Physicochemical Characterization of PEG-based Comb-like Amphiphilic Copolymer Structures for Possible Imaging and Therapeutic Applications

by

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Abstract

Comb-like copolymer structures, also known as graft/comb copolymers, have obtained a significant amount of attention in biomedical and industrial applications because of their unique compositional flexibility, which can lead to versatile structures in bulk, melt, and solution states. With biomedical applications (cancer diagnosis and treatment) as the context, this thesis is aimed at characterizing a series of polyethylene glycol (PEG) –based highly adaptable amphiphilic comb copolymer structures in their solution state that can serve as carriers and potentially contrast enhancement agent in magnetic resonance imaging (MRI). To successfully develop and implement such a delivery/contrast agent system, an adequate understanding is needed concerning their physicochemical properties: stability, size, morphology, local structural information, and magnetic resonance characteristics.

The stability of these copolymer structures was characterized by their critical micelle concentration (the lower this concentration, the higher the stability), which was determined by total intensity light scattering and surface tension measurement. The size, morphology, and detailed structural information were studied by a combination of techniques, i.e., dynamic light scattering,
transmission electron microscopy, cryogenic transmission electron microscopy, and small angle neutron scattering. Furthermore, solutions of polymer structure containing perfluorocarbon blocks were characterized by $^{19}$F magnetic resonance spectroscopy to evaluate their application for MRI contrast enhancement.

Perfluorocarbon-containing comb copolymers (i.e., PEG-PFC) in solution had a low CMC of about 2 μM. They were found to form two populations of particles—small micelles and large secondary aggregates. Hydrodynamic radius of micelles did not change with polymer concentration, PEG length, sample preparation method, or time after sample preparation. Large secondary aggregates were most likely compound micelles. Sample preparation method, polymer molecular weight, and time after sample preparation could change the proportion of micelles vs. aggregates. Due to its “perfluoroalkyl-philic” property, PEG-PFC copolymer was able to encapsulate a perfluorocarbon compound, 1H, 1H, 2H, 2H-Perfluoro-1-decanol. $^{19}$F-NMR spectroscopy of PEG-PFC polymer solution showed significant spectral line broadening and consequent signal to noise (SNR) decrease due to micelle formation. Furthermore, hydrocarbon-containing comb copolymers (i.e., PEG-HyC) in PBS had a CMC of about 12 μM. The micelles formed by PEG-HyC copolymer had an $R_h$ of about 4-5 nm that did not change with polymer concentration.

Because of the formation of nano-size micelles, both PEG-PFC and PEG-HyC copolymers are good candidates to be developed as delivery vehicles for imaging and therapeutic agents. Their low CMC is an indication of their potential ability to maintain micelle integrity in situations of massive dilution. PEG-PFC copolymers could also be used to encapsulate insoluble fluorinated drugs. Though micelle formation of PEG-PFC copolymer caused significant $^{19}$F-NMR spectral line broadening and consequent SNR reduction, the copolymer can be modified to act as smart $^{19}$F-MRI probes for cancer diagnosis.
Dedication

To Jesus Christ my beloved Lord,
My life, my light, my way, and my strength,

“For of His fullness we have all received, and grace upon grace.”
John 1:16

“To the only wise God through Jesus Christ, to Him be the glory forever and ever. Amen.”
Romans 16:27

To the Body of Christ my family,
My goal, my love, my joy, and my crown,

“But to Him who is able to do superabundantly above all that we ask or think, according to the power which operates in us, to Him be the glory in the church and in Christ Jesus unto all the generations forever and ever. Amen.”
Ephesians 3:20-21
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I would like to express my sincere appreciation to my thesis advisor Professor Clark K. Colton for the support and instruction that I have received during the pursuit of this degree. I have received much perfecting and education under his tutelage. I would also like to thank all my thesis committee members for their guidance and supervision: Professor Anna V. Moore (Martinos Biomedical Imaging Center), Professor Susan Bonner-Weir (Joslin Diabetes Center), Professor Paula T. Hammond (Department of Chemical Engineering, MIT), Professor Robert J. Fisher, Professor Arthur C. Watterson (University of Massachusetts – Lowell), and Professor Jerome L. Ackerman (Martinos Biomedical Imaging Center). I am especially grateful to Professor Jerome L. Ackerman for teaching me and helping me with magnetic resonance in understanding and experiments.

I am grateful to many colleagues and scholars at MIT, from whose experience and knowledge I have benefited tremendously. In particular, I would like to thank Doctor Aleksey Lomakin for helping me with dynamic light scattering both in theory and experiments. Professor Daniel Blankschtein and Doctor Arthur Goldsipe have helped me much in obtaining insights and understanding into polymeric micellar systems. I thank Doctor Shujun Chen (Department of Chemical Engineering, MIT) for invaluable discussions and help with transmission electron microscopy (TEM). I thank Doctor Kazuyoshi Murata for training me in cryogenic TEM. I would like to thank all my lab mates in the Colton Research Group for their continued care and encouragement.
In addition, I want to thank our wonderful collaborators. The Watterson group at University of Massachusetts – Lowell has provided us with materials for testing. Doctor Steven R. Kline at National Institute of Standards and Technology has extended his generous help for small angle neutron scattering experiments. Kevin P. Davis in Professor Frank S. Bates group at University of Minnesota has helped us to obtain further evidence with cryogenic TEM.

Finally, I would like to acknowledge the indispensable support that I have received from my husband and my family. Their love, care, consolation, and encouragement have been woven into this thesis and even more into my being.
Biography

Jin Zhou (周瑾) grew up in a typical Chinese family in Huainan, Anhui Province, China. She is the daughter of Zhengquan Zhou (周正泉) and Qi Zhao (赵齐). Jin has always been found to be one of the most energetic, diligent, and curious students, which enabled her to excel in almost every area she would step into. In addition to her outstanding academic performance, she has developed a wide variety of interests, e.g., athletics, singing, dancing, and philosophy. By the end of her freshman year in high school, Jin has decided to choose the field of science and technology to be a most intriguing and rewarding path for her.

In 1998, Jin was enrolled into the nation's best university in science and technology – Tsinghua University, Beijing. She continued to be the top student in the four-year undergraduate study in the Department of Chemical Engineering. At the same time, she was active in serving the student community, especially in athletics and arts. In 2002, Jin graduated summa cum laude among her class of 120 brilliant students and received her Bachelor of Science in Chemical Engineering. Shortly after her graduation, Jin came to Massachusetts Institute of Technology (MIT) to pursue her advanced degrees.

In 2003, Jin was awarded with Outstanding Achievements in the program of Master of Science in Chemical Engineering Practice, MIT. In her Ph.D. research, Jin has pleasantly enjoyed the power and beauty of interdisciplinary collaboration. The crossing of experts from different fields (e.g., physics, chemistry, biology, and engineering) has provided many more opportunities for learning, exploration, and discovery. This unique experience has equipped Jin very much beyond the wealth of knowledge.

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Nomenclature

\( a \)  Major axis of a spheroid
\( b \)  Minor axis of a spheroid
\( D \)  Diffusion coefficient
\( D_k \)  Diffusion coefficient of type “k” particles
\( I_0 \)  Total scattered light intensity
\( I_k \)  Intensity of scattered light by type “k” particles
\( k_B \)  Boltzmann constant
\( m \)  Copolymer polymerization number
\( n \)  Micelle aggregation number
\( q \)  Scattering vector
\( R_h \)  Hydrodynamic radius
\( SNR \)  Signal-to-noise ratio
\( T \)  Temperature in Kelvin
\( V_s \)  Partial specific volume
\( z \)  Number of repeating units in polyethylene glycol (PEG)

\( \eta \)  Solvent viscosity
\( \theta \)  Scattering angle
\( \tau \)  Time of photodetection
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**Abbreviations**

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<td>Phosphate Buffer Solution</td>
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<td><strong>PEG</strong></td>
<td>Polyethylene Glycol</td>
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<tr>
<td><strong>PEG-HyC</strong></td>
<td>Copolymer with hydrocarbon side chains</td>
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<td><strong>NMR(S)</strong></td>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
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<tr>
<td><strong>SANS</strong></td>
<td>Small Angle Neutron Scattering</td>
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<td><strong>TEM</strong></td>
<td>Transmission Electron Microscopy</td>
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1 Background and Significance

1.1 Cancer Diagnosis and Treatment

Cancer has been one of the greatest threats to human health worldwide. In the United States, American Cancer Society reported that in 2005 there were 559,312 deaths caused by cancer, occupying 22.8% of all deaths, ranking number 2 after heart diseases. It is noteworthy that cancer death rate decreased only slightly in 2004 compared to that in 1950; whereas rates for other major chronic diseases (e.g., heart diseases, cerebrovascular diseases, influenza) have decreased substantially over the same period of time [1]. Some of the major challenges that remain to be resolved are (1) small primary tumors go undetected; (2) metastatic cancers are grossly underdiagnosed, in which cases many patients routinely go on to develop and die from metastases; (3) treatment response to therapy is poorly measured; and (4) the use of therapeutic agents is limited or annulled by their side effects causing severe collateral damage to healthy tissues/organs in patients. Various microscopic structures (such as polymeric micelles, liposomes, viral vectors) that can carry contrast agents and/or drugs offer unique potential to improve the use of imaging modalities and therapeutic drug delivery.

In collaboration with University of Massachusetts Lowell and Martinos Biomedical Imaging Center, our research group has initiated a project to develop a nano-scale delivery system utilizing polyethylene glycol (PEG) –based amphiphilic comb-like copolymers, which is aimed to improve disease management in cancer. The project overview is presented in the next section, which serves as the background of the research work included in this thesis.
1.2 Project Overview

1.2.1 Copolymer delivery system

PEG-based amphiphilic comb-like copolymers (comb polymers) have been synthesized via a two-step chemo-enzymatic synthetic route [2]. The fundamental unit of the polymer backbone is PEG (MW 600-1,500 Da) coupled to a trifunctional, hydrophobic linker molecule. Hydrophobic side chains can be attached to the linker via an ether, ester, or peptide bond. This side chain can be terminated by a hydrogen or an amino, hydroxyl, or carboxyl functional group. Because of their amphiphilic nature, i.e., hydrophilic and hydrophobic portions coexisting with one another in one entity, these copolymers will form micellar structures in a selective solvent (e.g., water) when the polymer concentration exceeds a certain threshold, which threshold is called the critical micelle concentration (CMC). The hydrophobic side chains constitute the micellar core and the hydrophilic PEG groups make up the shell/corona. Figure 1.1 represents the chemical synthesis and self-assembly of this copolymer system. To target cancer cells with certain biomarkers, ligands can be incorporated into the copolymer structures with the goal to present these targeting ligands on the surface of the polymeric assemblies. These ligand-attached copolymers can form into nano-scale vehicles to recognize cancer cells specifically and to deliver into them various therapeutic or imaging agents (Figure 1.2).

Due to the flexibility of the synthetic chemistry, this copolymer system can be altered and/or functionalized for various purposes. For example, by choosing appropriate side chain terminal group, therapeutic or imaging agents can be encapsulated in the micellar core (i.e., encapsulation). In stead of alkyl side
chains, perfluorocarbon-containing side chains can be attached (i.e., fluorination) [3]. In addition, iodine (\(^{123}\text{I}, ^{124}\text{I}, ^{125}\text{I}, ^{131}\text{I}\)) substitution can take place at free carbons on the aromatic linker groups and certain moieties of attached ligands (i.e., radioiodination). Figure 1.3 represents these various modifications of the copolymer system. Ultimately, a multi-modal imaging/therapeutic probe could be constructed that has the capability to specifically recognize and enter into cancer cells \textit{in vivo}. By delivering into them desired agents, therapeutic treatment can achieve high efficacy without severe side effects and modern imaging techniques can be applied for diagnosis and treatment assessment. Figure 1.4 presents the construct of a probe containing three functionalities that can be used for multiple \textit{in vivo} imaging modalities: (1) radioiodine for nuclear imaging (NI), i.e. single-photon emission computed tomography (SPECT) and positron emission tomography (PET), (2) fluorophores (e.g., Cy5.5) for near infrared fluorescence (NIRF) imaging, and (3) perfluorocarbon-containing side chains for \(^{19}\text{F}\) magnetic resonance imaging (MRI).

\section*{1.2.2 \(^{19}\text{F}\)-MRI for contrast enhancement}

Among non-invasive imaging techniques, MRI has demonstrated its unique and outstanding advantages, e.g., low incidence of side effects for patients, no penetration limit, and high-resolution three dimensional images. The most commonly seen MRI utilizes proton nuclei (\(^1\text{H}\)), i.e., \(^1\text{H}\)-MRI, because they are the most abundant nuclei in the human body. In order to differentiate between normal tissues and neoplastic tissues, appropriate image contrast is needed, which is achieved by the application of various contrast agents (e.g., gadolinium conjugates, iron oxide nanoparticles) in conjunction with \(^1\text{H}\)-MRI.
$^{19}$F-MRI is a unique method that can be used to obtain contrast enhancement for conventional $^1$H-MRI. The advantages of utilizing $^{19}$F nuclei for MR contrast enhancement are (1) the MR sensitivity of $^{19}$F is 84% that of $^1$H (ranking second among all MR responsive nuclei) [4]; (2) $^{19}$F is not present in the human body except in bones (solid-state $^{19}$F is invisible to MR scans in application); (3) $^{19}$F MR signal intensity is proportional to local $^{19}$F concentration, which enables quantitative analysis if possible; and (4) the gyromagnetic ratios of $^{19}$F and $^1$H are so close [4] that it is possible for existing $^1$H-MRI scanners to include $^{19}$F-MRI. The challenge of $^{19}$F-MRI is to achieve sufficient signal-to-noise ratio (SNR) because the abundance of $^{19}$F nuclei is limited by how much contrast agent can be delivered to and retained at the tumor site.

An illustration of contrast enhancement utilizing $^{19}$F-MRI is shown in Figure 1.5. Both $^1$H-MR and $^{19}$F-MR images were taken 27 minutes after the mouse gavaged 0.3 ml perfluorononane [5]. When the two images of the stomach of a mouse are overlaid, $^1$H-MRI renders anatomic information and $^{19}$F-MRI delineates the duodenum (D) and the small intestine (SI) by a bright image without background signal.

1.2.3 Targeting strategy illustration

For diagnosis and treatment of cancers, targeting strategies are crucial to enable probes to recognize cancer cells at various developing stages and deliver into them imaging/therapeutic agents with high specificity. An illustration of targeting strategy for early tumor detection by $^1$H-MRI is presented in this section based on the research work of our collaborator (Moore et. al., Martinos Biomedical Imaging Center) [6].
It has been discovered that mucin-1 (MUC-1) is overexpressed on almost all human epithelial cell adenocarcinomas (e.g., breast, ovarian, pancreatic, lung, prostate, and colon carcinomas), some nonepithelial cancer cell lines (e.g., melanoma and neuroblastoma), certain hematological malignancies, and β-cell non-Hodgkin lymphomas. These cancer types in total constitute more than 50% of all cancer cases in human. More interestingly, MUC-1 is heavily glycosylated in normal cells whereas MUC-1 is underglycosylated in cancer cells, making it a good target for specific targeting. In this particular work, a synthetic peptide was used to target the binding site on uMUC-1 with the sequence of YCAREPPTTRTFAYWG, designated as EPPT1. The image contrast was accomplished by using the superparamagnetic iron oxide nanoparticles, which shortens the transverse relaxation time (T2) of the precession of proton nuclei. The probe is thus constructed by attaching the EPPT1 peptide to cross-linked iron oxide (CLIO) nanoparticles, designated as CLIO-EPPT.

uMUC-1-positive and uMUC-1-negative tumors were injected into nu/nu mice bilaterally and grew to about 0.5 cm in diameter. Then in vivo 1H-MRI was performed on animals before and 24 hours after CLIO-EPPT was injected. T2 maps of 1H-MRI are shown in Figure 1.6, from which we can see that there is no change in signal intensity of T2-weighted images in uMUC-1-negative tumors but significant signal reduction in some regions of uMUC-1-positive tumors (marked by white arrows). The fact that the targeted contrast agent (CLIO-EPPT) only darkens the receptor-positive tumor in vivo indicates that the targeting strategy is successful.
1.2.4 Combining $^{19}$F-MRI and targeting strategy

The perfluorocarbon (PFC) -containing copolymers are designed to serve as delivery vehicles with targeting ability, but they also have the potential to enhance MR image contrast through the utilization of $^{19}$F-MRI. Figure 1.7 shows the potential effect of combining the aforementioned targeting strategy and contrast enhancement by $^{19}$F-MRI. After the probe accumulates in neoplastic tissues, $^1$H-MRI will provide the structural information of the anatomy and $^{19}$F-MRI can identify tumors as bright areas with no background signals, thus rendering clear and unambiguous diagnosis.

Here lies the motivation for the research work included in this thesis. To successfully apply this delivery system in practice, it is necessary to obtain adequate understanding of the physico-chemical properties of the copolymer system in dilute solutions. Furthermore, the magnetic resonance response of the polymer solution needs to be examined in order to test its suitability for MR contrast enhancement.

1.3 Thesis Overview

This thesis is devoted to the study of the micellization behavior of this PEG-based amphiphilic copolymer system in dilute solution and its magnetic resonance response. The perspective is to utilize it as a delivery system for imaging and therapeutic agents and potentially as an MR contrast agent. This chapter has provided the background and motivation of the research. Chapter 2 presents a literature review on the most relevant research topics, i.e., delivery systems, $^{19}$F-MR spectroscopy and imaging, nonlinear copolymers, and
fluorinated polymers. Chapter 3 covers the experimental section including materials and different characterization methods, i.e., CMC determination, dynamic light scattering (DLS), transmission electron microscopy (TEM), cryogenic TEM (Cryo-TEM), small angle neutron scattering (SANS), and $^{19}$F-MR spectroscopy. The experimental results and implications of copolymer characterization are discussed in Chapter 4. Chapter 5 concludes this thesis and presents recommendations for future research.
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Figure 1.1 Representation of the chemical synthesis and self assembly of PEG-based amphiphilic comb-like copolymer system in a selective solvent

*The flower-like micellar structure was first suggested by Professor Watterson's group.*
Figure 1.2 Copolymers with attached ligands and encapsulated agents being specifically internalized by cancer cells via receptor-mediated endocytosis.
Radioiodination: Iodine ($^{123}$I, $^{124}$I, $^{125}$I, $^{131}$I) substitution (arrows) at free carbons on aromatic linker groups and certain moieties of attached ligands

Figure 1.3 Examples of modifications of the copolymer system – encapsulation, fluorination, and radioiodination
Figure 1.4 Representation of the construct of a multi-modal imaging probe

* = Possible sites for radioiodination (SPECT/PET)

= Fluorophore (e.g. Cy5.5) (NIRF)

= Perfluorocarbon, i.e., PFC ($^{19}$F-MRI)
Images acquired 27 min after gavage of 0.3 ml perfluorononane in mice

Figure 1.5 An illustration of contrast enhancement by $^{19}$F-MRI

$^1$H-MRI renders anatomic information and $^{19}$F-MRI delineates the duodenum (D) and the small intestine (SI) by a bright image without background signal

Reference: [5]; reprinted with permission from John Wiley & Sons, Inc.
No change in signal intensity of $T_2$-weighted images in uMUC-1-negative tumors but significant signal reduction in some regions of uMUC-1-positive tumors (marked by white arrows), indicating that the targeted contrast agent (CLIO-EPPT) darkens only receptor-positive tumor \textit{in vivo}.

Reference: [6]; reprinted with permission from the American Association for Cancer Research, Inc. and permission from authors.
After probe accumulation in neoplastic tissues, $^1$H-MRI provides the structural information of the anatomy and $^{19}$F-MRI identifies tumors as bright areas with no background signal.
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2 Literature Review

2.1 Introduction

The PEG-based amphiphilic copolymer system is designed as a delivery system for therapeutic and imaging agents. Its comb-like structures (examples shown in Figure 2.1) qualify them to belong to the category of nonlinear copolymers in contrast to ‘AB’ di-block or ‘ABC’ tri-block linear copolymers. Copolymers with perfluorocarbon side chains are designated in this thesis as PEG-PFC and those with hydrocarbon side chains as PEG-HyC. The length of PEG will be specified when necessary, e.g., PEG1500-PFC, PEG900-HyC.

The literature review in this chapter covers four most relevant research areas: (1) delivery systems for biomedical applications, (2) $^{19}$F-MR imaging and spectroscopy, (3) nonlinear copolymers, and (4) fluorinated polymers.

2.2 Delivery Systems

Most commonly used drug administration methods, i.e., oral and intravenous routes, have manifested certain disadvantages, e.g., (1) the drug bioavailability is low at target site; (2) normal tissues are adversely affected by the toxic side effects of drugs; (3) drugs degrade before they reach the desired site of action [7]. Drug delivery systems are designed to address the above issues by (1) encapsulating drugs inside and thus providing them protection before they reach the desired site of action; (2) changing the size and molecular weight of the “effective drugs” and thus optimizing their biodistribution and
pharmacokinetics; and (3) utilizing various targeting schemes and thus minimizing the side effects to normal/healthy tissues. Hydrophobic drugs, which are not soluble in the blood and do not reach their target, can thereby be administered via the use of a suitable delivery system. In fact, a variety of drug delivery systems have been developed to include small molecules, proteins and large DNA fragments [8]. This section is devoted to an overview of five categories of delivery systems with sizes ranging from nanometers to microns. The last subsection is given to review the current applications of nanotechnology in cancer diagnosis and therapy.

2.2.1 Polymer-based systems

Polymeric nanoparticles

Polymers offer great flexibility as delivery systems in terms of their synthesis and preparation methods, types of agents that can be encapsulated, and their versatility (e.g., biocompatibility, biodegradability, surface modifiability) [9]. Some natural polymers that have been used to construct delivery systems are: albumin [10], gelatin [11], alginate [12], collagen [13], and chitosan [14]. A few examples of synthetic polymers are: poly lactic acid (PLA) [15], poly glycolic acid (PGA) [16], their copolymers poly lactide-co-glycolide (PLGA) [17], polyacrylates [18], poly caprolactone (PCL) [19], and polyethylene oxide (PEO) [20-23]. The methods used to prepare polymeric nanoparticles include single (oil-in-water) emulsion [17-19, 24, 25], double emulsion (water-in-oil-in-water) [26, 27], emulsification solvent diffusion method [28-30], self-assembly [31-34], etc. The drug release profile from the polymeric nanoparticles can be modulated by polymer/drug properties and external conditions such as pH, temperature, and magnetic field.
A classic representation of polymeric nanoparticles as versatile delivery systems can be seen in the case of polymeric micelles. Figure 2.2 [32] schematically highlighted the features of polymeric micelles utilized as delivery vehicles. Micelle core formation can be driven by different forces (e.g., hydrophobic interactions, electrostatic interactions); micelle shell often serves for biocompatibility and steric stabilization; the surface of the micelles can be modified to include targeting moieties, (e.g., peptides, antibodies). The wide variety of tunable parameters of polymeric nanoparticles has enabled them to be used as delivery systems in numerous biomedical applications. A few of the most important applications are cancer chemotherapy [35, 36], drug delivery to brain [37-39], and gene delivery [40-43].

Dendrimers

Dendrimers are highly branched macromolecules with repeated units (Figure 2.3 [44]). The first dendrimers were synthesized by Vögtle in 1978 [45] with “a divergent method”, followed by others such as Tomalia [46]. In 1990 Fréchet introduced the “convergent” approach [47] to synthesize well-defined dendritic molecular architectures. Since then, dendrimers have drawn tremendous attention due to their unique molecular architecture. Some of their outstanding features are [46-51]: (1) highly branched structures giving rise to multivalency, (2) well-defined molecular weight with low polydispersity index, (2) tunable core structure and folding branches creating cavities of hydrophilic or hydrophobic nature, and (3) surface groups amenable for modification for desired applications. As a result, delivery systems formed by dendrimers have well-controlled size, shape, density, polarity, reactivity, and solubility. Bioactive agents can be incorporated by being encapsulated into the dendrimer core or chemically attached or physically adsorbed onto the dendrimer surface.
Among more than 50 families of dendrimers, poly amidoamine (PAMAM) dendrimers are the first that are synthesized, characterized, and commercialized [51]. PAMAM has been utilized to incorporate and to deliver genes [52-56], anti-tumor drugs [57-59] (e.g., 5-fluorouracil), anti-inflammatory drugs [60-63] (e.g., ketoprofen), and antimalarial drugs (e.g., artemether [64]).

**Nanogels**

Nanogels are networks of polymeric particles formed by cross-linking, whose size is in the submicron range. Nanogels can be prepared by two different methods: (1) emulsion polymerization [65] and (2) cross-linking of preformed polymer fragments [66]. Emulsion polymerization is the most commonly used method for nanogel preparation, but because the polymerization takes place in a mixture (usually an emulsion) of monomers, cross-linking agents, and surfactants, the final products are often toxic and not suitable for biomedical applications unless purified after the synthesis.

The advantages of using nanogels as drug delivery systems are their high drug loading capacity and their ability to respond reversibly to change in external conditions, e.g., temperature, pH, ionic strength, and solvent property [67-69]. Temperature-responsive nanogels are mostly constructed by poly N-isopropylacrylamide (PNIPAAm) and its derivatives [66]. The mechanism is based on polymer phase separation phenomenon that occurs when the temperature is raised to its lower critical solution temperature (LCST), above which nanogels tend to shrink/collapse and below which they are swollen. These nanogels have manifested controlled and sustained release of drug when subject to temperature changes [70-72].

pH-sensitive nanogels made of poly methacrylic acid-grafted-ethylene glycol [P(MAA-g-EG)] have been used for protein delivery. Insulin have been
incorporated into P(MAA-g-EG) nanogels and tested via oral administration [73, 74]. In an acidic environment like that of the stomach, the gels are not swollen because of the formation of intermolecular complexes, protecting insulin from degradation by proteases. In basic and neutral environments like the intestine, the intermolecular complexes dissociate, causing rapid gel swelling and consequent insulin release. Other examples include glucose-sensitive nanogels [75, 76], gene delivery [77, 78], and anti-tumor drug delivery [79].

2.2.2 Liposomes and lipid nanoparticles

Liposomes and lipid nanoparticles are spherical vesicles, whose membrane is composed of phospholipid bilayer. They can be made by different methods, e.g., extrusion [80], reversed-phase evaporation [81], detergent-based procedures [82], high pressure homogenization [83, 84], micro-emulsion method [85, 86], high speed stirring and/or ultrasonication [87], water-oil-water double emulsion method [88], solvent emulsification evaporation/diffusion [89]. Interested readers can refer to a good review on liposome synthesis by Watwe and Bellare [90].

Liposomes have been extensively studied as delivery systems. There are four mechanisms of liposome-cell interactions: (1) adsorption, (2) endocytosis, (3) fusion, and (4) lipid exchange [91]. Liposomes have great flexibility with regard to their size, structure, composition, and modification [92, 93]. Figure 2.4 [94] is an illustration of liposomes as versatile delivery systems. Bioactive agents can be encapsulated in the aqueous environment of the lipid bilayer vesicle (e.g., hydrophilic drugs and DNA). Lipid-soluble drugs can be solubilized in the lipid bilayer. Surface modifications can prevent them from being captured by the reticuloendothelial system (RES). Homing peptides can help them to actively
target pathological tissues for diagnosis and treatment of diseases. Unmodified liposomes are preferentially taken up by the RES; therefore they have been used to encapsulate drugs with toxic side effects and to passively target the RES. An example is the use of antibiotic amphotericin B to treat systemic fungal infections [95]. Amphotericin B has extensive renal toxicity; whereas liposomal amphotericin B (Ambisome) reduces the renal toxicity of the drug at normal doses while treating the liver and spleen by passive targeting [96]. Other applications include using liposomes to enhance immunological response (immunoadjuvants) [97], to deliver genes into specific cells in the body [98, 99], and to deliver active agents to brain [100, 101].

2.2.3 Viral vectors and virus-like particles

Another category of delivery systems that has been extensively studied is viral vectors and virus-like particles, which are designed to mimic viral behavior in infecting cells. Viruses are very efficient in transfec{ing their own DNA into specific host cells and use the machinery of the host cells to reproduce themselves [102]. This behavior is ideal in drug or gene delivery, but because viruses are pathogenic, they must be used in modified forms. Recombinant viral vectors and virus-like particles (VLPs) are such modified delivery systems.

Recombinant viral vectors

A recombinant viral vector is designed to retain the efficiency of gene transfer and expression but to eliminate the pathogenicity of the virus. The nonessential genes of the viruses (for their replication phase) are replaced by foreign genes of interest so as to disable the innate viral infection in the host. But
the modified viruses are still capable of transfecting the desired cell types with the foreign genes of interest and induce gene expression in the host.

There are many different types of recombinant viral vectors, e.g., adenovirus vectors, retrovirus vectors, adeno associated virus vectors, vaccinia virus vectors, herpes simplex virus vectors, etc. Adenovirus vectors contain linear double-stranded DNA's with no envelopes. They can be produced cost-effectively and consistently with high infectious ability into both dividing and non-dividing cells [103, 104]. Though they are widely used for gene delivery in vivo and are in clinical trials for cancer therapy [105], they often stimulate immune response to the cells transfected and thus cause loss of gene expression 1-2 weeks after injection [106].

Retrovirus vectors are modified from retroviruses that have single-stranded RNA's and envelopes, which contain proteins that specifically interact with surface receptors of the target cells [107]. The viral replication genes are replaced with foreign genes of interest. After cell infection, the viral genome is reverse transcribed into double-stranded DNA, integrated into the host genome, and expressed as proteins. Figure 2.5 shows the infection process of a subtype of the retrovirus vector (lentiviral vector) [108]. Two major advantages of using retroviral vectors in gene delivery are (1) stable long-term integration in the host genome and (2) lowest clinical toxicity [106, 109]. Therefore, they are most suitable for treatment of genetic diseases where permanent gene expression is desirable.

**Virus-like particles (VLPs)**

Unlike recombinant viral vectors, virus-like particles (VLPs) contain no viral genome at all but only the viral capsid proteins so as to mimic the structural
confirmation of the actual viruses, which enables them to efficiently transfect cells.

Papilloma VLPs have been used for immune therapy for papillomavirus-related diseases. For example, long-term protection against the rabbit papilloma virus has been stimulated by the papilloma VLPs [110, 111]. In addition, different types of papilloma VLPs have been shown to induce immune responses from B and T lymphocytes [112-114] and thus demonstrated the potential of using VLPs for immunization against different types of papillomaviruses. Another major category of VLPs is polyomavirus-like particles. By encapsulating plasmid pCMV-β-gal as its genomic information, this system has successfully transfected monkey kidney cell lines and caused consequent expression of functional β-galactosidase [115]. Furthermore, a fluorescent protein and a low molecular weight drug methotrexate have been encapsulated by the polyoma VLPs and delivered into mouse fibroblasts in vitro [116], giving promise to their applications in not only gene delivery but also delivery of therapeutics and vaccines.

2.2.4 Nanofibers

Nanofibers can be made from carbon [117], organometallic compounds [118], inorganic compounds [119], and polymers [120]. Figure 2.6 is a transmission electron micrograph of carbon nanofibers [121]. They have a diameter of a few to hundreds of nanometers [122]. Because of the biocompatibility, biodegradability, and ease of formation, polymeric nanofibers have been extensively examined for biomedical applications [120, 123-126]. As delivery systems, nanofibers have a few outstanding characteristics: (1) large surface area, (2) ease of surface functionalization, and (3) controlled pore size
enabling modifiable release kinetics by changing the composition and morphology of the nanofibers. Different methods can be used to produce polymer nanofibers, e.g., drawing [122], template synthesis [127], self-assembly [128], and electrospinning [129], among which electrospinning is the most attractive method for biomedical applications with the capability of large-scale production. A good review of different processing and characterization techniques of nanofibers is by Jayaraman et.al. [126].

Nano-fibrous scaffolds containing various growth factors have been extensively investigated in tissue engineering and have demonstrated controlled release of the growth factors. These results hold promise for bone repair and regeneration [130-135] and for treating Alzheimer’s disease and Parkinson’s disease, where peripheral nerve regeneration is needed [136-138]. Other applications of polymeric nanofibers include the delivery of DNA [139, 140] and small drug molecules (e.g., antibiotic tetracycline hydrochloride [141, 142], antituberculosis drug rifampin [142]).

2.2.5 Inorganic nanoparticles

Various inorganic nanoparticles have drawn significant attention in biomedical applications due to their unique structural, spectroscopic, or magnetic properties. They have expanded the armory of nanotechnology as novel diagnostics and therapeutics. Five categories of inorganic nanoparticles are included in this section: (1) carbon nanotubes and fullerenes, (2) quantum dots, (3) nanoshells, (4) gold nanoparticles, and (5) paramagnetic nanoparticles.

Carbon nanotubes and fullerenes
The backbone of carbon nanotubes (CNTs) is composed only of carbon atoms, which are arranged in benzene-ring conformation as graphite sheets. The carbon graphite sheets are then rolled up to form seamless cylinders, which can be either single-walled CNTs or multi-walled CNTs. Figure 2.7 is a 3D model of single-walled carbon nanotubes [143]. Fullerenes are also made of carbon atoms. They are considered to be one of the allotropes of carbon. The structure of fullerenes resembles that of a soccer ball (Figure 2.8 [144]). Their diameter can be as small as 2 nm [145].

Carbon nanotubes can be produced by three different methods: chemical vapor deposition [146], electric arc discharge [147], and laser ablation [148]. After the CNTs are produced, a significant amount of residues (e.g., metal catalyst and amorphous carbon) are left in the final product. Therefore, purification is necessary for subsequent applications. Various purification techniques include oxidation, chromatography, centrifugation, filtration, and chemical functionalization [149-154]. Furthermore, because CNTs are completely insoluble in aqueous solutions by themselves, they need to be functionalized in order to be dispersed and stabilized in solution for biomedical applications. Two approaches have been used to modify the CNT surface to increase its solubility – noncovalent and covalent. Suitable noncovalent modifications include the use of polysaccharides [155], peptides [156], proteins [157], and nucleic acids [158]. Covalent modifications include (1) the use of acids to add hydrophilic functional groups to the CNT surface by oxidation [159] and (2) the addition reaction that CNTs undergo to become functionalized CNTs (f-CNTs), which are soluble in various solvents [160, 161]. Functionalized CNTs (f-CNTs) have a few attractive features for biomedical applications: (1) they have large inner volume relative to the tube dimensions, which can be loaded with desired bioactive agents for delivery; they have low toxicity, and (3) they are non-immunogenic [155].
example, CNTs have been double functionalized with fluorescein and an antibiotic drug (amphotericin B, AmB), which enabled both the tracking of the uptake of CNTs and the delivery of AmB as an antifungal treatment [162]. Other application of CNTs include the delivery of nucleic acids [163, 164], proteins [165, 166], and vaccines [167]. Interested readers can refer to the review article by Bianco, Kostarelos, and Prato [168].

Similar to CNTs, fullerenes can also be functionalized on the surface to become soluble in aqueous solutions [169, 170]. Their hollow structures allow loading of bioactive agents for drug and gene delivery applications. Fullerenes are themselves strong antioxidants. They are capable of removing free radicals that are associated with certain diseases. For example, in neurodegenerative diseases, oxygen free radicals break chemical bonds in critical molecules (e.g., nucleic acids) due to the presence of their unpaired electrons and thus cause cell damage and possible apoptosis [171]. Dugan et. al. showed that carboxylic acid functionalized fullerenes are water soluble and can efficiently scavenge free radicals [172], which demonstrated their potential in treating neurodegenerative diseases. In the case of cancer treatment, intracellular uptake of fullerene-pyropheophorbide a complexes in Jurkat cells has been reported, in which photo-induced cytotoxicity was observed in culture [173]. Furthermore, fullerene-paclitaxel conjugate was reported to have significant anticancer activity with slow drug release kinetics [174]. Ashcroft et. al. synthesized and characterized a water-soluble fullerene derivative that is covalently attached to an antibody to recognize human tumor cell antigen, which opened up the opportunity of using fullerenes as active targeting delivery systems [175]. Other applications of fullerene derivatives include delivery of antibacterial agents [176], plasmid DNA [177], nuclear medicine [178], and magnetic resonance imaging contrast agents [179, 180].
Quantum dots

Quantum dots (QDs) are nano-scale semiconductors with many superior optical properties compared to conventional fluorescent dyes. The emission fluorescent spectra of QDs are tunable by changing the composition and size of the QDs. Their spectra have narrow and discreet frequencies from ultraviolet to the infrared range. QDs are very efficient in absorbing and emitting light, making them sensitive light sensors and excellent light emitters. QDs are found to be 10-20 times brighter than organic dyes. QDs are also one order of magnitude more resistant to photobleaching than their organic fluorescent dye counterparts. QDs exhibit cytotoxicity both in vitro and in vivo, which hinders their biomedical applications. But QDs can be modified on the surface with hydrophilic polymers and biological ligands, e.g., antibodies, peptides, oligonucleotides [181-183]. Therefore, they have the potential to be developed into probes with specific targeting capabilities.

Han et. al. reported the use of well-controlled different-sized QDs embedded in polymeric microbeads for multicolor optical coding in vitro, which can be used for gene expression study, high-throughput screening, and medical diagnostics [184]. Furthermore, Gao et. al. encapsulated semiconductor QDs with an ABC triblock copolymer and linked to a monoclonal antibody that specifically target human prostate cancer cells. This QD-based multifunctional probe demonstrated cancer targeting and imaging abilities in live animals [185]. Other applications of QDs include lung imaging [186] and human breast cancer imaging [187]. With the advancement of conjugation chemistry and surface technology, QDs hold much potential as novel biomedical diagnostic labels.
**Nanoshells**

Similar to quantum dots, nanoshells also have tunable optical properties with emission/absorption spectra expanding from the ultraviolet to the infrared frequencies [188]. They are constructed with a dielectric core (usually silica) with a thin metal shell (typically gold) [189]. Nanoshells have no heavy metal in their composition and therefore are not toxic. But their sizes are bigger than QDs, which is the major disadvantage for their biomedical applications.

Nanoshells with polyethylene glycol (PEG) coating have been used *in vivo* as long-circulating imaging contrast agent with optical coherence tomography and photoacoustic tomography [190-192]. More interestingly, nanoshells have been designed to serve as photo-absorbers, which can generate effective thermal energy in photo-thermal ablation therapy [191, 193, 194]. AuroShell™ (Nanospectra) particles belong to this nanoshell therapeutic family. After these nanoparticles are delivered to neoplastic tissues, a near-infrared laser light is illuminated externally at the tumor site, AuroShell™ then act as specific heat generators by absorbing the light energy and converting it to heat, thus destroying the cancerous tissues.

**Gold nanoparticles**

Gold nanoparticles are another category of promising nanotechnology platform. They are easy to fabricate and they can strongly absorb and scatter light at desired wavelengths. Gold nanoparticles are less toxic compared to quantum dots and the metal gold is approved by FDA for some therapeutic applications. Copland et. al. conjugated gold nanoparticles to a monoclonal antibody to target human breast cancer cells. The *in vitro* experiments demonstrated efficient selective targeting and imaging by optoacoustic tomography of human SK-BR-3 breast cancer cells in a gelatin phantom that
optically resembled breast tissue. The limit of detection concentration at a depth of 6 cm was 109 nanoparticles per ml [195]. Paciotti et. al. developed a gold nanoparticle based drug delivery system that has attached PEG and recombinant human tumor necrosis factor on its surface. In vivo animal tests showed that these nanoparticles, after intravenous administration, rapidly accumulated in colon carcinomas but not in the livers, spleens, or healthy organs, indicating that the particles escaped the RES system and had selective targeting ability. The system was further developed to include paclitaxel as a multifunctional nanoscale delivery platform [196, 197]. Gold nanoparticles were further investigated in radiotherapy [198], vital reflectance imaging [199], and photo-thermal cancer therapy [200, 201].

**Paramagnetic nanoparticles**

Paramagnetic nanoparticles have been extensively studied alongside with the fast advancement of MRI. MRI has 3D high spatial resolution as its advantage but lower sensitivity compared to nuclear imaging. The successes of utilizing MRI for diagnosis and therapy assessment depend to a large extend on the contrast-to-noise ratio obtainable, which necessitates the use of contrast agents, e.g., gadolinium-based conjugates, iron oxide nanoparticles. Iron oxide nanoparticles have attracted much attention because of their superparamagnetic property (i.e., high magnetic susceptibility) that enables them to produce substantially high contrast.

Ultra-small superparamagnetic iron oxide (USPIO) has been found to be small enough to migrate across the capillary wall via vesicular transport and through inter-endothelial junctions [202]. There have been numerous applications of this class of nanoparticles in conjunction with both passive and active targeting strategies. In the case of passive targeting, USPIO has been used
for MRI of cardiovascular diseases [203-205], MRI of the lymphatic system and associated cancers and metastases [206-210], MRI of arthritis [211], MRI of transplanted pancreatic islets [212, 213], etc. For active targeting, iron oxide nanoparticles have been conjugated to different targeting moieties (e.g., antibodies, peptides) to detect cancers [214-219], atherosclerotic plaques where apoptosis takes place [220], and even in combination with delivery of chemotherapeutic drugs [221, 222]. There also have been several commercialized iron oxide nanoparticles for cancer diagnosis, e.g., ferumoxtran-10, AMI-227, and Combidx® developed by Advanced Magnetics Inc., and Sinerem® by Laboratoire Guerbet.

2.2.6 Nano-scale cancer diagnostics and therapeutics

As I have briefly summarized in the previous sections, the development of nano-scale delivery systems has been a very active field of research. Some of these research areas have stepped into various stages of clinical trials or have resulted in products in the market for biomedical applications either in vitro or in vivo. Specifically, some of these products are summarized in Table 2.1 and Table 2.2 in the applications of cancer diagnosis and therapy [223].

2.3 19F-MR Imaging and Spectroscopy

Most commonly used MRS/MRI mapping nuclei are 1H, 13C, 19F, and 31P. Table 2.3 gives the magnetic properties of these nuclei [4]. 19F has a sensitivity that is the second highest next to 1H. It is naturally abundant but almost absent in biological systems so that if contrast agents containing 19F are introduced into
animals or humans, the signal intensity in a pixel is proportional to the corresponding local concentration of $^{19}\text{F}$ and the resulting contrast-to-noise ratio (CNR) is only limited by the signal-to-noise ratio (SNR), which potentially can be higher than the CNR obtainable with conventional $^1\text{H}$-MRI contrast agents. Combined with $^1\text{H}$-MRI that provides anatomic information, $^{19}\text{F}$-MRI/MRS has been used in several important fields: (1) cancer imaging, (2) gastrointestinal imaging, (3) lung imaging, (4) studies of drug pharmacokinetics, and (5) measurement of oxygen partial pressure.

2.3.1 Cancer imaging

In 1987, Shimizu et. al. reported the use of $^{19}\text{F}$-MRI for detection of tumors with a 2-Tesla NMR system [224]. Perfluorotributylamine (FTBA) was conjugated with anticarcino-embryonic antigen (CEA) and administered to nude mouse that was infused with LOVO, a CEA-producing human cancer cell line. Tumors were excised from the animals and examined by $^{19}\text{F}$-MRI in vitro. The imaging results of the antibody distribution matched the immuno-histological results. In 1988, Ratner et. al. intravenously administered an emulsion of perfluoroocetyl bromide (PFOB) into mice that were implanted with RIF-1 tumors [225]. In the generated $^{19}\text{F}$-MR images, they were able to see the RIF-1 tumor, the liver and the spleen that were identified anatomically with $^1\text{H}$-MRI. This is because PFOB emulsion was captured by the reticuloendothelial system (RES) that resulted in the accumulation in the liver and spleen; the emulsion also entered the interstitial space of the tumors because of the leaky nature of the tumor vasculature.

In 1993, Meyer et. al. reported a quantitative $^{19}\text{F}$-MRI technique using an emulsion of perfluorotributylamine (FTBA) as a vascular contrast agent [226].
The results demonstrated that this technique was effective for in vivo assessment of tumor vascular volume and the changes in vascular volume with tumor growth. More recently, Oishi et. al. reported in 2007 pH-sensitive PEGylated polyamine nanogels containing $^{19}$F compounds to be utilized as tumor-specific smart $^{19}$F-MRI probes (Figure 2.9) [227]. These probes demonstrated remarkable on-off regulation of $^{19}$F-MR signals in response to the extracellular pH (6.5) of the tumor environment. The mechanism is through the increase of the molecular motion of the $^{19}$F compounds via the phase transition of the polyamine nanogel core in its volume. Furthermore, an enhanced $^{19}$F MR signal was achieved appreciably at a $^{19}$F-containing compound concentration of about 55 $\mu$M.

2.3.2 Gastrointestinal imaging

Gastrointestinal (GI) tract is one of the major organs in the abdomen. The knowledge of GI transit times, motility, and drug release is crucial to maximize the pharmaceutical potential of oral drugs. MRI method has been shown to provide better temporal or spatial resolution than a variety of methods, including X-ray and scintigraphic techniques, metal detectors, magnetic field detectors, and dyes [228]. $^{19}$F-MRI of fluorinated agents has been especially more successful than conventional $^1$H-MRI because $^1$H-MRI cannot trace small objects in the bowel due to poor contrast caused by large and intricate local signal changes [5]. An illustration of gastrointestinal tract imaging by $^{19}$F-MRI can be seen in Figure 2.10 [5]. After mice successively gavaged 0.3 ml of perfluorononane every 30 min over a period of 2.5 hr, $^{19}$F-MR coronal projections were taken immediately after the last gavage and after another hour. Schematic drawings based on these $^{19}$F-MR images are also presented, which describe the
three-dimensional sequence and arrangement of the intestinal loops for the two situations captured.

2.3.3 Lung imaging

Like most diseases, early detection of lung diseases (e.g. lung cancer, emphysema) is important for successful treatment of patients. MRI as a noninvasive imaging technique has developed rapidly over the past few decades, but MRI of lungs is behind the advancements of MRI of other organs and tissues because lungs are difficult to image with MRI in that: (1) there is not much water in the lungs, which results in a low spin density of protons for imaging and a much lower $^1$H-MRI sensitivity than in other organs; (2) strong susceptibility differences exist between lung tissue and lung air space, which causes severe signal loss in MR images, and (3) as the lung moves during respiration and cardiac pulsation, further signal loss occurs due to loss of spin phase coherence within several milliseconds and motion artifacts are difficult to eliminate.

Many different strategies have been developed to challenge these difficulties pertaining to lung MRI, for example, (1) $^{19}$F-MRI of lungs using inert fluorinated gases [229, 230] and perfluorocarbons (PFC) [231, 232]; (2) $^1$H-MRI of lungs using water-in-PFC emulsions [233, 234] during partial liquid ventilation (PLV); and (3) the use of hyperpolarized noble gases, e.g., $^{129}$Xe [235] and $^3$He [236, 237]. The use of inert fluorinated gases (e.g., CF$_4$, C$_2$F$_6$, SF$_6$) has two advantages for $^{19}$F-MRI [230]: (1) the longitudinal relaxation time ($T_1$) is very short (on the order of milliseconds), which permits the use of rapid repetition of radio frequency (RF) pulses and a large number of signal averages can thus be used to compensate for the low spin density of the gases; (2) each molecule has
multiple equivalent $^{19}$F atoms, which all resonate at the same Larmor Frequency and thus render a relatively strong MR signal.

Perfluorocarbons (PFC) (1) have low surface tension and are biologically inert in the respiratory system; (2) have high solubility of gases like O$_2$ and CO$_2$. Furthermore, by utilizing the linear relationship between the $^{19}$F spin-lattice relaxation rate ($R_1=1/T_1$) and the local oxygen partial pressure [238], there is the possibility of simultaneous measurement of pulmonary architecture, function, local oxygenation status, and oxygen transport properties \textit{in vivo} via $^{19}$F-MRI. Two forms of perfluorocarbons have been delivered to lungs as imaging agents – perfluorocarbon aerosols [231] and liquid perfluorocarbon during partial liquid ventilation [232]. Thomas et. al. delivered PFC aerosols to rat models and found that these aerosols were filtered and did not reach the lungs; but their surgically-placed tracheotomy tube inhalation protocol resulted in PFC aerosol deposition in the lungs by bypassing nasal breathing [231]. Laukemper-Ostendorf et. al. utilized the neat liquid perfluoro-octyl-bromide (PFOB, CF$_3$-(CF$_2$)$_6$-CF$_2$Br) to visualize the intrapulmonary distribution of PFC at high resolution and to measure oxygen partial pressure in porcine lungs during partial liquid ventilation (PLV) [232]. Figure 2.11 presents the high-resolution axial $^{19}$F-MR images of the PFOB distribution in the lungs of pig lying in supine position. The MR signals were acquired only from the end -CF$_3$ groups but not the other -CF$_2$-groups, which eliminated the chemical shift artifacts pertaining to $^{19}$F-MRI with PFCs. In this study, information concerning pulmonary structure, local oxygenation status, and oxygen transport properties was extracted simultaneously via \textit{in vivo}$^{19}$F-MRI.
2.3.4 Studies of drug pharmacokinetics

Drug pharmacokinetics has always been a critical issue in drug delivery studies. Fluorinated drugs self carry a natural marker \( ^{19}\text{F} \) for NMR detection. The most extensively studied drug is 5-FU, an anticancer drug. MRS started earlier than MRI studies [239-242] and then progressed into MRS studies with spatial localization [243-245]. With the rapid development in NMR, MRI is capable of tracking 5-FU at high magnetic field strength (9.4T) and with advanced imaging techniques [246]. Pharmacokinetic study using MRS at 11.7T was also reported [247].

It is worth mentioning that Bolo, et. al. used magnetic resonance spectroscopic imaging (MRSI) for the pharmacokinetics study after fluvoxamine or fluoxetine treatment [248]. In addition, Kimura et. al. reported the study of the biodistribution of a toxin, perfluorooctanoic acid (PFOA), using \(^{19}\text{F}-\text{MRI}\) in order to understand its potential effects on plant workers who are exposed to substances containing PFOA [249]. They were able to track the time course of PFOA accumulation in the mouse liver (Figure 2.12), which demonstrated the potential of \(^{19}\text{F}-\text{MRI}\) as an effective method toward further pharmacological and toxicological studies of perfluorocarboxylic acids.

2.3.5 Measurement of oxygen partial pressure

The reason that PFCs can be used for in vivo oxygen tension measurements is that there exists a linear relationship between \(^{19}\text{F}\) spin-lattice relaxation rate (R\(_1\) or 1/T\(_1\)) and the local oxygen concentration [250], which allows the non-invasive tissue oxygenation measurements using \(^{19}\text{F}-\text{MRI}\). The higher the oxygen concentration (pO\(_2\)), the greater the relaxation rate of \(^{19}\text{F}\), the brighter the pixel in the \(^{19}\text{F}-\text{MR}\) images.
Many of the applications depend on the distribution of PFC-related materials in the regions of interest to measure oxygen levels both \textit{in vivo} and \textit{in vitro}. Some examples of the applications of $^{19}$F-MRI combined with $^1$H-MRI in various areas are: (1) tissue oxygen tension measurement in rat breast and prostate tumors with hexafluorobenzene in order to assess tumor growth and development [251]; (2) pulmonary pO$_2$ measurement in rats with aerosols made of perfluorotributylamine to evaluate the respiratory function and the physiologic/pathologic condition of the lung [252]; (3) visualization of the intrapulmonary distribution of PFC and quantification of regional pO$_2$ in porcine lungs during partial liquid ventilation (PLV) using perfluoron [253]; (4) pO$_2$ measurement in PFC (\textit{trans}-1,2-bis(perfluorbutyl)-ethylene, i.e. F-44E) loaded alginate capsules implanted into different tissues to estimate the oxygen levels in the capsules and to find the optimal transplantation site [254]; and (5) mapping of oxygen tension in a hollow-fiber bioreactor with perfluorotributylamine emulsion to explore the relationship between oxygen concentration and cell density and thus optimize the reactor design and operation [255].

\section*{2.4 Nonlinear Copolymers}

Over the past few decades, block copolymers have drawn significant interest both scientifically and economically due to their ability to self-assemble into various ordered structures both in bulk and in solution [256, 257]. In particular, the micellization behavior of block copolymers in a selective solvent has been studied both experimentally and theoretically [258-263] because of their numerous applications, e.g., microreactors [264], colloidal stabilization [265-267],
surface modification [268-271], delivery of therapeutic and imaging agents [31, 272-281].

Among various copolymers systems, the micellar properties (e.g., critical micelle concentration, aggregation number, micellar size and morphology, core and shell dimensions) of linear copolymers (di-block, tri-block) [282-284] have been extensively examined as different factors are varied, including polymer concentration, molecular weight, chemical composition and chemical nature of blocks, solvent quality, pH, and temperature [285-288]. Recently, due to the advancement of synthetic methodologies, well-defined complex copolymer architectures can be prepared [289-291]. As presented in Figure 2.13, these complex architectures include graft (comb) copolymers [291-296], star-like copolymers [297-305], cyclic copolymers [306-308], super H-, and π-shaped copolymers [309-311]. As a result, studies of micellization behavior as a function of copolymer architecture have entered into a new stage [292], the understanding of which will potentially promote the applications of new nano-materials.

This section presents some examples in the synthesis and characterization of nonlinear copolymers, with a special focus on their solution behavior and the synthesis and characterization methods that were utilized. A comparison of the most relevant literature work with our copolymer system will be presented in Chapter 4 – Results and Discussion. Five categories of nonlinear copolymers are included: (1) graft/comb copolymers, (2) star-like copolymers, (3) cyclic copolymers, (4) super-H, super-\(\pi\) shaped copolymers, and (5) other architectures.

2.4.1 Graft/comb copolymers

Comb polymers are consisted of a backbone with side chains ("comb teeth") emanating from it in regularly spaced intervals, in which the chemical
composition of the side chain is different from that of the backbone. Their official
name is “graft (co)polymers”, but in textbooks and in the literature they are often
referred to as “comb (co)polymers” [312]. Comb copolymers have potential
applications such as protein absorbers [312], compatibilizers in polymer blend
interfaces [313, 314], rheology modifiers [315], specific solubilizers in solution
[316], and antifouling membrane additives [317]. Recently, the synthesis and self-
assembly of amphiphilic comb copolymers were studied extensively due to the
advancement of polymer synthesis and their interesting properties [290-293, 317-
320].

Zhou et. al. investigated amphiphilic comb copolymers of maleic
anhydride (MA) and stearyl methacrylate (SMA) [291]. The copolymers were
prepared through the reversible-addition-fragmentation-transfer (RAFT)
polymerization method. The RAFT polymerization method has been shown to be
able to construct multi-component polymer structures in a well-defined and
predictable fashion. This method has successfully produced copolymer
architectures such as alternating block copolymers [321], comb copolymers [290],
star copolymers [322], and polymer brushes on silicate substrates [323]. The
aggregates of these copolymers in a mixture of solvents tetrahydrofuran (THF)
and water were studied by dynamic light scattering and transmission electron
microscopy. The aggregates were found to change their morphologies and
dimensions with polymer concentration, water content, and pH. Furthermore,
these aggregates have potential applications as new biomaterials and drug
delivery vehicles because they provide reactive carboxylic groups on their
surfaces.

Zamurovic et. al. reported a study, in which the micellization behavior of
various complex comb-like copolymers was compared to their linear copolymer
counterparts [292]. The ultimate goal of such kind of studies is to find principles
regarding the influence of the macromolecular architecture on the micellization behavior of nonlinear block copolymers. Specifically, comb-like block copolymers of different architectures containing polystyrene (PS) and polyisoprene (PI) or polybutadiene (PB) were synthesized by anionic polymerization high-vacuum techniques using the macromonomer strategy. The micellization behavior was studied in two different selective solvents: (1) n-decane, selective for the PI (or PB) blocks, and (2) N,N’-dimethylacetamide, DMA, selective for the PS blocks. Static and dynamic light scattering methods were employed to obtain the aggregation number and the hydrodynamic radius of the micellar structures with estimated core and corona radii by calculations. A general trend has been observed based on this study and reports of other nonlinear block copolymer architectures (e.g., miktoarm-star-shape, super-H-shape) with the same building blocks (i.e., PS and PI) [304, 311, 324, 325]. Copolymers with more complicated structures have greater steric hindrance for both the soluble and insoluble components, which results in smaller micellar structures with lower aggregation numbers.

2.4.2 Star-like copolymers

Another category of complex copolymer architectures that has been extensively examined over the past decade is the star-like copolymers [297-305]. Mountrichas et. al. studied the micellization properties of two star block copolymers constructed with polystyrene (PS) and polyisoprene (PI) in dilute solutions of two selective solvents for the PS blocks, i.e., dimethylacetamide (DMA) with higher selectivity and ethyl acetate (EA) with lower selectivity [298]. The star copolymers were synthesized by anionic polymerization high-vacuum techniques and their diblock copolymer counterparts were synthesized by
sequential polymerization. The solution behavior of the copolymers was studied by static and dynamic light scattering and viscometry. Star copolymers and their diblock copolymer counterparts formed micelles of essentially the same structure. In solvent DMA, the star copolymers formed micelles of smaller sizes with lower aggregation numbers and thinner coronas compared to the diblock copolymers. In solvent EA, both star copolymers and diblock copolymers of low molecular weight formed unimolecular micelles (micelles made of a single polymer molecule), whereas copolymers of higher molecular weight formed multimolecular micelles (micelles made of multiple polymer molecules). Temperature changes could shift the micelle-unimer equilibrium to either side with the increase of temperature causing micelle dissociation and decrease of temperature facilitating micelle formation.

Among self-assembling polymeric systems, ion-containing block copolymers, also known as ionic block copolymers, have exhibited unique properties. They are composed of hydrophobic blocks linked to hydrophilic blocks that bear ionic functions, e.g., poly(4-vinylpyridinium alkyl halides), poly(metal acrylates), poly(metal methacrylates), and sulfonated polystyrene [326]. There exists a strong driving force for microphase separation due to the extreme difference between the ionic and hydrophobic blocks. As a result, ionic block copolymers have very low critical micelle concentrations (CMC) in both organic and aqueous solutions. In addition, the micelle cores are often compact, glassy spheres with the insoluble chains locked at kinetically “frozen” equilibrium conditions well below their glass transition temperatures.

The construct of ionic copolymers can be diblock, triblock, star-like, etc. Voulgaris et. al. reported a study of ionic star-like copolymers made of polystyrene (PS) and poly acrylic acid (PAA) [300], in which field most of the research has been devoted to ionic diblock and triblock copolymers. The ionic
star copolymers were produced by quantitative hydrolysis of the poly(tert-butyl acrylate) (PtBA) arms of the corresponding PSmPtBAn star copolymer precursors, which were synthesized by anionic polymerization. The aggregation properties of these copolymers were studied in solvents of mixtures of 1,4-dioxane (selective for PS blocks) and water. Copolymers with nearly symmetrical PS and PAA arms formed reverse spherical micelles of low aggregation number in 1,4-dioxane. Whereas in an 80:20 (vol) 1,4-dioxane/water mixture, these micelles were transformed into regular micelles with a high aggregation number and an elongated rod-like structure. Compared to similar studies of the morphological behavior of various aggregates formed by PS-block-PAA in 1,4-dioxane/water mixtures [327, 328], the same trend was observed, i.e., as the water content increases, the aggregate morphology changes from spheres to rods.

2.4.3 Cyclic copolymers

The effects of the cyclization of linear copolymers on copolymer micellization behavior were also examined recently [306-308]. Minatti et. al. synthesized cyclic copolymers and diblock copolymers constructed of polystyrene (PS) and polyisoprene (PI) with exactly the same degree of polymerization [306]. Their solution behavior in heptane (a selective solvent for PI) was studied by dynamic light scattering (DLS) and atomic force microscopy (AFM). In the case of the linear diblock copolymer, spherical micelles were formed and maintained their morphology as polymer concentration was increased. For the cyclic copolymers, at low concentrations, they formed small “sunflower-like” micelles. But as the polymer concentration increased, these “sunflower-like” micelles started to stack together to become giant wormlike micelles with varying size and polydispersity as functions of polymer
concentration (Figure 2.14). Some of these wormlike micelles can be longer than 1 μm. Furthermore, the cross-section diameter of these wormlike micelles was smaller than the diameter of the micelles formed by the diblock copolymer and did not change with polymer concentration. When the solvent was changed (e.g., the length of the n-alkane solvent series), the micelle size of the diblock copolymer remained the same, whereas that of the cyclic copolymer changed significantly. There has not been much research in this specific area. More work is needed to obtain further understanding of the effects of cyclization of linear copolymers on their micellization behavior.

2.4.4 Super-H, super-π -shaped copolymers

The super-H and super-π -shaped block copolymers can be viewed as two star-like copolymers bridged with a common arm. Iatrou et al. synthesized a model super-H-shaped block copolymers with protonated polyisoprene (PI) and deuterated polystyrene (PS) by anionic polymerization using tetrachlorosilane as the linking agent [311]. The micellization behavior of this model copolymer was examined in n-decane, a selective solvent for the PI block. Small angle neutron scattering (SANS), light scattering, and viscometry were used to characterize the micelles. Super-H-shaped copolymers with high polystyrene content (> 33%) form near-monodisperse, spherical micelles; those with low polystyrene content (< 14%) did not form aggregates under the experimental conditions examined. Compared to linear PS-PI diblock copolymers, the super-H-shaped structure increased the solubility and reduced the micelle aggregation number by one order of magnitude.

Pispas and Hadjichristidis constructed well-defined model copolymers of super-H and super-π architecture by anionic polymerization high-vacuum
techniques [309]. These structures have polyisoprene as the backbones and polystyrene as the branches. The copolymer solution behavior of both shapes was examined in two selective solvents: n-decane (selective for polyisoprene backbones) and ethyl acetate (selective for polystyrene branches). In n-decane, both copolymer structures formed multimolecular micelles. Their hydrodynamic properties resembled that of hard spheres. In ethyl acetate, super-H-shaped copolymers formed multimolecular micelles above the critical micelle concentration via a transient state of large, loosely bound aggregates. For super-π-shaped copolymers, they formed spherical unimolecular micelles at low concentrations (< 2 mg/mL) and multimolecular micelles of near-spherical morphology at higher concentrations. In all cases, the aggregation numbers were found to be lower than those of the diblock and triblock copolymers of similar compositions in selective solvents. In addition to experimental work, Kim et. al. reported a Brownian dynamics simulation approach to study the micellization behavior of π-shaped copolymers in a selective solvent [310].

2.4.5 Other architectures

Other copolymers of complex architectures were also studied in the same manner. For example, Bayer and Stadler synthesized a “dumbbell-shaped” copolymer with a long polystyrene chain linking two poly ethylene oxide “stars” [329]. The synthesis was realized by anionic grafting of ethylene oxide from a hydroxylated polybutadiene-polystyrene-polybutadiene triblock copolymer. These dumbbell-shaped molecules have various number and length of PEO branches. The solution behavior study of these copolymers in a nonpolar solvent toluene indicates that the hydroxyl groups at the end of the branch chains are responsible for aggregation. Xu et. al. investigated “comb-coil-shaped”
copolymers made of polystyrene backbones and polyisoprene branches [330]. The synthesis was accomplished via a combination of free radical polymerization and anionic polymerization with the "grafting-onto" strategy. As a selective solvent for the polystyrene block (e.g., dimethyl phthalate) is added into these copolymers, their micro-structures changed from disordered or weakly ordered structures into well-organized lamellar structures in the intermediate polymer concentration range.

2.5 Fluorinated Polymers

Fluorinated polymers have drawn much attention due to their unique properties, e.g., hydrophobicity, oleophobicity, low adhesion, nontoxicity, and high oxygen solubility [331, 332], which enabled them to be used as blood substitutes [333-335], surface modifiers [336-338], surfactants [339], etc. Over the past decade, many research groups have endeavored in the synthesis and characterization of new amphiphilic fluorinated copolymers to develop novel materials for various applications [332, 336, 338-354]. Due to the scope of this thesis, this section focuses on the solution behavior of fluorinated copolymers with a brief summary of various synthetic schemes reported in the literature.

2.5.1 Synthesis of fluorinated copolymers

Fluorinated blocks in copolymer structures bring unique properties in their ordered micro-phase behavior because the fluorinated block is different from both hydrophilic and oleophilic blocks [332]. For example, when a fluorine containing polymer is linked to a hydrocarbon polymer, typical surfactant
properties can be observed: surface induced phase separation and micellar association [355-357]. In the literature, there have been a few reports on the synthesis of copolymers containing fluorinated blocks. The chemistry used for synthesis includes anionic polymerization [341], group transfer polymerization [345], cationic polymerization [339, 351, 352], ring opening metathesis [358, 359], and living radical polymerization [360]. Interested readers can refer to the cited articles for further details.

2.5.2 Characterization of fluorinated copolymers

Krupers et. al. synthesized semifluorinated diblock copolymers consisting of methyl methacrylate and 1H, 1H, 2H, 2H- perfluoroocetyl methacrylate by nucleophilic catalyzed group transfer polymerization [345, 346]. In a solvent that is selective for the poly methyl methacrylate (PMMA) blocks (e.g., toluene and tetrahydrofuran), these diblock copolymers formed micelles of core-shell structures with the fluorinated blocks as the inner core and the PMMA blocks as the corona. Interestingly, the pure diblock copolymers formed cylindrical micelles; whereas upon addition of PMMA homopolymers, spherical aggregates were observed. The change of micellar morphology is believed to be due to the effective increase of the volume fraction of PMMA blocks, which enlarged the interface between the fluorinated blocks and the PMMA blocks. This impedes the formation of cylindrical micelles and facilitates the formation of spherical micelles.

Matsumoto et al. synthesized fluorine-containing amphiphilic block copolymers by sequential living cationic polymerization and subsequent hydrolysis [351, 352]. The copolymers are constructed with poly(2-hydroxyethyl vinyl ether) and poly(2-(2,2,2-trifluorooethoxy ethyl vinyl ether) with different
degrees of polymerization and exhibit amphiphilic characteristics both in water and nonpolar solvents (e.g., toluene). These copolymers have been shown to have high surface activity (e.g., their surface tension in aqueous solutions can be as low as 30 mN/m). Their critical micelle concentrations were found to be around 10 ~ 100 µM. SAXS measurements of their aqueous solutions revealed the formation of core-shell type spherical micelles at concentrations of 1.0 wt %. The aggregation number increased with the increase of the length of the fluorinated segments. Furthermore, these fluorinated amphiphilic block copolymers have greater ability to solubilize fluorinated compounds than their non-fluorinated counterparts.

Ito et. al. produced two different fluorinated diblock copolymers: poly(methacrylic acid)-block-poly(perfluorooctylethyl methacrylate) (PMAA-b-PFMA) and poly(t-butyl methacrylate)-block-poly(perfluorooctylethyl methacrylate) (PtBMA-b-PFMA) [361]. They confirmed the formation of micelles of both copolymers in water and ethanol by TEM and light scattering. Micelle size of PMAA-b-PFMA is affected by pH, ionic strength, and solvent nature. PtBMA-b-PFMA molecule in ethanol formed micelles of smaller sizes than PMAA-b-PFMA, which is considered to be the result of the higher steric hindrance of the PtBMA block.

Imae et al. used anionic block copolymerization to produce poly(methyl methacrylate)-block-poly(2-perfluorooctylethyl methacrylate) (PMMA-b-PFMA) block copolymers [353]. In acetonitrile and chloroform, they form spherical micelles with a PFMA core and a PMMA corona/shell. Busse et al. recently reported that triblock copolymers containing fluorinated blocks also formed micelles as confirmed by light scattering, small angle X-ray scattering, and TEM [354]. Zhang et. al. investigated the aggregation behavior of a series of 2-(N-ethylperfluorooctanesulfonamido) ethyl acrylate copolymerized with
polyacrylamide in water by a combination of static and dynamic light scattering methods [342]. It was estimated that each aggregate contains 5 ~ 9 individual polymer chains on average. Loppinet and Gebel studied the aggregation behavior of short pendant chain perfluorosulfonated ionomer solutions by small angle X-ray and neutron scattering [362]. They have found that these polymers in aqueous solutions form rod-like aggregates with a radius of about 17 Å. Xu et al. synthesized and characterized polyethylene glycols end-capped with fluorocarbon hydrophobes [363]. The rheological study suggested that these triblock copolymers formed flowerlike micellar structures and extended networks connected by bridging fluorocarbon-capped polymer chains.

2.5.3 Summary

In summary, fluorinated block copolymers have been produced via various synthetic routes and their unique solution properties have been recently investigated with a combination of techniques (e.g., transmission electron microscopy, light scattering, neutron scattering). It has been found that the fluorinated segments have strong interactions with one another and a "perfluoroalkyl-philic" property, causing these copolymers to have lower critical micelle concentration (CMC) and to be able to preferentially solubilize fluorinated compounds. Furthermore, the micellar morphology is influenced by various factors, e.g., the chemical composition and chemical nature of blocks, polymer concentration, pH, ionic strength, solvent nature, and additives.
### Tables

#### Table 2.1 Nanotechnology-based diagnostics for early detection of cancer

<table>
<thead>
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<th>Technology</th>
<th>Application</th>
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<th>Company</th>
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<td>Cell assay detection</td>
<td>In market</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Biopixle</td>
<td>Quantum dots</td>
<td>Cancer diagnostics</td>
<td>In market</td>
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<td>In market</td>
<td>Crystalplex</td>
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<tr>
<td>Verigene</td>
<td>DNA functionalized gold nanoparticles</td>
<td>Cancer diagnostics</td>
<td>In market</td>
<td>Nanosphere</td>
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<tr>
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<td>Iron oxide nanoparticles</td>
<td>Diagnostics of lymph cancer</td>
<td>In market</td>
<td>Advanced magnetics</td>
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<tr>
<td>Qdot 800</td>
<td>Near IR conjugated QDs</td>
<td>Lymph node mapping of colon, lung and uterine cancer</td>
<td>Preclinical</td>
<td>Quantum dot</td>
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</table>

Reference: [223]
Table 2.2 Nanotechnology-based pharmaceuticals for cancer treatment

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<td>Cytarabine</td>
<td>DepoCyt</td>
<td>Lymphomatous meningitis</td>
<td>In market</td>
<td>SkyePharma</td>
</tr>
<tr>
<td>Liposome Vaccine</td>
<td>BLP25</td>
<td>Non-small cell lung cancer</td>
<td>Phase III</td>
<td>Biomira Inc</td>
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<tr>
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<td>Marqibo</td>
<td>Non-Hodgkin’s lymphoma</td>
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<tr>
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<td>NX211</td>
<td>Ovarian cancer</td>
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<tr>
<td>Irinotecan HCl and floxuridine</td>
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<td>Advanced colorectal carcinoma</td>
<td>Phase II</td>
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<td>Telintra</td>
<td>Myelodysplastic syndrome</td>
<td>Phase II</td>
<td>Telik</td>
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<tr>
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<td>–</td>
<td>Lymphocytic leukemia</td>
<td>Phase II</td>
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<tr>
<td>Vinorelbine</td>
<td>–</td>
<td>Solid tumor</td>
<td>Phase I</td>
<td>Yakult Honsha</td>
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<tr>
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<td>ThermoDox</td>
<td>Breast and liver cancer</td>
<td>Phase I</td>
<td>Celsion</td>
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<tr>
<td><strong>Polymeric Nanoparticles</strong></td>
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<td></td>
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<td>Genexol</td>
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<td>Phase II</td>
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<td>Cisplatin</td>
<td>Nanoplatin</td>
<td>Treat wide range of cancer</td>
<td>Phase I</td>
<td>NanoCarrier</td>
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Reference: [223]
Table 2.3 Magnetic properties of nuclei $^1\text{H}$, $^{13}\text{C}$, $^{19}\text{F}$, and $^{31}\text{P}$

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Z</th>
<th>$I$</th>
<th>$\mu_m/\beta_m$ (rad gauss$^{-1}$ sec$^{-1}$)</th>
<th>$\gamma$</th>
<th>$\omega_0/2\pi$ (MHz/10$^4$ gauss)</th>
<th>Natural abundance (%)</th>
<th>Relative sensitivity *</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1\text{H}$</td>
<td>1</td>
<td>$\frac{1}{2}$</td>
<td>4.84</td>
<td>26,753</td>
<td>42.6</td>
<td>99.98</td>
<td>1.000</td>
</tr>
<tr>
<td>$^{13}\text{C}$</td>
<td>6</td>
<td>$\frac{1}{2}$</td>
<td>1.22</td>
<td>6,728</td>
<td>10.7</td>
<td>1.11</td>
<td>0.016</td>
</tr>
<tr>
<td>$^{19}\text{F}$</td>
<td>9</td>
<td>$\frac{1}{2}$</td>
<td>4.55</td>
<td>25,179</td>
<td>40.1</td>
<td>100</td>
<td>0.834</td>
</tr>
<tr>
<td>$^{31}\text{P}$</td>
<td>15</td>
<td>$\frac{1}{2}$</td>
<td>1.96</td>
<td>10,840</td>
<td>17.2</td>
<td>100</td>
<td>0.066</td>
</tr>
</tbody>
</table>

* Relative sensitivity is given for equal numbers of nuclei in equal external fields.

Reference: [4]
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Figures

(a) PEG-PFC

(b) PEG-HyC

Comb-like Structure

Figure 2.1 Examples of PEG-based amphiphilic comb-like copolymer structures and their schematic representations
Figure 2.2 Features of polymeric micelles that make them versatile delivery systems

Reference: [32]
Figure 2.3 Schematic representation of the architecture of dendrimer and dendron

Reference: [44]; reprinted with permission
Bioactive agents can be encapsulated inside of the lipid bilayer vesicle (e.g., hydrophilic drugs and DNA) and lipid-soluble drugs in the lipid bilayer. Surface modifications can prevent them from being captured by the reticuloendothelial system (RES) and help them to actively target pathological areas for diagnosis and treatment of diseases.

Reference: [94]; reprinted with permission
Figure 2.5 Packaging and infection by a lentiviral vector

Reference: [108]; reprinted with permission
Figure 2.6 Transmission electron micrograph of carbon nanofibers

Reference: [121]; reprinted with permission
Figure 2.7 3D model of three types of single-walled carbon nanotubes

Reference: [143], used under the GNU Free Documentation License
Figure 2.8 Structure of the icosahedral fullerene $C_{540}$

Reference: [144]; reprinted with permission
The PEGylated nanogels have a polyamine core, which undergo volume phase transition between physiological pH and cancerous pH. This phenomenon enables the on-off regulation of $^{19}$F-MR signal based on the molecular motion of the $^{19}$F-containing compounds in the nanogel core. The nanogel has a shrunk hydrophobic core at the physiological pH, which restricts the molecular motion of the $^{19}$F atoms, which corresponds to the OFF state of the probes. At the pH typical of tumor environments, the nanogel obtains a hydrophilic core through volume phase transition and renders the $^{19}$F atoms freedom to precess, which corresponds to the ON state of the smart probes.

Reference: [227]; reprinted with permission from the American Chemical Society
Figure 2.10 $^{19}$F-MR coronal projections and corresponding schematic drawings of the gastrointestinal tract of a mouse

After mice successively gavaged 0.3 ml of perfluorononane every 30 min over a period of 2.5 hr, $^{19}$F-MR coronal projections were taken immediately after the last gavage (A) and after another hour (B). Schematic drawings (C and D) based on these $^{19}$F-MR images are also presented, which describe the three-dimensional sequence and arrangement of the intestinal loops for the situation depicted in A and B, respectively.

L, left-hand side; S, stomach; D, duodenum; SI, small intestine; C, cecum; Cn, colon

Reference: [5]; reprinted with permission from John Wiley & Sons, Inc.
Figure 2.11 High-resolution axial $^{19}$F-MR images of the PFOB distribution in the lungs of pig lying in supine position

Images at three different slice positions (caudal, central, and cranial) are shown. Each image was obtained during a single end-expiratory breath-holding period of 34 sec. RL and LL: right and left lung, respectively. Small arrows: PFOB-filled main bronchi at the central slice position in the cranial slice. Big arrow: the ventilated extrapulmonary reference phantom. Star: trachea.

Reference: [232]; reprinted with permission from John Wiley & Sons, Inc.
Figure 2.12 *In vivo* $^1$H- and $^{19}$F-MR coronal images of a mouse administered with PFOA

Time (hr) after the administration of PFOA is indicated below each image. From these images, the time course of PFOA accumulation in the mouse liver is clearly shown. PFOA was initially in the mouse stomach; 1 hour after the administration of PFOA, it began to accumulate in the liver; after 2.7 hours, the transfer was almost completed.

*Reference:* [249]
Figure 2.13 Schematic representation of the structures of linear copolymers and some nonlinear copolymers
Figure 2.14 Illustration of spherical micelles formed by diblock copolymer and "sunflower-like" micelles formed by cyclic copolymer and their subsequent stacking to form wormlike structures that can be longer than 1 μm

PS = polystyrene; PI = polyisoprene;

Reference: [306]; reprinted with permission from the American Chemical Society
3 Experimental Section

3.1 Introduction

This chapter presents the experimental details included in the research work of this thesis in order to understand the micellization behavior of the PEG-based amphiphilic comb polymers in dilute solution and its magnetic resonance response. The materials used are covered in section 3.2. Different characterization methods are covered from section 3.3 - 3.8: critical micelle concentration determination, dynamic light scattering, transmission electron microscopy, cryogenic transmission electron microscopy, small angle neutron scattering, and $^{19}$F-NMR spectroscopy. The necessary basics of each method precede the experimental procedure in order to facilitate the interpretation of the experimental results.

3.2 Materials

3.2.1 Polymer synthesis

Polymer synthesis was carried out by Professor Arthur Watterson's group at University of Massachusetts, Lowell [2, 3]. The synthesis of copolymers studied in this thesis is detailed in Appendix I. The structure of perfluorinated PEG-based amphiphilic comb copolymers is shown in Figure 2.1 (a) PEG-PFC. Briefly, the perfluorinated amphiphilic polymer was synthesized using a chemo-enzymatic strategy. The backbone polymer was obtained by the reaction between dimethyl 5-hydroxyisophthalate and polyethylene glycol (PEG) catalyzed by
Novozyme-435 (Candida antarctica lipase B immobilized) at 90°C under vacuum. The side chain precursors (perfluorinated bromoesters) were synthesized by the acylation of the hydroxyl group of 1H, 1H, 2H, 2H-perfluorodecanol with bromoacetic acid catalyzed by Novozyme-435 under solventless conditions (70°C, mild vacuum). Then, perfluorododecyl bromoacetates were attached to the phenolic hydroxyl of the backbone polymer using potassium carbonate in acetone to produce the perfluorinated amphiphilic comb-like copolymers. Their analog copolymers with hydrocarbon side chains were synthesized via the same chemo-enzymatic strategy, whose structure is shown in Figure 2.1 (b) PEG-HyC. Another analog copolymer with 95% of perfluorinated side chains and 5% of FITC (a fluorescent dye) was also prepared, the structure of which is shown in Figure 3.1 (a) PEG900-PFC-FITC.

After synthesis, polymers were purified via dialysis for 1~2 weeks then and freeze dried. These polymers are gel-like with a color that is light yellow to brown.

3.2.2 Solvents and supplementary material

Phosphate buffer solution (PBS, 1x, pH = 7.4), a selective solvent for the PEG blocks, was purchased from Mediatech, Inc (Herndon, VA) and used as received. Acetonitrile (ACN), a good solvent for both PEG and PFC blocks, was purchased from VWR International (> 99.5%) and used as received. Deuterium oxide, D₂O, (D, 99.9%) was purchased from Cambridge Isotope Laboratories (Andover, MA) and made into D₂O PBS (1x, pH = 7.4) for sample preparation in small angle neutron scattering. 1H, 1H, 2H, 2H-Perfluoro-1-decanol (97%) was purchased from Sigma-Aldrich and used for dynamic light scattering and ¹⁹F-NMR experiments, the structure of which is shown in Figure 3.1 (b) PFD.
3.2.3 Micelle solution preparation methods

Five different methods were used to prepare micelle solutions. (1) Direct: gel-like polymer was dissolved in PBS at desired concentrations without any agitation over a period of 3-4 hours. (2) Sonication: after PBS was added to polymer, the sample was sonicated (Model S-450A, Branson Sonifier, Danbury, CT) with a series of 2-sec pulses with 2-5 min of pausing time in between pulses until no polymer was visible to the eye. For more details of the sonication protocol, see Appendix II. (3) "Acetonitrile": polymer was dissolved in acetonitrile (ACN) over a period of a few minutes and then diluted in PBS with 2 vol% of ACN in the final solution (e.g., 20 μL of ACN in 1 mL of solution). (4) Orbital Shaker: polymer in PBS at desired concentration was set on an orbital shaker (Junior Orbit Shaker, Lab-Line Instruments, Melrose Park, IL) and shaken at about 60 RPM overnight. (5) Dialysis: polymer was dissolved in ACN, put into a dialysis bag (MWCO = 1000, Spectrum Laboratories Inc.), and dialyzed against PBS for 24 hours with PBS changed twice.

All samples were kept at room temperature and allowed to stabilize for at least 3 days before measurements.

3.2.4 Encapsulation of a perfluorocarbon compound

For the study of encapsulation of 1H, 1H, 2H, 2H-Perfluoro-1-decanol (PFD) by PEG900-PFC copolymer, both compounds were firstly dissolved in ACN and then mixed at PFD:PEG900-PFC molar ratios of 0, 0.5, 1, 2, 4, and 8. The samples were then diluted in PBS with 3 or 4 vol% of ACN in the final solutions with PEG900-PFC concentration of 1 mg/mL. Control samples were
made without polymer, i.e., PFD was dissolved in ACN and then diluted in PBS at the same weight concentrations of PFD.

3.3 Critical Micelle Concentration (CMC) Determination

Critical micelle concentration (CMC) is an intrinsic property observed for amphiphiles and surfactants in their selective solvents. When a small amount of amphiphile is placed into a selective solvent, it preferentially adsorbs onto the solvent surface so as to reduce the Gibbs free energy. This is what is observed as surface activity of such molecules, which causes the surface tension to decrease. When the content of the amphiphiles is continually increased causing the available surface to be limited, the amphiphilic molecules dissolved in the bulk will arrange themselves spontaneously into various geometries, in which the solvent-philic blocks will shield the solvent-phobic blocks from the solvent. These geometries are called micelles. The concentration above which micelles start to form is called the critical micelle concentration (CMC). Above CMC, further addition of amphiphiles will not reduce the surface tension significantly but increase the number of micelles in the bulk solution.

At CMC, many physical properties change abruptly, such as surface tension, turbidity, electrical conductivity, and osmotic pressure. CMC can be determined by measuring any property that is influenced by micelle formation, e.g., surface tension, electrical conductivity, light scattering, pulse radiolysis, density, viscosity, refractive index, spectrofluorometry, dye solubilization, ultrasonic absorption, and rheological measurements [364]. In this section, we present two different methods that were tested for our copolymer system – total intensity light scattering and surface tension measurement.
3.3.1 Total intensity light scattering

Because the light intensity scattered by a particle is proportional to the square of its mass [365], the appearing of micelles (where CMC is defined) will cause scattered light intensity to have a sudden increase. This is manifested as an increase in the slope of total scattered light intensity vs. the amphiphile concentration above the CMC compared to the slope below CMC. Utilizing this phenomenon, the relationship between scattered light intensity (collected at 90°) and polymer concentration can be used to determine the CMC.

PEG-PFC copolymer was dissolved in PBS at 1 mg/mL using the “direct” method. Subsequent samples were made by series of dilution. All samples for light scattering experiments (0.125 mg/mL - 1 mg/mL) were centrifuged at 13k relative centrifugal force (rcf), i.e., xg for 40 min to rid of dusty particles. The light scattering system (Benedek Group, department of Physics, MIT) is an in-house built system [366] with a He-Ne laser (wavelength 633 nm, power 50 mW; Model 127, Coherent, Santa Clara, CA) as the light source and an avalanche photodiode as the photodetector.

3.3.2 Surface tension measurement

As amphiphiles/surfactants are added into its selective solvent, surface tension decreases gradually in the low concentration range. Above CMC, the addition of amphiphiles does not decrease surface tension significantly but increases the number of micelles in the bulk solution. Figure 3.2 depicts this process. Therefore, by measuring the surface tension at varying polymer
concentrations, the abrupt change in the relationship of surface tension vs. concentration can be identified as the CMC.

PEG-PFC copolymer was dissolved in PBS at 1 mg/mL using the "Direct" method. Subsequent samples were made by series of dilution. Surface tension measurements were performed on sample concentrations from $5 \times 10^{-2} - 5 \times 10^{-3}$ mg/mL. Surface tension of polymer solution was measured by the pendant drop method with Drop Shape Analysis System (Model DSA10-Mk2, Kruss GmbH, Germany). Surface tension was obtained by Drop Shape Analysis software (version 1.80.0.2) utilizing the Young-Laplace equation:

$$\gamma = (\rho_1 - \rho_2)gR_0^2 / \beta$$

where $\gamma$ = surface tension, $(\rho_1 - \rho_2)$ = fluid density difference at interface, $g$ = gravitational constant, $R_0$ = radius of drop curvature at apex, and $\beta$ = shape factor, expressed as three dimensionless first order equations:

$$\frac{dx}{ds} = \cos \phi$$
$$\frac{dz}{ds} = \sin \phi$$
$$\frac{d\phi}{ds} = 2 + \beta z - \sin \phi / x$$

The definitions of $x, s, z, \phi$ are shown in Figure 3.3.

This method requires very small amounts of samples and can measure very low surface tensions. Before each measurement, the instrument was calibrated with distilled deionized water that had been passed through 0.02 μm filters.
3.4 Dynamic Light scattering (DLS)

Dynamic light scattering (DLS) is the most extensively used method in this thesis work. Three important aspects of DLS are presented in sections 3.4.1-3.4.3: basic principle, polydisperse systems, and practical issues. Sample preparation and experimental details are covered in sections 3.4.4 and 3.4.5.

3.4.1 Basic principle

Dynamic light scattering (DLS), also known as photon correlation spectroscopy or quasielastic light scattering, is based on the time-dependent fluctuations in scattered light intensity (instead of the total scattered light intensity) due to the Brownian motion of particles suspended in solution. DLS is a non-invasive method that is relatively simple and fast. It measures particle sizes in the bulk solution that is statistically advantageous compared to TEM and Cryo-TEM. Furthermore, DLS measurement directly represents solution properties of the sample because the necessary sample preparation procedure does not change the solution structures but only rids of the impurities.

The autocorrelation function of the measured scattered light intensity is directly related to particle diffusion rate in solution, from which particle sizes can be estimated. Figure 3.4 shows the basic building blocks necessary for DLS in particle size measurement: light scattering, photodetection, autocorrelation, and data analysis. A laser light is incident on a sample. The scattered light is collected and then its autocorrelation calculated. It can be rigorously shown [367] that the normalized autocorrelation function $|g(\tau)|$ is related to the particle diffusion coefficient $D$ by Equation (3.1) for a mono-disperse system:

$$|g(\tau)| = \exp(-Dq^2\tau) \quad \text{Equation (3.1)}$$
where $q = \frac{4\pi n}{\lambda} \sin \frac{\theta}{2}$ is the scattering vector with three constants for a particular experiment, $n$ = solvent refractive index, $\lambda$ = wavelength of light, and $\theta$ = scattering angle.

It is customary to relate the diffusion coefficient to the hydrodynamic radius $R_h$ of spherical particles defined by the Stokes-Einstein equation:

$$R_h = \frac{k_B T}{6\pi \eta D}$$

Equation (3.2)

where $k_B$ = Boltzmann’s constant, $T$ = temperature in Kelvin, and $\eta$ = solvent viscosity. For non-spherical particles, $R_h$ is often called the apparent hydrodynamic radius.

### 3.4.2 Polydisperse systems

Most sample solutions are not mono-disperse in particle size. Therefore, the autocorrelation function contains more than one characteristic decay constants, or equivalently diffusion coefficients. The normalized autocorrelation function of a polydisperse system can be written as:

$$|g(\tau)| = \frac{1}{I_0} \sum I_k \exp(-D_k q^2 \tau)$$

Equation (3.3)

where $I_0$ = total scattered light intensity, $I_k$ = intensity of light scattered by type “k” particles, and $D_k$ = diffusion coefficient of type “k” particles.

$|g(\tau)|$ is measured as a function of $\tau$ as shown in Equation (3.3). The goal is to solve this equation to obtain a relationship between $D_k$ and its coefficient $I_k$ or $\frac{I_k}{I_0}$, which is the desired particle size distribution function. The mathematical
solution is through a deconvolution operation of the correlation function $|g(\tau)|$ expressed in Equation (3.3), i.e., to find the best fit for the experimental data. However, this problem is mathematically ill-posed, meaning that there can be unlimited and drastically different distributions that fit to the same correlation function and are equally valid. There are three major approaches that have been utilized to treat this ill-posed problem: direct fit, cumulants, and regularization [368].

### Direct Fit

The direct fit method is the simplest approach, which is based on an assumed size distribution function (e.g., single modal, bimodal, Gaussian). The parameters are then determined by finding the best fit to the measured autocorrelation function. The more the parameters the assumed function has, the better the fitting is to the experimental data, but the less meaningful the values of these parameters are. This is because typical DLS data in practice only allows reliable determination of about three independent parameters for the particle size distribution function. The accuracy of this method depends almost entirely on how close the guessed distribution function mimics the true particle size distribution. Often times, this method can be misleading for samples of unknown size distributions.

### Cumulants

The method of cumulants does not assume a size distribution function but determines the moments of particle size distribution. Cumulants are stable characteristics of a distribution that are insensitive to the spikes the distribution may contain. The first cumulant (moment) can be determined from the initial
slope of the autocorrelation function expressed in Equation (3.3), which renders
the average diffusion coefficient \( D \), i.e.,
\[
- \frac{d}{dr} \ln g(r)_{r=0} = \frac{1}{I_0} \sum k I_k D_k q^2 = \bar{D} q^2
\]
Equation (3.4)

The second cumulant is the curvature (second derivative) of the initial
section of the autocorrelation function, which represents the width of the particle
size distribution. The third cumulant describes the asymmetry of the particle size
distribution. As mentioned earlier, typical DLS data only allows the
determination of three independent parameters, which means that only three
meaningful cumulants can be obtained. The first cumulant, the average diffusion
coefficient \( \bar{D} \), can be obtained with better than ±1% accuracy. The second
cumulant, the width of the distribution, can be determined with an accuracy of
±5-10%. The third moment, representation of the asymmetry of the distribution,
can only be estimated with an accuracy of about ±100%. This method does not
carry an intrinsic tendency of bias because there is no pre-assumed size
distribution function, but the description of the particle size distribution obtained
by this method often lacks necessary details.

**Regularization**

The regularization method does not assume a size distribution function,
thus eliminating the tendency of bias. This approach is based on the assumption
that the distribution is a smooth function and the fact that there cannot be
negative components. Several different algorithms have utilized this approach to
reconstruct the particle size distribution from DLS data, among which CONTIN
developed by Provencher [369] is the most popular one used in the literature and
commercial instruments. These different methods all impose some parameter of
smoothness to obtain the distribution but differ in their mathematical specifics. All the regularization methods produce similar results.

The choice of the smoothness parameter is one of the most difficult and important factors in the regularization approach. Smoothing too strongly produces very stable results but the distributions produced will lack desired details. Smoothing too weakly causes false spikes to appear in the distribution. The principle of choosing the smoothing parameter is that it should be as small as possible while producing stable and reproducible distributions in repetitive measurements of the same correlation function. Two conditions help to render fine details in the particle size distribution obtained. (1) The narrower the particle size distribution is, the less the smoothing needs to be and the better the reconstructed results are. (2) The less the statistical errors the measurements have, the less the smoothing needs to be without losing stability in size distribution reconstruction.

In summary, the regularization method produces particle size distributions with unbiased function shape, which cannot be obtained through the direct fit method or the cumulant analysis.

3.4.3 Practical issues

In DLS, there are some practical issues that need special attention in order to obtain meaningful and accurate measurements. Three major aspects are presented in this section: sample purity, optical set-up, and photodetection.
Sample purity

The optical purity (different from chemical purity) of the samples is the most important factor in obtaining desired size measurements in DLS. As mentioned in section 3.3.1, the scattered light intensity is proportional to the square of the particle mass, which means that a few large scatterers can completely dominate over the light scattered from a population of relatively small particles. It is therefore necessary to rid of the impurities in the samples and avoid collecting dusty particles from the air during sample preparation process.

Two commonly used sample purification methods are filtration and centrifugation. In filtration, it is necessary to make sure the filter is compatible with the sample solution, e.g., there is no chemical reaction between the filter material and the solvent or the solute. In addition, filtration could cause loss of material from the sample, thus possibly changing the sample concentration and causing insufficient light scattering intensity in the measurements. Without knowing the size distribution of the sample, it is often necessary to try filters with various pore sizes until satisfactory results are produced. As a “rule of thumb”, there should only be no or little resistance when passing samples through filters while eliminating undesirable large particles. Centrifugation is milder way to clean DLS samples. It removes large impurities effectively and is less likely to alter sample concentrations. Since this method is based on sedimentation, loose particles with low densities can remain in solution.

Lastly, keep in mind that it is not possible to rid of sample impurities absolutely. Extraordinarily high scattered light intensity is an indication of impurities entering into the scattering volume. Scattered light intensity by impurities should be rejected. Measurements that are heavily influenced by impurity scattering intensities should be discarded.
Optical set-up

As the light source, DLS laser must be well-stabilized and generate sufficient light intensity for scattering experiments. An effective way in optical set-up to minimize the influence of stray light and large impurities is to establish as small a scattering volume as possible. The scattering volume is the intersection of the illuminated volume and the collection volume in the sample as shown in Figure 3.5. A small scattering volume reduces stray light that can enter the photodetector, which decreases the noise level in photodetection. Furthermore, a small scattering volume reduces the possibility of large impurities entering into the scattering volume and helps the measurement of desired particle sizes to be meaningful and accurate.

Photodetection

As any other signal detection, photodetection in DLS need to meet two requirements (1) sufficient photo counts and (2) usable signal-to-noise ratio. DLS hardware system is often fixed with the only user-adjustable component as the pinhole size (Figure 3.5). Larger pinhole size allows higher photo counts to be collected but at the same time reduces the signal-to-noise ratio. For samples with strong light scattering characteristics, these two requirements are readily met simultaneously. For weak light scattering samples, sometimes it is not possible to find a usable pinhole size. In this case, another instrument should be tested for its suitability. It is also possible that DLS is not suitable at all to determine particle size distribution for certain samples.
3.4.4 Experiments and data analysis

All polymer solutions in PBS were centrifuged at 13k relative centrifugal force (rcf), i.e., \( xg \) for 40 min to rid of dusty particles before measurements. Each sample was measured at least five times. DLS measurements were conducted using an in-house built system [366] with a He-Ne laser (wavelength 633 nm, power 50 mW; Model 127, Coherent, Santa Clara, CA) as a light source. Light scattered at 90° was detected by an avalanche photodiode built into a 256 channel correlator (Precision Detectors, Bellingham, MA). The size distribution of scattering particles was reconstructed using “PrecisionDeconvolve” deconvolution software developed by Dr. Aleksey Lomakin utilizing the regularization method [368]. Readers who are interested in the details of the deconvolution method can refer to Braginskaya et. al. [370].

3.5 Transmission Electron Microscopy (TEM)

3.5.1 Introduction

Transmission electron microscopy (TEM) is a microscopy technique to directly visualize the micro-structure of ultra thin specimen by passing a beam of electrons through the specimen. Atoms of higher atomic weights are more able to stop or deflect the beam of electrons whereas those of low weights allow the electrons to pass through relatively unimpeded. This is how TEM image contrast is generated. For biological samples, specimen is often selectively stained with elements of high atomic weight so that the biological material can be differentiated from the embedding resin. In unstained samples, the intrinsic difference of atomic weights in the elements contained in the specimen produces
the image contrast. For example, TEM of the fluorinated diblock copolymers in a selective solvent only displayed the micellar core composed of the fluorinated blocks because the fluorine atom has higher atomic weight and higher electron density than the elements contained in the other block [361].

There are also a few limitations in TEM technique: (1) sample preparation can be difficult and complicated causing TEM to be a time-consuming and low throughput technique; (2) micro-structure of samples could be altered during sample preparation process; (3) the analysis of obtained TEM images may not be representative of the bulk properties of the whole sample because the field of view is relatively small; and (4) samples could be damaged by the passing electron beam, especially in the case of biological materials.

3.5.2 Experiments and data Analysis

PEG-PFC comb polymer was dissolved in PBS at 4 mg/mL using the “Direct” method. A 3 μL drop of this solution was applied to a carbon-coated copper grid, and allowed to air-dry at room temperature for 2 hours before TEM examination. A JEOL 200CX transmission electron microscope was used to characterize the morphology of PEG-PFC comb polymer at 200 kV accelerating voltage. Software “Image Tool” (version 3.0, University of Texas Health Science Center at San Antonio) was used for image analysis.
3.6 Cryogenic Transmission Electron Microscopy (Cryo-TEM)

3.6.1 Introduction

Cryogenic transmission electron microscopy (Cryo-TEM) is an advanced TEM technique. It has been utilized to examine the micro-structure of various materials, e.g., proteins, DNA, DNA-lipid structures, nano-structured fluids, polymeric micelles [371-374]. The key of this method to examine solution samples is the rapid vitrification of the thin layer of sample on a carbon-coated grid after the blotting procedure. The sample grid is plunged into a cryogen (usually liquid ethane) very rapidly, which prevents the solution from forming crystallized structures. The desired structures can be therefore visualized in an amorphous background. This sample preparation method is believed to preserve the micro-structures at their solution state.

Cryo-TEM has the same limitations as TEM except that its sample preparation method is considered to preserve the solution structures rather than change them. Practically, Cryo-TEM has some further constraints: (1) it could not allow high-intensity electron beams to pass through the vitrified samples because this would damage/melt the sample, giving Cryo-TEM a limited magnification; (2) since the structures are embedded in amorphous ice, the image contrast is often lower than that of TEM; (3) the observation time for Cryo-TEM cannot be too long because the sample is gradually melting away as the electron beam passes through; and (4) the images obtained might be out of focus because the field of view in focus is damaged as it is being observed and the captured image is actually the neighboring field of view, which may need a different focus setting.
3.6.2 Experiments and data Analysis

PEG-PFC comb polymer was dissolved in PBS at 4 mg/mL using the “Direct” method. Vitreous samples were prepared within a controlled environment vitrification system (CEVS) [375] – details presented in Appendix III. A droplet of solution (~10 µl) was deposited on a copper TEM grid coated with a holey polymer film (Ted Pella). A thin film of solution (< 300 nm) was obtained by blotting with filter paper. After allowing the sample sufficient time to relax from any residual stresses imparted during blotting (~30 s), the grid was plunged cooled in liquid ethane at a temperature close to -183°C, its freezing point, resulting in vitrification of the aqueous film. The sample grids were examined at -179°C in a JEOL 1210 transmission electron microscope operating at 120 kV. Images were recorded with a GATAN 724 multi-scan digital camera and analyzed using “Image Tool” software (version 3.0, University of Texas Health Science Center at San Antonio).

3.7 Small Angle Neutron Scattering (SANS)

3.7.1 Introduction

Small angle neutron scattering (SANS) began to be applied in the study of colloid micro-structures in the late 1970s and has been shown to be a unique technique in elucidating the structure, interaction, and phase transitions in micellar and microemulsion systems [376]. A schematic illustration of SANS experimental set-up is shown in Figure 3.6.

Compared to small angle X-ray scattering and light scattering techniques, SANS has three outstanding advantages that make it very powerful in the study
of colloid association behavior [376]. (1) Scattering of radiation is most sensitive to objects that are on the same length scale as the wavelength of the radiation itself. Neutron radiation has short wavelengths (5-20 Å); therefore dust particles and sample container imperfections do not have significant influence on its scattering intensities even at small angles where the scattering is the strongest. In addition, sample of interest can be kept in quartz cells, whose thickness can be adjusted so that neutron transmission is higher than 80%, under which conditions multiple scattering events are negligible. (2) The solvent neutron density (and therefore its scattering length density) can be varied to a large extent and in a continuous fashion by simply changing the D$_2$O/H$_2$O ratio of the solvent because deuterium has a neutron in its nucleus whereas hydrogen has none. In addition, D$_2$O and H$_2$O are chemically identical and thus their mixture would not cause changes in solvent-solute interactions, which can result in alterations of the solution structures. This method is called the contrast variation method, which has a wide range of applications, e.g., investigation of the growth of microemulsion droplet [377], study of the aggregation composition in catanionic surfactant mixtures [378]. At contrast matching points, this method reduces the number of phases in colloid systems and thus easily elucidates the structure and dimensions of each phase (Figure 3.7). (3) It is also possible to selectively deuterate particular functional groups in amphiphilic molecules so as to enhance the spatial resolution of SANS measurements.

3.7.2 Scattering Length Density

The intensity of neutron scattering of the solute relative to the solvent is directly proportional to the square of the difference in scattering length densities
between the two (also called "the contrast"). The scattering length density \( \rho \) of a molecule is defined by equation (3.5):

\[
\rho = \frac{\rho_m N_A}{M} \sum n_i b_i 
\]

Equation (3.5)

where \( \rho_m \) = mass density, \( N_A \) = Avogadro's number, \( M \) = molecular weight, \( n_i \) = number of atoms of type \( i \), and \( b_i \) = scattering length of atoms of type \( i \). \( \rho \) has units of \((\text{length})^2\), often as \( \text{cm}^2 \) or \( \text{Å}^2 \).

3.7.3 Experiments and data Analysis

PEG900-PFC and PEG1500-PFC were measured with SANS. Solutions of PEG-PFC comb copolymers were dissolved at 10 mg/mL using the "Direct" method in three different solvents (D\(_2\)O PBS, H\(_2\)O PBS, and D\(_2\)O PBS : H\(_2\)O PBS at 1:1 volume ratio) to provide variation in the solvent scattering length density (SLD). The scattering length densities of D\(_2\)O, H\(_2\)O, and D\(_2\)O:H\(_2\)O (1:1 by volume) are \( 6.34 \times 10^{-6} \text{ Å}^2 \), \( -0.56 \times 10^{-6} \text{ Å}^2 \), and \( 2.9 \times 10^{-6} \text{ Å}^2 \), respectively. The scattering length density of the fluorocarbon core is calculated to be \( 3.7 \times 10^{-6} \text{ Å}^2 \), approximated as perfluorooctane with the density of 1.76 g/ml. The scattering length densities are obtained through the NIST scattering length density calculator [379].

Neutron scattering experiments were performed on the NG7 30m-SANS instrument (NIST, Gaithersburg, MD). Neutrons of wavelength \( \lambda = 6 \text{ Å} \) with a distribution of \( \Delta \lambda / \lambda = 15\% \) were incident on samples held in quartz cells of 1 mm path length. The overall scattering vector range covered was \( 0.003 \text{ Å}^{-1} < q < 0.3 \text{ Å}^{-1} \), where \( q = \frac{4 \pi n}{\lambda} \sin \frac{\theta}{2} \) is the magnitude of the scattering vector. Sample
scattering was corrected for background and empty cell scattering. All data were analyzed by a global non-linear least-squares fit of the contrast variation data to a common core-shell model, using software supplied by NIST [380].

The core-shell model was selected because physically the “partial PEG and partial perfluorocarbon” architecture of the polymer is expected to cause the solution structure to segregate into different micro domains. In addition, the core-shell model was the simplest model structure that fit the data. Other structural models were tested as well – spheres, cylinders, disks, ellipsoids, and the core-shell versions of each of these. The core-shell sphere model was considered to fit the date the best.

3.8 \textsuperscript{19}F-NMR Spectroscopy

In order for the PEG-PFC polymer solution to act as MRI contrast agents, the NMR properties of the solution have been tested \textit{in vitro}. Specifically, the signal-to-noise (SNR) obtainable is of vital importance because the challenge of utilizing \textsuperscript{19}F-MRI for contrast enhancement lies in the limit of SNR that can be achieved. The experiments designed are to (1) investigate the influence of micellization on SNR and (2) mimic biological samples \textit{in vitro} to estimate the obtainable SNR.

3.8.1 Generation of NMR signal

Nuclear magnetic resonance (NMR) is a phenomenon that is observed with atoms that possess nuclear spin of \( \frac{1}{2} \) (Table 2.3). When these nuclei are immersed in an external static magnetic field, they can be viewed as
“microscopic magnets” that precess at a particular frequency called the Larmor frequency determined by the gyromagnetic ratio of the nuclei. These “microscopic magnets” will align themselves either along or against the direction of the applied static magnetic field, corresponding to a lower and a higher energy state respectively. There are slightly more spins that occupy the lower energy state, which results in a macroscopic effect of a net magnetization that is in the same direction as the static magnetic field.

If the system is excited with an electromagnetic wave, whose energy matches the difference between the two spin energy states, the nuclear spins in the lower energy state will undergo a transition to get to the higher energy state. The electromagnetic wave needed is in the radio frequency (RF) range, which could also be viewed as an oscillating magnetic field that is applied in addition to the static magnetic field. The macroscopic effect is a rotation of the net magnetization. When this excitation is removed, the system will spontaneously return to its initial equilibrium state, during which process the relaxation of the spins will radiate radio frequency electromagnetic waves, which is the NMR signal. Figure 3.8 is a schematic illustration of how NMR signal is generated.

If the relaxation of the nuclear spins is free of constraints (e.g., in a liquid sample), the process to return to equilibrium is slow, which corresponds to sharp line shape in the obtained NMR spectrum. If the molecular motion is constricted (e.g., in a solid sample), the relaxation process is fast, which produces broad spectral line shape.

3.8.2 Chemical shift

When samples are place in a static magnetic field, the electrons that surround the nuclei will also circulate around the direction of the externally
applied magnetic field, which results in a local microscopic magnetic field that opposes the external field. The effective magnetic field for the nuclear spins is therefore different from the externally applied static field and this difference changes with the local environment that surrounds the nuclei. For example, the local environment varies with the type of nuclei and the type of bonds in the molecule. Therefore, the same nuclei at different positions of the molecule experience different effective magnetic fields and precess at different frequencies. This phenomenon is called the "chemical shift", which is seen in NMR spectroscopy as peaks at different positions.

3.8.3 Experimental design – influence of micellization

PEG900-PFC copolymer was dissolved in PBS and acetonitrile (ACN) at 50 mg/mL; 1H, 1H, 2H, 2H-Perfluoro-1-decanol (PFD) was dissolved in ACN at 14.6 mg/mL. All three samples have a total [^{19}F] of 0.54 M, filling 5-mm NMR tubes. NMR spectroscopy was performed on a 14.1 Tesla (600 MHz) spectrometer (Avance, Bruker BioSpin, Rheinstetten, Germany; Magnex Scientific, Oxford, UK) with a ¹H/¹⁹F dual probe using acquisition software XwinNMR (version 3.6, Bruker BioSpin, Rheinstetten, Germany). The spectra were obtained with 8 scans and 10 sec of delay time to allow sufficient time for ¹⁹F atoms to relax. The effect of micellization was studied by three different analyses. (1) The NMR spectral characteristics and chemical shifts of all three samples were compared. (2) The signal-to-noise ratio (SNR) of the -CF₃ group of each spectrum was obtained by software “NUTS” (version 95, Acorn NMR) and compared with one another. (3) The relative ratio of the area under the ¹⁹F peaks (-CF₃ group vs. -(CF₂)ₓ groups) was obtained to see if it matches the molecular structures.
3.8.4 Experimental design – mimicking biological samples in vitro

In order to mimic NMR experiments with cells that have taken up PEG-PFC copolymers, small amounts of PEG900-PFC copolymer in PBS (50 mg/mL) were tested. As shown in Figure 3.9, sample (1) contains 27 μL sitting on a layer of agarose gel in a 5-mm NMR tube; sample (2) contains 39 μL at the bottom of the NMR tube. NMR spectra of sample (1) were taken twice: one at 0.5 hour after the sample was prepared (8 scans, 10 sec of delay time), the other between the 2nd hour and the 3rd hour after sample preparation (360 scans, 10 sec of delay time). Sample (2) spectra were also taken twice: 8 scans and 360 scans with 10 sec of delay time. The experiments took place 0.5-2 hours after sample preparation. For sample (2), obtained NMR spectra are not sensitive to when the scans take place because the sample is stable. The SNR of the -CF₃ group of each spectrum was obtained by software “NUTS” and compared with one another.
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Figure 3.1 Structure of PEG-based FITC-containing perfluorinated comb copolymer (a) and structure of 1H, 1H, 2H, 2H-Perfluoro-1-decanol (PFD)
Figure 3.2 Critical micelle concentration (CMC) determined by surface tension

(1) As amphiphiles/surfactants are added into its selective solvent, surface tension decreases gradually in the low concentration range. (2) Above CMC, the addition of amphiphiles does not decrease surface tension significantly but increases the number of micelles in the bulk solution.
\[ \gamma = (\rho_1 - \rho_2) g R_0^2 / \beta \]

\[ \gamma = \text{surface tension} \]

\[ (\rho_1 - \rho_2) = \text{fluid density difference at interface} \]

\[ g = \text{gravitational constant} \]

\[ R_0 = \text{radius of drop curvature at apex} \]

\[ \beta = \text{shape factor} \]

\( \beta \) is defined through the Young-Laplace equation expressed as 3 dimensionless first order equations

\[ \frac{dx}{ds} = \cos \phi \]

\[ \frac{dz}{ds} = \sin \phi \]

\[ \frac{d\phi}{ds} = 2 + \beta z - \sin \phi / x \]

Figure 3.3 Surface tension measurement by the pendant drop method
Figure 3.4 Building blocks for dynamic light scattering: light scattering, photodetection, autocorrelation, and data analysis.
Figure 3.5 Definition of scattering volume in dynamic light scattering
Figure 3.6 Experimental set-up of small angle neutron scattering (SANS)
By externally changing the solvent neutron density, the number of phases that is observed in SANS can be reduced at the contrast matching points, which decreases the complexity of SANS data analysis and enables the determination of the dimensions of different phases. As shown above, when the micellar core neutron density equals that of the solvent, only the micellar shell is visible. When the micellar shell neutron density matches that of the solvent, only the micellar core is visible.
Figure 3.8 Schematic illustration of how NMR signal is generated

When randomly oriented nuclei are immersed in an externally applied static magnetic field $B_0$, they align with $B_0$ and precess at the Larmor frequency. The macroscopic effect of this phenomenon is a net magnetization $M$ along the direction of $B_0$. When a second oscillating magnetic field $B_1$ (i.e., and excitation at radio frequency, RF) is added, the net magnetization $M$ is rotated to a certain position. After the $B_1$ is removed, the net magnetization $M$ will precess around the axis along the $B_0$ direction, which process generates the NMR signal to be measured.
Figure 3.9 NMR experimental design with PEG-PFC copolymers to mimic experiments with cells that have taken up PEG-PFC copolymers

Sample (1) contains 27 μL of PEG900-PFC in PBS (50 mg/mL) sitting on a layer of agarose gel in a 5-mm NMR tube. Sample (2) contains 39 μL at the bottom of the NMR tube.
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4 Results and Discussion

4.1 Introduction

The amphiphilic nature of the PEG-based copolymers enables them to form micelles spontaneously with the hydrophobic blocks as the micellar core and the hydrophilic PEG blocks as the corona/shell. This chapter discusses their micellization behavior based on the copolymer characterization experiments. Critical micelle concentration, a measure of micelle stability, is determined by light scattering and surface tension measurement. The size, morphology, and kinetics of the micelle solution are studied by a combination of techniques: dynamic light scattering, transmission electron microscopy, cryogenic transmission electron microscopy, and small angle neutron scattering. In addition, available theoretical models are applied to these results in order to obtain further insight into the micellar morphology based on the molecular structure of the copolymers. Finally, the NMR response of the polymer solution is measured by $^{19}$F-NMR spectroscopy and its implications presented.

4.2 Copolymer Molecular Weight

The molecular weight (MW) of copolymers was estimated by atomic mass and the polymerization number of the backbone polymer before side chain attachment ($n$, Figure 2.1). The most extensively tested PEG900-PFC and PEG1500-PFC comb copolymers were constructed with the backbone polymer that had a polymerization number of about 5.4. The polymerization number was
estimated by \(^1\)H-NMR in the Watterson Lab at University of Massachusetts - Lowell (details presented in Appendix IV). There is no available data for the polymerization number of PEG900-HyC copolymer, the polymerization number was assumed to be the same as that of PEG-PFC copolymers (5.4).

In addition, PEG-based fluorinated copolymers were synthesized utilizing molecular sieves to increase the polymerization number to about 7.5 (determined by \(^1\)H-NMR), which will be designated as PEG900-PFC* and PEG1500-PFC*. These PEG-PFC* copolymers were only tested by DLS.

Table 4.1 summarizes the polymerization numbers and MW of the various PEG-based comb copolymers.

### 4.3 Critical Micelle Concentration (CMC)

Total intensity light scattering method was first used to measure the critical micelle concentration (CMC) of PEG900-PFC copolymer in PBS. Below 0.125 mg/mL, polymer solutions gave scattered light intensities at the same level as the PBS buffer solution. In the concentration range of 0.125~1 mg/mL, the scattered light intensity scales linearly to polymer concentration and no discontinuity is observed as shown in Figure 4.1. Therefore, the CMC of PEG900-PFC copolymer in PBS was estimated to be below 0.125 mg/mL. Surface tension measurement further revealed that the CMC of PEG900-PFC in PBS is about 0.016 mg/mL, i.e., 2 \(\mu\)M (Figure 4.2).

The CMC of PEG900-HyC, the analog of PEG900-PFC, was also measured by total intensity light scattering and surface tension. Below 0.5 mg/mL, polymer solutions gave scattered light intensities at the same level as the PBS buffer solution. In the concentration range of 0.5~4 mg/mL, the scattered light intensity
scales linearly to polymer concentration and no discontinuity was observed as shown in Figure 4.3. Therefore, the CMC of PEG900-HyC copolymer in PBS was estimated to be lower than 0.5 mg/mL. Surface tension measurement suggested that the CMC of PEG900-HyC in PBS is about 0.07 mg/mL, i.e., 12 μM (Figure 4.4).

Among fluorinated block copolymers, the triblock copolymer produced by Hussain et. al. [381] is most similar to our copolymers in composition. The triblock copolymer is composed of PEG10000 as the middle block and poly perfluorohexylethyl methacrylate (PFMA) as the two end blocks, the structure of which is shown in Figure 4.5, designated as PFMA-PEG-PFMA. The CMC of triblock copolymer PFMA-PEG-PFMA in aqueous solution is about 64 μM with the fluorinated block content of 9 wt%. The PFC block in the PEG900-PFC comb copolymer is about 28 wt% with a CMC of about 2 μM. It has been found that CMC decreases when fluorinated block content increases [345, 381]. Even though these two polymers are different, especially in their architectural structures, our result seemed to agree well with this trend.

A similar surfactant to the PEG900-HyC copolymer is Tergitol® 15-S-7, CH₃-(CH₂)₁₄-(OCH₂CH₂)₇H, trademark of Union Carbide Chemicals & Plastics Technology Corp. Its CMC is 76 μM [382], higher than that of PEG-HyC (12 μM) but the two CMC’s are on the same order of magnitude.

The existence of CMC indicates that micelles formed by the copolymer chains are not unimolecular micelles but multimolecular micelles. Both PEG-PFC and PEG-HyC have low CMC’s in PBS, especially in the case of PEG-PFC copolymer with a CMC of 2 μM. The low CMC gives promise to these copolymer micelles to preserve their integrity in case of massive dilution, e.g., intravenous injection in biomedical applications.
4.4 Dynamic Light scattering (DLS)

DLS is the most extensively used method in studying the micellization behavior of the PEG-based amphiphilic copolymers. This section will first present the size distribution of the polymer solution and related factors, then the study of the hydrodynamic radius ($R_h$) of the micelles, thirdly the encapsulation of a perfluorocarbon compound, fourthly the observations with micelle solution formed by PEG-HyC copolymer in PBS, and lastly a discussion of the mechanism for secondary aggregate formation.

4.4.1 Size distribution

The effect of sample preparation

The size distribution obtained by DLS suggested the existence of two populations of particles – small micelles and large aggregates. Different sample preparation methods produced micelles of the same size but different proportions between small micelles and large aggregates (Figure 4.6). Direct, sonication, and “acetonitrile” methods essentially produced samples with the same size distribution. Orbital shaker produced a sample with a wider and more continuous distribution. However, dialysis seemed to have produced a sample of a different nature, in which significantly more large aggregates are present. The micellization behavior can be different during dialysis process because instead of exposing both blocks to a selective solvent simultaneously, dialysis allows a gradual exposure of both blocks dispersed in a good solvent to a selective solvent, which could encourage bigger aggregates and even other structures to form, e.g., vesicles and extended networks. The fact that ultracentrifugation did
not sediment the micron-size aggregates gives us evidence that these structures have low densities.

**The effect of polymer molecular weight (MW)**

PEG900-PFC* and PEG1500-PFC* were tested by DLS and compared to the results of PEG900-PFC and PEG1500-PFC. As shown in Figure 4.7, the hydrodynamic radius of micelles (filled columns) is the same for these copolymers with different MW. But the proportion of large aggregates (empty columns) increases with the increase of polymer MW, especially in the case of polymers with PEG1500.

**The effect of time after sample preparation**

After polymer solutions are kept at room temperature for a long period of time (e.g., 1 year), there is precipitation at the bottom of the solution. Therefore, size distribution of PEG900-PFC in PBS solution was measured by DLS after the sample was kept at room temperature for a few days and then for about half a year. As shown in Figure 4.8, size distribution did not change a few days after sample preparation. However, after half a year, the proportion of aggregates has increased significantly; in the meantime, there were still micelles in solution with unchanged hydrodynamic radius. This could be an indication of a slow process of micelles aggregating to become large aggregates.

**4.4.2 Hydrodynamic radius of micelles**

**The effect of time after sample preparation**

Table 4.2 presents detailed data of the hydrodynamic radius ($R_h$) of small micelles vs. time after sample preparation. Within experimental error, $R_h$ of
micelles formed by PEG900-PFC copolymer in PBS did not change with time significantly.

It seems that there are at least two processes in the polymer solution: (1) exchanges between monomers and micelles and (2) micelles possibly agglomerating to become secondary aggregates. The first process should be in a dynamic equilibrium as is typical for surfactant systems. The second process seems to proceed toward the direction of the secondary aggregates in a slow fashion (time scale on the order of months).

**The effect of polymer concentration**

The $R_h$ of micelles formed by PEG-PFC copolymers in PBS is about 8-9 nm. As shown in Figure 4.9, $R_h$ of small micelles does not change with polymer concentration for either PEG900-PFC or PEG1500-PFC in PBS. This is an indication that these micelles are not growing micelles, e.g., worm-like micelles.

**The effect of PEG length**

There is also no significant change with $R_h$ of micelles when PEG length is varied in the copolymer structure PEG-PFC from PEG600 to PEG1500 (Table 4.3). The volume of PEG1500 is 2.5 times that of PEG600 (i.e., 1500/600). In solution, PEG in the micellar corona most likely would take on a 3D globular shape. As a first-order estimation, the thickness the micellar corona made of PEG1500 is 1.08 times, i.e., $(2.5/2)^{1/3}$, that made of PEG600. Consequently, variations in PEG length did not change the overall hydrodynamic radius of micelles significantly.
4.4.3 Encapsulation of a perfluorocarbon compound

Figure 4.10 shows the hydrodynamic radius of micelles in the case of encapsulation of 1H, 1H, 2H, 2H-Perfluoro-1-decanol (PFD) by PEG900-PFC. It can be seen that the micelle size increases with increasing PFD content, which seems to reach a plateau at PFD:PEG900-PFC molar ratio of 8 at mixing. The scattered light intensity increased initially with the increase of PFD content (micelle size) but started to decrease when the PFD:PEG900-PFC molar ratio exceeded 2 at mixing (Figure 4.11). This is most likely caused by loss of material due to overloading of PFD. (Since only the top layer of solution was taken and used for DLS measurements after centrifugation, excess amounts of insoluble PFD with some amount of polymer were separated from the solution tested.) Control samples with PFD alone showed scattered light intensity at the same level as PBS buffer solution because PFD is insoluble in aqueous environment.

This experiment demonstrated that the PEG-PFC copolymers can encapsulate perfluorocarbon compounds, most likely due to its “perfluoroalkylphilic” property.

4.4.4 Micelles formed by PEG900-HyC

The PBS solution of PEG900-HyC copolymer, an analog of PEG-PFC, was also tested by DLS. Figure 4.12 shows the size distribution of PEG-HyC polymer solution. The dominant component is the individual micelles with a hydrodynamic radius ($R_h$) of 4-5 nm, which does not change with polymer concentration (Table 4.4). There are only very few larger particles present; and as a result, it is difficult to measure their sizes accurately. Qualitatively, the larger aggregates have a size that is on the order of tens to hundreds of nm.
The size difference of micelle formed by PEG-PFC and PEG-HyC lies in the difference of the hydrophobic blocks. The extended PFC side chains are longer than the hydrocarbon side chains by about 1 nm (details in section 4.8). The PFC chains are also “bulkier” in nature than their hydrocarbon analogs [383]. Furthermore, the aggregation number of PEG-PFC is higher than that of PEG-HyC (details in section 4.8). Therefore, micelles formed by PEG-PFC copolymers are bigger than those formed by PEG900-HyC copolymer.

4.4.5 Mechanism for secondary aggregate formation

Xu et. al. reported the formation of micelles and aggregates of polyethylene glycol (PEG-35000) end-capped with short fluorocarbon tails [363]. Micelle formation was driven by the strong interactions of the hydrophobic fluorocarbon portions, whereas aggregates formed by the polymer chains bridging together individual micelles as shown in Figure 4.13 (A). In the same way, we propose that PEG-based comb copolymers also could bridge together individual micelles to form secondary aggregates, also reported as compound micelles [381]. This mechanism is illustrated in Figure 4.13 (B). With this proposed mechanism, we could explain some of the phenomena observed in particle size distribution measured by DLS.

The effect of polymer molecular weight (MW)

As can be inferred from Figure 4.13 (B), longer polymer chains (higher MW) have greater capacities in bridging together individual micelles, which could be the reason why there is a greater portion of aggregates for higher MW copolymers.
**Difference between PEG-PFC and PEG-HyC copolymers**

PFC-containing side chains have stronger interactions with one another than hydrocarbon side chains, which could also issue in greater bridging abilities. Therefore, micelles formed by PEG-PFC copolymers have greater tendency to be brought together to form secondary aggregates than PEG-HyC copolymers.

**4.5 Transmission Electron Microscopy (TEM)**

TEM of PEG1500-PFC in PBS also revealed that there are two populations of particles – small micelles and secondary aggregates, as shown in Figure 4.14. Heterogeneities exist in the TEM images depending on the selection of field of view. The small micelles are represented by the small dark spots and secondary aggregates by the large regions seen as conglomerations of dark spots. Because the PFC side chains have higher electron density than PEG, the dark spots only represent the micellar core which is made up of PFC blocks, which is consistent with the observation reported in the literature [361].

The large aggregates are conglomerations of small micelles, which are also called compound micelles [291, 381, 384]. Similar to our comb-like copolymer structure, comb-like amphiphilic copolymer poly(maleic anhydride-alt-stearyl methacrylate)-b-poly(stearyl methacrylate) produced by Zhou et. al. [291] formed individual micelles and compound micelles. Furthermore, fluorinated triblock copolymers PFMA-PEG-PFMA made by Hussain et. al. [381] were also found to form micelles with the fluorinated block as the core and PEG as the corona. These micelles also coexist with “clusters” formed by individual micelles, i.e., compound micelles.
A close examination of the TEM micrograph revealed that the micellar core is approximately spherical in shape (Figure 4.15). The secondary aggregates take on various shapes, the size of which is approximated by the diameter of the conglomerated dark dots as shown in Figure 4.15. Image analysis of a collection of TEM images rendered the micelle core diameter to be 3.1 ± 0.6 nm, the histogram of which is shown in Figure 4.16. The aggregates, i.e., compound micelles, have a wide size distribution (74.6 ± 54.0 nm) with the histogram shown in Figure 4.17.

4.6 Cryogenic Transmission Electron Microscopy (Cryo-TEM)

Cryo-TEM of PEG1500-PFC in PBS confirms that there are two populations of particles – small micelles and large aggregates, as shown in Figure 4.18. Due to the fact that particles are embedded in amorphous ice, image contrast is not as high as TEM. Micellar core of PFC still appears to be dark spots in an approximately spherical shape (Figure 4.19). The aggregates appear to be darker regions containing different shades (Figure 4.20). Image analysis of a collection of Cryo-TEM images indicates that the micellar core has a diameter of 7.0 ± 0.8 nm and the aggregates have a diameter of 22.7 ± 10.5 nm, with the histograms shown in Figure 4.21 and Figure 4.22, respectively.

If we compare the results obtained by TEM and Cryo-TEM, the micellar core shown in TEM is smaller than that observed by Cryo-TEM; the aggregates seen in TEM are bigger with a wider size distribution. This is probably due to the drying process needed for TEM sample preparation, which has altered the structures in solution. Cryo-TEM, on the other hand, most likely preserved the solution structures in amorphous ice by plunge cooling the sample in liquid.
ethane. In the literature, such an advantage of cryo-TEM vs. traditional TEM has been reported for the preservation of various solution structures, e.g., viruses [385], micelles formed by block copolymers [386].

The structure of another copolymer analog (PEG900-PFC-FITC), useful in fluorescence-based biological assays, is shown in Figure 3.1 (a), in which 5% of the PFC side chains are replaced by a fluorescent dye (FITC). As shown in Figure 4.23, Cryo-TEM reveals that PEG900-PFC-FITC still forms micelles with a near-spherical micellar core. Image analysis shows that the micellar core has a diameter of $6.7 \pm 1.2$ nm with the histogram shown in Figure 4.24. This result is very close to that of micelles formed by PEG1500-PFC in PBS, implying that the replacement of FITC in 5% of the side chains did not change the micellar structure significantly. In the Cryo-TEM experiments that were performed on PEG900-PFC-FITC, we did not observe larger aggregates in the various fields of view that were obtained. We could not determine whether or not there are larger aggregates formed by this copolymer because one shortcoming of TEM techniques lies in its statistical disadvantage that it only observes a certain sampling of particles in the bulk solution, given a limited number of experiments.

Cryo-TEM with PEG-HyC copolymer solution did not observe structures because (1) these micelles are even smaller ($R_h$ of 4-5 nm) and (2) there are no electron-dense components to render high image contrast.
4.7 Small Angle Neutron Scattering (SANS)

Dynamic light scattering gives us the overall hydrodynamic radius of the micelles but not the local dimensions of the core and the shell. SANS with contrast variation can measure the local structure of the micelles. The results of the SANS measurements with PEG1500-PFC and PEG900-PFC are shown in Figure 4.25. The choice of different solvents highlights or diminishes the scattered intensity from different regions of the structure. In H2O, the scattering length density (SLD) of the PEG corona is nearly contrast matched to the solvent, and in 1:1 H2O:D2O, the SLD of the solvent is much closer to the fluorinated core.

The scattering curves of the two samples are nearly identical over the full q-range, which indicates that the structures formed by these two copolymers are very similar with only slight differences. Therefore, data analysis of one sample is representative of both. PEG900-PFC data sets were analyzed by fitting all three data sets with a global model as shown in Figure 4.26. At lower q (\(< 0.01 \text{ Å}^{-1}\)), the data shows an upturn, indicating large aggregates, with a radius of at least 20-40 nm. They cannot be quantified accurately by SANS because of their large size (Ultra SANS would be needed).

The scattered intensity from a dilute solution of aggregates can be written as: \(I(q) = n \cdot P(q)\), where \(n\) = number density of aggregates and \(P(q)\) = form factor containing details of the structure of aggregates.

The form factor used to model the aggregates is one of a spherical structure with a polydisperse core and constant shell thickness, details of which are given elsewhere [387]. The core of the micelles is assumed to contain the fluorinated side chains, while the shell (or corona) of the micelles is the hydrated hydrophilic backbone of the polymer. For this analysis, a fixed scattering contribution from a small population (\(\phi = 10^{-4}\)) of spheres 30 nm radius was
added to account for the scattering at low q (< 0.01 Å⁻¹). The presence of these clusters is corroborated by TEM images and has no impact on the structural parameters of the aggregates. To reduce the number of free parameters in the fitting, the following parameters were held fixed: all solvent SLD's, the SLD of the fluorocarbon core, and the polymer concentration as prepared. Thus, there were only four fitted parameters: core radius, core polydispersity, shell thickness, and shell SLD. The global analysis reveals a micellar structure containing a core of radius (3.1 ± 0.04) nm with a polydispersity of (0.9 ± 0.03) nm and a shell of hydrated PEG of (1.9 ± 0.04) nm in thickness. The SLD of the shell is different for each data set (different solvents) and falls between 0.80 x 10⁻⁶ Å⁻² and the SLD of the solvent. The SLD of the shell depends on the level of hydration, but no estimate of hydration has been made.

From SANS results, we confirmed that Cryo-TEM measurements represent the micelle core size more truthfully than TEM measurements.

Table 4.5 summarizes the micellar dimensions determined by different experimental methods. Measurements by Cryo-TEM, DLS, and SANS agree well with one another. We will now compare the experimental observations with copolymer molecular structure to obtain further insights into micelle morphology and aggregation number.
4.8 Study of Micelle Morphology and Aggregation Number

4.8.1 Spheroidal morphology

Given the PEG-based comb copolymer structures (Figure 2.1), the extended length \( L \) of the PFC-containing side chains is calculated from the knowledge of the C-C bond length in a fluorocarbon chain and the van der Waals radius of the terminal \(-\text{CF}_3\) group by Equations (4.1) [383]:

\[
(C\text{F}_2)_{n-1}\text{CF}_3 \\
L = 1.30 \times (n - 1) + 2.04 \text{ (angstrom)} \tag{4.1}
\]

Similarly, the extended length \( L \) of the hydrocarbon side chains can be computed from the knowledge of the C-C bond length in a hydrocarbon chain and the van der Waals radius of the terminal \(-\text{CH}_3\) group by Equation (4.2) [388]:

\[
(CH_2)_{n-1}\text{CH}_3 \\
L = 1.27 \times (n - 1) + 1.54 \text{ (angstrom)} \tag{4.2}
\]

The PFC-containing side chain is about 2.0 nm in length, which is shorter than the average core radius measured by SANS (3.1 nm). According to the nature of these soft micellar structures, it is conceivable that they can take on a spheroidal shape in solution. However, the small size of these micelles makes it difficult to ascertain their exact shape in solution. The goal of the discussion in this section is to explore whether it is reasonable for the synthesized copolymer chains to form solution structures that we have observed in experiments. The calculations utilizing the spheroidal shape serve as an example of such explorations, which by no means rules out the possibility of the micellar core assuming other morphologies.
There exists a well-established relationship [389] between the dimensions of the prolate and oblate spheroids and the hydrodynamic radius ($R_h$) measured by DLS, which is given in Equations (4.3) and (4.4):

Oblate spheroids (major axis = $a$, minor axis = $b$):

$$\lambda = \frac{b}{a} < 1$$  \hspace{1cm} \text{Equation (4.3)}

$$\beta = \frac{\cos^{-1}\lambda}{\lambda\sqrt{1 - \lambda^2}}$$

$$R_h = \frac{a}{\lambda^2 \beta}$$

Prolate spheroids (major axis = $a$, minor axis = $b$):

$$\lambda = \frac{a}{b} > 1$$  \hspace{1cm} \text{Equation (4.4)}

$$\beta = \frac{\ln(\lambda + \sqrt{\lambda^2 - 1})}{\lambda\sqrt{\lambda^2 - 1}}$$

$$R_h = \frac{a}{\lambda^2 \beta}$$

As shown in Figure 4.27, the extended length of the PFC-containing side chains is regarded to be the length of the minor axis (2.0 nm) of the micellar core. Since it is difficult to accurately estimate the hydrated PEG shell thickness utilizing available theoretical tools, we will use the shell thickness obtained from SANS (1.9 nm). Therefore, the length of the minor axis of the spheroidal micelle is 3.9 nm. Figure 4.27 also shows the calculated relationship of $R_h$ vs. the length of the major axis of oblate and prolate spheroids based on Equations (4.3) and (4.4). To obtain an $R_h$ of 8 nm, the major axis of an oblate spheroid is about 6.8 nm, rendering an aspect ratio of 1.7; whereas the major axis of a prolate spheroid needs to be 18 nm (result not shown), giving an aspect ratio of 4.6. It is obvious
that the oblate spheroidal shape is more realistic based on the molecular structure and experimental observations of the PEG-PFC copolymers.

Figure 4.28 (A) is a schematic illustration showing how the comb copolymer chains pack themselves into a spheroidal micelle. The hydrophobic side chains form a close-packed spheroidal core with a minor axis close to the extended chain length (2.0 nm) and a major axis of about 4.9 nm. The hydrophilic PEG groups are distributed in an outer shell with a shell thickness of about 1.9 nm. This is similar to the packing pattern of micelles formed by surfactant DiC₆ (1,2-dihexanoyl-sn-glycero-3-phosphorylcholine) as shown in Figure 4.28 (B) [376].

4.8.2 Micelle aggregation number

Micelle aggregation number is number of polymer chains that constitute one micelle. Since the micellar core is composed of the hydrophobic portion of the copolymers, we can estimate the aggregation number by dividing the micelle core volume with the partial specific volume of the hydrophobic portion of the copolymer.

Based on the dimensions of the oblate spheroids calculated above, we can assume that the major axis of the micellar core is \(6.8 - 1.9 = 4.9\) nm, knowing that this could be an overestimation because a dynamic method as DLS intrinsically gives larger size measurements. The micellar core volume can be thus calculated, assuming that the minor axis of the micellar core is 2.0 nm. In addition, partial specific volume \(\left( V_s \right) \) of the hydrophobic portion of PEG-PFC copolymers (Figure 4.29) can be calculated utilizing Equation (4.5) and tabulated volume increment for any atom or atomic group \(\left( V_i \right)\) [390]:
where $V_i = \text{calculated partial specific volume}$ and $V_i = \text{volume increment for any atom or atomic group}$.

We can roughly estimate the aggregation number ($n$) using Equation (4.6) with micelle core volume of 201 nm$^3$ and partial specific volume of 0.572 nm$^3$/molecule. The aggregation number is given in Figure 4.31 as a function of the polymerization number ($m$).

\[ n = \frac{4\pi}{3} \frac{a^2 b}{(V_s \times m)} \quad \text{Equation (4.6)} \]

where $n = \text{aggregation number}$, $a = \text{major axis of micelle core}$, $b = \text{minor axis of micelle core}$, $m = \text{polymerization number}$, and $V_s = \text{partial specific volume of the hydrophobic portion of copolymer}$.

A similar analysis of micelles formed by PEG900-HyC in PBS can be done assuming that the minor core radius is the extended chain length of the hydrocarbon side chains, which is about 1.2 nm calculated by Equation (4.2) [388]. As shown in Figure 4.30, the estimated major axis of micelles is about 4.7 nm, indicating that the length of the major axis of the micellar core is 2.8 nm. Therefore, the volume of the micellar core is about 39.4 nm$^3$. The partial specific volume ($V_s$) [390] of the hydrophobic portion of each repeating unit in the PEG-HyC copolymer is estimated to be 0.415 nm$^3$/molecule. Using Equation (4.6), the aggregation number ($n$) for micelles formed by PEG-HyC copolymer in PBS is given in Figure 4.31 as a function of the polymerization number ($m$).
4.9 Comparison with Similar Linear Block Copolymers

In summary, the PEG-based amphiphilic comb copolymers form "flower-like" micelles [363] in a spheroidal shape above the CMC through the association of the hydrophobic side chains. Micelles formed by the PEG-PFC copolymers have an $R_h$ of 8-9 nm, which further conglomerate to form secondary aggregates, i.e., compound micelles (Figure 4.32). Micelles formed by copolymer PEG-HyC have an $R_h$ of 4-5 nm. Very few aggregates exist in the PEG-HyC solution systems.

Because of the uniqueness of the PEG-based comb copolymers, it is difficult to find ideal analogs that have the same components and compositions for comparison. Nonetheless, it is valuable to make an effort to find the most similar analogs reported in the literature and compare the micellar properties. For example, a comparison can be made between PEG-PFC comb copolymer and PFMA-PEG-PFMA triblock copolymer [381], and between PEG-HyC comb copolymer and Tergitol® 15-S-7 diblock polymer/surfactant [382]. Table 4.6 lists their micellar properties, i.e., CMC, hydrodynamic radius ($R_h$), and aggregation number ($n$).

As can be seen from Table 4.6, CMC, micelle size, and aggregation number for the PEG-based comb copolymers are all smaller than the listed analogs. Notice that these analogs are diblock and triblock copolymers. As mentioned in chapter 2, it has been reported in the literature [292] that non-linear copolymers form micelles that have lower aggregation numbers and smaller sizes compared to linear copolymers with similar components and compositions. The reason is regarded to be the complexity of the copolymer architecture, which increases the steric hindrance of both the soluble and insoluble blocks, resulting
in fewer polymer chains forming smaller micelles. The results obtained for the PEG-based comb copolymer structures have followed the same trend. It is conceivable that because fewer chains are needed to form a micelle, the onset of micelle formation took place at a lower concentration, which is why the CMCs are lower than the listed analogs.

4.10 $^{19}$F-NMR Spectroscopy

The experiments designed in $^{19}$F-NMR spectroscopy are to (1) investigate the influence of micellization on SNR and (2) mimic biological samples in vitro to estimate the obtainable SNR. Interestingly, the second experimental design also revealed the influence of sample environment on SNR.

4.10.1 Influence of micellization

Figure 4.33 shows the $^{19}$F-NMR spectra obtained from three samples with the same total [$^{19}$F]: (a) PEG900-PFC in PBS with micelle formation, (b) PEG900-PFC in ACN with no micelle formation, and (c) a small molecule, PFD, in ACN. The single peak downfield represents the -CF$_3$ group; the multiple peaks upfield represent the -(CF$_2$)$_7$- groups. The area under the -CF$_3$ peak of all three spectra is the same within experimental error, confirming that the total [$^{19}$F] is the same. Both the spectrum of PEG900-PFC in ACN and that of PFD in ACN have sharp spectral lines, which is an indication of free molecular motion in small molecular assemblies. A close examination shows only very slight line broadening from PFD in ACN to PEG900-PFC in ACN, which indicates that the formation of copolymer at this polymerization number did not restrict molecular
motion appreciably and thus did not change its NMR properties significantly. In addition, the $^{19}\text{F}$ peak integral ratio of the -CF$_3$ group vs. the -(CF$_2$)$_7$- groups is 3:14, which matched the chemical structures of both compounds.

The spectrum of PEG900-PFC in PBS (aqueous micelle solution) has an upfield chemical shift of about 2.5 ppm due to micelle formation, which can be viewed as a solvent effect since the PFC-containing side chains are effectively dissolved in PEG in aqueous solutions. Furthermore, this spectrum manifested significant spectral line broadening, which resulted in decreased signal-to-noise ratio (SNR). This is due to restricted molecular motion of the PFC side chains in micellar structures. Furthermore, the $^{19}\text{F}$ peak integral ratio of the -CF$_3$ group vs. the -(CF$_2$)$_7$- groups is 3:10, which did not match the copolymer structure. This implies that out of the seven -(CF$_2$)$_7$- groups, two of them may have experienced severe restriction of molecular motion and thus could not contribute to the total $^{19}\text{F}$-NMR signal.

This phenomenon has been discovered in the 1970's [391, 392]. Researchers reported the same observations that molecular motion was "severely inhibited" due to micelle formation, leading to the broadening of NMR spectral lines and a decrease in the SNR.

4.10.2 Mimicking biological samples in vitro

Figure 4.34 shows the $^{19}\text{F}$-NMR spectra of two samples with small amounts of PEG900-PFC in PBS at 50 mg/mL with two different experimental set-ups. For sample (2), the signal-to-noise ratio (SNR) has been verified to follow the relationship $\text{SNR} = \text{mass} \times \sqrt{\text{scan \ #}}$. Using this relationship and assuming that the polymer concentration in biological samples could be as high as 1 mg/mL,
even if the measurement has 3600 scans, the SNR is only 1.4, which is too low to be detected by $^{19}$F-NMR.

If we compare sample (1) and sample (2) for measurements with the same scan number, after the polymer mass is compensated, the SNR of sample (1) is lower than that of sample (2), especially for the spectrum measured between the 2$^{nd}$ and 3$^{rd}$ hr after sample preparation. The spectral lines were broadened, causing the SNR to decrease. The reason lies in the diffusion of polymer into the agarose gel, which is an environment that restricts molecular motion more than a solution environment. The longer this diffusion continues, the more prominent the broadening of the spectral lines and the decrease of SNR.

4.10.3 Summary

In summary, micellization causes PEG-PFC copolymer solution to have broadened spectral lines as measured by $^{19}$F-NMR, which results in SNR reduction. Consequently, it is difficult to utilize $^{19}$F-NMR to measure biological samples loaded with PEG-PFC polymers in vitro because the SNR is too low. Furthermore, the local environment of the polymer influences its molecular motion and therefore can change the SNR significantly.
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Tables

Table 4.1 Polymerization number and MW of PEG-based Comb Copolymers

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>Polymerization Number (m)</th>
<th>MW (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG900-PFC</td>
<td>5.4</td>
<td>8391</td>
</tr>
<tr>
<td>PEG900-PFC*</td>
<td>7.5</td>
<td>11642</td>
</tr>
<tr>
<td>PEG1500-PFC</td>
<td>5.4</td>
<td>11718</td>
</tr>
<tr>
<td>PEG1500-PFC*</td>
<td>7.5</td>
<td>16262</td>
</tr>
<tr>
<td>PEG900-HyC</td>
<td>5.4</td>
<td>6426</td>
</tr>
</tbody>
</table>

Polymerization number (m) was determined by $^1$H-NMR measurements. MW is calculated by polymerization number and atomic mass based on copolymer structures shown in Figure 2.1.
Table 4.2 Hydrodynamic radius ($R_h$) of micelles formed by PEG900-PFC copolymer in PBS vs. time after sample preparation

<table>
<thead>
<tr>
<th>Days after sample preparation</th>
<th>Hydrodynamic radius ($R_h$) of micelles (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>9.4 ± 1.4</td>
</tr>
<tr>
<td>5</td>
<td>8.7 ± 0.7</td>
</tr>
<tr>
<td>8</td>
<td>9.1 ± 1.3</td>
</tr>
<tr>
<td>10</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>174</td>
<td>8.5 ± 1.8</td>
</tr>
</tbody>
</table>

$R_h$ is represented by (mean ± standard deviation). Measurements were done for the same sample, repeated 5-10 times.
Table 4.3 Hydrodynamic radius ($R_h$) of micelles vs. PEG length for PEG-PFC in PBS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hydrodynamic radius ($R_h$) of micelles (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG600-PFC</td>
<td>8.8 ± 1.3</td>
</tr>
<tr>
<td>PEG900-PFC</td>
<td>7.9 ± 0.3</td>
</tr>
<tr>
<td>PEG1500-PFC</td>
<td>8.3 ± 0.5</td>
</tr>
</tbody>
</table>

$R_h$ is represented by (mean ± standard deviation). Measurements were done for the same sample, repeated 5-10 times.
Table 4.4 Hydrodynamic radius ($R_h$) of micelles vs. polymer concentration for PEG900-PFC in PBS

<table>
<thead>
<tr>
<th>Polymer Concentration (mg/mL)</th>
<th>Hydrodynamic radius ($R_h$) of micelles (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>5.4±1.0</td>
</tr>
<tr>
<td>2</td>
<td>4.4±0.6</td>
</tr>
<tr>
<td>1</td>
<td>4.8±1.2</td>
</tr>
<tr>
<td>0.5</td>
<td>4.7±1.5</td>
</tr>
</tbody>
</table>

$R_h$ is represented by (mean ± standard deviation). Measurements were done for the same sample, repeated 5-10 times.
Table 4.5 Micelle dimensions formed by PEG-PFC copolymers in PBS determined by different characterization methods

<table>
<thead>
<tr>
<th></th>
<th>TEM</th>
<th>Cryo-TEM</th>
<th>( R_h ) (DLS)</th>
<th>SANS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micelle Radius (nm)</td>
<td></td>
<td></td>
<td>7.9 ± 0.3 (PEG900)</td>
<td>5.0 ± 0.9 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.3 ± 0.5 (PEG1500)</td>
<td></td>
</tr>
<tr>
<td>Core Radius (nm)</td>
<td>1.6 ± 0.3</td>
<td>3.5 ± 0.4</td>
<td></td>
<td>3.1 ± 0.9</td>
</tr>
</tbody>
</table>

* PEG900-PFC and PEG1500-PFC measured by SANS should have very similar dimensions with only slight differences in their local structures because the scattering curves of the two samples are nearly identical over the full q-range as shown in Figure 4.25.
Table 4.6 Comparison between PEG-PFC comb copolymer and PFMA-PEG-PFMA triblock copolymer, and between PEG-HyC comb copolymer and Tergitol<sup>®</sup> 15-S-7 diblock polymer/surfactant

<table>
<thead>
<tr>
<th>Compound</th>
<th>CMC (μM)</th>
<th>Hydrodynamic Radius</th>
<th>Aggregation Number (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$R_h$ (nm)</td>
<td></td>
</tr>
<tr>
<td>Micelles</td>
<td>Compound Micelles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG-PFC</td>
<td>2</td>
<td>8-9</td>
<td>~100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PFMA-PEG-PFMA'</td>
<td>64</td>
<td>18</td>
<td>~100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PEG-HyC</td>
<td>12</td>
<td>4-5</td>
<td>–</td>
</tr>
<tr>
<td>Tergitol&lt;sup&gt;®&lt;/sup&gt;</td>
<td>76</td>
<td>11</td>
<td>–</td>
</tr>
</tbody>
</table>

* reference [381]
# reference [382]

<sup>a</sup> on the order of 100 nm
<sup>b</sup> aggregation number is based on polymerization number of 5
Below 0.125 mg/mL, polymer solutions gave scattered light intensities at the same level as the PBS buffer solution (sample 0 mg/mL). In the concentration range of 0.125–1 mg/mL, no discontinuity was observed. Therefore, the CMC of PEG900-PFC copolymer in PBS was estimated to be below 0.125 mg/mL. Molecular weight of PEG900-PFC is about 8000 Da.
Figure 4.2 CMC determination of PEG900-PFC in PBS by surface tension

The CMC of PEG900-PFC in PBS determined by surface tension measurement is about 0.016 mg/mL, i.e., 2 μM. Molecular weight of PEG900-PFC is about 8000 Da.
Below 0.5 mg/mL, polymer solutions gave scattered light intensities at the same level as the PBS buffer solution (sample 0 mg/mL). In the concentration range of 0.5-4 mg/mL, no discontinuity was observed. Therefore, the CMC of PEG900-HyC copolymer in PBS was estimated to be lower than 0.5 mg/mL. Molecular weight of PEG900-HyC is about 6000 Da.
The CMC of PEG900-HyC in PBS determined by surface tension measurement is about 0.07 mg/mL, i.e., 12 μM. Molecular weight of PEG900-HyC is about 6000 Da.
PFMA-PEG-PFMA

Figure 4.5 Chemical structure of fluorinated triblock copolymer PFMA-PEG-PFMA

Hussain et al. [381] produced a triblock copolymer that is made of PEG 10000 ($z = 227$) as the middle block and poly perfluorohexylethyl methacrylate (PFMA) as the two end blocks ($y = 1$).
Figure 4.6 Size distribution measured by DLS vs. sample preparation methods of PEG900-PFC copolymer in PBS
Figure 4.7 Size distribution measured by DLS for PEG-PFC and PEG-PFC* copolymers with different molecular weights in PBS.

PEG-PFC copolymers (top two graphs) are made via the “Sonication” method. PEG-PFC* copolymers (bottom two graphs) are made via the “Direct” method. See Table 4.1 for MW of polymers.

The hydrodynamic radius of micelles (filled columns) is the same for different polymer MW. But the proportion of large aggregates (empty columns) increases with the increase of polymer MW, especially in the case of copolymers containing PEG1500 blocks.
Figure 4.8 Size distribution measured by DLS vs. time after sample preparation for PEG900-PFC copolymer in PBS.

The smallest size components in the graphs are artifacts caused by short noise when the signal to noise ratio in photodetection is not high.
Figure 4.9 Hydrodynamic radius of micelles vs. polymer concentration for copolymers PEG900-PFC and PEG1500-PFC in PBS
Figure 4.10 Hydrodynamic radius of micelles in the case of encapsulation of 1H, 1H, 2H, 2H-Perfluoro-1-decanol (PFD) by PEG900-PFC at mixing molar ratios of PFD:PEG900-PFC = 0, 0.5, 1, 2, 4, 8
Figure 4.11 Scattered light intensity in the case of encapsulation of 1H, 1H, 2H, 2H-Perfluoro-1-decanol (PFD) by PEG900-PFC at mixing molar ratios of PFD: PEG900-PFC = 0, 0.5, 1, 2, 4, 8
The majority of the material is in the form of small micelles (with \( R_h \) of 4-5 nm). Only very few large particles exist; in some experiments, no large particles were seen (bottom right graph). Therefore, it is difficult to measure their sizes accurately. Qualitatively, the larger aggregates have a size that is on the order of tens to hundreds of nm.
Figure 4.13 Illustration of the mechanism of secondary aggregate formation: (A) PEG-35K with end-capped fluorocarbon tails and (B) PEG-based comb-like copolymers

(A) reference: [363]
Heterogeneities exist in the TEM images depending on the selection of field of view. Two populations of particles can be seen – small micelles represented by the small dark spots and secondary aggregates by the large regions as conglomerations of dark spots.
An example of a micellar core is circled by solid line. Secondary aggregates are circled by dashed lines. Within the secondary aggregates, some dark spots seem to be bigger than others. This could be due to the nature of TEM observations – a 2D projection of 3D objects. These bigger dark spots might represent multiple micellar cores overlapping one another.

A close examination of the TEM micrograph reveals that the micellar core is approximately spherical in shape. The secondary aggregates take on various shapes, the size of which is approximated by the diameter of the regions circled by the dashed lines.
Figure 4.16 Histogram of micelle core diameter obtained in TEM (PEG1500-PFC in PBS)

Image analysis of TEM images shows that micelles formed by PEG1500-PFC in PBS have a core diameter of 3.1 ± 0.6 nm.
Image analysis of TEM images shows that aggregates formed by PEG1500-PFC in PBS have a diameter of $74.6 \pm 54.0$ nm.
Figure 4.18 Cryogenic transmission electron micrographs with PEG1500-PFC in PBS – different fields of view of the same sample grid

Black arrows: carbon frame of the lacy carbon film on sample grid
White arrows: ice crystals formed accidentally
Because sample solution is contained in the “holes” provided by the lacy carbon film on the sample grid, the solution is formed into a meniscus due to capillary effect. Therefore, most often particles are found close to the carbon frames on the edge of the carbon film holes.

*These images were obtained by Kevin Davis in Professor Frank Bates group at University of Minnesota.*
Figure 4.19 Close examination of micellar core formed by PEG1500-PFC in PBS shown by Cryo-TEM

Cryo-TEM shows that the micellar core made of PFC still appears to be dark spots in an approximately spherical shape, as marked out by the circles in the image above.

Black arrow: carbon frame of the lacey carbon film on sample grid
White arrows: ice crystals formed accidentally
Figure 4.20 Close examination of aggregates formed by PEG1500-PFC in PBS shown by Cryo-TEM

Due to the fact that particles are embedded in amorphous ice, image contrast is not as high as TEM. The aggregates appear to be darker regions containing different shades, as marked out by the circles in the image above.
Figure 4.21 Histogram of micelle core diameter obtained in Cryo-TEM (PEG1500-PFC in PBS)

Image analysis of Cryo-TEM images shows that micelles formed by PEG1500-PFC in PBS have a core diameter of 7.0 ± 0.8 nm.
Figure 4.22 Histogram of aggregate size obtained in Cryo-TEM (PEG1500-PFC in PBS)

Image analysis of Cryo-TEM images shows that aggregates formed by PEG1500-PFC in PBS have a diameter of $22.7 \pm 10.5$ nm.
Figure 4.23 Micellar core formed by PEG900-PFC-FITC in PBS shown by Cryo-TEM

An example of the micellar core formed by PFC-PFC-FITC is circled by solid line, which appears to be approximately spherical in shape.
Image analysis of Cryo-TEM images shows that micelles formed by PEG900-PFC-FITC in PBS have a core diameter of $6.7 \pm 1.2$ nm. This result is very close to that of micelles formed by PEG1500-PFC in PBS, implying that the replacement of FITC in 5% of the side chains did not change the micellar structure significantly.
Figure 4.25 SANS results with copolymers PEG1500-PFC and PEG900-PFC utilizing contrast variation method

SANS of PEG900-PFC (open symbols) and PEG1500-PFC (filled symbols) at each of the three solvent compositions: D₂O (circles), H₂O (squares), and 1:1 H₂O:D₂O (triangles). Error bars represent one standard deviation of the scattered intensity.
Figure 4.26 Model fit to SANS data sets for PEG900-PFC

SANS of PEG900-PFC at each of the three solvent compositions: D$_2$O (circles), H$_2$O (squares), and 1:1 H$_2$O:D$_2$O (triangles). The solid lines are the model fit. Error bars represent one standard deviation of the scattered intensity.

**Acknowledgements:**

The SANS work utilized facilities supported in part by the National Science Foundation under Agreement No. DMR-9986442. The mention of commercial products does not imply endorsement by NIST, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.
Figure 4.27 Calculated relationship of hydrodynamic radius $R_h$ vs. length of the major axis of oblate and prolate spheroids for micelles formed by PEG-PFC

The length of the minor axis is assumed to be 3.9 nm.

*The 3D images of spheroids are from reference [393]; used under the GNU Free Documentation License*
Figure 4.28 Illustration of comb copolymer packing scheme in micelles (A) and its similarity to the packing pattern in micelles formed by surfactant DiC₆ (1,2-dihexanoyl-sn-glycero-3-phosphorylcholine) (B)

(A) In the spheroidal micelle, the hydrophobic side chains form a close-packed spheroidal core with a minor axis close to the extended chain length (2.0 nm) and a major axis of about 4.9 nm. The hydrophilic PEG groups are distributed in an outer shell with a shell thickness of about 1.9 nm.

(B) Micelle core formed by surfactant DiC₆ is also spheroidal in shape [376], with a minor axis of 7.8 Å, which equals the extended length of the hydrophobic tail, and a major axis of 24 Å. The outer shell made of the head groups has a thickness of 10 Å along the minor axis and 6 Å along the major axis.
Figure 4.29 Estimation of aggregation number using micelle core volume and partial specific volume ($V_s$) of the hydrophobic portion of PEG-PFC copolymer

Partial specific volume ($V_s$) is calculated from reference [390]
Figure 4.30 Calculated relationship of hydrodynamic radius $R_h$ vs. length of the major axis of oblate and prolate spheroids for micelles formed by PEG-HyC.

The length of the minor axis is assumed to be 3.1 nm.

*The 3D images of spheroids are from reference [393]; used under the GNU Free Documentation License*
Figure 4.31 Estimation of micelle aggregation number as a function of polymerization number for PEG-PFC and PEG-HyC copolymers

If polymerization number $m = 5$, aggregation number $n$ is 70 for PEG-PFC and 19 for PEG-HyC.
Figure 4.32 Representation of PEG-PFC copolymers forming small micelles and compound micelles in PBS
Figure 4.33 $^{19}$F-NMR spectroscopy with (a) PEG900-PFC in PBS, 50 mg/mL; (b) PEG900-PFC in acetonitrile (ACN), 50 mg/mL; and (c) 1H, 1H, 2H, 2H-Perfluoro-1-decanol (PFD) in ACN, 14.6 mg/mL. Total [$^{19}$F] for all three samples is 0.54 M.
Figure 4.34 $^{19}$F-NMR spectroscopy with PEG900-PFC in PBS at 50 mg/mL with two different experimental set-ups, demonstrating the influence of sample environment
5 Conclusions and Recommendations

In conclusion, characterization of PEG-base amphiphilic copolymers has obtained understanding of their solution behavior and $^{19}$F-NMR response. This chapter summarizes the physical chemistry of the polymer solution for PEG-PFC and PEG-HyC copolymers and discusses the implications for their biomedical applications.

5.1 Physical Chemistry

5.1.1 PEG-PFC copolymers

PEG-PFC copolymers in solution have been found to form two populations of particles — small micelles with a hydrodynamic radius ($R_h$) of about 8-9 nm and secondary aggregates with sizes on the order of tens and hundreds of nanometers. The same phenomenon was observed for fluorinated triblock copolymer PFMA-PEG-PFMA [381].

PEG-PFC copolymers have a low CMC (about 2 $\mu$M) as a result of the strong interactions between PFC-containing side chains. The hydrodynamic radius of the micelles does not change with polymer concentration, PEG length, sample preparation method, or time after sample preparation. These micelles are near-spherical in shape, most likely spheroidal with PFC forming the core and hydrated PEG as the corona.

The secondary aggregates are most likely compound micelles. Sample preparation method, polymer molecular weight, and time after sample preparation can change the proportion of micelles vs. aggregates. There seems to
be a slow dynamic process of micelles aggregating to become large secondary aggregates.

Furthermore, due to its “perfluoroalkyl-philic” property, PEG-PFC copolymer can encapsulate a perfluorocarbon compound, 1H, 1H, 2H, 2H-Perfluoro-1-decanol.

$^{19}$F-NMR spectroscopy of PEG-PFC polymer solution has shown significant spectral line broadening and consequent SNR reduction because of micelle formation. In addition, the local environment of the sample can significantly alter the spectral characteristics (e.g., spectral line width, SNR) by influencing the molecular motion.

5.1.2 PEG-HyC copolymers

The CMC of PEG-HyC copolymer in PBS (about 12 μM) is higher than that of PEG-PFC copolymers. This is most likely due to the weaker interactions between hydrocarbon side chains compared to PFC-containing side chains. This could also be the reason why PEG-HyC solution system contains very few large aggregates formed by individual micelles bridged together by copolymer chains.

The micelles formed by PEG-HyC copolymer have an $R_s$ of about 4-5 nm that does not change with polymer concentration. These micelles could be spheroidal in shape with the hydrocarbon side chains constituting the core and hydrated PEG forming the corona/shell.
5.2 Recommendations for Biomedical Applications

Based on the findings of the physical chemistry of copolymer solutions, their potential biomedical applications are presented.

5.2.1 Delivery systems

Both PEG-PFC and PEG-HyC copolymer systems are good candidates to be developed as delivery vehicles for imaging and therapeutic agents. Their low CMC is an indication of their ability to maintain micelle integrity in situations of massive dilution (e.g., intravenous injection). The micelles formed by these polymers possess nano-scale sizes, which give them a few advantages [8]: (1) to be easily engulfed by various cells in the body; (2) to interact with biomolecules on the cell surface and within the cells without adversely affecting their normal behavior and properties; and (3) to overcome tight anatomical barriers to reach desired physiological targets, e.g., the blood-brain barrier, the pathways of the pulmonary system. Furthermore, the PEG-PFC copolymer system could be used to encapsulate insoluble perfluorocarbon compounds due to their “perfluoroalkyl-philic” property.

The present focus of the research group is to incorporate targeting moieties into the copolymer structures so that the copolymer systems are equipped to target a variety of cancer cells.

5.2.2 Magnetic resonance contrast enhancement

It has been shown that micellization of PEG-PFC copolymer causes significant $^{19}$F-NMR spectral line broadening and consequent SNR reduction. It is therefore not feasible to directly use such copolymer systems for magnetic
resonance contrast enhancement. However, if the copolymer is chemically or bio-
chemically modified so that they would release the PFC-containing side chains in
a tumorous environment by the change of pH or the presence of certain enzymes,
these copolymers can act as smart $^{19}$F-MRI probes similar to the probe reported in
[227]. The key lies in the release of the PFC-containing side chains, which renders
them the opportunity to have unconstrained molecular motion. Figure 5.1
illustrates the concept of such a smart $^{19}$F-MRI probe based on the comb-like
copolymer system. Of course, such applications depend on the delivery of probes
to the desired site of action in vivo in sufficient amounts.
Micelles formed by modified PEG-PFC copolymers

Tumorous environment

Probe activation

Release of PFC blocks

NMR signal

Figure 5.1 Illustration of the concept of a smart $^{19}$F-MRI probe based on the comb-like copolymer system

When the PFC blocks are constricted in the micellar core, they cannot render sufficient NMR signal. But when these micelles release the PFC blocks by the change of pH or the presence of certain enzymes in a tumorous environment, the PFC blocks can have non-constricted molecular motion that enables them to give amplified NMR signal. This is the activation mechanism of this smart $^{19}$F-MRI probe.
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6 Appendix I – Polymer Synthesis

6.1 Introduction

The polymer synthesis was carried out by Professor Watterson’s group at University of Massachusetts – Lowell. The following records are based on papers [2, 3] published (or to be published) by the Watterson group and the Colton group and the doctoral thesis of Rahul Tyagi.

6.2 Synthesis of PEG-PFC Copolymers

6.2.1 Synthesis of backbone polymer

Equimolar of dimethyl 5-hydroxyisophthalate (1.0 mmol) and PEG (1.0 mmol) were place in a round-bottom flask (25 mL capacity). Novozyme-435 (10% by weight w.r.t. monomer, 0.80-1.7 g) to this mixture and the reaction flask was then placed in a constant temperature oil bath maintained at 90 °C under vacuum. The reaction was allowed to proceed for 48 hrs and then quenched by adding chloroform and filtering away the enzyme under vacuum. The organic solvent was evaporated under vacuum and the residue was dialyzed using membrane (MWCO 6000). After the completion of dialysis, the obtained backbone polymer [poly (oxyethylene)-oxy-5-hydroxyisophthaloyl] was freeze-dried (Figure 6.1).
6.2.2 Synthesis of 1H,1H,2H,2H-perfluorododecyl 2-bromo acetate

1H,1H,2H,2H-perfluorodecanol (2.32 g, 5 mmol) and bromoacetic acid (2.08 g, 15 mmol) were placed in a round bottom flask. To this mixture was added Novozyme-435 (Candida antarctica lipase B immobilized, 10% by weight w.r.t. monomer) and the reaction flask was placed in a constant temperature oil bath maintained at 70 °C degree under mild vacuum. The reaction was allowed to proceed for 12 hrs. The completion of the reaction was checked by TLC using a gradient solvent system of 10% methanol in chloroform. The TLC plate was developed by dipping in alkaline KMnO₄ and heating it to visualize the spots. After completion, the reaction was quenched by adding chloroform and filtering away the enzyme. The filtrate obtained was washed with water to remove excess bromoacetic acid. The filtrate was dried over anhydrous sodium sulphate, and concentrated by removing the solvent under vacuum to obtain 1H,1H,2H,2H-perfluorododecyl 2-bromo acetate as a solid (Figure 6.2).

6.2.3 Synthesis of PEG-PFC copolymer

The synthesis of PEG-PFC copolymer (Figure 6.2) is achieved by the coupling of 1H,1H,2H,2H-perfluorododecyl bromo acetate with the backbone polymer [poly (oxyethylene)-oxy-5-hydroxyisophthaloyl]. Equimolar quantities of 1H,1H,2H,2H-perfluorododecyl bromo acetate (0.685 g, 1 mmol) and [poly (oxyethylene)-oxy-5-hydroxyisophthaloyl] (0.780 g, 1 mmol) were dissolved in dry acetonitrile and to the resultant solution was added two equivalent of anhydrous potassium carbonate. The reaction mixture was refluxed at 70 °C and the progress of the reaction was monitored by the disappearance of 1H,1H,2H,2H-perfluorododecyl bromo acetate on TLC using hexane as a solvent. After completion, the reaction was quenched by filtering away potassium carbonate.
carbonate by simple filtration and the filtrate obtained was concentrated by
purging with nitrogen gas to obtain poly (oxyethylene)-oxy-5-acetyl
1H,1H,2H,2H perfluorodecyl isophthaloyl, i.e., PEG-PFC copolymer.

6.3 Synthesis of PEG-PFC-FITC Copolymer

6.3.1 Synthesis of terpolymer

Terpolymer (compound 4 in Figure 6.3) was synthesized by the enzymatic
reaction of dimethyl 5-amino-isophthalate (compound 3, 0.05mmol), dimethyl 5-
hydroxy-isophthalate (compound 1, 0.95 mmol) and PEG900 (compound 2, 1.0
mmol). This mixture was placed in a round-bottom flask, Novozyme-435 (10
wt% wrt monomers) was added, and the reaction vial was then placed in a
constant temperature oil bath maintained at 90 °C under vacuum. The reaction
was allowed to proceed for 48 hrs, after which it was quenched by adding water
and filtering away the enzyme and any unreacted monomer (1 and 3) under
vacuum. The filtrate was dialyzed using a membrane (MWCO 6000). After the
completion of dialysis, the terpolymer was obtained as a semisolid by freeze-
drying.

6.3.2 Attachment of FITC (dye) on terpolymer

FITC (fluorescein-5-isothiocyanate) dye (compound 5, 0.067 mmol) was
added to a three-neck round bottom flask containing terpolymer (4, 1.0 mmol)
dissolved in anhydrous DMF (10 ml) under a nitrogen environment. The
resulting mixture was stirred at room temperature for four hours. Upon
completion of the reaction, DMF was removed by washing several times with excess hexane. Obtained residue was further dried under vacuum and then subjected to dialysis using membrane (MWCO 6000-8000) to get rid of unreacted FITC. The obtained compound 6 is shown in Figure 6.3.

6.3.3 Attachment of 1H,1H,2H,2H-perfluorododecyl bromo acetate

1H,1H,2H,2H-perfluorododecyl bromo acetate and FITC-attached backbone polymer were dissolved in dry acetonitrile and anhydrous potassium carbonate was then added. The reaction mixture was refluxed at 70 °C and the progress of reaction was monitored by TLC. After completion of the reaction, salts were filtered away and acetonitrile was removed to give the PEG-PFC-FITC copolymer (Figure 6.4).

6.4 Synthesis of PEG-HyC Copolymers

The backbone polymer [poly (oxyethylene)-oxy-5-hydroxyisophthaloyl] (1.0 mmol) was dissolved in anhydrous acetonitrile and anhydrous potassium carbonate (3.0 mmol) was added in it under nitrogen. To this mixture was added nonanoyl chloride (1.2 mmol) dissolved in anhydrous acetonitrile. The reaction mixture was refluxed and the progress of the reaction was monitored by TLC using ethyl acetate in petroleum ether (30%). After completion, the potassium carbonate was removed by filtration and the solvent was removed under vacuum to give the PEG-HyC copolymer (Figure 6.5).
Figure 6.1 Synthesis of backbone polymer [poly (oxyethylene)-oxy-5-hydroxyisophthaloyl] via enzymatic reaction of PEG and dimethyl 5-hydroxyisophthalate
Figure 6.2 Chemo-enzymatic synthesis of PEG-PFC Copolymer

(1): 1H,1H,2H,2H-perfluorodecanol; (2) bromoacetic acid; (3) 1H,1H,2H,2H-perfluorododecyl 2-bromo acetate; (4) poly (oxyethylene)-oxy-5-hydroxyisophthaloyl; (5) poly (oxyethylene)-oxy-5-acetyl 1H,1H,2H,2H perfluorodecyl isophthaloyl, i.e., PEG-PFC copolymer
Figure 6.3 Synthesis of terpolymer by enzymatic reaction and attachment of FITC

1: dimethyl 5-hydroxy-isophthalate; 2: PEG900; 3: dimethyl 5-amino-isophthalate; 4: terpolymer; 5: FITC dye (fluorescein-5-isothiocyanate); 6: FITC-attached terpolymer
Figure 6.4 Reaction of FITC-attached terpolymer with 1H,1H,2H,2H-perfluorododecyl bromo acetate to produce the PEG-PFC-FITC copolymer
Figure 6.5 Reaction of nonanoyl chloride with backbone polymer [poly (oxyethylene)-oxy-5-hydroxyisophthaloyl] to produce PEG-HyC copolymer.
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Appendix II – Sonication Protocol

7.1 Introduction

The instrument used for sonication is BRANSON Digital Sonifier located in Room 56-454 at MIT. This instrument is equipped with a 3 mm diameter tapered microtip (amplitude range 116-494, liquid volume 1-10 mL, part # 101-148-062). The Sonifier operation procedure and sample handling guidelines are included in this appendix.

7.2 Sonifier Operation Procedure

1. Spray 70% ethanol over KIM wipes to wipe the probe tip clean.
2. Get ice (from the autoclave room) and sit the sample into the ice to keep it cool (Figure 7.1).
3. Set the tip into the liquid of the sample (Figure 7.1) and make sure that the tip end does not touch the bottom of the tube. Otherwise, the tube could get burned.
4. Turn on the Sonifier and select time (e.g., 2 sec), power using the diode on the right side (e.g., 40%), and pulse timing (e.g., 0.5 sec on and 59.9 sec off). The time selected is the total pulse-on time.
5. Test for 2 sec if you would like to (optional). Then press start.
6. At the end, turn the Sonifier off. Take out the sample and wipe the probe clean again.
7.3 Sample Handling Guidelines

1. Samples are usually made at 1.0 mg/mL (in PBS for light scattering). The solvent is added to the polymer and sits still for about 60 min. Then the sample is taken for sonication.

2. Sonication power level is usually 40%. Pulse sequence is 0.5 sec pulse on and 59.9 sec pulse off. Total pulse-on time is 10-18 sec, depending on if there is un-dissolved polymer that is visible to the eye.

3. The sonication is done in 2-2-2-2-2… sec fashion, with 2 - 5 min of resting time between sets of 2-sec runs.

4. After the sample is dissolved by appearance, it sits still in the ice-water for a few hours and then is kept at room temperature.
Figures

Figure 7.1 Illustration of sample set-up for the sonication protocol
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8 Appendix III – CEVS System

8.1 Introduction

The controlled environment vitrification system (CEVS) is developed and used in Professor Frank Bates group at the University of Minnesota. This record of CEVS is based on the material provided by Kevin P. Davis from the Bates group.

8.2 Hardware Overview

The CEVS is made of three major units: (1) polycarbonate outer chamber (transparent) with an opening door and side ports; (2) sample loading unit, including a pair of tweezers with a locking O-ring and a stainless steel shaft that slides vertically; and (3) temperature and humidity control unit, including a cryogen reservoir, a lamp, and a temperature monitor for temperature control, and small reservoirs with sponges for chamber humidity control, a fan to achieve temperature and humidity uniformity in the chamber.

8.3 Outer Chamber

As shown in Figure 7.1 and 7.2, the CEVS chamber is made of polycarbonate. Its walls are assembled into two parts, each in a C form. The two assemblies slide together in grooves machined in the faces. The chamber can be easily opened to gain access to its interior and then sealed shut again. Side ports,
each covered with a split rubber septum, allow the entry of forceps, pipettes, and filter medium into the system.

8.4 Sample Loading Unit

The outer chamber encloses a stainless steel shaft that slides vertically. A pair of tweezers with a locking O-ring is attached to the bottom of the shaft. The tweezers holds the TEM grid upon which the sample solution for plunge freezing is applied. The shaft can be locked in the “up” position (Figure 7.3 and 7.4) or released to the “down” position (Figure 7.5 and 7.6) with a shutter control. Initial pressure to the shutter control will activate the shutter release and open the shutter. Further pressure will activate the shaft release causing the shaft/tweezers/specimen to plunge. Closing of the shutter is manual.

8.5 Temperature and Humidity Control

Uniformity of temperature and humidity is achieved with a circulating fan located behind/above the specimen (Figure 7.2).

8.5.1 Temperature control

There is a cryogen reservoir (Figure 7.2) on the back of the chamber to cool the CEVS down. Likewise, there is lamp in the back of the chamber used to increase temperature. The top wall has a port through which a temperature monitor (Figure 7.1) is inserted.

Controlling/monitoring the temperature of the CEVS is accomplished with the Dixell unit (Figure 7.7). Turning on the unit will turn on the CEVS fan.
Holding down the “Set” button for several seconds will allow the user to set the chamber temperature via the lamp.

8.5.2 Humidity control

On the back wall are mounted small reservoirs with sponges (Figure 7.2) that are used for chamber humidity control.
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Figures

Figure 8.1 Side view of CEVS chamber with side ports and the door (open)

Temperature monitor is shown.
Figure 8.2 Side view of CEVS chamber with side ports and the door (closed)

Cryogen reservoir, fan, and sponges for humidity control are shown.
Figure 8.3 Side view of CEVS with sample loading unit in the "up" position
Figure 8.4 Shutter in closed position while sample loading unit in the “up” position
Figure 8.5 Sample loading unit in the “down” position
Figure 8.6 A close look of sample loading unit in the "down" position

If sample grid is loaded, the O-ring would be at the lower tip of the tweezers to secure the sample grid.
Figure 8.7 Dixell unit to control and monitor CEVS chamber temperature
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9 Appendix IV – Polymerization Number by NMR

9.1 Introduction

Molecular weight of PEG-based copolymers is calculated by atomic mass and the polymerization number of the backbone polymer before side chain attachment. The polymerization number is determined by $^1$H-NMR spectroscopy. This record is based on material provided by Professor Watterson's group at University of Massachusetts – Lowell with special thanks to Mukesh Pandey and Rahul Tyagi.

9.2 Basic Principle

Polymerization number ($m$) is equivalent to the number of diester isophthalate (represented as Ph) as the linker. In $^1$H-NMR spectroscopy, the two $\text{–CH}_2$ groups that are directly attached to a diester isophthalate have a distinct triplet peak at chemical shift of 4.50 as shown in Figure 8.1. Because diester isophthalate in the first unit is attached to PEG only on one end, the total number of hydrogen in these groups is calculated by Equation (8.1):

$$n(H) = 4m - 2$$  \hspace{1cm}  \text{Equation (8.1)}

In addition, the end group $\text{–OCH}_3$ (MeO- in Figure 8.1) has a singlet peak at chemical shift of 3.87, which contains 3 hydrogen atoms. By integrating the area under the peaks in $^1$H-NMR, we could obtain the number ratio of “H” at different chemical shifts, i.e.,

$$\frac{n(H)}{3} = \frac{4m - 2}{3} = \frac{\text{Peak area at chemical shift of 4.50}}{\text{Peak area at chemical shift of 3.87}}$$  \hspace{1cm}  \text{Equation (8.2)}
9.3 Sample Calculation

Utilizing the $^1$H-NMR spectrum given in Figure 8.1 and Equation (8.2), we have:

$$\frac{n(H)}{3} = \frac{4m - 2}{3} = \frac{3.9}{0.6}$$

Therefore, polymerization number $m \approx 5.4$. 

Total number of "H" at chemical shift of 4.50: \( n(H) = 4m - 2 \)

- 3 "H" at chemical shift of 3.87
- Polymerization number
- Peak area is designated by the number beneath each peak at different chemical shifts.

Figure 9.1 Polymerization number determined by \(^1\)H-NMR for backbone polymer

Ph = isophthalate
Peak area is designated by the number beneath each peak at different chemical shifts.
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10 References


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