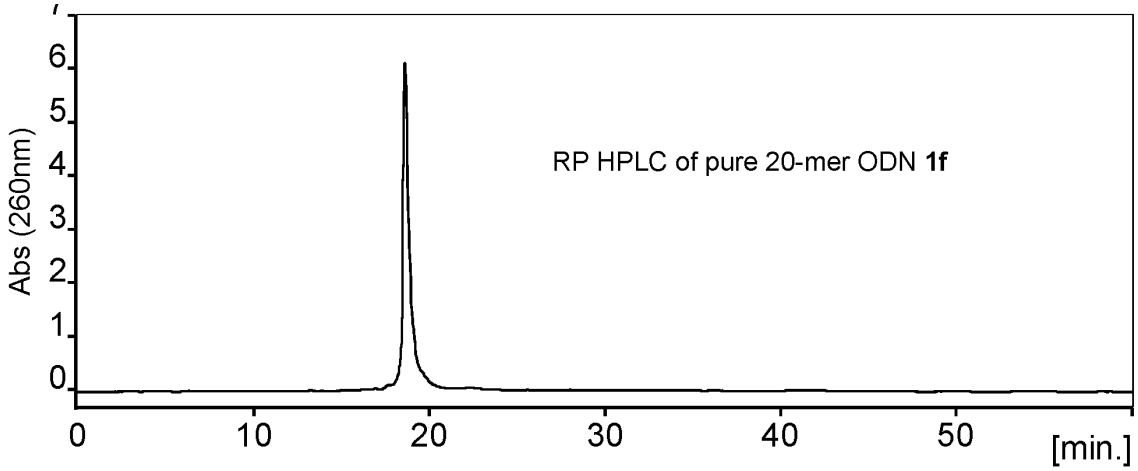


Figure A.12:RP HPLC profile of pure 20-mer **1f**

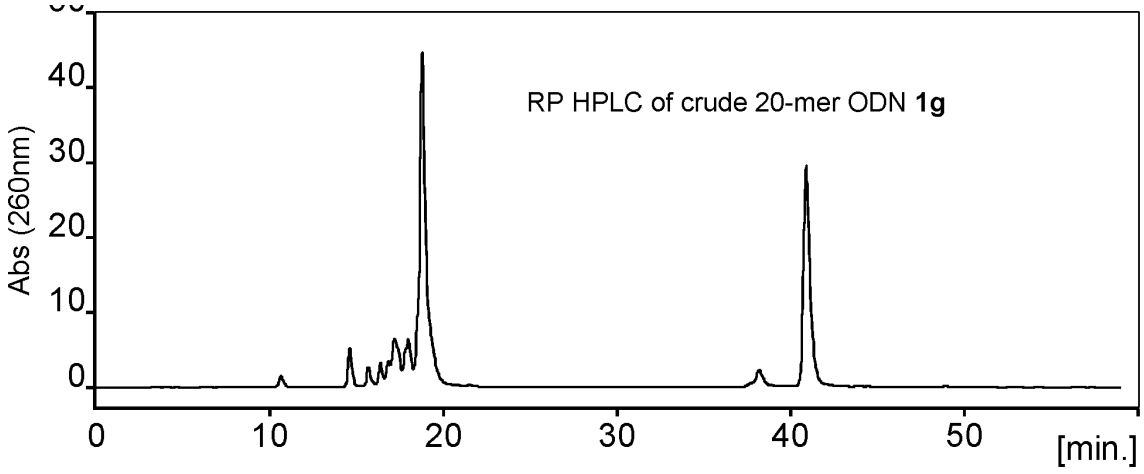


Figure A.13:RP HPLC profile of crude 20-mer **1g**

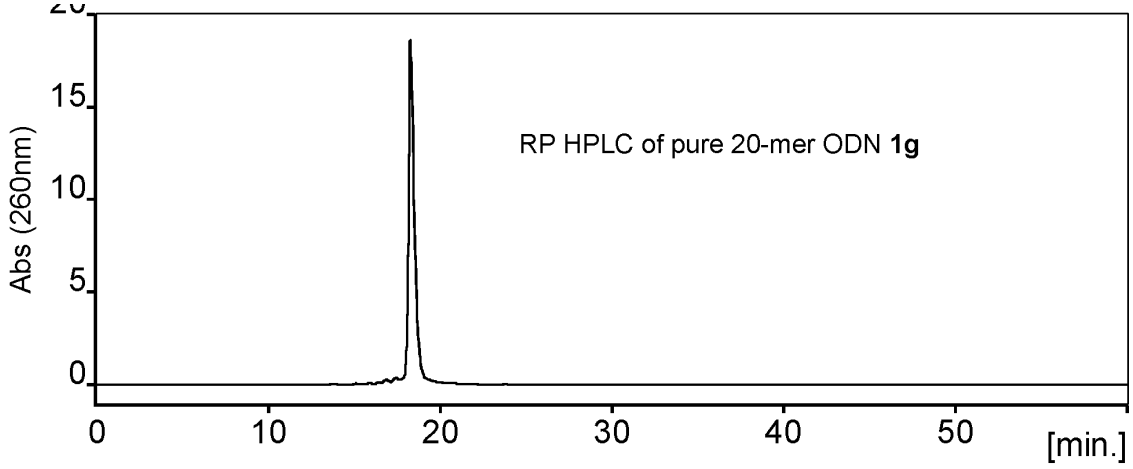


Figure A.14:RP HPLC profile of pure 20-mer **1g**

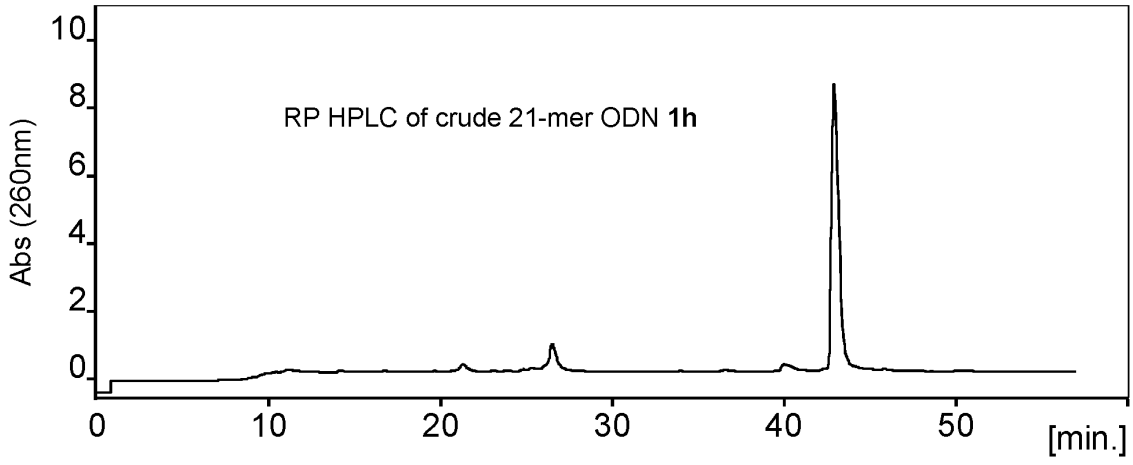


Figure A.15:RP HPLC profile of crude 21-mer **1h**

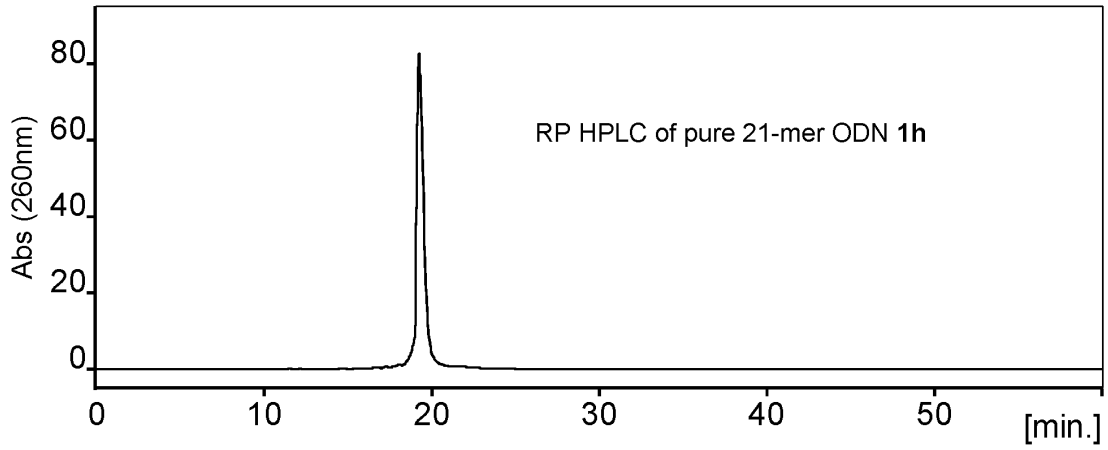


Figure A.16:RP HPLC profile of pure 21-mer **1h**

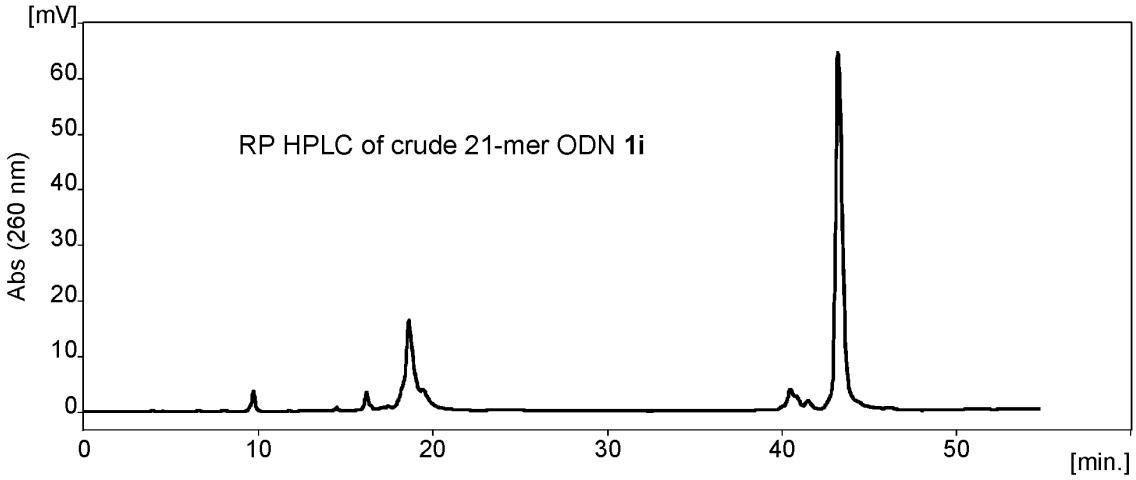


Figure A.17:RP HPLC profile of crude 21-mer **1i**

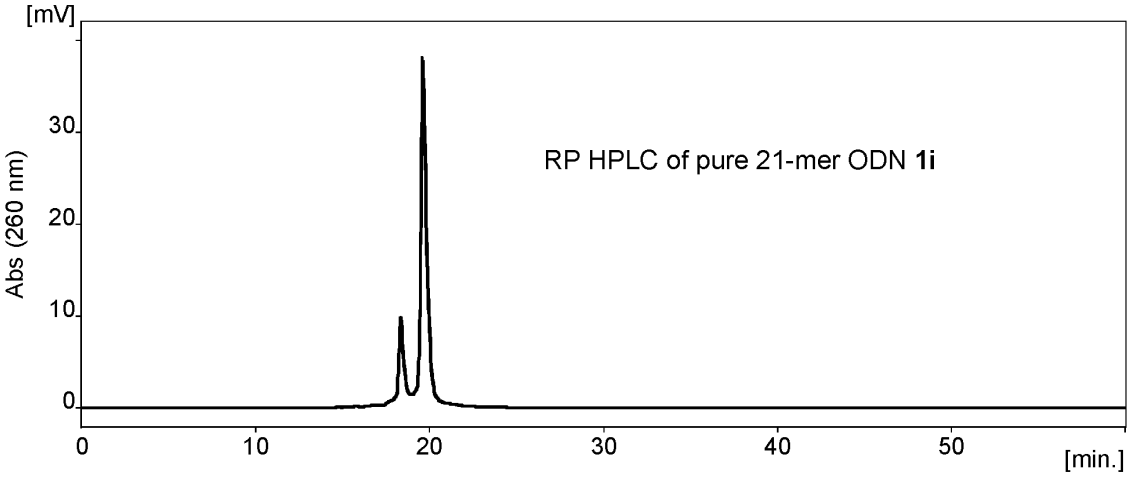


Figure A.18:RP HPLC profile of pure 21-mer **1i**

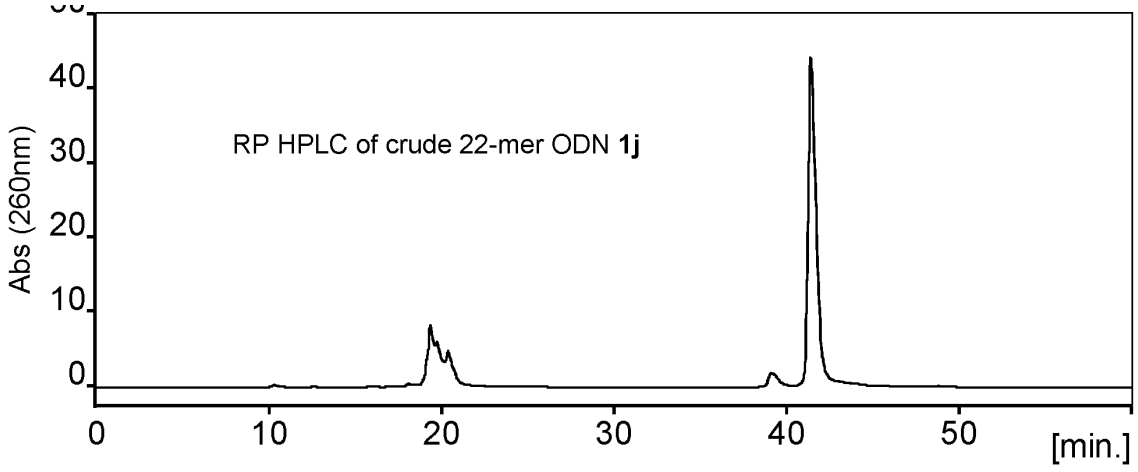


Figure A.19:RP HPLC profile of crude 22-mer **1j**

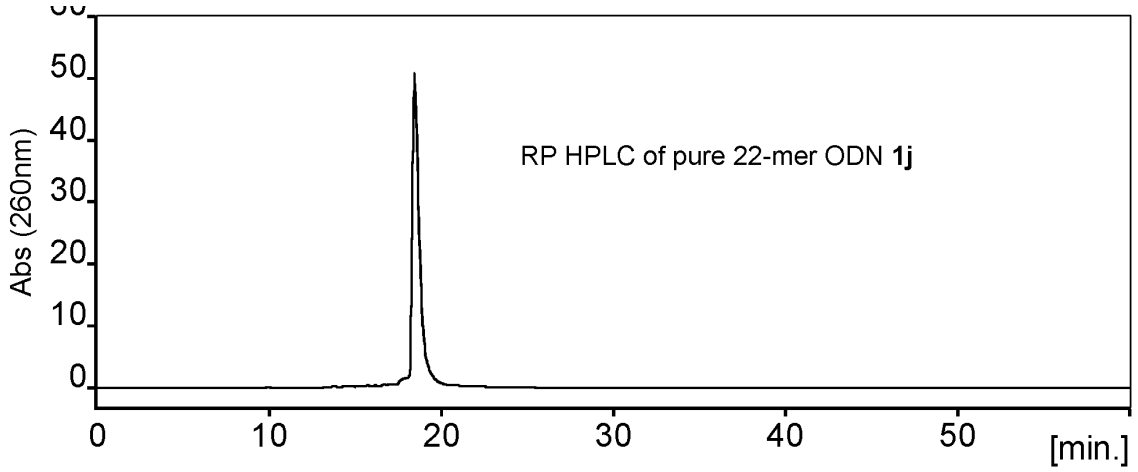


Figure A.20:RP HPLC profile of pure 22-mer **1j**

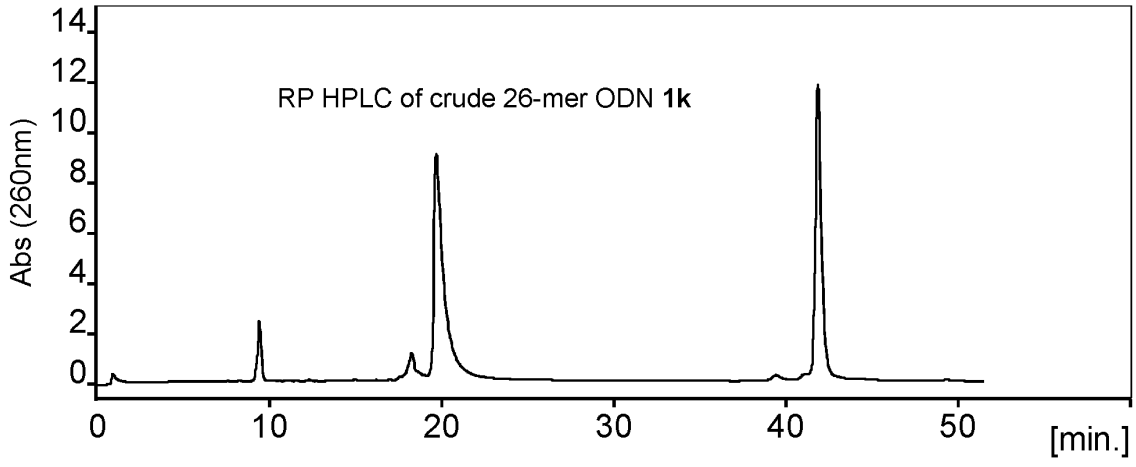


Figure A.21:RP HPLC profile of crude 26-mer **1k**

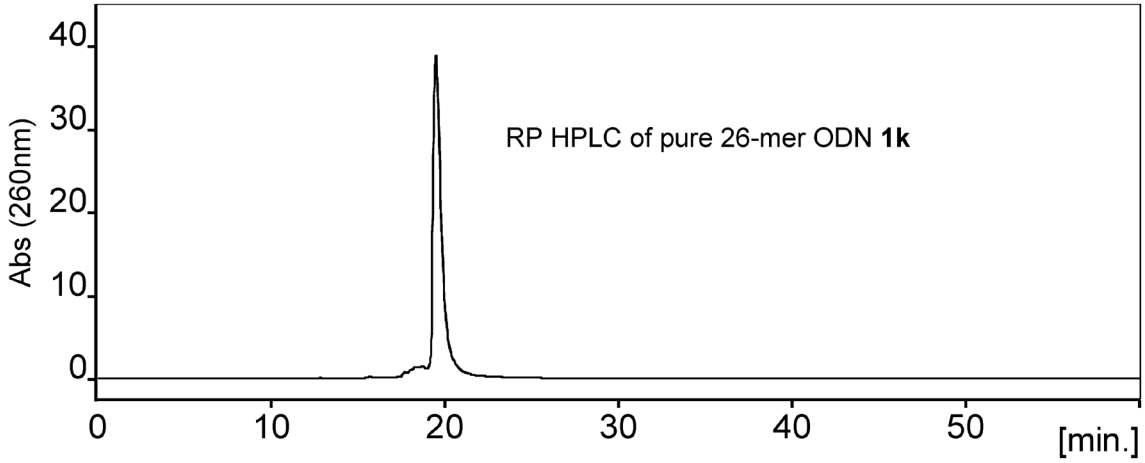


Figure A.22:RP HPLC profile of pure 26-mer **1k**

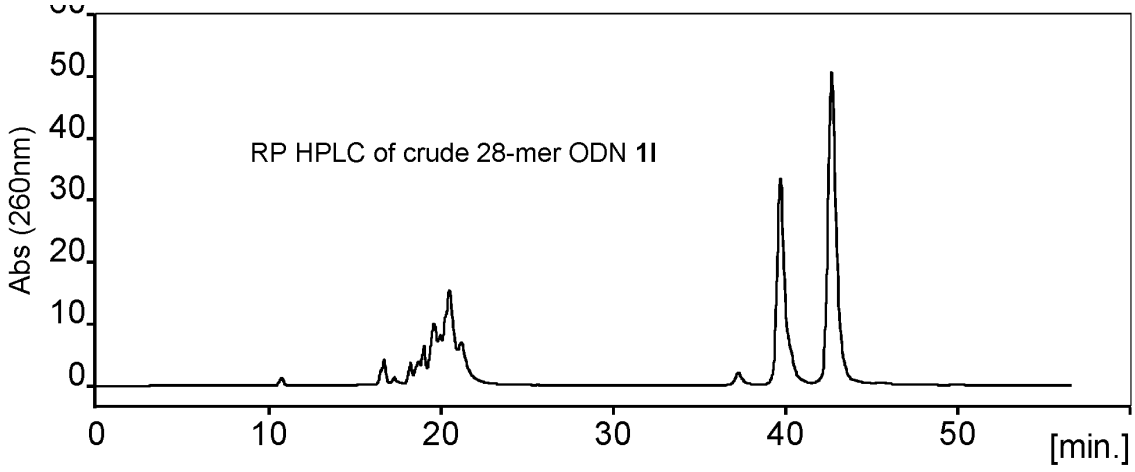


Figure A.23:RP HPLC profile of crude 28-mer 1l

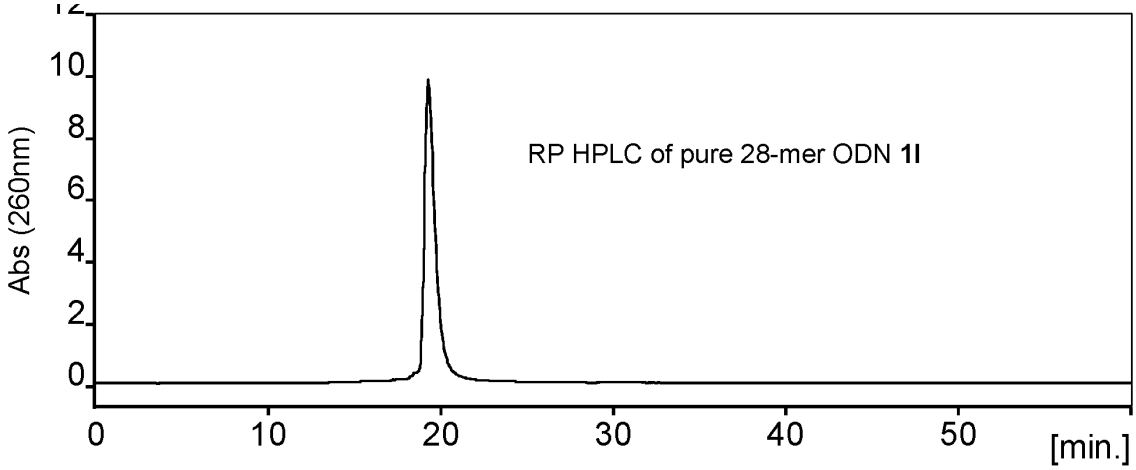


Figure A.24:RP HPLC profile of pure 28-mer **1l**

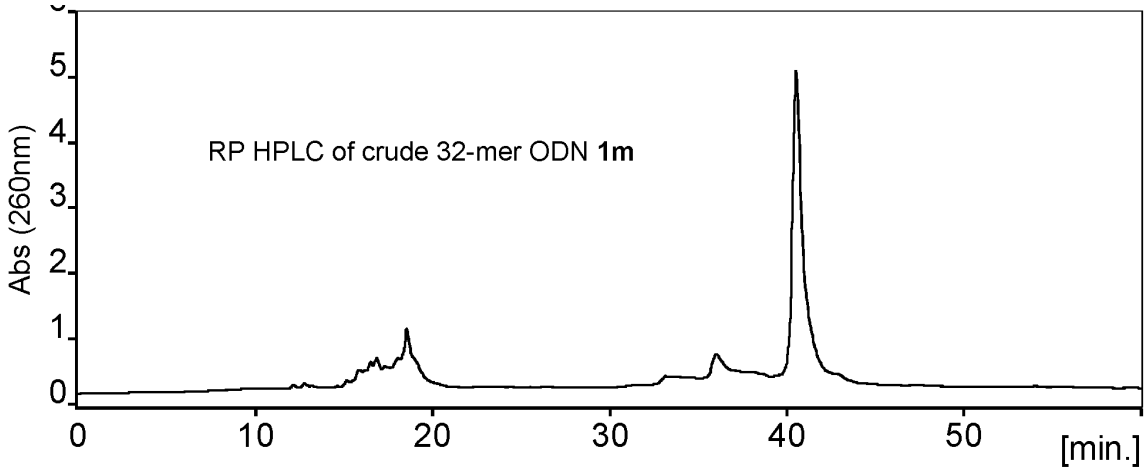


Figure A.25:RP HPLC profile of crude 32-mer **1m**

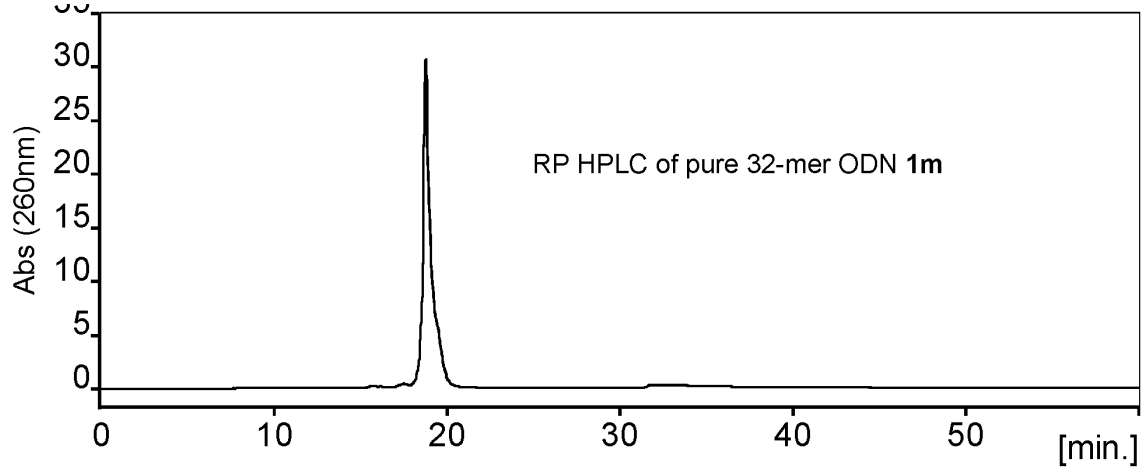


Figure A.26:RP HPLC profile of pure 32-mer **1m**

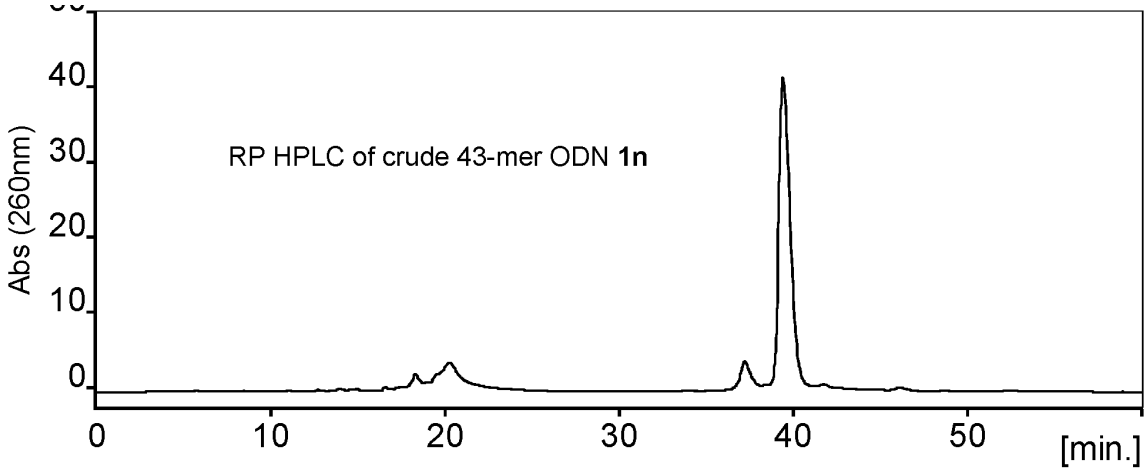


Figure A.27:RP HPLC profile of crude 43-mer **1n**

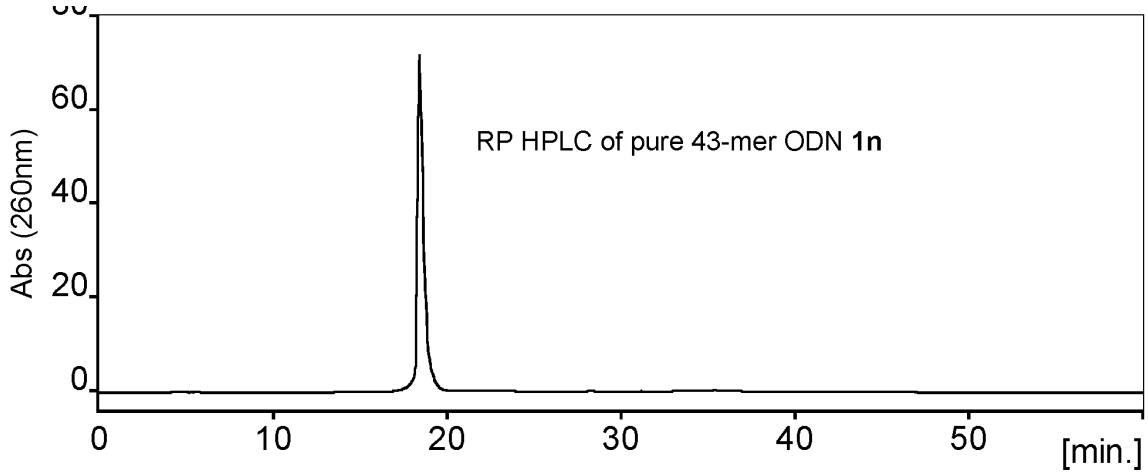


Figure A.28:RP HPLC profile of pure 43-mer **1n**

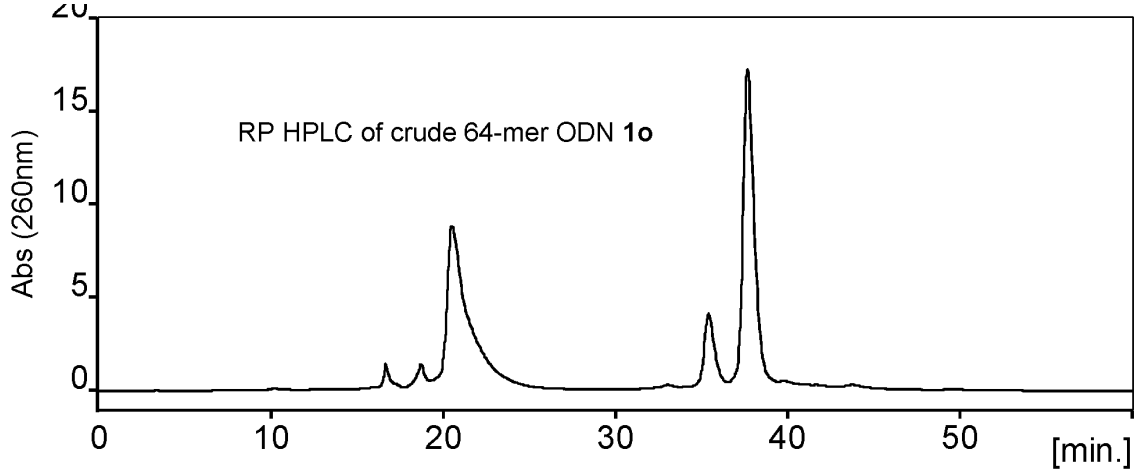


Figure A.29:RP HPLC profile of crude 64-mer **1o**

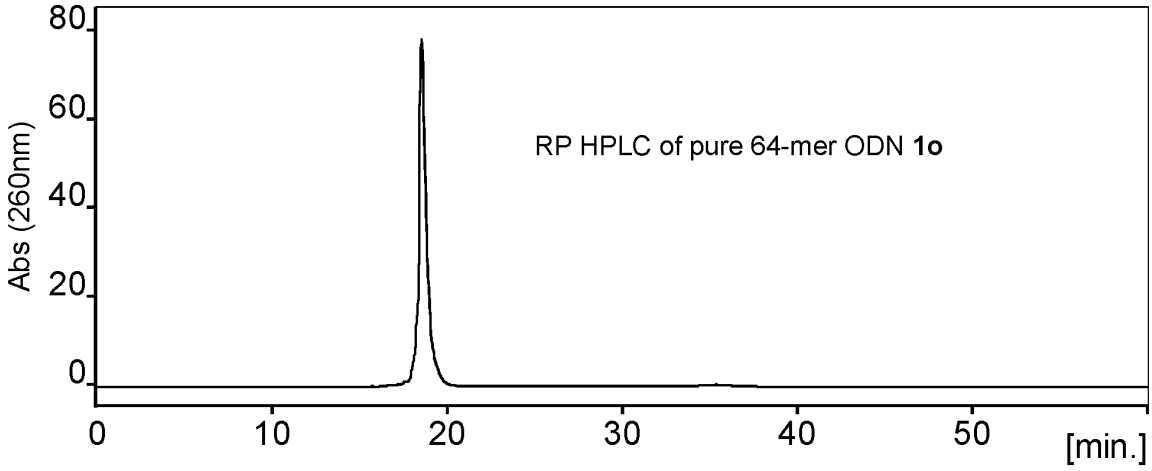


Figure A.30:RP HPLC profile of pure 64-mer **1o**

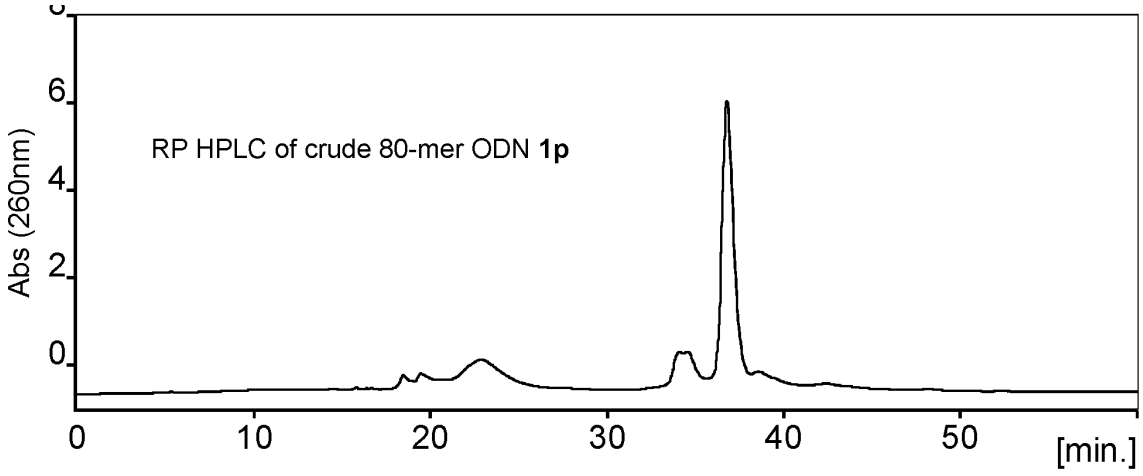


Figure A.31:RP HPLC profile of crude 80-mer **1p**

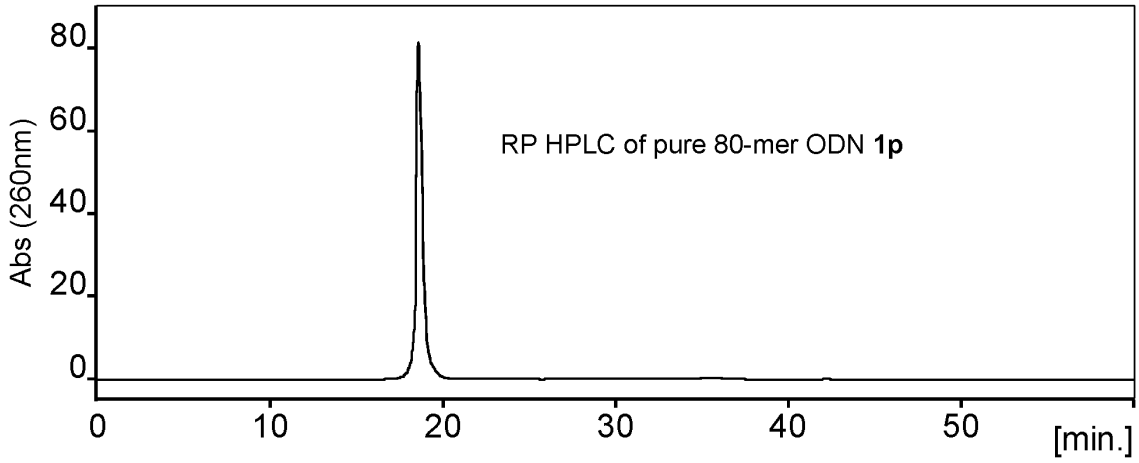


Figure A.32:RP HPLC profile of pure 80-mer **1p**

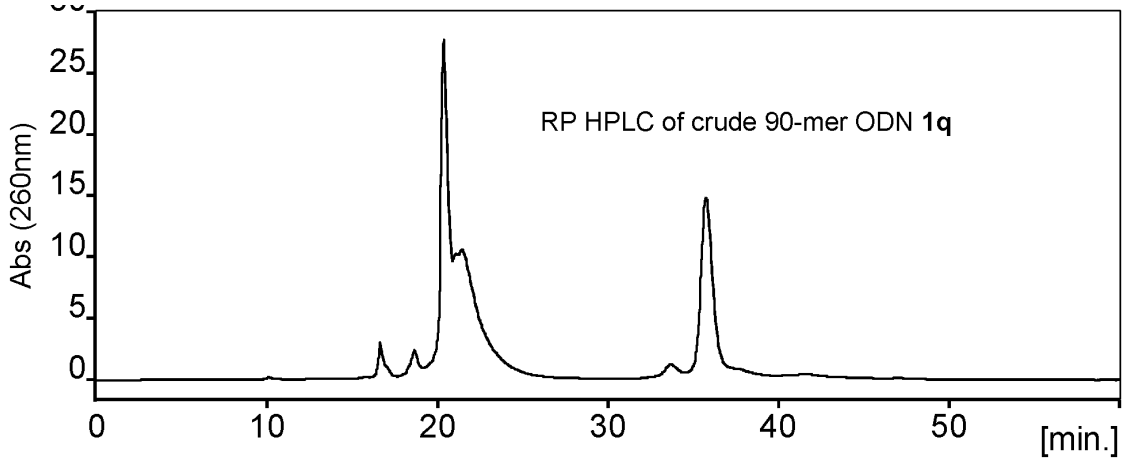


Figure A.33:RP HPLC profile of crude 90-mer **1q**

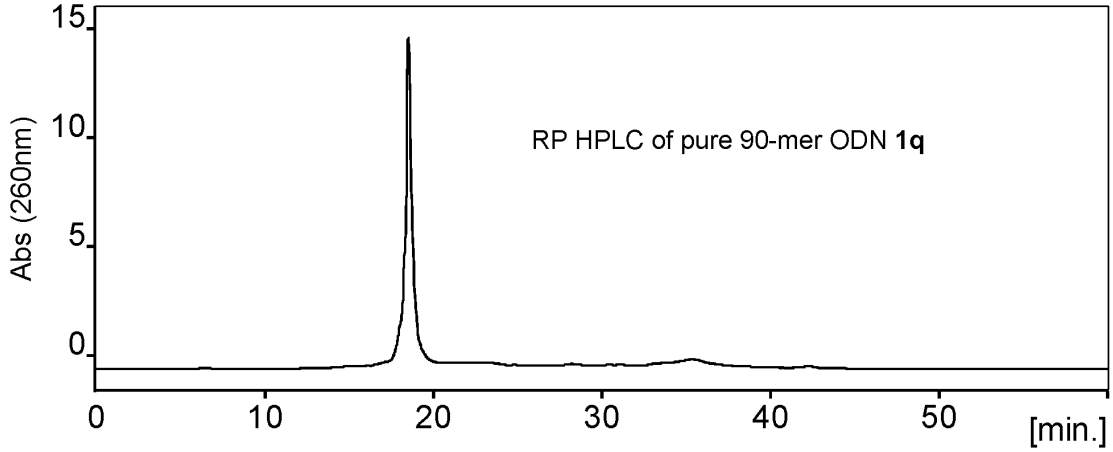


Figure A.34:RP HPLC profile of pure 90-mer **1q**

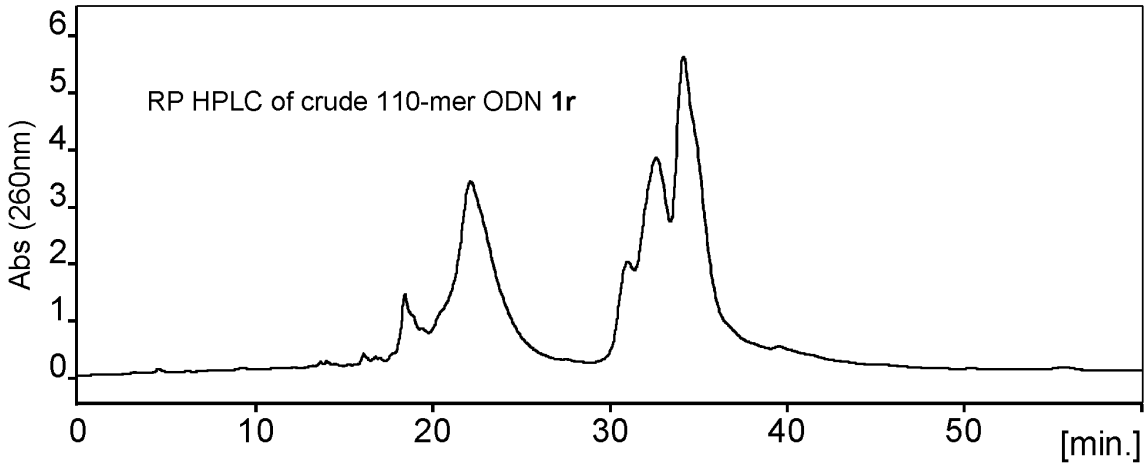


Figure A.35:RP HPLC profile of crude 110-mer **1r**

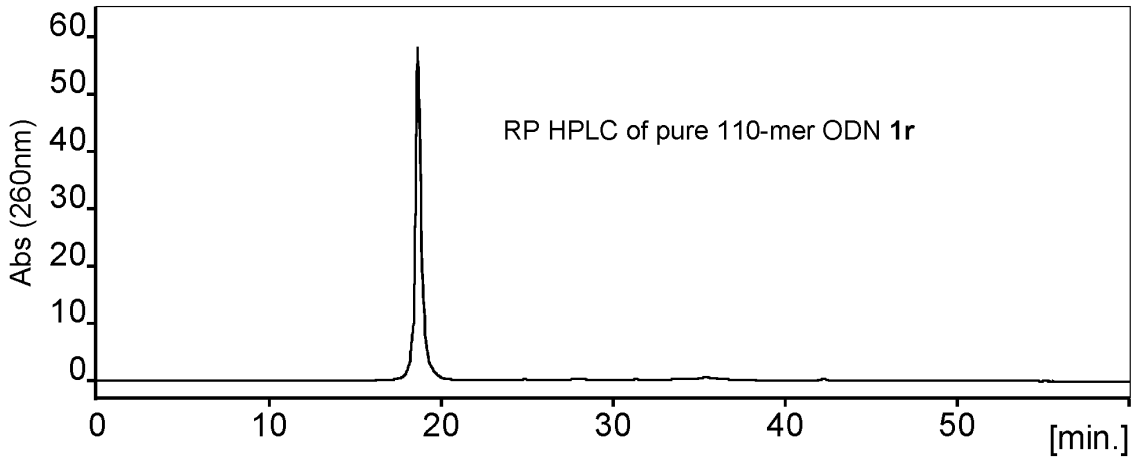


Figure A.36:RP HPLC profile of pure 110-mer **1r**

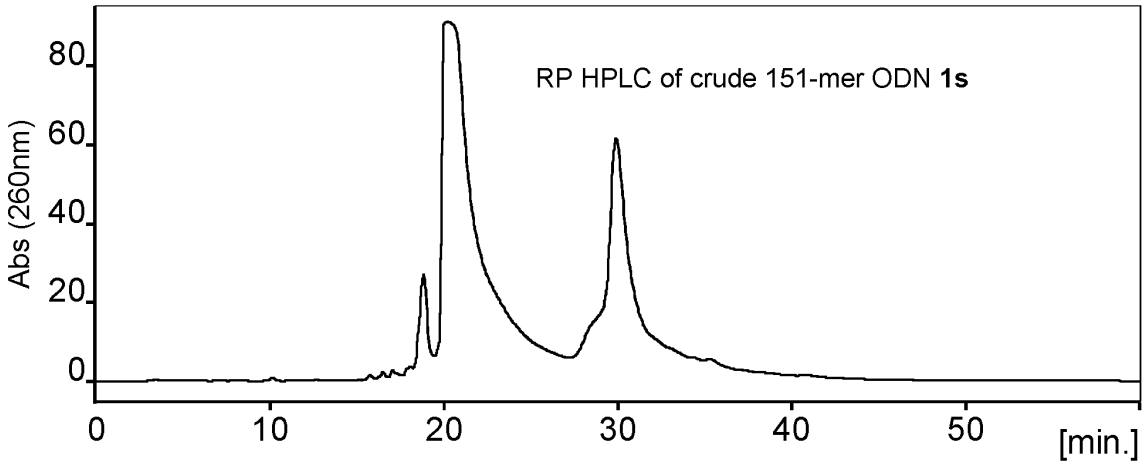


Figure A.37:RP HPLC profile of crude 151-mer **1s**

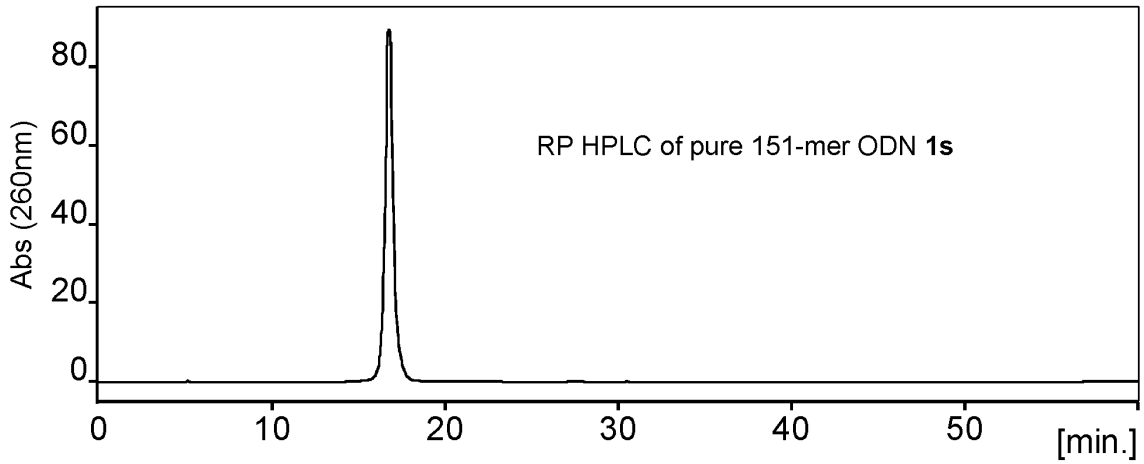


Figure A.38:RP HPLC profile of pure 151-mer **1s**

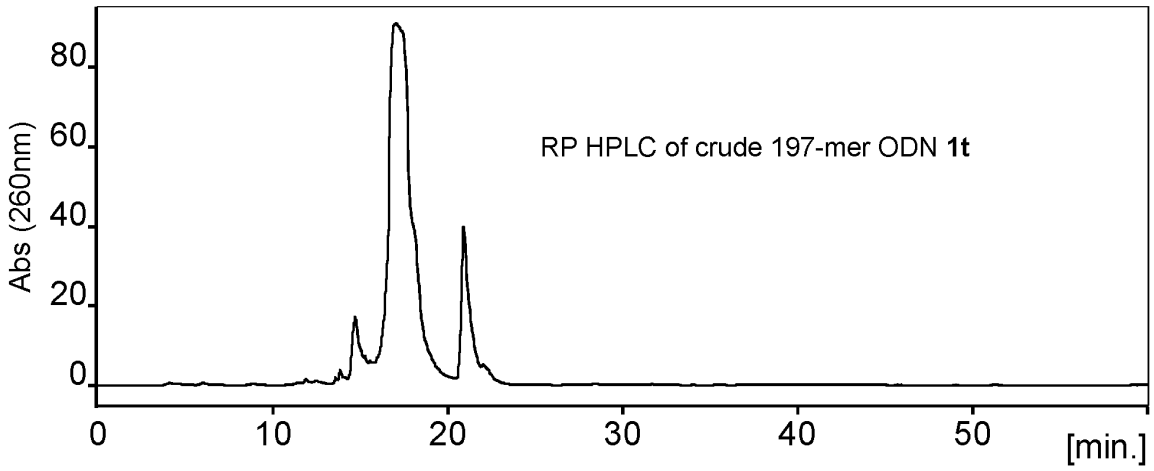


Figure A.39:RP HPLC profile of crude 197-mer **1t**

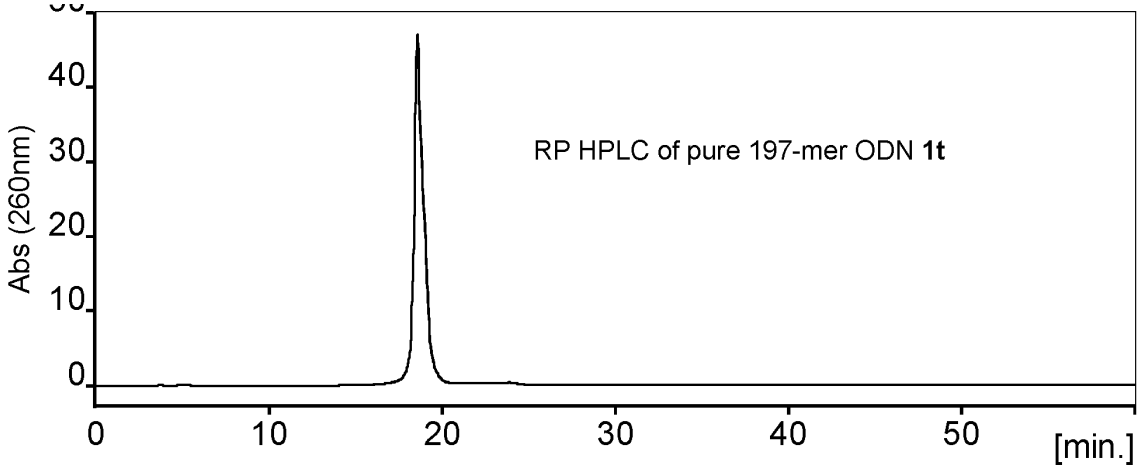


Figure A.40:RP HPLC profile of pure 197-mer **1t**

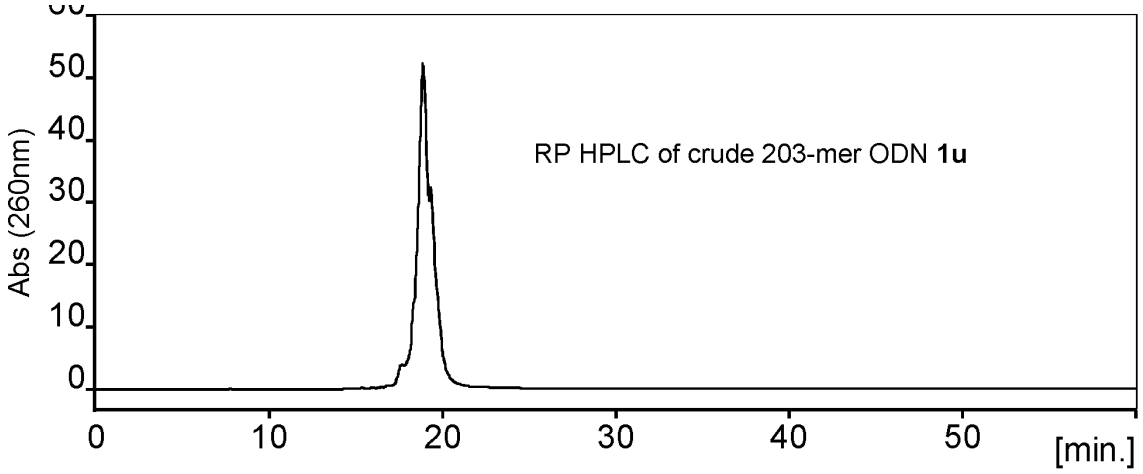


Figure A.41:RP HPLC profile of crude 203-mer **1u**

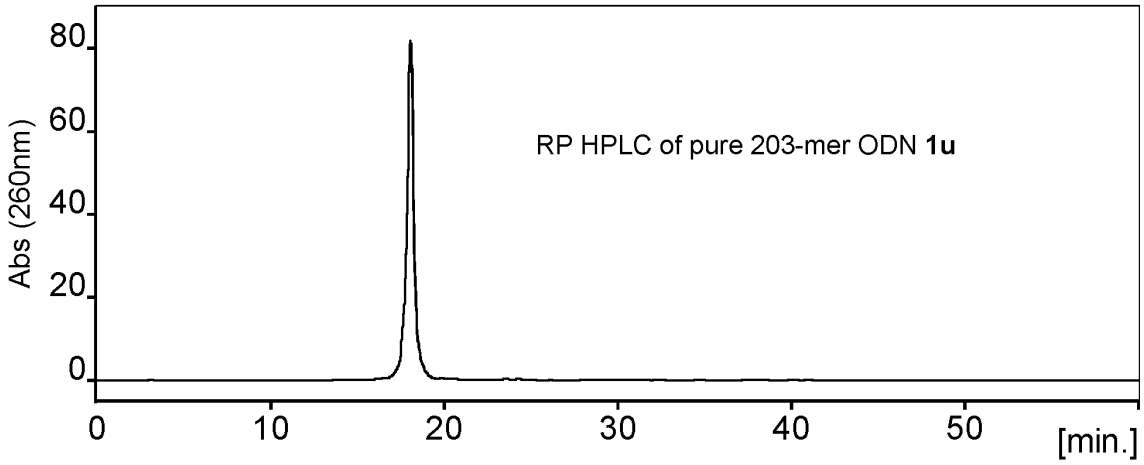


Figure A.42:RP HPLC profile of pure 203-mer **1u**

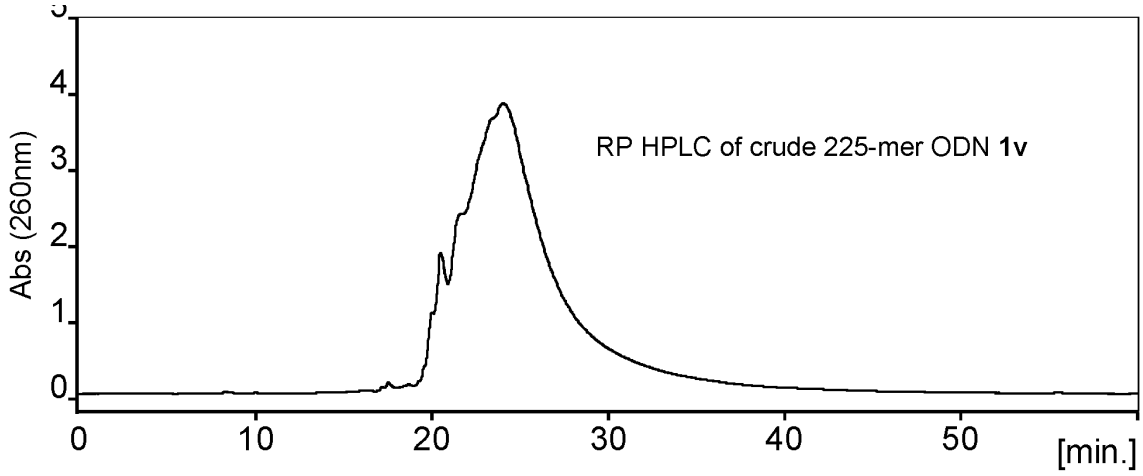


Figure A.43:RP HPLC profile of crude 225-mer **1v**

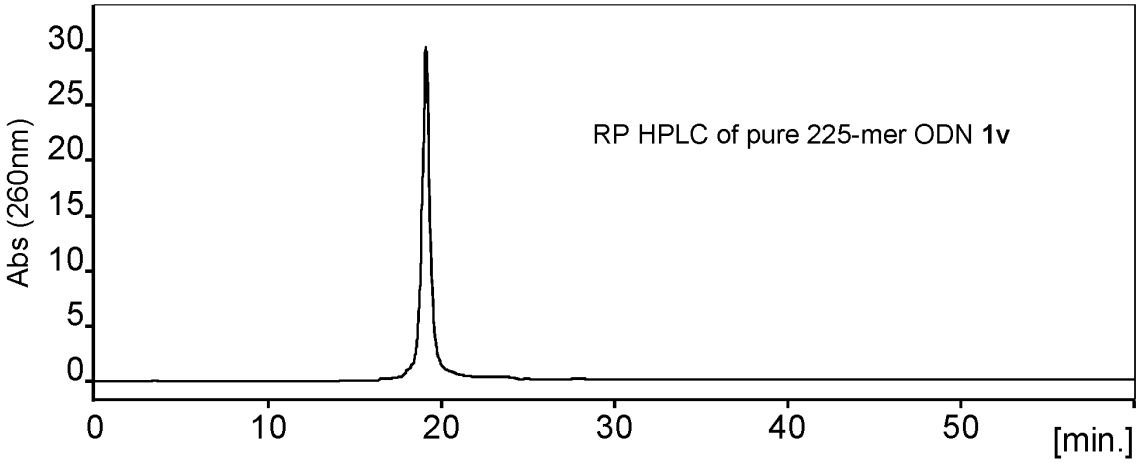


Figure A.44:RP HPLC profile of pure 225-mer **1v**

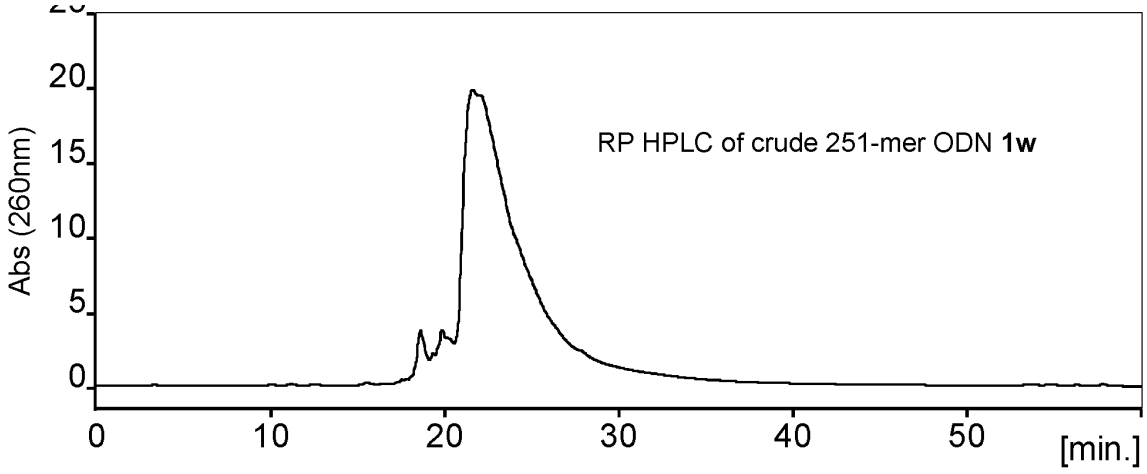


Figure A.45:RP HPLC profile of crude 251-mer **1w**

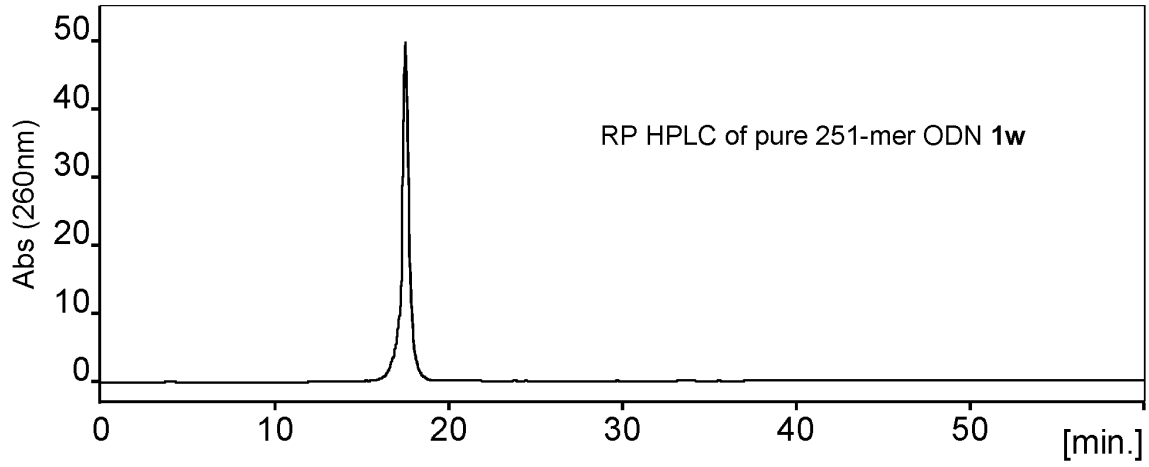


Figure A.46:RP HPLC profile of pure 251-mer **1w**

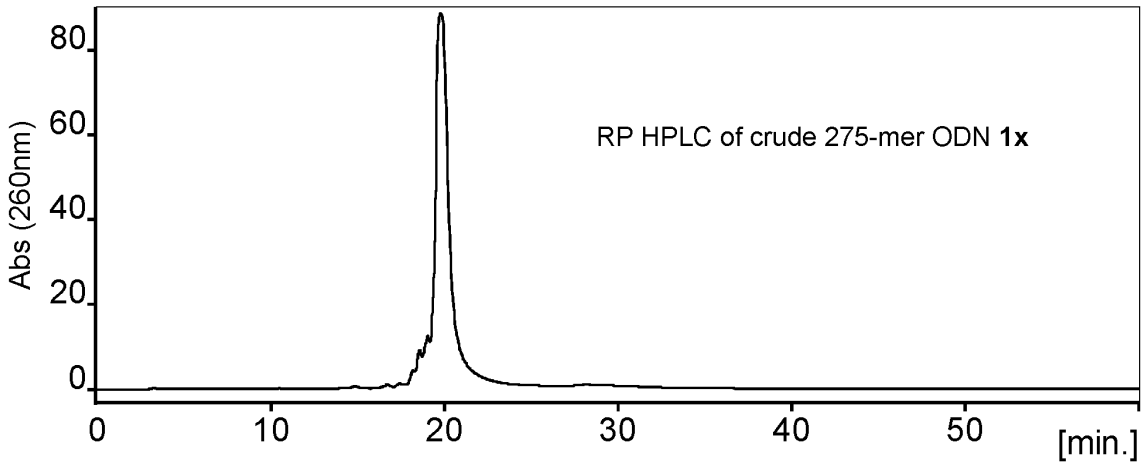


Figure A.47:RP HPLC profile of crude 275-mer **1x**

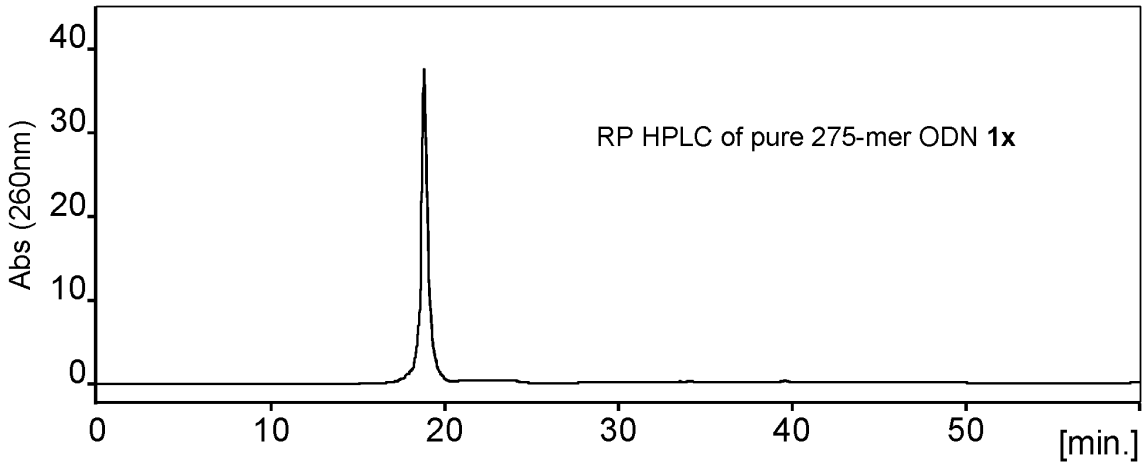


Figure A.48:RP HPLC profile of pure 275-mer **1x**

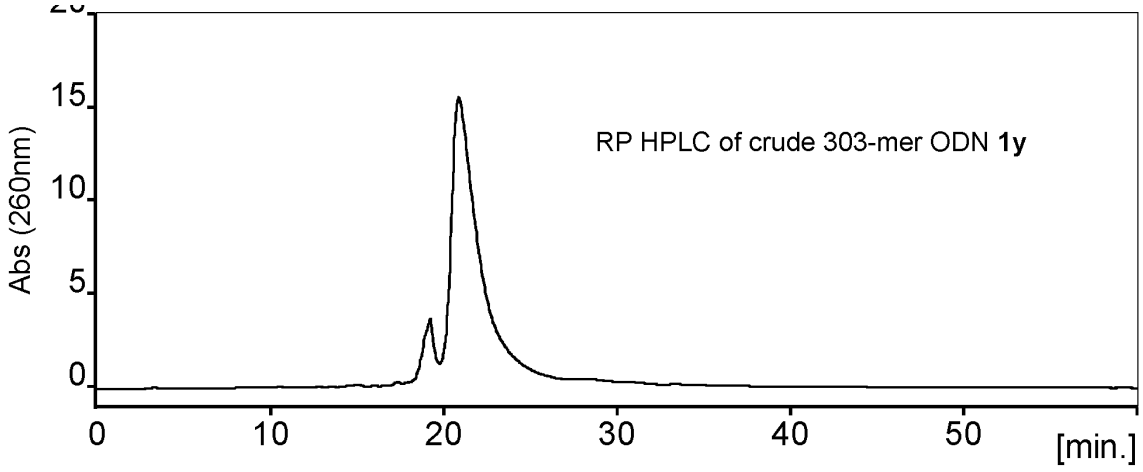


Figure A.49:RP HPLC profile of crude 303-mer **1y**

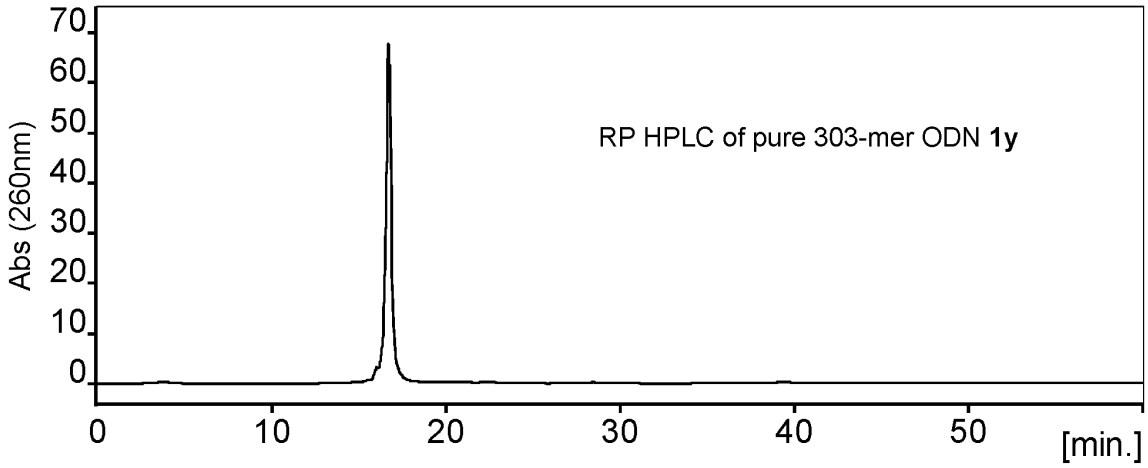


Figure A.50:RP HPLC profile of pure 303-mer **1y**

A.1.1 MALDI-TOF MS of ODNs

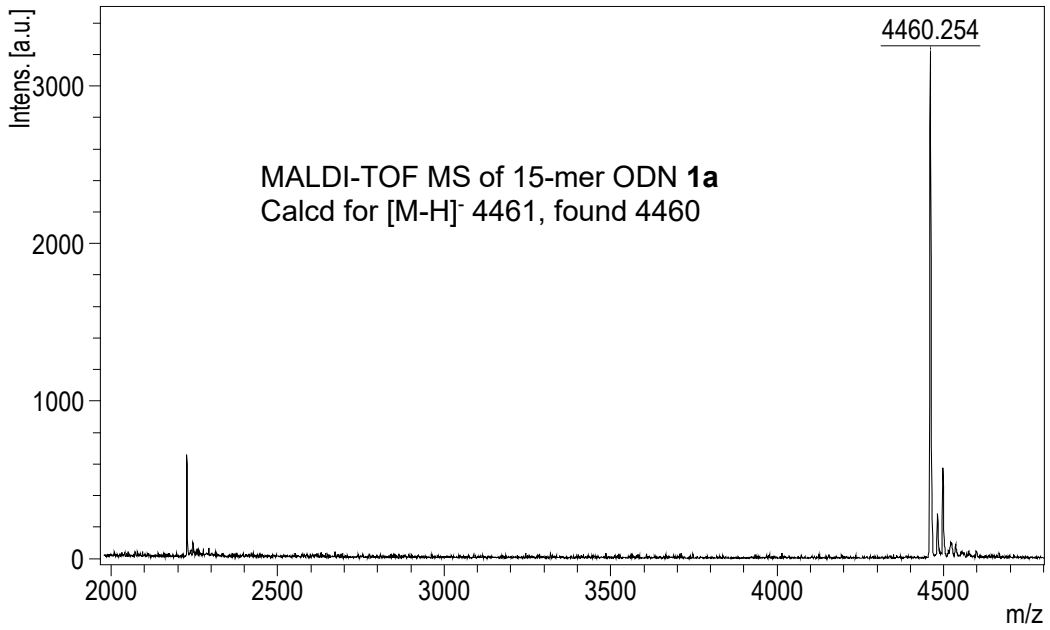


Figure A.51:MALDI-TOF MS of 15-mer ODN **1a**

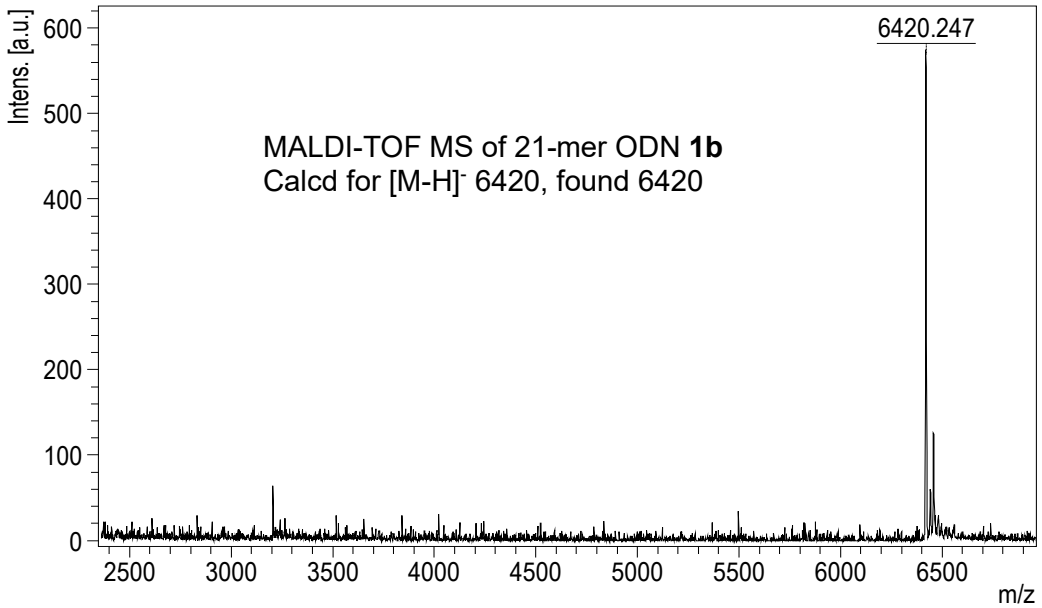


Figure A.52:MALDI-TOF MS of 21-mer ODN **1b**

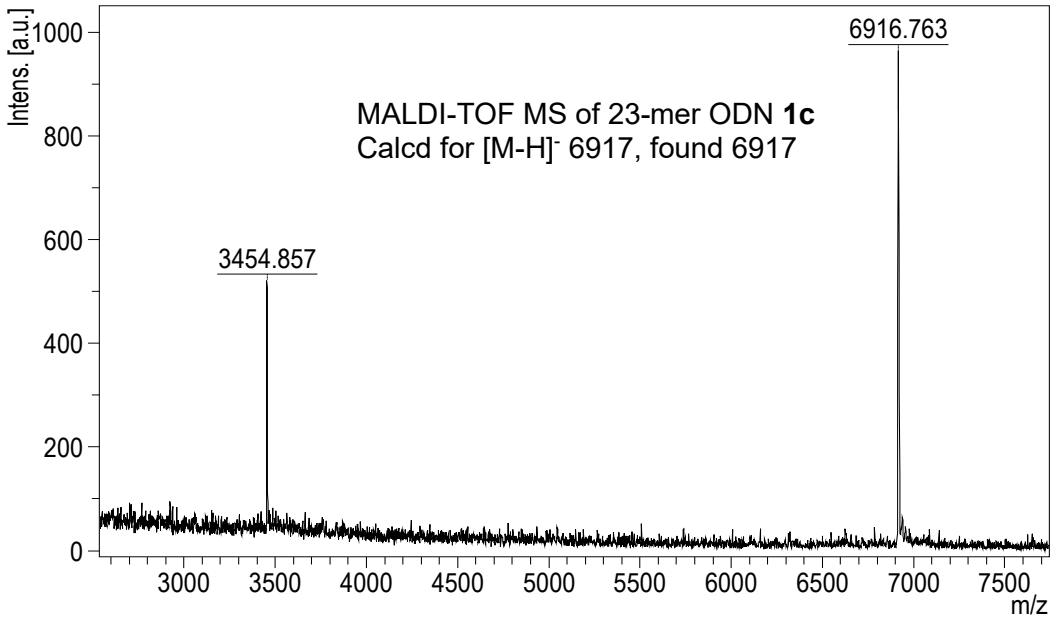


Figure A.53:MALDI-TOF MS of 23-mer ODN **1c**

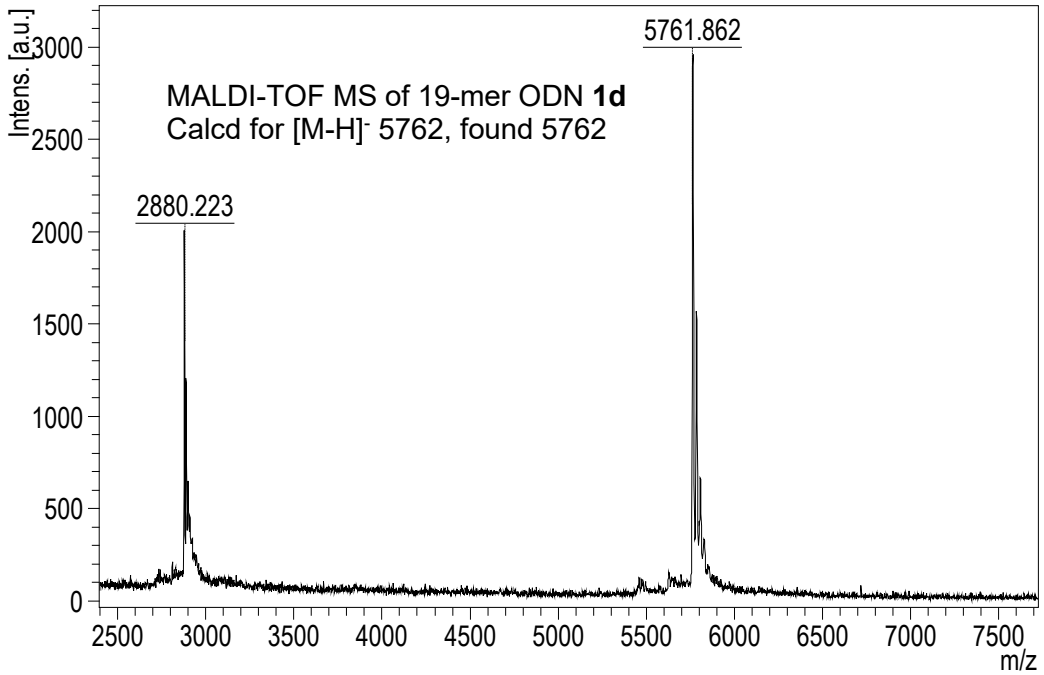


Figure A.54:MALDI-TOF MS of 19-mer ODN **1d**

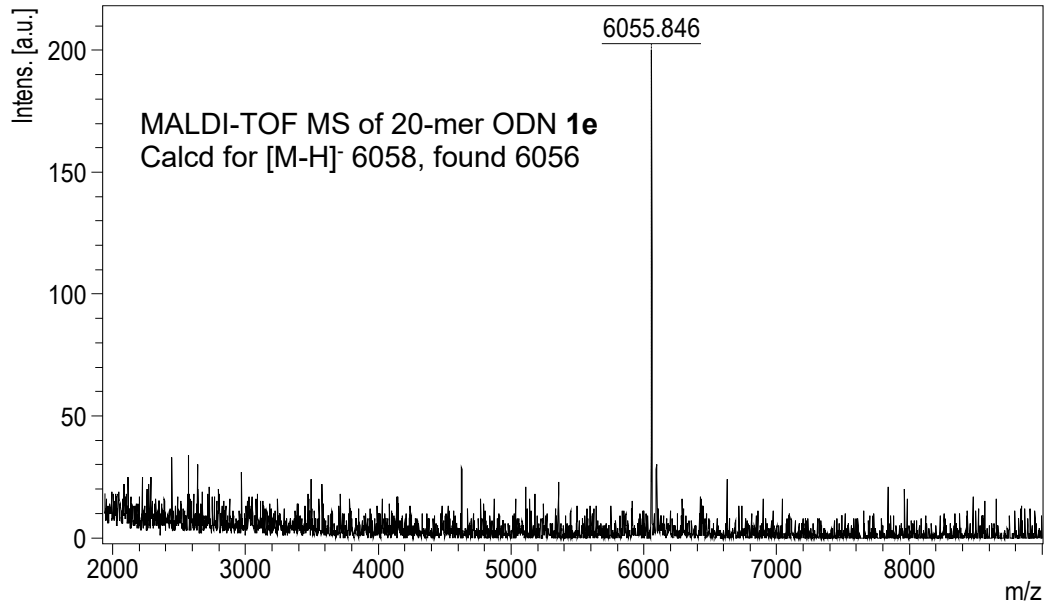


Figure A.55:MALDI-TOF MS of 20-mer ODN **1e**

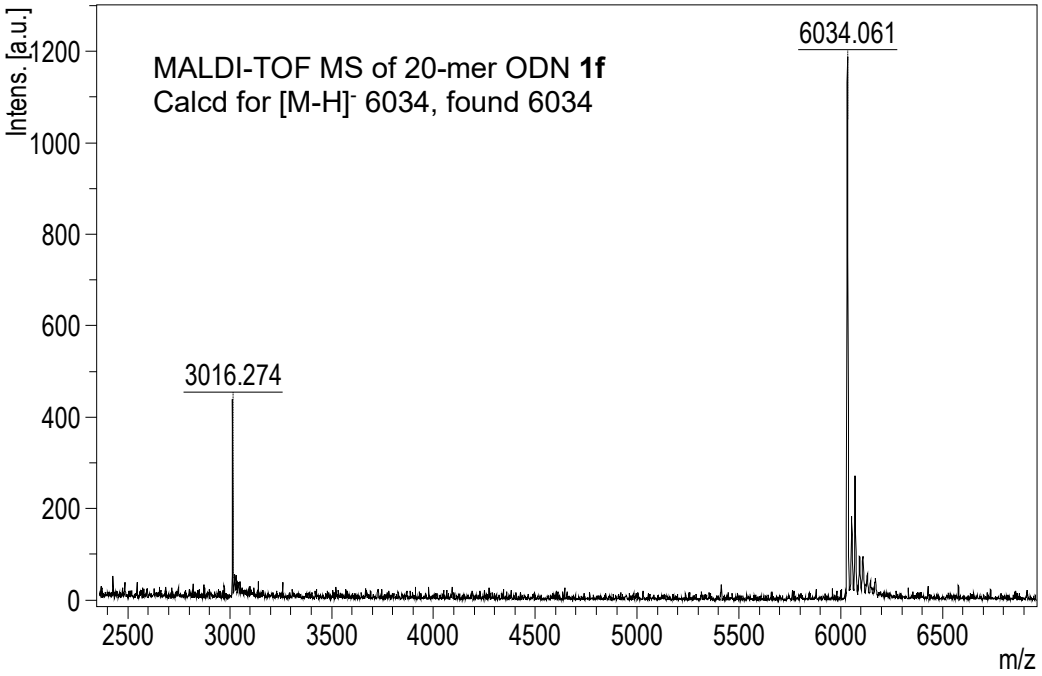


Figure A.56:MALDI-TOF MS of 20-mer ODN **1f**

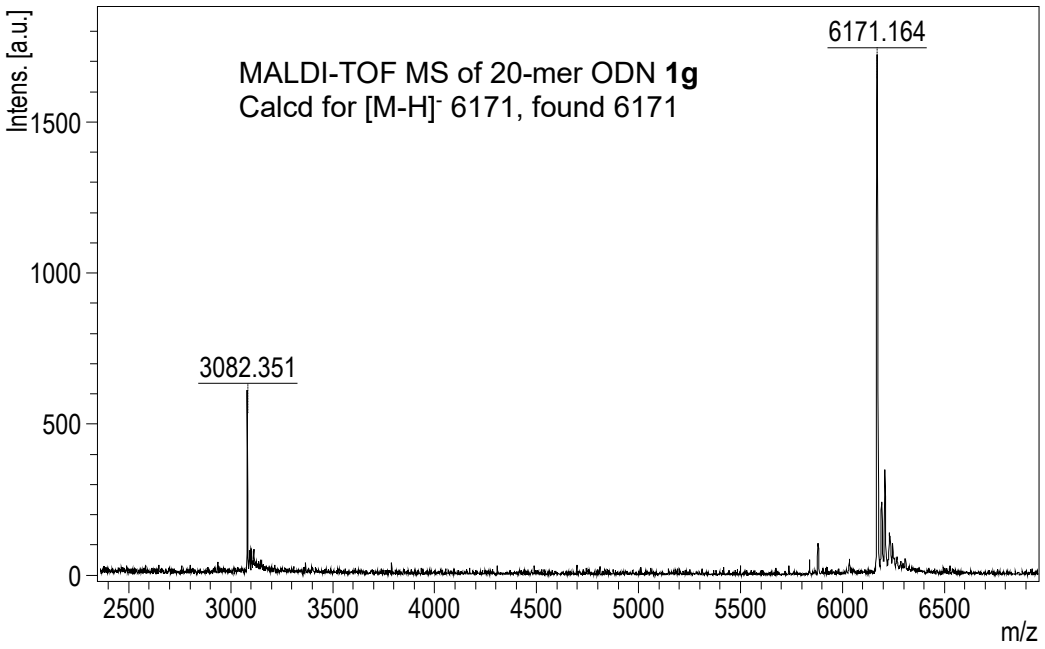


Figure A.57:MALDI-TOF MS of 20-mer ODN **1g**

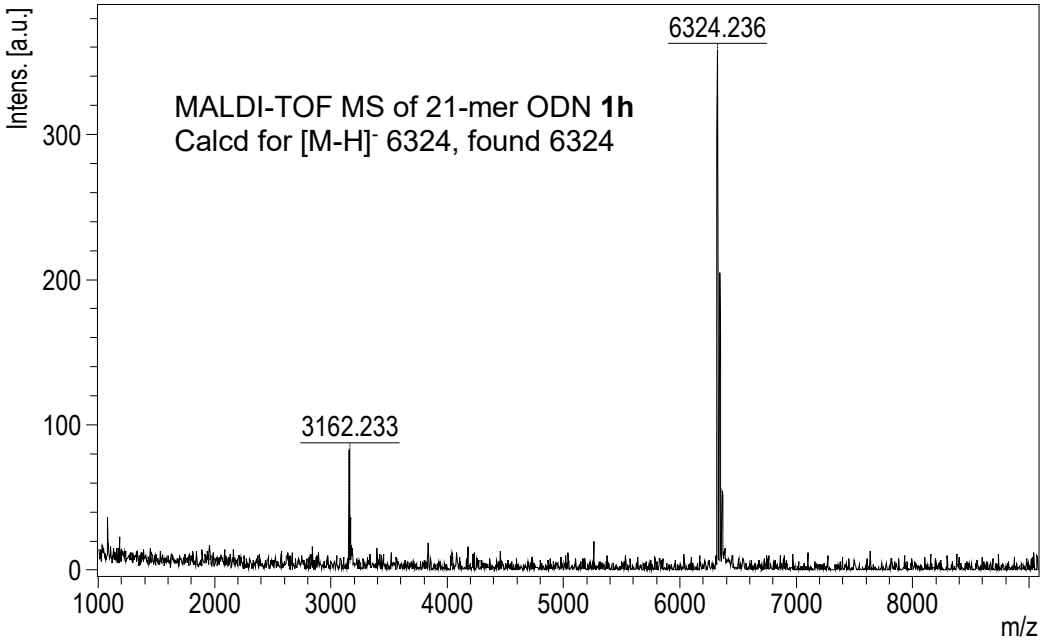


Figure A.58:MALDI-TOF MS of 21-mer ODN **1h**

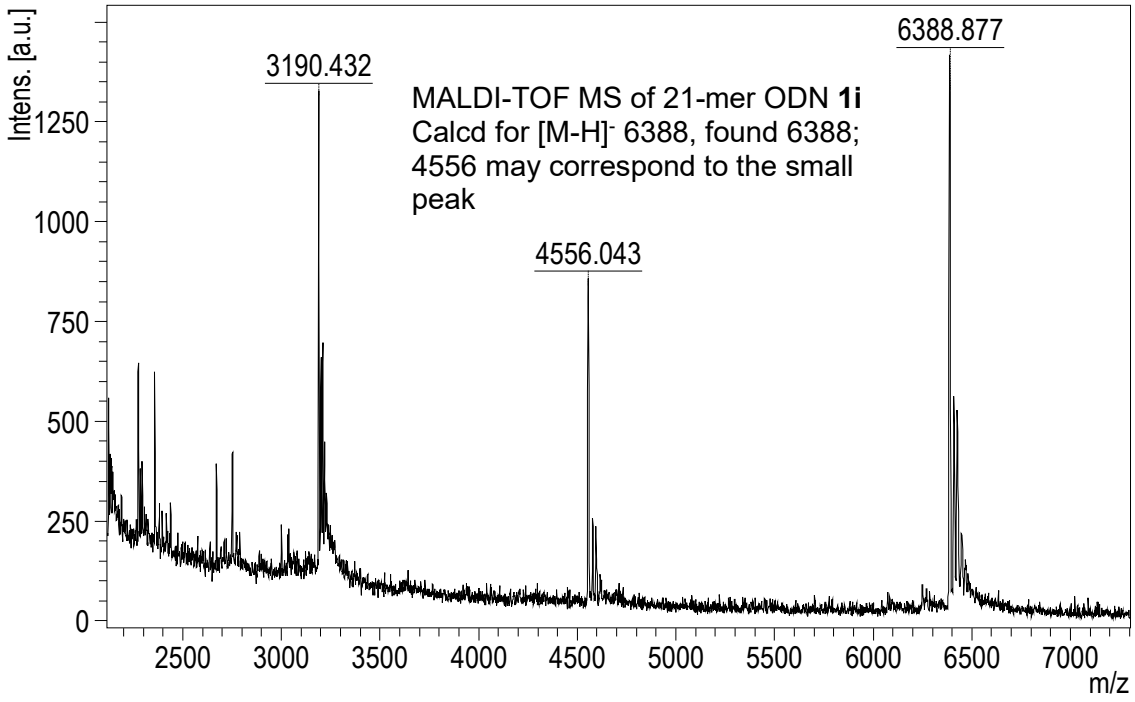


Figure A.59:MALDI-TOF MS of 21-mer ODN **1i**

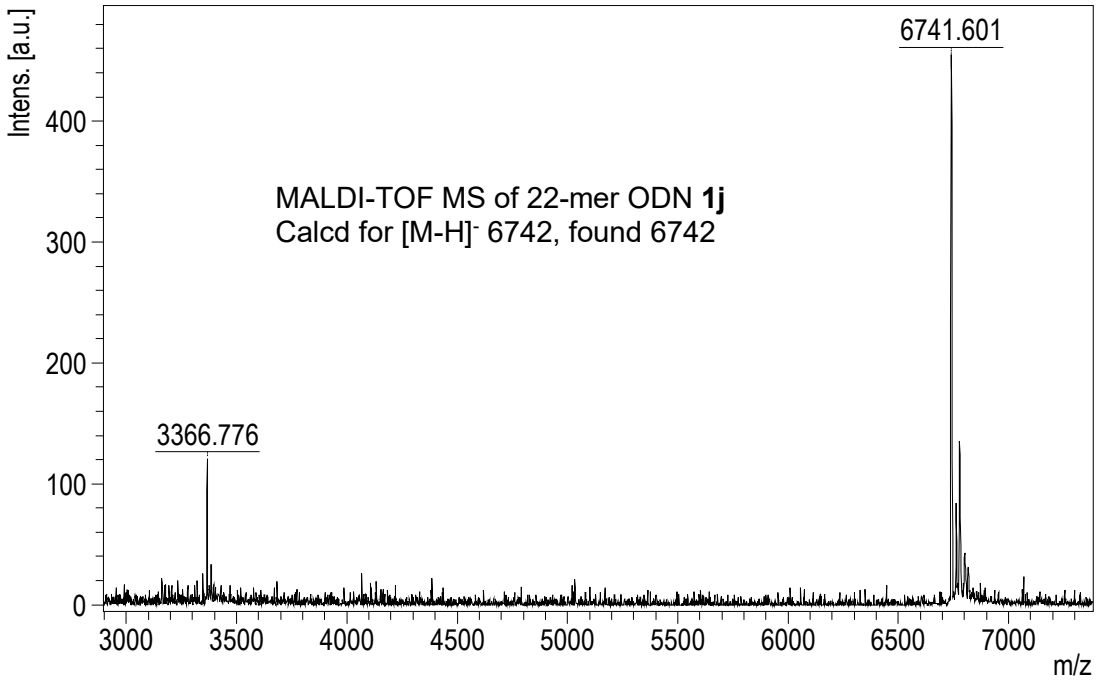


Figure A.60:MALDI-TOF MS of 22-mer ODN **1j**

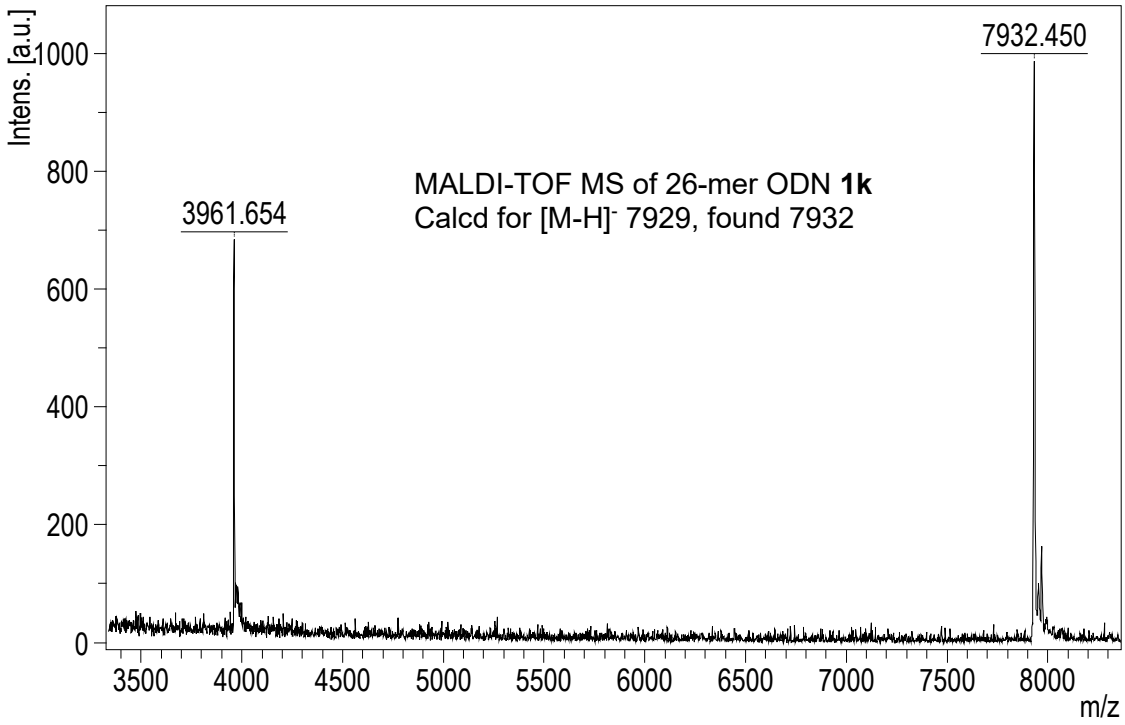


Figure A.61:MALDI-TOF MS of 26-mer ODN **1k**

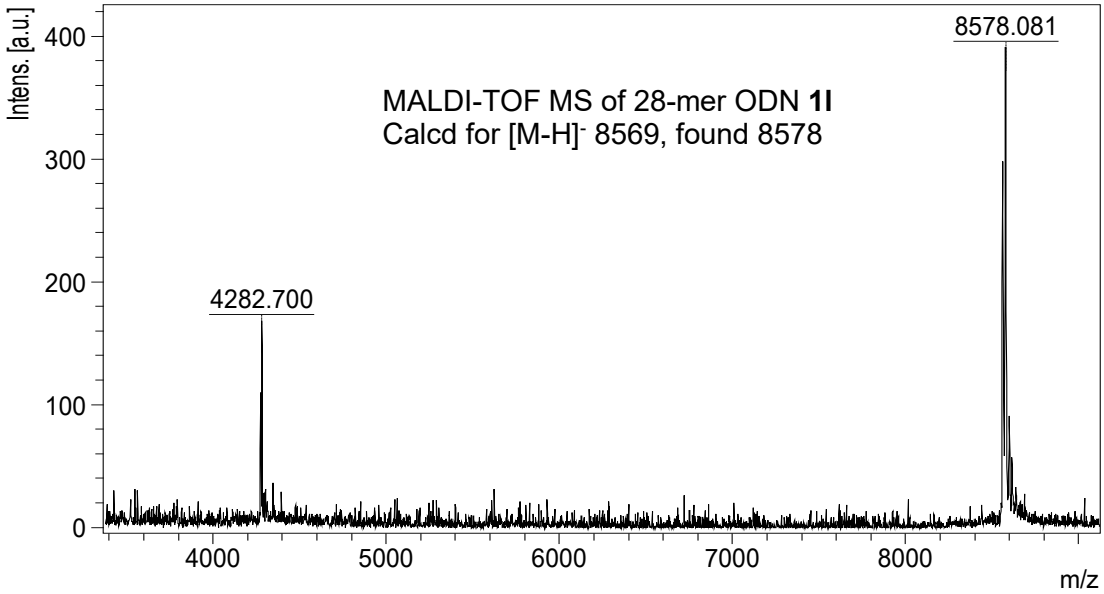


Figure A.62:MALDI-TOF MS of 28-mer ODN **1l**

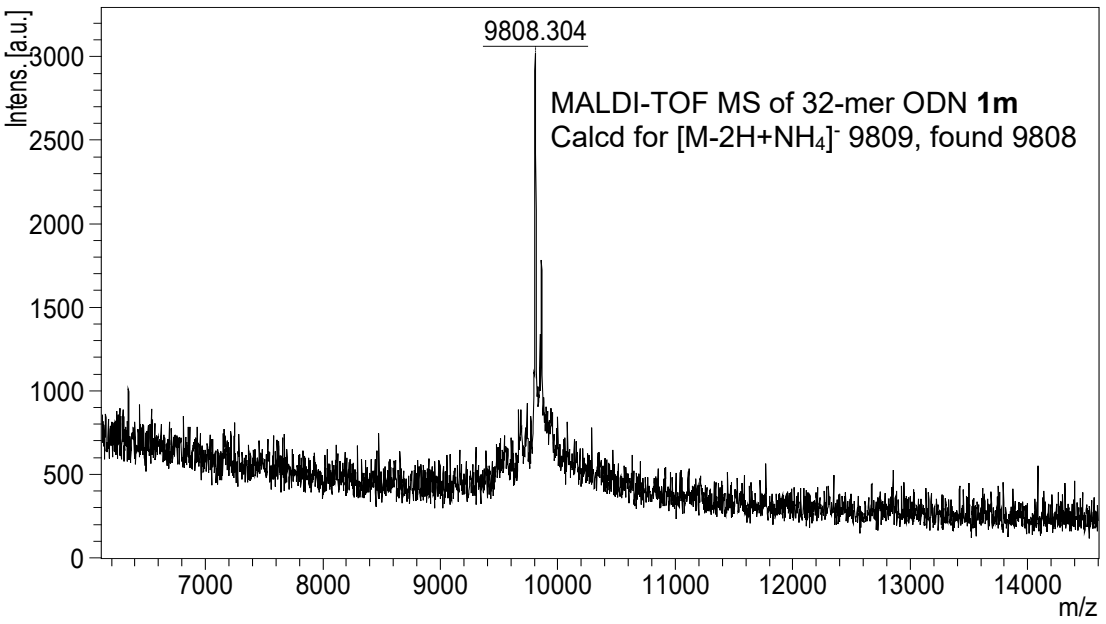


Figure A.63:MALDI-TOF MS of 32-mer ODN **1m**

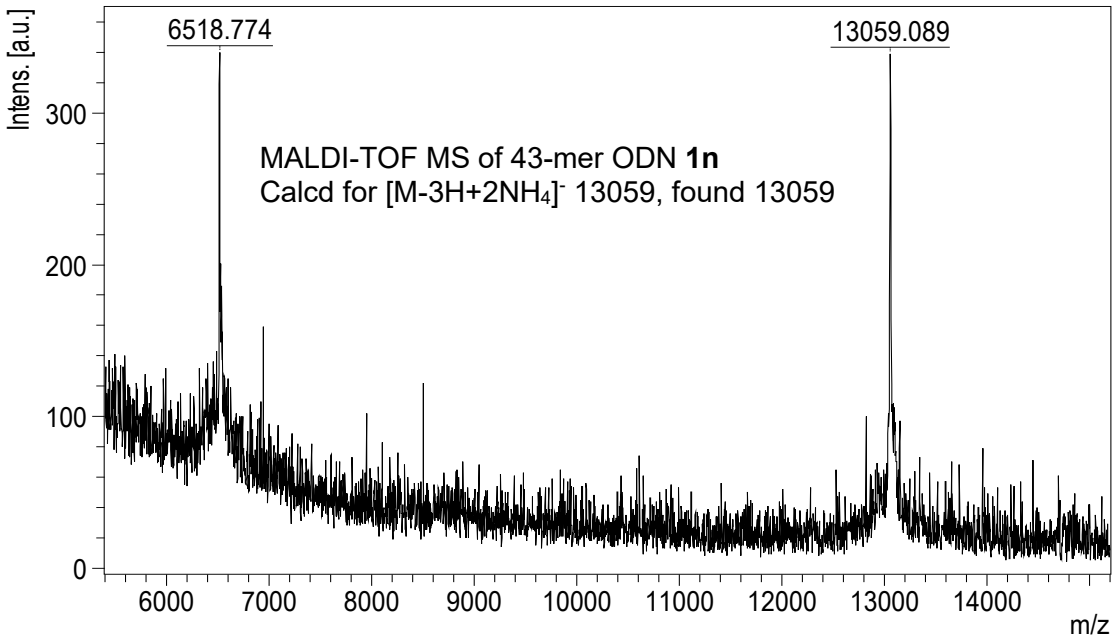


Figure A.64:MALDI-TOF MS of 43-mer ODN **1n**

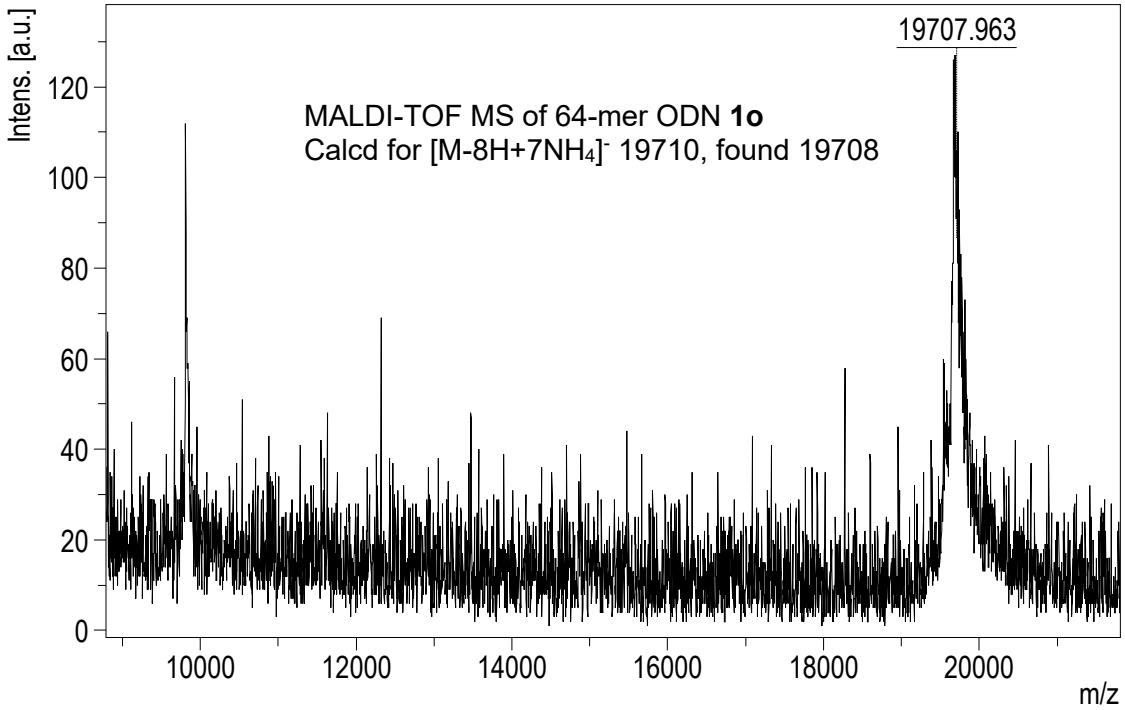


Figure A.65:MALDI-TOF MS of 64-mer ODN **1o**

A.1.2 Photo of ODN 1m

Figure A.66:Photo of ODN **1m** from large scale purification using the catching full-length sequence by polymerization technology

A.1.3 LC-MS data for ODN 1a-c

The following pages (122-127) contain LC-MS data for ODN **1a**

Table 2:LC-MS data for ODN **1a**

Column	**Agilent Extended C-18, 1.8 µm, 50 × 2.1 mm**
Mobile phase	A: 200 mM HFIP, 8.1 mM TEA in water B: Methanol
Gradient	Time: 0-1-11-11.5-14.5-15 (min) B%: 10-10-70-90-90-10 Post run: 10 min
Flow rate: (mL/min)	0.2
Column temp	40 °C
Injection Volume	2 uL
Detection	Agilent 1200 Series HPLC with Agilent 6224 Time-of-Flight LC/MS Electrospray Ionization (ESI), Negative Mode, 400-3200 mAu, data collection started at 2 min after injection.

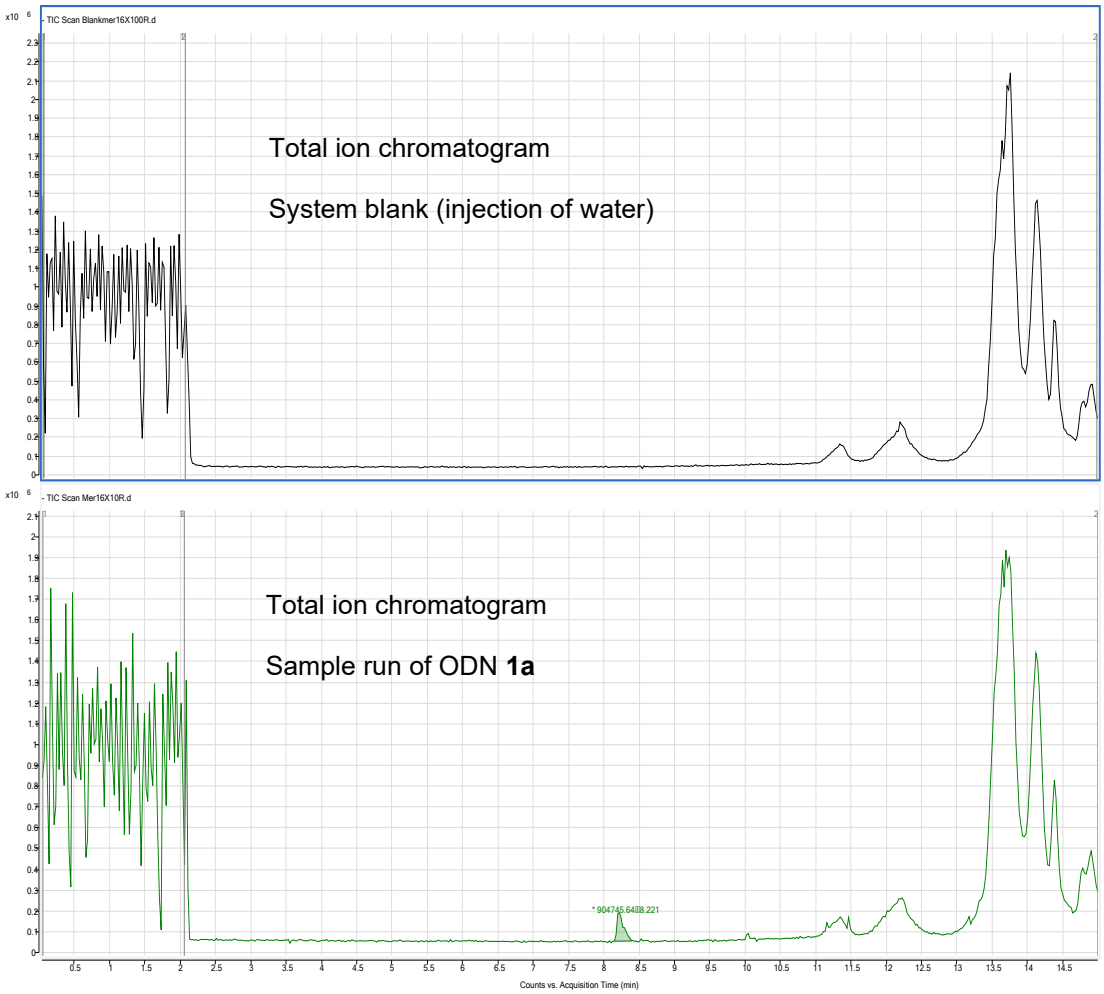


Figure A.67:Total ion chromatogram

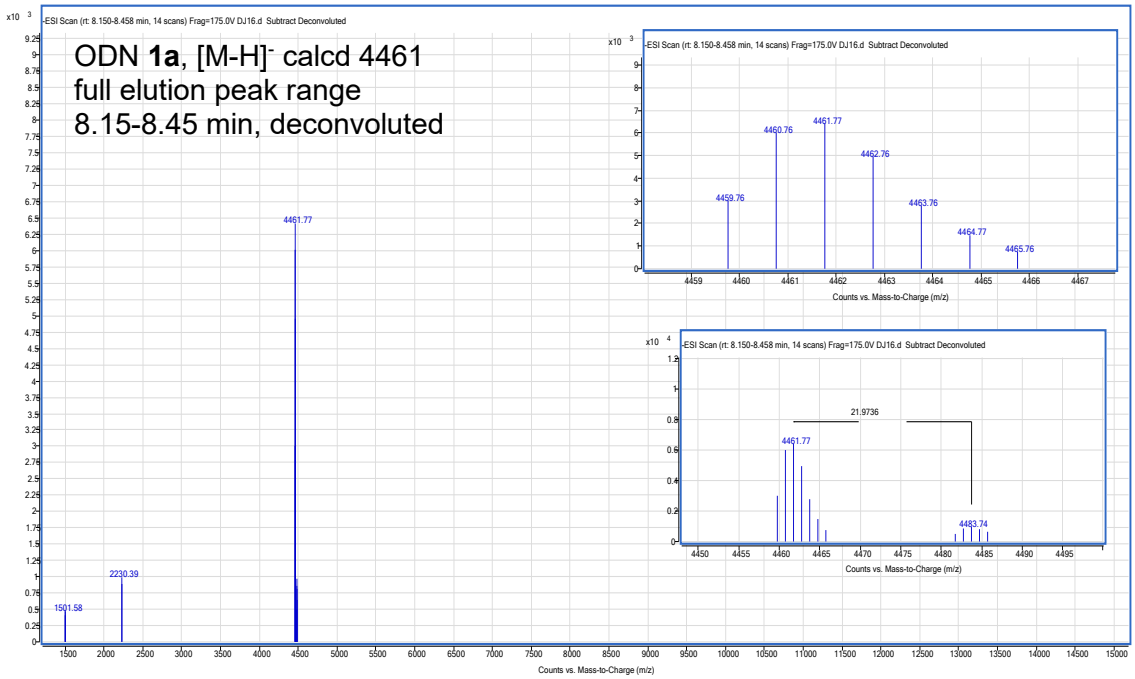


Figure A.68:ODN **1a** (peak rang 8.15-8.45 min)

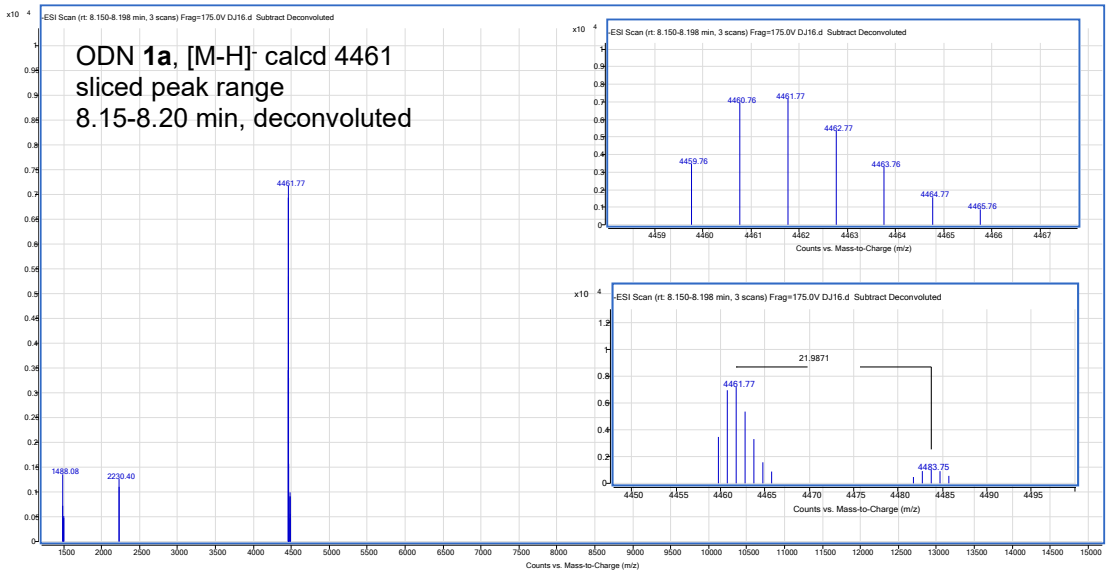


Figure A.69:ODN **1a** (peak range 8.15-8.20 min)

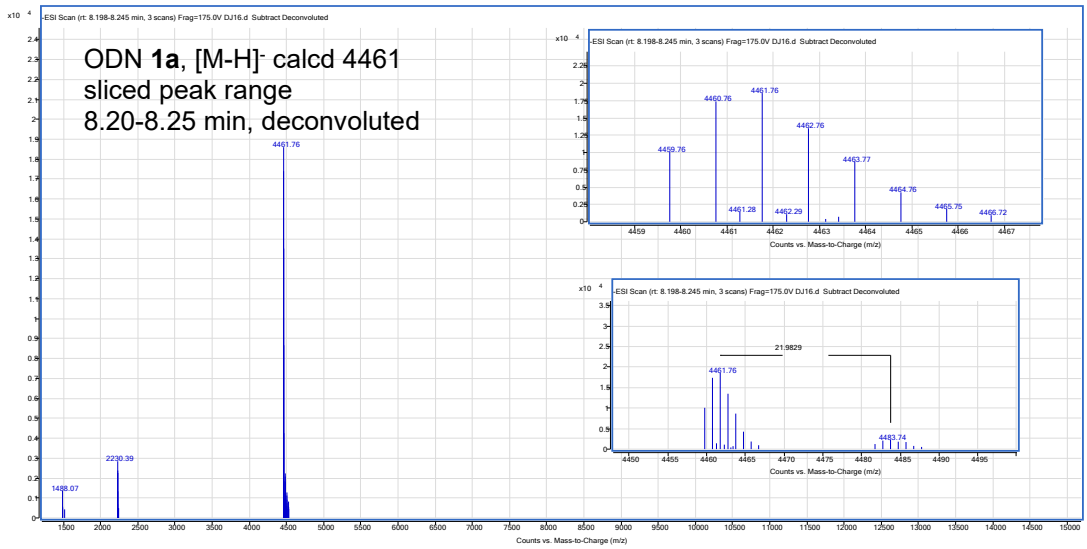


Figure A.70:ODN **1a**, (peak range8.20-8.25 min)

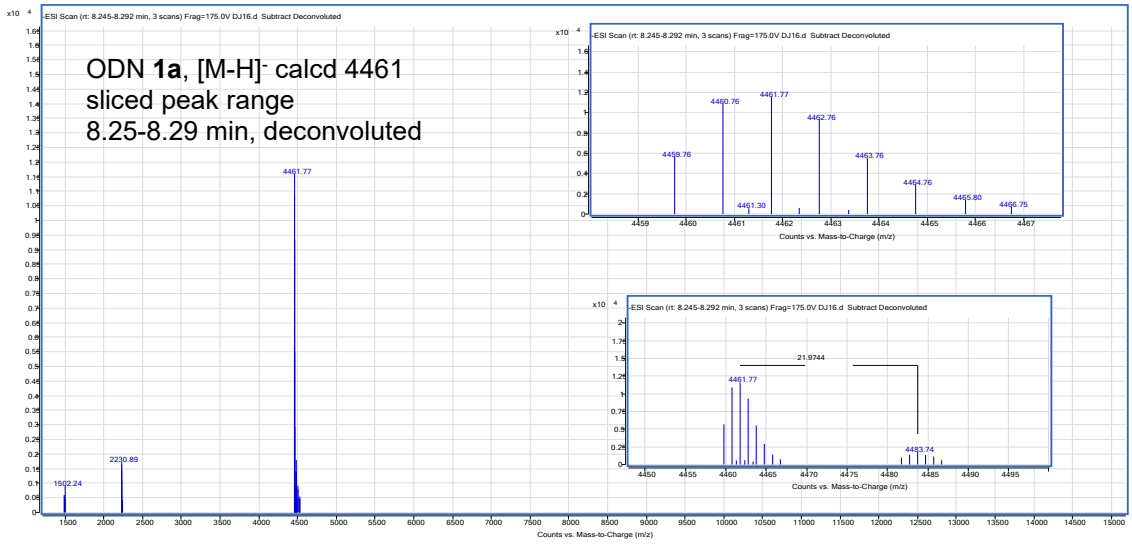


Figure A.71:ODN **1a**, (peak range 8.25-8.29 min)

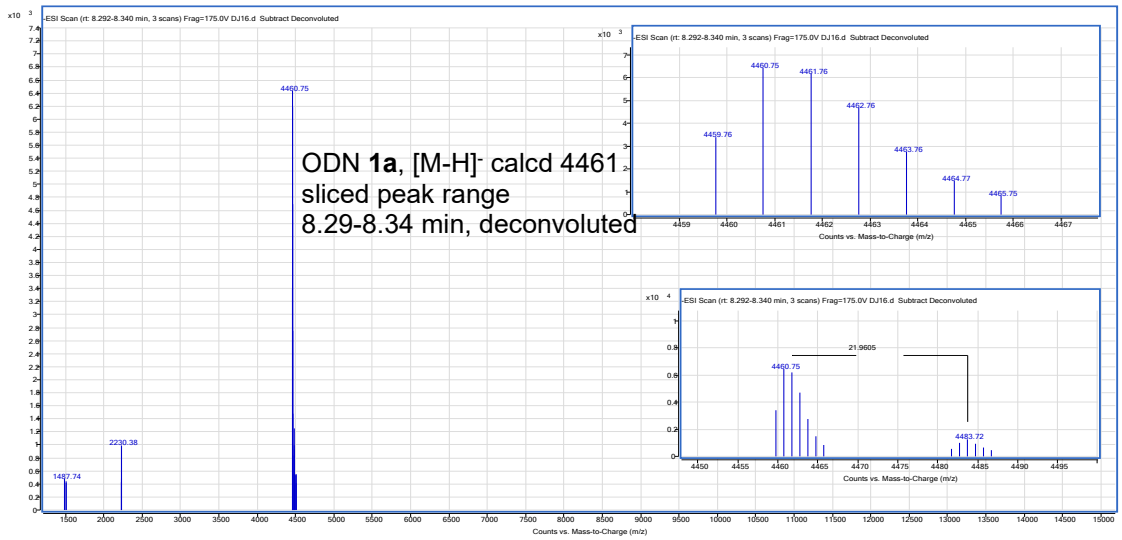


Figure A.72:ODN **1a**, (peak range 8.29-8.34 min)

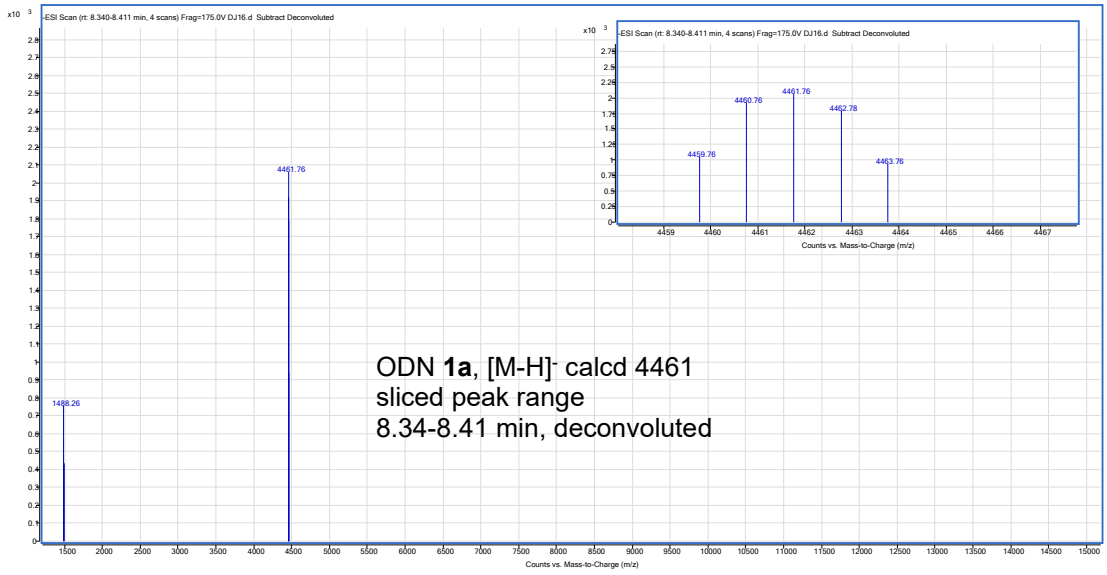


Figure A.73:ODN **1a**, (peak range 8.34-8.41 min)

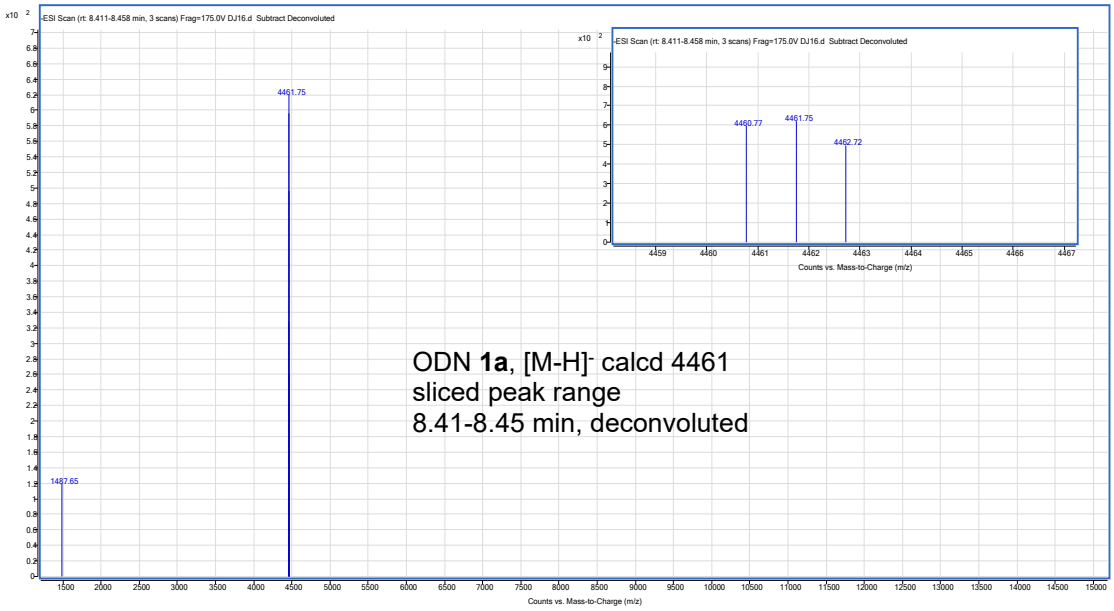


Figure A.74:ODN **1a**, (peak range 8.41-8.45 min)

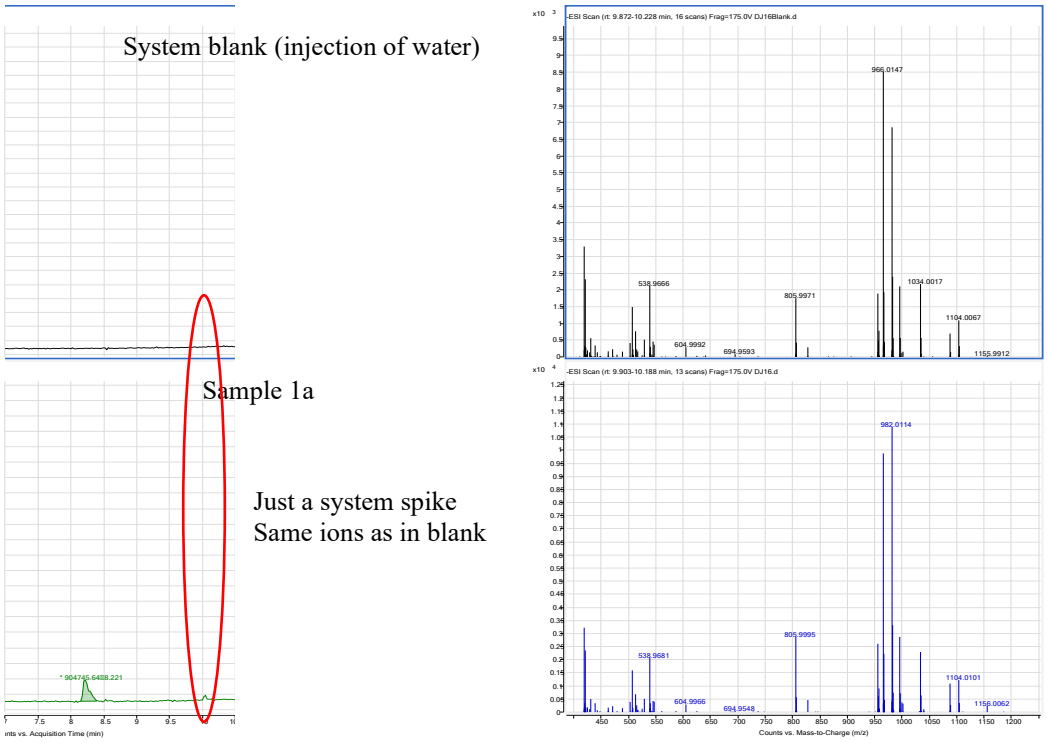


Figure A.75:System blank (injection of water)

The following pages (128-133) contain LC-MS data for ODN **1b**

Table 3:LC-MD data for ODN **1b**

Column	**Agilent Extended C-18, 1.8 µm, 50 × 2.1 mm**
Mobile phase	A: 200 mM HFIP, 8.1 mM TEA in water B: Methanol
Gradient	Time: 0-1-11-11.5-14.5-15 (min) B%: 10-10-70-90-90-10 Post run: 10 min
Flow rate: (mL/min)	0.2
Column temp	40 °C
Injection Volume	2 uL
Detection	Agilent 1200 Series HPLC with Agilent 6224 Time-of-Flight LC/MS Electrospray Ionization (ESI), Negative Mode, 400-3200 mAu, data collection started at 2 min after injection.

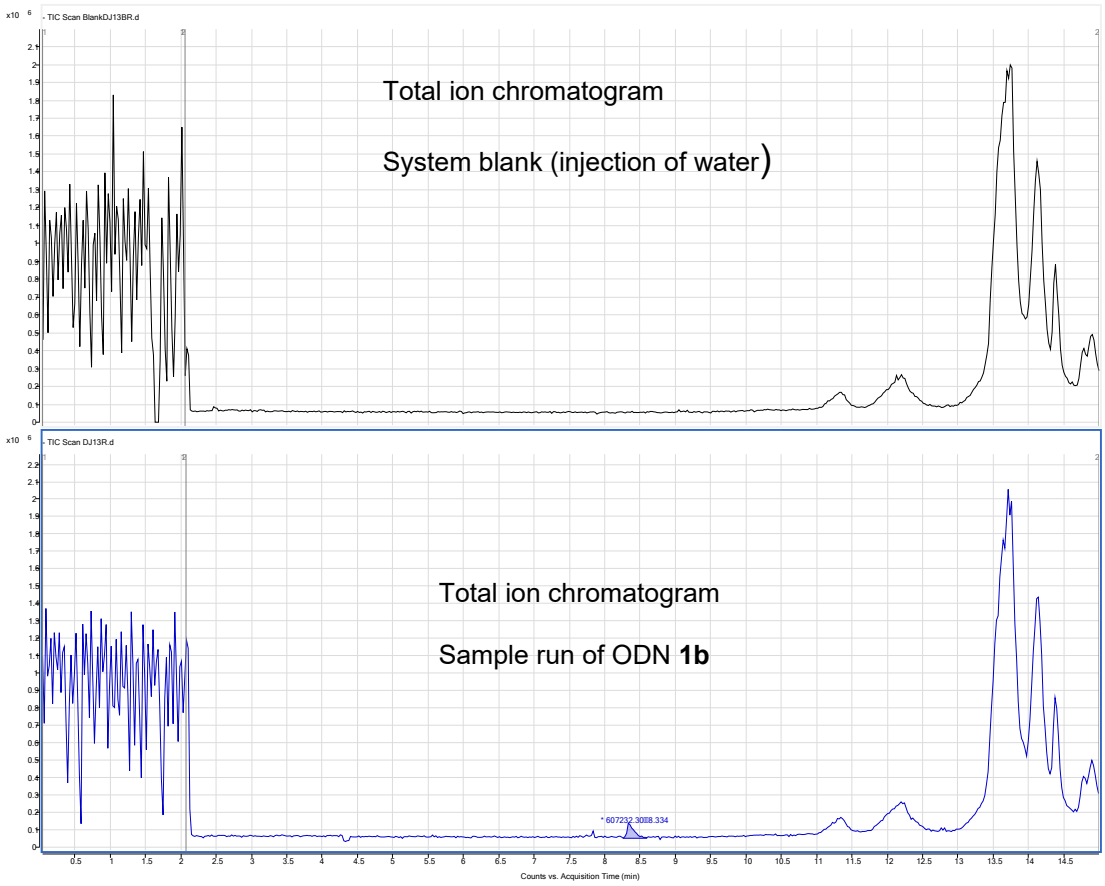


Figure A.76:Total ion chromatogram Sample run of ODN **1b**

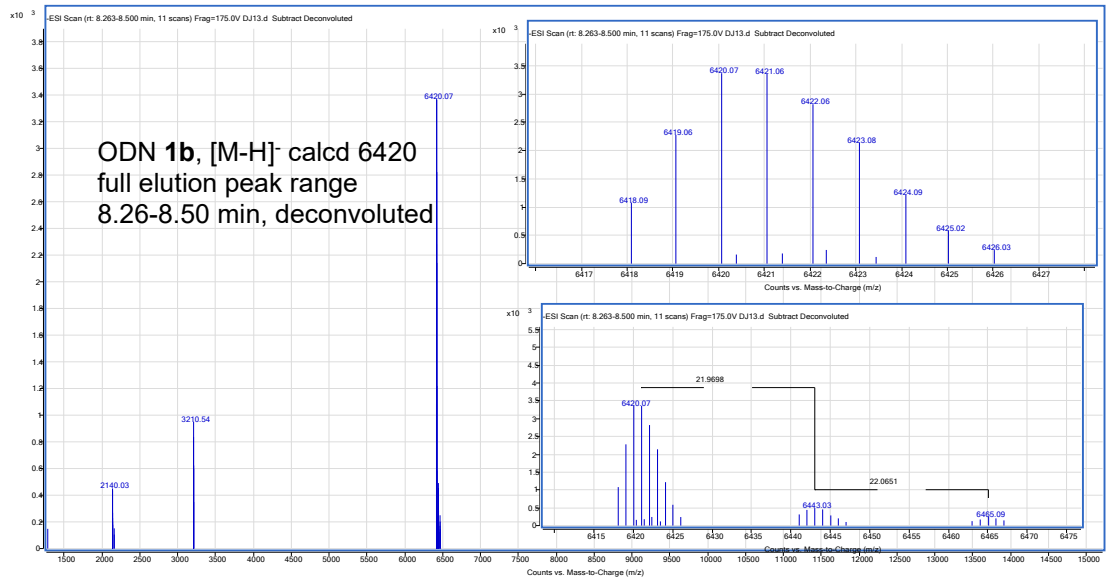


Figure A.77:ODN **1b**, (peak range 8.26-8.50 min)

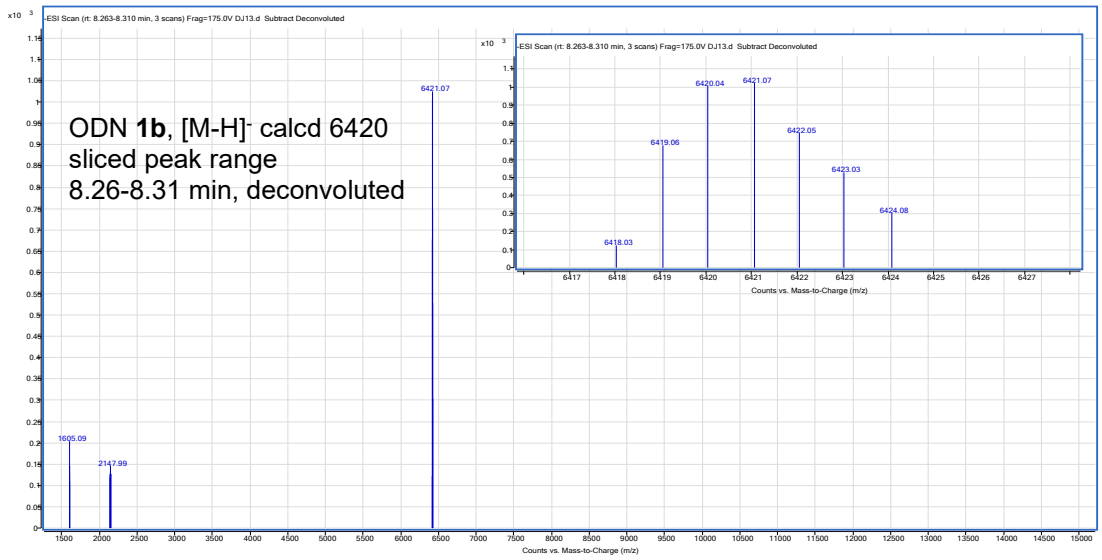


Figure A.78:ODN **1b**, (peak range 8.26-8.31 min)

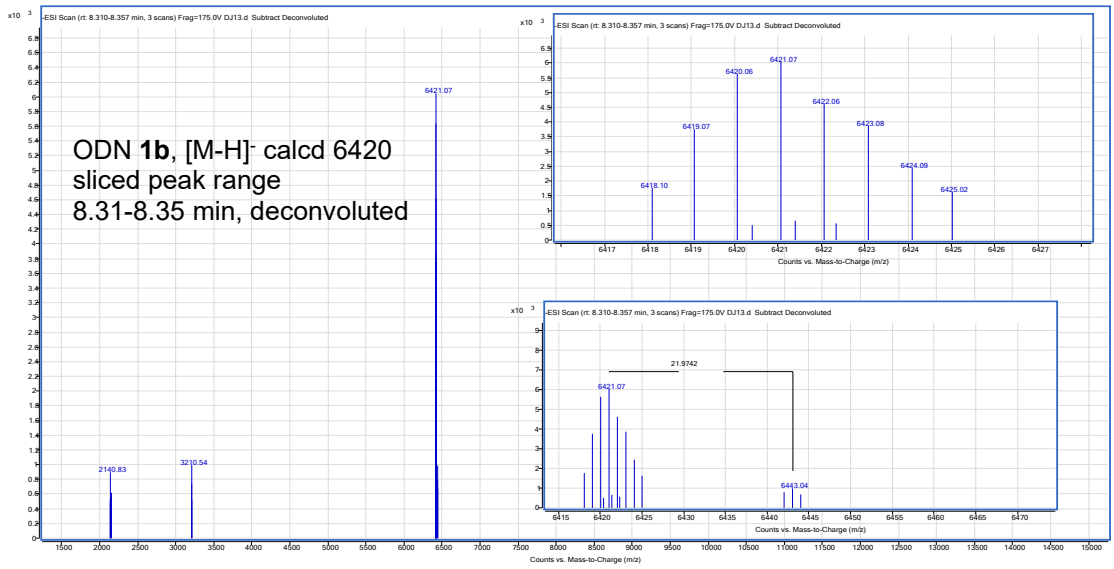


Figure A.79:ODN **1b**, (peak range 8.31-8.35 min)

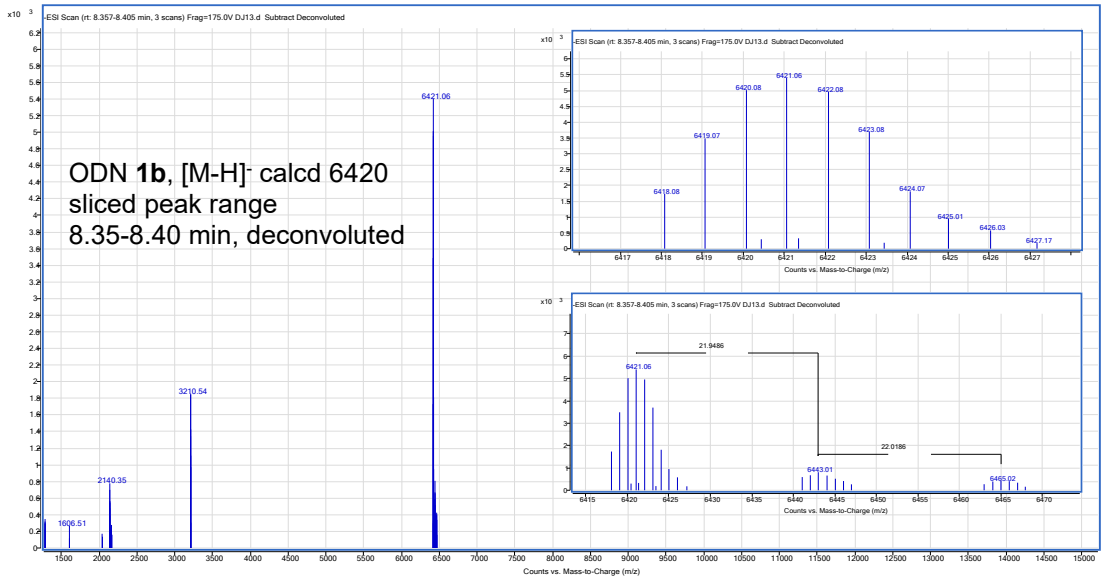


Figure A.80:ODN **1b**, (peak range 8.35-8.40 min)

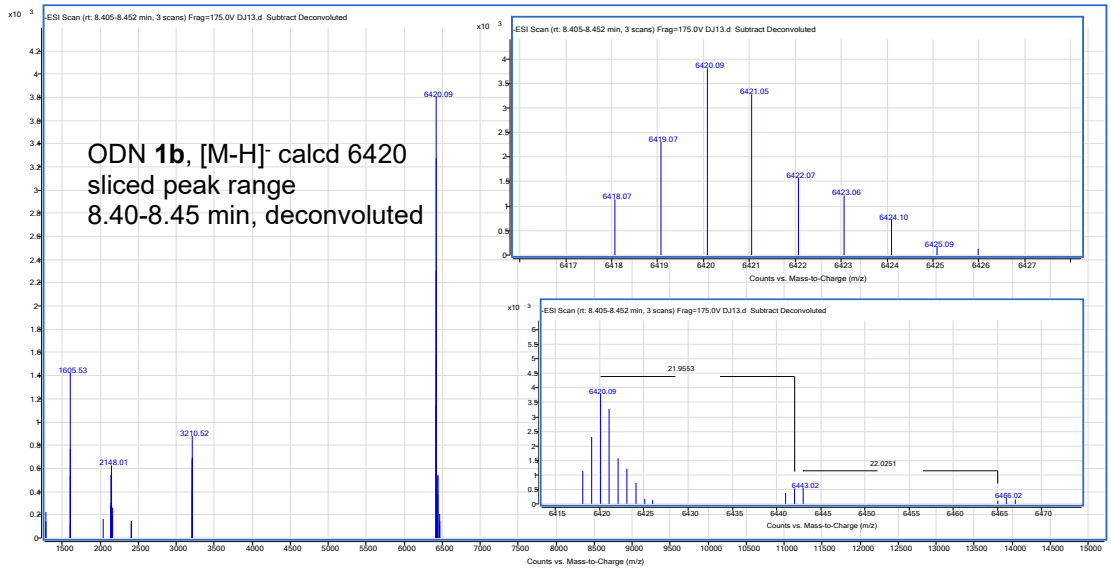


Figure A.81:ODN **1b**, (peak range 8.40-8.45 min)

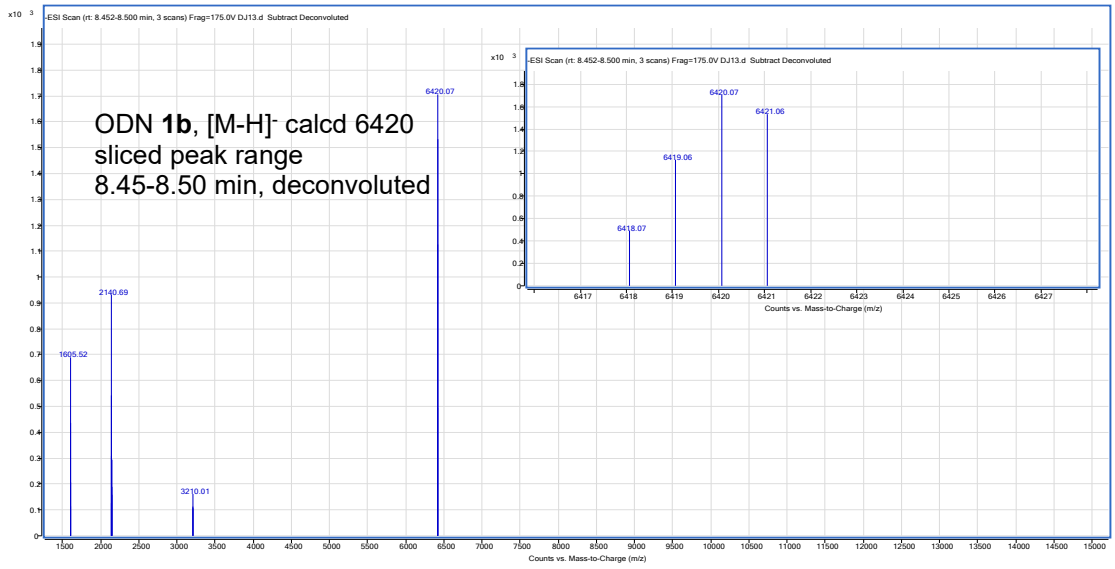


Figure A.82:ODN **1b**, (peak range 8.45-8.50 min)

The following pages (133-137) contain LC-MS data for ODN **1c**

Table 4:LC-MS data for ODN **1c**

Column	**Agilent Extended C-18, 1.8 µm, 50 × 2.1 mm**
Mobile phase	A: 200 mM HFIP, 8.1 mM TEA in water B: Methanol
Gradient	Time: 0-1-11-11.5-14.5-15 (min) B%: 10-10-70-90-90-10 Post run: 10 min
Flow rate: (mL/min)	0.2
Column temp	40 °C
Injection Volume	2 uL
Detection	Agilent 1200 Series HPLC with Agilent 6224 Time-of-Flight LC/MS Electrospray Ionization (ESI), Negative Mode, 400-3200 mAu, data collection started at 2 min after injection.

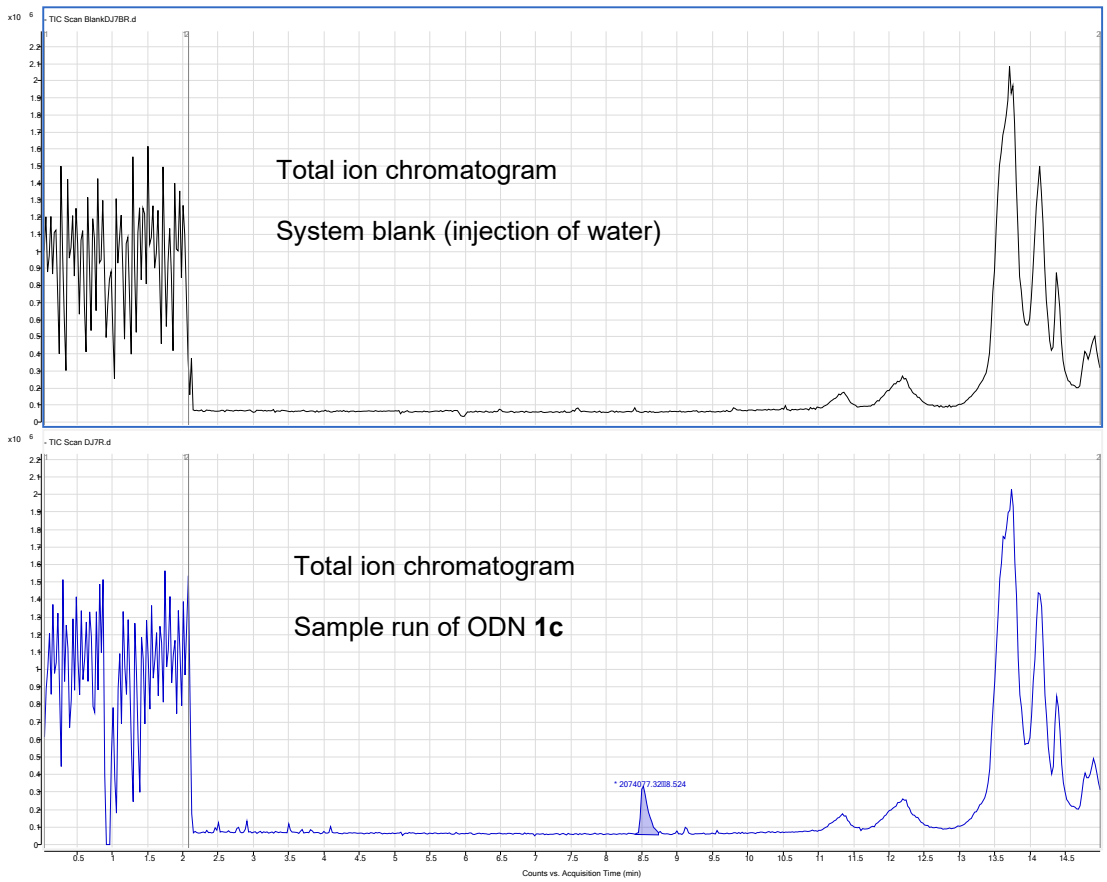


Figure A.83:Total ion chromatogram **1c**

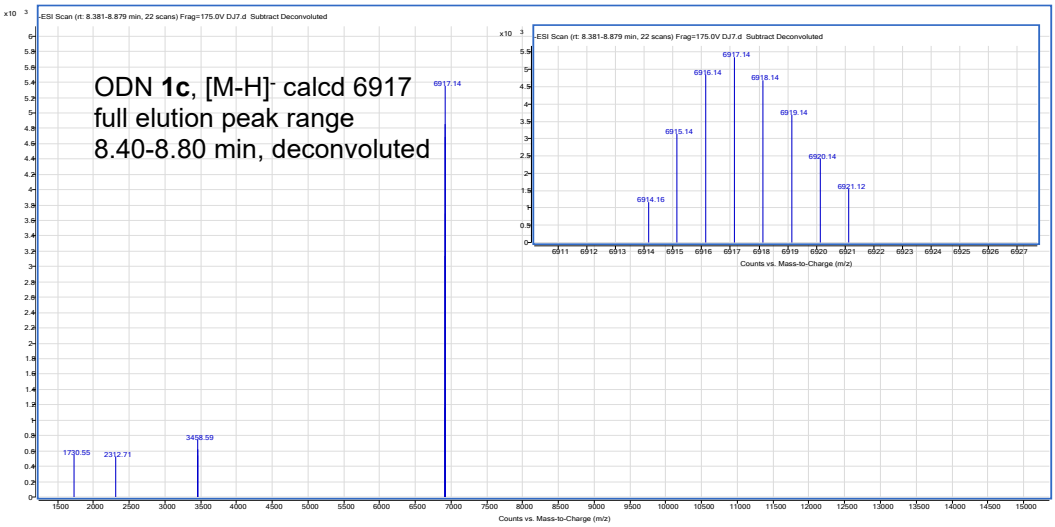


Figure A.85:ODN **1c**, (peak range 8.40-8.80 min)

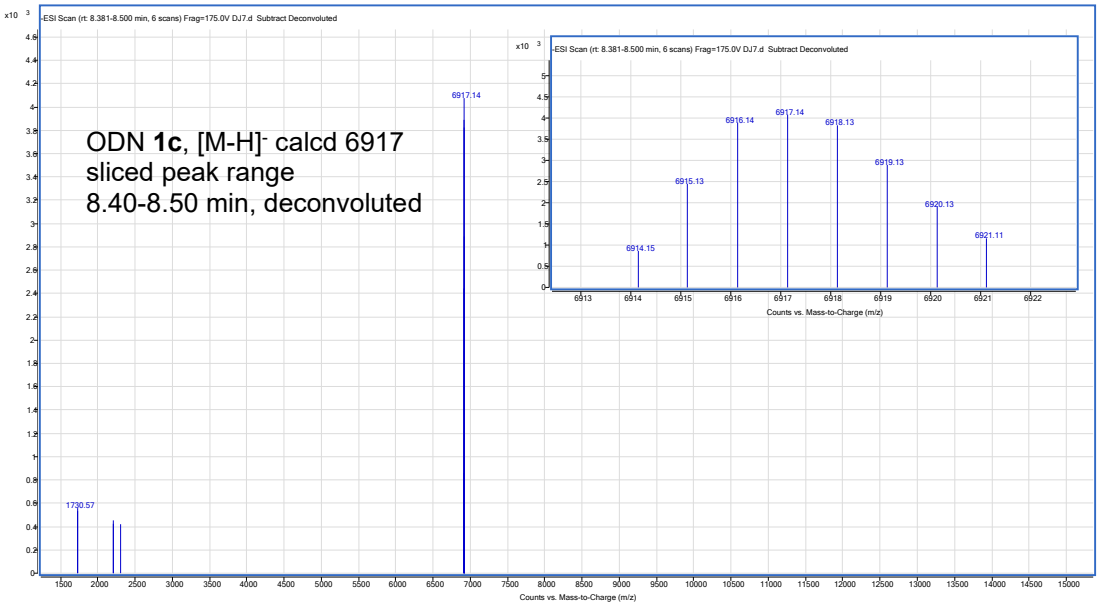


Figure A.86:ODN **1c**, (peak range 8.40-8.50 min)

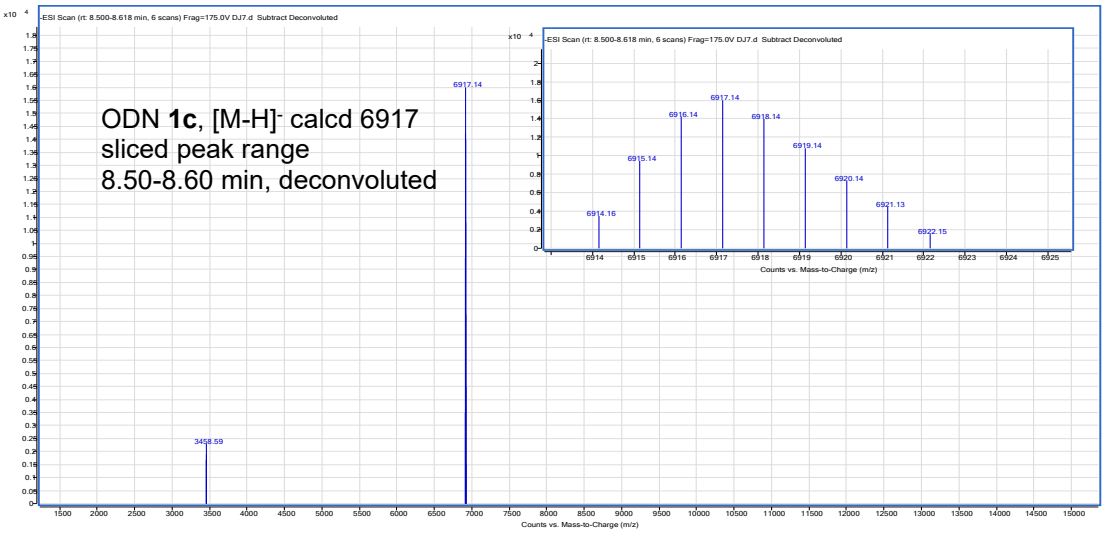


Figure A.87:ODN **1c**, (peak range 8.50-8.60 min)

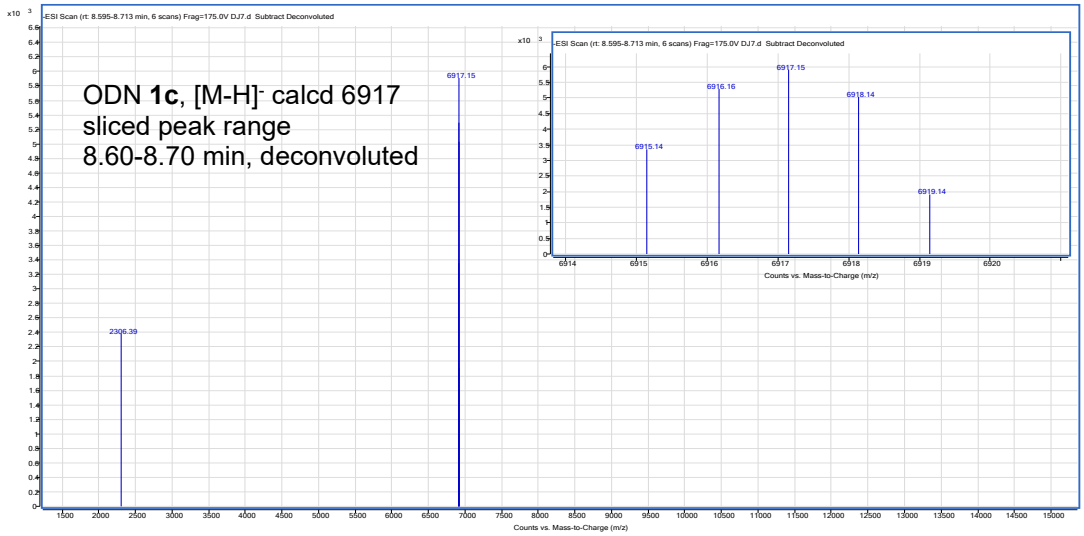


Figure A.88:ODN **1c**, (peak range 8.60-8.70 min)

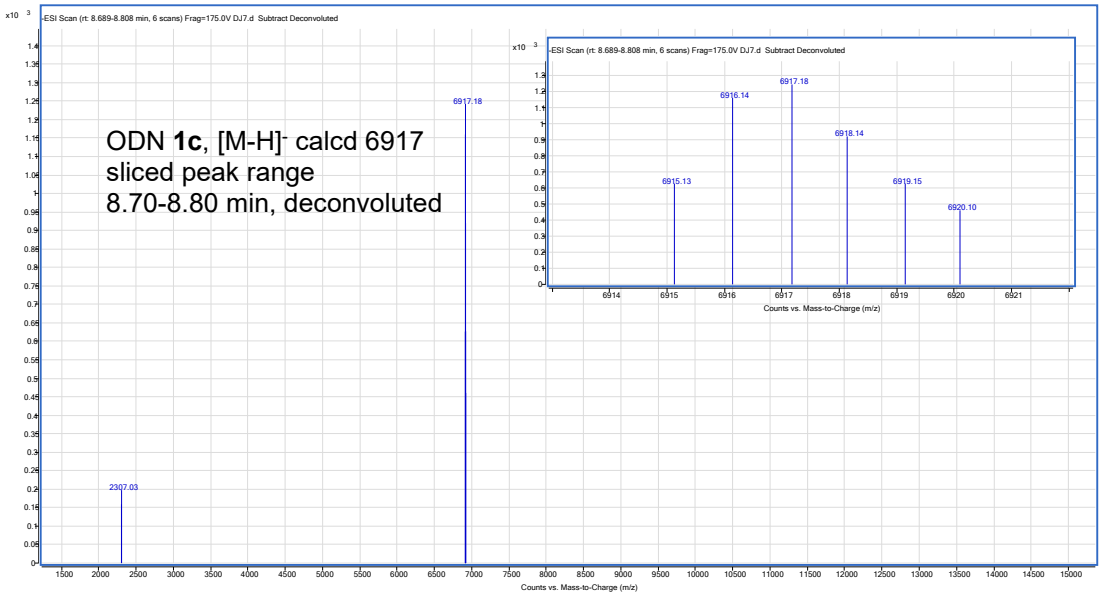


Figure A.89:ODN **1c**, (peak range 8.70-8.80 min)

Other small elution peaks: same ions as in solvent blank

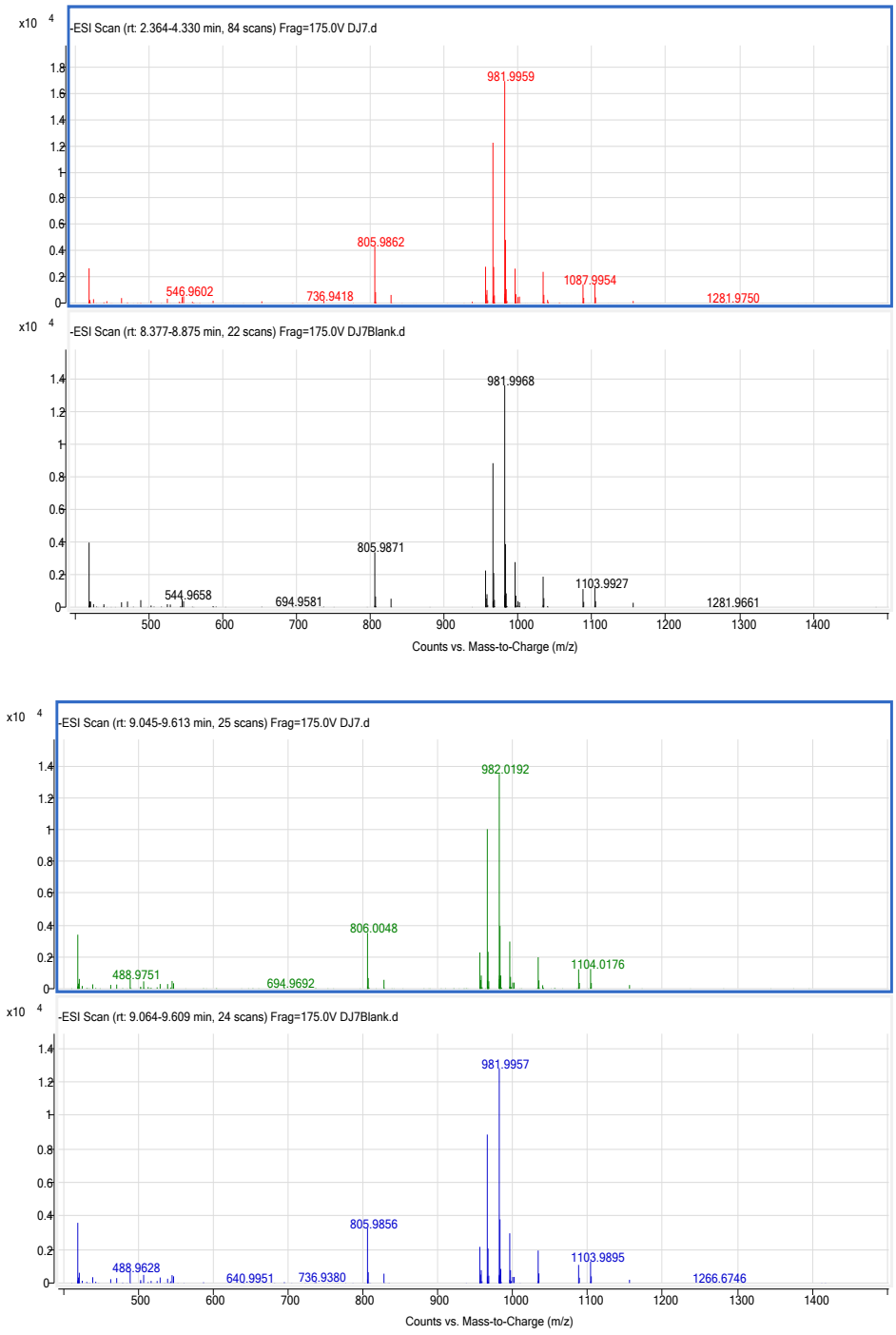


Figure A.90:Other small elution peaks: same ions as in solvent blank

A.1.4 UV spectra and OD_{260} of ODNs

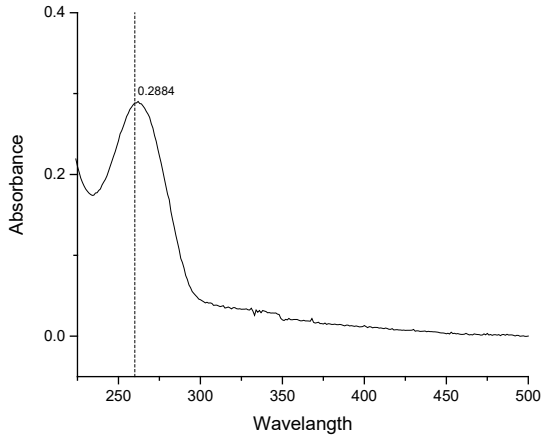


Figure A.91:UV of 15-mer ODN **1a**

UV of 15-mer ODN **1a**. The ODN was synthesized on a 1 μmol scale. One fifth of the CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 100 μL of water, and 10 μL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 1 μmol synthesis and purification was calculated to be 28.84 (0.2884 × 5 × 100 μL ÷ 10 μL × 2 mL ÷ 1 mL).

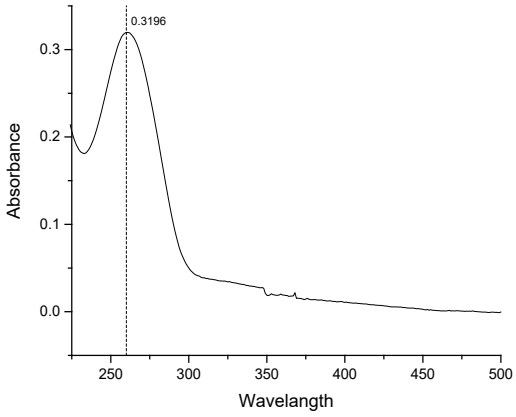


Figure A.92:UV of 21-mer ODN **1b**

UV of 21-mer ODN **1b**. The ODN was synthesized on a 1 μmol scale. One fifth of the CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 100 μL of water, and 10 μL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 1 μmol synthesis and purification was calculated to be 31.98 (0.3198 × 5 × 100 μL ÷10 μL × 2 mL ÷ 1 mL).

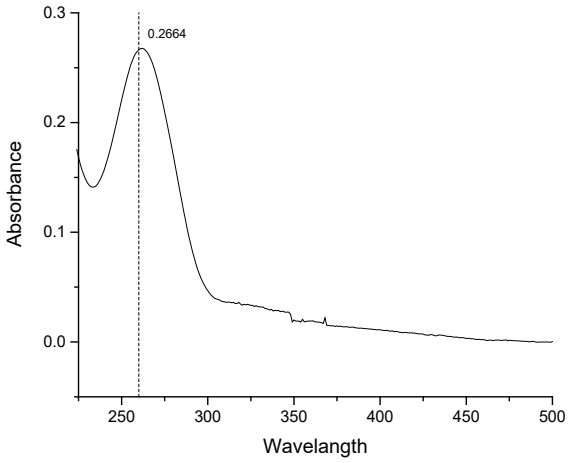


Figure A.93:UV of 23-mer ODN **1c**

UV of 23-mer ODN **1c**. The ODN was synthesized on a 1 μmol scale. One fifth of the CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 100 μL of water, and 10 μL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 1 μmol synthesis and purification was calculated to be 26.64 (0.2664 × 5 × 100 μL ÷ 10 μL × 2 mL ÷ 1 mL).

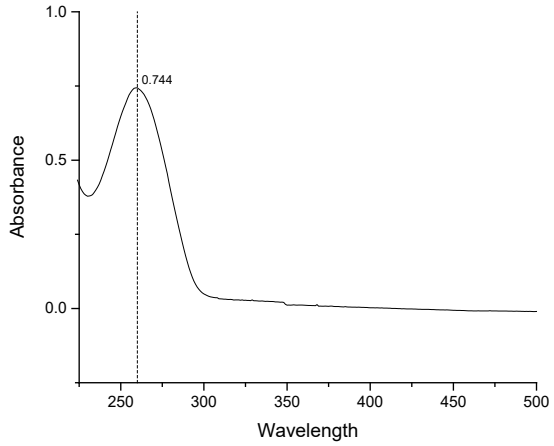


Figure A.94:UV of 19-mer ODN **1d**

UV of 19-mer ODN **1d**. The ODN was synthesized on a 1 μmol scale. One fifth of the CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 100 μL of water, and 10 μL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 1 μmol synthesis and purification was calculated to be 74.4 (0.744 × 5 × 100 μL ÷ 10 μL × 2 mL ÷ 1 mL).

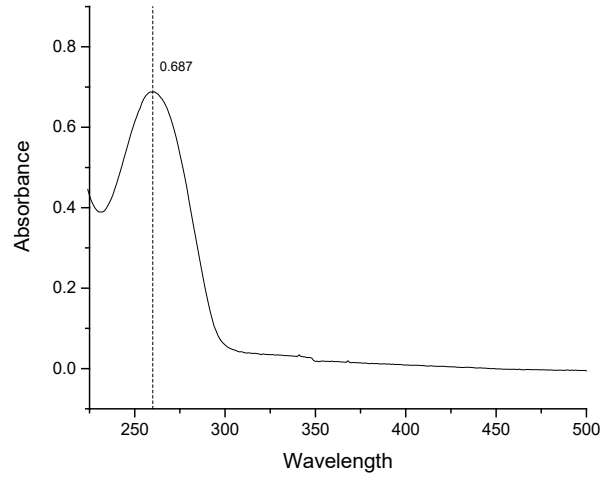


Figure A.95:UV of 20-mer ODN **1e**

UV of 20-mer ODN **1e**. The ODN was synthesized on a 1 μmol scale. One fifth of the CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 100 μL of water, and 10 μL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 1 μmol synthesis and purification was calculated to be 68.7 (0.687 × 5 × 100 μL ÷ 10 μL × 2 mL ÷ 1 mL).

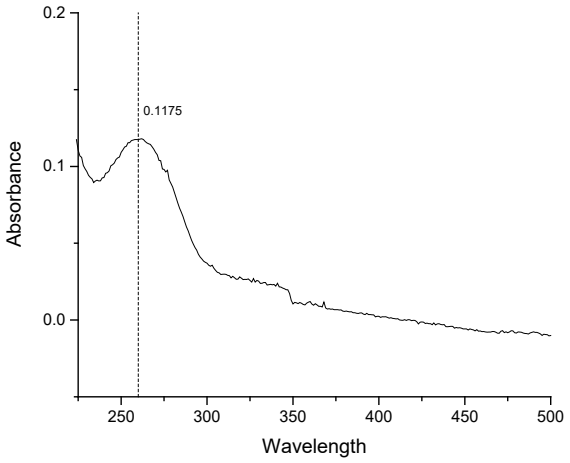


Figure A.96:UV of 20-mer ODN **1f**

UV of 20-mer ODN **1f**. The ODN was synthesized on a 1 μmol scale. One fifth of the CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 100 μL of water, and 10 μL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 1 μmol synthesis and purification was calculated to be 11.75 (0.1175 × 5 × 100 μL ÷ 10 μL × 2 mL ÷ 1 mL).

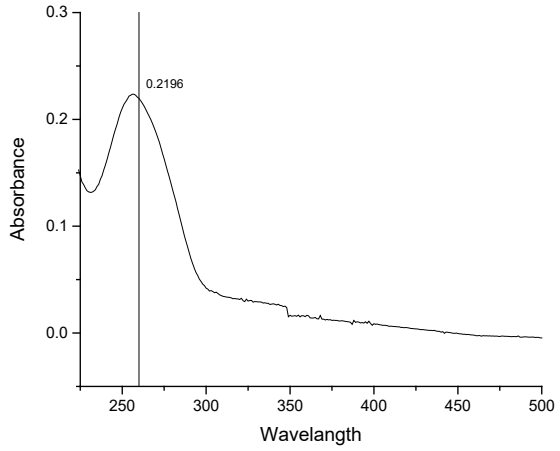


Figure A.97:UV of 20-mer ODN **1g**

UV of 20-mer ODN **1g**. The ODN was synthesized on a 1 μmol scale. One fifth of the CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 100 μL of water, and 10 μL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 1 μmol synthesis and purification was calculated to be 21.96 (0.2196 × 5 × 100 μL ÷ 10 μL × 2 mL ÷ 1 mL).

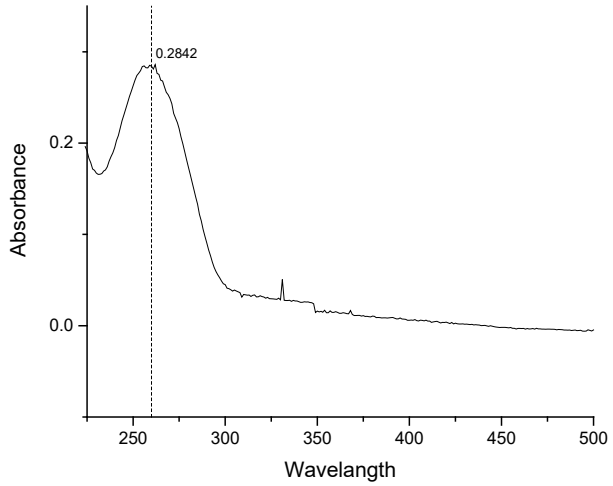


Figure A.98:UV of 20-mer ODN **1h**

UV of 20-mer ODN **1h**. The ODN was synthesized on a 1 μmol scale. One fifth of the CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 100 μL of water, and 10 μL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 1 μmol synthesis and purification was calculated to be 28.42 (0.2842 × 5 × 100 μL ÷ 10 μL × 2 mL ÷ 1 mL).

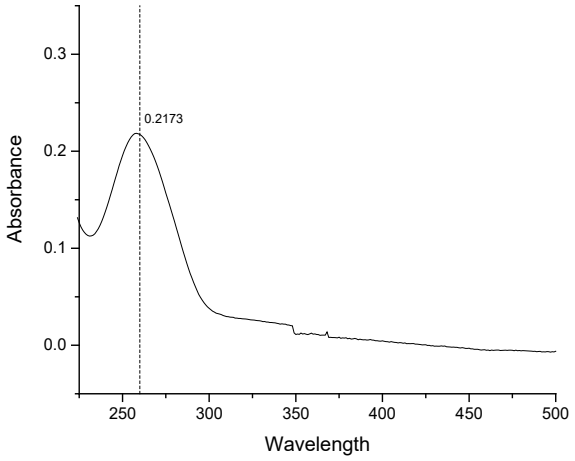


Figure A.99:UV of 22-mer ODN **1j**

UV of 22-mer ODN **1j**. The ODN was synthesized on a 1 μmol scale. One fifth of the CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 100 μL of water, and 10 μL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 1 μmol synthesis and purification was calculated to be 21.73 (0.2173 × 5 × 100 μL ÷ 10 μL × 2 mL ÷ 1 mL).

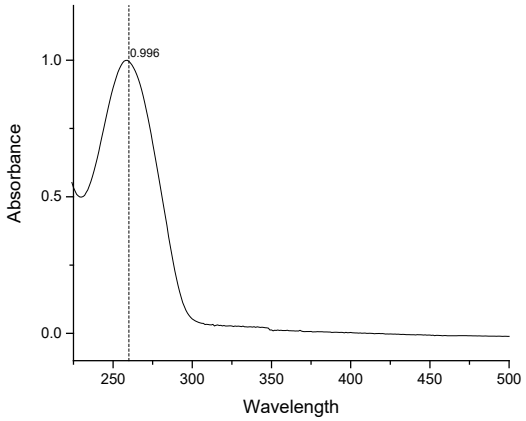


Figure A.100:UV of 26-mer ODN **1k**

UV of 26-mer ODN **1k**. The ODN was synthesized on a 1 μmol scale. One fifth of the CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 100 μL of water, and 10 μL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 1 μmol synthesis and purification was calculated to be 99.6 (0.996 × 5 × 100 μL ÷ 10 μL × 2 mL ÷ 1 mL).

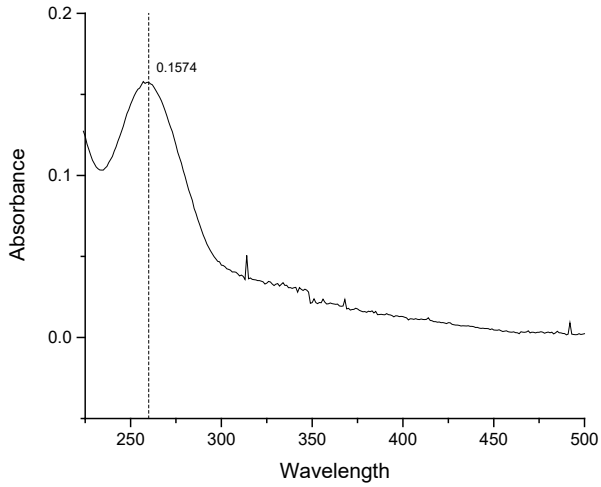


Figure A.101:UV of 28-mer ODN **1l**

UV of 28-mer ODN **1l**. The ODN was synthesized on a 1 µmol scale. One fifth of the CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 100 µL of water, and 10 µL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 1 µmol synthesis and purification was calculated to be 15.74 (0.1574 × 5 × 100 µL ÷ 10 µL × 2 mL ÷ 1 mL).

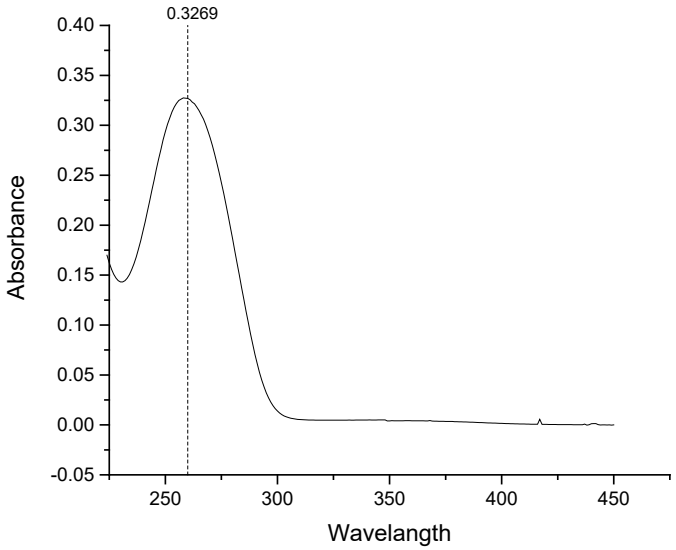


Figure A.102:UV of 32-mer ODN **1m**

UV of 32-mer ODN **1m**. The ODN was synthesized on a 60 µmol scale. All CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 5 mL of water, and 5 µL was taken out and diluted to 40 mL to obtain the UV spectrum. The OD_{260} of the 60 µmol synthesis and purification was calculated to be 13,076 (0.3269 × 5 mL ÷ 5 µL × 40 mL ÷ 1 mL).

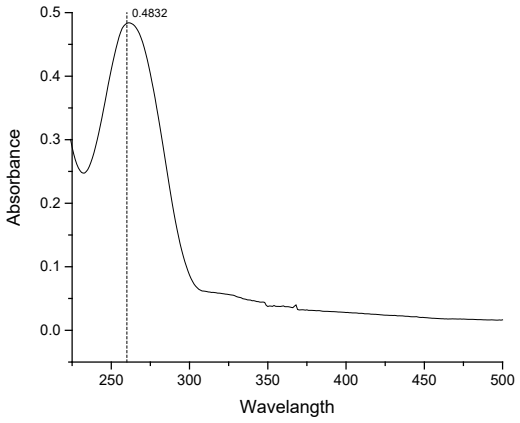


Figure A.103:UV of 43-mer ODN **1n**

UV of 43-mer ODN **1n**. The ODN was synthesized on a 0.2 µmol scale. All CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 200 µL of water, and 20 µL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 0.2 µmol synthesis and purification was calculated to be 9.664 (0.4832 × 200 µL ÷ 20 µL × 2 mL ÷ 1 mL).

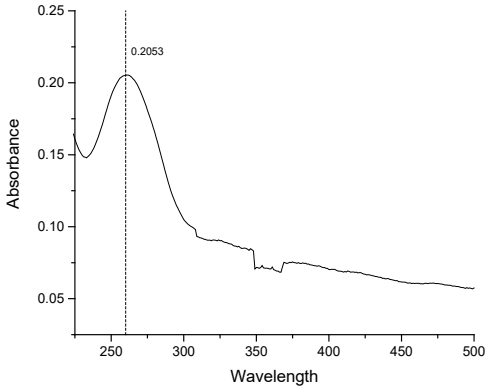


Figure A.104:UV of 64-mer ODN **1o**

UV of 64-mer ODN **1o**. The ODN was synthesized on a 0.2 µmol scale. All CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 200 µL of water, and 20 µL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 0.2 µmol synthesis and purification was calculated to be 4.106 (0.2053 × 200 µL ÷ 20 µL × 2 mL ÷ 1 mL).

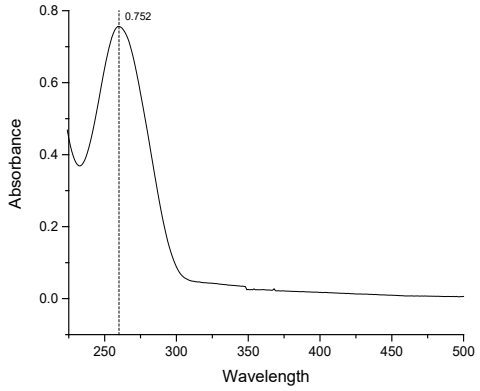


Figure A.105:UV of 80-mer ODN **1p**

UV of 80-mer ODN **1p**. The ODN was synthesized on a 0.2 µmol scale. All CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 200 µL of water, and 20 µL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 0.2 µmol synthesis and purification was calculated to be 15.04 (0.7520 × 200 µL ÷ 20 µL × 2 mL ÷ 1 mL).

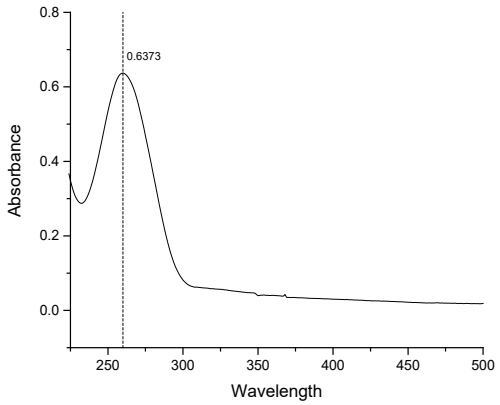


Figure A.106:UV of 90-mer ODN **1q**

UV of 90-mer ODN **1q**. The ODN was synthesized on a 0.2 µmol scale. All CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 200 µL of water, and 20 µL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 0.2 µmol synthesis and purification was calculated to be12.746 (0.6373 × 200 µL ÷ 20 µL × 2 mL ÷ 1 mL).

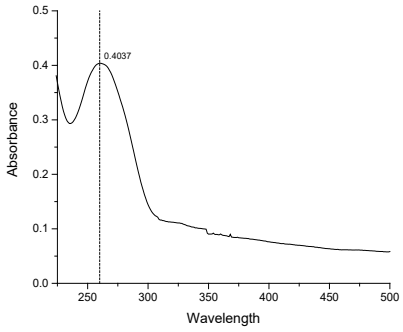


Figure A.107:UV of 110-mer ODN **1r**

UV of 110-mer ODN **1r**. The ODN was synthesized on a 0.2 µmol scale. All CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 200 µL of water, and 20 µL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260}

of the 0.2 μmol synthesis and purification was calculated to be 8.074 (0.4037 × 200 μL ÷ 20 μL × 2 mL ÷ 1 mL).

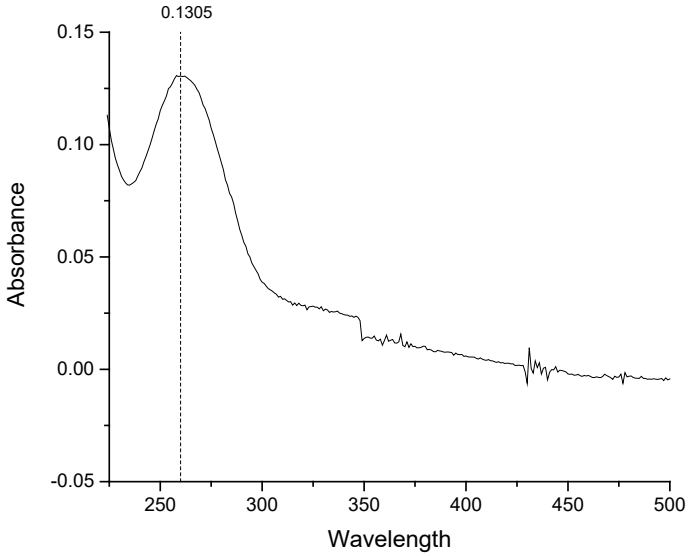


Figure A.108:UV of 151-mer ODN **1s**

UV of 151-mer ODN **1s**. The ODN was synthesized on a 0.2 μmol scale. All CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 200 μL of water, and 20 μL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 0.2 μmol synthesis and purification was calculated to be 2.61(0.1305 × 200 μL ÷ 20 μL × 2 mL ÷ 1 mL).

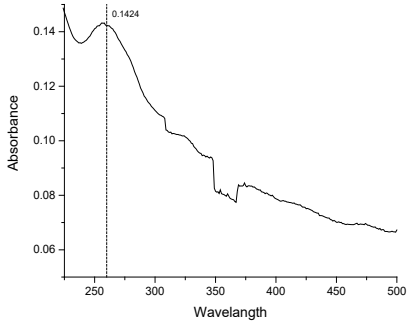


Figure A.109:UV of 197-mer ODN **1t**

UV of 197-mer ODN **1t**. The ODN was synthesized on a 0.2 μmol scale. All CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 200 μL of water, and 20 μL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 0.2 μmol synthesis and purification was calculated to be 2.848 (0.1424 × 200 μL ÷ 20 μL × 2 mL ÷ 1 mL).

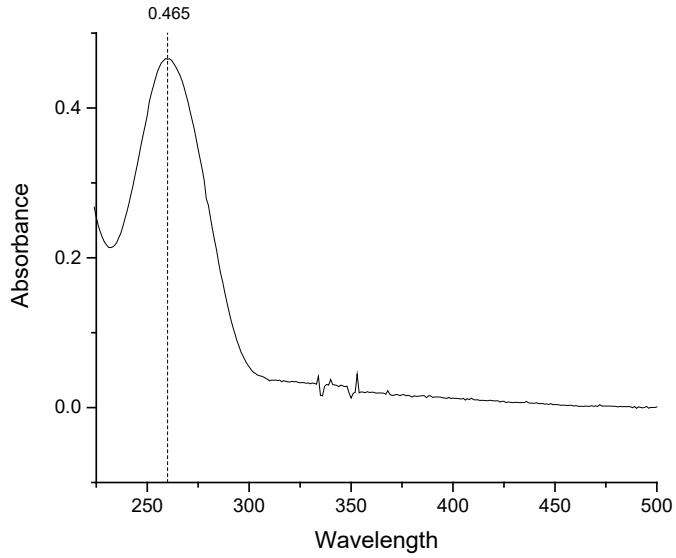


Figure A.110:UV of 203-mer ODN **1u**

UV of 203-mer ODN **1u**. The ODN was synthesized on a 0.2 μmol scale. All CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 200 μL of

water, and 20 µL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 0.2 µmol synthesis and purification was calculated to be 9.3 (0.465 × 200 µL ÷ 20 µL × 2 mL ÷ 1 mL).

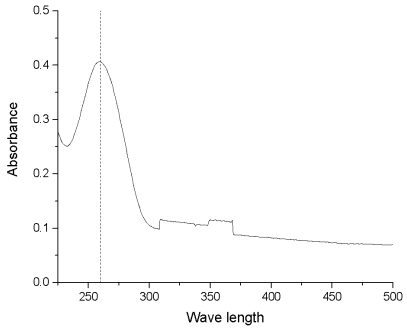


Figure A.111:UV of 225-mer ODN **1v**

UV of 225-mer ODN **1v**. The ODN was synthesized on a 0.2 µmol scale. All CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 200 µL of water, and 20 µL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 0.2 µmol synthesis and purification was calculated to be 6.688 (0.3344 × 200 µL ÷ 20 µL × 2 mL ÷ 1 mL).

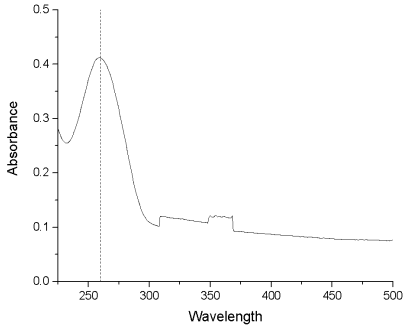


Figure A.112:UV of 251-mer ODN **1w**

UV of 251-mer ODN **1w**. The ODN was synthesized on a 0.2 µmol scale. All CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 200 µL of

water, and 20 µL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 0.2 µmol synthesis and purification was calculated to be 7.316 (0.3658 × 200 µL ÷ 20 µL × 2 mL ÷ 1 mL).

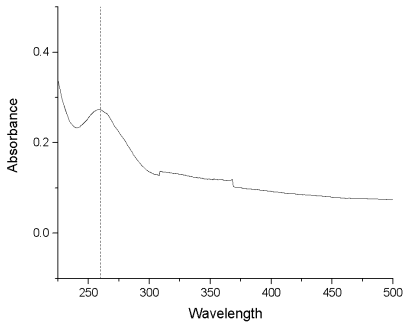


Figure A.113:UV of 275-mer ODN **1x**

UV of 275-mer ODN **1x**. The ODN was synthesized on a 0.2 µmol scale. All CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 200 µL of water, and 20 µL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 0.2 µmol synthesis and purification was calculated to be 5.456 (0.2728 × 200 µL ÷ 20 µL × 2 mL ÷ 1 mL).

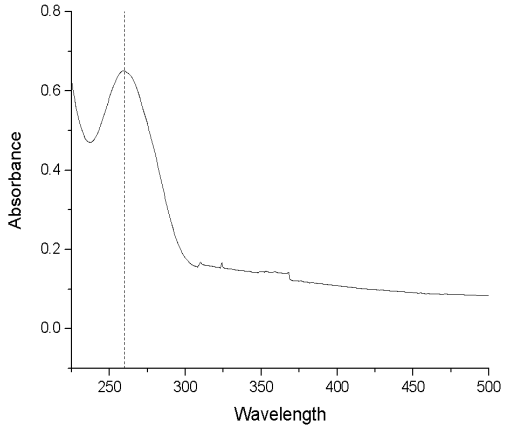


Figure A.114:UV of 303-mer ODN **1y**

UV of 303-mer ODN **1y**. The ODN was synthesized on a 0.2 μmol scale. All CPG was subjected to deprotection and cleavage. The purified ODN was dissolved in 200 μL of water, and 20 μL was taken out and diluted to 2 mL to obtain the UV spectrum. The OD_{260} of the 0.2 μmol synthesis and purification was calculated to be 13.012 (0.6506 × 200 μL ÷ 20 μL × 2 mL ÷ 1 mL).

B Supporting information for chapter 4

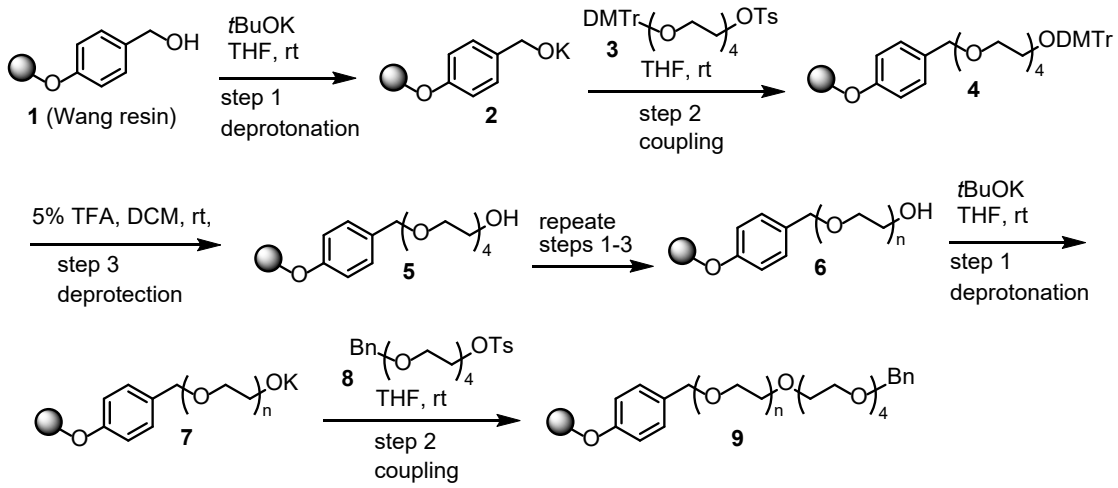


Figure B.1:Automated synthesis scheme for PEG synthesis

B.1 Suggested bottle assignment:

R1 (5 L): Dry THF (side A)

R2 (2 L): Wet THF (side B)

R3 (2 L): THF/water (v/v 1:1) (Side B)

R4 (1 L): Dry DMF (side A)

R5 (1 L): *t*BuOK in THF (0.5 M concentration)

R6 (0.5 L): Dry NMP (side A)

R7 (0.5 L): Dry DMSO (side A)

R8 (2 L): 10% TFA in DCM (Side B)

R9 (2 L): DCM (Side B)

AA (0.1 L): Monomer **8** in dry THF (side A)

B.2 Automated synthesis procedure

1. Deliver dry THF to Wang resin (**1**) in RV
2. Shake at rt for 10 min
3. Remove THF from RV
4. Repeat 1-3 for four additional times
5. Deliver dry DMF to Wang resin (**1**) in RV
6. Shake at rt for 10 min
7. Remove DMF from RV
8. Repeat 5-7 for four additional times
9. Deliver dry DMSO to Wang resin (**1**) in RV
10. Shake at rt for 10 min
11. Remove DMSO from RV
12. Repeat 9-11 for four additional times
13. Deliver dry NMP to Wang resin (**1**) in RV
14. Shake at rt for 10 min
15. Remove NMP from RV
16. Repeat 13-15 for four additional times
17. Deliver *t*BuOK in DMF to **1** in RV (goal: deprotonate **1** to give **2**)
18. Shake for 5 min at rt (ideally lower T)
19. Remove liquid from RV
20. Repeat 17-19 one more time
21. Deliver dry DMF to RV
22. Shake for 3 min
23. Remove DMF from RV
24. Deliver dry DMF to RV
25. Shake for 3 min
26. Remove DMF from RV
27. Deliver monomer **3** in DMF (0.6 M) to RV (goal: couple **2** with **3** to give **4**)
28. Shake for 6 h at rt
29. Remove liquid from RV
30. Deliver wet THF to RV

31. Shake for 10 min
32. Remove THF from RV
33. Repeat 30-32 for four additional times
34. Deliver water/THF (v/v 1:1) to RV
35. Shake for 10 min
36. Remove liquid from RV
37. Repeat 34-36 for four more times
38. Deliver wet THF to RV
39. Shake for 10 min
40. Remove THF from RV
41. Repeat 38-40 for four times
42. Repeat 1-49 for two more times (goal: couple more times to ensure complete coupling)
43. Deliver DCM to RV
44. Shake for 1 min
45. Remove DCM from RV
46. Deliver 5% TFA in DCM to RV (goal: remove DMTr from **4** to give **5**)
47. Shake for 10 min
48. Remove liquid from RV
49. Repeat 43-48 for 14 more times (goal: complete detritylation)
50. Deliver DCM to RV
51. Shake for 10 min
52. Remove liquid from RV
53. Repeat 50-52 for four more times
54. Repeat steps 1-53 until PEG **6** with desired "n" is reached

B.3 PEG cleavage from the Wang resin

1. Detritylation with TCA in DCM until color disappear
2. Wash with DCM 3 times
3. Dry in a desiccator overnight
4. Treat with pure TFA for 30 min
5. Wash with TFA and collect the supernatant
6. Wash the resin with THF/H2O
7. Combined and evaporate to dryness
8. Add ~ 300 µL water and vortex and spin(precipitation) (two times)
9. Evaporate water
10. Add ~ 300 µL toluene and vortex and spin(precipitation) (two times)
11. Evaporate toluene
12. Treat with ~ 300 µL of concentrated ammonia in water
13. Evaporate ammonia and water
14. Add ~ 300 µL of toluene
15. Evaporate toluene

    Clear pale yellow colored oil.

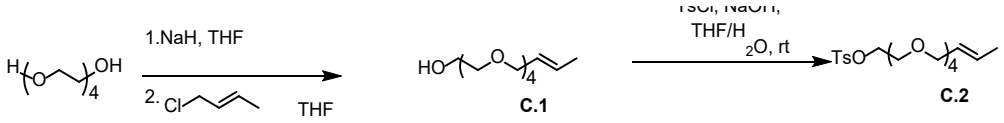
C Supporting information for chapter 5

C.1 Testing base-labile monomers for automated solid-phase synthesis

Prior to choosing the 2pheneethyl group as the base-labile protecting group, two other groups were tested. The solution phase reaction kinetics are different from the solid-phase kinetics, and therefore, we synthesized those monomers and used them for automated solid-phase PEG synthesis.

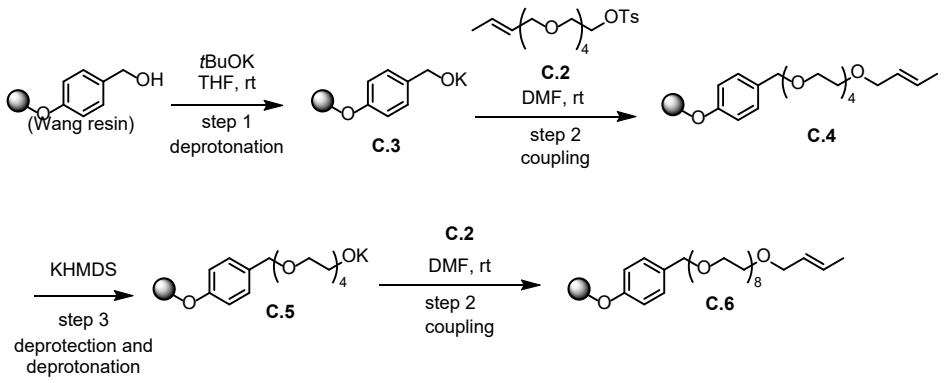
C.2 Testing (*E*)-but-2-ene group as base-labile protecting group

The synthesis of the monomer is shown in Scheme C.1. First the PEG_4 starting material is deprotonated with sodium hydride, and it was reacted with (*E*)-1-chlorobut-2-ene to obtain compound **C.1.** Then the compound was reacted with tosyl chloride to obtain the monomer **C.2**.



Scheme C.1:Synthesis of base-labile monomer **C.2**

The automated PEG synthesis was carried out using Scheme C.2. After obtaining PEG_8, **C.6**, the PEG was cleaved off from the Wang resin and analyzed using ESI-MS.



Scheme C.2:Automated solid-phase synthesis scheme

The cleaved PEG_8 molecule (**C.7**, Figure C.1) is shown in Figure C.1. The expected mass of the cleaved PEG_8 sample is 424.

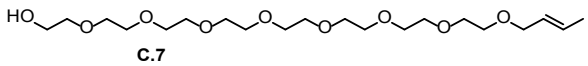


Figure C.1:PEG_8 obtained after the cleavage of **C.6**

The ESI-MS obtained for PEG sample is shown in figure C.2. The MS shows the product (442= M+ NH_4^+) and another peak at 618. This corresponds to the molar mass of PEG_{12}. This means the protecting group was removed prematurely, during the coupling step, and couped one more time. Therefore, this monomer is not suitable for the automated solid-phase PEG synthesis.

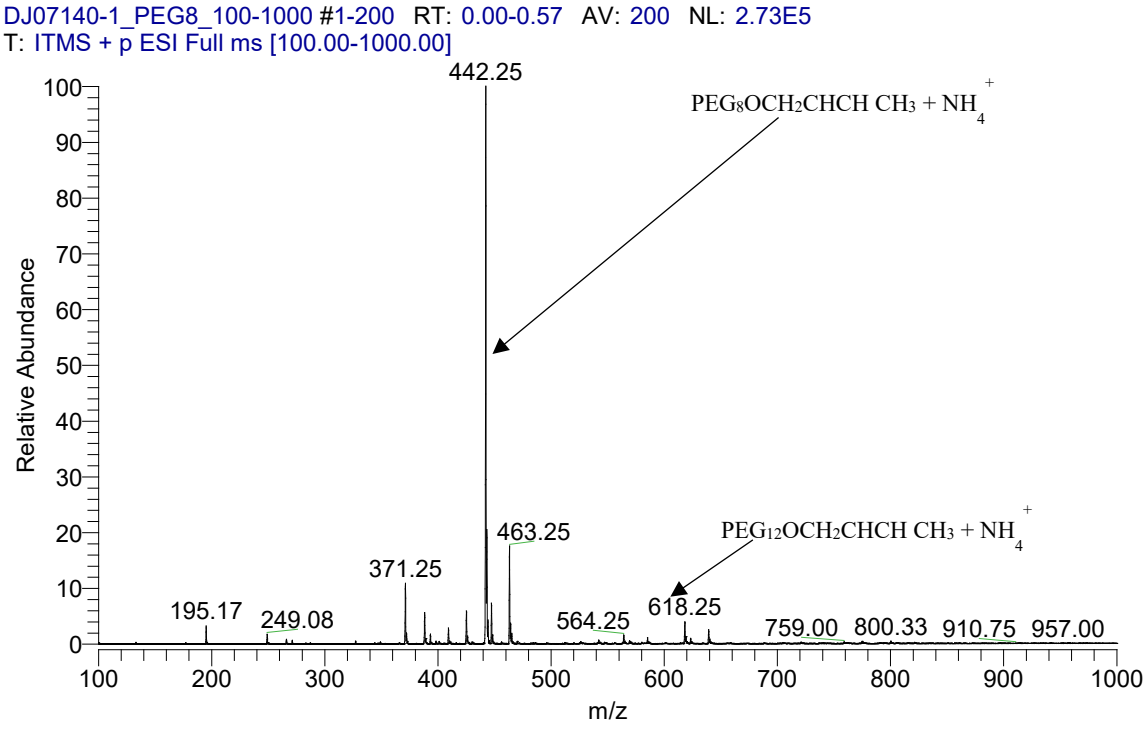
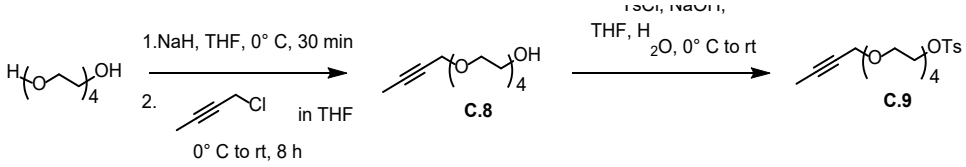


Figure C.2:ESI-MS of PEG_8

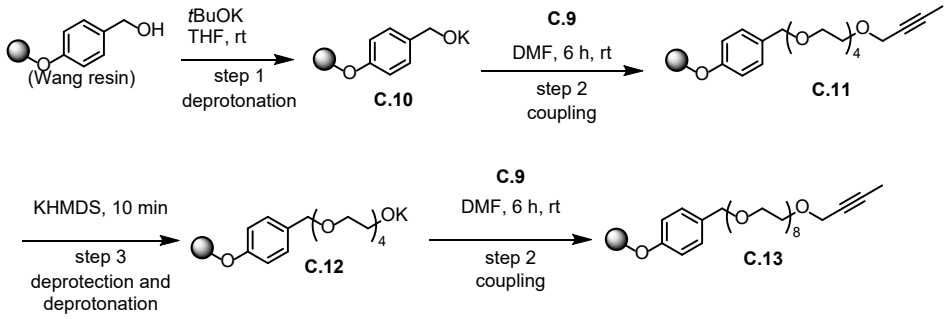
C.3 Testing but-2-yne group as base-labile protecting group

The synthesis of the monomer is shown in Scheme C.2. First the PEG_4 starting material is deprotonated with sodium hydride, and it was reacted with 1-chlorobut-2-yne to obtain compound **C.8**. Then the compound was reacted with tosyl chloride to obtain the monomer **C.9**.



Scheme C.3:Synthesis of the base-labile monomer **C.9**

The automated PEG synthesis was carried out using Scheme C.4. After obtaining PEG_8, **C.13**, the PEG was cleaved off from the Wang resin and analyzed using ESI-MS.



Scheme C.4:Automated solid-phase synthesis

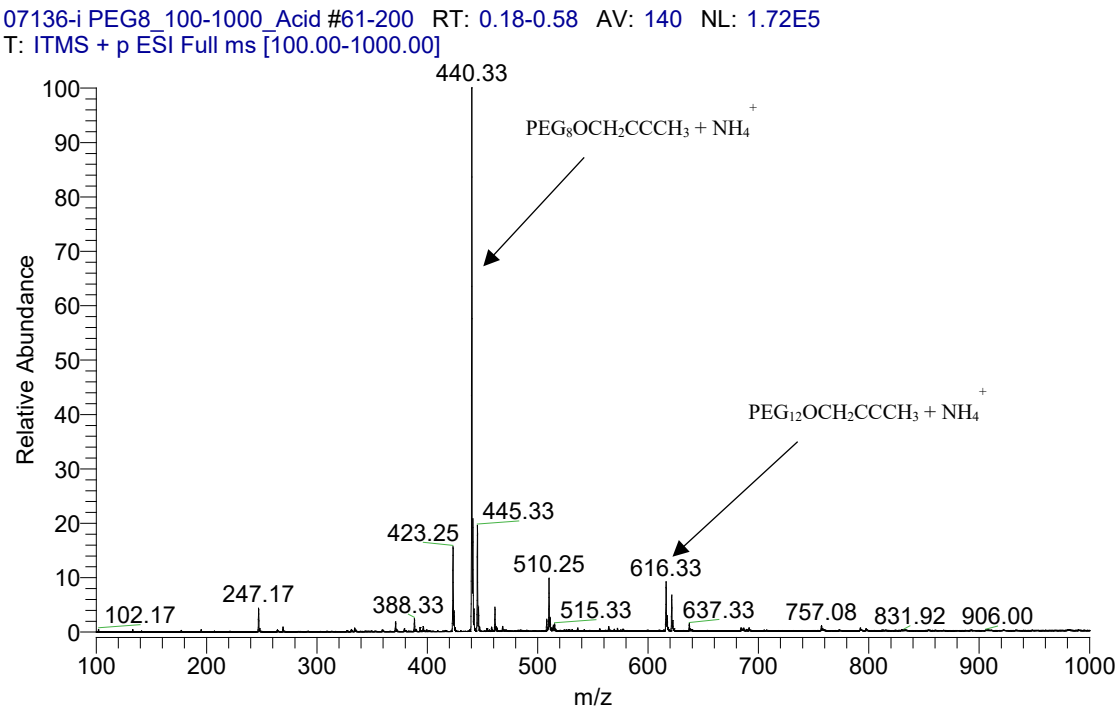


Figure C.3:ESI-MS of PEG_8

The ESI-MS obtained for PEG sample is shown in Figure C.3. The MS shows the product (440= M+ NH4) and another peak at 616. This corresponds to the PEG12 moiety. This means the protecting group was removed prematurely, during the coupling step, and couped one more time. Therefore, this monomer is not suitable for the PEG synthesis.

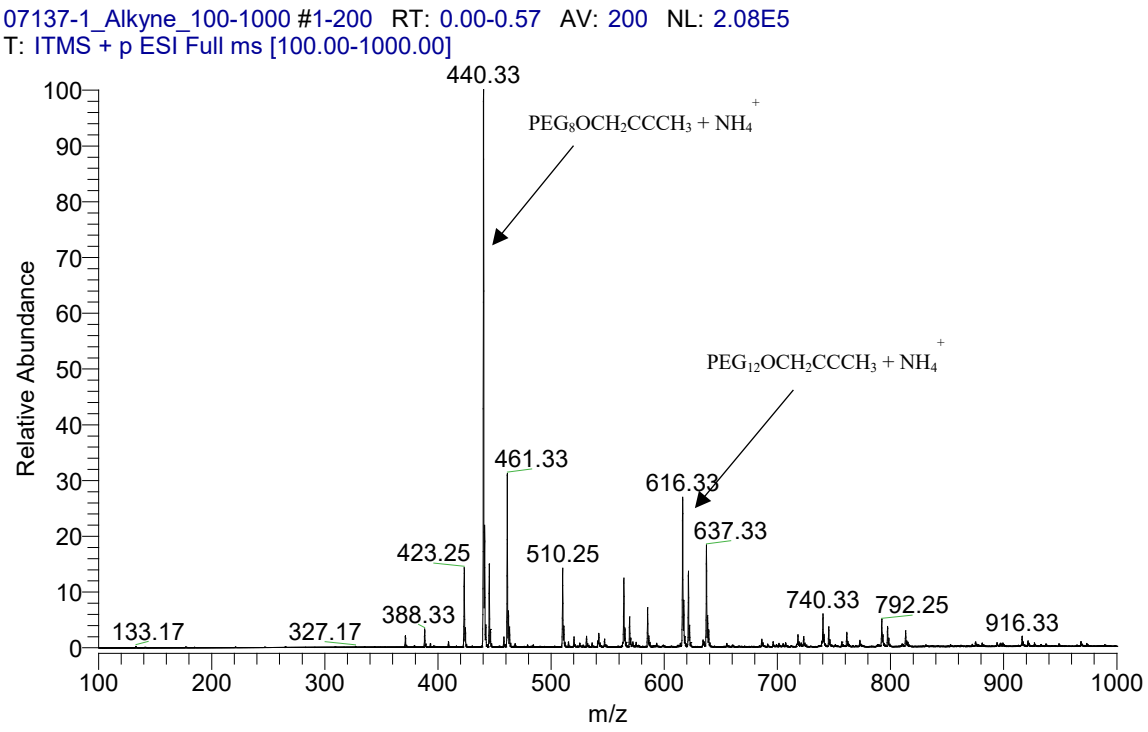
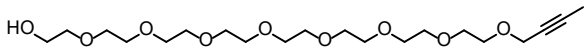
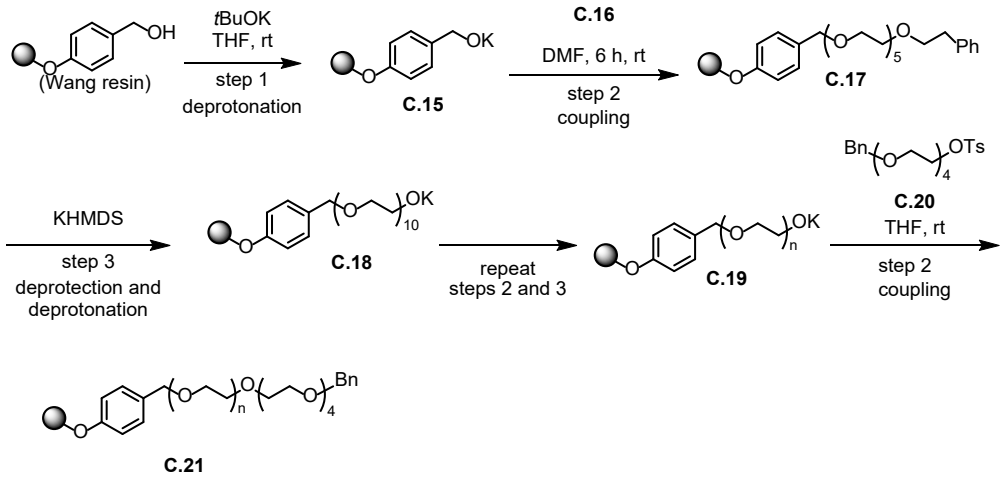


Figure C.4:ESI-MS of PEG_8



C.14 Exact Mass: 422.25

However, the 2 phenethyl protecting group gave us the best results, and that was used as base-labile protecting group for automated PEG synthesis. The synthesis scheme is shown in Scheme C.3. This was described in detail in chapter 5. The bottle arrangement and the detailed script for the automated synthesis is shown in next two sections.



Scheme C.3:Automated PEG synthesis scheme

C.4 Suggested bottle arrangement

R1 (5 L): Dry THF

R2 (2 L): Wet THF (side B)

R3 (2 L): THF/water (v/v 1:1) (Side B)

R4 (1 L): Dry DMF

R5 (1 L): KHMDS in THF (0.25 M concentration)

R6 (0.5 L): Dry NMP

R7 (0.5 L): Dry DMSO

R8 (2 L):

R9 (2 L): DCM (Side B)

AA (0.1 L): Monomer C.16 in dry THF

C.5 Automated synthesis procedure

1. Deliver dry THF to Wang resin (**1**) in RV
2. Shake at rt for 10 min
3. Remove THF from RV
4. Repeat 1-3 for four additional times
5. Deliver dry DMF to Wang resin (**1**) in RV
6. Shake at rt for 10 min
7. Remove DMF from RV

8. Repeat 5-7 for four additional times
9. Deliver dry DMSO to Wang resin (**1**) in RV
10. Shake at rt for 10 min
11. Remove DMSO from RV
12. Repeat 9-11 for four additional times
13. Deliver dry NMP to Wang resin (**1**) in RV
14. Shake at rt for 10 min
15. Remove NMP from RV
16. Repeat 13-15 for four additional times
17. Deliver KHMDS in THF to **1** in RV (goal: deprotonate Wang resin to give **C.15**)
18. Shake for 5 min at rt (ideally lower T)
19. Remove liquid from RV
20. Repeat 17-19 one more time
21. Deliver dry DMF to RV
22. Shake for 3 min
23. Remove DMF from RV
24. Deliver dry DMF to RV
25. Shake for 3 min
26. Remove DMF from RV
27. Deliver monomer **C.16** in DMF to RV (goal: couple **C.15** with **C.16** to give **C.17**)
28. Shake for 6 h at rt
29. Remove liquid from RV
30. Deliver wet THF to RV
31. Shake for 10 min
32. Remove THF from RV
33. Repeat 30-32 for four additional times
34. Deliver water/THF (v/v 1:1) to RV
35. Shake for 10 min
36. Remove liquid from RV
37. Repeat 34-36 for four more times

38. Deliver wet THF to RV
39. Shake for 10 min
40. Remove THF from RV
41. Repeat 38-40 for four times
42. Deliver DCM to RV
43. Shake for 10 min
44. Remove DCM from RV
45. Repeat 42-44 for four more times
46. Deliver dry THF to Wang resin (**1**) in RV
47. Shake at rt for 10 min
48. Remove THF from RV
49. Repeat 1-3 for four additional times
50. Deliver dry DMF to Wang resin (**1**) in RV
51. Shake at rt for 10 min
52. Remove DMF from RV
53. Repeat 5-7 for four additional times
54. Deliver dry DMSO to Wang resin (**1**) in RV
55. Shake at rt for 10 min
56. Remove DMSO from RV
57. Repeat 9-11 for four additional times
58. Deliver dry NMP to Wang resin (**1**) in RV
59. Shake at rt for 10 min
60. Remove NMP from RV
61. Repeat 13-15 for four additional times
62. Deliver KHMDS in THF to **1** in RV (goal: deprotect and deprotonate **C.17** to give C.**18**)
63. Shake for 5 min at rt (ideally lower T)
64. Remove liquid from RV
65. Repeat 62-64 one more time
66. Deliver dry DMF to RV

67. Shake for 3 min
68. Remove DMF from RV
69. Deliver dry DMF to RV
70. Shake for 3 min
71. Remove DMF from RV
72. Deliver monomer **C.16** in DMF to RV {goal: couple **C.18** with **C.16** to give **C.19** (n=10)}
73. Shake for 6 h at rt
74. Remove liquid from RV
75. Deliver wet THF to RV
76. Shake for 10 min
77. Remove THF from RV
78. Repeat 75-77 for four additional times
79. Deliver water/THF (v/v 1:1) to RV
80. Shake for 10 min
81. Remove liquid from RV
82. Repeat 79-81 for four more times
83. Deliver wet THF to RV
84. Shake for 10 min
85. Remove THF from RV
86. Repeat 83-85 for four times
87. Deliver DCM to RV
88. Shake for 10 min
89. Remove DCM from RV
90. Repeat 87-89 for four more times
91. Repeat steps 1-90 until PEG **C.19** with desired “n” is reached

C.6 PEG cleavage from Wang resin

1. Treat with pure TFA for 30 min
2. Wash with TFA and collect the supernatant
3. Wash the resin with THF/H2O
4. Combined and evaporate to dryness
5. Add ~ 300 µL water and vortex and spin(precipitation) (two times)
6. Evaporate water
7. Add ~ 300 µL toluene and vortex and spin(precipitation) (two times)
8. Evaporate toluene
9. Treat with ~ 300 µL of concentrated ammonia in water
10. Evaporate ammonia and water
11. Add ~ 300 µL of toluene
12. Evaporate toluene

Clear pale yellow colored oil.

C.7 NMR spectra

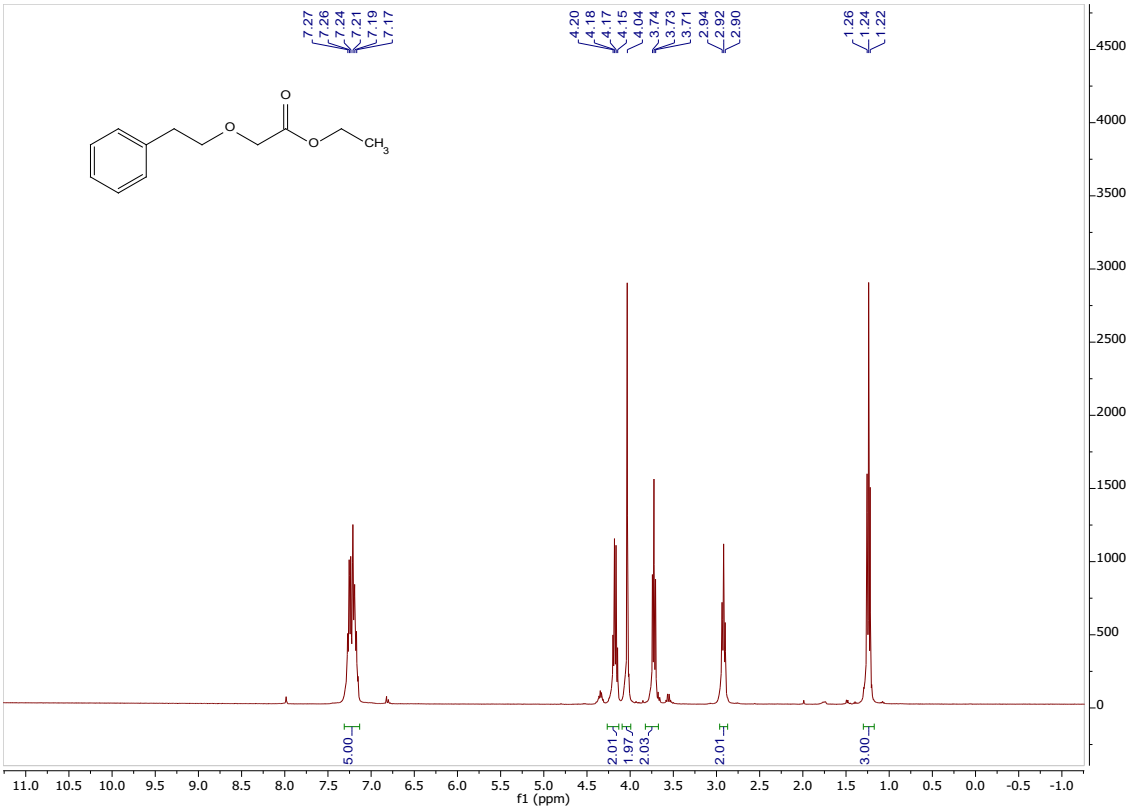


Figure C.5:HNMR spectrum of compound **5.1**

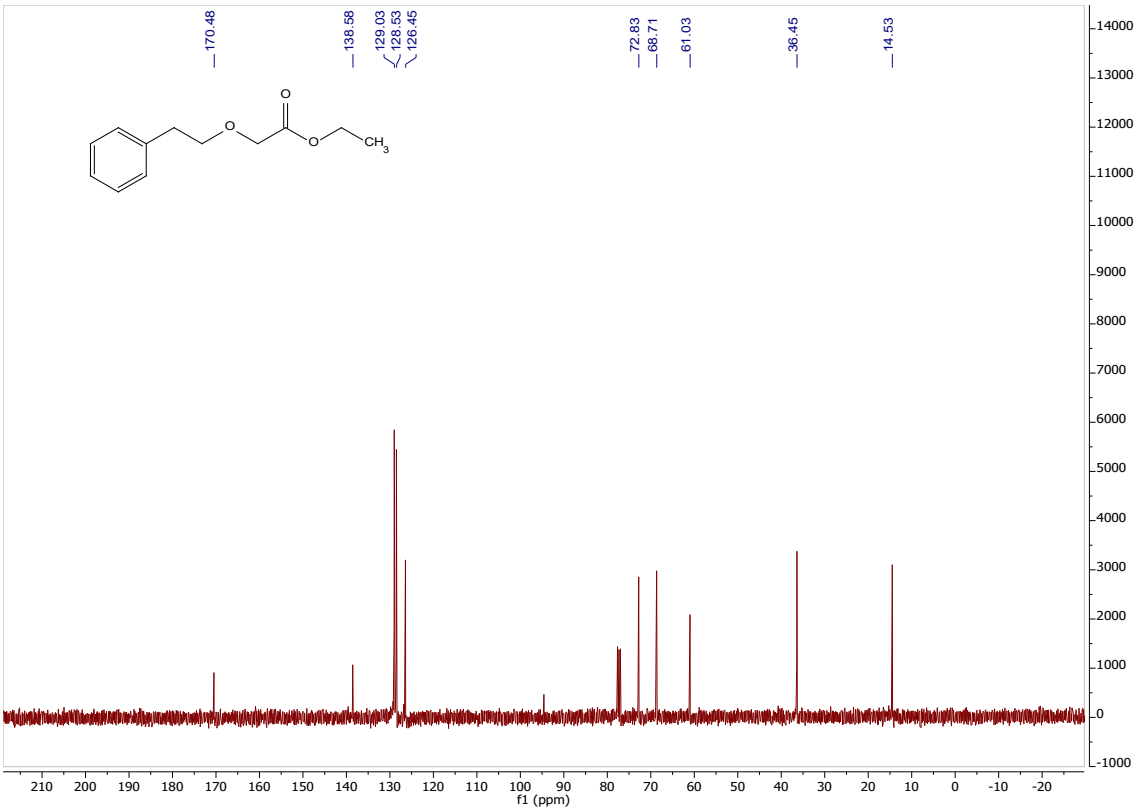


Figure C.6:CNMR spectrum of compound **5.1**

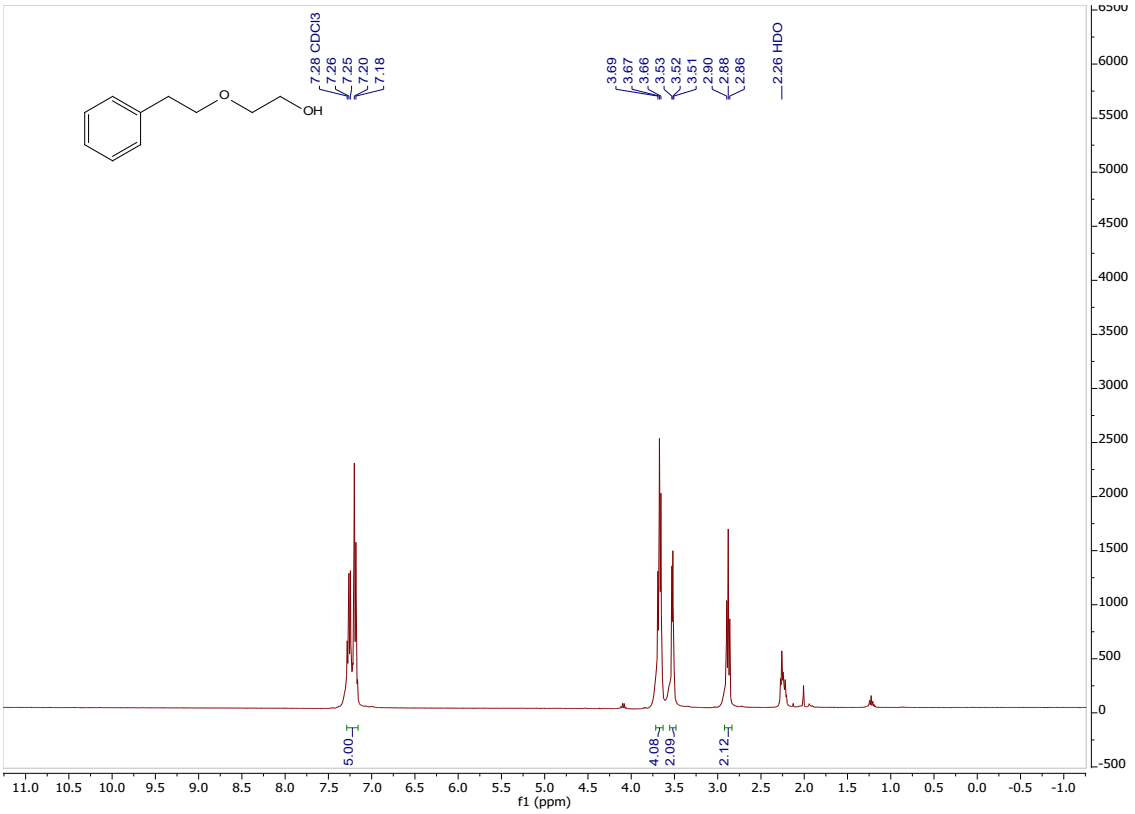


Figure C.7:HNMR spectrum of compound **5.2**

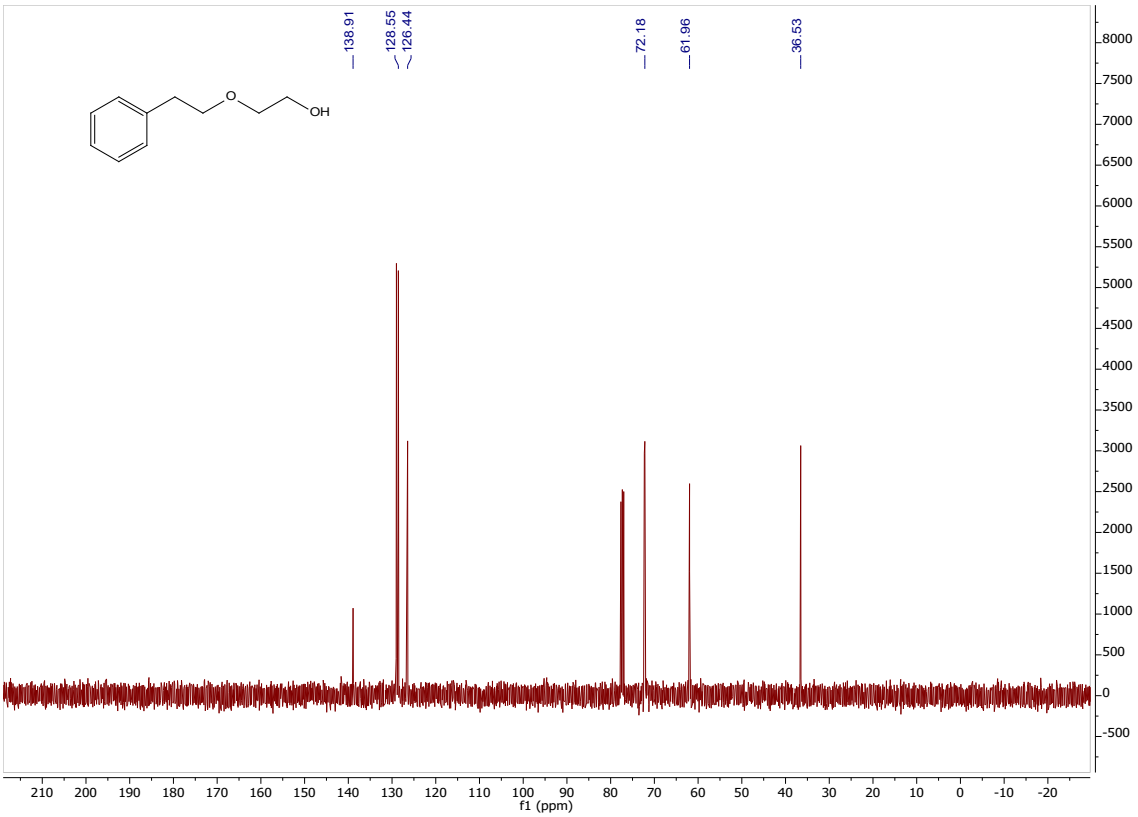


Figure C.8:CNMR spectrum of compound **5.2**

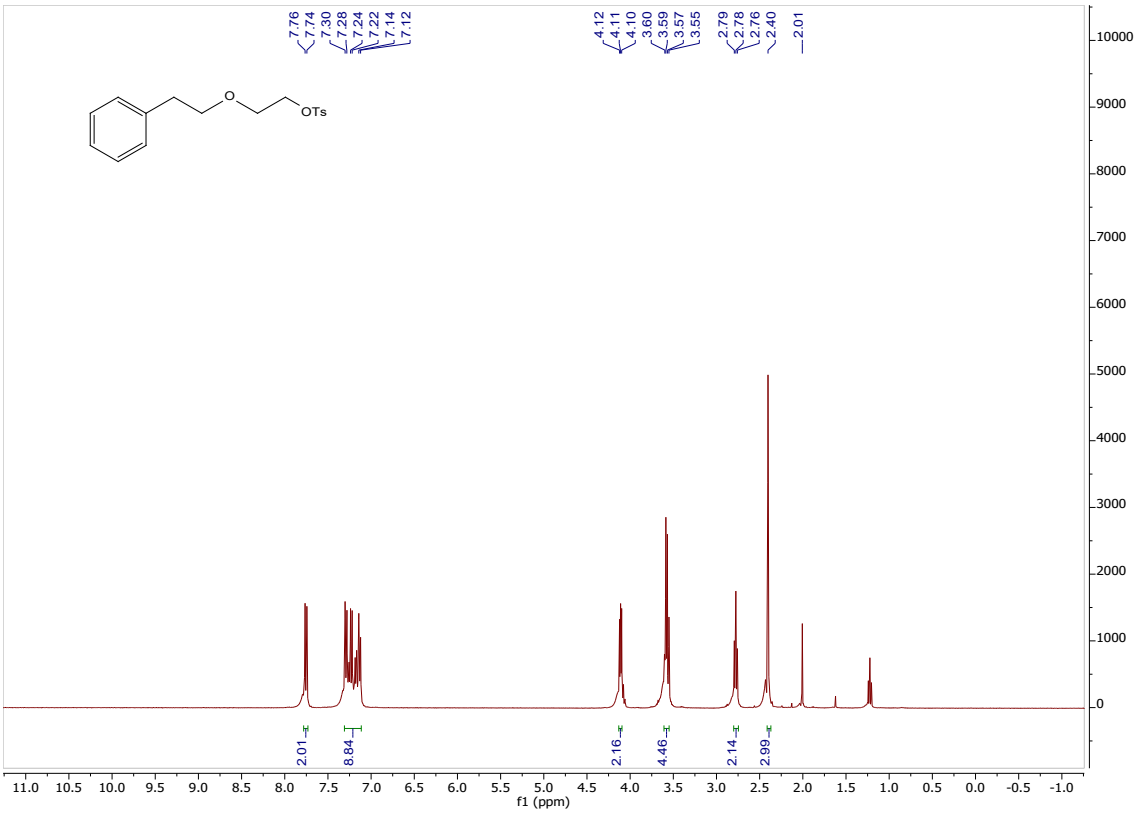


Figure C.9:HNMR spectrum of compound **5.3**

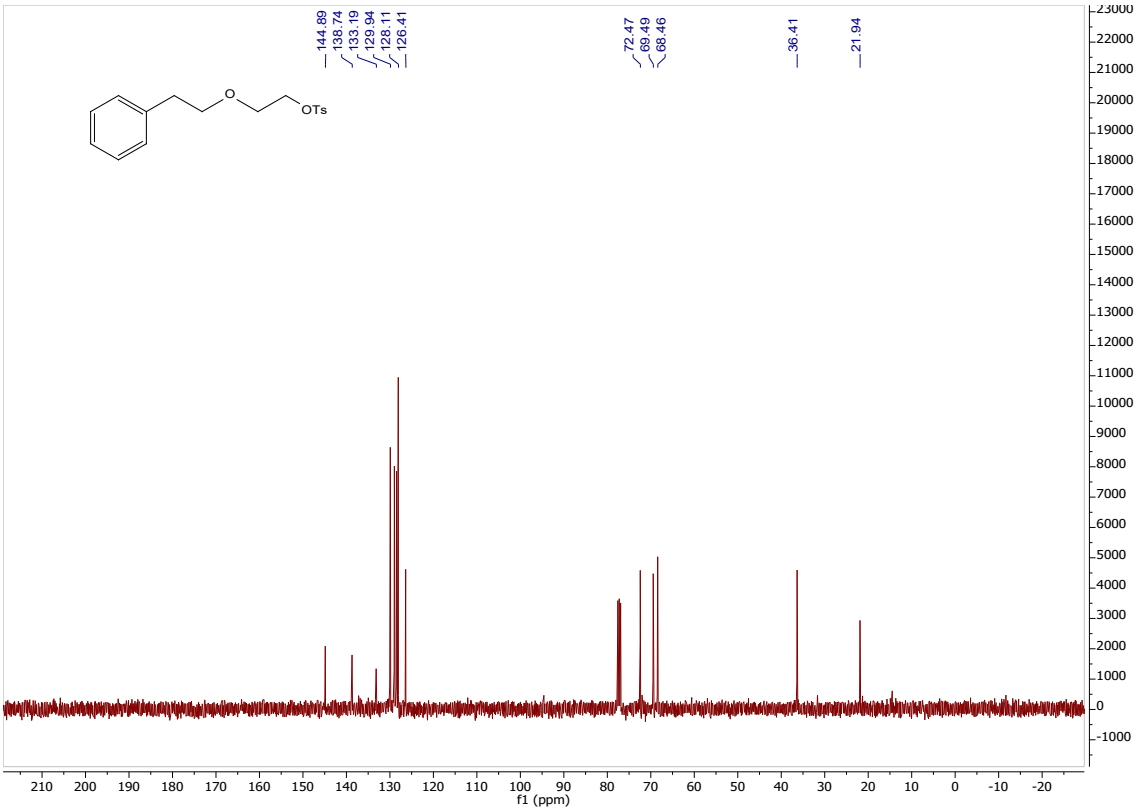


Figure C.10:CNMR spectrum of compound **5.3**

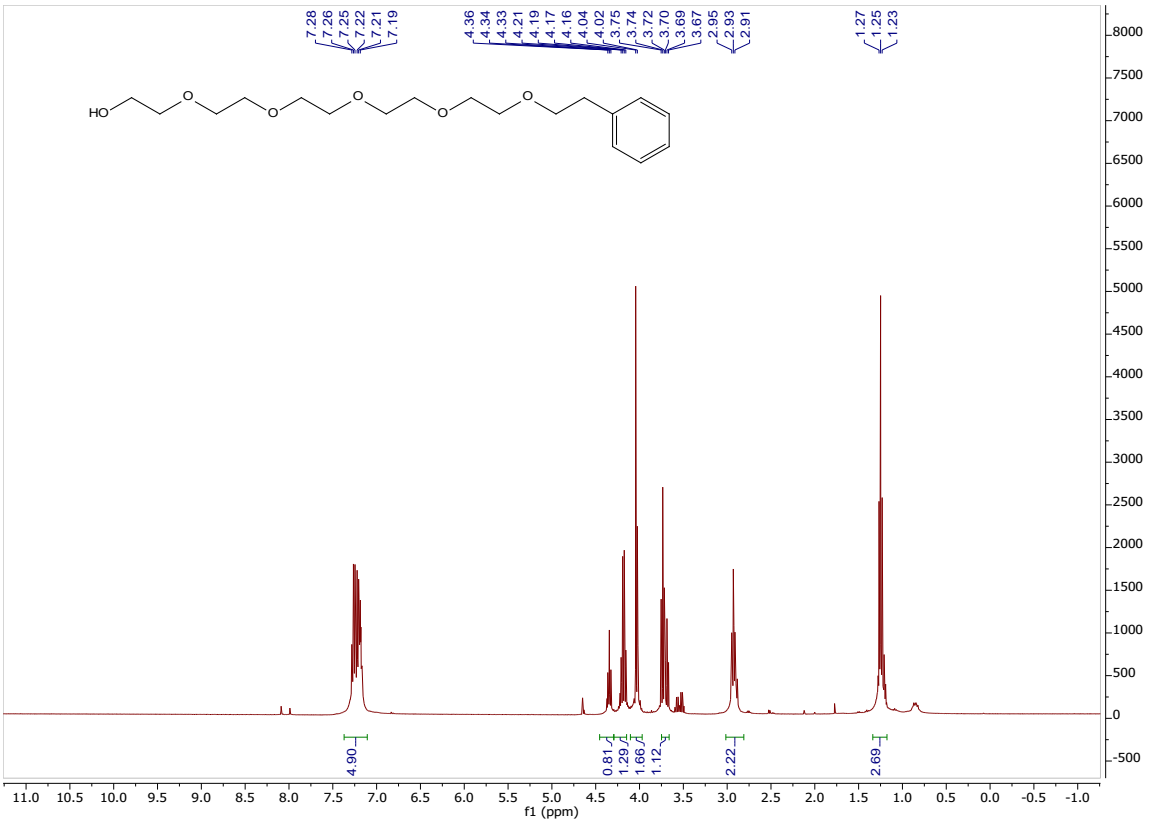


Figure C.11:HNMR spectrum of compound **5.4**

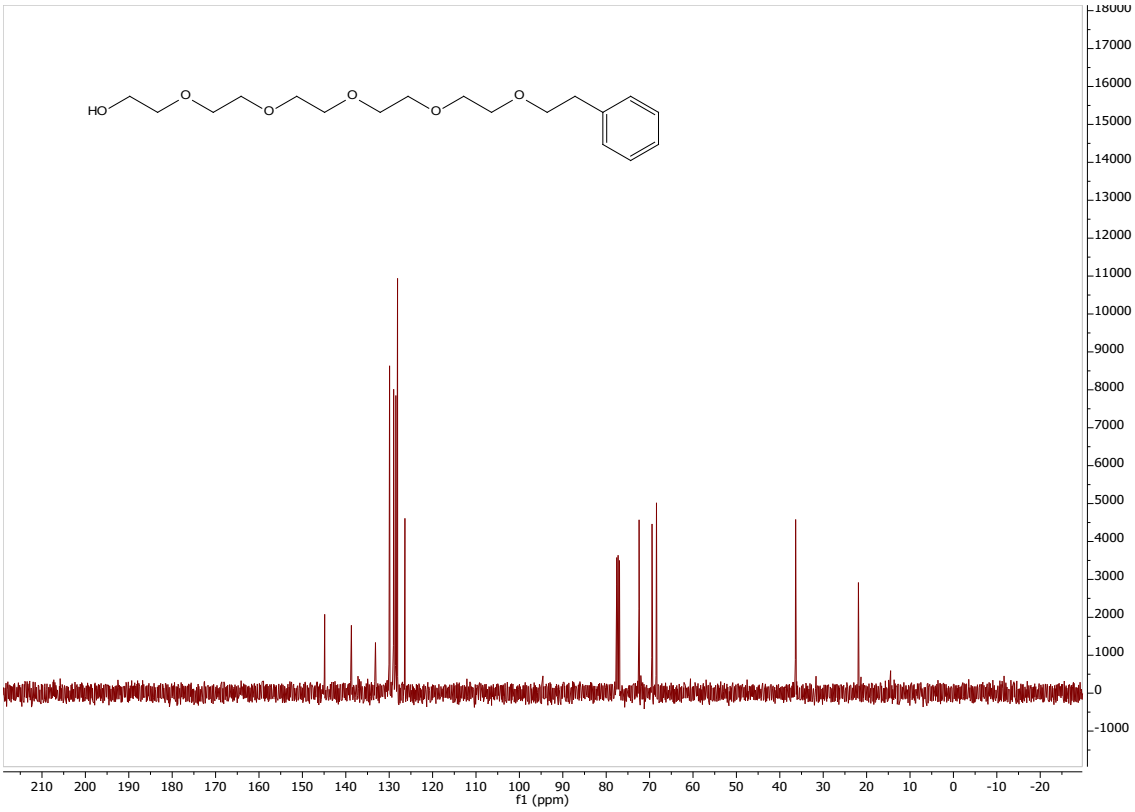


Figure C.12:CNMR spectrum of compound **5.4**

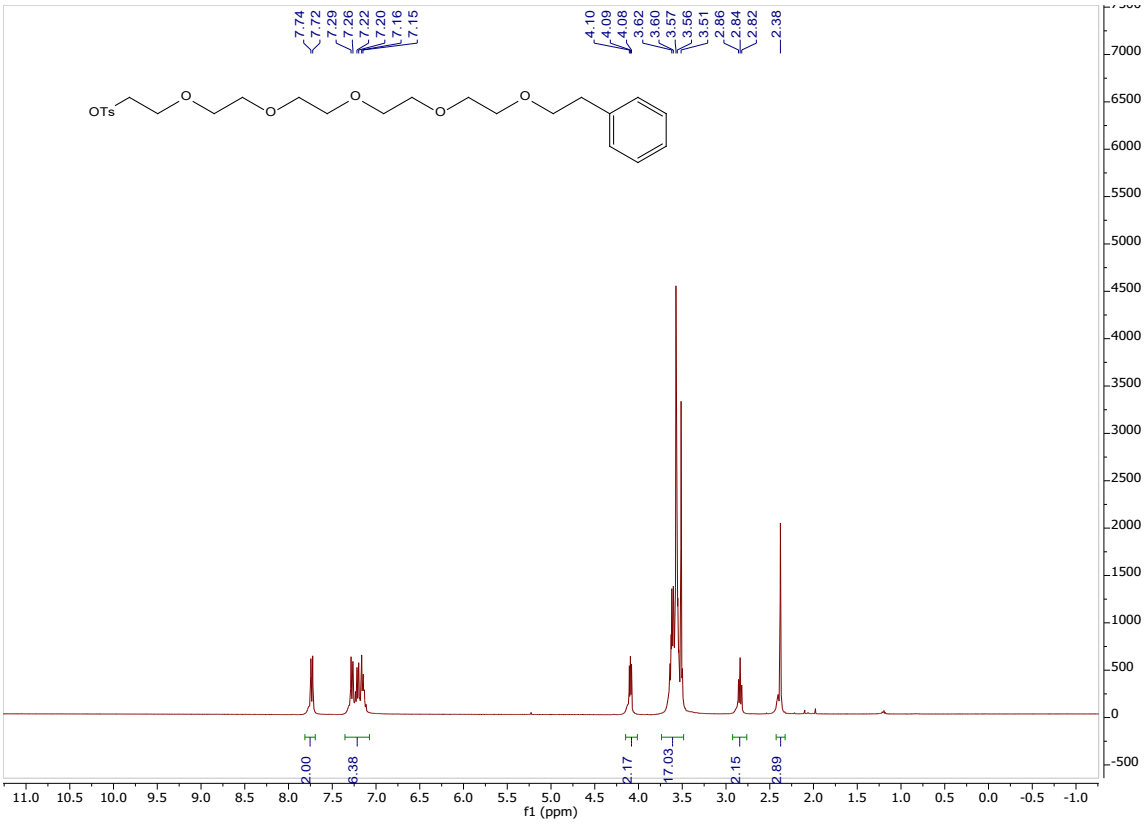


Figure C.13:HNMR spectrum of compound **5.5**

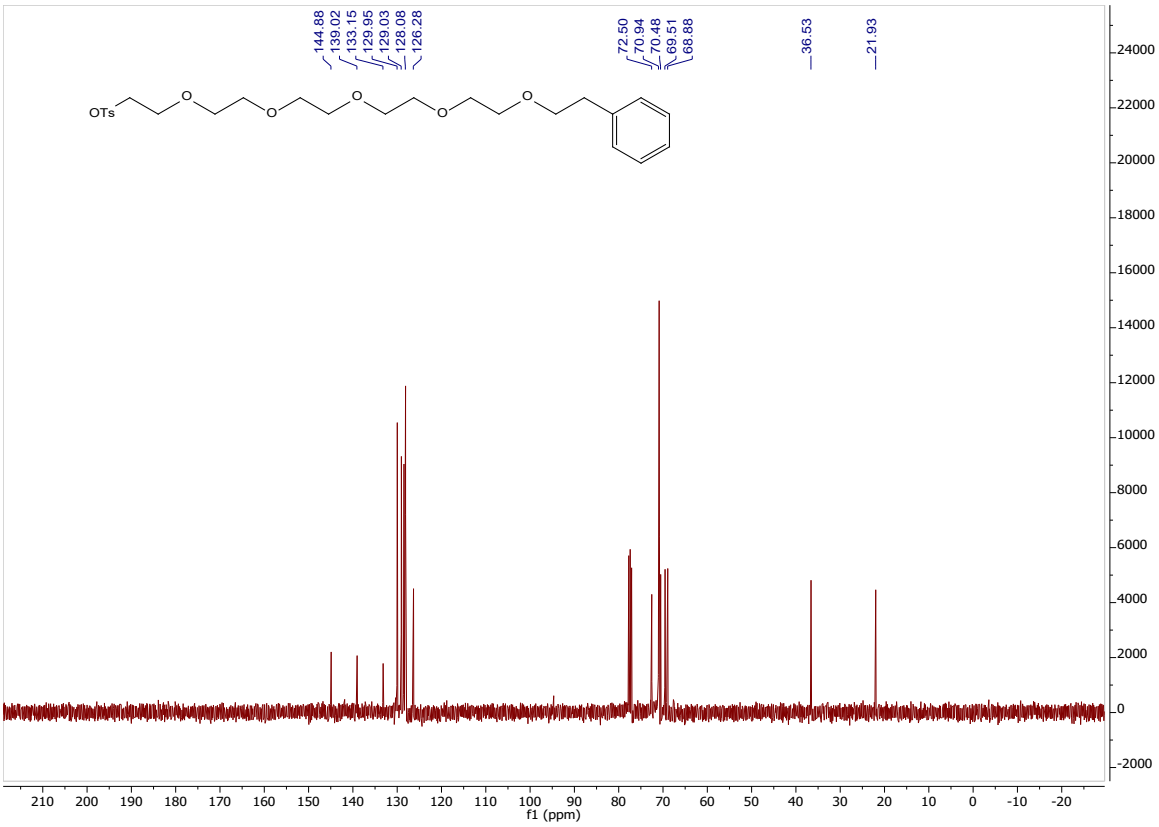


Figure C.14:CNMR spectrum of compound **5.5**

D Copyright documentation

Chapter 2 of this document is previously published in OPRD journal in 2018.