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Synthesis and Characterization of Poly(ethylene oxide) Star Molecules for Biological and Medical Applications

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B.S. Chemical Engineering

Rutgers University, 1993

SUBMITTED TO THE DEPARTMENT OF CHEMICAL ENGINEERING IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN CHEMICAL ENGINEERING

AT THE

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

JUNE 1998

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SYNTHESIS AND CHARACTERIZATION OF POLY(ETHYLENE OXIDE) STAR MOLECULES FOR BIOLOGICAL AND MEDICAL APPLICATIONS

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Submitted to the Department of Chemical Engineering on May 21, 1998 in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemical Engineering

ABSTRACT

Linear poly(ethylene oxide) (PEO) has been of interest for a long time due to its many unusual properties, one being its unique ability to repel proteins and other polymers when in aqueous solution. Interest in PEO star molecules has recently been spurred by the possible advantages that they hold over linear PEO molecules in many biomedical applications. A star polymer is a specific form of a branched polymer. It consists of linear polymer chains, referred to as the arms, all connected by a common branch point, known as the core. This thesis describes a new method for synthesizing PEO star molecules: using preformed dendrimers as the core and reacting them with preformed linear PEO which after attachment become the arms. The advantage this method has over previous methods of synthesizing PEO star polymers is that it allows for precise control over the number of arms, the length of the arms, and the reactable group on the outer ends of the arms. It also provides the ability to add more than one type of PEO arm to a particular star molecule core while maintaining control over the ratio of the different types of PEO arms attached.

The outer ends of the linear PEO (PEG) chains used in this investigation had different functions: methoxy (nonreactable), hydroxyl (reactable), or blocked amino (subsequently reactable). All samples have a narrow molecular weight distribution around predetermined values ranging from 2,000 to 20,000. Dendrimers of the PAMAM Starburst™ type served as the cores. These have terminal amino groups in precise numbers: 16, 32, 64, 128, 256. Every type of modifed PEG used to make the star molecules had as the terminal group, on what became the inner end, the N-hydroxy succinimidyl ester of propionic acid, which in contact with amino groups on the dendrimer leads to amide bond formation. Because of steric crowding, it was found that the number of PEG arms that became attached to the core decreased systematically from 100% for 32 amino dendrimers to about 50% for the 256 amino dendrimers. However the polydispersity index remained remarkably close to unity (1.15 or less).

There were two underlying motivations for synthesizing these molecules. One was to create a material that could be used for a variety of biomedical applications. The other was to gain an an understanding of how branching architecture affects the properties of PEO. To satisfy the second motivation, the dilute solution properties were measured for

samples of methoxy ended stars with 16, 30 and 53 arms, these arms ranging in molecular weight from 2000 to 20000. The second virial coefficient (A_2) and the diffusion coefficient (D_o) of the star molecules were measured using static and dynamic light scattering measurements. The intrinsic viscostity was determined using a series of measurements taken with a Ubbelhode viscometer. These measurements were used to calculate how the physicochemical properties of these star molecules depend on both the number of and molecular weight of their arms. The values obtained were then compared to those of linear PEO of equivalent molecular weight so that the effects of branching could be quantified. Relationships were developed between the molecular weight of the star molecules and these dilute solution properties providing the capability to predict these properties for star molecules not yet synthesized.

Lastly, this new method of synthesis was used to create a new type of PEO star molecule, one containing two different types of arms, a shorter arm containing a hydroxyl group at its terminus, and a longer one containing a methoxy group. One of the intended uses for this unique star molecule is as a drug delivery vehicle, with the drug of interest being attached to the shorter arms with the longer arms acting to isolate the drug from either the immune system or cells of the body to which it could cause harm. By attaching biotin to the ends of the shorter arms, and then exposing the biotin bound molecules to avidin, it was shown that the ability for proteins to reach a molecule attached to the shorter arms is hindered as the ratio of the number of long to short arms increases.

Thesis Supervisor: Edward W. Merrill

ACKNOWLEDGMENTS

Wow, how do I begin this. Its kind of hard to believe I'm actually writing this (and less than 24 hours before my defense, nothing like waiting until the last minute). Its been a long (but not as long as I feared it might become) time since I first got here. I've had some really good times, and some very definitely not so good times. But, for better or for worse, I guess I got through it (how's that for optimism!) So, I guess this is where I thank everyone who helped me get through it with my sanity in tact.

First off I would like to thank my advisor, Professor Merrill, without whom this thesis would never exist. Or at least not with any resemblance to the current one being turned in. It has been an enjoyable experience working for you these past four years. Both the breadth and depth of your knowledge is overwhelming, and has never ceased to amaze me. I'm just sorry there will be no one else after me to have the pleasure of having you for an advisor.

I want to thank Sue Sofia and Premnath for making the lab an enjoyable environment. Sue was always a great help in sharing her knowledge with me. Even after she graduated and moved on to greener pastures, I always knew I could call her for advice. In addition to being a great lab mate, she has also been a great friend and running partner (do you know how bad I've been about going running since you left, I don't know how I'll ever keep in shape in NJ w/out you guilting me into going in 30 degree weather). Thanks ! Unfortunately there wasn't a group after Premnath left, but I was fortunate to have people from other groups who were willing to help me out both with experiments and advice. For that I'd like to thank Jeff Sperinde, Lily Chu and Nancy Diggs. Lily and Nancy, you've also been great friends and I'm looking forward to hanging out with you in NJ!

Of course, before I even got started on my thesis research I had to get through the my first semester at MIT, definitely not on of my better experiences in life. I kind of want to thank almost every one of the first years who started with me for helping to make that experience much more fun that it deserved to be (and also for helping me pass). I truly think we had a great class, and we still have to get together for one last party before we all go our separate ways. I especially want to thank John Konz, Randy Weinstein and Kane Jennings (in no particular order). You guys have been great friends, and actually managed to making going to hell fun!

Before my parents, if they ever read this, decide I'm completely ignoring them, let me mention you now. Thanks for everything you've done for me through the years (yes, even for encouraging me to come to MIT, assuming you're right about me realizing twenty years from now it was worth it). I know I don't say it enough (actually, maybe never). You've been wonderful parents and I consider myself lucky to have you. There, its in writing, but no holding it in front of me the next time I blame you for everything wrong in my life.

Finally I want to thank John (my John), for being a wonderful husband and friend. You've always given me a shoulder to cry on (and I know there were periods I needed it a lot), been supportive, and just plain gone above and beyond what I ever expected from you. If I were to list everything you've done for me, I'd never finish this. I just want you to know that you are the best thing that ever happened to me and I love you very much (shoot, more stuff in writing). Now aren't you psyched its all over?

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CHAPTER ONE

Introduction and Background

1.1 Star Polymers

Star branched polymers represent the simplest form of a branched polymer. A model star molecule can be viewed as consisting of a central core from which several linear polymer chains, "arms", are attached, with all branches being of equal molecular weight (see Figure 1-1). Variations on this model star polymer include molecules containing on the same core polymer arms of different lengths and also arms of different polymer species. Star molecules were first synthesized in 1948 by Schaefgen and Flory[1] who polymerized ε-caprolactam in the presence of either a tetra or octofunctional carboxylic acid to produce four and eight armed polyamide stars of the type $R\{-CO(NH(CH_2)_5CO)_n\text{-}OH\}_f$.

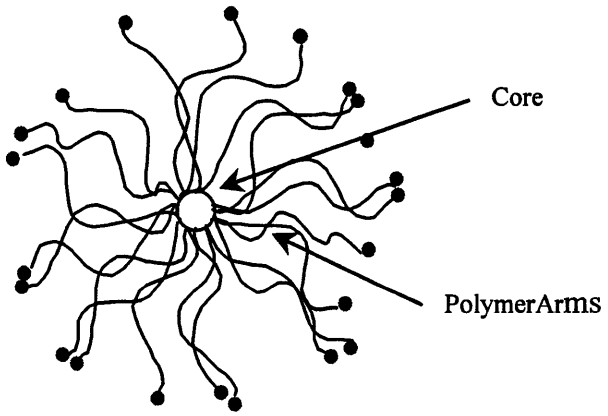

Figure 1-1. Model Star Polymer

1.1.1 Synthesis

One of the challenges involved in synthesizing star molecules is to devise a protocol allowing for precise control over both the molecular weight, M_{arm}, and the number of arms, *f*, on a star molecule. Once synthesized it is very difficult to measure

both the length and the number of arms on a particular star polymer without having any knowledge of either. For example, light scattering can be used to measure the molecular weight, but there are many combinations of f and M_{arm} that result in stars of identical molecular weight. The analytical situation becomes even more complex if there is a broad distribution in the size of linear molecules that make up the arms, it is for this reason that anionic polymerization methods are often used in their synthesis. Due to its termination-free nature, anionic polymerization leads to a narrow molecular weight distribution.[2] In addition, if the polymer is not deliberately terminated, its chain ends possess highly reactive organo-alkali metal groups. Another challenge in synthesizing star molecules lies in discovering ways to attach a large number of linear polymers to one branch point. Most methods for synthesizing star polymers fall under four general categories.

Method one: The arms are polymerized first via anionic or cationic polymerization. The living ends of the arms are then used to initiate polymerization of a suitable bis-unsaturated monomer (usually divinyl benzene), which becomes the core. By taking a sample of the arms prior to core initiation, the length of the arms on a star can be determined. Thurmond and Zimm[3] used this method to synthesize polystyrene star molecules. This method has been used more recently by Marsalko et al.[4,5] to synthesize multi-arm polyisobutylene star molecules. They discovered however that as they increase the number of arms on the star molecules, the molecular weight distribution of the star molecules synthesized broadens. Another downside to this method is that there is no way to theoretically predict the number of arms apriori.

Method two: Again the arms are polymerized first via ionic polymerization. The anionic living polymers are then deactivated by means of a plurifuncional electrophile, which is to be the core. As in the first method, the length of the arms can be independently

measured by taking a sample of the linear polymer chains synthesized prior to reacting with the core. Additionally, the quantity of arms on the star can be predicted based on the number of reactive groups on the core. Polyisoprene stars containing up to 18 arms have been synthesized using this method.[6] As methods of synthesizing multifunctional chlorosilane cores have become more refined, this method has been used to create polybutadiene star molecules containing as many as 128 arms.[7] As a result of using well defined cores, star molecules synthesized in this manner have been shown to have a very narrow molecular weight distribution.

Method three: The first two methods of synthesis are often referred to as "arm first" methods. One disadvantage of these techniques is they do not allow for synthesis of star molecules containing functional groups on their outer ends. This issue is circumvented by using what is referred to as the "core first" method. In this method a plurifunctional anionic initiator core is formed first and is used to initiate the anionic polymerization of the arms. This approach was first used to synthesize polystyrene star molecules.[8] Rempp et al used this method to synthesize PEO star molecules.[9] The main drawback to this technique is that it does not allow for control of either the length or the number of arms. In addition, this method of synthesis often results in star molecules with a broad size distribution.[10] This will be discussed further in the section describing the application of this method to the synthesis of polyethylene oxide star molecules.

Method 4: One interesting technique combines the first and third methods described above. Star molecules synthesized using method one result in a living, star shaped polymer bearing within its core a number of active sites that is equal to the number of its branches.[11] This newest method uses these active sites to initiate the polymerization of another monomer of suitable electroaffinity. If this monomer is different than the initial one used, a heteroarm star molecule is formed. This method has been used to synthesize

star block copolymers containing styrene/butyl methacrylate),[12] styrene/2-vinylpyridine[13] and styrene/polyethylene oxide.[14]

1.1.2 Characterization

In general, branching of polymer molecules significantly modifies their properties. This change can be explained by two basic changes to the environment branching elicits. First, the local average polymer density increases relative to that in a linear chain. Second, the constrained segmental motion within branches and loops restricts cooperative motion of the chain as a whole.[15] Due to their simple geometry, many researchers have studied the model star polymer in an attempt to predict quantitatively how branching affects the characteristics of polymers.[3,5,15-18] Dilute solution properties are those most often measured in making these comparisons between branched molecules and linear ones.

The techniques most often used to measure the dilute solution properties of star molecules include static light scattering, small angle neutron scattering, dynamic light scattering, and Ubbelhode viscometry.[19-21] Static light scattering is used to measure the weight average molecular weight (M_w), the second virial coefficient (A_2) and the radius of gyration (R_G); neutron scattering is often used to measure R_G when this value is too small to be measured using light scattering; dynamic light scattering measures the diffusion coefficient at infinite dilution (D_o); and measurements taken with a Ubbelhode viscometer allows for calculation of the intrinsic viscosity [η] and the Huggins coefficient (k_H).

One way to compare star molecules with linear molecules is to convert some of the above values to their equivalent radii based on the following equations:

$$R_V = 5.41 \times 10^{-9}([\eta]M)^{1/3} \quad (1\text{-}1)$$

$$R_T = 4.63 \times 10^{-9}(A_2M^2)^{1/3} \quad (1\text{-}2)$$

$$R_s = 5.31 \times 10^{-2} kT/\eta_S D_O \qquad (1\text{-}3)$$

where R_V is the viscometric radius, R_T is the thermodynamic radius, and R_s is the stokes radius. It has been shown in a number of papers[18] that as the number of arms *f* on a star molecule increases its properties diverge increasingly from those of its linear counterpart. Specifically, the star molecule has a much smaller radius than a linear molecule of the same molecular weight. Therefore, in comparing a linear polymer molecule having n structural units and a star polymer molecule of equal hydrodynamic volume, the star molecule contains a much greater number of structural units than n, by up to a factor of 10 or more.

For monodisperse spheres all three methods of measuring the radius should give the same value. Therefore, if star molecules do act as hard spheres, researchers should find $R_V = R_T = R_S = R$. Many of the results found on star polymers show that as the number of arms on a star increase R_T / R_V reaches an asymptotic value greater than one.[15,18] Because R_T is measured statically (it doesn't involve movement of the molecule) whereas the measurement of R_V is based on flow of the sample in a solvent; these results have been interpreted as suggesting that star molecules behave as "fuzzy" spheres having greater hydrodynamic penetration than thermodynamic.

The sphere-like behavior of star molecules can also be investigated by comparing the radius of gyration to the other radii. For spheres of uniform density it is known that $R_G = R^*(3/5)^{1/2}$; therefore R_T/R_G should equal 1.291. There have been conflicting reports on what this value is for star molecules, some researchers have found that for stars of 20 or more arms R_T/R_G reaches a limiting value of around 1.291.[18] Other researchers have found R_T/R_G to be greater than 1.29, suggesting the possibility of a sphere with a dense core, thus making R_G smaller than would be found for a sphere of uniform density.[15]

Another way to compare star molecules to spheres is by examining the Huggins constant. An expansion of Einstein's equation for the effective viscosity of a suspension

of hard spheres to order ϕ^2, where ϕ is the volume fraction of the star molecule in solution, has been found to be[22]

$$\eta_s = \eta_o(1 + 2.5\phi + 6.2\phi^2). \tag{1-4}$$

A comparison of this equation to the Huggins equation

$$\eta_{sp}/c = [\eta] + k_h[\eta]^2 c \tag{1-5}$$

leads to $k_H=6.2/(2.5)^2=0.99$ for hard spheres, where k_H is the Huggins coefficient. The dependence found by Bauers[18] et al of the Huggins coefficient on functionality shows that it reaches the hard sphere limit of 0.99 at high functionalities. This result is consistent with those found by others.[7]

1.1.3 Theoretical Work

A variety of theoretical methods have been used to predict the dependence of star polymer molecular properties on the number of arms. Monte Carlo simulations,[20,23-25] renormalization group methods,[26] scaling theory[27-29], and mean field calculations[30] are among many of the theoretical methods used. Most of the models and simulations apply only to star molecules containing 12 arms or less. For those models that deal with star molecules containing larger number of arms, the bulk of the work is based on polymers in theta solvents. However most interest lies in how PEO star molecules with large numbers of arms behave in aqueous solution, which is a very good solvent. Therefore, experimental work is needed to get the desired information.

1.2 Polyethylene oxide

Poly(ethylene oxide) has gathered much attention recently in the field of biomaterials due to its unique property of rejecting proteins and other polymers when in aqueous solutions.[31,32] This leads to surfaces composed of PEO being biologically inert. Because PEO is readily water soluble, there are two methods of achieving PEO surfaces suitable for *in vivo* applications. One method is the cross-linking of PEO to form hydrogels, and there has been much experimental work done synthesizing and characterizing PEO hydrogels.[33-35] The other way to achieve a PEO surface is to immobilize PEO molecules onto a water insoluble surface. The terminal hydroxyl group on PEO allows for a convenient point of attachment to surfaces and other molecules. Again there has been much experimental work studying methods to achieve high surface densities of PEO.[36-40]

It has also been shown that molecules covalently attached to PEO usually remain active.[41,42] For example, Tay et al[43] have shown that heparin bound to PEO hydrogels had nearly ten-fold greater activity than when bound to polyvinyl alcohol. The long, hydrated "leash" that PEO provides allows the heparin to move out into solution giving it more access to the thrombin-antithrombin pair than does the tight bond to PVA. This has led to work examining the use of PEO to create surfaces with specific biological functions.

Not only do molecules attached to PEO remain biologically active, it has also been demonstrated that the covalent attachment of PEO to enzymes increases their *in vivo* half life.[44] This is done by two consequences of PEO's attachment. By adding large numbers of short PEO chains to the enzyme its effective size in solution is increased, thereby decreasing its rate of filtration through the kidney. In addition, the PEO acts as a "shield", hiding the enzyme from the immune system of the body there by rendering them nonimmunogenic and nonantigenic.[45]

1.3 PEO Star Molecules

1.3.1 Applications

Interest in PEO star molecules has been sparked by the possible advantages that they hold over linear PEO molecules in many biomedical applications. For example, it has been shown that higher surface densities, and therefore better protein rejecting properties, can be obtained by immobilizing star PEO onto surfaces as compared to using linear PEO.[36] An additional advantage of using PEO star molecules to cover surfaces is that not only do the large number of arms of the PEO star allow for greater points of attachment to the surface, they also provide additional points of attachment for linking to other bioactive molecules which might, for various reasons, be desirable to have attached to the surface. For example, PEO star molecules have been used to study cell response to immobilized endothelial growth factors.[46]

While PEO hydrogels can be formed by irradiation induced cross-linking of either star or linear PEO, irradiation of star PEO enables the synthesis of hydrogels containing much greater concentrations of terminal hydroxy groups than is attainable using linear PEO.[47,48] Additionally, certain occasions call for the ability to form gels in situ. For such applications irradiation induced cross-linking would be impractical and end linking would be the preferred method to forming gels. For such uses a multifunctional PEO molecule would be required. Such a method has been demonstrated using a tetra functional PEO,[49] and the use of PEO stars with even greater functionality would allow for the incorporation of ligands into the gel.

Many other uses for PEO star molecules have been proposed,[50] but have not been investigated due to the lack of reliable material. In addition, the lack of well-defined samples of PEO star molecules has restricted the ability to perform quantitative analysis on some of studies described above. For example, authors of a recent quantitative

analysis of the dependence of protein adsorption on PEO grafted surfaces were able to develop correlations between the size and concentration of linear PEO attached to a surface, and its ability to reject proteins.[36] While they were able to compare grafted linear PEO to grafted star PEO in their study, the lack of well defined monodisperse samples of star PEO did not allow them to correlate the protein rejection of the PEO star grafted surfaces with such factors as the number of arms on the stars and the molecular weight of the arms. They, and other investigators[51] have had to rely on average values of the properties of the PEO star molecules used in their experiments to develop models based on their results. The samples of star molecules used in all studies on PEO star molecules to date, were synthesized by anionic polymerization using a core-first method.[9] This method is discussed in detail in the following section.

1.3.2 Synthesis

PEO molecules containing three arms have been synthesized using triethylpropane as the core.[9] To synthesize PEO star molecules having a greater number of arms they used the "core first" method.[52] Their technique is shown schematically in Figure 1-2. Living cores are produced by adding a solution of divinylbenzene dropwise, at -40°C, to a dilute solution of potassium naphthalene in tetrahydrofuran under efficient stirring. Oxirane is added to the cores thereby converting the carbanions to oxanions. The mixture is then slowly heated to 30 or 35°C while the oxirane continues to polymerize. The reaction is terminated by addition of acidified methanol. Upon protonation of the alkoxide sites the branches carry hydroxy functions at their outer end which can be utilized for further reactions.

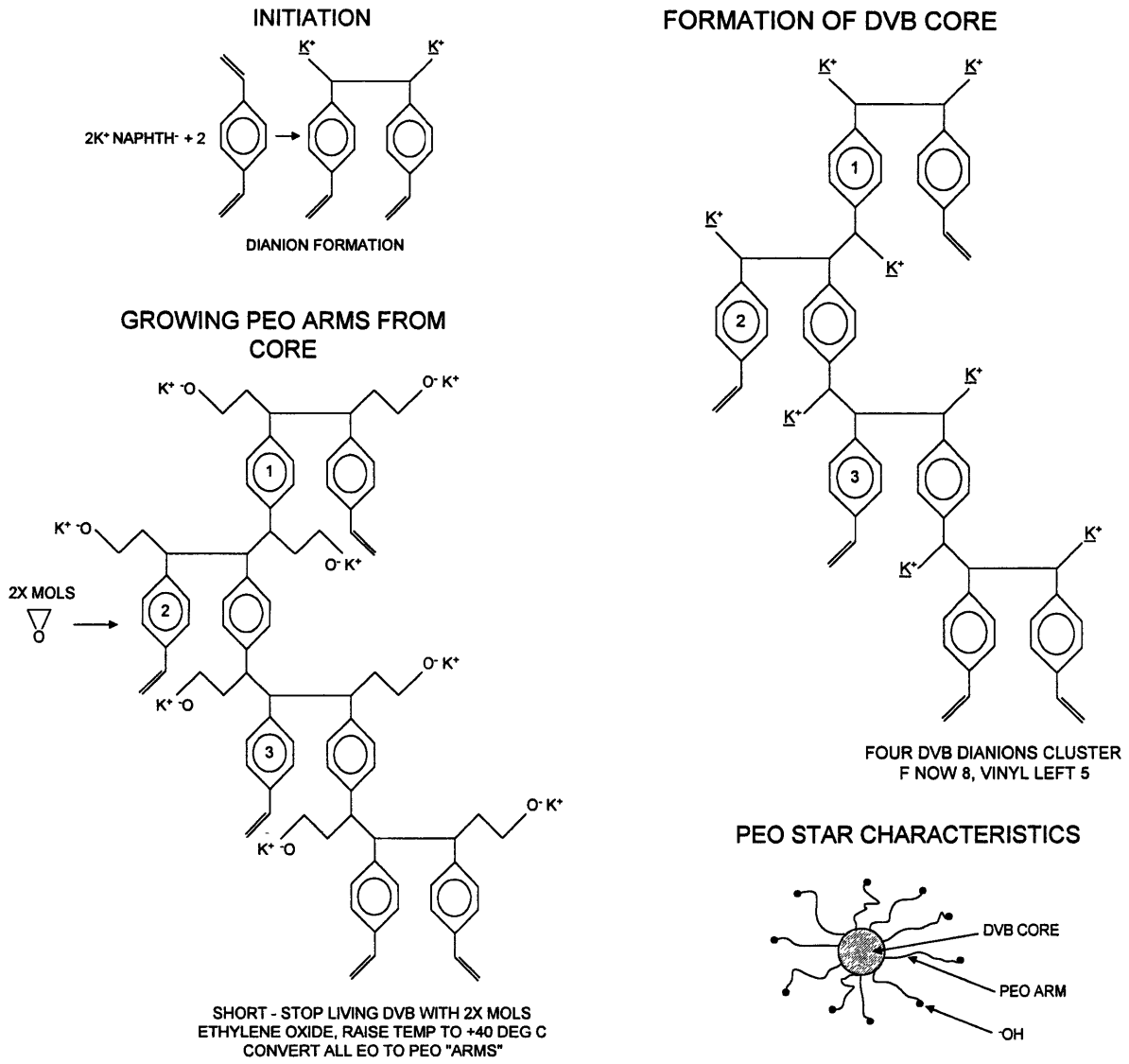

Figure 1-2. Synthesis of PEO Star Polymers Using Core-First Method

The moles of ethylene oxide that were polymerized were determined by subtracting the quantity of unused ethylene oxide following termination from the quantity initially injected. The total number of arms present in solution is assumed to be equal to the number of potassium naphthalene ion pairs added. The number average degree of polymerization of each arm, P_n, was calculated by dividing the moles of ethylene oxide consumed by the total number of arms. The number average molecular weight of the

individual branches, M_{arm}, was then calculated as the molecular weight of the monomer multiplied by P_n. The weight average molecular weight M_w was determined by classical multiangle light scattering with the assumption that the refractive index increment for the PEO star is the same as that of linear PEO in the same solvent. The average functionality was then calculated as M_{star}/M_{arm}.

The stars prepared in this way tend to be polydisperse as has been shown by analytical gel permeation chromatography.[53] Because the arms are synthesized via anionic polymerization, it is believed that the arms of the star molecules synthesized are all of the same length and therefore not the cause of the observed polydispersity. Rather, it is thought that the DVB cores grow at differing rates resulting in a sample of star molecules containing stars with differing numbers of arms. This hypothesis is supported by results obtained on polystyrene star molecules prepared by a similar method.[10] For these molecules it has been shown that the formation of the "cores" by random coupling between radical sites involves a broad distribution of sizes and number of carbanionic sites per initiating "core" resulting in stars with different numbers of arms. Attempts to fractionate PEO star molecules synthesized by the Rempp method using classical temperature manipulation proved to be cumbersome and inefficient. (see Appendix A).

Another method used to synthesize PEO star molecules was described earlier as the "in, out" method. The downside to this method is that it produces star molecules with both PEO and polystyrene chains as arms. Polystyrene is neither biocompatible nor water-soluble. So while the amphiphilic nature of these polymers makes them of scientific interest to study, they are not suitable for biological applications.

1.4 Scope of thesis

While there have been many studies done on the dilute solution properties of other star molecules, there has been little or no work done on similar characterization studies involving PEO star molecules. Much of the reason for this is that there has been no reliable method of synthesizing these molecules. This thesis describes a new methodology for synthesizing PEO star molecules that allows for precise control over both the number and length of the arms. These molecules were then used in a systematic study of the dilute solution properties of PEO star molecules in aqueous solution as a function of the molecular weight of the arm M_{arm} and of the functionality *f*, i.e. the number of arms.

While previous studies on the dilute solution properties of other star molecules have been done in the past, all of these studies have involved polymers in organic solution. In addition, the major goal of those studies was to advance scientific understanding of how branched molecules behave in solution. The only application proposed for those molecules involved being as viscosity modifiers.[54] The ability for those molecules to undergo reactions with other species was never an issue. While learning how branching architecture affects the properties of PEO star molecules is of interest, the ultimate goal is to use them for biomedical purposes, especially with bioactive species attached to the outer ends of the arms. With this goal in mind it was important that the method chosen for synthesizing these molecules should enable them to have functional groups on their outer ends to allow for binding to surfaces and other molecules, including enzymes, antibodies, anticoagulants, and other biologically active species. Not only does this method meet that criterion, it also allows for synthesis of a star molecule having on the same core arms of two or more different lengths and/or different outer end groups. In addition to the synthesis and characterization of the model PEO star molecules synthesized, this thesis also describes the synthesis of these "dual

armed" star molecules along with a study of the ability of the shorter arms to interact with other molecules. Moreover, it specifies some potential applications for these molecules.

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CHAPTER TWO

Synthesis of PEO Star Molecules

2.1 Introduction

One goal of this study was to devise a method for synthesizing PEO star molecules that would allow for precise control over both the number of arms on the molecule and the molecular weight of the arms. To achieve this objective it was decided to use preformed cores that were monodipersed and well characterized, and react them with preformed linear PEG chains that were also monodispersed and well characterized. Unexpectedly, despite the fact that the arms are PEO of molecular weight measured in thousands, it has been found possible to attach preformed arms in large number to preformed cores, creating star molecules having a low polydispersity index (<1.15).

2.2 Experimental

2.2.1 Cores

Polyamidoamine (PAMAM) Starburst® dendrimers synthesized by Dendritech were chosen as the preformed cores. A dendrimer is a dense, hyperbranched molecule built up generation by generation. Their synthesis, which is described in detail by Tomalia et al,[1,2] results in spherical molecules containing specific numbers of surface primary amino groups. Briefly, ethylene diamine is reacted with methyl acrylate forming what is referred to as a -1/2 generation dendrimer containing 4 carboxyl groups. The carboxyl groups are reacted with ethylene diamine resulting in a zero generation dendrimer with 4 primary amine groups (see Figure 2-1). The next generation is formed by repeating the methyl acrylate/ethylene diamine reaction series. The molecule is built

up in this manner, generation by generation with each successive generation having twice the number of surface primary amines as the generation before. It is these primary amine groups which are used as the point of attachment for the PEO chains which are to be the "arms" of the star molecule.

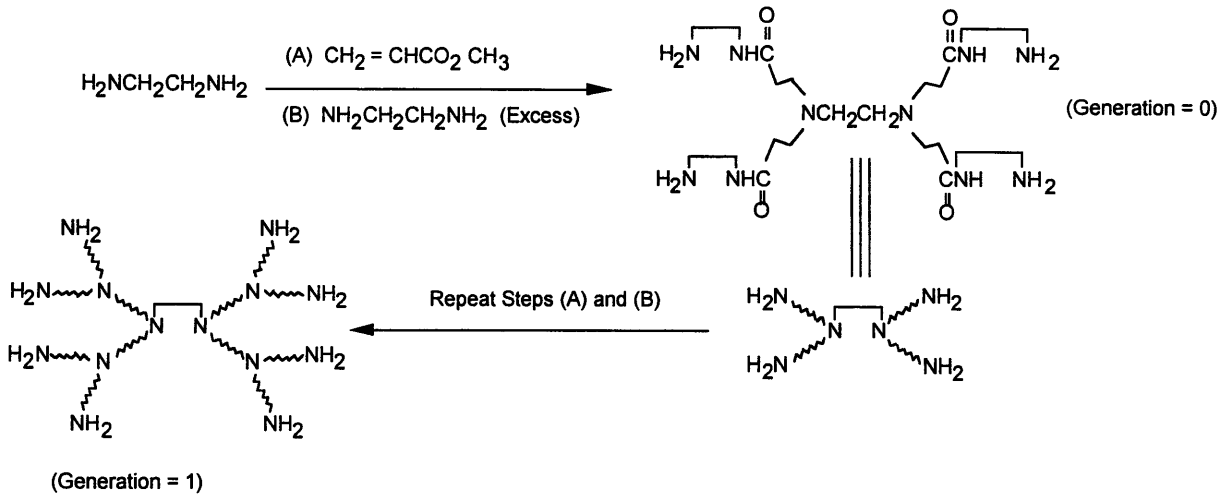

Figure 2-1. Schematic of PAMAM Dendrimer Synthesis

Table 2-1. Physical Characteristics of Starburst Dendrimers

Generation	Molecular Weight	Number of Amine Groups	Radius (nm)
1	1204	8	1.1
2	3252	16	1.45
3	6900	32	1.8
4	14196	64	2.25
5	28826	128	2.7
6	57654	256	3.35

Dendrimers containing 8, 16, 32,or 64 surface primary amine groups were purchased from Aldrich. Dendrimers containing 128 and 256 primary amine groups were donated by Dendritech. The dendrimers were received as either a 10, 20, 24 or 27 w/v%

solution in methanol. The physical characteristics of the dendrimers are shown in Table 2-1.

2.2.2 Arms

The arms consisted of linear chains of heterofunctional PEG that were synthesized prior to their attachment to the dendrimer core. Whereas the rigorous meaning of poly(ethylene glycol) (PEG) is α, ω dihydroxypoly(ethylene oxide), we use the abbreviation PEG in the following to denote a PEO molecule with two different ends. For all functionalized PEG molecules used in this study one of these ends was the N-succinimidyl ester of propionic acid (NHS). This group reacts with primary amine groups in the following manner:

$$\text{PEG-O-CH}_2\text{-CH2-CO}_2\text{-NHS} + \text{R-NH2} \longrightarrow \text{PEG-O-CH}_2\text{-CH}_2\text{-CONH-R}$$

Because the resulting linkage is an amide bond, it is expected to be stable over time under all conditions it was subjected to throughout the course of this investigation. Three different types of functionalized PEGs, depicted in Figure 2-2 were used to synthesize PEO star molecules. All were provided at cost by Shearwater Polymers, Huntsville, AL.

Type 1 Arm

Succinimidyl derivative of PEG propionic acid (Methoxy-SPA-PEG) whose structure is shown in Figure 2-2a. Four different molecular weights of this molecule were used. The molecular weights of the molecules used were reported by Shearwater to be 1847, 5000, 10000, and 21469. The methoxy end is unreactive. Reactions between this PEG and the dendrimer results in PEO star molecules with methoxy terminated ends.

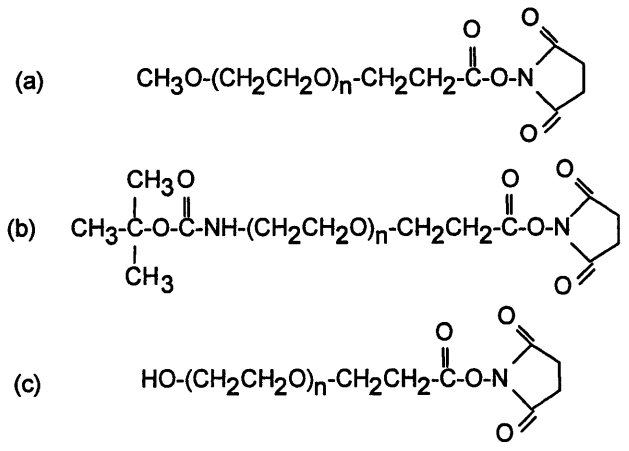

Figure 2-2. Functionalized linear PEG molecules reacted with dendrimer cores to synthesize PEO star molecules: (a) MeO-PEG-NHS (b) t-boc-PEG-NHS (c) HO-PEG-NHS

Type 2 Arm

PEG with a t-boc protected amine on one end and an N-succinimidyl group on the other end, depicted in Figure 2-2b. The molecular weight used of this PEG was reported by Shearwater Polymers to be 3400. Again the NHS group on the PEG reacts with the primary amine on the dendrimer cores. Reactions between this PEG and the dendrimer result in PEO star molecules terminating in t-boc protected amines. The t-boc protecting group can be removed by addition of dilute hydrochloric acid. This results in arms on the star molecules terminating in primary amine groups. If it is desired to increase the length of the polymer chains on the star molecules, additional NHS functionalized PEG can be reacted with these amine terminated star molecules.

Type 3 Arm

The last type of PEG used consists of a hydroxyl group on one end and an N-succinimidyl group on the other, Figure 2-2c.PEO star molecules were synthesized using

PEG of this type with molecular weights varying between 600 and 10000 as reported by Shearwater Polymers. Once again the NHS group on the PEG reacts with the primary amine on the dendrimer cores. Reactions between this PEG and the dendrimer result in arms on the star molecules terminating in hydroxyl groups.

2.2.3 Synthesis Protocol

Two different solvents were tried for the synthesis reaction. The stars were synthesized in either aqueous solutions with sodium bicarbonate buffer, or in dichloromethane with methanol added to dissolve the dendrimer.

2.2.3.1 Aqueous Solvent

A known quantity of dendrimer was dissolved in 0.1M sodium bicarbonate buffer. It was assumed that one PEG molecule would react with each primary amine group on the dendrimer's surface. Based on this assumption the dendrimer solution was then added to a 1.6x molar excess of PEG. The solution was made up so that the concentration of PEG was 70 mg/ml. The reason for adding the dendrimer to the buffer before dissolving the PEG in buffer is based on the high rate of hydrolysis of the NHS group. Therefore it was desired to avoid contact of the PEG with water until the dendrimer was present. The solution was then left stirring for a minimum of 2 hours, after which it was believed the reaction had gone to completion.

2.2.3.2 Dichloromethane

Dichloromethane and methanol were dried overnight over molecular sieves. All glassware used was dried overnight in an oven. A known mass of PEG was dissolved in

the dichloromethane. Dendrimer was then added to the PEG/dichloromethane mixture and stirred until the suspension was well mixed. The quantities of PEG and dendrimer used were such that the ratio of PEG to primary amine group was 1.05. The dried methanol was then added to dissolve the dendrimer. After the reaction was complete, as determined by constancy of the star molecular weight, explained later, the dichloromethane was evaporated off.

2.2.4 Removing Unreacted PEG

The stars prepared in the above manner contained excess unreacted linear PEG. This PEG was removed via ultrafiltration using an Amicon stirred ultrafiltration cell. The system is depicted in Figure 2-3. The cell was pressurized with nitrogen which provided the driving force through the membrane. Concentration polarization was minimized by stirring of the solution just above the membrane. The membranes used depended on the size of the star synthesized. For example, a star synthesized by reacting a dendrimer containing 64 amine groups with methoxy PEG (MW=5000), is completely retained by an Amicon YM100 (100,000 molecular weight cutoff (MWCO), regenerated cellulose) membrane. Therefore this membrane was used to separate these stars from the methoxy PEG. However, a star prepared using a dendrimer containing only 16 amino groups would pass through a YM100 membrane. Therefore a YM30 (30,000 MWCO, regenerated cellulose) membrane was used to separate this star from its linear PEG counterpart.

The ultrafiltration process was performed as follows: the reaction mixture was diluted to 50 ml with the desired final solvent. If the stars were going to undergo another reaction to extend the length of their arms the desired solvent would be 0.1 M sodium bicarbonate buffer. If the stars were in their final preparation state the desired solvent was MilliQ water. The solution was then concentrated to a final volume of 5 ml. Solvent

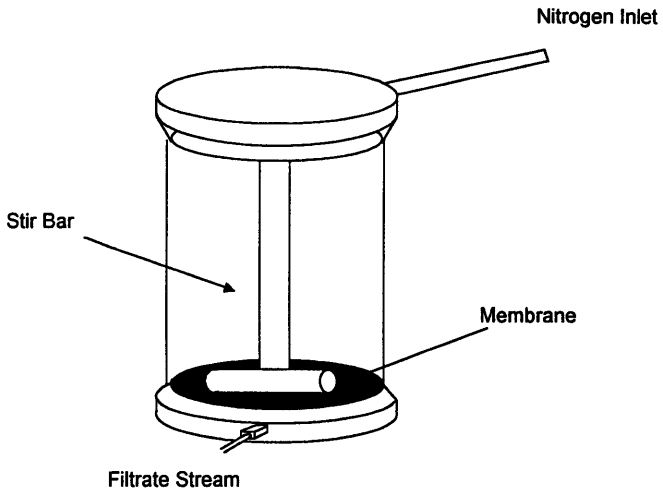

Figure 2-3. Ultrafiltration Apparatus

was again added to the solution bringing the volume back to 50 ml. This process was repeated until there was no more linear PEG left in the reaction mixture. The presence of unreacted PEG was monitored by injecting a sample of both the permeate (solution passing through the membrane) and the retentate (solution retained by the membrane) into the GPC/LS. Stars diafiltered into water were then filtered using a 0.5 micron filter and the water was lyophilized off.

2.2.5 Removal of t-boc Protecting Group

PEO stars synthesized using t-boc-PEG-NHS had on the outer ends a t-boc protected amine. This protecting group was in some cases removed to yield a free primary amino group capable of binding various bioactive molecules. After the dendrimer/PEG reaction had gone to completion, removal of this group was achieved by adding a concentrated solution of hydrochloric acid to the reaction mixture. The solution was then left stirring overnight. The t-boc protecting group was then removed along with excess linear PEG as described above using ultrafiltration. In some cases it was desired

to extend the length of the arms by reacting the terminal amine groups with additional t-boc-PEG-NHS. If this was the case, the star molecules were diafiltered into 0.1M sodium bicarbonate buffer. Additional t-boc-PEG-NHS in a 1.6x molar ratio was then added to the solution of amine terminated PEO stars. The reaction mixture was again left stirring for at least 2 hours.

2.2.6 Reaction Kinetics

The hydrolysis half life of the NHS group on the linear PEG molecules is reported to be 16.5 minutes in pH 8 buffer at 25° C.[3] Because the half life decreases as pH is increased, and because aminolysis is faster than hydrolysis, it was assumed that the reaction was > 99% complete after 2 hours. This assumption was confirmed using GPC/LS measurements.

To ensure that the reaction proceeded when dichloromethane was the solvent, the reaction was monitored in the following manner. At various time intervals after the reaction commenced, a small sample was removed. Dichloromethane was evaporated off and hydroxyamine was added to react with any remaining NHS groups on the PEG. The sample was then run on the GPC to quantify the amount of unreacted PEG. The results showed the reaction to be 90% completed after one hour.

2.3 Analysis

The extent of reaction was monitored two different ways. The method used most often was gel permeation chromatography in series with light scattering (GPC/LS). This technique was used to monitor the both the weight averaged (M_w) and number averaged (M_n) molecular weights of the star molecules synthesized. The number of arms was calculated by dividing M_n by the molecular weight of the linear PEG used in the reaction.

2.3.1 Gel Permeation Chromatography/Light Scattering

Determination of both the molecular weight and polydispersity of all samples was made using gel permeation chromatography (GPC) in conjunction with light scattering (LS). The system setup is shown in Figure 2-4. The GPC used was a Waters Model 150C containing two Tosohaas TSK-gel columns in series, G6000PW and G4000PW. The GPC eluate from the columns pass through a Wyatt Dawn Model F laser photometer and then through the refractive index detector contained within the Model 150C system. Voltage measurements taken from the detectors are recorded every second and converted to light intensity and refractive index measurements respectively. Since the weight fraction of PEO is greater than 0.95 for the star molecules synthesized, the differential refractive index increment *dn/dc* for the PEO stars was assumed to be 0.135, equal to that of linear PEO at a wavelength of 632.8nm.. All calculations were made through ASTRA, a software package designed for use with the Wyatt Dawn Model F, which is run on a CUI 386 PC.

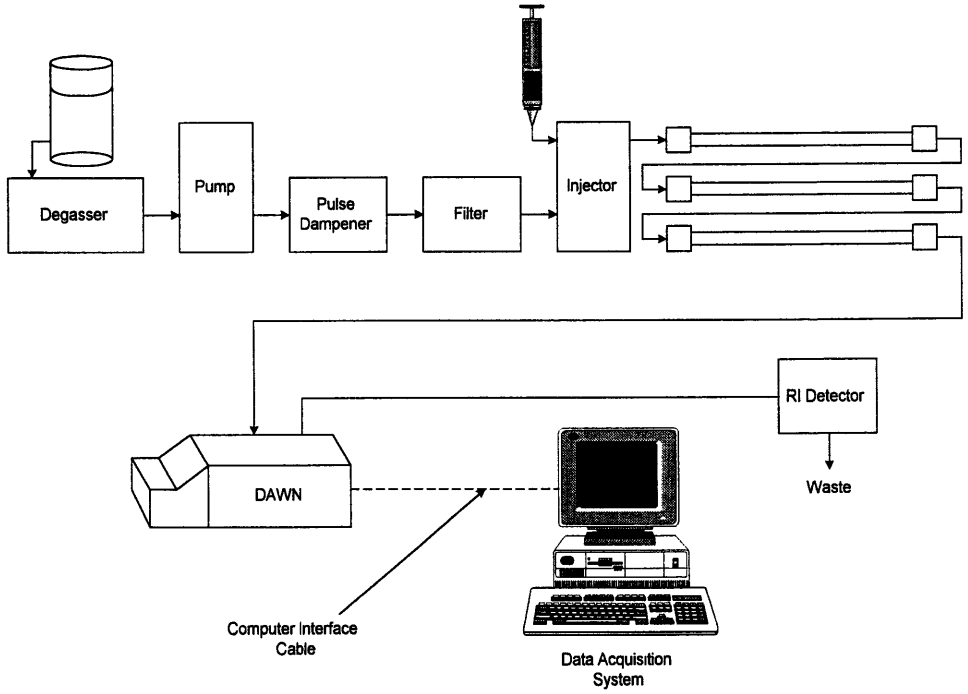

Figure 2-4. Gel Permeation Chromatography/Light Scattering System

2.3.1.1 Refractive Index Detector

RI Detector Instrument Constant

The RI detector instrument constant α is the number necessary to convert the changes in voltage output of the RI detector into refractive index units. This number was calculated by injecting a series of PEO standards of known concentration. The instrument was then allowed to calculate the total mass injected using a dummy value of α. The real value of α was calculated as

$$\alpha = \frac{injected\ mass}{calculated\ mass} \times \alpha_{dummy} \tag{2-1}$$

Sample Concentration

Sample concentrations were calculated from the RI detector output using the *dn/dc* method. The sample peaks were divided into slices. For each slice the change in refractive index compared to pure solvent was calculated according to

$$\Delta n_j = \alpha\left(V_{RI,j} - V_{RI,baseline}\right) \tag{2-2}$$

$V_{RI,j}$ RI signal voltage for the jth slice

$V_{RI,baseline}$ RI baseline voltage

The concentration of solute in each slice was then calculated by dividing Δn_j by *dn/dc* . Once the concentration was known, the mass for each slice, w_j,was determined by multiplying it by the volume of the slice. The total mass for each peak was then calculated according to

$$W = \sum_{peak} w_j \tag{2-3}$$

2.3.1.2 Flow through Light Scattering Device

The theory behind light scattering will be dealt with more thoroughly in Chapter 3. This section describes the details behind how the flow through light scattering device that is used in series with GPC works.

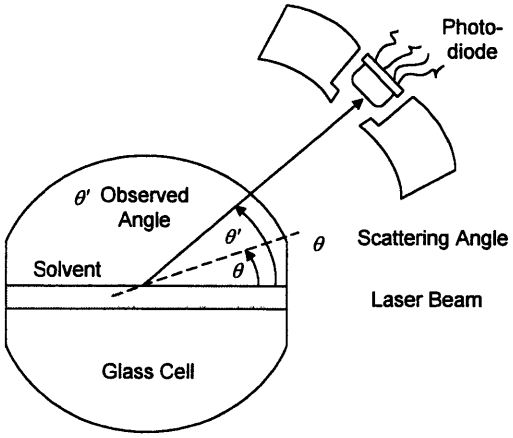

Figure 2-5. Schematic of Wyatt Dawn F Flow Through Light Scattering Cell. θ is the actual scattering angle while θ' is the observed angle due to differences in refractive index between the cell and the solvent. The detector shown is one of 17 surrounding the cell.

Calculation of Molecular Weight

The molecular weight of the samples was calculated using the following equation from Zimm[4]:

$$\frac{R_\theta}{K^* c} = MP(\theta) - 2A_2 cM^2 P^2(\theta) \qquad (2\text{-}4)$$

A_2 second virial coefficient

K^* an optical constant which equals $2\pi^2 n_o^{\,2}(dn/dc)^2 N_A^{-1}\lambda_o^{-4}$, where n_O is the refractive index of the solution, N_A is Avogadro's number, equal to

6.022×10^{23} and λ_s is the wavelength of incident light in air expressed in nanometers.

$P(\theta)$ Particle scattering factor approximately equal to $1-(16\pi^2/3\lambda_s^2)\overline{r_g^2}\sin^2(\theta/2)$ where $\overline{r_g^2}$ is the z-average mean-square radius of gyration for random-coil polymers and λ_s is the wavelength of light in the solution, λ_o/n.

R_θ The excess Raleigh ratio

The data were analyzed by constructing a plot of R_θ/K^*c *vs.* $\sin^2(\theta/2)$ and using a linear fit to obtain the intercept at zero angle, R_o/K^*c. As θ approaches zero, P(q) approaches unity and equation (2-4) becomes

$$\frac{R_0}{K^*c} = M - 2A_2cM^2. \tag{2-5}$$

The data were analyzed by assuming A_2 is equal to 0 so that M can be estimated according to

$$M = \frac{R_0}{K^*c}. \tag{2-6}$$

If A_2 is known M can be calculated exactly according to

$$M = \frac{2(1-\sqrt{8A_2c(R_0/K^*c)})}{8A_2c} \tag{2-7}$$

After the value of A_2 for the star molecules were calculated using static light scattering (see Chapter 3) this equation was used to recalculate the molecular weight estimated using equation 2-6. It was found that the estimated value assuming A_2=0, undervalued

the actual molecular weight by 5% for the sample with the highest second virial coefficient.

Calibration Constant

As seen from the above equations. The angle-dependent scattering of light is a function of the Raleigh ratio R_θ of the solution being measured, therefore R_θ, is the value we are trying to measure using the light scattering device. The Raleigh ratio is defined as follows:

$$R_\theta = \frac{I_\theta r^2}{I_0 V} \tag{2-8}$$

I_θ: Scattered intensity

I_o: Intensity of the incident beam

V: Volume of the scattering medium

r: Distance between the scattering volume and the detector

Because the quantities measured directly are detector voltages and not light intensities, an additional term is needed to relate R_θ to detector voltage. This term, referred to as the Configuration Specific Calibration Constant (A_{cscc}), absorbs the geometrical volume and solid angle factors in addition to the detector sensitivity.

$$R_{90} = A_{CSCC} \times \frac{V_{90} - V_{90,dark}}{V_{laser} - V_{laser,dark}} \tag{2-9}$$

V_{90} 90° detector signal voltage

$V_{90,\ dark}$ 90° detector dark offset voltage

V_{laser} laser monitor signal

$V_{laser,\ dark}$ laser monitor signal dark offset

The calibration constant was measured by passing pure filtered toluene, Raleigh ratio of 1.406×10^{-5} cm^{-1} at a wavelength of 632.8 nm, through the cell at a flow rate of 1 ml/min and measuring the voltage signal on the 90° detector. The shutter on the laser was then closed and the laser monitor was disconnected so that the dark offset signals could be measured.

Due to the geometry of the flow through cell, A_{cscc} is dependent on the solvent type and the cell type. The manufacturers of the Wyatt Dawn F have developed the following relationship relating the A_{cscc} to a true instrument constant (A_{inst}) which is independent of those changing factors.

$$A_{inst} = \frac{F}{n_s n_g} \times A_{CSCC} \tag{2-10}$$

where n_s and n_g are the solvent and cell refractive indices, respectively and F is a Fresnel factor describing reflection losses at the various interfaces in the cell, given by

$$F = \left[1 - \left(\frac{n_g - n_s}{n_g + n_s}\right)^2\right]^2 \left[1 - \left(\frac{n_g - 1}{n_g + 1}\right)^2\right] \tag{2-11}$$

Normalization Coefficients

Because each detector has its own geometry and sensitivity, a set of "normalization coefficients" were calculated to relate each detector to the A_{CSCC} calculated for the 90° detector. Algebraically

$$R_\theta = N_\theta \times A_{CSCC} \times \frac{V_\theta - V_{\theta,baseline}}{V_{laser} - V_{laser,dark}} \tag{2-12}$$

Here $V_{\theta,baseline}$ is used because the sample being analyzed is in solution and therefore it is the excess Rayleigh ratio that is of interest. By definition the N_{90} is equal to unity. The normalization coefficient for the 90° detector is by definition zero. The normalization coefficients were measured by running a ~0.4% solution of a 27,000 molecular weight PEO standard through the columns. Using the known radius of gyration of this molecule as well as the calculated R_{90}, R_θ was calculated for the remaining angles, which was in turn used to calculate N_θ.

2.3.1.2 Calculation of Molecular Weights

The RI and LS peaks obtained were divided into slices and both M and c were calculated as described above for each slice. The weight average (M_w) and number average (M_n) molecular weights of all samples synthesized in this study were then calculated according to the following well-known equations[5]

$$M_n = \frac{\sum c_j}{\sum\left(\frac{c_j}{M_j}\right)} \tag{2-13}$$

$$M_w = \frac{\sum c_j M_j}{\sum c_j} \tag{2-14}$$

the polydispersity index (pdi) was calculated according to

$$pdi = \frac{M_w}{M_n} \tag{2-14}$$

2.4 Results

2.4.1 Solvent Choice

Because the reactivity of PEG propionic acid in aqueous solution has been documented,[3] the first attempt at synthesizing PEO star molecules used a 0.1 M sodium bicarbonate buffer solution. Although the reaction proceeded to completion under those conditions, a large (1.6x) excess of PEG was required to compensate for the hydrolysis of the NHS group. To eliminate the need for adding so much excess PEG, the reaction was attempted using dichloromethane as a solvent. Unexpectedly, a large degree of crosslinking appeared to take place under these conditions. This crosslinking was observed by a combination of noticing a white precipitate forming after the reaction proceeded for a number of hours, combined with peaks on the light scattering chromatogram corresponding to molecules with molecular weights twice as large as the maximum possible assuming complete reaction of the amine groups on the dendrimers.

It was believed that a small fraction (~1%) of the PEG supplied by Shearwater Polymers contained the species NHS-PEG-NHS which was resulting in crosslinking of a small fraction of our star molecules. When the reaction took place under aqueous conditions, hydrolysis of the extra NHS group lessened the probability of crosslinking. To support this theory, the dendrimers were reacted with a less than stoichiometric amount of PEG in buffer solution to increase the likelihood of one PEG molecule reacting with two dendrimers. Figures 2-6 and 2-7 show the results of this experiment along with results of an experiment when an excess of PEG was used. In addition, a sample of 20K methoxy-PEG-NHS that was reported to contain no NHS-PEG-NHS, was reacted in less than stoichiometric ratio with dendrimers using dichloromethane as the solvent. The chromatogram of the stars produced are shown in Figure 2-8. These results seem to support the hypothesis and led to the remainder of the star synthesis being carried out under aqueous conditions.

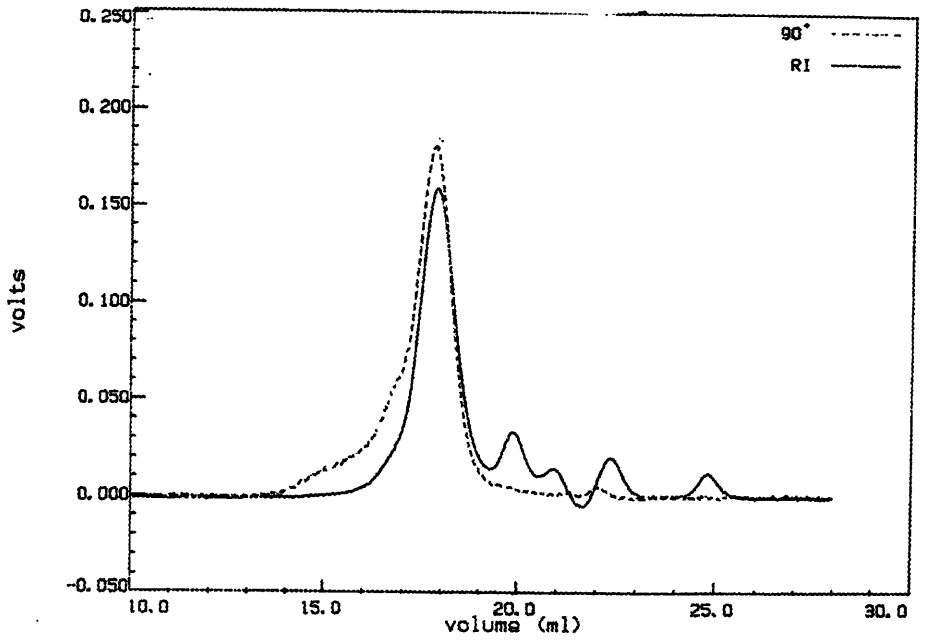

Figure 2-6. GPC/LS chromatogram of Sample 2-25-1. Dendrimers reacted with less than stoichiometric quantity of PEG in aqueous solvent

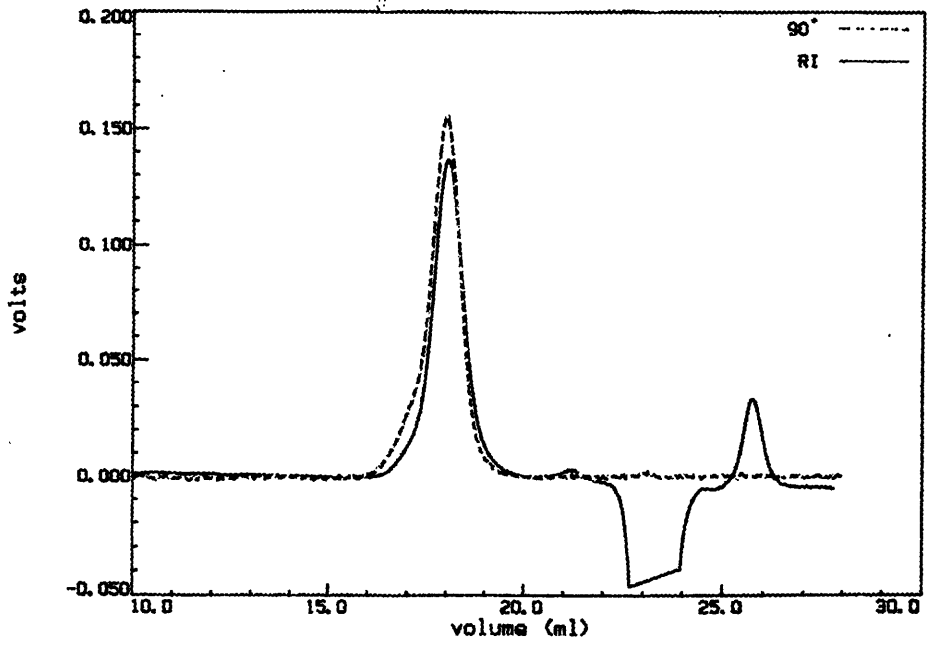

Figure 2-7. GPC/LS chromatogram of Sample 2-47-1. Dendrimer reacted with excess PEG in aqueous solvent

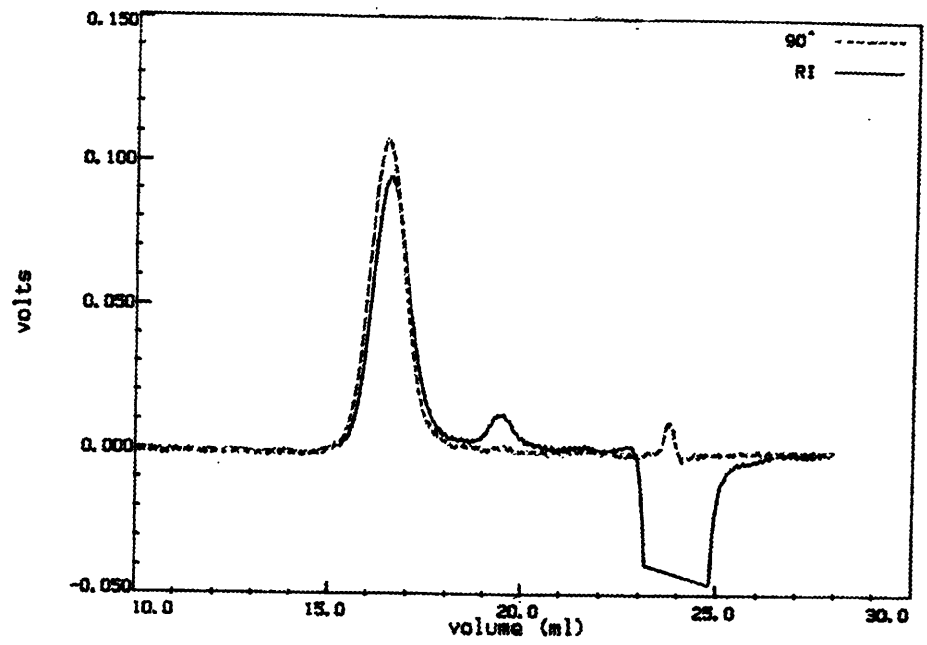

Figure 2-8. GPC/LS chromatogram of Sample 3-20-1. Dendrimer reacted with excess PEG in dichlormethane

2.4.2 Extent of Reaction of Dendrimer Amino Groups

It was decided to determine the maximum number of arms that could be reacted with one dendrimer. To do this dendrimers having from 8 to 256 primary amine groups were reacted with 5000 molecular weight methoxy-PEG-NHS in $NaHCO_3$ buffer. The number average molecular weights (M_n), polydispersity indexes (pdi - weight divided by number average molecular weight) and functionalities for all the star molecules is summarized in Table 2-2. The expected molecular weight was calculated by multiplying the number of amine groups on the dendrimer by the molecular weight of the PEG and adding the molecular weight of the dendrimer used. From these results we can see that when reacted with dendrimers of low functionality (<32), the functionalized PEGs react

with all the amine groups to create a relatively monodisperse molecule. However, as the functionality exceeds 32, steric hindrances preclude the PEGs from being able to react with all the amine groups on the dendrimers and M_n is less than expected, although we are still able produce star molecules with up to 140 arms. It is important to note that even when not all the amine groups on the dendrimer are reacted with PEG, a nearly monodisperse sample of star molecules still results. Based on these results dendrimers containing 16,32 and 64 functional groups were used to synthesize the star molecules used in the remaining experiments.

Table 2-2. Comparison of the expected Mn with that measured as well as the pdi and number of arms for the PEO star molecules synthesized

PEO Type	M PEO Note a	Dendrimer functionality (f_d) Note b	Dendrimer Mol Weight (M_d) Note b	Star Mn expected Note c	Star Mn found Note d	pdi Note d	Star functionality f_s Note e	Fraction of Dendrimer functions used f_s/f_d
MeO-PEO-NHS	5000	16	3206	83256	88700	1.09	17	1.06
MeO-PEO-NHS	5000	32	6909	166909	161000	1.06	31	0.97
MeO-PEO-NHS	5000	64	14215	334215	268000	1.01	51	0.80
MeO-PEO-NHS	5000	128	28000	668000	496000	1.06	94	0.73
MeO-PEO-NHS	5000	256	50845	1336000	778000	1.03	144	0.56

Notes:

(a) M_{PEO}, molecular weight of the PEO, g-mol-1, as reported by supplier, Shearwater Polymers, Inc
(b) Number of amino functions, f_d, and molecular weight, M_d, of the dendrimer as reported by supplier, Aldrich Chemical Co as selling agent for Dendritech, Inc
(c) Calculate by the formula: $M_{n,ex} = M_{PEO}f_d + M_d$, where $M_{n,ex}$ = expected molecular weight of the PEO star macromolecule
(d) Determined by GPC-LS, M_n, f_d is the experimentally determined molecular weight of the PEO star macromolecule
(e) Apparent star functionality, f_s, as calculated by the formula: $f_s = (M_n - M_d)/M_{PEO}$

2.4.3 Elution Volume vs. Molecular Weight

Usually, it is standard practice to calculate the molecular weight of a polymer based on the time it takes for it to pass through a gel permeation chromatography column.

In this method, polymer standards of known molecular weight are passed through a column and a standard curve is calculated by plotting the log of their molecular weight as a function of their elution volume. This standard curve is then used to calculate the molecular weight of the unknown sample.

As stated earlier, star polymers are much more compact than are their linear counterparts of equivalent molecular weight. Therefore it was expected that using such a standard curve to calculate the molecular weight of the star polymers synthesized in this study would lead to an underestimation of their molecular weight. Using the data obtained through GPC in series with light scattering, it is possible to compare just how the elution profile of star molecules compares with that of linear polymers. Figure 2-9 shows plots of molecular weight as a function of elution volume for both linear PEO standards and the star molecules synthesized in these studies. It can be clearly seen that star molecules that elute at the same elution volume as do linear polymers have much greater molecular weights. The greater the number of arms on a star molecule, the more their standard curves diverge.

It is interesting to look at the plots with the lines drawn through stars of constants arm molecular weight, as opposed to stars with constant arm number, Figure 2-10. These lines have been extrapolated to determine the elution volume at which they intersect the standard curve for linear PEO, and the corresponding molecular weights have been calculated and presented in Table 2-3, along with the number of arms that would correspond to stars of that molecular weight. Because elution volume corresponds to the hydrodynamic volume, these values are interpreted as being the maximum number of arms that can exist on a star before steric effects prohibit it from behaving as a random coil.

Table 2-3. Elution volumes at which the star and linear PEO elution profiles intersect

M_{arm}	Intersecting Ve	Corresponding Molecular Weight	Corresponding f
1847	20.3	13000	6.9
5000	18.7	33500	6.7
10000	17.6	66000	6.6
21469	16.7	116000	5.4

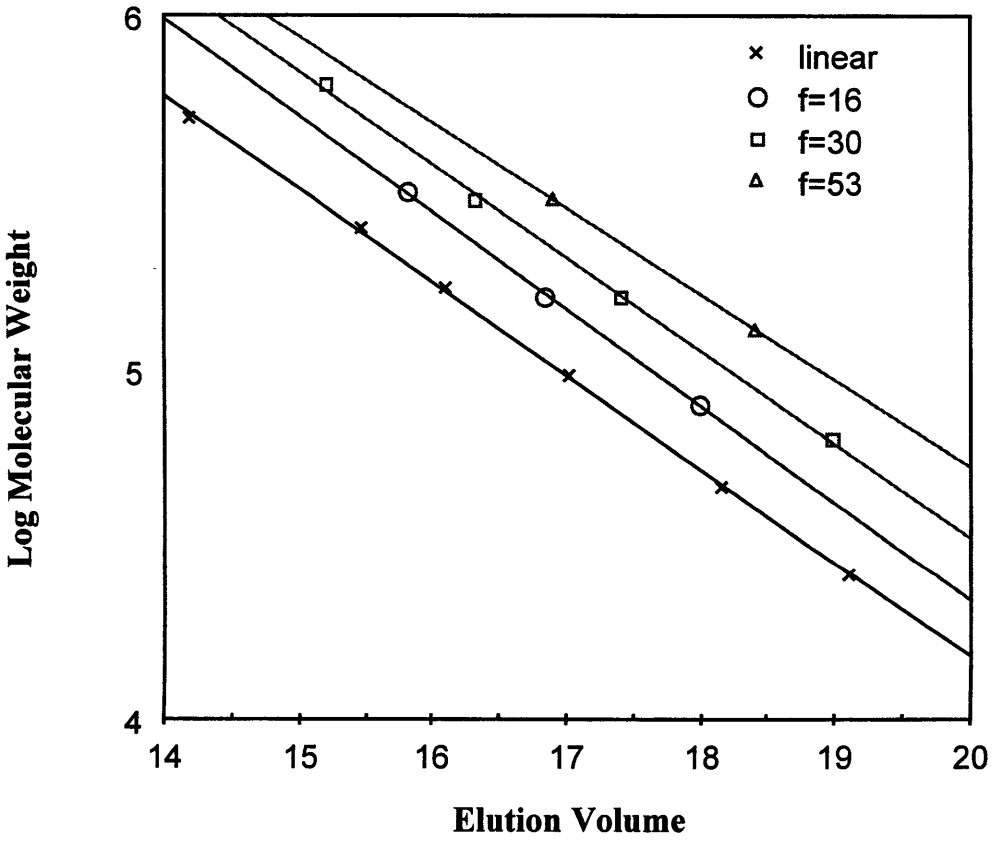

Figure 2-9. Molecular weight as a function of elution volume for bothe star and linear PEO. Lines drawn through molecules of constant arm number

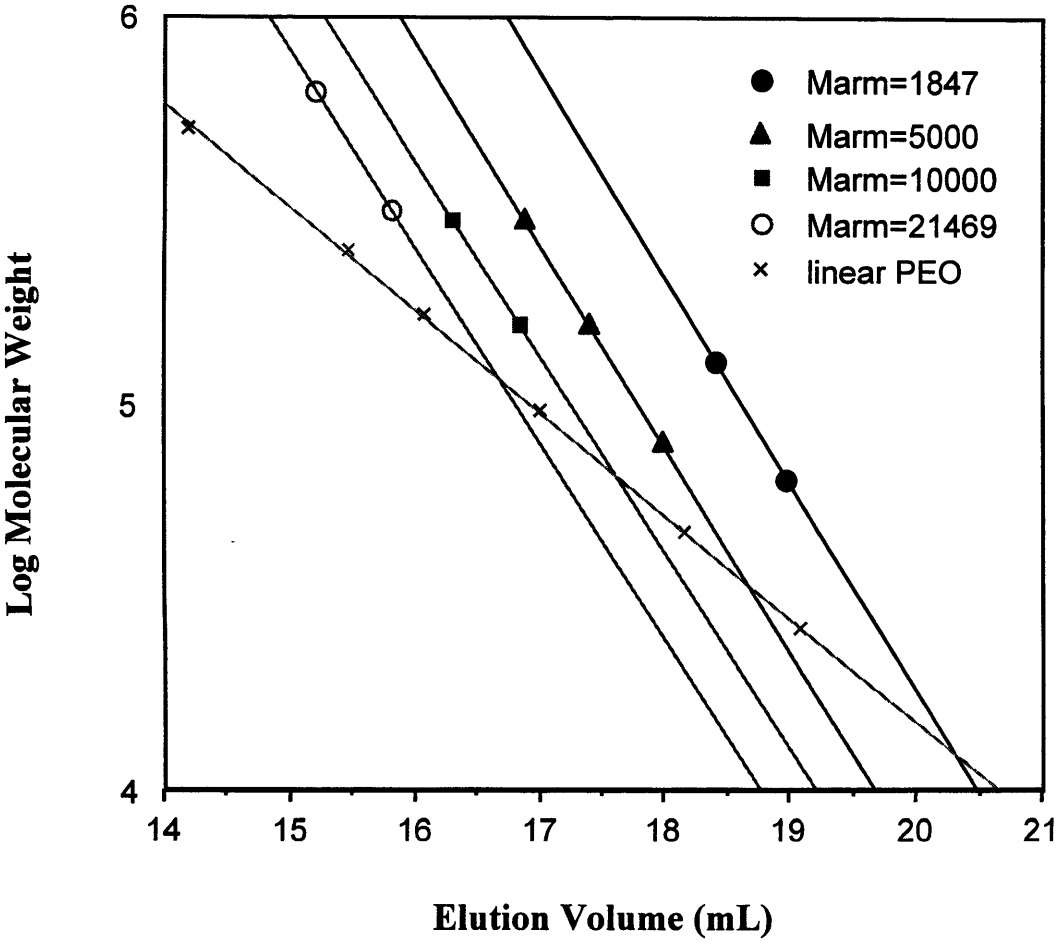

Figure 2-10. Molecular weight as afunction of elution volume for both star and linear PEO, lines drawn through molecules of constant arm molecular weight

2.5 References for Chapter Two

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CHAPTER THREE

Dilute Solution Properties

3.1 Introduction

While much work has been done characterizing the dilute solution properties of other star molecules, no such study has been done on PEO star molecules. Such a study is of interest for many reasons. The increasing interest in using PEO star molecules for biomedical application has made it the subject of many experimental investigations. Data on the dilute solution properties is necessary to correlate the results obtained with the properties of star molecules used. In addition, as researchers are developing new theoretical methods to model the behavior of star molecules with increasing functionality, experimental results are necessary to check the accuracy of these models. The reason no systematic experimental study on the physical properties of PEO star molecules has been undertaken is that until now there was no controlled method for synthesizing these molecules. The synthetic method described in the previous section provides the controlled method for synthesizing PEO star molecules needed for such a study.

3.2 Theory

3.2.1 Static Light Scattering

Static light scattering provides a means for measuring the weight averaged molecular weight M_W, the second virial coefficient A_2, and the radius of gyration R_G of polymer molecules. The standard method for obtaining these values is by using a Zimm plot.[1] This method utilizes a slightly different form of equation 2-4 shown below

$$\frac{K^* c}{R_\theta} = \frac{1}{MP(\theta)} + 2A_2 c \qquad (3\text{-}1)$$

the variables were defined in chapter 2 but are repeated here for convenience

A_2 second virial coefficient

K^* an optical constant which equals $4\pi^2 n_o^2 (dn/dc)^2 N_A^{-1} \lambda_o^{-4}$, where n_0 is the refractive index of the solution, N_0 is Avogadro's number, equal to 6.022×10^{23} and λ_0 is the wavelength of incident light in air expressed in nanometers.

$P(\theta)$ Particle scattering factor resulting from the diminished intensity of light scattered from large particles due to the fact that light scattered from one portion of the particle interferes with light scattered from another portion. It is equal to the scattered intensity for a large particle divided by the scattered intensity without interference

R_θ The excess Raleigh ratio

For particles with all molecular dimensions much less than the wavelength of the light being used ($<\lambda_S/20$), the particle scattering factor term is approximately equal to one and equation 3-1 can be approximated as

$$\frac{K^* c}{R_\theta} = \frac{1}{M} + 2A_2 c \qquad (3\text{-}2)$$

This equation is the basis of the Debye plot in which the Raleigh ratio is measured for a variety of concentrations at a constant angle. The second virial coefficient is obtained from the slope of K^*c/R_θ plotted as a function of c. The intercept yields M.

For larger molecules it has been shown that in the limit of low concentration and low angle the following relation exists

$$\lim_{\theta \to 0} \frac{1}{P(\theta)} = 1 + (\frac{16\pi^2}{3\lambda_s^2}) R_G^2 \sin^2(\frac{\theta}{2}) \tag{3-3}$$

and equation 3-1 can be rewritten as

$$\frac{K^* c}{R_\theta} = \frac{1}{M} + \frac{1}{M}\left(\frac{16\pi^2}{3\lambda_s^2}\right) R_G^2 \sin^2(\frac{\theta}{2}) + 2A_2 c \tag{3-4}$$

According to the method of Zimm,[1] the Raleigh ratio is measured at a number of angles for a range of concentrations of the sample being measured. The quantity $K^*c/\Delta R_\theta$ is plotted as a function of $\sin^2(\theta/2)+kc$ where k is an arbitrary constant chosen so that the product kc is typically between 0.2-0.4. Straight lines are fitted (1) through points with varying c at constant θ and extrapolated to c=0; and (2) through points with varying θ at constant c and extrapolate to θ=0. Straight lines are then drawn through the extrapolated points of zero concentration and zero angle and extrapolated to a common intercept at the axis of ordinates. The zero angle line gives a plot of K^*c/R_0 vs kc which gives 1/M as an intercept and $2A_2/k$ as the limiting slope. The zero concentration line also has 1/M for the intercept, with a limiting slope equal to $(16\pi^2/3M\lambda^2)R_G^2$.

It is important to note that the extrapolation of the line through points extrapolated to zero concentration is only applicable over a limited range of dimensions, $0.05 \pm R_G/\lambda \pm 0.5$. If R_G falls below the lower limit, $P(\theta)$ becomes too small for accurate estimation.

3.2.2 Dynamic Light Scattering

While for static light scattering techniques it is the total intensity of scattered light that is being measured, dynamic light scattering measures the real-time fluctuations in the intensity of scattered light which contains information relating to the Brownian motion of the polymer molecules. The technique used is known as photon correlation spectroscopy and involves measuring the autocorrelation function, G'(τ) of the intensity, i_θ, which is defined by[2]

$$G'(\tau) = \lim_{T \to \infty}\left[\frac{1}{T}\int_0^T i_\theta(t) i_\theta(t+\tau)dt\right] \tag{3-5}$$

The correlation time τ is the separation in time between two particular photon countings and T is the integration time. The measured autocorrelation function can be related to the normalized first-order electric field correlation function g(τ) by[3] (Brookhaven Manual)

$$b^{1/2} g(\tau) = [g'(\tau) - 1]^{1/2} \tag{3-6}$$

where b is an optical constant and g'(τ) is obtained by dividing G'(τ) by the baseline B. The decay of the normalized autocorrelation function g(τ) with increasing τ can be fitted by the following exponential function

$$\ln(g^{(1)}(\tau)) = -\Gamma\tau \tag{3-7}$$

where Γ is the characteristic decay rate. If there is more than one exponential decay contributing to the autocorrelation function then the above single exponential fit can be inaccurate. This is the case for all but very monodisperse samples, and therefore it is

usually more appropriate to use the method of cumulants in which the correlation function is expanded about an average linewidth

$$\ln(g^{(1)}(\tau)) = -\overline{\Gamma}\tau + \frac{\mu_2\tau^2}{2} + \frac{\mu_3\tau^3}{6} + \ldots \tag{3-8}$$

Finally, the characteristic decay rate is related to the mutual diffusion coefficient, D(c), of the scattering bodies by[2]

$$\overline{\Gamma} = D(c) \times q^2 \tag{3-9}$$

where q is the scattering wave vector given by

$$q = \left(\frac{4\pi n}{\lambda_o}\right) \times \sin\frac{\theta}{2} \tag{3-10}$$

The value of the diffusion coefficient is independent of the shape of the particle. It is only assumed that it represents translational diffusion.

The value of D(c) includes the effects of interparticle interactions. That is, when interparticle interactions are present, the movement of one particle is affected by the presence of neighboring particles, which may increase or decrease its overall diffusion. D(c) can be related to the self diffusion coefficient of the isolated polymer D_o and the concentration coefficient k_D^c as follows

$$D(c) = D_o(1 + k_D^c c) \tag{3-11}$$

3.2.3 Intrinsic Viscosity

Measurements of the viscosity of dilute polymer solutions can be used to provide information concerning the effects of polymer structure on chain dimensions. One parameter of particular importance for the purpose of polymer characterization is intrinsic viscosity. The intrinsic viscosity of a polymer is independent of concentration and relates the intrinsic ability of a polymer to increase the viscosity of a particular solvent at a given temperature. As will be discussed, it also provides a measurement for the effective size of a polymer in a particular solvent at a given temperature.

Table 3-1: Nomenclature of Dilute Solution Viscometry

Common Name	Symbol	Defining Equation
Relative viscosity	η_r	$\eta/\eta_o \sim t/to$
Specific viscosity	η_{sp}	$\eta_r - 1 = (\eta-\eta_o)/\eta_o$
Reduced viscosity	η_{red}	η_{sp}/c
Inherent viscosity	η_{inh}	$(\ln \eta_r)/c$
Intrinsic viscosity	$[\eta]$	$\lim_{C\to 0} (\eta_{red}) = \lim_{C\to 0} (\eta_{inh})$

The defining equations for the terminology used in this section are provided in Table 3-1, where η and η_o represent the viscosity of the solution and the solvent respectively. Intrinsic viscosity is conveniently measured by use of a capillary viscometer. The time it takes for the solution to flow between two points in the capillary is measured and the viscosity is determined by the following equation

$$\eta = ctd - \frac{Ed}{t^2} \tag{3-12}$$

Where t is the efflux time, d is the density of the solution, and C and E are constants specific to the viscometer used. For large flow times the relative viscosity η_r can be

estimated as the ratio of the efflux time for the solution, t, to that of the solvent, t_0. The specific viscosity expresses the incremental viscosity attributable to the polymeric solute, and the ratio η_{sp}/c is a measure of the specific capacity of the polymer to increase the relative viscosity. The intrinsic viscosity is the limiting value of this ratio at infinite dilution.[4] The specific viscosity of a solution of concentration c is related to $[\eta]$ by the following power series

$$\eta_{sp} = [\eta]c + k_1[\eta]^2c^2 + k_2[\eta]^3c^3 + \ldots \tag{3-13}$$

for dilute solutions this equation can be truncated and rearranged to the following form[5]

$$\frac{\eta_{sp}}{c} = [\eta] + k_H[\eta]^2 c \tag{3-14}$$

which is known as the Huggin's equation. The experimentally observed Huggins constant k_H for randomly coiled linear polymer molecules is approximately 0.35. As stated in chapter one, for a suspension of hard spheres the value of the Huggins constant has been calculated to be 0.99.[6]

Intrinsic viscosity may also be defined by the Kraemer equation[4]

$$\frac{\ln(\eta_r)}{c} = [\eta] + k_K[\eta]^2 c \tag{3-15}$$

where $k_K = k_H - 1/2$. Therefore the intrinsic viscosity can be calculated by extrapolating either the reduced viscosity or the inherent viscosity to zero concentration. It has been found that for star polymers of high functionality, the higher order terms in equation 3-13 are non negligible resulting in a plot of reduced viscosity as a function of concentration exhibiting upward curvature.[7]

3.2.4 Converting Dilute Solution Properties to Their Respective Radii

Typically characterization involves various measures of molecular "size". This can be done by converting the values obtained for [η], A_2, and D_o, into equivalent radii based on the respective equations for hard spheres .

3.2.4.1 Viscometric Radius

The viscometric radius, also known as the Einstein equivalent radius, is derived form Einstein's equation for the relative viscosity of a suspension of spheres of volume fraction ϕ

$$\frac{\eta}{\eta_o} = 1 + \frac{5}{2}\phi \tag{3-16}$$

By taking the volume of a single sphere to be equal to $(4/3)\pi R_V^3$, the volume fraction to be equal to $(c/M)N_A(4/3)\pi R_V^3$ and rearranging, the above equation can be rewritten in the following form

$$\frac{\frac{\eta}{\eta_o} - 1}{c} = \left(\frac{5}{2}\right)\left(\frac{cN_A}{M}\right)\left(\frac{4}{3}\pi R_V^3\right) \tag{3-17}$$

Substituting in numerical values for the constants, dividing both sides by c and taking the limit of infinite dilution leads to the defining equation

$$R_V = 5.41 \times 10^{-9}([\eta]M)^{\frac{1}{3}} \tag{3-18}$$

with [η] measured in dL/g, the radius calculated is in centimeters.

3.2.4.2 Stokes Radius

The Stokes radius is based on the equation for the frictional coefficient of an impermeable hydrodynamic sphere of radius R_S

$$f_o = 6\pi\eta_o R_S \tag{3-19}$$

where the frictional coefficient is equal to kT/D_0 and k is Boltzmann's constant. Rearranging leads to

$$R_S = 5.31 \times 10^{-2} \frac{kT}{\eta_o D_o} \tag{3-20}$$

With D_0 measured in cm^2/s, the radius calculated using this formula is in centimeters.

3.2.4.3 Thermodynamic Radius

The thermodynamic radius is based on the following second virial relation for hard spheres[8]

$$V = \frac{A_2 M^2}{4N_A} \tag{3-21}$$

Rearranging and substituting in values for the constants leads to the following equation for the thermodynamic hard sphere equivalent radius

$$R_T = 4.63 \times 10^{-9} (A_2 M^2)^{\frac{1}{3}} \tag{3-22}$$

With A2 reported in ml mol/g2, Rt is also calculated in centimeters.

3.3 Experimental Apparatus and Conditions

3.3.1 Dynamic Light Scattering

Dynamic (quasielastic) light scattering was performed using an apparatus consisting of a Lexel model 95 2W argon laser (λ=514.5 nm), a goniometer, and an autocorrelator (model BI-9000AT, Brookhaven Instruments, Holtsville, NY). The temperature of the samples was maintained at 30°C within ±0.1°C by a circulating ethylene glycol bath. The alignment of the instrument was checked before each measurement using I sinθ measurements of toluene.[3] Measurements were made at three different scattering angles to ensure that the only contribution to the correlation function was from the polymer center of mass mode. While no angular dependence was found, for consistency all the data reported here was obtained at a scattering angle of 90°.

All samples were prepared in aqueous solutions containing 0.02% NaN_3 as a bacteriostat. The solutions were made up using water that was purified using a Milli-Q ion-exchange system. To minimize interference from dust, each sample was filtered five times through a 0.2 μm syringe filter into a scattering cell that had been rinsed with acetone .

The diffusion coefficient D(c) was extracted from the measured autocorrelation function using the cumulants analysis method with a quadratic fit. Typically twenty intensity autocorrelation functions $G'(\tau)$ were obtained at each concentration for each molecular weight the average diffusion coefficients were plotted as a function of polymer concentration. The plots were then fitted via a linear least squares regression to obtain D_0 and k_d^c. The regressions were weighted using the standard deviation of the average autocorrelation functions calculated.

3.3.2 Static Light Scattering

In the static light scattering measurements the total scattered light intensity was measured using the same apparatus that was described above with the temperature being maintained at 30°C. The experimental parameters were set so that that at least 10 sample measurements were made at a duration of 0.5 seconds per intensity measurement. The dust rejection ratio was set at 3.[3] The refractive index of the sample cell and the index matching vat liquid (decalin) were used to compensate for reflections. The power on the laser was adjusted so that the intensity at 30° for the highest concentration being measured was 750,000 counts per second. The calibration constant for the instrument was calculated prior to each sample's measurements using toluene as the reference liquid. The refractive index increment was estimated to be 0.135[9], the value for linear PEO in water at λ = 514.5nm and 30°C.

Measurements were typically taken at ten angles between 30° and 145° for all samples. Due to an inability to remove dust from the solvent, its intensity was calculated for all other scattering angles from a single measurement at 90°. All data were originally analyzed using a Zimm plot, a sample of which is shown in Figure 3-1. For samples whose hydrodynamic diameter was later calculated to be less than 20 nm ($< 0.05*\lambda_s$) the data were reanalyzed using a Debye plot at a scattering angle of 90°.

3.3.3 IntrinsicViscosity

The intrinsic viscosities of the samples were measured using a Cannon-Ubbelhode micro capillary viscometer immersed in a water bath having a temperature stability of ±0.1°C. The diameter of the capillary of the viscometer was chosen so as to have flow times greater than 200 seconds, thus eliminating the possibility of any shear rate dependence as well as justifying neglecting the kinetic energy term in equation 3-12.

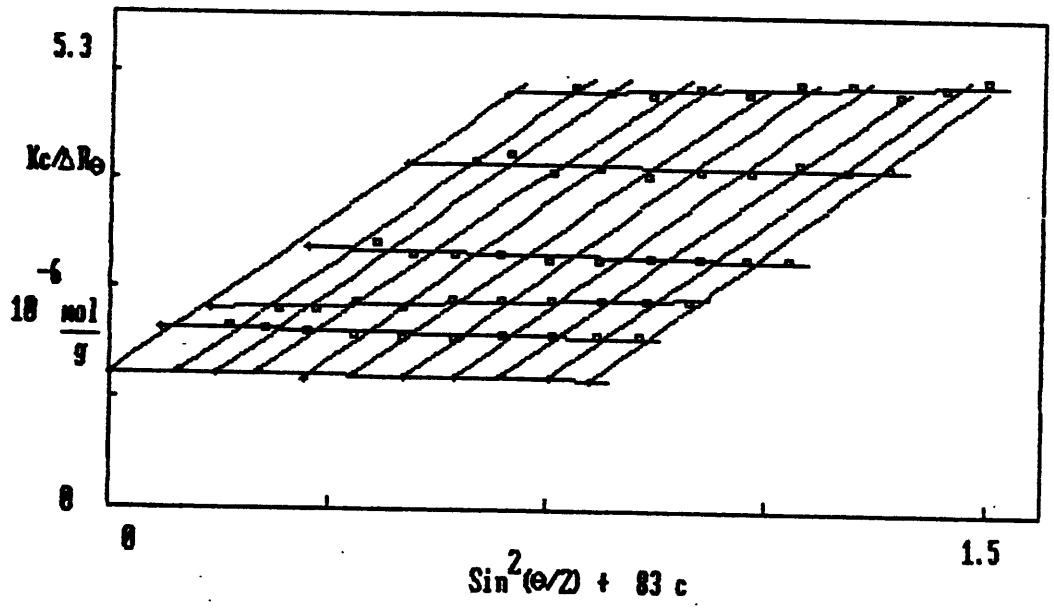

Figure 3-1. Zimm plot of light scattering data for sample 3-1 taken in water at 30 °C. λ_0=514.5 nm.

Dilutions were prepared directly within the viscometer by the addition of filtered solvent. To allow the solution to come to thermal equilibrium, the viscometer containing the sample was immersed in the water bath for a minimum of 15 minutes prior to taking a measurement. The time it took for the sample to flow through the capillary was measured to the nearest 0.01s. Measurements were repeated for each dilution until three readings were obtained which agreed within 0.1% of their mean.

All samples were prepared in aqueous solution containing 0.02% NaN_3 as a bacteriostat. Concentrations were chosen so that the most concentrated solution had a flow time approximately twice that of the solvent. At least five different concentrations were run for each sample analyzed. All solutions used were filtered at least once through a 0.2 μm syringe filter.

The data was analyzed by plotting both the inherent and reduced viscosity as functions of concentration. The intrinsic viscosity was estimated using a second order polynomial fit of the reduced viscosity plot as well as a linear fit of the inherent viscosity. A sample plot is shown in Figure 3-2.

3.4 Results

Samples of methoxy terminated PEO star molecules were synthesized by reacting methoxy-PEG-NHS of molecular weights varying between 1847 and 21467 with dendrimers containing 16, 32 and 64 functional groups. Methoxy terminated PEGs were chosen for this study so as to exclude the possibility of extraneous effects due to interactions between functional end groups. The above analytical techniques were used to calculate the dilute solution properties of the aforementioned star molecules.

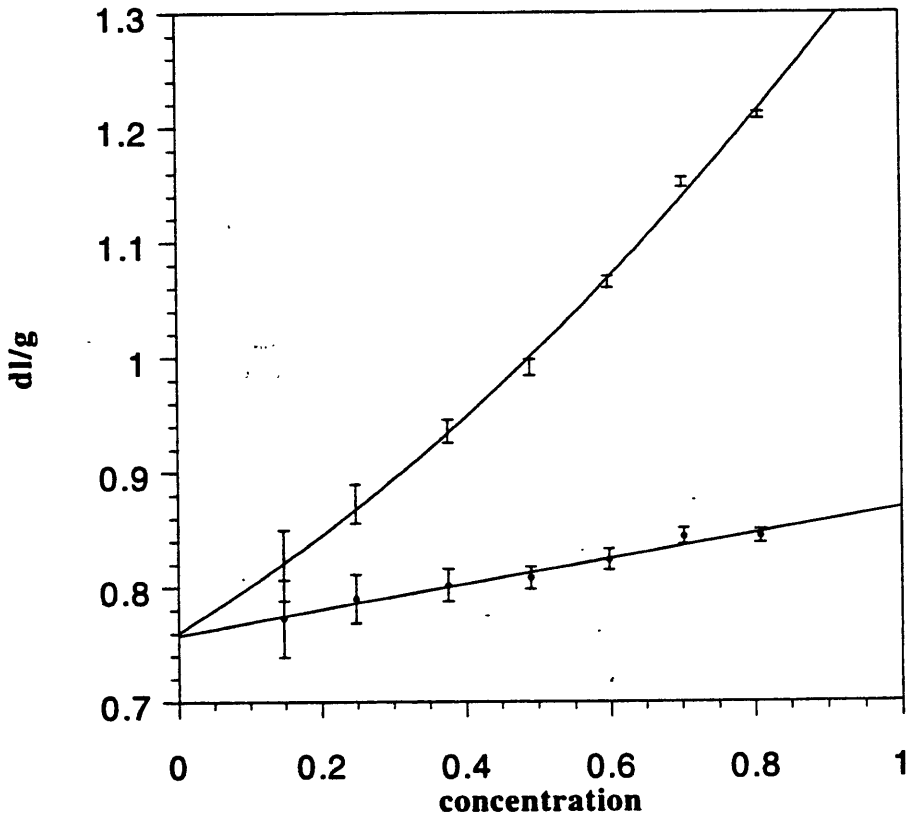

Figure 3-2. Determination of the intrinsic viscosity of 2-82-1 in water at 30 °C

3.4.1 Dynamic Light Scattering

As stated above, dynamic light scattering data was used to determine the diffusion coefficient for the polymer molecules synthesized. The data are summarized in Table 3-2. The molecular weight dependence of the diffusion coefficient of polymer molecules is well described by the power law

$$D_o = K_D M^{-a_D} \tag{3-23}$$

where K_D and a_D are constants for each polymer-solvent system at given values of temperature and pressure. For linear PEO the coefficients are 1.8875e-4 and 0.56992 respectively when D_o is measured in cm^2/s.[10]

Table 3-2. Summary of Dynamic Light Scattering Results

Sample	M_{arm}	M_W	f_{calc}	$D_o \times 10^7$ cm^2/s	k_d^c (ml/mg)	k_d^ϕ
3-24-1	1847	32700	15.9	6.26 (±0.23)	0.01	1.53
2-36-1	5000	84300	16.2	4.14 (±0.18)	0.01	1.54
2-84-1	10000	165000	16.2	2.76 (±0.04)	0.03	1.65
2-67-1	21469	330000	15.2	1.77 (±0.04)	0.11	3.60
2-76-1	1847	65600	31.8	5.18 (±0.21)	0.01	1.41
2-47-1	5000	160000	30.6	3.65 (±0.15)	0.00	-0.44
2-82-1	10000	300000	29.3	2.34 (±0.04)	0.02	1.68
2-66-1	21469	630000	29.0	1.49 (±0.02)	0.05	1.86
2-89-1	1847	114000	54.0	4.38 (±0.11)	0.01	1.42
2-31-1	5000	270000	51.2	2.90 (±0.10)	0.02	2.09
3-1-1	10000	580000	56.6	1.69 (±0.02)	0.01	0.44

For star polymers the molecular weight varies as a function of both the number of arms *f* and the molecular weight of the arms M_{arm}. A log-log plot of D_O as a function of

star molecular weight is shown in Figure 3-3. Lines are fitted through star polymers all containing the same number of arms. It can be seen from these results that the above relationship holds only for stars of constant *f*. As the number of arms on the star molecule increases, the curves shift upward. The values for the different constants obtained are summarized in Table 3-3. It is interesting to note that the scaling behavior appears to be similar for all the star molecules in this study, with all having similar values of a_D. The error in the intercept is too great to be able to draw any quantitative results.

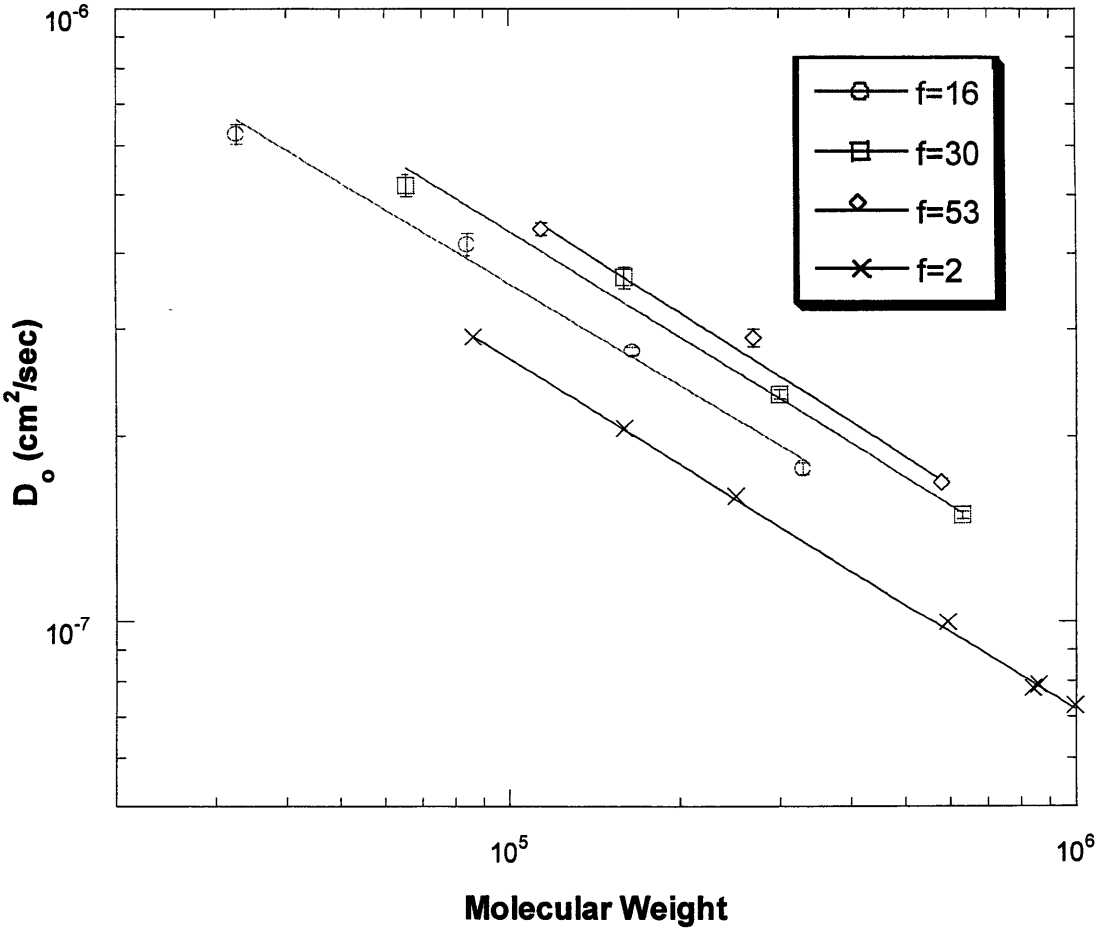

Figure 3-3. Log-Log plot of the diffusion coefficient against molecular weight for linear and star shaped PEO in water at 30 °C.

Table 3-3. Coefficients Describing the Molecular Weight Dependence of the Diffusion Coefficient (cm^2/s) for Linear and Star PEO

f	$K_D \times 10^4$	a_D
2	1.9	0.57
16	1.7	0.54 (± 0.02)
30	3.3	0.57 (± 0.02)
53	3.1	0.56 (± 0.02)

The measured values of k_D^c from the virial expansion for the concentration dependence of the diffusion coefficient expressed in equation 3-11 were converted to volume fraction units, k_D^ϕ. These values are shown in Table 3-2. While there are a couple of outliers, within error all are at or near the value that is predicted assuming the only interactions between the polymer molecules are from hard body repulsions.[11] Therefore it was assumed there were no additional interactions between the star molecules that need to be accounted for in the remaining experiments.

3.4.2 Static Light Scattering

3.4.2.1 Second Virial Coefficient Measurements

Values for A_2, were calculated for all PEO star molecules synthesized using the Zimm Plot method. For samples whose diameters were less than 20nm, the data were reanalyzed using a Debye plot. The values for the two methods were shown to be equal to one another within experimental error. Therefore all values discussed in this section, which are presented in Table 3-4 are those determined using Zimm plots. The decrease in A_2 with both increasing f and increasing M_{arm} is expected.[8]

Table 3-4. Summary of Second Virial Coefficients

Sample	M_{arm}	M_W	f_{calc}	$A_2 \times 10^3$ (±17%) ml mol/g^2
3-24-1	1847	32700	15.9	1.15
2-36-1	5000	84300	16.2	0.76
2-84-1	10000	165000	16.2	0.76
2-67-1	21469	330000	15.2	0.72
2-76-1	1847	65600	31.8	0.52
2-47-1	5000	160000	30.6	0.51
2-82-1	10000	300000	29.3	0.50
2-66-1	21469	630000	29.0	0.36
2-89-1	1847	114000	54.0	0.30
2-31-1	5000	270000	51.2	0.29
3-1-1	10000	580000	56.6	0.15

As with the diffusion coefficients, the second virial coefficients were fit to log-log plots against molecular weight (Figure 3-4). The following relation was established by using a direct power law fit of A_2 to M_W

$$A_2 = K_A M^{a_A} \tag{3-24}$$

Again the data were fit to lines of constant *f*. Values of K_A and a_A are shown in Table 3-5. While there is much error in the estimated values of the scaling parameter a_A, it is encouraging to see that it is within range of that found for linear PEO, -0.20±0.06.[10]

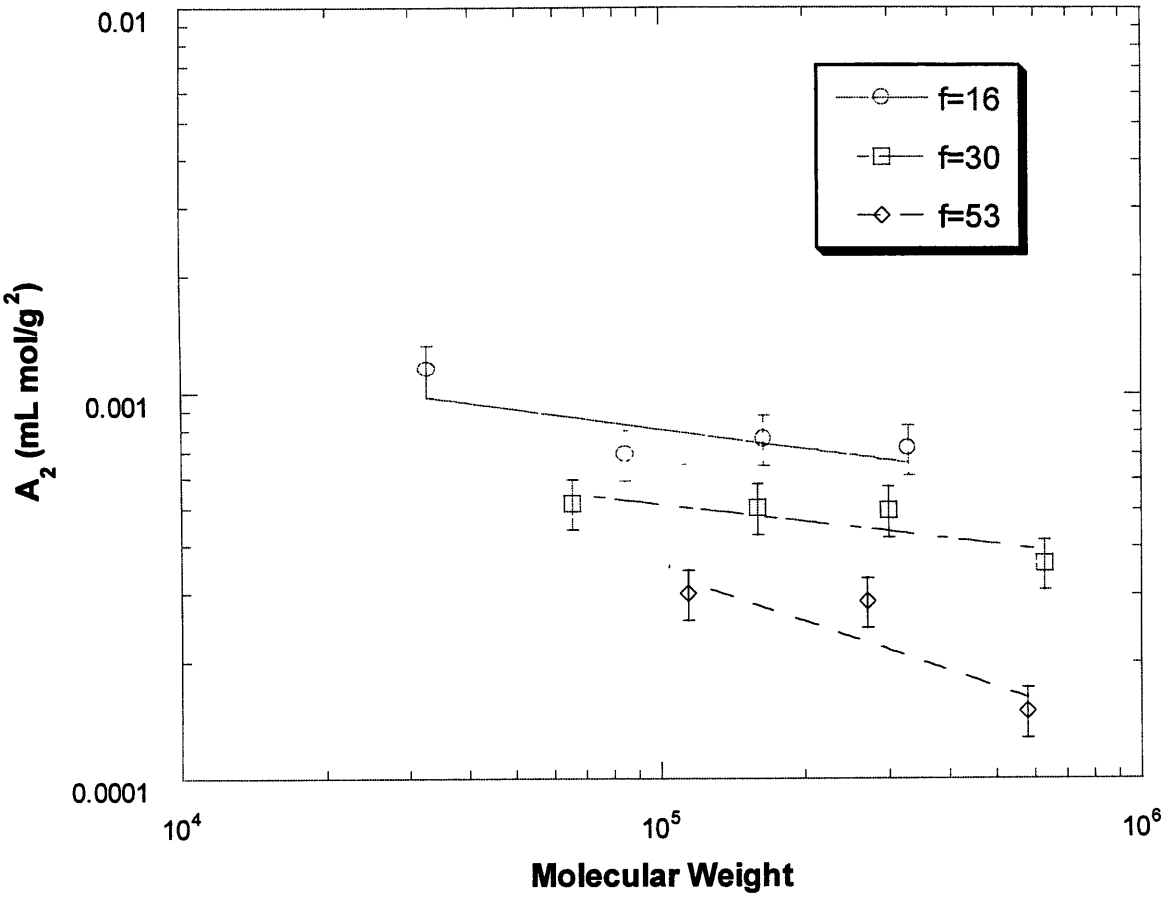

Figure 3-4. Log-log plot of the second virial coefficient against molecular weight for linear and star shaped PEO

Table 3-5. Coefficients Describing the Molecular Weight Dependence of the Second Virial Coefficient (mL mol/g^2) for Linear and Star PEO

f	K_A	a_A
2	1.84	-0.20 (± 0.06)
16	0.57 (± 0.6)	-0.17 (± 0.10)
30	0.28 (± 0.2)	-0.15 (± 0.08)
53	4.48 (± 6.8)	-0.42 (± 0 .12)

3.4.2.2 Radius of Gyration

The values obtained for the radius of gyration using the Zimm plot method appeared to have a large amount of scatter. Closer analysis of the expected radii of

gyration explains why. The Stokes radius of the largest star molecules synthesized was found to be 20 nm. Using the estimated hard sphere relationhip between R_S and R_G (R_S=1.291*R_G), it is expected that the radius of gyration should be approximately 15.5 nm. As stated earlier, the expression for P(θ) used in analyzing the Zimm Plot is not valid for molecules whose radii is less than 1/20th of the wavelength of light being used. Since a 514.5 nm light source was used in this investigation, R_G data are not valid for any sample with R_G less than 20nm. Since R_G data as obtained were invalid, radius of gyration will not be addressed further.

Table 3-6. Summary of Intrinsic Viscosity Results

Sample	M_{arm}	M_W	f_{calc}	[η] (dL/g)	k_H	k_K
3-24-1	1847	32700	15.9	0.16	1.02 (± 0.07)	0.08(± 0.06)
2-36-1	5000	84300	16.2	0.25	1.14(± 0.03)	0.75 (± 0.25)
2-84-1	10000	165000	16.2	0.45	0.94 (± 0.03)	0.42 (± 0.18)
2-67-1	21469	330000	15.2	0.74	0.92 (± 0.20)	0.28 (± 0.20)
2-76-1	1847	65600	31.8	0.14	1.44 (± 0.12)	0.43 (± 0.11)
2-47-1	5000	160000	30.6	0.24	0.94 (± 0.29)	0.51 (± 0.40)
2-82-1	10000	300000	29.3	0.40	1.31 (± 0.02)	0.71 (± 0.02)
2-66-1	21469	630000	29.0	0.66	1.24 (± 0.21)	0.68 (± 0.27)
2-89-1	1847	114000	54.0	0.13	1.12 (± 0.03)	0.37 (± 0.005)
2-31-1	5000	270000	51.2	0.22	1.55 (± 0.44)	0.47(± 0.07)
3-1-1	10000	580000	56.6	0.37	0.75 (+-0.35)	0.59 (+-0.08)

3.4.3 Intrinsic Viscosity

The values obtained for the intrinsic viscosity as well as those calculated for both the Huggins and Kraemer constants are summarized in Table 3-6. The plots obtained using the Huggins equation exhibited upward curvature and were therefore fit to a second

order polynomial. For linear random coiled polymers the intrinsic viscosity increases with increasing molecular weight according to the empirical Mark-Houwink equation.

$$[\eta] = K_{[\eta]} M^{a_{[\eta]}} \tag{3-25}$$

For the star molecules of constant *f*, as the molecular weight of arms of the star molecule increases, the intrinsic viscosity increases as expected. However, if the change in [η] is examined as a function of molecular weight, whereby the molecular weight of the arms is held constant and *f* is varied, it can be seen that [η] actually decreases with increasing molecular weight (ie increasing arm number). This is due to the star molecules becoming denser in polymer segments as the number of arms is increased.

The intrinsic viscosity as a function of molecular weight was plotted on a log-log plot and lines were drawn through data from stars of constant *f* via a direct power-law fit. The Mark-Houwink relation appears to provide a good fit, and the parameters found are shown in Table 3-7. While the intercept varies depending on the number of arms, within error the scaling parameter is nearly the same for all the star molecules synthesized.

Table 3-7. Coefficients Describing the Molecular Weight Dependence of the Intrinsic Viscosity (dL/g) for Linear and Star PEO

f	$K_{[\eta]} \times 10^5$	$a_{[\eta]}$
2	12.5	0.78
16	7.3 (4.1)	0.73 (0.04)
30	5.8 (1.7	0.70 (0.02)
53	9.3(5.2)	0.62 (0.04)

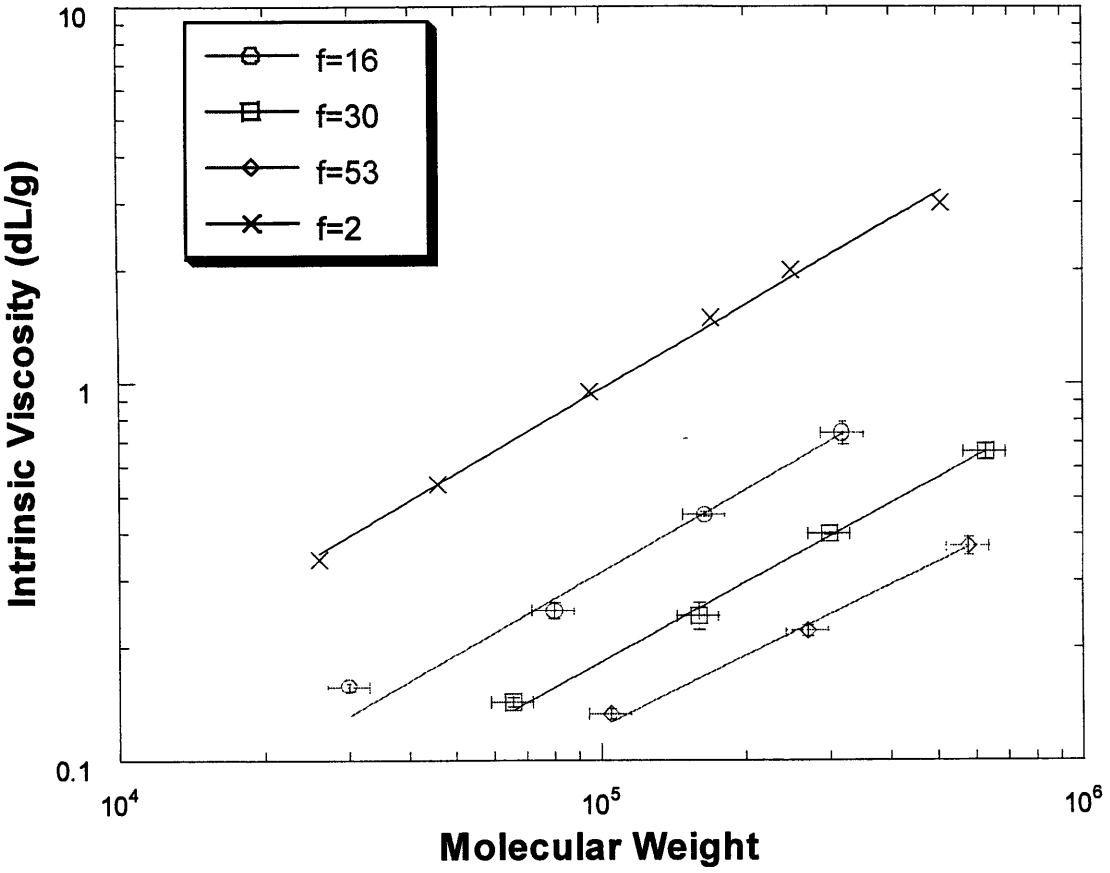

Figure 3-5. Log-log plot of the intrinsic viscosity against molecular weight for linear and star branched PEO

3-5 Star Molecules as Hard Sphere

Values of R_S, R_V, and R_T are summarized in Table 3-8. The various ratios of radii were also calculated for individual samples. Since all the radii are defined for non-draining, impenetrable spheres, these ratios should all be equal to one if the star molecules behave as hard spheres.

The average value of the ratio of dynamically determined radii, R_V/R_S, does turn out to be equal to 0.99 ± 0.06. This is in agreement with other results found by researchers for star polymers of high functionality.[7,12] Comparison of the statically determined radio R_T to the dynamically determined R_V yields ratios greater than one with

an average value of 1.22 ± 0.08. These results support the more realistic picture of star molecules behaving as fuzzy, as opposed to impenetrable spheres, having greater hydrodynamic than thermodynamic penetration.

Table 3-8. Summary of Radii Calculated

Sample	M_{arm}	M_W	f_{calc}	$R_{[\eta]}$ (nm)	R_S (nm)	R_{A2} (nm)	R_S/R_V	R_T/R_V
3-24-1	1847	32700	15.9	4.32	4.455	4.96	1.03	1.15
2-36-1	5000	84300	16.2	6.94	6.74	7.89	0.97	1.14
2-84-1	10000	165000	16.2	10.55	10.125	12.72	0.96	1.21
2-67-1	21469	330000	15.2	15.68	15.73	19.81	1.00	1.26
2-76-1	1847	65600	31.8	5.30	5.39	6.05	1.02	1.14
2-47-1	5000	160000	30.6	8.50	7.65	10.87	0.90	1.28
2-82-1	10000	300000	29.3	12.38	11.92	16.44	0.96	1.33
2-66-1	21469	630000	29.0	18.74	18.67	24.27	1.00	1.30
2-89-1	1847	114000	54.0	6.22	6.365	7.30	1.02	1.17
2-31-1	5000	270000	51.2	9.81	9.61	12.77	0.98	1.30
3-1-1	10000	580000	56.6	15.05	16.52	17.11	1.10	1.14
						Average =	**0.99**	**1.22**

As stated earlier, for linear, random coiling polymer molecules the experimentally observed value of k_H is ~0.35 and the experimentally observed value of k_K is ~-0.15. It has been found that for star polymers with *f* greater than 18 these values hover around those predicted for hard spheres, 0.99 and 0.5.[7,12] The data on k_H and k_K found in this investigation, shown in Table 3-6, have much error in them due to very low values for the intrinsic viscosity. However they clearly show deviations from behavior exhibited for linear PEO, with the plot obtained from the Kraemer equation having a positive slope, and the values of k_H calculated hovering around 1.

3.6 Comparison With Results on Other Star Polymers

One common method for examining the effects of branching on star architecture is to by comparing the dimensions of the molecule with those of linear polymers of the same molecular weight. Three dimensionless parameters that are often calculated are[8]

$$g_{[\eta]} = [\eta]/[\eta]_{lin} \tag{3-26}$$

$$g_S = R_S/(R_S)_{lin} \tag{3-27}$$

$$g_{A2} = A_2/(A_2)_{lin} \tag{3-28}$$

Values of $g_{[\eta]}$, g_S, and g_{A2} for the PEO star molecules synthesized are collected in Table 9. The values for linear PEO were calculated using the empirical correlations found in the literature.[10,13] Douglas and Freed[14] calculated a semiempirical correlation for these values which is shown for comparison in Table 9. As the number of arms on the star molecules increase, the experimental results found in this study deviate further and further from their predictions. As the number of arms goes above 50, their results for $g_{[\eta]}$ obviously no longer apply as they predict the physically impossible values of less than zero.

Ideally the set of g ratios of branched to linear polymer properties provide a "fingerprint", specifying uniquely the branching architecture.[7] It is encouraging to see that the numerical results results found for PEO star polymers compare very closely to those found for star molecules composed of other polymers.[7,14]

Another comparison of radii can be made by comparing the ratio of R_V for a star polymer to $(R_V)_a$, the viscometric radius for the corresponding unattached arm. For other star polymers investigated this ratio has been found to depend only on *f*.[12] Values of $(R_V)_a$ were calculated using the known values of M_a and the [η] vs M relationships for linear PEO.[13] The results are given in Table 3-10 . A log-log plot depicting the variation

of $R_V/(R_V)_a$ with f is shown in Figure 3-6. The parameters found for the following expression obtained from regression analysis of the data obtained for PEO stars

$$\frac{R_V}{(R_V)_a} = (1.45 \pm 0.11) f^{(0.30 \pm 0.02)} \tag{3-29}$$

are within experimental error of those found for polyisoprene star molecules in good solvents.[12]

$$\frac{R_V}{(R_V)_a} = (1.36) f^{(0.304)} \tag{3-30}$$

Table 3-9. Variation of Hydrodynamic Ratios with Chain Architecture

				Experiment			Theory	
Sample	M_{arm}	M_W	f_{calc}	g_S	$g_{[\eta]}$	g_{A2}	g_S	$g_{[h]}$
3-24-1	1847	32700	15.9	0.81	0.38	0.50	0.66	0.25
2-36-1	5000	84300	16.2	0.72	0.29	0.37	0.65	0.25
2-84-1	10000	165000	16.2	0.73	0.31	0.46	0.65	0.25
2-67-1	21469	330000	15.2	0.77	0.29	0.50	0.67	0.27
2-76-1	1847	65600	31.8	0.66	0.20	0.26	0.48	0.07
2-47-1	5000	160000	30.6	0.56	0.17	0.30	0.49	0.09
2-82-1	10000	300000	29.3	0.61	0.17	0.34	0.50	0.10
2-66-1	21469	630000	29.0	0.63	0.16	0.29	0.51	0.11
2-89-1	1847	114000	54.0	0.57	0.12	0.17	0.32	-0.03
2-31-1	5000	270000	51.2	0.52	0.10	0.19	0.34	-0.02
3-1-1	10000	580000	56.6	0.58	0.09	0.12	0.31	-0.03

Another ratio which gives more physical insight to the behavior of star polymers is $R_V/(2*R_{Varm})$, this ratio is a measurement of how stretched the polymer chains are in the star polymer as compared to their randomly coiled state. Values of this ratio are tabulated in Table 3-11. The viscometric radius used in this calculation has been adjusted by subtracting the portion of the radius due to the dendrimer core. As can be seen by the

results, stretching of the linear chain increases as a function of both *f* and M_{arm}.

Table 3-10. Variation of R_V/R_{varm} with Chain Architecture

Sample	M_{arm}	M_W	f_{calc}	R_V/R_{varm}
3-24-1	1847	32700	15.9	3.28
2-36-1	5000	84300	16.2	3.32
2-84-1	10000	165000	16.2	3.56
2-67-1	21469	330000	15.2	3.36
2-76-1	1847	65600	31.8	4.11
2-47-1	5000	160000	30.6	4.07
2-82-1	10000	300000	29.3	4.17
2-66-1	21469	630000	29.0	4.02
2-89-1	1847	114000	54.0	4.83
2-31-1	5000	270000	51.2	4.70
3-1-1	10000	580000	56.6	5.07

Table 3-11. Variation of $R_V/(2*R_{varm})$ with Chain Architecture

Sample	M_{arm}	M_W	f_{calc}	$R_V/2R_{Varm}$
3-24-1	1847	32700	15.9	1.08
2-36-1	5000	84300	16.2	1.31
2-84-1	10000	165000	16.2	1.53
2-67-1	21469	330000	15.2	1.52
2-76-1	1847	65600	31.8	1.36
2-47-1	5000	160000	30.6	1.60
2-82-1	10000	300000	29.3	1.78
2-66-1	21469	630000	29.0	1.82
2-89-1	1847	114000	54.0	1.54
2-31-1	5000	270000	51.2	1.81
3-1-1	10000	580000	56.6	2.16

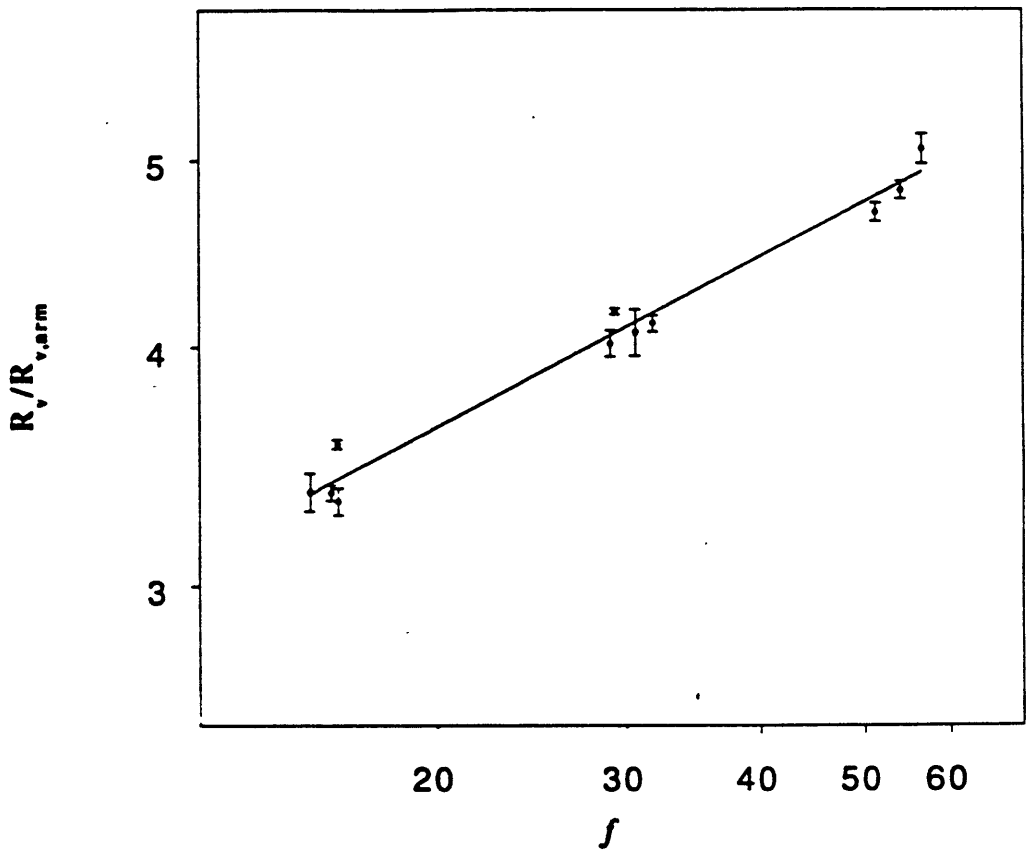

Figure 3-6. Ratio of viscometric radii for star and unattached arm as a function of arm number

3.7 Density Comparison with Linear PEO

Earlier it was stated that star molecules have higher density of polymer segments than do linear polymers. The data obtained on the radii of PEO star molecules allows for a direct comparison of densities of the two forms of PEO. The density of polymer segments for the star molecules was calculated according to

$$d = \frac{M_{seg}}{V_{seg} N_A} \tag{3-31}$$

The segmental volume V_{seg} was calculated using the Stokes radius to calculate the volume of the star and then subracting from that number the volume of the dendrimer. The molecular weight of the dendrimer was subtracted from the molecular weight of the dendrimer to get M_{seg}. N_A is Avogadro's number. The density for linear PEO of the same molecular weight as the star polymers was calculated according to the same equation using the known value of R_S. The results are shown in Table 3-12, along with the ratio of the two densities. These results clearly show that the star polymers have a much higher segment density than do their linear counterparts. As expected, the ratio of star density to linear density also increases as the number of arms on the star molecule increases.

According to the Daoud Cotton scaling model[16] for star molecules, the density of the polymer segments decreases the as their distance from the core increases. This hypothesis can also be verified by taking the data obtained on the segmental densities of

the star molecules. For example, by subtracting the volume of the star molecule containing arms of 1847 molecular weight from the volume of the star containing 5000, the density in the outer half of the 5000 molecular weight star molecule can be estimated. This procedure can then be repeated for the star molecules with arms of 10000 and 21469 molecular weight. These values were calculated for the star molecules containing 16 and 30 arms. The results are shown in Figure 3-7. These results clearly show that the segmental density of the star molecules increases as the polymer segments get further away from the core.

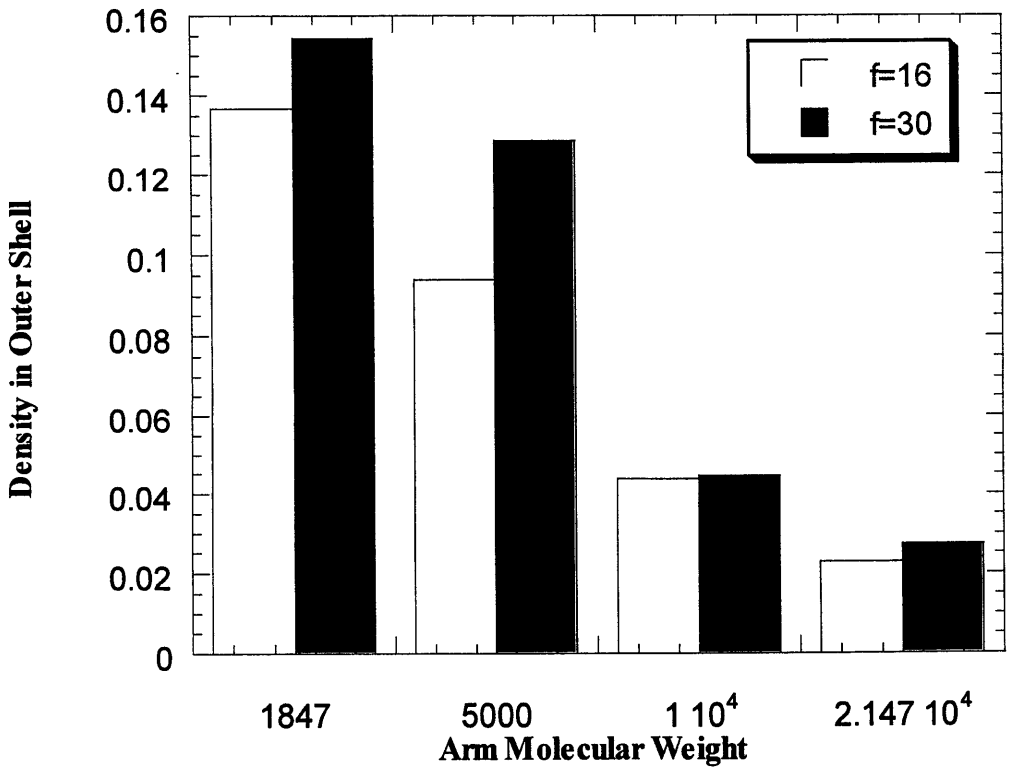

Figure 3-7. Density of polymer segments moving outward from the core

Table 3-12. Comparison of Polymer Segment Density (g/ml) in Star PEO with that of Linear PEO of Equivalent Molecular Weight

Sample	f	M_{arm}	Molecular Weight	Star Density	Linear PEO Density	d_{star}/d_{lin}
3-24-1	16	1847	32700	0.137	0.071	1.93295
2-36-1	16	5000	84300	0.106	0.038	2.75685
2-84-1	16	10000	165000	0.062	0.024	2.55121
2-67-1	16	21469	330000	0.033	0.015	2.22573
2-76-1	32	1847	65600	0.154	0.043	3.60726
2-47-1	32	5000	160000	0.137	0.024	5.66855
2-82-1	32	10000	300000	0.069	0.016	4.35594
2-66-1	32	21469	630000	0.038	0.009	4.03024
2-89-1	64	1847	114000	0.161	0.028	5.68601
2-31-1	64	5000	270000	0.116	0.017	7.006
3-1-1	64	10000	580000	0.050	0.010	5.05737

3-8 References for Chapter Three

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CHAPTER FOUR

Dual Armed Star Molecules

4.1 Introduction

Another goal of this investigation was to examine the possibility of using PEO star molecules as a drug delivery vehicle. This was achieved by taking advantage of the fact that the new method of synthesis described in Chapter 2 also enables the synthesis of a unique type of PEO star molecule containing more than one type of arm, shown in Figure 4-1. No other method developed so far for synthesizing PEO star molecules enables the synthesis of such a molecule. The arm first method synthesizes star molecules with all methoxy terminated arms. The core-first method synthesizes star molecule whose arms are all the same length and all contain the same outer functional group.

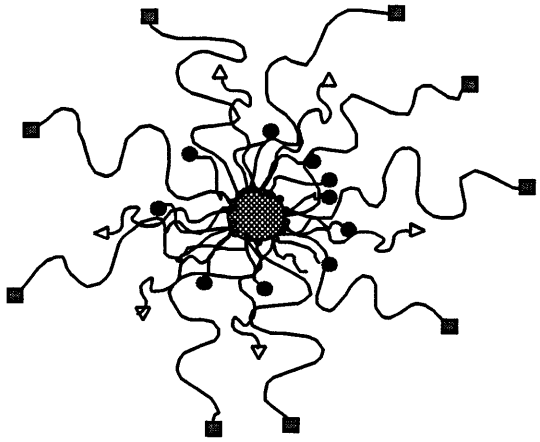

Figure 4-1. Multiarmed Star Polymer

This chapter describes the synthesis of star molecules containing both long (~20000 MW) and short (~2000 MW) arms. The long arms have a methoxy group at

their terminus while the shorter arms have a hydroxy group. It is believed that if some type of drug molecule is attached to the shorter arms, the longer arms would then wrap around the drug isolating either it from the immune system, or if the molecule of interest is toxic, isolating the body from the drug until it has reached its intended target.

4.2 Synthesis

4.2.1 Experimental Method

Synthesis of the dual armed star molecules was attempted in both aqueous and organic solvents. In order to have more precise control over the ratio of long to short arms on the star polymers synthesized, the synthesis protocols for both solvents involved sequential reactions. That is, the longer arms were reacted with the dendrimer first. The shorter PEO molecules were added to the reaction mixture only after all the long arms had reacted.

4.2.1.1 Cores

Generation three polyamidoamine dendrimers were used as the core for all the dual armed stars synthesized in this investigation. These dendrimers contain 32 primary amine groups.

4.2.1.2 Arms

Two different heterofunctional linear PEG molecules were reacted with the dendrimer core.

Long Arms

PEG having a methoxy group at one end and an N-succinimidyl group at the other, with a molecular weight of 21469 was used as the longer arm. Because reactions between this PEG and the dendrimer result in PEO star molecules with methoxy terminated arms, it was assumed that these arms would not participate in any future reactions.

Short Arms

PEG containing a hydroxyl group on one end and an N-succinimidyl group on the other were used as the shorter arm. The molecular weight of this PEG was reported by Shearwater to be 2025. Reactions between this PEG and the dendrimer result in arms on the star molecules that terminate in hydroxyl groups. The hydroxyl groups on these arms were then used in subsequent reactions to attach other molecules to the dual armed star molecule.

4.2.1.3 Synthesis in Aqueous Solution

A known quantity of dendrimer was dissolved in 0.1M sodium bicarbonate buffer. The linear PEG chains, which were to become the longer arms of the star molecules, were then added. The quantity of PEG to be added was determined by first calculating the ratio of PEG to dendrimer desired (ie the number of long arms desired). To compensate for loss of the succinimidyl group on the PEG due to hydrolysis, the actual amount of PEG added was 15% greater than the number calculated. The solution was made up so that the concentration of PEG was 70mg/ml. After the reaction had gone to completion a sample was removed for GPC analysis. An excess (~ 100%) of the linear PEG which was to become the shorter arms of the star molecule was then added. Any remaining unreacted PEG was removed via diafiltration as described in Chapter 2.

4.2.1.4 Synthesis in Dichloromethane

Dichloromethane and methanol were dried overnight over molecular sieves. All glassware used was dried overnight in a convection oven at 120°C. The methoxy terminated PEG which was to become the longer arms of the star was dissolved in dichloromethane. Dendrimer was added to the PEG/dichloromethane mixture and stirred until the suspension was well mixed. The quantity of dendrimer added to the PEG was determined in the same manner as describe above, however only a 4% excess of PEG was used since hydrolysis was not a major concern. Methanol was then added to dissolve the dendrimer. After the reaction was complete the hydroxy terminated linear PEG, which was to become the shorter arms of the star molecule, was added in excess. Again, prior to addition of the hydroxy terminated PEG, a sample of the reaction mixture was taken for GPC analysis. After the second reaction had gone to completion, the dichloromethane was evaporated off and the star molecules were dissolved in deionized water. Any remaining unreacted PEG and byproducts were removed by ultrafiltration

4.2.2 Results

The GPC chromatograms of the PEO star molecules synthesized in the above manner appeared to show some very strange results. The chromatogram taken from the star molecules synthesized in water after addition of the longer arms, but prior to the addition of the shorter arms, showed star molecules with higher molecular weights than were possible based on the quantity of PEG reacted with the dendrimers. In contrast, chromatograms taken from the star molecules synthesized in dichloromethane, prior to addition of the smaller molecular weight PEG, showed no peaks at all. These results turned out to be due to ionic interactions occurring between the GPC columns and the star molecules.

It was discovered that when star molecules were synthesized to have much less arms than the number of primary amines on the dendrimer used to synthesize it, the positive charges on the remaining primary amines caused them to adhere to the GPC columns. This was because the GPC columns used contain a number of carboxyl groups. This problem was remedied by switching the mobile phased used for GPC analysis from just an aqueous 0.04 w/v% NaN_3 solution, to one consisting of 0.8 M sodium nitrate with 0.02 w/v% NaN_3 azide.[1]

4.2.2.1 Solvent Choice

The dual armed PEO star polymers synthesized in buffer solution were more polydispersed than those synthesized in dichloromethane. As stated above, the chromatogram of a sample taken prior to the addition of the shorter arms exhibited star molecules having higher molecular weights than were thought possible. A mass balance revealed only a small portion of the star molecules injected actually appeared on the chromatogram. Apparently some of the dendrimers were reacting with more of the linear PEG than were others. For those dendrimers enough of their primary amines had undergone reaction to avoid their adherence to the column, therefore they appeared on the chromatogram. The remaining dendrimers, with fewer linear PEO attached, adhered to the column and therefore were not visible on the chromatogram. Figure 4-2 shows the chromatogram taken prior to the addition of the smaller arms, while Figure 4-3 shows a chromatogram taken afterward. The results from the second chromatogram reveal that the dual armed star molecules synthesized according to this method are not homogenous. There appear to be two populations of star molecules synthesized, one containing many long arms and fewer short arms and one containing fewer long arms and more shorter arms.

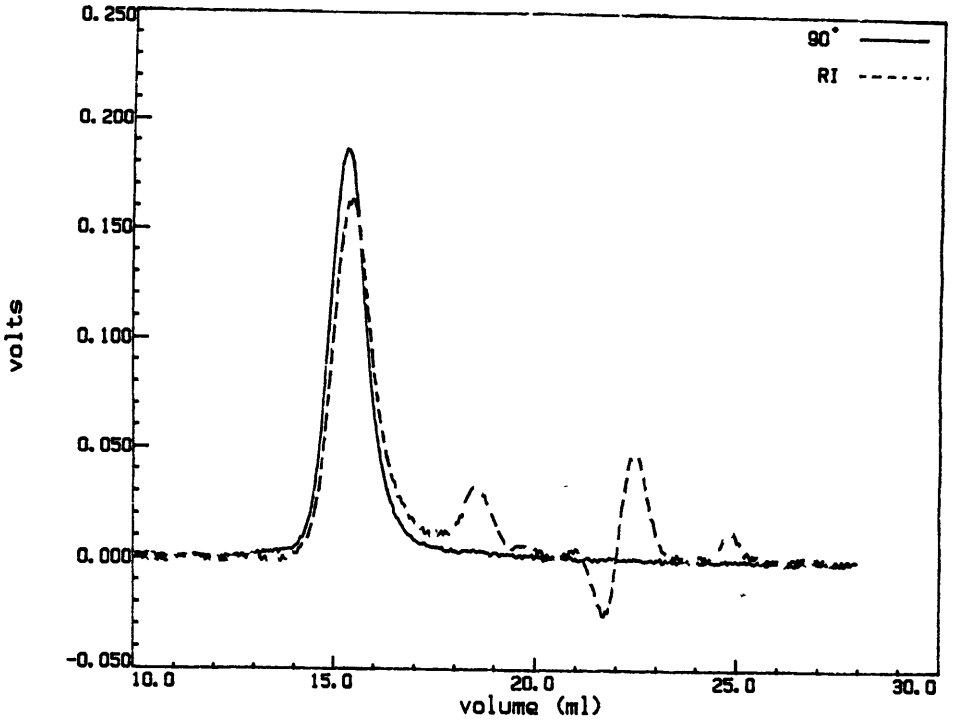

Figure 4-2. GPC/LS chromatogram of sample 3-8-1. M_w 530,000

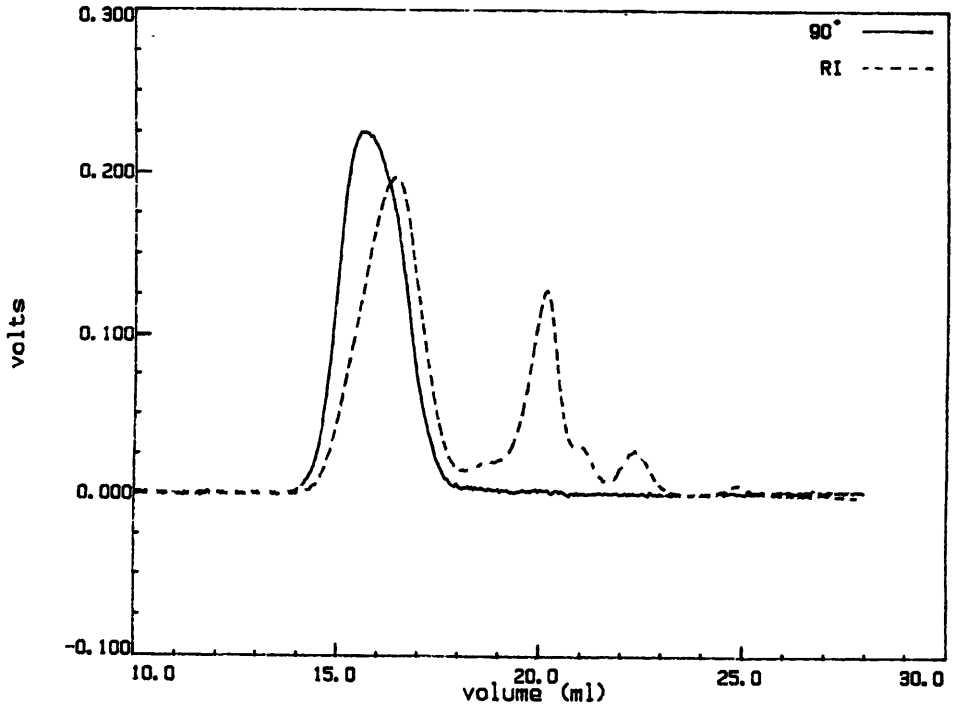

Figure 4-3. GPC/LS chromatogram of sample 3-8-2, M_w calculated to be 270,000

In contrast to the dual armed star molecules synthesized in aqueous solution, those synthesized in dichloromethane were more monodisperse. As stated above, when mobile phase containing no sodium nitrate was used, chromatograms of samples taken prior to the addition of the shorter arms revealed no peaks corresponding to star molecules. However, when sodium nitrate was added to the mobile phase a peak was observed which corresponded to a monodisperse star molecule with the molecular weight expected based on the ratio of PEG to dendrimer reacted. GPC chromatograms taken of a sample prior to and after reacting with the smaller molecular weight PEG are shown in Figures 4-4 and 4-5 respectively. Unlike the dual armed star polymer synthesized in buffer, this method results in a monodisperse sample, with all of the star molecules containing the same amount of both long and short arms. It is interesting to note that while the molecular weight increases slightly after the addition of the 2025 molecular weight PEG, the elution volume does not. Adding the shorter arms to the star molecule does not affect the volume of the star molecule to an appreciable degree.

It is believed that this difference in star polymers synthesized in the different solvents is due to the dendrimers insolubility in dichloromethane as well as the fast reaction between dendrimers and PEG. In aqueous solution the PEG molecules begin reacting with the dendrimer as soon as they start to dissolve and before the solution has time to become well mixed. Because the dendrimers are insoluble in dichloromethane, the suspension has the opportunity to become well mixed before the addition of methanol solvates the dendrimer.

4.2.2.2 Control Over Number of Long and Short Arms

Based on the above results all subsequent synthesis of dual armed stars took place in dichloromethane. With the assumptions that (1) the dendrimer core molecular weight

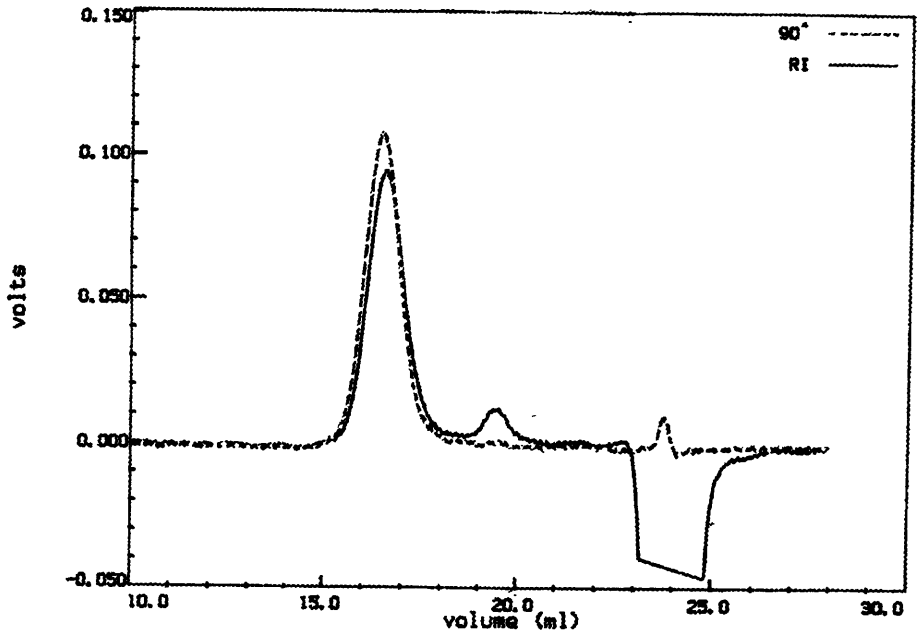

Figure 4-4. GPC/LS chromatogram of sample 3-20-1, M_w calculated to be 265,000

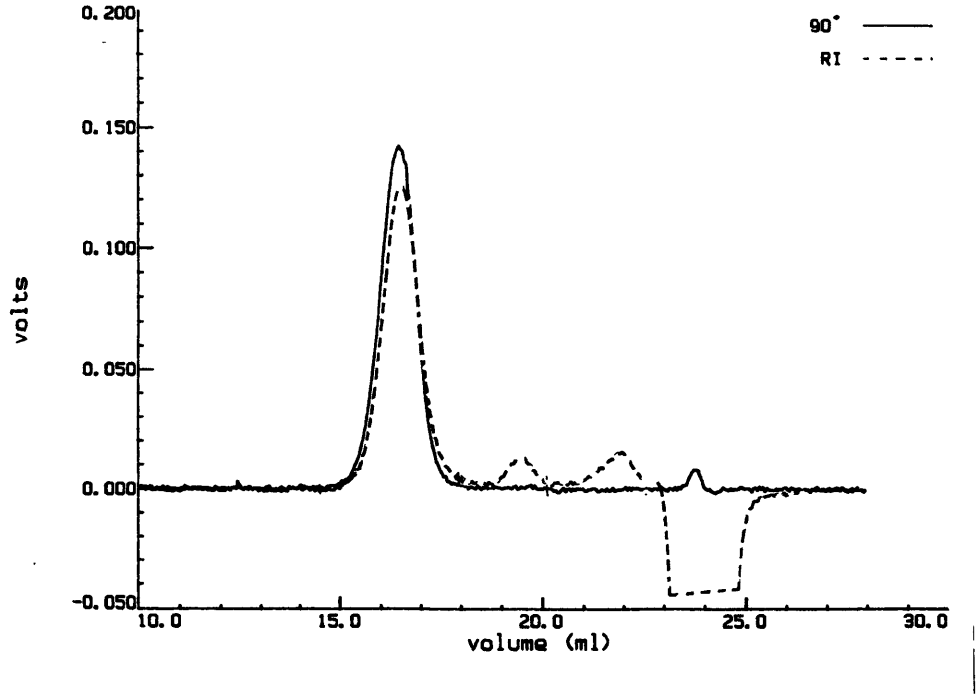

Figure 4-5. GPC/LS chromatogram of sample 3-20-2, M_w calculated to be 304,000

could be neglected and (2) the amines on the dendrimer react, the final molecular weight was estimated to be given by:

$$M_{final} = (32 - x) \times 21469 + 2030x \tag{4-1}$$

where x represents the number of dendrimer amino groups which attached 2025 molecular weight PEG and 32-x is equal to the number of dendrimer amino groups that attach 21469 molecular weight PEG. The ratio of high molecular weight PEG to dendrimer used in the synthesis, as well as the characteristics of the resulting star molecules produced are summarized in Table 4-1. Since the number of dendrimer amino groups which attached 21,469 molecular weight PEG is close to the initial charge ratio, it can be concluded that the coupling reaction of the NHS ester on PEG to the amino group on the dendrimer is very efficient and that consequently it is possible to predetermine the ratio of the two types of PEG arms to be attached to the dendrimer. Additionally, the dual armed stars produced in this manner have a very narrow polydispersity indicating all stars in a batch have the same number of long and short arms. This is very important for some of the uses proposed for these molecules.

Table 4-1. Summary of Properties of Dual Armed Star Molecules Synthesized

Molecular Weight	20kPEG/dendrimer ratio	Polydispersity Index	# of long arms	# of short arms	Diameter (nm)
170,000	6:1	1.10	6	26	22
295,000	11:1	1.05	12	20	28
353,000	15:1	1.03	15	17	32

4.2.2.3 Dynamic Light Scattering

Dynamic light scattering was performed on the above samples. The results are

included in Table 4-1. The results are as expected, with diameter of the star molecules increasing as the ratio of longer arms to shorter arms on one molecule increases.

4.3 Physical Interpretation

The dualed arm stars can be represented as consisting of a sphere whose surface is covered with immobilized linear PEG, see Figure 4-6. The number of short arms is f_s and the number of long arms is f_l. M_S represents the molecular weight of the short arms and M_l represents the molecular weight of the longer arms. The inner sphere has a radius equal to that of a star molecule containing f arms of molecular weight M_S. The molecular weight of the linear PEG "immobilized" on the surface of the sphere is equal to M_l-M_S. One goal is to determine the ratio of long arms to short arms (of molecular weight M_l and M_s respectively) necessary to shield a molecule attached to the ends of the shorter arms from other large molecules in solution.

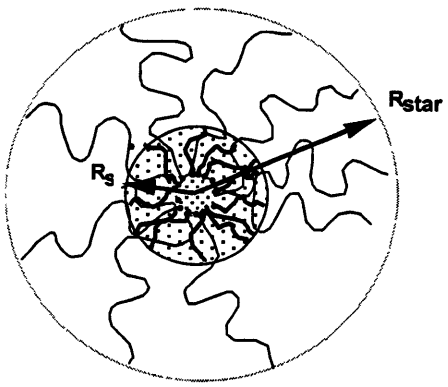

Figure 4-7. Dual armed star containing f_s short arms of molecular weight M_s and f_l long arms of molecular weight M_l. The radius of the "inner sphere" is equal to that of a star containing f arms of molecular weight M_s

For the system used in this study M_l is equal to 21469, M_s is equal to 2025 and the total number of arms f is 32. Therefore any small molecules bound to the shorter arms

can be envisioned as sitting on the surface of a sphere whose radius is approximately 5.3 nm, protected by immobilized linear PEG with molecular weight equal to 19444. The question that needs to answered is how many of these chains are needed to prevent proteins from reaching the inner sphere?

4.4 Determining the Accessibility of the Shorter Arms

Many of the uses that are being proposed for these dual armed star molecules involve attaching some sort of bioactive species to the shorter arms. Therefore it is necessary that the presence of the longer arms does not preclude the end groups on the shorter arms from undergoing reactions with other species. However, while it is necessary that the functional groups on the shorter arms have the ability to react with small bioactive species, another desirable feature of these dual armed star molecules is that the longer arms hinder larger molecules, like immune system proteins, from reaching what is attached to the shorter arms.

4.4.1 Tresylation

To prove that the longer methoxy terminated arms would not hinder the shorter hydroxyl terminated arms ability to react with other molecules, they were reacted with 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride, TrCl), a molecule known for its ability to activate hydroxyl groups for covalent modification to primary amines.

Trifluoroethanesulfonyl chloride (tresyl chloride, TrCl) and triethylamine (TEA) were purchased from Aldrich Chemical Inc. (Milwaukee, WI). The reaction procedure was a slight modification of that published by Nilsson and Mosbach[2,3]. The reaction vessel (30 mL round-bottom flask) with stir bar was dried overnight in a convection oven

at 120°C. The PEO to be tresylated was dissolved in dichloromethane (~ 10% w/v) followed by the addition of molecular sieves. The solution was allowed to finish bubbling with the cap opened slightly. The mixture was then sealed and refrigerated at 4°C overnight. TEA and extra dichloromethane were also dried over molecular sieve and refrigerated overnight.

The polymer solution was decanted into the reaction vessel and the remaining molecular sieves were rinsed twice with the dried dichloromethane in an effort to recover all the polymer. Stirring was then begun as TEA and then TrCl were added to the reaction mixture. The quantity of TrCl and TEA to be added was determined by first calculating the number of moles of hydroxyl groups on the star molecule (approximated as being equal to [mass PEO][f_s]/[M_W Star). The amount of TEA and TrCl to be added was three times the number of moles of OH calculated.

The reaction was allowed to proceed for at least 90 minutes before the dichloromethane was removed under vacuum. The polymer was then dissolved in 20 mL of methanol with 150 μL concentrated HCl and placed in a centrifuge tube at -20°C overnight to precipitate the tresylated PEO. For some of the star polymers of higher molecular weight it was necessary to warm up the solution slightly to get the PEO to dissolve in the acidified methanol. After precipitation of the PEO the solution was centrifuged at -20°C for 25 minutes, the supernatant poured off, and the PEO dissolved again in 20 ml of methanol containing 20 μL of concentrated HCl. This process of precipitation, centrifugation, and redissolving in acidified methanol was repeated until a total of at least 6 precipitations were performed. Note that only the first precipitation was left overnight at -20°C, the remaining were centrifuged after 2 hours. Once the polymer

was recovered from the last precipitation the methanol was removed under vacuum. Any tresylated polymer not used immediately was stored under nitrogen, dessicated, at -20°C.

The quantity of tresyl groups attached were then measured by elemental analysis on Fluorine performed by Quantitative Technologies Inc. The results showed the star molecules to be approximately 100% tresylated.

4.4.2 Avidin-Biotin

Avidin is a glycoprotein containing four identical subunits having a combined molecular weight of 67,000-68,000. Each subunit binds one molecule of biotin, a 244 molecular weight vitamin found in tissue and blood. The avidin-biotin interaction is the strongest known noncovalent biological interaction [4] ($K_a=10^{15}M^{-1}$) between protein and ligand. This system was used to test the ability of the longer arms to preclude larger molecules from reaching a bioactive species attached to the shorter arms.

Biotin was attached to the tresyl-activated stars. These molecules were then exposed to avidin, and the amount of avidin that was able to bind to the bound biotin was measured.

4.4.2.1 Reacting Biotin to Tresyl Activated Star

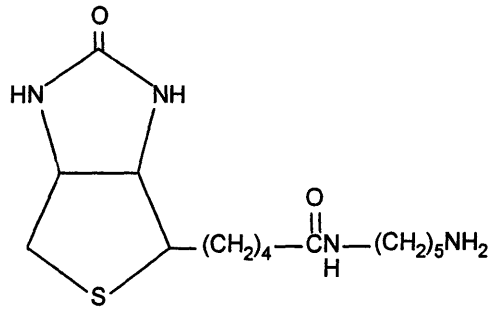

Figure 4-7. 5-(biotinamido)pentylamine

5-(Biotinamido) pentylamine was purchased from Pierce Chemical Co. Its structure is shown in Figure 4-8. The reaction proceeded at 4°C in phosphate buffer solution (100 mM, pH 8.0). The quantity of biotin to be reacted with the PEO was calculated assuming 100% tresylation of the star molecules. The amount of biotin added was then 5, 10 or 15x the number of moles of tresyl groups calculated, depending on the ratio of long to short arms on the star.

The reaction was allowed to proceed for 18 hours after which all the tresyl groups should have either reacted with the biotin or been hydrolyzed.[5] Any remaining unreacted biotin was removed via dialysis using 10K MWCO Slide-A-Lyzer cassettes purchased from Pierce Chemical Co. The purified biotinylated star molecules were recovered after lyophilization.

4.4.2.2 HABA/Avidin Assay

The number of biotin molecules accessible to the avidin protein was quantified using HABA/Avidin reagent purchased from Sigma. This reagent contains the dye 4-hydroxyazobenzene-2-carboxylic acid (HABA) bound to avidin. The assay is based on the ability of biotin to displace the dye in stoichiometric proportions.[6] This displacement of dye is accompanied by a change in absorbance at A_{500} which has a known extinction coefficient. The following procedure was based on instructions provided by Sigma:

The HABA/Avidin reagent was reconstituted by the addition of 10 ml deionized water. Four hundred fifty microliters of the reconstituted reagent was pipetted into a 0.5 ml cuvette, and the absorbance at 500 nm was read. Fifty microliters of the biotinylated star in solution was added and the contents mixed by pipetting in and out. The mixture was allowed to react for 2 minutes, after which the absorbance was read.

The data were analyzed by first calculating the change in absorbance

$$\Delta A_{500} = 0.9(A_{500}^{HABA/Avidin}) - A_{500}^{HABA/Avidin+sample} \qquad (4\text{-}2)$$

where the factor 0.9 is a dilution factor of HABA/Avidin upon addition of sample. The change in absorbance was then used to calculate the concentration of avidin bound to the sample added to the reagent

$$\frac{\mu\text{mole avidin bound}}{\text{ml sample}} = \frac{\Delta A_{500}}{34} \times 10 \qquad (4\text{-}3)$$

where 34 is the mM extinction coefficient at 500nm and 10 is the dilution factor of sample into the cuvette. That number was then used to calculate the number of avidin molecules bound per star molecule

$$\frac{\text{mole avidin bound}}{\text{mole star}} = \frac{\mu\text{mole avidin bound}}{\text{ml sample}} \div \frac{\mu\text{mole star}}{\text{ml sample}} \qquad (4\text{-}4)$$

4.4.3 Results

Three different star molecules were synthesized and reacted with biotin. A summary of the stars used, as well as the results obtained using the HABA/Avidin Reagent is shown in Table 4-2. These results demonstrate that as the ratio of long to short arms on the star molecule increases, the number of moles of avidin able to bind the the star decreases. At first glance the fact that only six moles of avidin bound per mole of star molecule, when the star contained 32 hydroxyl groups, appeared troubling. Closer inspection of the data appears to offer an explanation, specifically an examination of the size of an avidin molecule as compared to the size of the star molecule.

Table 4-2. Number of Avidin Molecules Bound per Star Molecule

f_l	f_s	moles avidin bound
0	32	6
12	20	4.5
20	12	1.6

Assuming the density of avidin to be equal to 0.9g/ml, the volume of an avidin molecule can be calculated from the following equation

$$\frac{1ml}{0.9g} \times \frac{68000g}{\text{mole}} \times \frac{1\,\text{mole}}{6.022 \times 10^{23}\,\text{molecules}} \qquad (4\text{-}5)$$

to be $1.25e^{5}$ $Å^{3}$. Taking the shape of avidin to be an ellipsoid with the minor axis equal to 40 Å[7], the major axis can be calculated to be 149.2 Å. The avidin molecules are attached to the ends of the shorter arms, they can be envisioned as being bound to a sphere whose radius is equal to that measured in chapter 3 for a star molecules containing 32 arms of 1847 molecular weight. To get an estimate of the maximum number of avidin molecules that can physically fit on the surface of such a sphere, the surface area of that sphere was divided by the projected area of avidin. Using a radius of 53 Å, the surface area is calculated to be 35300 Å. Since the projected area of the avidin molecule is estimated to be 4687 Å, only 7.5 avidin molecules could physically bind to the star molecule despite the fact that there are 32 chemical points of attachment. This number is slightly higher than the six measured, but this difference can be explained by certain characteristics not taken into account in the above estimations. For instance, the binding site for biotin is not on the surface of the avidin molecule, but rather at a depth of ~15 Å.[8] In addition the hydroxyl groups on the star molecule are also most likely not at the surface, but rather within the star molecule. Both of these facts should result in lowering the number of avidin molecules that could bind to the star molecule.

According to the model presented, as the ratio of long to short arms increases, even though the number of hydroxyl groups is decreasing, since the total number of arms remains constant at 32, the radius of the inner sphere should remain constant. Therefore the number of avidin molecules that can physically bind to the star molecule should remain constant based on the above analysis. Any decrease in avidin bound below that

bound to the star containing all hydroxy-ended arms, should be a result of steric hindrances due to the presence of the longer arms.

4.4.4 Comparison to Theory

For linear PEG immobilized on surfaces, studies comparing grafting density to protein rejection have demonstrated decreasing amounts of protein adsorbed on surfaces with increasing densities of linear PEG grafted. Nonadsorption of proteins did not occur until there was complete coverage of the surface corresponding to the point where the chains were roughly half-overlapping[9] as defined by the distance between the center of the polymers being equal to R_G. The radius of gyration of linear PEG can be calculated according to the well-known Flory equation.[10]

$$\left(R_G^{\,2}\right)^{1/2} = \frac{\alpha l}{\sqrt{6}}\left(C_\infty n\right)^{1/2} \qquad (4\text{-}6)$$

where α is the intramolecular expansion coefficient of PEO in water,

l is the average bond length in a monomer unit (l[PEO] = 1.47 Å)

C_∞ is the characteristic ratio for the polymer (C_∞ [PEO] = 4)

n is the number of main chain bonds, for PEO $n = 3(M/M_0)$

M is the molecular weight of the PEO

M_0 is the molecular weight of a monomer unit (M_0[PEO] = 44 g/mol)

For linear PEO of molecular weight 19444, R_G is calculated to be 63.8 Å. The surface area that this polymer chain is covering has already been shown to be 35300 $Å^2$. For a chain overlap equal to R_G, the projected area can be calculated to be $\pi(R_G/2)^2$ which equals 3197 $Å^2$. Dividing this number into 35300 results in a prediction of 11 long arms

being necessary to achieve complete protein rejection. However, from the results shown in Table 4-2 it is shown that while there was a decrease in the amount of avidin bound to the biotin, 11 long arms were not enough to prevent binding. Even 20 long arms still allowed some avidin through. This discrepancy can be explained as a result of two other factors that were not taken into account in the above analysis. One is the size of the molecule attached to the shorter arms. This in effect increases the radius of the surface which is being protected. This has two consequences. The surface area that needs to be covered increases, and the effective size of the linear PEG "immobilized" on it decreases. The other factor that needs to be taken into account is that these are spherical surfaces, as compared to planar surfaces, which were used in the other experimental investigations. [9] Therefore the surface area is increasing proportional to r^2, in accounting for the area which needs to be protected, the corrected radius might not be at the inner radius, but rather at a radius somewhere between the inner and outer radius.

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CHAPTER FIVE

Conclusions and Recommendations for Future Work

5.1 Synthesis

5.1.1 Solvent Choice

Based on the results found in this investigation, if there is no difunctional PEG present (ie NHS-PEG-NHS), dichloromethane is the preferred solvent for synthesizing regular PEO star molecules because the NHS group does not undergo hydrolysis. In aqueous solution hydrolysis of the NHS group requires an excess of linear PEG be used for the reaction which leads to loss of this costly starting material. However, if the PEG hasn't been purified sufficiently to remove all difunctional PEG, aqueous solvent must be used to prevent cross-linking of the star molecules. If aqueous solvent needs be used either for that or another unforeseen reason, it might be possible to recycle the excess linear PEO used for the reaction. Hydrolysis of the NHS groups results in the formation of carboxyl groups. While it was not attempted in this investigation it should be possible to regenerate the NHS groups via 1 ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) mediated coupling with n-hydroxysuccinimide[1].

Dichloromethane is also the preferred solvent for synthesizing dual armed star molecules. As demonstrated, the dual armed star molecules synthesized in dichloromethane using the sequential method were more monodispersed than those synthesized using the same procedure in aqueous solution. If the linear PEG used contains difunctional material, the sequential method for synthesizing dual armed stars will not work in either solvent. In that case a synthesis procedure that involves the addition of both types of PEG at the same time would need to be examined.

5.1.2 Extending the Number of Arms

Assuming the proper starting material is used, the method described for synthesizing dual armed stars could be extended to produce a variety of multiarmed stars. That is, one star molecule containing on its core more than two types of arms varying in functional end group, molecular weight, or both. For example, if the intended use of the star molecules is for a targeted delivery system, a third arm of higher molecular weight than the other two and containing a different functional group could be attached to the dendrimer.

5.1.3 Degradable Stars

The reaction between the amine groups on the dendrimer and the NHS group on the linear PEG chains that was used in this investigation was chosen because of the stability of the amide bond formed. For some uses however it might desirable to have a star molecule that degrades over time. An example would be a hydrogel, formed by cross-linking PEO star molecules via their end groups, that degrades over time. This could be achieved by attaching the linear PEG to the dendrimer using a labile linkage. An example of a functionalized linear PEG whose reaction with amine groups results in a such a linkage is succinimidyl succinate (SS-PEG), Figure 5-1. This molecule has been shown to react with amine groups within a short period of time under mild conditions.[2]

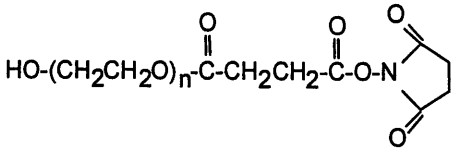

Figure 5-1. Succinimidyl Succinate PEG

The ester linkage between the polymer and the succinic ester residue has limited stability in aqueous media.[3] Therefore PEO star molecules formed using this polymer should exhibit slow hydrolytic cleavage between the arms and the dendrimer core. Other labile linkages that would be of interest to investigate include ones that either degrade at specific pHs or are cleaved by physiological enzymes. Such linkages could be desirable for a variety of applications.

Another variation would be a star consisting of long arms, of which the outer half is attached by a labile linkage. For example, the star would be synthesized by reacting the dendrimer with t-boc-PEG-NHS. After removal of the t-boc protecting group, this star would contain primary amines on its outer ends that could be reacted with SS-PEG. This molecule could be designed to be too large to pass through the glomerulus, thereby extending the *in vivo* half-life of enzymes attached to its outer ends. The portion of PEG arms connected via the SS linkage would cleave off over time leaving the much smaller PEO star molecule that is left to pass easily through the kidneys. Such a star molecule would be useful if the dendrimers alone prove to be biologically incompatible.

5.2 Characterization

The dilute solution properties of the PEO star molecules synthesized in this investigation were analyzed using static light scattering, dynamic light scattering and viscometric techniques. All analysis was performed in aqueous solution at 30°C. The data obtained was used to develop numerical relationships, within the range of the properties of the star molecules synthesized, between the dilute solution properties measured, f, and M_{arm}. Based on the results it is clear that PEO star molecules do not behave as random coils. Instead they appear to act as fuzzy spheres.

5.2.1 Other Solvents

All the data presented in Chapter 3 were from measurement made in water, a very good solvent for PEO.[4] Much of the experimental work on other types of star polymers, as well as the many theoretical studies performed, has examined the dilute solution property behavior of star molecules in theta solvents.[5-8] While data on the behavior of PEO star molecules in a theta solvent isn't necessary for many of the applications being proposed for PEO star molecules, it is of scientific interest to see how branching affects the properties of star molecules and could be beneficial in testing the accuracy of the many theoretical investigations undertaken. It would also be of interest to compare the properties of star branched PEO with those of other star branched polymers under theta conditions.

While examining the dilute solution properties under theta conditions is of interest from a scientific point of view, it would also be of interest with respect to biomedical applications to study the dilute solution properties under physiological conditions of 37°C using phosphate buffered saline (PBS) solution at pH 7.4. For linear PEO, water becomes a poorer solvent as the temperature increases or as the salt content increases.[3] Therefore PEO tends to "shrink" under those conditions. As many of the biomedical application proposed depend on the size of the PEO star molecule, it would be of interest to determine the percent decrease in the various radii (R_V, R_θ, R_S) as the molecule is transferred from pure water to PBS.

5.2.3 Small Angle Neutron Scattering

Most of the theoretical work published on star polymers involves calculations and simulations that provide information only on the radius of gyration for star molecules. Unfortunately, due to the small size of the star molecules studied in this investigation,

light scattering was not an adequate technique for obtaining R_G data. Therefore the experimental work described in Chapter 3 could not be used to test the validity of those investigations.

One method for measuring the radius of gyration of the PEO star molecules synthesized in this investigation would be to use neutron scattering. Because the wavelength of neutrons, typically 0.1 nm to 2.0 nm, is much smaller than that of visible radiation, they are able to provide size information on a much smaller dimensional scale. The basic equations resulting from the theory of scattering of visible radiation presented in Chapter 3 can also be applied to the scatting on neutrons. Since, the scattering of neutrons results from neutron scattering length differences[9] the optical constant K is redefined to take into account the different origins of the scattering.

5.3 Multi Armed Star Molecules

As demonstrated, the new synthetic method allows for the creation of multi armed PEO star molecules. Two potential medical applications for these novel star polymers are:

(1) Stars with short arms carrying enzymes protected by long arms terminated by non-reactive methoxy groups to protect recognition by the immune system.

(2) Stars with long arms carrying a cell recognition moiety, whereby the star adheres to, or is incorporated by, a specific cell, as well as shorter arms that are fitted with a cytotoxic compound through a labile linker.

In these and similar examples the longer arms carry one type of reactable group, e.g., hydroxyl, while the shorter arms carry a different reactable group, e.g., amine, so as to permit coupling of one class of active molecules to the longer arms and a different class to the shorter arms.

These examples bring into focus the criteria for choosing arm molecular weight and dendrimer functionality. The molecular weights and ratios of the longer and shorter arms need to be chosen so that certain molecules can diffuse through the "barrier" of longer arms while other molecules are repelled. These issues need to be investigated after it is decided what enzyme is to be attached and what is its intended substrate.

Two other issues that need to be investigated once a specific "drug" to be delivered is chosen include choosing the optimal coupling procedure and performing *in vivo* biodistribution studies. In choosing a coupling method between star polymer and "drug" one specific issue that needs to be decided is whether or not the linkage should be labile or permanent. If labile, then the release mechanism must be determined, ie specific pH, another enzyme, or just time. *In vivo* biodistribution studies should be done to see where, if anyplace, the PEO star molecules localize as well as the *in vivo* half lives of these molecules. In addition, if it is desired to attach a targeting moiety to the star molecule it would be necessary to examine how that molecule affects the biodistribution of the molecules. If the molecule to be delivered is attached via a labile linkage, it would also be desirable to conduct a similar biodistribution study on that molecule to see when and where it is released, as well as whether or not it aggregates to the specific area of interest in the body.

5.4 Additional Uses for PEO Star Molecules

PEO star molecules hold promise for a variety of applications. The following describes some of the potential uses as well as the advantages of using the star molecules created using the new synthetic method.

5.4.1 Creating PEO Surfaces

Applications for surfaces consisting of PEO star molecules fall under two broad categories. One entails using the star molecules to create a biomaterial, the other is using the star molecule surface as a tool to gain an understanding about other phenomena such as cell/ligand interactions or network theory.[10,11] As a biomaterial star molecules have an advantage over linear molecules in that the large number of arms provide not just points of attachment to surfaces, but also points of attachment to other molecules, see Figure 5-2. This allows for the synthesis of protein resistant, biologically active surfaces.

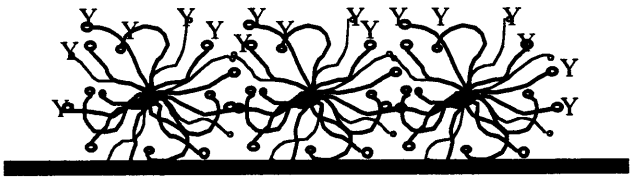

Figure 5-3 End-liking PEO stars to surfaces. Some of the arms are used as points of attachment between the star molecule and the surface rendering it protein resistant. Other arms are use to bind any bioactive species of interest

Similar surfaces can be made by crosslinking of PEO star molecules to form hydrogels. An advantage to using star molecules compared to linear molecules is that hydrogels synthesized via e-beam irradiation of PEO star molecules have a higher density of end groups as compared to those obtained using linear PEO.[10] In addition, multifunctional star molecules can form hydrogels via endlinking with each other, which obviously bifunctional linear PEO cannot. Hydrogels formed in this manner have the potential to be degradable. Both of these surfaces would be desirable for *in vivo* and *ex vivo* applications in which blood contact is required. Potential applications for such surfaces include small diameter vascular prosthesis, angioplastic stents, cardiovascular sutures, metabolic support catheters, angioplastic balloon catheters, artificial hearts and

ventricular assist device.[12] For the latter category, using PEO star molecules incorporated into surfaces to gain an understanding of other phenomena, the method for synthesizing PEO star molecules that is described in this thesis holds a great advantage over other star molecules due to the high level of control that it allows. An example of this can be found for the case of using PEO star molecules to develop network theory involving multifunctional crosslinks. Previous experiments based all their results on average values, when in reality the star molecules encompassed a broad range.[11] In addition, those researchers were constrained by the limited samples of the star molecule synthesized. Because this new method would allow researcher to choose the properties of the star molecules, it would allow researchers to be able to control the variables in the equations, such as arm length and functionality, enabling the ability to test and refine the theory. Similar arguments can be made for using these new star molecules to study the effects of immobilized ligands on cell receptors. These new PEO star molecules would allow researchers to control the number and concentration of ligands on a surface.

5.4.2 Free in Solution

While there has been a variety of work done investigating the synthesis and uses of PEO star molecules on surfaces. There has been little work done exploiting the many possible uses for PEO star molecules free in solution. For example, PEO star molecules fitted with antibodies so as to amplify antigen-antibody reactions could be used as a diagnostic tool.[12] The well characterized size of these molecules as well as their ability to undergo reactions with other molecules make them a good candidate to be used as carriers of fluorescent dyes to probe the integrity of the kidney glomerulus or as carriers in affinity escort ultrafiltration.

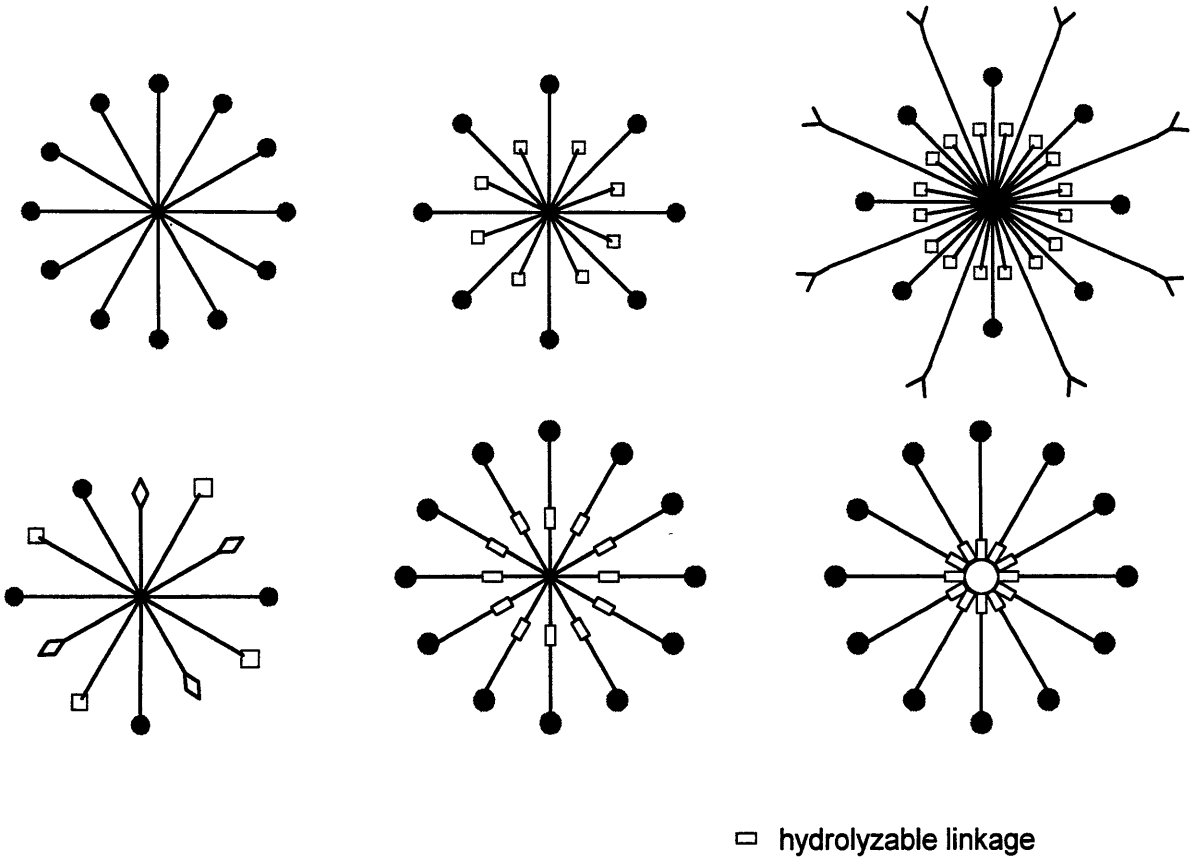

Figure 5-3. Potential structures of PEO star molecules that can be formed using the new method of synthesis

5.5 Conclusions

To summarize, perhaps the greatest advantage the new method for synthesizing PEO star molecules offers is that it allows for the possibility to "engineer" them for a specific application. Figure 5-3 illustrates some of the different types of PEO star molecules that can now be synthesized. It gives researchers the ability to create star molecules containing a controlled number of arms with predefined molecular weights and containing specific functional groups at their outer ends. In addition it allows for the synthesis of star molecules containing more than one type of arm with a controlled ratio of the various arms. Therefore for any of the above uses envisioned, one can synthesize a star molecule with the specific characteristics desired. The work described in chapter 3, in which the dilute solution properties of the star molecules synthesized are measured,

gives researchers information on some of the characteristics of the star molecules they want to use.

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APPENDIX A

Fractional Precipitation of Star Poly(ethylene oxide)

A.1 Introduction

Prior to developing the new synthetic method described in Chapter 2, the need for monodisperse samples of polyethylene oxide star molecules instigated a study into possible fractionation methods to obtain such samples from an initially polydisperse preparation of these molecules. The molecules under study were prepared using the core first method described in Chapter 1.[1] As determined by GPC-light scattering, these molecules have a polydispersity index ranging from 2 to 15 for the various samples synthesized. Because the arms of the star molecules are synthesized via anionic polymerization of ethylene oxide initiated by divinyl benzene cores, the poly(ethylene oxide) arms of these molecules should all be of the same molecular weight. Therefore it is believed that the polydispersity is a result of the cores growing at different rates resulting in a population of cores having a broad distribution of active carbanion sites from which the arms are subsequently grown.

The method for fractionation that was investigated in this study is that of classical temperature manipulation. The basis for temperature fractionation lies in the liquid lattice theory of polymer solutions developed by Flory[2] for linear polymer molecules. While star molecules cannot be fitted into the logic of Flory's model, it was thought to be of interest to examine experimentally how star shaped PEO molecules compared to linear PEO. Fractionation of linear PEO using organic theta solvents does not work if θ is much below the crystalline melting point (66 °C) and thus one would be forced to seek high temperature θ solvents. Instead advantage has been taken of PEO's inverse solubility - temperature behavior in aqueous solutions.[3] The lower critical solution temperature,

LCST, of linear PEO in water is 95 °C for PEO of infinite molecular weight and increases above the boiling point of water as molecular weight decreases. The LCST is systematically lowered by the addition of salts to water.[4]

This investigation sought to obtain star PEO of more narrow molecular weight distribution by precipitation fractionation in aqueous solutions of sodium carbonate. The precipitation temperature of star PEO in pure water was determined along with its dependence on salt concentration and compared with data found by other investigators on linear PEO. This information was then used as a basis for fractionation of star PEO in aqueous solutions of sodium carbonate (Na_2CO_3)

A.2 Experimental

A.2.1 Cloud Point Dependence of Star PEO on Salt Concentration

The characteristics of the anionically polymerized PEO star molecules which were studied are given in Table A-1. One percent w/vol solutions of star 3510 were prepared. One ml was placed in a capped test tube and immersed in an oil bath. The temperature was slowly raised until the solution became cloudy. These experiments were run using ion free MilliQ water as well as solutions of sodium carbonate, potassium chloride, and sodium phosphate at various concentrations. The results were then compared with those of Bailey and Callard[4] for linear PEO.

Table A-1. Anionically Polymerized Core First PEO Star Molecules: Characteristics

Code	Source	M_w[c]	M_{arm}[d]	$f=M_w/M_{arm}$[e]
3510	a	350,000	5200	67
73	b	173,000	10,000	17

[a] Gift of Dr. Paul Rempp, Centre de Rescherches sur les Macromolecules, Strasbourg France. [b] Purchased from Shearwater Polymers Inc., Huntsville, AL. [c] Weight average molecular weight as determined by GPC/LS. [d]Arm molecular weight as reported by Dr. Rempp or Shearwater Polymers Inc. [e]Number of arms.

A.2.2 Fractionation of PEO Star Molecules

Once it was determined that star PEO, like linear PEO, will precipitate in aqueous salt solutions, it was decided to determine the molecular weight dependence of this precipitation. A 1% solution of star 073 in 0.375 M sodium carbonate was placed in a water bath and the temperature raised until the cloud point was reached. The solution was then left undisturbed at this temperature overnight or longer until the two phases were separated. The supernatant was then poured off and placed back into the water bath. The temperature of the bath was raised again until the next cloud point was reached. The procedure was repeated until no appreciable amount of polymer was left in the supernatant phase. The gel phase at each step was redissolved in water and analyzed using gel permeation chromatography (GPC) in series with light scattering (LS). Note the term gel is used tot note the heavier and highly viscous phase. In the context of this discussion it does not imply network formation.

While these experiments clearly demonstrated that higher molecular weight star PEO polymers do precipitate at lower temperatures than do lower molecular weight star molecules, these successive fractionations did not lead to monodisperse fractions. This result is not surprising considering that according to theory, while the highest molecular weight species is more predominant in the more concentrated phase, all species are present in both phases. Therefore it was decided to undertake a rigorous "pyramid" of fractionation based on the work of Thurmond and Zimm.[5] This fractionation method is based on combining supernatants and gels, which have similar molecular weights, taken from the successive fractionation of a sample as described above. Each of these newly created samples then undergo one cloud point separation. Again gels and supernatants of similar molecular weights are combined and the process is repeated. It was believe that using such a scheme of combining successive gels and supernatants would result in a

sample of star molecules with a polydispersity index closer to unity. For all fractionations the initial polymer concentration was brought to 0.5% PEO concentration and 0.375 M Na_2CO_3 by concentrating the solution using an Amicon stirred ultrafiltration cell with a PM10 membrane and by adding the necessary quantity of a 2.0 M Na_2CO_3 solution.

A.3 Results and Discussion

A.3.1 Dependence of Cloud Point on Salt Concentration

The results of these experiments are shown in Figure A-1. A 1.0 % solution of linear PEO of molecular weight 200,000 or greater, precipitates at 95 °C in ion free water[4]. However, for the PEO star molecules at zero salt concentration no cloud point was reached, even after raising the temperature to 100 °C, so the data were used to extrapolate one. The addition of salts does indeed lower the cloud point temperature with Na_2SO_4 having the greatest effect and KCl having the least. Bailey and Koleske using linear PEO observed the same trends.[4]

A.3.2 Fractionation of Star 073

The first cloud point of star 073 in 0.375 M Na_2CO_3 occurred at 46 °C and resulted in a gel with a weight averaged molecular weight equal to 423,000 and a polydispersity index of 2.05. After six more successive fractionations of the supernatant our last gel precipitated at 54 °C, had a molecular weight of 202,000 and a polydispersity index of 1.31. After this we found there was not enough polymer left in our initial 100 mg sample to collect any more fractions. As stated earlier, these results clearly show that

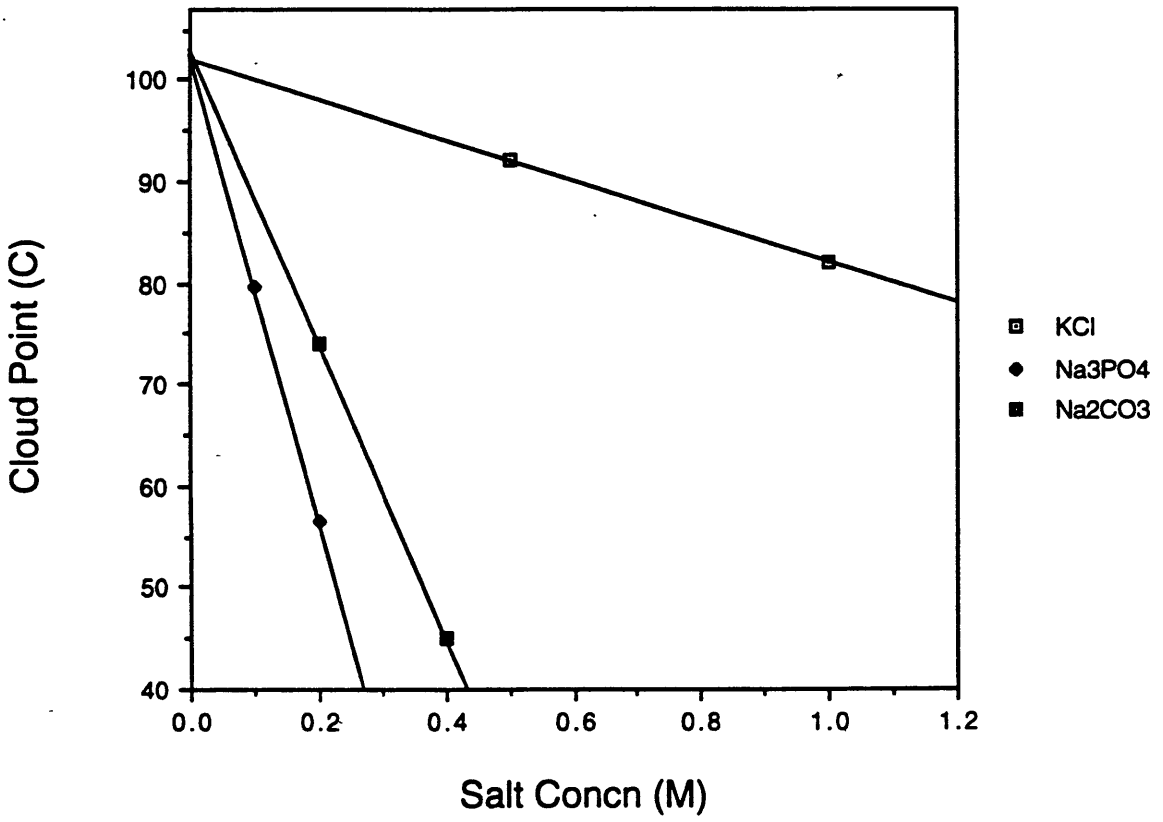

Figure A-1. Cloud point of star 3510 as a function of salt concentration. PEO concentration is 1 wt %

higher molecular weight star molecules precipitate out of solution at lower temperatures than do lower molecular weight PEO star molecules. The molecular weights, polydispersity indices and mass fractions of the final samples attained after combining and refractionating the gels and supernatants of the first fractionation are summarized in Table A-2. By using this method we were able to more cleanly separate out the higher molecular weight samples from the solution, with the molecular weight of our highest molecular weight sample being 960,000. However, we were unable to obtain samples with a polydispersity index less than 1.2.

Table A-2. Samples Obtained After Combining the Gels and Supernatants from Star 73 and Refractionating

M_w	mass fraction	M_w	mass fraction	M_w	mass fraction
960000	0.044	393000	0.028	248000	0.03
728000	0.013	381000	0.014	219000	0.057
538000	0.016	349000	0.041	189000	0.096
508000	0.002	286000	0.004	185000	0.038
455000	0.038	278000	0.039	166000	0.414
403000	0.049	270000	0.076		

A.3.3 Dependence of Cloud Point on Star Molecular Weight

A plot of the cloud point temperature as a function of molecular weight of the star PEO polymers is shown in Figure A-2. These data were collected during the second attempt at fractionation described above. Bailey and Callard found that, for linear PEO, at molecular weights greater than 50,000 and concentrations greater than 0.3% the upper consulate temperature becomes independent of both variables[4]. These facts in themselves are at variance with the theory of fractionation as presented for example in ref 2 chapter 13, since the theory requires that the consulate temperature approach the theta temperature as molecular weight goes to infinity, and that the critical concentration should move to progressively lower values as molecular weight increases. While our

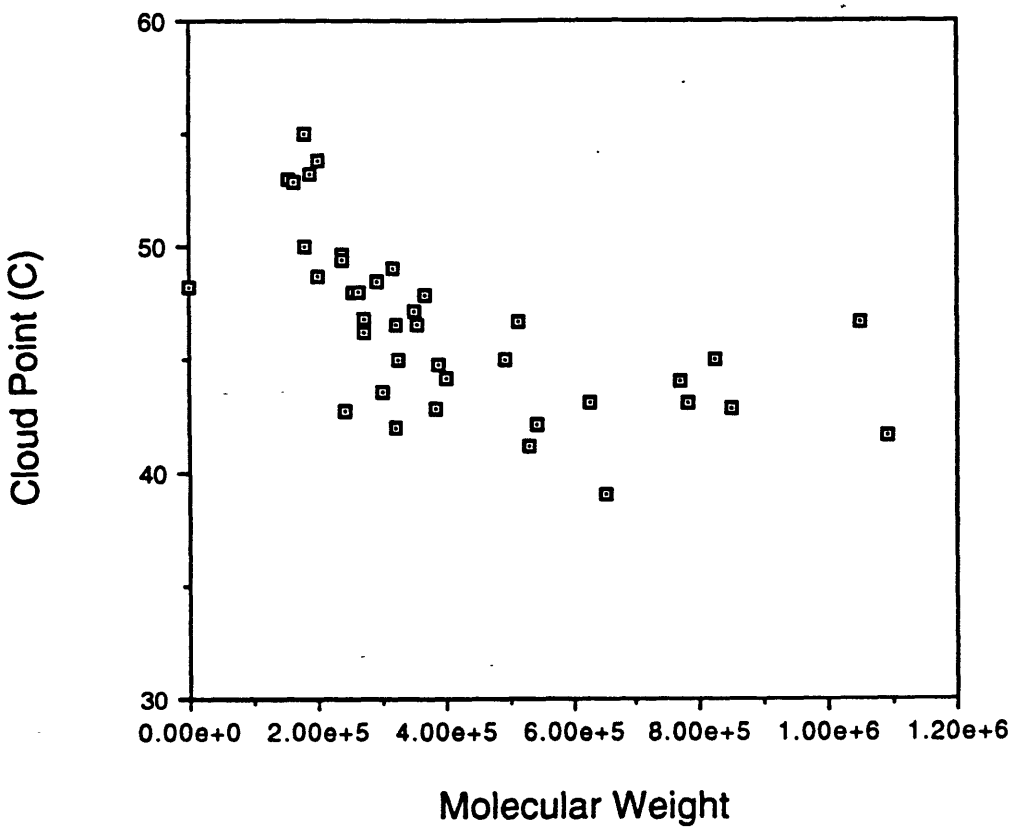

Figure A-2. Cloud point of PEO star molecules as a function of molecular weight, PEO concentration is 0.5 wt% in 0.375 M Na_2CO_3

results do not show this same insensitivity starting as low as 50,000, we do see the dependence of cloud point on molecular weight diminish greatly once the molecules exceed around 250,000 g/mole. This discrepancy is most likely due to the branching architecture of the star molecules and the fact that while the total molecular weight might be large, the molecule is made up of linear PEO arms whose molecular weights are less than 10,000.

Instead of comparing the precipitation behavior of PEO star molecules with linear PEO based on molecular weight, more insight might be gained by comparing the two using intrinsic viscosity as a measure of their respective sizes. Using this method Bailey and Callard showed that in salt free solutions of linear PEO the cloud point initially decreases as intrinsic viscosity increases, but then becomes independent of molecule size when intrinsic viscosity is greater than 1 dl/g.[4] Because PEO star molecules have lower intrinsic viscosities than linear polymers of equivalent molecular weight, it is expected that star PEO macromolecules would precipitate at higher temperatures than their linear molecular weight counterparts would.

A.4 Conclusions

Fractional precipitation was found to be an inefficient method for obtaining a sample of monodisperse star molecules. However this study did provide some insight into how the thermodynamic properties of PEO star molecules differ from those of linear molecules. More insight could be gained by performing a similar investigation using the star molecules synthesized by the method given in Chapter 2. Specifically, it would be of scientific interest to study how changing the arm number and arm molecular weight affects the cloud point of these molecules. If more information is known regarding their cloud points at different salt concentrations, fractional precipitation could prove to be a convenient separation method for some applications. For example, in large scale

synthesis the separation of star molecules from unreacted arms might be accomplished in this way, instead of by ultrafiltration.

A.5 References for Appendix A

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(3) Bailey, F. E.; Powell, G. M.; Smith, K. L. *Ind. Eng. Chem.* **1958**, *50*, 8.

(4) Bailey, F. E. and Callard, P. W. *J. Appl. Polymer Sci.* **1959**, *1*, 56.

(5) Thurmond, C. D. and Zimm, B. H. *J. Polym. Sci.* **1952**, *8*, 47.

APPENDIX B

In Vitro Toxicity Testing

B.1 Introduction

Before the PEO star molecules synthesized in this investigation can be used in many of the biomedical applications proposed, they must be proven to be biocompatible. Linear PEG has been shown to be poorly immunogenic. In addition it has been proven to be nontoxic and has already received FDA approval for use in pharmaceuticals intended for delivery into the bloodstream.[1] The quantity of work done examining the biocompatibility of the PAMAM dendrimers used as the core of the PEO star molecules has not been nearly as extensive. However in a recent investigation, a preliminary study was made of generations 3,5 and 7 to assess their *in vitro* toxicity, *in vivo* toxicity, immunogenicity, and biodistribution.[2] Their results found no evidence for immunogenicity of any of the generations tested. Their *in vitro* studies found that the PAMAMs exhibited some toxicity to the cells which was dose and generation dependent, which they hypothesized might be solely a reflection of their cationic behavior.[2]

Because the PEO star molecules synthesized consist of the dendrimers covered by linear polyethylene glycol at such a density as to prevent recognition by molecules of the immune system, it is believed that there should be no biocompatibility or toxicity problems. Of course *in vivo* investigations will have to be done before they can be used in humans. This investigation describes some preliminary *in vitro* studies on the biocompatibility of the PEO star molecules synthesized. Chinese Hamster Ovary cells were grown in media containing PEO star molecules, dendrimers, or linear PEO. The samples grown under the different conditions were monitored daily for a period of 7 days,

and cell viability and their ability to divide was determined by counting the increase in cells each day. These numbers were compared to a control media.

B.2 Experimental

B.2.1 Materials

The star molecules used in these experiments were synthesized by reacting methoxy-PEG-NHS, molecular weight 5000, with generation 4 dendrimers (containing 64 primary amine groups). The resulting star molecules had a molecular weight of 260,000 suggesting each star molecule contained 50 arms. Linear PEO of molecular weight 200,000 was purchased from Scientific Polymer Products. The above material was sterilized by first dissolving in a 70% EtOH/water solution that was evaporated off. The cells were grown in media prepared according to the following recipe: 90 ml Dubelco's Modified Eagle Medium, 10 mL fetal bovine serum, 2 mL L-glutamine, 1 mL sodium pyruvate, and 1mL penstrep. The cells were grown in a humidified 10% CO_2 atmosphere at 37°C and passaged every 3 to 4 days.

B.2.2 Procedure

Twelve well tissue culture plates were plated with approximately 20000 CHO cells in 1 mL of medium and allowed to adhere overnight. The cells were then exposed to either 400 nM or 4μM of G4 dendrimers, 200Klinear PEO or star PEO for 22-95 hours. At the end of the designated exposure period the cells were trypsinized and counted using a coulter counter. Cells from one plate were trypsinized 30 minutes after the replating so that a baseline could be obtained. To compare cell growth after more than 96 hours of exposure, the cells were plated onto 35mm plates, allowed to adhere

overnight, and the media replaced with that containing 4μM of either G4 dendrimers, 200Klinear PEO or star PEO. Samples of these cells were taken after 96 and 120 hours, trypsinized, and counted using the coulter counter.

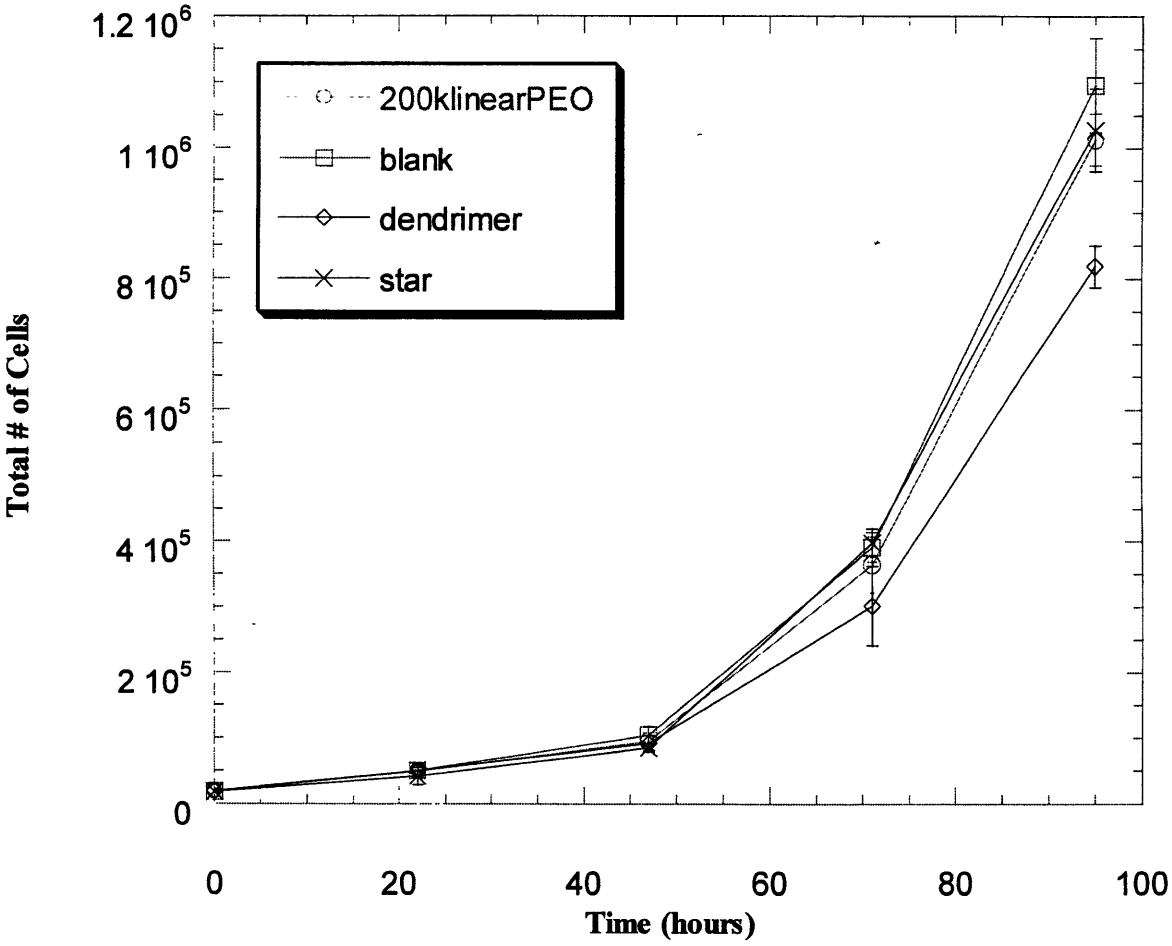

Figure B-1. Total # of cells per well after exposure to a concentration of 4 μM

B.3 Results

Figure B-1 displays the results taken from the samples exposed to 4 μM of each material being tested. As can be seen from the results, after 60 hours the cells exposed to the dendrimers began to show deleterious effects. However there is no difference, within

the error of the experiments, among the cell cultures exposed to linear PEO, star PEO or the control group. Similar results can be seen with the samples exposed to 400 nM of the different polymers. The data obtained for these samples after being exposed to the materials in question for 96 and 120 hours is displayed in Figure B-2. Again, the number of cells in the cultures exposed to both forms of PEO does not differ from that of the control cell culture, while the quantity of cells in the cultures exposed to the dendrimer is much less.

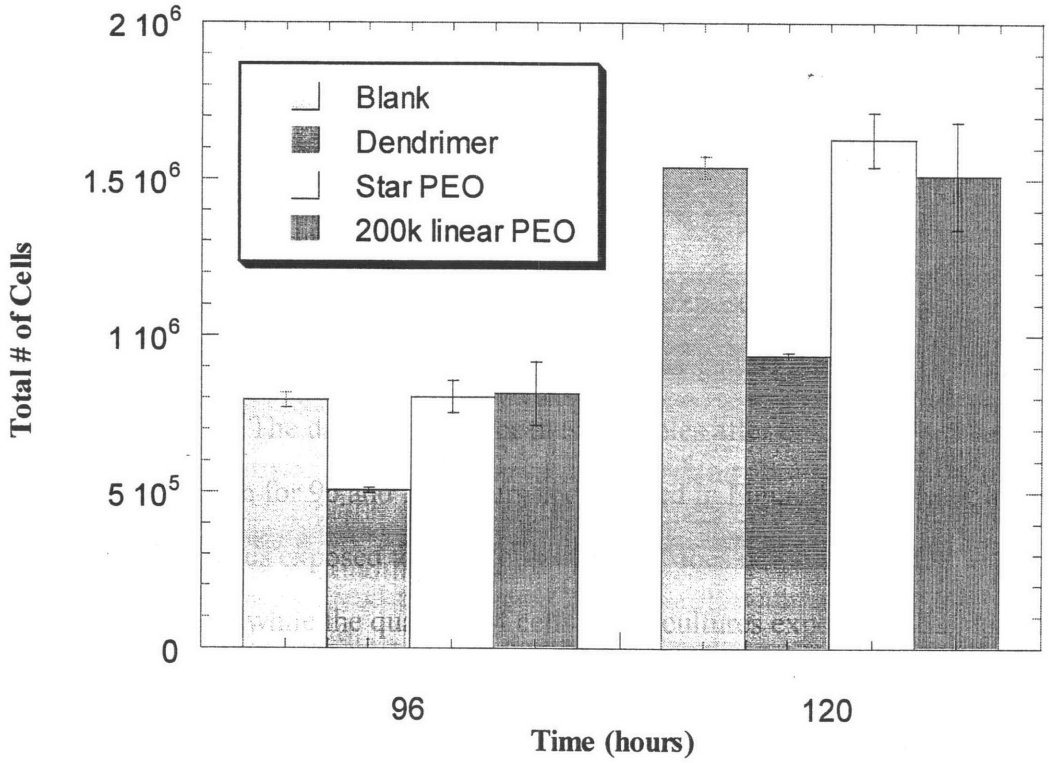

Figure B-2. Total # of cells per plate after exposure to a concentration of 400 nM

B.4 Discussion

The results shown in this investigation concur with those found by Roberts et al (ref), which suggested that PAMAM dendrimers have a toxic effect on *in vitro* cell cultures. However, it is encouraging that the star molecules synthesized using those dendrimers do not appear to have any negative effects on the cell cultures.

B.5 References for Appendix B

(1) Harris, J.M.; *Poly(ethylene Glycol) Chemistry: Biotechnical & Biomedical Applications*; Plenum Press; New York, 1992.

(2) Roberts, J. C.; Bhalgat, M. K.; Zera, R. T. *J. of Biomedical Materials Research* **1996,** *30*, 1996.