OPTIMIZATION AND TROUBLESHOOTING OF POLYMER SYNTHESIS FOR SELF-ASSEMBLY OF TYROSINE-BASED NANOSPHERES

by

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And approved by

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ABSTRACT OF THE THESIS

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Thesis Director:

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Synthesis of poly(ethylene glycol)-block-oligo(DTO suberate)

-block-poly(ethylene glycol), a tyrosine-based ABA triblock copolymer was conducted in an effort to match specifications of a previous batch of polymer used in to self-assemble into nanospheres for drug delivery in previously published work. Though synthesis was successful and the polymer's physical and thermal specifications were met, the resulting nanospheres did not meet the size specifications of past samples. Polymer synthesis was optimized both by varying the amount of coupling agent used in the reaction as well as varying the monomeric excess of suberic acid using the Carothers's equation. This work resulted in tunable nanospheres based on polymer molecular weight. Varying of coupling agent was found to produce polymers of different molecular weights albeit in a random fashion. Use of monomeric excess was found to reproducibly produce polymers of varying molecular weights. Additionally nanosphere preparation was also studied

to determine whether the use of sucrose during nanosphere self-assembly would result in an increased polymer yield. The use of a sucrose solution during nanosphere preparation was found to cause no improvement in polymer yield and is in fact believed to have the opposite effect.

PREFACE

All that is gold does not glitter,

Not all those who wander are lost;

The old that is strong does not wither,

Deep roots are not reached by the frost.

From the ashes a fire shall be woken,

A light from the shadows shall spring;

Renewed shall be blade that was broken,

The crownless again shall be king.

-J. R. R. Tolkien

DEDICATION

This work is dedicated to my daughter Scarlett Isabel Davie for making me a mother and giving me the profound privilege of raising a woman strong enough to change the world.

ACKNOWLEDGEMENTS

I want to thank my parents Dagmar and Carlos Agüero for their unwavering support through my variety of careers, and my sister Kristin Agüero for being my best friend and biggest fan. I also want to thank my husband Scott Davie for believing in me when I decided to turn both our lives upside down by returning to school, and most recently, for sharing with me the joys and frustrations of parenting while working full time.

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LIST OF ABBREVIATIONS

%	Percent	DMSO-d_6	Deuterated dimethyl
~	Approximately		sulfoxide
°	Degrees	DPTS	4-(dimethylamino)
°C	Degrees Celsius		pyridinium 4-toluene
±	Plus or minus		sulfonate
δ	Chemical shift	DSC	Differential scanning
µL	Microliter		calorimetry
^{1}H	Proton (when describing	FT-IR	Fourier transform
	NMR)		infrared spectroscopy
ACN	acetonitrile	g	Gram
cm^{-1}	Wavenumber units	GPC	Gel permeation
Da	Dalton		chromatography
dd	Doublet of doublets	H_2O	Water
ddd	Doublet of doublets of	hr/hrs	hours
	doublets	IPA	2-propanol
DIC	N,N'-diisopropyl	kDa	Kilodalton
	carbodiimide	m	Multiplet
DTO	desamino tyrosyl tyrosine	MeOH	methanol
	octyl ester	mg	Milligram
DCM	dichloromethane	MHz	Megahertz
DMF	dimethylformamide	min	Minute
DLS	Dynamic light scattering	mL	Milliliter

mM	Millimolar	RT	room temperature
M_n	Average molecular number	rpm	Revolutions per minute
		s	Singlet, second
mPEG	Polyethylene glycol monomethyl ether	t	Triplet
		TFA	Trifluoroacetic acid
M_w	Molecular weight	TGA	Thermogravimetric analysis
nm	Nanometers		
NMR	Nuclear magnetic resonance	T_g	Glass Transition Temperature
PBS	Phosphate Buffered Saline	THF	tetrahydrofuran
PDI	Polydispersity Index	T_m	Melting temperature
PEG	poly(ethylene glycol)	UV-Vis	Ultraviolet and Visible Light Spectroscopy
ppm	Parts per million		

CHAPTER1. Introduction

The field of drug delivery has been strongly aided by the use of polymers as drug delivery vehicles.[1] Polymers have evolved from simple carrier systems to highly tunable instruments for obtaining controlled release of a wide variety of drugs.[1, 2] Their importance in the field stems from polymers' innate versatility. They can be altered synthetically through structural or molecular weight changes, but can also be formulated in ways to best suit the desired application.

Formulation involves the creation of a "device" such as a film, scaffold, fiber or nanomaterial, which can serve as a drug delivery device while benefiting from the chemical properties of the polymeric source material.[3] Formulating can offer a tunability of drug release, targeting and biocompatibility that the polymer itself may not have. Nanomaterials formulated from polymers are of particular relevance for this thesis.

Nanomaterials in general have enjoyed great popularity of late within the drug delivery research community as their size (typically no larger than 100 nm) make them ideal for cellular interaction and uptake, they can uptake or adsorb other substances on their surfaces and generally result in reduced toxicity as compared to traditional drug dosing methods.[4] While much of the focus of nanomaterial research is on metal nanoparticles, polymers capable of self-assembling into micellar nanoparticles offer many of the same benefits as metal without some of metal's inherent drawbacks.[5-7] Specifically, amphiphilic block copolymers, consisting of a hydrophobic and hydrophilic domains, are able to self-assemble in aqueous media,

and in doing so, encapsulate hydrophobic drugs.[4] The use of polymeric nanoparticles increases the drug's stability, concentration, and targeting capability.

Kohn et. al have previously published the synthesis, characterization and application of a tyrosine-derived biodegradable polymer capable of self-assembling into a nanosized micelle.[8] These nanospheres, dubbed Tyrospheres™, consist of an ABA triblock copolymer in which the "A" block is a hydrophilic poly(ethylene glycol) moiety and the "B" block is a hydrophobic tyrosine based polyester that self-assemble into Tyrospheres™ when exposed to aqueous media.

Tyrospheres™ have been shown to be highly stable and to successfully encapsulate a series of hydrophobic drugs with high drug loading. [9-11] Moreover, they have been shown to enhance the skin penetration of lipophilic compounds making them ideal drug carriers for topical skin applications.[12] In order to continue ongoing research into drug encapsulation using Tyrospheres™, it has recently become necessary to repeat the synthesis of the ABA triblock copolymer from which they are assembled. Though synthesis appeared successful by initial characterization methods, the newly synthesized polymer, and Tyrospheres™ made from it, did not exhibit the same properties as previously reported. The investigation into the chemical differences that could lead to this discrepancy is the topic of this thesis.

CHAPTER 2. Polymer Synthesis and Tyrosphere™ Formulation

2.1 Introduction

Tyrospheres™ are self-assembled from a triblock copolymer of desaminotyrosyl tyrosine octyl ester (DTO), suberic acid (SA) and 5,000 Da polyethylene glycol monomethyl ether (PEG(5k)). This triblock polymer is designed to have a hydrophobic block consisting of an oligomer of DTO and SA hereby referred to as oligo(DTO suberate) and two hydrophilic blocks of PEG, one on each end of the hydrophobic block (Figure 2.1).

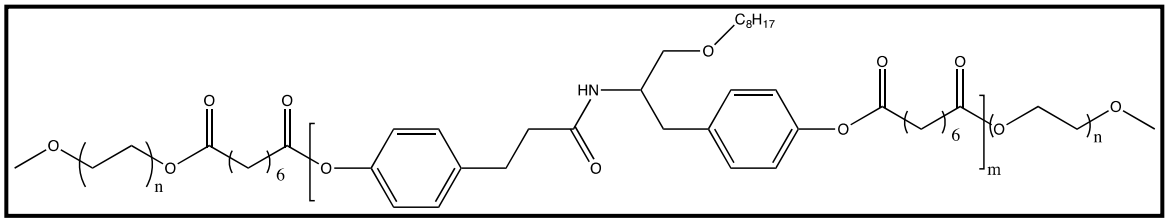


Figure 2.1. Structure of poly(ethylene glycol)-block-oligo(DTO suberate)-block-poly(ethylene glycol).

Synthesis of poly(ethylene glycol)-block-oligo(DTO suberate)-block-poly(ethylene glycol) hereby referred to as DTO/SA-PEG(5k) was conducted in an effort to match an existing batch of polymer (hereby referred to as Batch L) from previously published work. The size characterization properties of Batch L, determined by gel permeation chromatography (GPC) are listed in Table 2.1. This data was collected in 2010 during the original synthesis of Batch L. Additionally;

GPC was conducted during the current research in order to ensure the size of Batch L remained unchanged over time. As indicated in Table 2.1, the size of DTO/SA-PEG(5k) for Batch L did not change in the time between the published work and current efforts.

Table 2.1. Gel permeation chromatography (GPC) data for Batch L of DTO/SA-PEG(5k) from its original synthesis[13] and during current research. It is not possible to obtain a value for oligo(DTO suberate) once the triblock polymer has been fully assembled. Therefore the assumption was made that matching values for the overall DTO/SA-PEG(5k) indicated matching values for oligo(DTO suberate).

Year	Polymer portion	Mn (kDa)	Mw (kDa)	PDI
2010	oligo(DTO suberate)	13.2	20.0	1.50
2010	DTO/SA-PEG(5k)	23.0	30.0	1.30
2016	DTO/SA-PEG(5k)	21.6	31.3	1.44

2.2 Results and Discussion

2.2.1 *Synthesis of poly(ethylene glycol)-block-oligo(DTO suberate)-block-poly(ethylene glycol)*

Synthesis of poly(ethylene glycol)-block-oligo(DTO suberate)-block-poly(ethylene glycol), hereby referred to as DTO/SA-PEG(5k), was carried out by carbodiimide coupling to form an ester between DTO and suberic acid, followed by

another carbodiimide coupling to form an ester between the acid containing ends of oligo(DTO suberate) and PEG (Figure 2.2) using a previously reported method.[8]

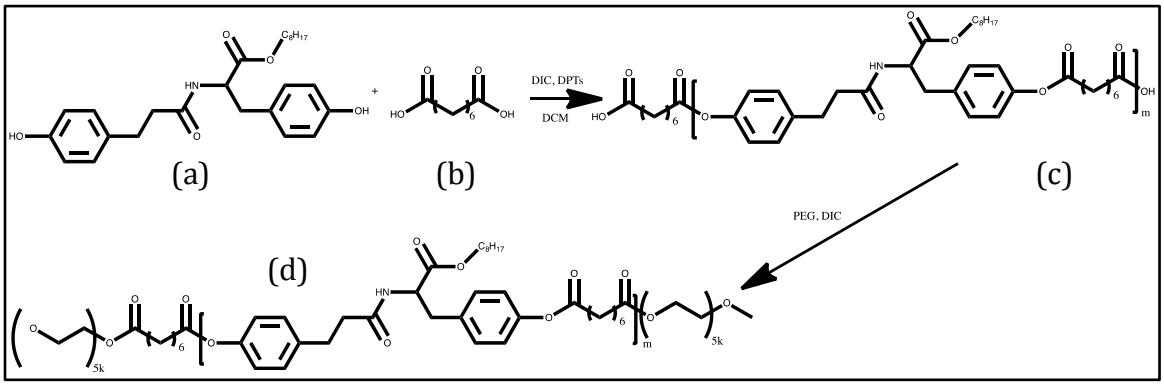


Figure 2.2. Synthetic route for DTO/SA-PEG(5k). DTO (a) and suberic acid (b) are reacted using carbodiimide coupling to form the hydrophobic domain, oligo(DTO suberate) (c). Additional DIC is then used to couple the hydrophilic domains of poly(ethylene glycol) and form the final polymer (d).

As indicated in the procedure, GPC monitoring is conducted every 30-40 minutes in order to monitor growth of oligo(DTO suberate). In an effort to reach the target value of 12-15 kDa without going over, the reaction vessel for Batch 1 was placed at 4°C at 40 minutes once the first GPC aliquot was taken. Lowering the temperature of the reaction effectively halts it, allowing time for the GPC data to be collected. Once the GPC indicated that the molecular number was 13.7 kDa, the reaction was brought back to room temperature and PEG was added.

The overall reaction of Batch 1 was monitored by GPC (Table 2.2) and a polymer of 27.9 kDa was obtained. While this value was slightly larger than the 21.6

kDa of Batch L, it was determined that the matching size of the oligo(DTO suberate) block would result in polymers with similar chemical properties.

Table 2.2. Gel permeation chromatography data for synthesis of Batch 1. Placing the vessel on ice at 40 minutes halted the reaction progress as evidenced by the lack of growth between 40 and 70 minutes. Additional aliquots were taken after each step of the reaction in order to monitor molecular weight changes.

Sample Time	Mn (kDa)	Mw (kDa)	PDI	Retention Time (min)
40 minutes	13.7	21.4	1.56	14.87
70min, just before PEG addition	13.0	19.6	1.51	15.02
6 hours (after PEG addition)	24.3	27.5	1.14	14.77
Post work-up and 1st precipitation	25.1	30.8	1.23	14.44
Post 2nd precipitation	27.9	33.3	1.19	14.36

Furthermore Batch 1 was analyzed by differential scanning calorimetry (DSC), thermogravimetric analysis (TGA) and ^{1}H-NMR. By DSC the glass transition temperature (T_g) of the polymer was -26 °C and the melting temperature T_m was 55 °C. By TGA, the decomposition temperature (T_d) was 140 °C. The polymer's ^{1}H-NMR spectrum was fully annotated (Figure 2.3) and shows all relevant peaks with integration matching expectation.

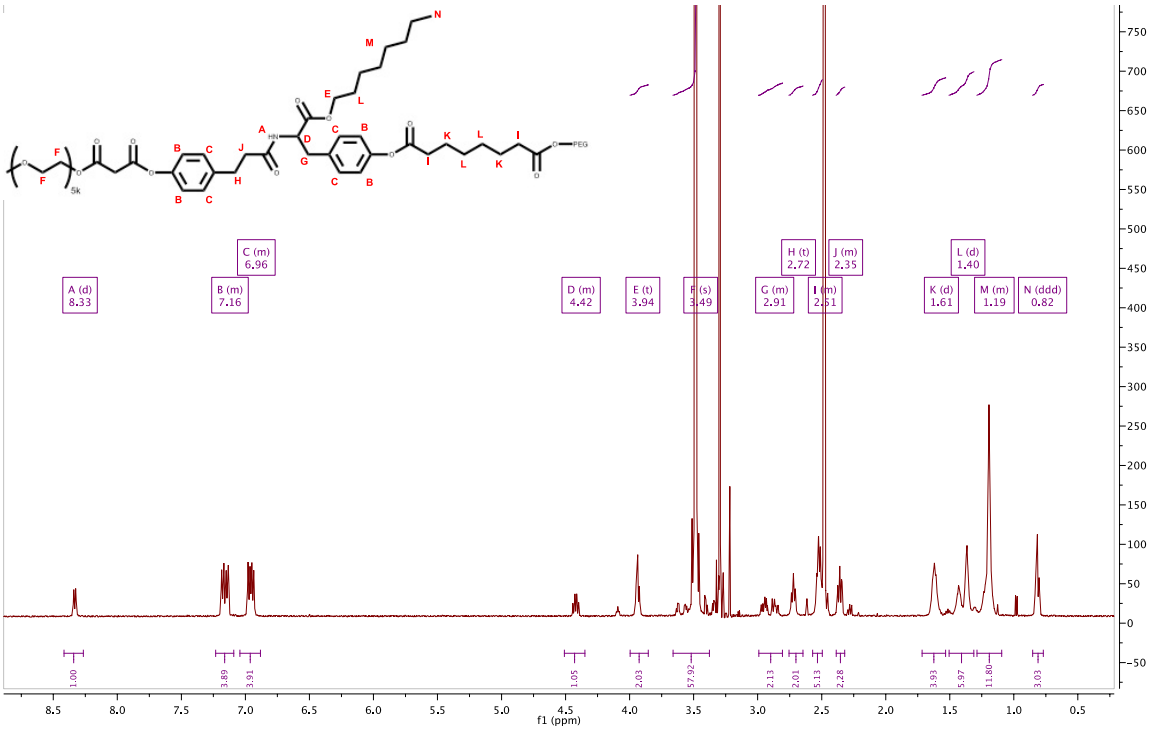


Figure 2.3. Fully annotated ^{1}H-NMR spectrum of DTO/SA-PEG(5k) in dmso-d_6.

The spectrum in Figure 2.3 also indicated that the product was pure (with the exception of a small amount of residual acetic acid visible at 0.99 ppm). This spectrum was compared to that of the starting materials, DTO and suberic acid (Figure 2.4) and formation of the desired product was confirmed by the disappearance of both the acid peak of suberic acid (a) and the phenolic peaks of DTO (e). A spectrum of PEG was not used for comparison as the characteristic PEG singlet at 3.5 ppm is the only major peak change that indicates the addition of PEG to oligo(DTO suberate). Finally, the spectrum was also compared to the ^{1}H-NMR of Batch L. The two spectra matched completely, with the exception of the previously mentioned acetic acid impurity (Figure 2.5).

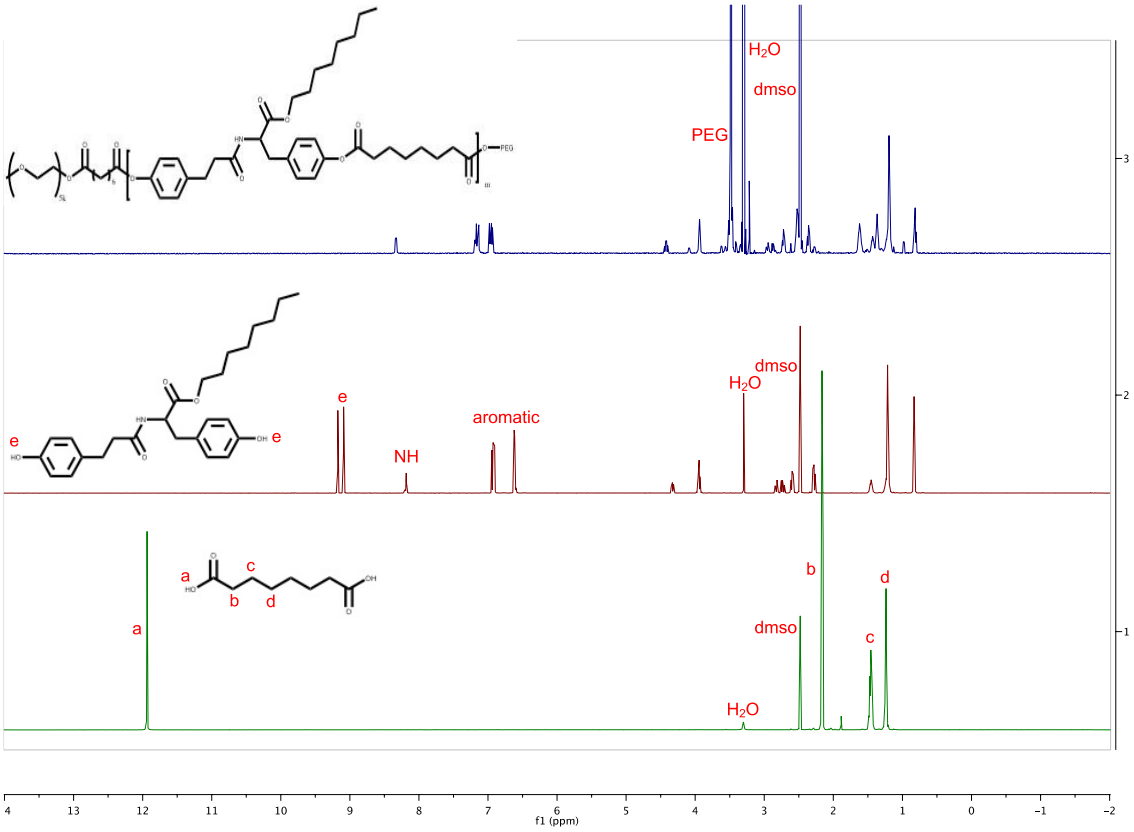


Figure 2.4. Comparative ^{1}H-NMR of DTO/SA-PEG(5k) (top) to its starting materials, DTO (middle) and suberic acid (bottom). Some peaks of particular interest are noted.

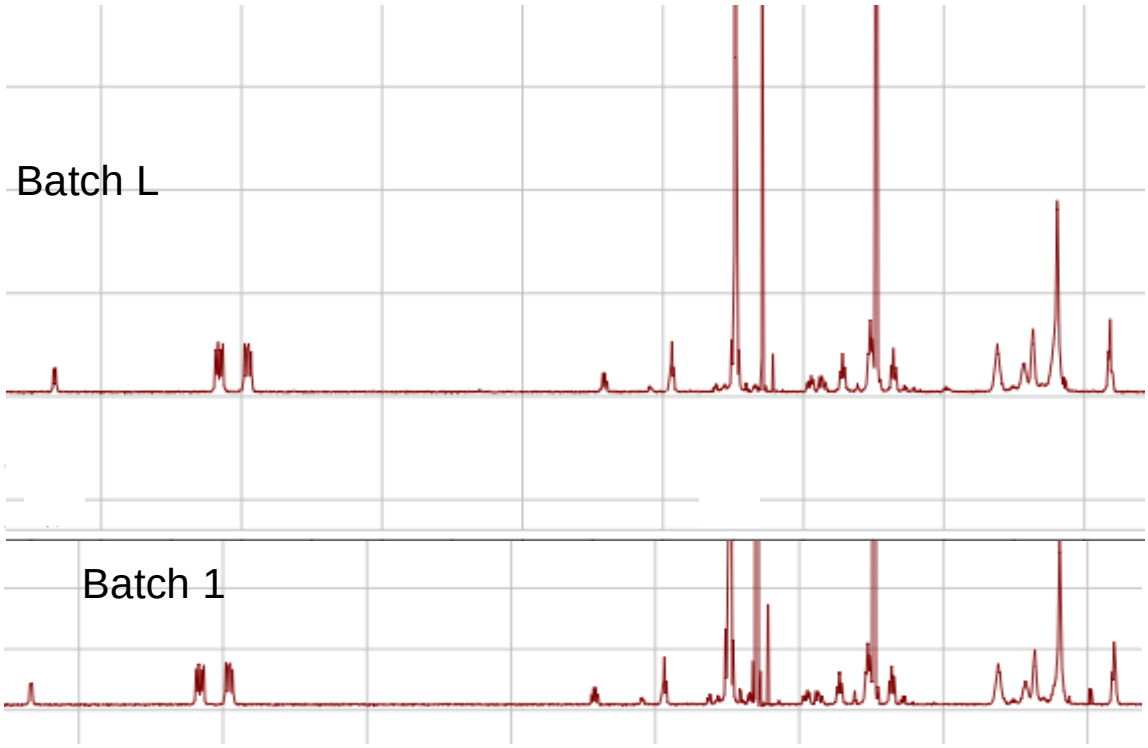


Figure 2.5. Comparative ^{1}H-NMR spectra of DTO/SA-PEG(5k) from Batch L and the newly synthesized, Batch 1.

2.2.2 *Preparation of Tyrospheres™*

Once it was confirmed that Batch 1 of DTO/SA-PEG(5k) met the necessary chemical specifications to match Batch L, the polymer underwent self-assembly into Tyrospheres™ (Figure 2.6). Once again there were specifications for these Tyrospheres™ based on the previous results from Batch L. Specifically, Batch L forms Tyrospheres™ with a hydrodynamic diameter of 66.1 nm as measured by dynamic light scattering (DLS).[13] The generally expected size therefore, for Tyrospheres™ made from Batch 1 was between 62-70 nm.

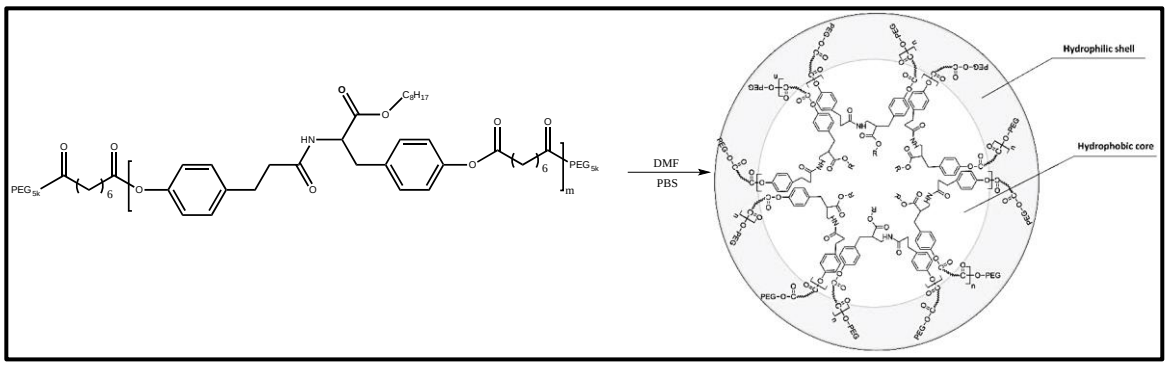


Figure 2.6. Scheme for self-assembly of DTO/SA-PEG(5k) into nanospheric micelles referred to as Tyrospheres™.

With these specifications in mind, Tyrospheres™ were self-assembled by dissolving polymer and adding it dropwise to aqueous media according to a previously reported method.[8] The polymer assembles into Tyrospheres™ upon contact with water, is then filtered and centrifugation is used to separate particles from solvent and residual aggregates (Figure 2.7). After re-suspension DLS is used to analyze the resulting spheres.

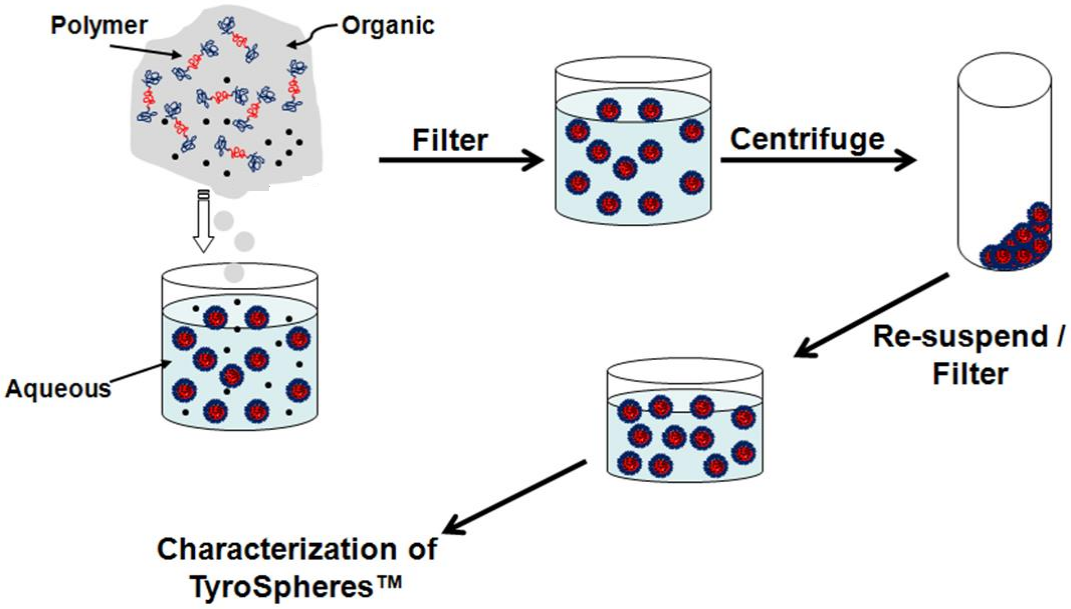


Figure 2.7. Step by step schematic representation of Tyrospheres™ self-assembly procedure.[11]

When characterized by DLS, the Tyrospheres™ resulting from Batch 1, hereby referred to as Batch 1-T, resulted in spheres of 28.6 nm. Given the much unexpected nature of this result, self-assembly was repeated for Batch 1 polymer. In parallel, self-assembly was conducted using polymer from Batch L (Batch L-T) to confirm that proper technique was being used. DLS results for Batch 1-T and Batch L-T were compared (Figure 2.8 and Table 2.3).

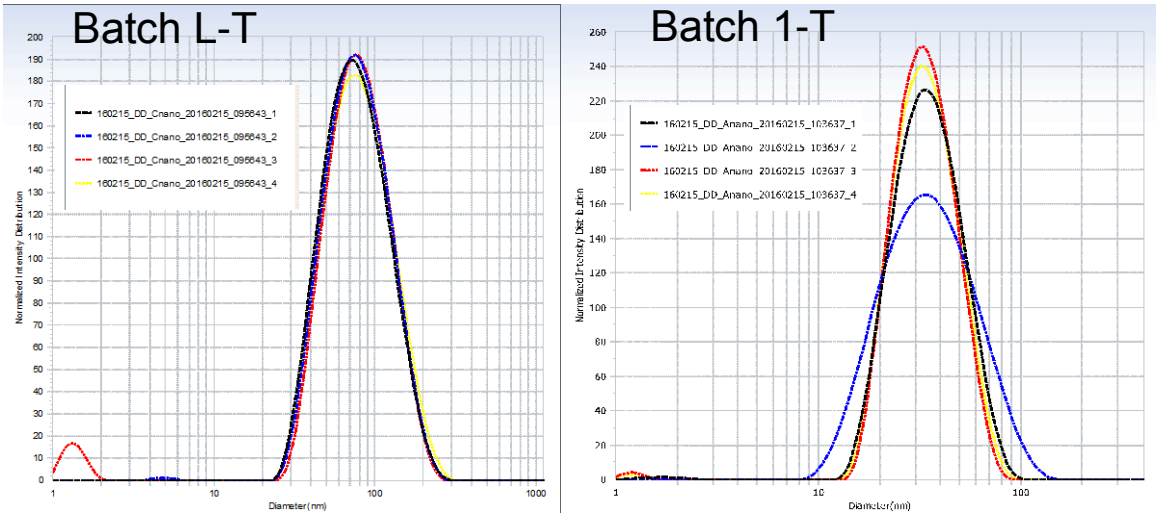


Figure 2.8. Dynamic light scattering data for Tyrospheres™ made from Batch L (on the left) with an average hydrodynamic diameter of 70.8 nm and Tyrospheres™ made from Batch 1 (on the right) with an average hydrodynamic diameter of 31.3 nm.

The DLS data for Batch L-T in Table 2.3 shows a hydrodynamic diameter of 70.8 nm. This value is slightly larger than those previously reported for Tyrospheres™ from Batch L but within an acceptable range. This indicates that no error in the self-assembly procedure is occurring. The DLS value for Batch 1-T in Table 2.3 shows a hydrodynamic diameter of 31.3 nm confirming the initial values for Tyrospheres™ made from Batch 1 and illustrating good reproducibility.

Table 2.3. Dynamic light scattering values measuring the hydrodynamic diameter of Tyrospheres™.

Sample Name	Run number	Diameter (nm)	Polydispersity Index	D (10%) (nm)	D (50%) (nm)	D (90%) (nm)
Batch 1-T	1	32.0	0.130	20.3	33.1	54.5
Batch 1-T	2	30.3	0.223	17.0	33.1	65.6
Batch 1-T	3	31.4	0.111	20.7	32.0	50.4
Batch 1-T	4	31.6	0.110	20.4	32.2	51.9
Batch 1-T	Average	31.3	0.144	19.6	32.6	55.6
Batch L-T	1	70.9	0.193	40.6	71.8	132.5
Batch L-T	2	70.5	0.161	41.7	73.9	134.3
Batch L-T	3	70.5	0.174	40.4	73.9	132.9
Batch L-T	4	71.1	0.168	41.3	74.6	140.3
Batch L-T	Average	70.8	0.174	41.0	73.6	135.0

The slight molecular weight difference between Batch 1 and Batch L (27.9 kDa versus 21.6 kDa) raised the question of whether it was possible that the larger polymer was resulting in larger spheres that were somehow aggregating or being removed during the filtration step. One last batch of Tyrospheres™ was made from Batch 1 and the filtration step was skipped. The resulting spheres were once again analyzed by DLS and showed an average hydrodynamic diameter of 33.2 nm. (Figure 2.9 and Table 2.4). This value is consistent with the diameter of the filtered

Tyrospheres™ from Batch 1-T and indicates that the ~32 nm spheres are the only ones forming during self-assembly. The lack of agreement between Batch 1-T and Batch L-T led to the next series of experiments.

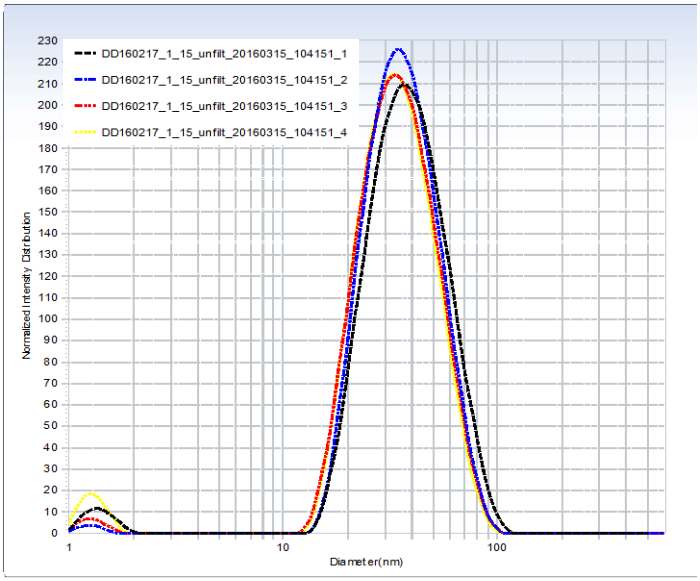


Figure 2.9. Dynamic light scattering data for Tyrospheres™ made from Batch 1 without completing the filtering step. They have an average hydrodynamic diameter of 33.2 nm.

Table 2.4. Dynamic light scattering values measuring the hydrodynamic diameter and polydispersity of Tyrospheres™.

Sample Name	Run number	Diameter (nm)	Polydispersity Index	D (10%) (nm)	D (50%) (nm)	D (90%) (nm)
Batch1-T unfiltered	1	34.5	0.148	20.8	35.9	61.5
Batch1-T unfiltered	2	34.1	0.186	20.9	34.0	56.5
Batch1-T unfiltered	3	32.4	0.196	19.4	32.6	55.5
Batch1-T unfiltered	4	31.7	0.219	18.6	31.9	54.2
Batch1-T unfiltered	Average	33.2	0.187	19.9	33.6	56.9

2.2.3 *Repeat Synthesis of DTO/SA-PEG(5k)*

The synthesis of DTO/SA-PEG(5k) was repeated various times to try and determine the cause of the discrepancy with Batch L. While the same basic procedure was used for each new batch, slight variations were applied in an effort to obtain polymer that resulted in the correct size of Tyrospheres™. Information about batch sizes and variations can be found in Table 2.5.

Table 2.5. GPC data for four batches of DTO/SA-PEG(5k) synthesized using minor (or no) variations to existing procedure.

Batch Number	Mn of oligo(DTO suberate (kDa)	Mn of DTO/SA-PEG(5k) (kDa)	Procedural variation
Batch 2	11.0	20.9	Placed on ice at 30 min
Batch 3	----*	21.1	Kept at RT, monitored by GPC every 10 minutes
Batch 4	----*	24.4	Kept at RT
Batch 5	13.5	26.5	Kept at RT

*THF based GPC instrument was unavailable at the time of oligo(DTO suberate) synthesis and therefore only final value is available.

The Mn of oligo(DTO suberate) and DTO/SA-PEG(5k) are consistently within the desired range to reproduce Batch L. Procedural variations however revealed inconsistencies in the synthesis. Previous descriptions of the reaction kinetics for this synthesis claimed that at room temperature, oligomer growth of the middle block occurred too rapidly to monitor and that the molecular weight was often too large by the time GPC data became available.[13] Current research however, indicated that the reaction self terminates after approximately 40 minutes (Table 2.6).

Table 2.6. GPC data for synthesis of DTO/SA-PEG(5k) monitored every 10 minutes.

Sample Name	Sample time (min)	Mn* (Da)	Mw (Da)	PDI
Batch 3	30	16456	18479	1.12
Batch 3	40	23965	28888	1.21
Batch 3	50	23614	27922	1.18

*This data was generated using a DMF GPC. It is included to indicate polymer growth as a function of time.

Based on the Carothers's equation (Figure 2.10), the use of excess suberic acid in the synthesis of oligo(DTO suberate) will theoretically terminate the reaction at a particular molecular weight.[14] As seen in the equation, the target molecular weight, or more specifically, the degree of polymerization, is directly controlled by the amount of excess material used. This theory is consistent with the current finding that oligo(DTO suberate) synthesis self-terminated after approximately 40 minutes, and disagrees with previous observations that the reaction would continue to grow uncontrollably if left unquenched. According to the Carothers's equation, the ratio necessary to obtain a 13 kDa oligomer of DTO and suberic acid is 1:1.096. This value disagrees with the procedural value of 1:1.125 that was used in the previously published synthesis.[8, 13] What is unclear is whether the previous syntheses were allowed to run to exhaustion to in fact determine where the reaction would self-terminate, and whether a smaller excess (1:1.096) was previously attempted.

Figure 2.10. The Carothers's equation for linear polymerization of two monomers with one monomer in excess.[14]

$$\tilde{X}_n = \frac{1+r}{1+r-2rp}$$

where $\tilde{X}_n$ is the degree of polymerization
r is the excess of a particular reagent
p is the extent of reaction

In the limit of a complete reaction where p=1, the equation simplifies to:

$$\tilde{X}_n = \frac{1+r}{1-r}$$

2.2.4 *Repeat preparation of Tyrospheres™*

Since GPC data continued to produce polymers that appeared to match Batch L in molecular weight, Tyrospheres™ were made from each new batch to determine whether the appropriate size was being obtained. The data for these Tyrosphere™ batches is summarized in Table 2.7.

Table 2.7. Tyrosphere™ size resulting from self-assembly conducted with the multiple batches of DTO/SA-PEG(5k) synthesized.

Batch Name	Resulting Tyrosphere™ diameter (nm)
Batch 2-T	none*
Batch 3-T	35.8
Batch 4-T	35.6
Batch 5-T	36.9

*DLS showed polydisperse aggregates and no sign of monodisperse spheres

Despite molecular weights that correspond with Batch L, each polymer used to make Tyrospheres™ continued to produce spheres much smaller than expected.

2.3 Conclusion

In order to continue past research, DTO/SA-PEG(5k) was once again synthesized through carbodiimde coupling of a diol (DTO) to a diacid (suberic acid) and then subsequent addition of PEG end-groups. Multiple polymer batches matching the size specification by GPC for Batch L, a previously synthesized and published polymer, were made. However, these batches all failed to produce Tyrospheres™ of expected size. Variation of procedural steps for this experiment revealed discrepancies between past descriptions of kinetics and polymer chain termination as compared to current experiments.

2.4 Experimental

2.4.1 *Materials*

Suberic acid – SA (Fluka 406527/2), Desaminotyrosyl tyrosine octyl ester - DTO (NJCBM), 4-dimethylaminopyridinium-p-toluene sulfate – DPTS (NJCBM), Methylene chloride – DCM (Fisher #D143-4), Diisopropylcarbodiimide – DIC (FSTBD4871V), Poly(ethylene glycol) monomethyl ether MW 5000 – PEG5K (BCBG9781V), 2- Propanol – IPA (Sigma Aldrich #190764), Dulbecco's phosphate buffered saline - PBS (Sigma Aldrich #D8537), *N*,*N*-dimethylformamide – DMF (Fisher #DX1726-1), Tetrahydrofuran –THF (Fisher #TX0282-1), Acetic acid (Fisher #AC22214). All materials were used as received.

2.4.2 *Characterization*

Polymer molecular weights (M_w) and number average molecular weight (M_n) were determined by gel permeation chromatography (GPC, Waters) in THF or where indicated specifically, DMF (0.1%TFA) as the eluting solvent. The calibration curve for GPC was created by using standards of polystyrene of Mw from 7.2 up to 526 kDa.

NMR spectra were obtained by conducting 64 scans on a Varian 500 MHz spectrophotometer. Samples were dissolved in deuterated dimethyl sulfoxide (DMSO-*d6*).

Glass transition and melting temperatures were determined using differential scanning calorimetry (DSC) Model DSC 823e (Mettler-Toledo Inc., Columbus, OH). A sample of approximately 5 to 15 mg was heated from -50 to 200 °C at a rate of 10 °C per minute, and then kept at 200 °C for 5 minutes , cooled and then heated again from -50 °C to 200 °C at a rate of 10 °C per minute.

Thermogravimetric analysis was conducted using a Thermogravimetric Analyzer (TGA) Model TGA/SDTA851e with STARe software version 19.10 (Mettler-Toledo Inc., Columbus, OH). Approximately 10 mg of polymer was heated from 25 to 250 °C at a rate of 10 °C per minute.

2.4.3 *Experimental*

2.4.3.1 *Synthesis of poly(ethylene glycol)-block-oligo(DTO suberate)-block-poly(ethylene glycol)*[8]

1 molar equivalent of desaminotyrosyl tyrosine octyl ester (DTO), 1.125 molar equivalents of suberic acid (SA) and 0.6 molar equivalents of 4-

dimethylaminopyridiniu-p-toluene sulfate (DPTs) were combined and dissolved in methylene chloride (DCM). The reaction mixture was stirred at room temperature until a homogenous solution was obtained. Next, 3 molar equivalents of diisopropylcarbodiimide (DIC) was added and solution continued stirring at room temperature. Molecular number was monitored by gel permeation chromatography (GPC) every 30-40 minutes to determine when target molecular number (12-14kDa) was obtained.

Once oligo(DTO suberate) reached appropriate molecular number, 0.25 molar equivalents poly(ethylene glycol) monomethyl ether (M_w 5000 Da) was added to the reaction mixture followed five minutes later by a second portion of 0.6 molar equivalents DIC. The reaction was then allowed to stir overnight.

The reaction was quenched with acetic acid, concentrated, re-dissolved in minimal DCM and precipitated over 2-propanol. Product was filtered and dried overnight to produce a white powder. Additional precipitations were performed to increase the molecular number and decrease the polydispersity index of the polymer.

Poly(ethylene glycol)-block-oligo(DTO suberate)-block-poly(ethylene glycol). Yield: 5.86 g, white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 833 (d, J = 7.4 Hz, 1H, NH), 7.23 – 7.09 (m, 4H, aromatic), 7.04 – 6.88 (m, 4H, aromatic), 4.51 – 4.35 (m, 1H, CH), 3.94 (t, J = 6.6 Hz, 2H, $COOCH_2$), 3.49 (s, 58H, PEG CH_2), 2.99 – 2.81 (m, 2H, CH_2), 2.72 (t, J = 7.6 Hz, 2H, benzyl CH_2), 2.57 – 2.50 (m, 4H, $COOCH_2$), 2.39 – 2.32 (m, 2H, CH_2), 1.61 (d, J = 12.8 Hz, 4H, CH_2), 1.40 (d, J = 31.9 Hz, 6H, CH_2), 1.29 – 1.09 (m, 12H, CH_2), 0.82 (ddd, J = 7.7, 6.0, 1.7 Hz, 3H, CH_3). M_N: 27.9 kDa, M_w: 33.3 kDa, PDI 1.19.

2.4.3.2 *Preparation of Tyrospheres™* [8]

600 mg of DTO/SA-PEG(5k) was dissolved in 600 µL of dimethylformamide (DMF). This solution was then added dropwise to 14.4 mL of phosphate buffered saline (PBS) under constant magnetic stirring. The resulting solution was stirred for an additional 5 minutes and then filtered (using a 0.22 µm filter) into an ultracentrifuge tube. The Tyrospheres™ underwent 3 hours of ultracentrifugation at 65000 RPM and 18°C. Afterwards the supernatant was discarded and the resulting pellet was washed twice with 1 mL PBS and was left in 1 mL PBS to re-suspend the Tyrospheres™. The tube was wrapped in parafilm and placed on an orbital shaker overnight for the re-suspension.

Tyrospheres™. Yield: Size 31.3 nm, 45% yield.

2.4.4 Dynamic Light Scattering

Particle size and polydispersity index were conducted on a Beckman Coulter Delsa™ Nano DLS. Samples consisted of approximately 10 mg/mL of polymer and were taken at 25 °C. The Tyrosphere™ suspensions were analyzed for cumulants, size distribution and polydispersity by a normalized intensity distribution.

CHAPTER 3. Molecular Weight Variation to Vary Tyrosphere™ Size

3.1 Introduction

Once it became clear that producing a polymer with molecular weight identical to Batch L (by GPC) would no longer yield Tyrospheres™ of the desired size, it became necessary to determine what kind of effect altering the size of the hydrophobic block would have on the resulting spheres and what molecular weight would produce the desired Tyrosphere™ hydrodynamic diameter. Through molecular modeling, it was previously determined that given the fixed size of the PEG hydrophilic domains, it is the size of the oligo(DTO suberate) hydrophobic block that affects the hydrodynamic diameter of the resulting Tyrosphere™.[15] By altering the molecular weight of this block it is possible to predictably control the resulting sphere size. However, those calculations were based on an absolute molecular weight in agreement with the values for Batch L. Given the issues with recreating Batch L, it became necessary to synthesize a series of polymers all with different size hydrophobic blocks in order to correlate their molecular weights with the Tyrosphere™ diameters they produced.

There are two methods available for increasing the molecular weight of an oligomer like DTO suberate. The first is to vary the amount of coupling agent (in this case DIC) added to the reaction. Presumably, reactions to which additional coupling agent is added will continue to react and result in a higher molecular weight. The second method to control molecular weight of a polymer involves use of the Carothers's equation introduced in Chapter 2.[14] By varying the amount of

monomeric excess used in the reaction, polymerization should self-terminate at a predetermined, calculated molecular weight. This method has the potential to offer more control than by varying coupling agent.

Therefore, both methods were used and Tyrospheres™ were self-assembled from the newly synthesized variety of polymer sizes. The resulting sphere sizes were compared to determine both the effect of both small and large molecular weight fluctuations as well as to identify the optimal molecular weight to recreate Tyrospheres™ like those in Batch L-T.

3.2 Results and Discussion

3.2.1 *Synthesis of DTO/SA-PEG(5k) with varying oligo(DTO suberate) molecular weight by varying amount of coupling agent*

When varying experimental conditions for Chapter 2, it was noted that adding additional aliquots of DIC to ongoing reactions of DTO and suberic acid, could increase the molecular weight of oligo(DTO suberate) obtained. To this end, synthesis of DTO/SA-PEG(5k), was carried out in triplicate and the amount of DIC added to each sample was varied slightly from the original synthesis.[8]

All reaction samples began identically and we run with no variation for 1 hour. At the 1 hour mark, sample 1 was “quenched” by adding PEG (and the aliquot of DIC typically added along with PEG) while an additional aliquot of DIC alone was added to samples 2 and 3. This terminated the growth of sample 1’s hydrophobic core while the additional DIC caused samples 2 and 3 to continue to grow. At hour 2, sample 2 was “quenched” with PEG while sample 3 received yet another aliquot of

DIC. Finally at hour 3, sample 3 was "quenched" with PEG. GPC analysis was conducted on each sample, at each hour time point in order to chart the growth of the hydrophobic block and the PEGylated final polymer (Table 3.1).

Table 3.1. GPC data obtained for DTO/SA-PEG(5k) synthesis in triplicate with varying amounts of DIC added. Color coordination was added for simplicity.

Sample name	Time point (min)	Mn (Da)	Mw (Da)	PDI
Sample 1	60	12310	20206	1.64
Sample 2	60	12514	19257	1.54
Sample 2	120	12876	19934	1.55
Sample 3	60	17018	24762	1.58
Sample 3	120	25177	41378	1.64
Sample 3	180	25630	41448	1.62
Sample 1	Post work up	28944	34273	1.18
Sample 2	Post work up	22856	29775	1.30
Sample 3	Post work up	43292	55062	1.27

Table 3.1 indicates that sample 2 did not continue to grow after the addition of DIC at 60 minutes while sample 3 did. Likewise sample 3 did not continue to grow after the addition of DIC at 120 minutes. Also clear from Table 3.1 is the fact that although PEG(5k) is added to both sides of the hydrophobic block, there is not

always an exact 10 kDa change in the Mn of the polymer. Therefore, sample 1 is larger after workup than sample 2 despite having the same size hydrophobic core.

In addition to GPC, all batches were analyzed by ^{1}H-NMR in order to confirm their structure.

3.2.2 *Synthesis of oligo(DTO suberate) of varying molecular weight by use of the Carothers's equation*

Varying the amount of DIC added proved to alter the molecular weight of the hydrophobic block as expected but did not do so in a predictable, nor reproducible way. To this end, another method of varying the hydrophobic block size was used. As described in Chapter 2, the Carothers's equation describes the amount of monomeric excess necessary to terminate a reaction at a desired molecular weight. (Equation 2.1). Therefore, synthesis of oligo(DTO suberate) was set up in quadruplicate and the molar equivalence of suberic acid was varied for each sample in order to achieve a molecular weight (and degree of polymerization, DP) predicted by the Carothers's equation.

For this experiment the desired DP's were based off of the molecular weight for oligo(DTO suberate) of Batch L (13 kDa) and the molecular weight for oligo(DTO suberate) that produced the most desirable results from samples 1-3 (25.6 kDa). Two additional points were selected in order to provide incremental variation to the molecular weights being tested. All desired molecular weights selected were divided by 596 Da (the mass of one DTO-SA monomer unit) to obtain the DP to be used in the Carothers's equation (Table 3.2).

Table 3.2. Carothers's equation predicted molecular weight and degree of polymerization for a given molar excess of suberic acid for quadruplicate synthesis of DTO/SA-PEG(5k) to vary the size of the hydrophobic block.

Sample Name	Desired Mn (kDa)	Desired DP	Molar excess of suberic acid
Sample A	13	21.8	1.096
Sample B	18	30.2	1.068
Sample C	23	38.6	1.053
Sample D	28	47.0	1.043

All four syntheses were carried out at the same time and the only variation between them was the monomeric excess of suberic acid calculated in Table 3.2. The samples were allowed to react for 3 days in order to ensure that polymerization self-terminated and that time was not a factor in their size. In order to ensure reaction termination, GPC aliquots were taken after 3 days and compared to additional aliquots taken at 4 days (Table 3.3). The agreement in size from day 3 to day 4 indicated that the reactions had terminated. All four reactions were quenched and worked up as oligo(DTO suberate) for characterization.

Table 3.3. Molecular weight data confirming reaction termination of oligo(DTO suberate) block and indicating post workup molecular weight information.

Day 3				Day 4			
Sample Name	**Mn (Da)**	**Mw (Da)**	**PDI**	**Sample Name**	**Mn (Da)**	**Mw (Da)**	**PDI**
Sample A	21796	41068	1.88	Sample A	21883	36582	1.67
Sample B	29393	53346	1.82	Sample B	28903	48679	1.68
Sample C	35614	64540	1.81	Sample C	36314	61969	1.71
Sample D	38381	69617	1.81	Sample D	39461	64958	1.65

Post Workup

Sample Name	**Mn (Da)**	**Mw (Da)**	**PDI**
Sample A	19048	33829	1.78
Sample B	24826	47549	1.92
Sample C	29596	57600	1.95
Sample D	30988	59968	1.94

The post workup values in Table 3.3 indicate that the molecular weight values are slightly smaller for oligo(DTO suberate) after precipitation. While these values are all larger than the value predicted by the Carothers's equation, Samples A, B and C all have increasing molecular weights as predicted. Sample D appeared larger than sample C before workup but is the same size as sample C in final analysis.

All samples were further characterized by ^{1}H-NMR (Figure 3.1). Since DTO and suberic acid polymerize in a one to one ratio, it is impossible to determine the degree of polymerization from ^{1}H-NMR. Therefore despite variation in molecular weight, all the spectra of oligo(DTO suberate) were found to be the same. All peaks were in agreement with the structure of oligo(DTO suberate) and after being subjected to an additional precipitation, all samples were pure as seen in Figure 3.1.

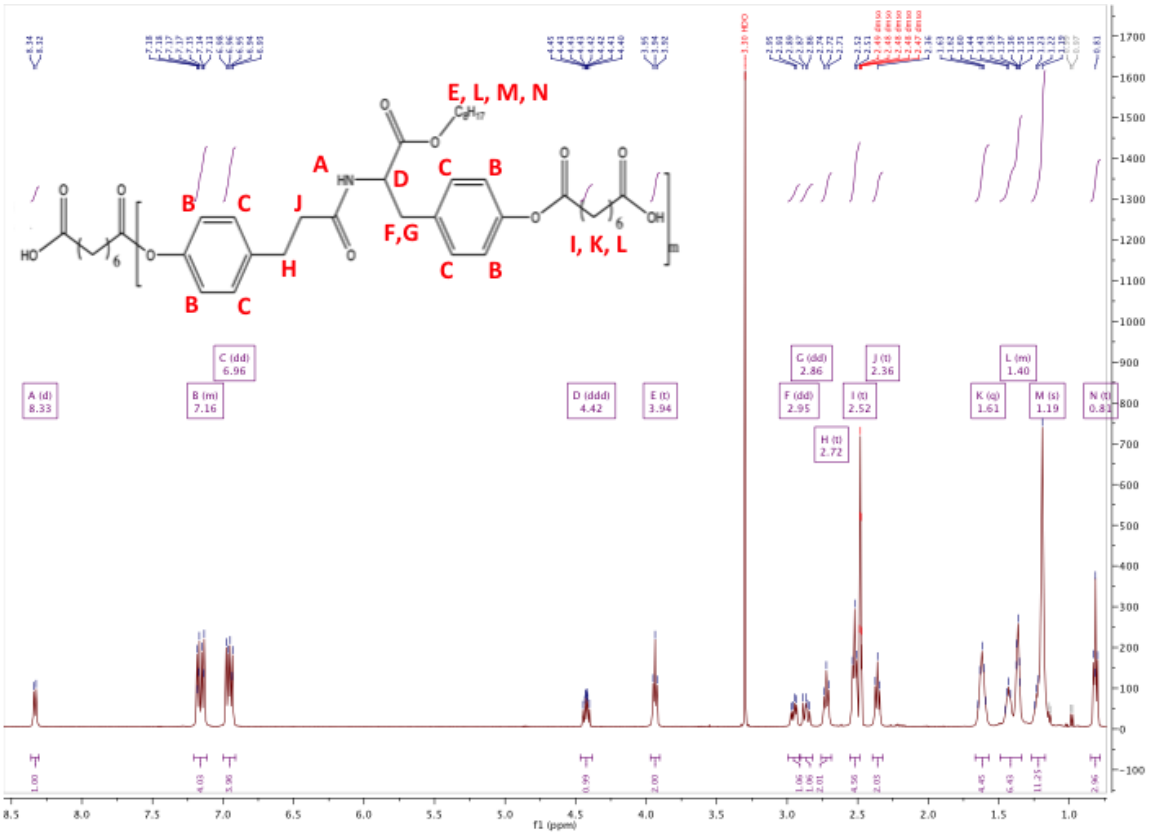


Figure 3.1. Fully annotated ^{1}H-NMR spectrum of oligo(DTO suberate) sample D in dmso-d_6. All samples have identical spectra.

3.2.3 *Synthesis of DTO-SA/PEG(5k) of varying molecular weight by use of the Carothers's equation*[16]

Once the Carothers's equation had been used to synthesize various oligo(DTO suberate) molecular weights, the synthesis was repeated in order to verify that comparable sizes would once again be obtained. This time however, each reaction was PEGylated before workup in order to obtain triblock polymer for use in making Tyrospheres™.

The same DP's listed in Table 3.2 were once again targeted by varying the excess of suberic acid used in the synthesis. Once again each reaction was allowed to go to exhaustion and GPC was used to indicate that polymerization of the hydrophobic block had terminated (Table 3.4). All four reactions were then PEGylated, worked up and characterized. The values obtained for oligo(DTO suberate) agreed with those obtained in samples A-D indicating that the Carothers's equation provides a reproducible and predictable molecular weight.

Table 3.4. Molecular weight data confirming reaction termination of oligo(DTO suberate) block as well as molecular weight of DTO/SA-PEG(5k).

Oligo(DTO suberate)[16]			
Sample Name	**Mn (kDa)**	**Mw (kDa)**	**PDI**
Sample W	22.5	42.7	1.89
Sample X	27.5	49.8	1.81
Sample Y	28.8	51.7	1.79
Sample Z	33.4	58.5	1.75
DTO-SA/PEG(5k)			
Sample Name	**Mn (kDa)**	**Mw (kDa)**	**PDI**
Sample W	29.3	35.7	1.22
Sample X	35.5	46.0	1.30
Sample Y	36.1	47.4	1.31
Sample Z	40.6	55.1	1.36

3.2.4 *Tyrosphere™ preparation*

Tyrospheres™ were prepared as previously described from seven polymer samples (Samples 1, 2 and 3 as well as Samples W, X, Y and Z) in order to determine the effect of oligo(DTO suberate) (hydrophobic core) size on the resulting Tyrosphere™ (Table 3.5). All self-assembly was carried out by identical methods as previously described and the results were compared both to Batch L-T, in order to

determine whether the original target size (70.8 nm) had been achieved, and to one another to understand the effect of oligo(DTO suberate) size on the resulting Tyrosphere™.

Table 3.5. Molecular weight of both hydrophobic block and overall polymer compared to the resulting size of Tyrosphere™ they produce. Polymers for samples 1-3 were made by varying coupling agent amount while polymers W-Z were made by varying monomeric excess according to the Carothers's equation.[16]

Sample Name	Oligo(DTO suberate) Mn (kDa)	DTO/SA-PEG(5k) Mn (kDa)	Average Tyrosphere™ hydrodynamic diameter (nm)
Sample 1	12.3	28.9	40.3
Sample 2	12.9	22.9	37.4
Sample 3	25.6	43.2	81.8
Sample W	22.5	29.3	45.1
Sample X	27.5	35.5	61.9
Sample Y	28.8	36.1	66.7
Sample Z	33.4	40.6	125.6

Table 3.5 illustrates a few key points. First, when comparing Samples 1 and 2 it becomes clear that when the hydrophobic block of two polymers synthesized at the same time are the same, they will result in very similar size Tyrospheres™. However, samples 1 and 2 also indicate that the overall size of the polymer may

affect the size of the spheres as sample 1 which is larger overall produced larger spheres. The second noteworthy point is the lack of agreement between samples 3, W and X. While sample 3 has a hydrophobic block molecular weight that falls between samples W and X, there seems to be no correlation or agreement on the resulting size of the spheres. This relationship can be seen clearly in Figure 3.2. Within each experiment there is a linear trend to the relationship between hydrophobic block size and sphere size. However, between experiments, there is no correlation.

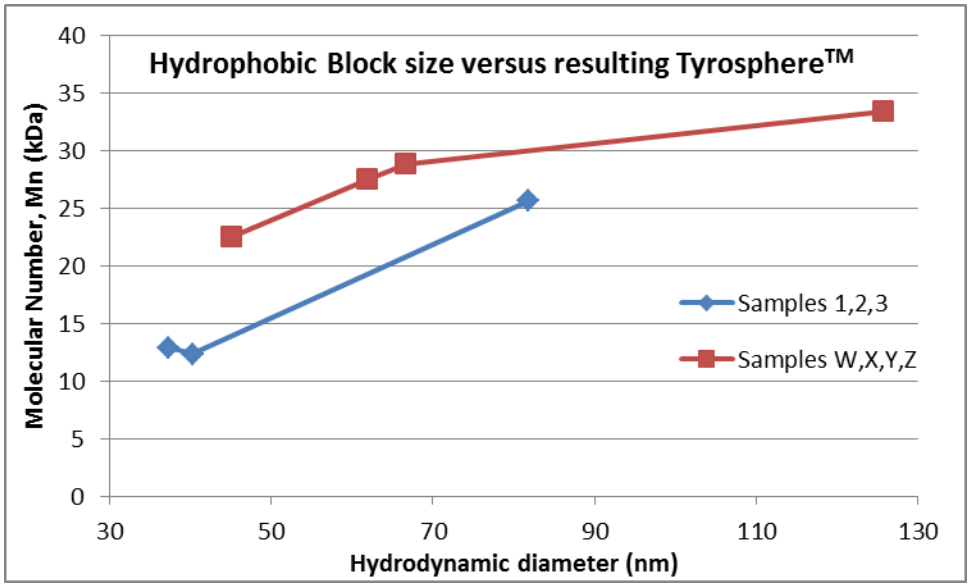


Figure 3.2. Relationship between the size of the hydrophobic block and the resulting sphere diameter for DTO/SA-PEG(5k).

It is worth noting that synthesis for samples 1-3 was carried out with a different batch of monomers than samples W-Z and that in the time between these syntheses the GPC was switched from THF to Water and back to THF, requiring a

recalibration with every change. These changes are enough to cause extreme variation between polymer batches. In fact, along with the lack of agreement between batches shown in Figure 3.2, this is understood to be the reason that although synthesis of DTO/SA-PEG(5k) was initially carried out following the same procedure used for Batch L, and even though the values by GPC seemed identical, the resulting polymers behaved very differently.

3.3 Conclusion

In order to explore the relationship between polymer molecular weight and resulting Tyrosphere™ size, a series of samples of DTO/SA-PEG(5k) were synthesized and the molecular weight of oligo(DTO suberate) (the hydrophobic block) was varied. This was done first by varying the amount of coupling agent and subsequently through use of the Carothers's equation to vary the amount of suberic acid used for the synthesis (monomeric excess). Although use of the equation showed reproducibility, producing oligo(DTO suberate) of comparable values across two experiments, these values were not in precise accordance with those predicted by the equation. A correction factor would have to be included in order to target a specific molecular weight.

In all cases, Tyrospheres™ were produced from these series of DTO/SA-PEG(5k) samples and the size of the spheres was generally seen to increase with an increase in oligo(DTO suberate) molecular weight. Here however it was noted that while this trend holds true between samples from a particular batch, there is no batch to batch agreement in size. Therefore, oligo(DTO suberate) of 25 kDa can

produce spheres of 50 nm for one batch and 82 nm for another. This batch to batch disagreement is believed to be the main reason that Batch L was unable to be replicated despite matching all necessary specifications during polymer synthesis.

3.4 Experimental

3.4.1 *Materials*

Suberic acid – SA (Alfa Aesar G05SO50), Desaminotyrosyl tyrosine octyl ester - DTO (NJCBM, BC15-66 and ML160628), 4-dimethylaminopyridinium-p-toluene sulfate – DPTS (NJCBM), Methylene chloride – DCM (Fisher #D143-4), Diisopropylcarbodiimide – DIC (Alfa Aesar E16X007), Poly(ethylene glycol) monomethyl ether MW 5000 – PEG5K (BCBG9781V), 2- Propanol – IPA (Sigma Aldrich #190764), Dulbecco's phosphate buffered saline - PBS (Sigma Aldrich #D8537), *N*,*N*-dimethylformamide – DMF (Fisher #DX1726-1), Tetrahydrofuran – THF (Fisher #TX0282-1), Acetic acid (Fisher #AC22214). All materials were used as received.

3.4.2 *Characterization*

Polymer molecular weights (M_w) and number average molecular weight (M_n) were determined by gel permeation chromatography (GPC, Waters) in THF as the eluting solvent. The calibration curve for GPC was created by using standards of polystyrene of Mw from 7.2 up to 526 kDa.

NMR spectra were obtained by conducting 64 scans on a Varian 500 MHz spectrophotometer. Samples were dissolved in deuterated dimethyl sulfoxide (DMSO-*d6*).

3.4.3 *Experimental*

3.4.3.1 *Synthesis of DTO/SA-PEG(5k) with varying oligo(DTO suberate) molecular weight by varying amount of coupling agent*

In three separate 20 mL scintillation vials, 1 molar equivalent of desaminotyrosyl tyrosine octyl ester (DTO), 1.125 molar equivalents of suberic acid (SA) and 0.6 molar equivalents of 4-dimethylaminopyridiniu-p-toluene sulfate (DPTs) were combined and dissolved in methylene chloride (DCM). The reaction mixtures were stirred at room temperature until a homogenous solution was obtained. Next, 3 molar equivalents of diisopropylcarbodiimide (DIC) was added to each solution and stirring continued at room temperature.

After 60 minutes a 200 μL aliquot was taken from each reaction mixture,, quenched with 3 drops of acetic acid and dried to completion. These aliquots were saved and analyzed along with all aliquots taken throughout the experiment by THF GPC. Once GPC aliquots were taken, 0.25 molar equivalents poly(ethylene glycol) monomethyl ether (M_w 5000 Da) was added to sample 1 followed five minutes later by a second portion of 0.6 molar equivalents DIC. Sample 1 was then allowed to stir overnight. Also at 60 minutes, 0.35 molar equivalents DIC were added to samples 2 and 3 and then both samples continued stirring at room temperature.

At 120 minutes, GPC aliquots were taken from samples 2 and 3. Immediately after this, 0.25 molar equivalents poly(ethylene glycol) monomethyl ether (M_w 5000 Da) was added to sample 2 followed five minutes later by a second portion of 0.6 molar equivalents DIC. Sample 2 was then allowed to stir overnight. Also at 120

minutes, 0.35 molar equivalents DIC was added to sample 3 and then sample 3 continued stirring at room temperature.

Finally, at 180 minutes a GPC aliquot was taken from sample 3 and to it was immediately added 0.25 molar equivalents poly(ethylene glycol) monomethyl ether (M_w 5000 Da) followed five minutes later by a second portion of 0.6 molar equivalents DIC. Sample 3 was then allowed to stir overnight.

Following overnight stirring, all three samples were quenched with acetic acid, concentrated, re-dissolved in minimal DCM and precipitated over 2-propanol. Product was filtered and dried overnight to produce a white powder

Sample 1 – DTO/SA-PEG(5k). Yield: 0.468 g, white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 833 (d, *J* = 7.4 Hz, 1H, NH), 7.23 – 7.09 (m, 4H, aromatic), 7.04 – 6.88 (m, 4H, aromatic), 4.51 – 4.35 (m, 1H, CH), 3.94 (t, *J* = 6.6 Hz, 2H, $COOCH_2$), 3.49 (s, 58H, PEG CH_2), 2.99 – 2.81 (m, 2H, CH_2), 2.72 (t, *J* = 7.6 Hz, 2H, benzyl CH_2), 2.57 – 2.50 (m, 4H, $COOCH_2$), 2.39 – 2.32 (m, 2H, CH_2), 1.61 (d, *J* = 12.8 Hz, 4H, CH_2), 1.40 (d, *J* = 31.9 Hz, 6H, CH_2), 1.29 – 1.09 (m, 12H, CH_2), 0.82 (ddd, *J* = 7.7, 6.0, 1.7 Hz, 3H, CH_3). M_N: 28.9 kDa, M_w: 34.3 kDa, PDI 1.18.

Sample 2 DTO/SA-PEG(5k). Yield: 0.527 g, white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 833 (d, *J* = 7.4 Hz, 1H, NH), 7.23 – 7.09 (m, 4H, aromatic), 7.04 – 6.88 (m, 4H, aromatic), 4.51 – 4.35 (m, 1H, CH), 3.94 (t, *J* = 6.6 Hz, 2H, $COOCH_2$), 3.49 (s, 58H, PEG CH_2), 2.99 – 2.81 (m, 2H, CH_2), 2.72 (t, *J* = 7.6 Hz, 2H, benzyl CH_2), 2.57 – 2.50 (m, 4H, $COOCH_2$), 2.39 – 2.32 (m, 2H, CH_2), 1.61 (d, *J* = 12.8 Hz, 4H, CH_2), 1.40 (d, *J* = 31.9 Hz, 6H, CH_2), 1.29 – 1.09 (m, 12H, CH_2), 0.82 (ddd, *J* = 7.7, 6.0, 1.7 Hz, 3H, CH_3). M_N: 22.9 kDa, M_w: 29.8 kDa, PDI 1.30.

Sample 3– DTO/SA-PEG(5k). Yield: 0.468 g, white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 833 (d, *J* = 7.4 Hz, 1H, NH), 7.23 – 7.09 (m, 4H, aromatic), 7.04 – 6.88 (m, 4H, aromatic), 4.51 – 4.35 (m, 1H, CH), 3.94 (t, *J* = 6.6 Hz, 2H, $COOCH_2$), 3.49 (s, 58H, PEG CH_2), 2.99 – 2.81 (m, 2H, CH_2), 2.72 (t, *J* = 7.6 Hz, 2H, benzyl CH_2), 2.57 – 2.50 (m, 4H, $COOCH_2$), 2.39 – 2.32 (m, 2H, CH_2), 1.61 (d, *J* = 12.8 Hz, 4H, CH_2), 1.40 (d, *J* = 31.9 Hz, 6H, CH_2), 1.29 – 1.09 (m, 12H, CH_2), 0.82 (ddd, *J* = 7.7, 6.0, 1.7 Hz, 3H, CH_3). M_N: 43.3 kDa, M_w: 55.1 kDa, PDI 1.27.

3.4.3.2 *Synthesis of oligo(DTO suberate) of varying molecular weight by use of the Carothers's equation*

In four 20 mL scintillation vials 1 molar equivalent of desaminotyrosyl tyrosine octyl ester (DTO) and 0.6 molar equivalents of 4-dimethylaminopyridiniu-p-toluene sulfate (DPTs) were combined and dissolved in methylene chloride (DCM). To sample A was added 1.096 molar equivalents of suberic acid. To sample B was added 1.068 molar equivalents of suberic acid . To sample C was added 1.053 molar equivalents of suberic acid. To sample D was added 1.043 molar equivalents of suberic acid. The reaction mixtures were stirred at room temperature until a homogenous solution was obtained. Next, 3 molar equivalents of diisopropylcarbodiimide (DIC) was added to each solution and they continued stirring at room temperature. All reactions stirred for 3 days in order to ensure complete exhaustion of the reaction.

After 3 days, a 200 μL aliquot was taken from each sample, dried completely and analyzed by THF GPC. This was repeated on day 4. GPC values from both days were compared to ensure no further polymer growth. Once reaction termination

was confirmed, the reaction mixtures were concentrated, re-dissolved in minimal DCM and precipitated in 2-propanol. Product was filtered and dried overnight to produce a white powder. Powder was once again dissolved in minimal DCM and precipitated in methanol to remove additional impurities. Once again product was collected by filtration and dried under vacuum.

Sample A - Oligo(DTO suberate). Yield: 0.284 g, white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.33 (d, J = 7.7 Hz, 1H, NH), 7.20 - 7.11 (m, 4H, aromatic), 6.96 (dd, J = 14.8, 8.2 Hz, 4H, aromatic), 4.42 (ddd, J = 8.8, 7.6, 6.2 Hz, 1H, CH), 3.94 (t, J = 6.5 Hz, 2H, $COOCH_2$), 2.95 (dd, J = 13.9, 6.1 Hz, 1H, chiral CH_2), 2.86 (dd, J = 13.9, 8.8 Hz, 1H, chiral CH_2), 2.72 (t, J = 7.6 Hz, 2H, benzyl CH_2), 2.52 (t, J = 7.4 Hz, 4H, $COOCH_2$), 2.36 (t, J = 7.7 Hz, 2H, CH_2), 1.61 (q, J = 6.9 Hz, 4H, CH_2), 1.49 - 1.34 (m, 6H, CH_2), 1.19 (s, 12H, CH_2), 0.81 (t, J = 6.8 Hz, 3H, CH_3). M_N: 19.1 kDa, M_w: 33.8 kDa, PDI 1.78.

Sample B - Oligo(DTO suberate). Yield: 0.105 g, white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.33 (d, J = 7.7 Hz, 1H, NH), 7.20 - 7.11 (m, 4H, aromatic), 6.96 (dd, J = 14.8, 8.2 Hz, 4H, aromatic), 4.42 (ddd, J = 8.8, 7.6, 6.2 Hz, 1H, CH), 3.94 (t, J = 6.5 Hz, 2H, $COOCH_2$), 2.95 (dd, J = 13.9, 6.1 Hz, 1H, chiral CH_2), 2.86 (dd, J = 13.9, 8.8 Hz, 1H, chiral CH_2), 2.72 (t, J = 7.6 Hz, 2H, benzyl CH_2), 2.52 (t, J = 7.4 Hz, 4H, $COOCH_2$), 2.36 (t, J = 7.7 Hz, 2H, CH_2), 1.61 (q, J = 6.9 Hz, 4H, CH_2), 1.49 - 1.34 (m, 6H, CH_2), 1.19 (s, 12H, CH_2), 0.81 (t, J = 6.8 Hz, 3H, CH_3). M_N: 24.5 kDa, M_w: 47.5 kDa, PDI 1.92.

Sample C - Oligo(DTO suberate). Yield: 0.182 g, white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.33 (d, J = 7.7 Hz, 1H, NH), 7.20 - 7.11 (m, 4H, aromatic), 6.96 (dd, J = 14.8, 8.2 Hz, 4H, aromatic), 4.42 (ddd, J = 8.8, 7.6, 6.2 Hz, 1H, CH), 3.94 (t, J = 6.5 Hz, 2H, $COOCH_2$), 2.95 (dd, J = 13.9, 6.1 Hz, 1H, chiral CH_2), 2.86 (dd, J = 13.9, 8.8 Hz,

1H, chiral CH_2), 2.72 (t, *J* = 7.6 Hz, 2H, benzyl CH_2), 2.52 (t, *J* = 7.4 Hz, 4H, $COOCH_2$), 2.36 (t, *J* = 7.7 Hz, 2H, CH_2), 1.61 (q, *J* = 6.9 Hz, 4H, CH_2), 1.49 - 1.34 (m, 6H, CH_2), 1.19 (s, 12H, CH_2), 0.81 (t, *J* = 6.8 Hz, 3H, CH_3). M_N: 29.6 kDa, M_w: 57.6 kDa, PDI 1.95.

Sample D - Oligo(DTO suberate). Yield: 0.263 g, white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.33 (d, *J* = 7.7 Hz, 1H, NH), 7.20 - 7.11 (m, 4H, aromatic), 6.96 (dd, *J* = 14.8, 8.2 Hz, 4H, aromatic), 4.42 (ddd, *J* = 8.8, 7.6, 6.2 Hz, 1H, CH), 3.94 (t, *J* = 6.5 Hz, 2H, $COOCH_2$), 2.95 (dd, *J* = 13.9, 6.1 Hz, 1H, chiral CH_2), 2.86 (dd, *J* = 13.9, 8.8 Hz, 1H, chiral CH_2), 2.72 (t, *J* = 7.6 Hz, 2H, benzyl CH_2), 2.52 (t, *J* = 7.4 Hz, 4H, $COOCH_2$), 2.36 (t, *J* = 7.7 Hz, 2H, CH_2), 1.61 (q, *J* = 6.9 Hz, 4H, CH_2), 1.49 - 1.34 (m, 6H, CH_2), 1.19 (s, 12H, CH_2), 0.81 (t, *J* = 6.8 Hz, 3H, CH_3). M_N: 31.0 kDa, M_w: 60.0 kDa, PDI 1.94.

3.4.3.3 *Synthesis of DTO/SA-PEG(5k) of varying molecular weight by use of the Carothers's equation*

This synthesis followed the same procedure as that of oligo(DTO suberate) until verification that initial polymerization had gone to exhaustion. At that point (Day 4) 0.25 molar equivalents poly(ethylene glycol) monomethyl ether (M_w 5000 Da) was added to each sample followed five minutes later by a second portion of 0.6 molar equivalents DIC. All samples were then allowed to stir overnight.

Following overnight stirring, all four samples were concentrated, re-dissolved in minimal DCM and precipitated over 2-propanol. Product was filtered and dried overnight to produce a white powder.

DTO/SA-PEG(5k).

Sample W - M_N: 29.3 kDa, M_w: 35.7 kDa, PDI 1.22.

Sample X- M_N: 35.5 kDa, M_w: 46.0 kDa, PDI 1.30.

Sample Y- M_N: 36.1 kDa, M_w:47.4 kDa, PDI 1.31.

Sample Z- M_N: 40.6 kDa, M_w: 55.1 kDa, PDI 1.36.

As previously denoted by citation, all synthesis, yield and characterization performed by Mariana Reis Noguiera de Lima.[16] Molecular weight data published with permission.

3.4.3.4 *Preparation of Tyrospheres™* [8]

600 mg of DTO/SA-PEG(5k) was dissolved in 600 µL of dimethylformamide (DMF). This solution was then added dropwise to 14.4 mL of phosphate buffered saline (PBS) under constant magnetic stirring. The resulting solution was stirred for an additional 5 minutes and then filtered (using a 0.22 µm filter) into an ultracentrifuge tube. The Tyrospheres™ underwent 3 hours of ultracentrifugation at 65000 RPM and 18°C. Afterwards the supernatant was discarded and the resulting pellet was washed twice with 1 mL PBS and was left in 1 mL PBS to re-suspend the Tyrospheres™. The tube was wrapped in parafilm and placed on an orbital shaker overnight for the re-suspension.

3.4.4 Dynamic Light Scattering

Particle size and polydispersity index were conducted on a Beckman Coulter Delsa™ Nano DLS. Samples consisted of approximately 10 mg/mL of polymer and were taken at 25 °C. The Tyrosphere™ suspensions were analyzed for cumulants, size distribution and polydispersity by a normalized intensity distribution.

CHAPTER 4: Effect of Sucrose on Tyrosphere™ Yield during Self-Assembly

4.1 Introduction:

When using Tyrospheres™ for drug delivery, three measurements are taken after formulation. The first, drug yield, refers to the amount of drug that remains in the Tyrospheres™ as compared to the amount of drug initially used for self-assembly. Drug yield varies highly from drug to drug and can be greatly impacted by the drug molecule itself. The second measurement taken is drug loading. This is a percentage comparison of the mass of drug found in the Tyrospheres™ to the mass of polymer in those same spheres. Drug loading can be affected both by the drug and the polymer. Finally, there is the measure of polymer yield. After Tyrosphere™ formulation, polymer yield is calculated to determine what percentage of the polymer used for self-assembly remains in the form of a nanosphere. Polymer yield is the only variable in self-assembly that does not depend on what drug is being encapsulated. While all three parameters are important, the challenges facing each of them are unique and must be optimized separately.

Polymer yield is typically conducted using lyophilization to obtain a dry sample.[11] Polymer yield for Tyrospheres™ averages around 55% after all filtrations and centrifugations are complete.[11, 17] In an attempt to further optimize the self-assembly process and improve polymer yield, the use of a sucrose solution during self-assembly was investigated.

Sucrose was first investigated as a cryoprotectant due to its role in protecting biological material during dehydration and rehydration in nature as well as food

products.[18] Based on these findings researchers began using sucrose as a cryoprotectant of biological materials undergoing freezing and thawing procedures in lab.[19] Tyrosphere™ formulations have been mixed with sucrose solutions for the process of freeze-drying and were found to retain their previous size when reconstituted from freeze-dried formulation.[11] In all these cases, sucrose was added after self-assembly as a cyroprotectant before freeze-drying. Recent studies involving sucrose have indicated however that when used during self-assembly, sucrose solutions can have effects on nanostructure and morphology as well as provide stability to the resulting nanomaterials.[20, 21] With this in mind, a study was conducted to determine whether the use of sucrose during the self-assembly process of Tyrospheres™ resulted in an improved polymer yield.

4.2 Results and Discussion

4.2.1. *Tyrosphere™ preparation*

Tyrosphere™ self-assembly was conducted using DTO/SA-PEG(5k) from Batch 5 (Mn = 26 kDa) once again using previously reported methods.[8] In order to study the effect of sucrose on polymer yield within the Tyrospheres™, self-assembly was conducted in triplicate. All three samples of polymer were dissolved in DMF. The first sample was then added dropwise to PBS, following existing protocol. The second sample was added dropwise to a 225 mM sucrose solution in DI water and the third sample was added dropwise to a 225 mM sucrose solution in PBS. This concentration of sucrose was chosen as it is the concentration used when sucrose is added to Tyrospheres™ as a cryoprotectant for freeze-drying.[11] All three samples

were then filtered, ultracentrifuged and re-suspended in PBS. Re-suspension in PBS was kept constant for all three samples in order to ensure that any variation in polymer yield would be the result only of the self-assembly solution.

There was no visual difference in the three samples during or immediately after dropwise addition. After ultracentrifugation, the sample made only in PBS appeared to have the largest resulting pellet. After re-suspension all three samples, once again appeared identical. DLS analysis was used to determine whether there was any change in size of the spheres as a result of the use of sucrose.

Figure 4.1 and Table 4.1 show the DLS data for Tyrospheres™ from each batch. Sucrose had no effect on Tyrospheres™ size or polydispersity index as each batch made spheres with an average hydrodynamic diameter of 36 nm and an average PDI less than 0.1.

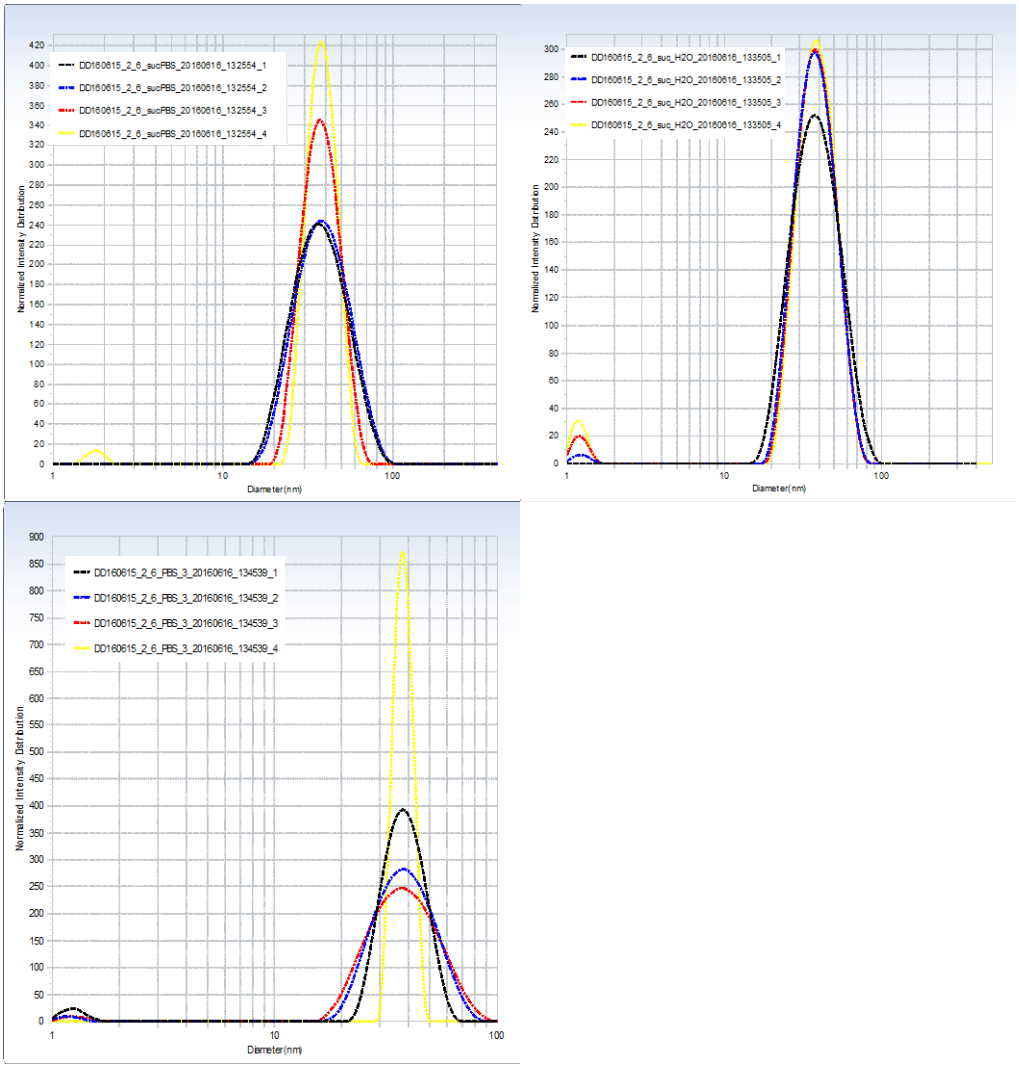


Figure 4.1. Dynamic light scattering values measuring the hydrodynamic diameter and polydispersity of Tyrospheres™ made in (clockwise from upper left) a 225 mM sucrose solution in PBS, a 225 mM sucrose solution in deionized water, and PBS with no sucrose.

Table 4.1. Average hydrodynamic diameter values for Tyrospheres™ made with and without sucrose.

Solution type	Average Diameter (nm)	Average Polydispersity Index
225 mM Sucrose in PBS	36.3	0.067
225 mM Sucrose in DI Water	36.5	0.077
PBS	36.9	0.077

4.2.2 *Polymer Yield Determination*

In order to determine whether the use of sucrose in the self-assembly process had any effect on the polymer yield, an aliquot of each sample was lyophilized and the weight of the aliquot was analyzed before and after lyophilization. In order to conduct this analysis, several controls had to be considered. When determining the polymer yield for Tyrospheres™ made in PBS, an aliquot of PBS alone is also lyophilized. The average mass of solid left over from PBS lyophilization indicates the mass of residual salt from PBS. This is then accounted for in calculations of polymer yield. For the two sucrose-containing samples however it was necessary to take into account the potential for residual sucrose as well as to consider the presence of salt in the sucrose/PBS but the lack thereof in sucrose/DI water. To this end, in addition to a sample of PBS, the following solutions were also lyophilized as controls:

1. 225 mM sucrose in PBS – accounts for a scenario in which all sucrose and salt are left over
2. 225 mM sucrose in DI water – accounts for a scenario in which all sucrose is left over
3. PBS alone in a tube first rinsed with sucrose in PBS – accounts for the potential residual sucrose and salts from self-assembly but assumes that only a minor amount will remain
4. PBS alone in a tube first rinsed with sucrose in DI water – accounts for the potential residual sucrose from self-assembly but assumes that only a minor amount will remain

The post-lyophilization masses of these controls (Table 4.2) were then compared with the masses for the Tyrosphere™ samples made via the sucrose and non-sucrose methods in order to determine polymer yield. While these controls were still somewhat inexact, they were sufficient to indicate whether residual sucrose would hinder calculations enough to pursue more stringent controls.

Table 4.2. Post-lyophilization masses for 0.1 mL aliquots of various control conditions.

Control conditions	Average Mass (mg)
225 mM sucrose in PBS	8.9
225 mM sucrose in DI water	8.8
PBS after rinsing with 225 mM sucrose in PBS	2.1
PBS after rinsing with 225 mM sucrose in DI Water	1.2
PBS	0.7

Each of the Tyrosphere™ samples had almost identical post-lyophilization masses (Table 4.3). Each of these values required a 0.7 mg adjustment for the presence of PBS salts. However, by comparing the values in Table 4.2 with those in Table 4.3 it became clear that sucrose did not improve the yield of Tyrospheres™.

Table 4.3. Post-lyophilization masses for Tyrosphere™ samples .

Sample	Average Mass in Vial (mg)
Tyrospheres™ made in 225 mM sucrose in PBS	5.2
Tyrospheres™ made in 225 mM sucrose in DI water	4.9
Tyrospheres™ made in PBS only	5.2

Taking the case of Tyrospheres™ made in sucrose/PBS for example, the residual mass remaining is 5.2 mg. In order for this sample to contain a large amount of sucrose from the original self-assembly solution, the residual mass would have to be compared to the 8.9 mg residual mass of the 225 mM sucrose solution in PBS. Even after rinsing the tube with this solution and then using only PBS, 2.1 mg of residual mass would remain. This indicates that if sucrose is present then the residual mass for this Tyrosphere™ sample is at best equal to that of Tyrospheres™ made in PBS alone, and at worst is far lower. The same case can be made for the Tyrospheres™ made in 225 mM sucrose in DI water, which also has a value comparable to the PBS only sample. Therefore, sucrose offers no improvement and in fact may lower polymer yield within the Tyrospheres™. If only the residual mass of PBS salts are considered, Table 4.4 summarizes the polymer yields for the three self-assembly methods. This once again reiterates that the use of sucrose during the self-assembly process does not improve polymer yield.

Table 4.4. Polymer yields for Tyrospheres™ made with and without sucrose as part of the self-assembly process.

Sample	Average Polymer Yield (%)	Standard Deviation
Tyrospheres™ made in 225 mM sucrose in PBS	91	2
Tyrospheres™ made in 225 mM sucrose in DI water	85	1
Tyrospheres™ made in PBS only	91	1

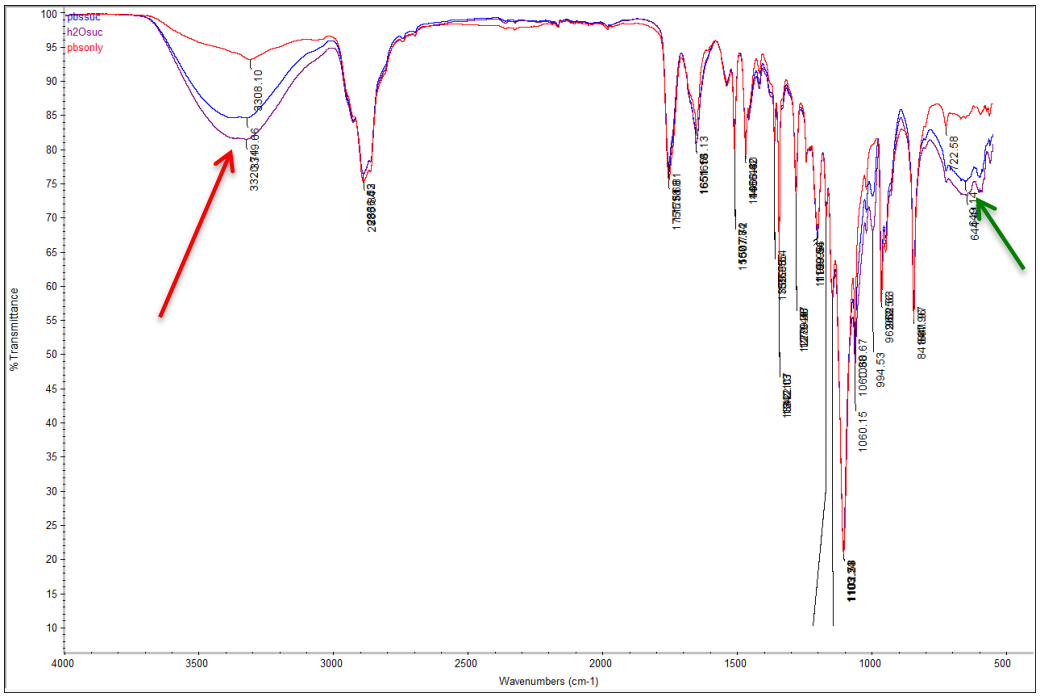
4.2.3 *Infrared Spectroscopy to characterize residual sucrose in Tyrospheres™*

The polymer yields in Table 4.4 were calculated by only accounting for the presence of PBS salts in the samples. This offered the best-case value for sucrose's effect on polymer yield. However, as previously mentioned some of the residual mass in each sample could be the result of sucrose, but based on the values from Table 4.2, this would dramatically reduce the polymer yield. In order to detect residual sucrose in the samples, Fourier transform infrared spectroscopy (FT-IR) was utilized.

The three overlapped FT-IR spectra are, as expected, nearly identical (Figure 4.2a). There are however two regions (highlighted by arrows) that indicate the presence of sucrose. The first (red arrow) is the stretch at 3320 cm^{-1}. A stretch in this region is indicative of the hydroxyl groups in sucrose hydrogen bonding (Figure 4.2b).[22] This is also the region where water appears, also as a result of hydrogen bonding. Therefore, there is a slight stretch in the red, PBS-only spectrum. However, the purple and blue spectra for the samples made in sucrose, show a much stronger stretch in that region. This is either indicative of the presence of sucrose, or that samples made in sucrose are more prone to water uptake. The second arrow (green) indicating the presence of sucrose is at 645 cm^{-1}. This is the stretch indicative of an out of plane hydroxyl bend.[22] In this region, the red, PBS-only spectrum shows no stretch while the purple and blue spectra each show a sizeable stretch in this region. Together these stretches indicate the presence of residual sucrose in these Tyrosphere™ samples and therefore bring the polymer yield for

these samples lower than the previously calculated 91±2% (sucrose in PBS) and 85±1% (sucrose in DI water)

(a)



(b)

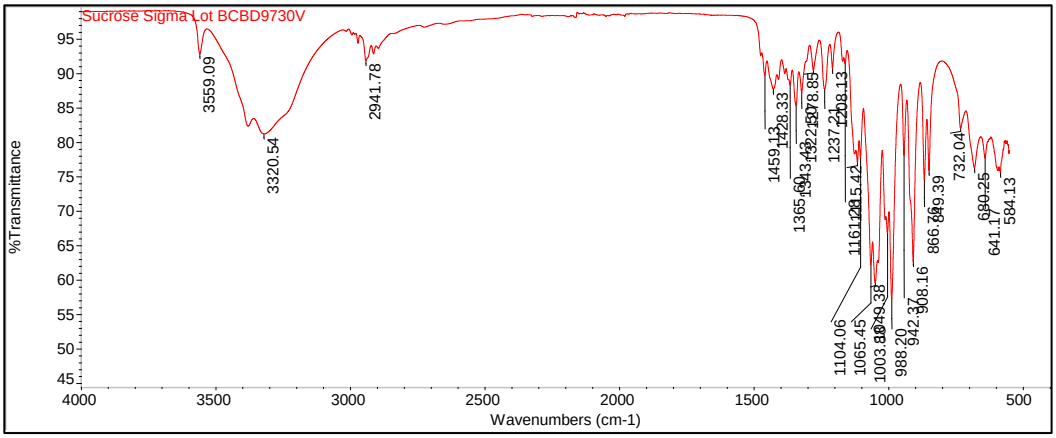


Figure 4.2. (a) Overlapped FT-IR spectra of Tyrospheres™ made in a 225 mM sucrose in PBS solution (blue), a 225 mM sucrose in DI water solution (purple) and in PBS alone (red). There are two regions on the spectra that indicate the presence of residual sucrose; denoted by arrows. (b) FT-IR spectra of sucrose for comparison.

4.3 Conclusion

Self-assembly of Tyrospheres™ was conducted in a sucrose solution in an attempt to improve polymer yield within the spheres. The samples made containing sucrose had the same average mass as the sample made following traditional methods and even if the potential for residual sucrose was ignored, polymer yield was not improved by the presence of sucrose during self-assembly. FT-IR was then used to further characterize the sample and it was found that the samples made in a sucrose solution did contain some residual sucrose therefore lowering their yield even further.

4.4 Experimental

4.4.1 *Materials*

Poly(ethylene glycol)-block-oligo(DTO suberate)-block-poly(ethylene glycol) - DTO-SA/PEG(5k) (NJCBM, Batch# 160601-VBD), Dulbecco's phosphate buffered saline - PBS (Sigma Aldrich #D8537), *N*,*N*-dimethylformamide – DMF (Fisher #DX1726-1), sucrose (Sigma-Aldrich – BCBD9730V), deionized water – DI water. All materials were used as received.

4.4.2 *Experimental*

4.4.3.1 *Preparation of Tyrospheres™* [8]

Three samples of 600 mg of DTO/SA-PEG(5k) were dissolved in 600 µL each of dimethylformamide (DMF). Two samples of 3.851 g of sucrose were dissolved, one in 50 mL of deionized water (DI water) and the other in phosphate buffered saline (PBS.) to obtain two 225 mM sucrose solutions. Each polymer solution was

then added dropwise to one of the following solutions: (1) 14.4 mL of phosphate PBS, (2) 14.4 mL of 225 mM sucrose in PBS, (3) 14.4 mL of 225 mM sucrose in DI water, all under constant magnetic stirring. The resulting solutions were stirred for an additional 5 minutes and then filtered (using a 0.22 μm filter) into ultracentrifuge tubes. The Tyrospheres™ underwent 3 hours of ultracentrifugation at 65000 RPM and 18°C. Afterwards the supernatants were discarded and the resulting pellets were washed twice with 1 mL PBS and was left in 1 mL PBS to re-suspend the Tyrospheres™. The tubes were wrapped in parafilm and placed on an orbital shaker overnight for the re-suspension.

Tyrospheres™. yield and size information in section 3.2.

4.4.3 *Dynamic Light Scattering*

Particle size and polydispersity index were conducted on a Beckman Coulter Delsa™ Nano DLS. Samples consisted of approximately 10 mg/mL of polymer and were taken at 25 °C. The Tyrosphere™ suspensions were analyzed for cumulants, size distribution and polydispersity by a normalized intensity distribution.

4.4.4 Polymer Yield determination

100 μL aliquots of each sample (Tyrospheres™ made in PBS, Tyrospheres™ made in 225 mM sucrose in PBS, Tyrospheres™ made in 225 mM sucrose in DI water) was placed in a pre-weighed scintillation vial (n=3). These aliquots were then frozen, covered with aluminum foil and lyophilized. Post-lyophilization, the vials were once again weighed to determine the residual mass of Tyrospheres™. Control samples were all handled in the same manner. Polymer yield was calculated

by subtracting the mass of any relevant control from the residual mass in the vials, accounting for dilution and comparing to the initial 60 mg polymer sample.

4.4.5 *Infrared Spectroscopy*

Infrared Spectroscopy was conducted on a ThermoFischer Scientific FT-IR.

CHAPTER 5. Suggestions for Future Work

5.1 Introduction

During the course of this work DTO/SA-PEG(5k) was synthesized 8 times. This allowed for determination of certain trends of behavior within batches of polymer and from batch to batch.

Within any given batch of polymer the size of the Tyrospheres™ produced was reproducible. Batch 5, for example, consistently formed spheres of ~ 36 nm through upwards of 10 self-assemblies. Moreover, though drug loading was not covered in this thesis, work done with a single batch of polymer on loading a given drug was also exceedingly reproducible. [23, 24] The same loading could be achieved batch after batch even though the value achieved differed greatly from that expected for Batch L. This indicates that DTO/SA-PEG(5k) has very good intra-batch reproducibility.

When comparing batch to batch however it is not as straight forward. In every case where the molecular weight of oligo(DTO suberate) was kept to approximately 13 kDa (in accordance with Batch L), Tyrospheres™ of approximately 35 nm were produced. Though none of these batches show consistency with Batch L, they all show good batch to batch consistency with one another. However, when molecular weight of oligo(DTO suberate) was varied in Chapter 3, there was no batch to batch agreement in the resulting Tyrosphere™ sizes. Therefore DTO/SA-PEG(5k) appears to suffer more from batch to batch irreproducibility. While the work of this thesis did indicate that variation in the GPC column as well as changes in reagents can affect the reproducibility of DTO/SA-

PEG(5k) synthesis, there is a yet unsolved question that would benefit from further research.

5.2 Residual PEG Content

When synthesizing DTO/SA-PEG(5k) an excess of PEG(5k) is added to the reaction vessel in order to ensure complete PEGylation of the hydrophobic block. This excess PEG is generally believed to be removed during precipitations of the polymer in IPA. However, it is possible that residual PEG is remaining in some polymer samples and affecting their behavior during self-assembly.

By ^{1}H-NMR, PEG is identified by a large characteristic peak at 3.5 ppm. When annotating the ^{1}H-NMR spectrum of DTO/SA-PEG(5k) this peak is integrated against that of the amide peak on DTO (Figure 5.1). This integration allows for the determination of the degree of polymerization (DP) of oligo(DTO suberate).

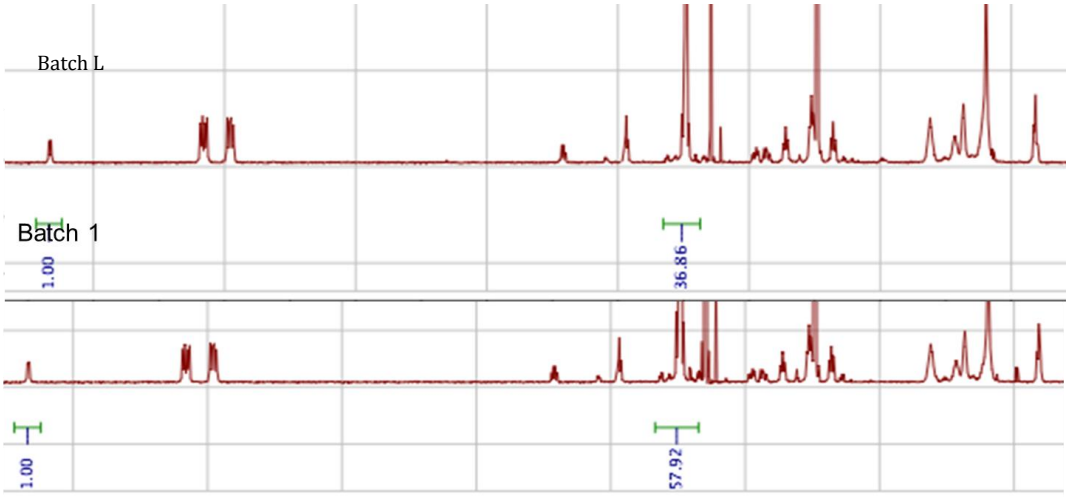


Figure 5.1. ^{1}H-NMR spectra for two batches of DTO/SA-PEG(5k) with both the amid peak (on the far left) and the PEG peak (on the right) integrated.

If two batches of polymer have the same molecular weight and therefore DP, for their hydrophobic blocks, this integration should be the same. The peaks of interest are (a) a doublet at 8.33ppm and (b) a large singlet at 3.49ppm. These correspond to the amine group on DTO and the PEG chains respectively as shown in Figure 5.2.

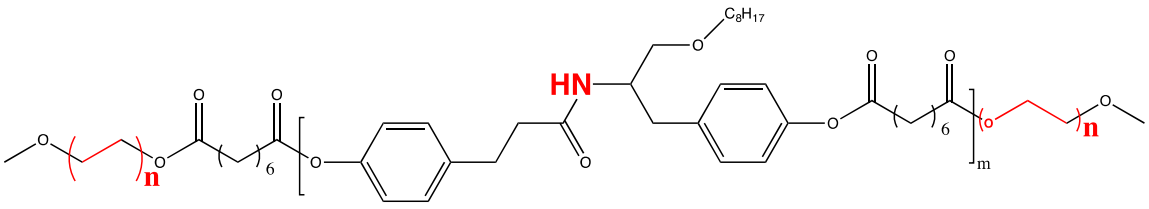


Figure 5.2. Illustration of the hydrogen that are relevant to determining the degree of polymerization for the hydrophobic block of DTO-SA(PEG5k) by ^{1}H-NMR.

In order to calculate the DP of the polymer, a few considerations must be made. First, the PEG chains are each 5 kDa. Therefore, if you divide the molecular weight of the polymer PEG by the molecular weight of one ethylene glycol unit (only the repeat unit OCH_2CH_2,) you get the number of monomers in each chain.

5,000/44.0 = 114 units per chain

Now, you take into account that each polymer will have one PEG chain on each end and that each unit contains 4 hydrogen atoms.

114 units x 2 chains x 4 hydrogen atoms = 909 units

At this point consider a particular integration of the PEG signal. For example for Batch 1 when integrated, the PEG peak shows a value of 58.55 hydrogen atoms. These 59 approximate hydrogen atoms have only one corresponding hydrogen atom from the amine in the polymer. Therefore a ratio can be set up (Figure 5.3). In the case of Batch 1 the DP is therefore 15.7 while Batch L results in a value of 24.6.

$$\frac{908\ H\ from\ PEG}{x\ H\ from\ DTO} = \frac{integration\ of\ PEG}{1.00}$$

$$x\ H\ from\ DTO = \frac{908\ H\ from\ PEG}{1.00(integration\ of\ PEG)}$$

Figure 5.3. Equation relating the 1H-NMR integration of PEG to that of the amide hydrogen from DTO.

The molecular weight of one DTO/SA unit (the portion above contained by the parentheses and the subscript “m”) is 596 Da. By multiplying the DP by the molecular weight of a single DTO/SA monomer, you obtain the molecular weight of the entire DTO/SA hydrophobic block. In the case of Batch 1 this results in a value of 9.4 kDa while Batch L results in 14.7 kDa. Not only do these values not agree with each other, but both polymers have a reported oligo(DTO suberate) of 13 kDa by GPC. Therefore there is a discrepancy between GPC and ^{1}H-NMR values for molecular weight.

This discrepancy may be the result of unbound or excess PEG remaining in the polymer. Since the ^{1}H-NMR signal in question is generated from the methylene hydrogen of the repeat unit of PEG, PEG that is not bound to the polymer can also contribute to this system. If unbound PEG is present it will artificially drive up the value of the PEG integration making the molecular weight appear lower than it may actually be. From this peak alone it is impossible to determine the difference between bound and unbound PEG.

There is however another peak within the spectra that might serve to better determine the nature of PEG in the polymer. When PEG reacts with the carboxylic acid group at the end of suberic acid, it forms an ester. This causes the hydrogen closest to the ester to shift downfield in the spectra and appear at 4.09 ppm (Figure 5.4).

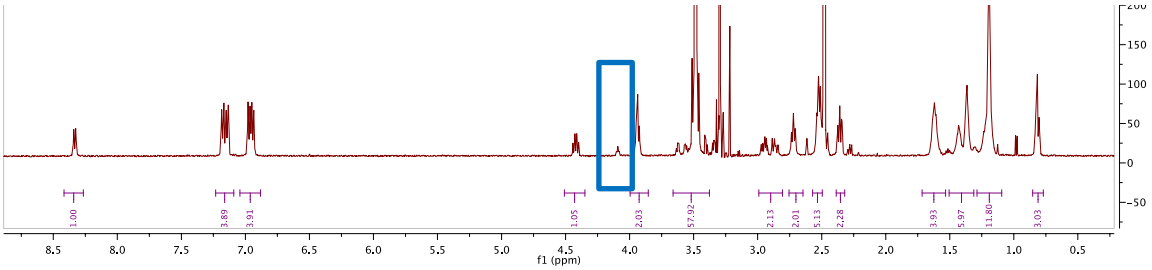


Figure 5.4. 1H-NMR spectra of DTO/SA-PEG(5k) indicating the position of an ester peak from bound PEG.

Since this peak is the result of only bound PEG it may prove useful in determining the amount of residual PEG in the sample and may indicate that some polymers have a molecular weight discrepancy.

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