March 2015

Design, Synthesis, and Bio Relevant Applications of Zwitterionic Amphiphilic Macromolecular Assemblies

Rajasekhar Reddy Rami Reddy

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DESIGN, SYNTHESIS, AND BIO RELEVANT APPLICATIONS OF ZWITTERIONIC AMPHIPHILIC MACROMOLECULAR ASSEMBLIES

A Dissertation Presented
by
RAJASEKHAR REDDY RAMI REDDY

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 2015
Department of Chemistry
DESIGN, SYNTHESIS, AND BIO RELEVANT APPLICATIONS OF ZWITTERIONIC AMPHIPHILIC MACROMOLECULAR ASSEMBLIES

A Dissertation Presented

by

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DEDICATION

Dedicated to my sister Ms. Aruna and to my loving parents
(Mrs. Anu Radha and Mr. R. Ayyavaru Reddy)
ACKNOWLEDGMENTS

I would like to take this opportunity to express my sincere gratitude to all the people who have supported, trusted, and actively helped me make this thesis possible.

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ABSTRACT

DESIGN, SYNTHESIS, AND BIO RELEVANT APPLICATIONS OF
ZWITTERIONIC AMPHIPHILIC MACROMOLECULAR ASSEMBLIES

FEBRUARY 2015

RAJASEKHAR REDDY RAMI REDDY
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Supramolecular nanoassemblies capable of reducing non-specific interactions with biological macromolecules, such as proteins, are of great importance for various biological applications especially for targeted drug delivery therapeutics. Recently, zwitterionic materials have been shown to reduce non-specific interactions with biomolecules, owing both to their charge neutrality and their ability to form strong hydration layer around zwitterions via electrostatic interactions. This dissertation focuses on design, synthesis, thorough characterization, and applications of zwitterionic amphiphilic dendrimers and polymeric materials. Firstly, A new triazole-based zwitterionic moiety was conceived and incorporated as the hydrophilic functionality in facially amphiphilic dendrons. Self-assembly characteristics and the structural and functional characteristics of the zwitterionic dendrons were then evaluated by spectroscopic techniques and by comparison with the corresponding charge-neutral PEG and anionic carboxylate-based dendrons respectively. Surface charge measurements, temperature sensitivity and evaluation of interactions of these assemblies with proteins form the bases for these comparisons. Because of the charge neutral characteristics of zwitterionic moieties exposed to outer aqueous phase, these dendritic assemblies evade non-specific interactions with biological milieu while
enhancing the responsiveness towards specific biological triggers, such as proteins.
We further simplified this molecular design by developing synthetically accessible
zwitterionic carboxybetaine based amphiphilic polymers. Zwitterionic amphiphilic polymers are synthesized in a simple one-pot reaction by treating reactive acrylate polymer with secondary amine and 2-bromoaceticacid. This zwitterionic polymer possesses hydrophilic glycinebetaine and hydrophobic decyl chains placed orthogonally and forms environment dependent self-assembled aggregates owing to its amphiphilicity. The zwitterionic assemblies stably encapsulate guest molecules and undergo pH-dependent zeta potential and size variations owing to the pH sensitivity of zwitterionic component. In a related project, we’ve developed degradable polycarbonate based amphiphilic polymers possessing hydrophilic peg and hydrophobic decyl components. This degradable polymers form self-assembled aggregates encapsulate hydrophobic guest molecules, undergo degradation upon changes in pH or in presence of enzymes (PLE, an esterase). Work is underway towards the design and synthesis of zwitterionic amphiphilic degradable polymers and their characterization. Over all the work presented in this thesis have implications in design and development of polymeric excipients for targeted drug delivery, controlled release of drugs, and stimuli-sensitive therapeutics.
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CHAPTER 1
INTRODUCTION

1.1 Introduction

Self-assembly processes are ubiquitous in nature, wherein various components organize autonomously into patterns or structures without any human intervention. In general, molecular self-assembly involves non-covalent interactions like hydrogen bonding, induced dipole-dipole, electrostatic and Vander Waals interactions. Nature proved itself as an unbeatable master in performing the art of self-assembly; formation of double helical DNA, ion channels and lipid bilayers in biomembranes are few examples in this regard. Widespread applicability of the macromolecular self-assembly processes gathered lot of attention and are tremendously explored in the recent years. Molecular components form various self-assembled structures depending on the information coded (shape, size, surface properties, charge, and polarizability) in them. Exploiting this key feature by designing components that organize themselves into desired patterns and functions is the main application of self-assembly. In this regard, artificial macromolecules provide excellent platform to mimic biological structures and functions.

Polymers and dendrimers by virtue of their size and shape can be compared with biological components like proteins; moreover, the ease of functional group manipulation provides an excellent opportunity in fine-tuning their properties. Small molecule surfactants are well known for their self assembling properties and have been used for various applications like wetting agents, emulsifying agents and solubilizing agents etc., but their higher critical micellar concentration (CMC) and low stability in dilute conditions severely hampers their applications. Amphiphilic macromolecules overcome the above-mentioned problems by offering lower CMC
and higher stability\textsuperscript{4}. Since most of their applications involve in drug delivery, having lower CMC is very advantageous, so that dilution in blood circulation does not precipitate encapsulated drug. Using nanoassemblies of macromolecular systems as targeted drug delivery vehicles has another advantage of Enhanced Permeation and Retention effect (EPR effect), especially when drug is delivered to diseased site\textsuperscript{5}. In the light of this various types of nanoassemblies such as micelles, amphiphilic dendritic unimolecular micelles, vesicles, polymersomes, and Polymer-drug conjugates (Figure 1.1) were engineered, developed, and their bio-relevant applications were explored. Nanoparticular systems based on micelles are particularly interesting because of their ability to encapsulate hydrophobic guest molecules (Most of the drugs are hydrophobic in nature).

1.2 Micelles

Macromolecular micelles (5-100 nm) have been of great interest as a versatile nanoparticular medicine platform, especially for cancer therapeutic applications because of their ability to contain hydrophobic (poorly water-soluble)
chemotherapeutic molecules in their hydrophobic interior\textsuperscript{6-9}. Amphiphiles consisting of hydrophilic and hydrophobic components covalently linked such as small molecule surfactants or amphiphilic polymers exhibiting phase separation between hydrophobic and hydrophilic segments to form nanoscopic supramolecular core/shell structures (Figure 1.2). Its hydrophobic container like phase retains hydrophobic drugs, while the hydrophilic shell imparts solubility in aqueous media. Currently, several micellar formulations are under consideration for anticancer therapy in clinical studies, of which Genexol-PM has been FDA approved for use in patients with breast cancer. It has been demonstrated in early clinical trials that the cancer drug-polymer micelle formulation can enhance the aqueous solubility and prolong their \textit{in vivo} half-lives with minimum toxicity\textsuperscript{10}. The free doxorubicin (Dox) has an elimination phase half-life ($t_{1/2}$) of 48 min, while the Dox encapsulated micelle formulation has roughly 2.3-2.8h\textsuperscript{6}, i.e. roughly three times more than free Dox.

\textbf{Figure 1.2}: Cartoon representation of micelles.

In micellar systems the hydrophilic components are exposed to the external medium in which they are solubilized and hence, the chemical characteristics of the hydrophilic components in the amphiphilic macromolecules play an important role in how these nanoparticular systems interact with biological media. In general, the hydrophilic groups used in most of the amphiphilic molecules are either charged
(positive or negative charges) or neutral polyethylene glycols\textsuperscript{11-14} (PEG) with varying chain lengths (Figure 1.3). Even though, charged hydrophilic groups endow water solubility and provide hydrophilic-hydrophobic balance, they bring in unnecessary non-specific interactions with biological macromolecules like proteins. It could lead to potential side effects when such charged assemblies are used for drug delivery applications. On the other hand, amphiphilic assemblies based on polyethylene glycols as hydrophilic groups provide water solubility and avoid non-specific interactions, but these rather long chain functionalities could cause steric blockade for protein from accessing ligand functionalities. Moreover, polyethylene groups lose their protein resistant properties and precipitate at elevated temperatures (above 35 °C), which is attributed to their lower critical solution temperature behavior\textsuperscript{15-18}. Recently it has been shown that PEG decomposes in the presence of oxygen and transition metal ions common in most biologically relevant conditions\textsuperscript{19,20}. Thus there is a need for materials which show ultra low or no non-specific interactions and also biocompatible.

1.3 Zwitterionic Materials
The interactions that govern chemical processes may be broadly categorized into specific interactions (high activity for a certain target molecule), and nonspecific interactions (mild activity for all targets). Zwitterionic materials are charge neutral ionic systems, that possess both cationic and anionic components in close proximity rendering the entire system charge neutral (Figure 1.4). Typically the cationic moiety is a quaternary ammonium, whereas the anionic moiety may be sulfonate (Sulfobetaine) \(^{21, 22}\), carboxylate (carboxybetaine) \(^{30, 31}\) or a phosphate (phosphocholines) \(^{32, 33}\). Owing to their charge neutrality zwitterionic materials were demonstrated to possess ultra-low fouling characteristics. Recently, zwitterionic polymer coated surfaces have been shown to greatly reduce non-specific interactions with biological systems\(^{23-25}\). The synthesis and solution properties of zwitterionic polymers are very well explored\(^ {26}\). Their biological and medicinal applications are started emerging very recently because of their remarkable anti-fouling properties. In case of both PEG and zwitterionic species, charge neutrality and surface hydration is generally considered as key reasons for their resistance to non-specific protein adsorption. Zwitterionic materials can bind water molecules even more strongly via electro statically induced hydration. Short hydrophilic functionalizable groups and temperature insensitivity are advantageous features of zwitterionic materials. Overall

**Figure 1.4:** Cartoon representation of zwitterionic materials and their chemical structures.
Zwitterionic materials possess highly desirable chemical characteristics sought after in fields of biomedical engineering, tissue engineering, and medicinal fields, in the next couple of sections we detail some key developments in zwitterionic materials.

Non-specific protein interactions with synthetic materials have adverse effects in several applications such as medical devices to protein therapeutics. This problem can be either mitigated or completely eliminated by chemically modifying the surface of materials by non-fouling materials. For the past few decades PEG has been the most commonly used non-fouling material\textsuperscript{27-29}; lately its successor zwitterionic polymers are replacing PEG as non-fouling material\textsuperscript{30-36}. Both PEG and zwitterionic materials are capable of resisting protein adsorption to surfaces, but via completely different mechanisms. PEG’s water hoarding capacity sterically prevents proteins interacting with the PEG surface (Steric repulsion model)\textsuperscript{37}; protein adsorption requires conformational transitions both in protein and PEG, which are both energetically and kinetically unfavorable\textsuperscript{37, 38}. On the other hand, zwitterionic materials prevent protein adsorption described based on Hofmeister series\textsuperscript{39-41} and strong electrostatics induced hydration layer formation around zwitterions.

**Figure 1.5:** Zwitterionic polycarboxybetaine, the structural relationship between poly(carboxybetaine) and ammonium and acetate ions, both of which are protein-stabilizing ions found in the Hofmeister series; advantageous features of zwitterionic materials are highlighted on the right.
Hofmeister series is an arrangement of ionic species based on their ability to stabilize (kosmotropic agents) or destabilize (chaotropic agents) protein structures. Zwitterionic species paired with high charge density anions and low charge density cations stabilize proteins. When proteins are stable (kept from unfolding) they will not adhere to the surface in effect reducing non-specific interactions. For example, zwitterionic polymer, polycarboxybetaine consists of an acetate and ammonium group, which is one of the most stabilizing monovalent ion pairs.

1.3.1 Zwitterionic Surface Coatings and Their Ultra-Low Fouling Properties

Considering the advantages of zwitterionic materials various surface platform technologies for surface engineering applications have been developed. Specifically, several synthetic techniques have been developed to coat zwitterionic materials to variety of surfaces and has been demonstrated to sensitively detect analytes even in complex media such as human plasma and serum. Here we present some examples of zwitterionic surfaces and their ultralow fouling properties. Jiang and his team pioneered this field of research and written several articles reviewing the literature and detailing their findings. Polysulfobetaine and polycarboxybetaines have been coated on to various surfaces via several common adhesive linkers, including thiols for gold surfaces, silanes for glass surface, hydrophobic interactions for hydrophobic surfaces. These materials were then exposed to complex biological media such as proteins, blood plasma and undiluted serum. These results clearly demonstrate that zwitterionic coatings resist non-specific interactions even under harsh conditions, stressing the plausible applications of zwitterionic moieties in myriad of biological applications. The utility of zwitterionic species in the preparation of nanomaterials for drug delivery applications is relatively underexplored. There are few reports in literature that are based on zwitterionic block.
copolymers that form self-assembled nanoparticles because of the mutual immiscibility of the hydrophilic and hydrophobic blocks in bulk solvent\textsuperscript{28, 31, 36, 49, 50}.

**Figure 1.6:** SPR response for nonspecific adsorption from a) 10 and 100% human blood serum and b) 10 and 100% human blood plasma on various SAM and polymer surfaces. An adsorbed protein monomer is equivalent to 250 ng/cm\textsuperscript{2}. Reproduced from ref 2. (SAM: self-assembled monolayer; OEG: Oligo Ethylene Glycol; pSB: Polysulfobetaine; pCB: polycarboxybetaine;
1.3.2 Zwitterionic Amphiphilic Macromolecules

Here in this thesis we envisioned the possibility of engineering amphiphilic polymers utilizing zwitterionic moieties to form nanoparticular aggregates. Towards this goal, we initially came up with few molecular designs that have hydrophilic zwitterionic groups as hydrophilic groups and lengthy alkyl chains as hydrophobic groups in a polymeric amphiphiles (Chart 1.1). To minimize the complexity of the design we chose to utilize well studied facially amphiphilic polymer platform developed in our lab (vide infra). We hypothesize that, the zwitterionic amphiphiles synthesized by this method possess some unique features such as: (i) self-segregate in aqueous solutions to form nanoparticular aggregates that possess zwitterionic hydrophilic groups as surface functionalities which reduce nonspecific interactions with biological media; (ii) provide the ability to install targeting moieties; (iii) from temperature independent assemblies; (iv) enhances/facilitates specific interactions between polymeric aggregates and receptors such as proteins (Figure 1.7). In the
coming sections we outline the facially amphiphilic dendrimers and polymers and possibility to introduce zwitterionic features on to this assemblies.

1.4 Facially Amphiphilic Macromolecules

Dendrimers are tree like branched, mono-disperse polymers that can be synthesized systematically with a precise molecular weights. Many attractive characteristics of dendrimers ability to control molecular weight, attainment of globular shape with increasing generations, and the presence of multiple peripheral functional handles to decorate with ligands and or drug molecules on their periphery providing high density of ligand functionalities on the surface, which makes dendrimers suitable candidates for various biological applications. An internal hydrophobic core together with a large number of functional groups on periphery portion provides micro environmental space is suitable for host-guest chemistry and targeting capabilities (Figure 1.8). Due to their globular nature, the hydrophobic moieties are tucked in giving the opportunity to achieve a unimolecular micelle. Thus the molecule shows the micellar properties without the necessity of an aggregation,

Figure 1.7:  Pictorial representation of amphiphilic dendron or polymer in equilibrium with its aggregates; on the right the advantages features of the aggregates outlined

<table>
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<th>Advantageous features</th>
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<td>- Hydrophobic guest encapsulation</td>
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<td>- Ability to solubilize poorly soluble drugs</td>
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<tr>
<td>- Stealth properties</td>
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<tr>
<td>- Prolonged blood circulation</td>
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<td>- Functional end groups</td>
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<td>- Handle to introduce ligands</td>
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<td>- Enhanced accessibility of ligands</td>
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consequently, it exhibits lower CMC’s than that of linear polydisperse macromolecule based micelles.

1.4.1 Facially Amphiphilic Carboxylate Dendrimers

In our group we have designed and synthesized amphiphilic dendrimers that display hydrophilic and hydrophobic functionalities orthogonally because of inherent twist in biaryl core and strategic placement of these functionalities\textsuperscript{14, 51}. The molecular structure for carboxylate G3 dendrimer is shown in Figure 1.6. In this design, amphiphilic-repeating unit (AB\textsubscript{2} type monomer) possesses hydrophobic carboxylate and hydrophilic lengthy alkyl chains at orthogonal positions (Scheme 1.1). We hypothesized that such strategic orthogonal placement of the amphiphilic functionalities would facilitate for this molecules to self-segregate possessing macromolecular surfactant features i.e. these molecules would be able to form either micelle or inverted micelle type assemblies with respect to the solvent polarity\textsuperscript{52, 53} (Figure 1.3). We were gratified to learn that indeed is the case with these dendrimers from various complimentary experiments.

\textbf{Figure 1.8:} Unimolecular micelle and its components
These carboxylate dendrimers are water-soluble and were demonstrated to encapsulate hydrophobic guest molecules such as (Nile red) suggested that these dendrimers form hydrophobic interior and hydrophilic exterior in aqueous solutions. Similarly, these dendrimers are also soluble in organic solvents such as toluene in the presence of minute amounts of water to form inverse micelles and has been shown to encapsulate hydrophilic dye proflavine indicated the presence of polar microenvironment in toluene. Over all, these results indicates that dendrimers form micelle type assemblies in water and inverted micelle type assemblies in apolar solvents like toluene. DLS studies further corroborated our studies indicated the formation of stable nanoparticles in the size range of 25 to 40 nm depending on the dendrimer generation, notice that this rather large sizes are due to formation of aggregates of aggregates not due to formation of unimolecular micelles. Similar aggregation characteristics were observed for dendrimers in organic solvents i.e. for inverted micellar assemblies in toluene. The carboxylate dendritic assemblies possess anionic surface characteristics and hence are prone to non-specific interactions with biological media, hence these dendritic aggregates are not suitable for certain biological applications, such as targeted-drug delivery applications.

**Scheme 1.1**: Pictorial representation of synthesis and environment dependent self-segregation of hydrophilic and hydrophobic components in amphiphiles
With carboxylate based dendrimers the key issue is non-specific interactions, particularly if this molecular design to be studied for drug delivery applications it is a major concern. To reduce the non-specific interactions associated with carboxylate dendrons, we introduced Poly ethylene glycol (PEG) as the hydrophilic group in every repeat unit of the dendrimer backbone (Figure 1.8). As mentioned in previous sections, PEG is known to be temperature sensitive, this has led to temperature sensitive dendritic micelles that show a generation dependent lower critical solution
temperature (LCST) behavior. Upon comparison with a linear PEG based amphiphilic polymer of similar repeat unit structure (no LCST behavior observed for a polymer), a dendritic effect which is thought to be the reason for dendrimer LCST behavior. The polyethylene glycol based amphiphilic dendrimers morphology observed via Transmission Electron Microscope (TEM) studies indicates spherical micellar type structures in aqueous and organic solvents. It has also been demonstrated that, these amphiphilic peg based dendrimers reduce non-specific interactions with biological milieu such as protein solution, but their temperature sensitivity at biologically relevant temperatures could be detrimental for biological applications. These structural characteristics and synthesis of dendritic assemblies are well understood, we took advantage of this and developed zwitterionic amphiphilic dendrimers.

**Figure 1.11:** Structure of temperature sensitive PEG based amphiphilic G3-dendron
1.4.3 Facially Amphiphilic Polymers

We further expanded the facially amphiphilic molecular design with dendrimers to polymers to expand the possibilities and to reduce the synthetic complexity associated with amphiphilic dendrimers mentioned above by designing and synthesizing facially amphiphilic homopolymers based on styrenic backbone and acrylate backbone\(^{54-56}\) (scheme 1.2). We demonstrated that these amphiphilic polymers spontaneously self-assemble to form nanosized aggregates\(^{57-59}\). These polymers possess ionic carboxylate group or quaternary ammonium group as hydrophilic components. Carboxylate amphiphilic polymeric assemblies are demonstrated to interact non-specifically with biological media in a predictable manner and this feature has been exploited for pattern sensing of bioanalytes\(^{29-30}\). Surprisingly, these polymers form kinetically trapped assemblies i.e. they self organize themselves to form micellar or inverse micellar assemblies depending on the nature of the solvent and are trapped in the solvent in which they are initially solublized which has been exploited to electrostatically extract peptide analytes\(^{59-64}\). This non-specific interaction feature of amphiphilic polymers is detrimental to drug delivery applications, but the structural features of these amphiphilic homopolymers are well studied in our laboratories and hence we exploiting this molecular design for engineering zwitterionic amphiphilic polymers, which will be discussed in detail in coming chapters.

**Scheme 1.2:** Chemical structures of facially amphiphilic polymers

![Chemical structures of facially amphiphilic polymers](image-url)
1.5 Conclusions

In this chapter, we have briefly discussed certain unique structural features of charged ionic materials, peg based materials, their advantages and shortcomings in bio relevant applications. We discussed about the advantageous features of both zwitterionic materials and the utility of zwitterionic species as hydrophilic functionalities in amphiphilic macromolecules and their non-specific interaction reducing capabilities were highlighted in this chapter. We discussed about facially amphiphilic dendrimers and polymers developed in our laboratories and how this platform can be used to make zwitterionic amphiphilic molecules their plausible applications of the resulting aggregates.

In coming chapters we will discuss synthetic details about the introduction of zwitterionic features on to the amphiphilic dendrimers and amphiphilic polymers, thorough characterization of this new class of macromolecules, self-assembly characteristics of these amphiphiles in chapters 2 to 4. In chapter 3, we detail the specific protein-ligand interaction induced disassembly of zwitterionic dendritic aggregates with ensued guest release. We also present the studies of interaction of these polymeric assemblies with biological macromolecules such as proteins in chapter 2. In chapter 5, we discuss design, synthesis and characterization of new degradable polycarbonate platform developed in our group. We further investigate degradation characteristics of these polymers in the presence of various stimuli. In last chapter, we summarize our findings and expand on the future directions of this work in our laboratories. We hope the findings in this dissertation provide insights that are useful to design and synthesize materials for various biological applications such as polymer therapeutics specifically nanoscopic materials for targeted drug delivery applications.
1.6 References


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CHAPTER 2
ZWITTERIONIC MOIETIES FROM THE HUISGEN REACTION-A CASE STUDY WITH AMPHIPHILIC DENDRITIC ASSEMBLIES

2.1 Introduction

There has been significant emphasis on the development of new anti-fouling materials, which reduce non-specific interactions with biological macromolecules.\textsuperscript{1-4} These types of materials can impact a variety of applications ranging from antifouling coatings in ships\textsuperscript{5} to biomedical applications such as drug delivery,\textsuperscript{6-8} implants,\textsuperscript{9} and sensors.\textsuperscript{10} Molecules that exhibit non-fouling features can be generally divided into two major classes: polyhydrophilics and polyzwitterionics. Popular polyhydrophilics include polyethylene glycol (PEG),\textsuperscript{11-14} polysaccharides,\textsuperscript{15} and polyhydroxy materials.\textsuperscript{16-19} Besides being hydrophilic and hence the requisite water solubility, the key functional groups in these molecules are also charge neutral and present hydrogen bond donor/acceptor characteristics. PEG, the most extensively studied among the polyhydrophilics, has the advantage of being easily accessible and exhibiting good non-fouling characteristics. However, although the temperature sensitivity (often referred to as lower critical solution temperature (LCST) behavior) of the PEG moieties has been taken advantage of,\textsuperscript{20-23} the phase and hydrophilicity changes associated with this transition can be deleterious to other applications.\textsuperscript{24, 25} Also, it has been shown that PEG is susceptible to auto-oxidation at biologically relevant conditions, which converts them into reactive aldehyde moieties.\textsuperscript{26, 27} These features have resulted in search for alternate functional groups that can reliably endow non-fouling characteristics and a significant part of that focus has been on polyzwitterionics.
Zwitterionics, such as sulfobetaines,\textsuperscript{28, 29} carboxybetaines,\textsuperscript{30, 31} and phosphobetaines,\textsuperscript{32, 33} possess both positive and negative charges on the same monomer unit. These polybetaines are gaining attention as potential alternatives to PEG, due to their overall charge neutrality, high water solubility, and their ability to bind water \textit{via} electrostatics. These features have been shown to decrease the rate of adsorption of proteins, cells, and bacteria to their surfaces.\textsuperscript{4, 34, 35} In general, the non-fouling nature of zwitterionics are studied by coating them on various surfaces and then measuring the extent of adsorption of proteins or cells upon exposure to biological milieu.\textsuperscript{36} Considering the versatility of the 1,3-Huisgen cycloaddition reaction, the so-called click chemistry, we were interested in developing zwitterionic moieties that can be conveniently introduced with this reaction. In addition, the versatility of this reaction allows for high fidelity incorporation of the zwitterionic moieties on multiple surface sites on a molecular surface. Moreover, due to the continuing interest in amphiphilic supramolecular assemblies and their use in applications such as delivery and sensing, we were particularly interested in evaluating such a functional group on surfaces of assemblies in solution rather than on surfaces.

Towards this goal, we envisaged the possibility of triazole based zwitterionic functionalities and introduce them as hydrophilic groups on the surfaces

\textbf{Figure 2.1:} Cartoon representation of zwitterionic dendrimer aggregation formation and its protein-resistant properties
of assemblies based on facially amphiphilic dendrons (Figure 2.1). These dendrons are known to form micelle-like assemblies in the aqueous phase, where the hydrophilic functional groups are presented on the surface of the spherical assembly.\textsuperscript{37} Most importantly, since both polyelectrolyte and PEG-based facially amphiphilic dendrons have been shown to provide similar assemblies, this architecture provides the foundation for direct evaluation of the targeted zwitterionic assemblies through comparisons.

\textbf{Chart 2.1:} Chemical structures of the zwitterionic dendrimers

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2.2 Results and Discussion

2.2.1 Design and Synthesis

Since the key objective here is to incorporate a zwitterionic moiety through the Huisgen cycloaddition chemistry, we targeted a triazolium-based zwitterionic moiety. With the cationic part of the zwitterion in the triazolium group, we envisaged a simple introduction of the anionic moiety through the click chemistry. Accordingly, we use a functional equivalent of azidoacetic acid to react with the alkyne-containing dendrons. The targeted facially amphiphilic dendrimers are based on a biaryl repeat unit (Chart 2.1). In most other amphiphilic dendrimers, the amphiphilicity arises from the difference in hydrophilicity between the functional groups at the periphery and the dendrimer backbone. In these facially amphiphilic dendrimers, both hydrophobic and hydrophilic units are placed in every repeat unit. The zwitterionic triazolium unit will be introduced on the hydrophilic face of the dendron.

Synthesis of the zwitterionic dendron G1 began by first preparing the biaryl monomer 1 in five steps using a previously reported procedure (Scheme 2.1). Alkylation of phenolic groups with 1-bromodecane and isolation of the mono-substituted product (43% yield) resulted in incorporation of the hydrophobic decyl moiety on one of the face of the monomer unit. The resultant mono-phenol was then treated with propargyl bromide to introduce the acetylenic moiety for the click chemistry in 83% yield. The methoxymethyl ether (MOM) protecting group was then removed by treatment with a Dowex resin to afford the biaryl monomer 2 in 64% yield. This biaryl molecule is the repeating unit for the dendron. The monomer incorporated at the periphery of these dendrons does not contain the biaryl units; these are based on the simple benzyl ether dendron monomers. Reaction between the bromomethyl version of the peripheral monomer 3 and the biaryl molecule 2 yielded
the G1 dendron 4 in 74% yield. The G2 dendron was prepared similarly by reacting the bromomethyl version of 4 with the repeat monomer unit 2 (see experimental section for details).

Scheme 2.1: Synthetic scheme for the clickable zwitterionic dendrimer, G1.

These acetylene-containing dendrons were then used for the Huisgen cycloaddition reaction (click chemistry) with azidot-t-butylacetate 5 (synthesized from azido-t-bromobutylacetate and sodium azide). This reaction afforded the G1-triazole ester 6 in 71% yield. Methylation of the triazole groups, using iodomethane in acetonitrile at 80 °C for 12 h, afforded the cationic dendron containing the N-methyl-triazolium esters. The tert-butyl ester moieties were then hydrolysed using trifluoroacetic acid (TFA) to provide the targeted zwitterionic dendron G1 (Scheme 2.1). The conversion of the propargyl moiety to the triazole ester and the subsequent conversion to the zwitterionic moiety were monitored by both IR and NMR spectroscopy (Scheme 2.2). The acetylenic proton in the precursor and the triazole proton in the clicked products were used as the key diagnostic peaks in NMR. Similarly, the disappearance of the acetylenic peaks and the change in the C=O stretching frequencies were used as the diagnostic peaks in IR. A similar approach
was used for the syntheses of $\textbf{G0}$ and $\textbf{G2}$ zwitterionic dendrons, the details of which are outlined in the experimental section.

2.2.2 Self-assembly Characteristics of Dendrimers

a). IR data

b). NMR data

Scheme 2.2: Spectral representation of conversion of propargyl/decyl dendrimer to zwitterionic dendrimer a) IR spectra; b) NMR spectra

We were first interested in identifying whether these (otherwise quite hydrophobic) dendrons can be solubilized (or dispersed) in the aqueous phase. We
were gratified to find that these dendrons were indeed quite soluble in water. Next, we were interested in investigating whether these dendrons do form the amphiphilic assemblies that we have previously observed. To probe the assembly characteristics, we first utilized the possibility of hydrophobic guest encapsulation by these assemblies. We used Nile red as a fluorescent hydrophobic guest for this purpose. Nile red is not soluble in aqueous solutions. However, in the presence of the dendrons, significant amounts of Nile red is solubilized in the aqueous phase, indicating that these molecules are accommodated in the hydrophobic pockets of self-assembled aggregates (Figure 2.2a). This feature was then used to calculate the critical aggregation concentrations (CAC) of G1 and G2 dendrons (Figure 2.2b). The

Figure 2.2: Self-assembly characteristics of zwitterionic dendrimers: a) Emission spectrum of an aqueous solution of Nile red in the presence and absence of G1 and G2 dendrimers ($\lambda_{ex} = 550$ nm); (b) CAC calculation for G1 by plotting fluorescence intensity of Nile red vs concentration of G1 dendrimer ($\lambda_{ex} = 550$ nm; $\lambda_{em} = 618$ nm). (c) Size of the aggregates determined by DLS at 25-µM concentrations of G1 and G2. (d) TEM images of G1 and G2 dendrimers confirming the formation of micelle assemblies.
CACs of the G1 and G2 dendrons were calculated to be ~ 6 µM and 8 µM respectively. We observed that, the solubility of G0 is very low; hence, we did not perform any further studies with this molecule. The size and shape of these assemblies were then investigated by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The sizes of the assemblies were found to be about 20 nm and 26 nm for the G1 and G2 dendrons respectively (Figure 2.2c). Finally, TEM experiments verified the micelle-like assembly formation of these dendritic aggregates above their CAC and that the shapes of these assemblies are spherical (Figure 2.2d). Overall, the combination of experiments unambiguously suggests that the zwitterionic dendrimers form self-assembled aggregates in aqueous solutions.

2.2.3 Surface Charge Studies

Surface charge plays an important role in an assembly’s non-specific interactions with proteins. The reason then for targeting the zwitterionic moieties is that these species would result in a neutral overall surface charge. For this reason, we evaluated the zeta potential of zwitterionic dendrimer assemblies at 25 µM concentration, well above their CAC. The zeta potential of the G1 and G2 dendrons were found to be -3 mV and -2 mV respectively (Figure 2.3). This clearly demonstrates the near charge neutral nature of the zwitterionic dendritic aggregates. To get a better perspective on the magnitude of these numbers, we compared the zeta potentials of structurally similar dendritic assemblies as controls. The anionic carboxylate G1 and G2 dendrons (7 and 8, Chart 2.2) exhibited much higher negative zeta potentials of -24 mV and -40 mV. On the other hand, the charge neutral PEG-based G1 and G2 dendrons (9 and 10, Chart 2.2) exhibited the zeta potentials of -2 mV and -10 mV respectively. PEG-based assemblies exhibit slightly negative surface
charges the reason for which is not clear\textsuperscript{48,49}. These comparisons further support the charge neutral features of the zwitterionic dendritic aggregates.

### 2.2.4 Temperature Sensitivity

One among the reasons for our interest in zwitterionic functionalities as hydrophilic moieties arises from their lack of temperature sensitivity and thus the stability of the assemblies in a broader range of temperatures. To test this, we investigated whether the turbidity of the solution increases with temperatures. It is known that the PEG-based assemblies do exhibit the so-called LCST behaviour.\textsuperscript{50}

Turbidity generation in solution can be conveniently measured by measuring the anomalous scattering that arises in solution. We used the high-tension voltage, arising due to the turbidity induced scattering, in a circular dichroism spectrometer to probe the LCST behaviour of these molecules. As seen in Figure 2.4, while the PEG-based dendrons 9 and 10 exhibits significant temperature dependent turbidity, the G1 and G2 zwitterionic dendrons remained clear in solution in the entire temperature range (25-50 °C) or even at higher temperatures (Figure 2.9).

### 2.2.5 Dendrimer-Protein Interactions

![Chart 2.2: Chemical structures of G1 and G2 dendrons]
Finally, to assess the non-specific interactions of the zwitterionic dendritic assemblies, we used binding-induced disassembly as the probe. We have previously shown that non-specific interaction between amphiphilic assemblies and proteins can result in binding induced disassembly that can be read by changes in the emission properties of an encapsulated fluorophore.\textsuperscript{51, 52} Accordingly, we exposed 20 µM concentration of dendritic assemblies, containing encapsulated Nile red, with equimolar concentrations of several proteins at pH 7.4. The emission changes were monitored over a 12-hour time period (Figure 2.5). We chose three different proteins, pepsin (pI = 2.8, Mn = 30 kDa), thrombin (pI = 7.2, Mn = 36 kDa), and chymotrypsin (pI = 9.2, Mn = 25 kDa), for this study. These proteins were chosen for their divergence in surface charge; these are anionic, neutral, and cationic respectively at

\begin{figure}[h]
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\caption{Zeta potential of G1 and G2 dendritic assemblies}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig2_4.png}
\caption{HT voltage measurements for a) PEG dendrimers, b) Zwitterionic dendrimers. c) LCST behavior of PEG G1 dendrimer at 40 °C, compared to zwitterionic dendrimers.}
\end{figure}
the experimental pH 7.4. Interestingly, the zwitterionic moieties did not show any significant release of the dye molecules (<20% change in emission). However, when these were compared with the corresponding carboxylate and PEG-based dendrons, we found that these dendrons also did not exhibit any significant changes in emission intensity with time. Considering that we have shown that anionic assemblies do interact very well with chymotrypsin and modify its enzymatic properties,\textsuperscript{53} we ascribed this result to the possibility that these interactions do not result in binding-induced disassembly and thus the dye molecules are not released. To test this possibility, we used metalloproteins instead of the non-metalloproteins above.

**Figure 2.5:** Interactions of G1 and G2 dendrimers with non-metalloproteins (at equal concentrations) monitored by fluorescence intensity decrease attributed to precipitation of released dyes over time.

We have previously shown that the proximity resulting from the binding interaction between metalloproteins and polyelectrolyte assemblies is sufficient to quench the emission of an encapsulated fluorophore.\textsuperscript{54} In this case, the binding-induced disassembly process is not required. The emission quenching should directly report the binding interaction between the proteins and the amphiphilic assemblies. The fluorescence quenching here would be due to the energy or electron transfer to
the cofactor functional groups in the proteins. We chose three metalloproteins, cytochrome C (Cyt C, pI = 10.2, Mn = 12 kDa), myoglobin (Myo, pI = 6.8, Mn = 18 kDa), and hemoglobin (Hb, pI = 7.2, Mn = 68 kDa). We were gratified to find that indeed the zwitterionic dendrons did not exhibit any appreciable fluorescence quenching with Cyt C and Myo (Figure 7). However, emission quenching was observed with Hb. The value of these results were further tested by comparing them with the charge neutral PEG and the anionic carboxylate dendritic assemblies using these three metalloproteins. Interestingly, the fluorophore in the PEG-based dendrons were also not quenched by both Cyt C and Myo, but these also exhibited quenching with Hb. However, in the case of assemblies based on the anionic dendrons, all three metalloproteins strongly quenched the emission of the encapsulated fluorophore. This

Figure 2.6: Interactions of G1 and G2 dendrimers with metalloproteins (at equal concentrations) monitored by fluorescence intensity decrease attributed to quenching by metalloproteins over time.

is attributed to the anticipated non-specific interactions between the polyelectrolyte assemblies and proteins. The reasons for the non-specific quenching with hemoglobin in the zwitterionic and the PEG based assemblies are not clear to us at this time.
Overall, the triazolium carboxylate based zwitterionic dendritic assemblies behave very similar to that of the popular PEG-based assemblies in their interactions with proteins.

2.3 Summary

In summary, we have designed and developed a clickable zwitterionic functional group, which has been tested for its synthetic and functional utility using the facially amphiphilic dendritic scaffold. We have shown that: (i) these dendrons based on the triazolium carboxylate zwitterionic moieties can indeed be solubilized in the aqueous phase; (ii) these dendrons form self-assembled aggregates in aqueous solutions with guest encapsulation capabilities; (iii) these assemblies are charge neutral, as confirmed by zeta potential measurements; (iv) these assemblies are stable aggregates over a wide temperature range and do not exhibit any LCST behavior, providing a promising alternative to PEG-based systems; (iv) the non-specific interactions of these zwitterionic assemblies are comparable to those from PEG-based assemblies. Although our initial demonstration has been with triazolium carboxylate, the design can be easily extended to a variety of other zwitterionic combinations and for a variety of polymeric scaffolds. Since biofouling is a major hurdle in multiple areas, the findings here will not only be useful in applications such as drug delivery and surface coatings, but also in areas such as controlled multivalent presentation of ligands for interactions with biological macromolecules.

2.4 Experimental Section

Materials and Methods

All chemicals and solvents were purchased from commercial sources and were used as such, unless otherwise mentioned. $^1$H NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer using the residual proton resonance of the solvent as
the internal standard. Chemical shifts are reported in parts per million (ppm). When peak multiplicities are given, the following abbreviations are used: s, singlet; bs, broad singlet; d, doublet; t, triplet; m, multiplet. $^{13}$C NMR spectra were proton decoupled and recorded on a 100 MHz Bruker spectrometer using carbon signal of the deuterated solvent as the internal standard.

2.4.1 Synthetic protocols for preparation of Zwitterionic dendrimers

Synthesis of $\alpha$-Azido $\text{t}$butyl ester, 12

A solution of NaN$_3$ (0.733 g, 11.27 mmol) and bromo compound 11 (2.0 g, 10.25 mmol) in DMSO (10 mL) was stirred at room temperature for 12 h. 70 mL water was added to quench the reaction and the water layer was extracted with 3 x 50 mL of diethyl ether. The combined organic layers were washed with 2 x 50 mL brine and dried over Na$_2$SO$_4$. The organic solvent was evaporated to dryness and the crude product 12 (1.51 g, 92 %) was used for next step without further purification, hence no further analysis was performed.

Syntheses of G0, G1, and G2 dendrimers
General procedure for the synthesis of propargyl G1-OH (4) and G2-OH (17) dendrimers

To a solution of biaryl monomer 2 (1.0 eq) and appropriate bromobenzyl compound (2-3 eq) in dry acetone, was added K$_2$CO$_3$ (3 eq) and 18-crown-6 (0.2 eq). The reaction mixture was refluxed under argon atmosphere for 12-24 h (12h for G1 and 24h for G2). The progress of the reaction was monitored by TLC. After completion of the reaction, acetone was evaporated and the crude reaction mixture was partitioned between ethyl acetate and water. The aqueous layer was extracted twice with ethyl acetate and the combined organic layer was dried over Na$_2$SO$_4$ and evaporated to dryness. The crude product was purified by silica gel column chromatography.

General procedure for click reaction

The mixture of dendritic acetylene compound (1.0 eq), azide, 12 (2.5 eq for 1 acetylene group), CuSO$_4$. 5H$_2$O (0.5 equiv.) and sodium ascorbate (0.5 eq.) in DMSO solvent mixture was heated at 50 °C for 12-36 h (12h for G0, 24h for G1, 36h for G2). The reaction progress was monitored by TLC. After completion of the reaction, the reaction mixture was partitioned between ethyl acetate and saturated aqueous NH$_4$Cl solution. The aqueous layer was extracted twice with ethyl acetate and the combined organic layer was dried over Na$_2$SO$_4$ and evaporated to dryness. The crude product was purified by silica gel column chromatography.

General procedure for N-methylation of triazole dendrimers

A solution of dendritic triazole compound (1.0 eq), Iodomethane (10 eq for 1 triazole ring) in dry acetonitrile was heated in a sealed tube at 60 °C for 12-24 h (12 h for G0, 24 h for G1 & G2. All volatile compounds were removed under vacuum with a rotary evaporater to give crude dendritic triazolium adduct, which then washed with
ethyl ether (3 x 10 mL), dried under vacuum afford the crude compound, which was used for next step without further purification.

**General procedure for hydrolysis reaction of N-methyl-triazolium ester dendrimers**

To a solution of n-methyl-triazolium ester dendrons (1.0 eq) in anhydrous dichloromethane was added TFA. The reaction mixture was allowed to stir for 12–36 h (12 h for G0, 24 h for G1 and 36 h for G2). The solvent was evaporated using rotary evaporator to afford the crude zwitterionic dendron, which was further purified by using reverse phase column chromatography.

**Synthesis of compound 14**

According to general procedure for click reaction, compound 13 (0.5 g, 1.57 mmol) was treated with azide 12 (0.492 g, 3.14 mmol) to give 0.605 g (81%) of 14.

$^1$H-NMR (400 MHz; CDCl$_3$): $\delta$ 9.91 (s, 1H), 7.79 (s, 1H), 7.07 (d, $J = 6.3$ Hz, 2H), 6.81 (s, 1H), 5.28 (s, 2H), 5.10 (s, 2H), 3.99 (t, $J = 6.6$ Hz, 2H), 1.80 (dt, $J = 14.5, 7.0$ Hz, 2H), 1.50-1.29 (m, 23H), 0.90 (t, $J = 6.9$ Hz, 3H). $^{13}$C-NMR (100 MHz; CDCl$_3$): $\delta$ 191.8, 165.1, 160.8, 159.7, 138.3, 124.1, 108.2, 108.1, 107.9, 84.0, 68.5, 62.2, 51.6, 31.9, 29.6, 29.3, 29.4, 29.1, 27.9, 26.0, 22.7, 14.1. HRMS (FAB+) calculated for C$_{26}$H$_{41}$N$_3$O$_5$ [M+H$^+$]: 476.62 found 476.30. HRMS (FAB+) calculated for C$_{26}$H$_{41}$N$_3$O$_5$ [M+H$^+$]: 476.62 found 476.30.

**Synthesis of compound 15**

According to general procedure for N-methylation of triazole, compound 14 (0.5 g, 1.05 mmol) was treated with Iodomethane (0.65 mL, 10.51 mmol) to yield 0.38 g of
compound 15. This compound is used as such for next reaction; hence no further analysis was performed for this molecule.

Synthesis of zwitterionic molecule, G0

According to the general procedure for hydrolysis reaction of t-butyl ester, G0 triazolium compound 15 (0.38 g, 0.77 mmol) was treated with TFA (1.2 mL) to yield 0.255 g of zwitterionic Dendron G0 (57 % for 2 steps). ESI-MS calculated for C_{23}H_{35}N_{3}O_{5} 433.54, found 433.26. ^1H-NMR (400 MHz; MeOD): δ 8.78 (s, 1H), 6.68-6.56 (m, 3H), 5.43 (s, 2H), 5.20 (s, 2H), 4.58 (s, 2H), 4.38 (s, 3H), 3.99 (t, J = 6.4 Hz, 2H), 1.80-1.76 (m, 2H), 1.49-1.30 (m, 14H), 0.92 (t, J = 6.9 Hz, 3H); ^13C-NMR (100 MHz; MeOD): δ 160.6, 158.4, 144.5, 139.5, 137.4, 130.5, 106.3, 104.7, 100.3, 67.7, 63.5, 57.9, 55.7, 37.3, 31.6, 29.3, 29.2, 29.1, 29.0, 28.9, 25.8, 22.3, 13.0. ESI-MS calculated for C_{23}H_{35}N_{3}O_{5} 433.54, found 433.26.

Synthesis of G0-Br

To a solution of 13 (1.8 g, 5.65 mmol) in dichloromethane (20 mL) was added PBr_{3} (1 mL, 11.31 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 12h. The reaction was quenched with aqueous NaHCO_{3} solution at 0 °C. The aqueous layer was extracted with dichloromethane 3 x 50 mL; the combined organic layers
were washed with water, brine and dried over Na$_2$SO$_4$. The evaporation of the solvent afforded crude product, which was further purified by using silica gel column chromatography to give G0-Br (1.43 g, 67 %). $^1$H-NMR (400 MHz; CDCl$_3$): δ 6.60 (dt, $J = 3.8$, 1.7 Hz, 1H), 6.48 (t, $J = 2.3$ Hz, 1H), 4.69 (d, $J = 2.4$ Hz, 1H), 4.44 (s, 1H), 3.95 (t, $J = 6.6$ Hz, 1H), 2.56 (t, $J = 2.4$ Hz, 1H), 1.82-1.75 (m, 1H), 1.46-1.30 (m, 1H), 0.91 (t, $J = 6.9$ Hz, 1H). $^{13}$C-NMR (100 MHz; CDCl$_3$): δ 160.4, 158.7, 139.7, 108.5, 107.6, 101.9, 75.7, 68.2, 55.9, 33.5, 31.9, 29.6, 29.4, 29.3, 29.2, 26.0, 22.7, 14.1; HRMS (FAB+) calculated for C$_{20}$H$_{29}$BrO$_2$ 381.35, found 381.14.

Synthesis of compound 4

According to the general procedure for synthesis of biaryl hydroxymethyl compound, 2 (0.6 g, 1.40 mmol) was reacted with G0-Br (1.18 g, 3.09 mmol) to afford 4 (1.06 g, 74%). $^1$H-NMR (400 MHz; CDCl$_3$): δ 6.80-6.59 (m, 9H), 6.49 (t, $J = 2.3$ Hz, 2H), 4.99 (s, 4H), 4.74 (d, $J = 5.9$ Hz, 2H), 4.69 (d, $J = 2.4$ Hz, 4H), 4.60 (d, $J = 2.4$ Hz, 2H), 3.94 (dt, $J = 12.3$, 6.3 Hz, 6H), 2.52 (t, $J = 2.4$ Hz, 2H), 2.47 (t, $J = 2.4$ Hz, 1H), 1.82-1.61 (m, 8H), 1.50-1.23 (m, 42H), 0.92-0.86 (m, 9H). $^{13}$C-NMR (75 MHz; CDCl$_3$): δ 160.4, 159.0, 158.7, 157.4, 155.6, 141.7, 139.5, 135.5, 119.8, 110.2, 106.7, 105.7, 104.8, 104.6, 101.2, 101.1, 78.9, 78.4, 75.6, 75.4, 69.8, 68.8, 68.1, 65.5, 56.3,
55.8, 31.9, 29.6, 29.5, 29.5, 29.3, 29.2, 29.2, 29.0, 26.0, 26.0, 22.6, 14.1; MALDI-ToF m/z calculated for C_{66}H_{90}O_{9} [M+Na^+]: 1050.42 found, 1050.34.

Synthesis of compound 6

According to general procedure for click reaction, compound 4 (0.3 g, 0.292 mmol) was treated with azide 12 (0.229 g, 1.46 mmol) to give 0.310 g (71%) of 6. \(^1\)H-NMR (400 MHz; CDCl\(_3\)): δ 7.72 (s, 2H), 7.35 (s, 1H), 6.77-6.48 (m, 12H), 5.21-4.94 (m, 17H), 4.70 (d, J = 4.7 Hz, 2H), 3.92 (q, J = 6.6 Hz, 6H), 1.77 (quintet, J = 6.9 Hz, 4H), 1.45-1.21 (m, 72H), 0.89 (dt, J = 8.2, 4.8 Hz, 9H). \(^13\)C-NMR (100 MHz; MeOD): δ 168.7, 168.6, 163.9, 162.9, 162.4, 160.7, 159.6, 148.1, 147.8, 145.7, 143.0, 139.4, 127.6, 127.6, 123.1, 113.7, 109.8, 109.2, 108.7, 108.3, 105.3, 104.5, 104.3, 97.1, 88.9, 87.3, 87.2, 83.9, 83.9, 82.8, 82.6, 81.9, 81.8, 81.5, 81.0, 80.3, 79.8, 79.7, 79.4, 78.6, 78.5, 77.4, 77.4, 73.3, 72.2, 71.6, 68.8, 67.5, 66.6, 65.4, 64.2, 61.9, 56.1, 55.0, 54.8, 35.3, 33.0, 32.8, 32.7, 32.7, 32.5, 31.4, 31.3, 29.5, 29.4, 26.1, 21.9, 17.5.

MALDI-ToF m/z calculated for C_{88}H_{123}N_{9}O_{15} [M+H^+]: 1498.93 found, 1499.89.

Synthesis of compound 16
According to general procedure for N-methylation of triazole, compound 6 (0.43 g, 0.29 mmol) was treated with Iodomethane (0.55 mL, 8.6 mmol) to yield 0.385 g of crude compound 16. This crude product 16 is used as such for next reaction without any further analysis.

Synthesis of zwitterionic Dendron G1

According to the general procedure for hydrolysis reaction of t-butyl ester, G1 trizonium compound 16 (0.385 g, 0.25 mmol) was treated with TFA (0.2 mL) in dichloromethane (8 mL) to yield 0.17 9 g of zwitterionic Dendron G1 (48% for 2 steps). $^1$H-NMR (400 MHz; MeOD): $\delta$ 8.81 (s, 2H), 8.43 (s, 1H), 6.91 (s, 1H), 6.83 (s, 1H), 6.91 (s, 1H), 6.83 (s,
1H), 6.78 (s, 2H), 6.72 (s, 2H), 6.64 (t, \( J = 2.2 \) Hz, 1H), 6.61 (t, \( J = 2.2 \) Hz, 2H), 6.46 (d, \( J = 2.1 \) Hz, 2H), 5.45 (s, 4H), 5.27 (s, 2H), 5.22 (s, 4H), 5.08 (s, 2H), 5.04 (s, 4H), 4.67 (s, 2H), 4.37 (s, 6H), 3.98 (t, \( J = 6.36 \) Hz, 4H), 3.90 (t, \( J = 6.08 \) Hz, 2H), 3.81 (s, 3H), 1.81-1.74 (m, 4H), 1.57-1.45 (m, 4H), 1.59-1.19 (m, 40H), 0.94-0.84 (m, 9H). 13-C NMR (100 MHz; MeOD): \( \delta \) 168.6, 164.0, 162.9, 162.4, 148.2, 147.8, 143.1, 127.6, 113.7, 109.9, 109.2, 105.3, 104.3, 87.3, 87.2, 84.0, 83.9, 82.8, 82.6, 81.9, 81.8, 81.5, 81.1, 79.8, 78.7, 78.6, 77.4, 77.4, 73.3, 72.3, 71.6, 68.8, 67.5, 65.4, 64.2, 61.9, 56.1, 55.0, 35.3, 33.0, 32.9, 32.8, 32.7, 32.5, 31.4, 31.3, 29.5, 29.4, 26.1, 21.9, 17.5.

MALDI-TOF m/z calculated for C\(_{75}\)H\(_{106}\)N\(_9\)O\(_{15}\) [M+H]: 1373.69; found 1374.84.

Synthesis of G1-Br

To a solution of carbon tetrabromide (0.23 g, 0.877 mmol) and compound 4 (0.6 g, 0.585 mmol) in dichloromethane (30 mL) was added PPh\(_3\) (0.291 g, 0.877 mmol) at 0 °C. After stir 12 h at room temperature, water was added to quench the reaction. Aqueous layer was separated and the organic layer was washed with excess of water. The organic layer was dried over Na\(_2\)SO\(_4\), concentrated using rotary evaporator to give crude product, which was purified by using silicagel column chromatography to afford G1-Br (0.51 g, 79%) \(^1\)H-NMR (400 MHz; CDCl\(_3\)): \( \delta \) 6.82-6.49 (m, 11H), 4.99 (s, 4H), 4.69 (d, \( J = 2.4 \) Hz, 4H), 4.60 (d, \( J = 2.4 \) Hz, 2H), 4.53 (s,
2H), 3.94 (dt, J = 14.3, 6.9 Hz, 6H), 2.50 (dt, J = 12.1, 2.4 Hz, 3H), 1.82-1.61 (m, 6H), 1.50-1.23 (m, 43H), 0.92-0.86 (m, 9H). $^{13}$C-NMR (75 MHz; CDCl$_3$): δ 160.4, 159.0, 158.7, 157.3, 155.5, 139.5, 138.1, 135.1, 120.7, 110.1, 107.1, 107.0, 106.7, 105.7, 101.4, 101.1, 78.7, 78.4, 75.7, 75.6, 69.8, 68.8, 68.1, 56.4, 55.8, 33.9, 31.9, 29.6, 29.4, 29.3, 29.2, 29.2, 29.0, 26.0, 26.0, 22.7, 14.1. MALDI-ToF m/z calculated for C$_{66}$H$_{89}$BrO$_8$[M+Na$^+$]: 1113.31 found, 1113.43.

Synthesis of compound 17

According to the general procedure for synthesis of dendritic hydroxymethyl compound, 2 (0.055 g, 0.131 mmol) was reacted with G1-Br (0.3 g, 0.275 mmol) to afford 17 (0.282 g, 89%). $^1$H-NMR (400 MHz; CDCl$_3$): δ 6.88-6.48 (m, 28H), 5.07 (s, 4H), 4.98 (s, 7H), 4.76-4.59 (m, 16H), 3.94 (dt, J = 4.5, 6.4 Hz, 13H), 2.52-2.41 (m, 7H), 1.82-1.62 (m, 15H), 1.48-1.22 (m, 98H), 0.88 (dt, J = 12.2, 6.9 Hz, 21H). $^{13}$C-NMR (75 MHz; CDCl$_3$): δ 162.7, 158.0, 157.0, 156.0, 156.5, 154.8, 142.2, 141.8, 137.1, 135.7, 133.3, 121.7, 121.6, 117.4, 107.7, 106.3, 103.9, 103.3, 98.3, 87.9, 85.3, 82.7, 81.4, 81.1, 81.1, 79.0, 75.8, 73.7, 72.6, 70.6, 68.5, 67.3, 66.9, 66.4, 65.6, 64.2,
Synthesis of compound 11

According to general procedure for click reaction, compound 17 (0.5 g, 0.204 mmol) was treated with azide, 12 (0.482 g, 3.0 mmol) to give 0.53 g (73%) of 18. $^1$H-NMR (400 MHz; CDCl$_3$): $\delta$ 7.73 (s, 3H), 7.44 (s, 1H), 7.35 (s, 1H), 6.90-6.49 (m, 26H), 5.22-4.90 (m, 36H), 4.12 (dq, $J = 19.6, 6.8$ Hz, 7H), 3.94 (t, $J = 6.5$ Hz, 13H), 2.07 (s, 9H), 1.76 (dd, $J = 14.4, 6.8$ Hz, 8H), 1.49-1.26 (m, 131H), 0.96-0.84 (m, 21H). $^{13}$C-NMR (100 MHz; CDCl$_3$): $\delta$ 160.4, 159.1, 159.0, 158.7, 157.3, 155.6, 155.5, 139.5, 137.8, 135.5, 120.1, 110.2, 106.7, 105.7, 105.6, 101.1, 98.5, 96.8, 93.6, 90.3, 81.3, 80.5, 78.8, 78.4, 78.3, 78.0, 77.5, 76.4, 76.2, 76.1, 76.1, 75.6, 75.6, 75.5, 75.5, 74.7, 73.9, 69.8, 68.8, 68.1, 65.4, 64.0, 60.8, 58.4, 57.5, 56.4, 55.9, 55.8, 51.8, 29.6, 29.5, 29.5, 29.4, 29.3, 29.2, 29.2, 29.1, 26.0, 22.6, 18.4, 14.1. MALDI-ToF m/z calculated for C$_{200}$H$_{287}$N$_{21}$O$_{35}$ [M+H$^+$]: 3545.54 found, 3548.91.
Synthesis of compound 19

According to general procedure for N-methylation of triazole, compound 18 (0.5 g, 0.14 mmol) was treated with Iodomethane (3 mL, 21.1 mmol) in acetonitrile (8 mL) to yield 0.7 g of crude compound 19. This crude product is used as such for next step; hence no further analysis was performed on this molecule.

Synthesis of zwitterionic dendrimer G2
According to the general procedure for hydrolysis reaction of t-butyl ester, G2 triazolium compound 9 (0.7 g, 0.20 mmol) was treated with TFA (2 mL) in dichloromethane (20 mL) to yield 0.087 g of zwitterionic Dendron G2 (19% for 2 steps). \(^1\)H-NMR (300 MHz; MeOD): \(\delta 8.83\) (s, 4H), \(8.44\) (d, \(J = 0.1\) Hz, 3H), 6.92-6.45 (m, 25H), 5.23 (m, 37H), 4.67 (s, 4H), 4.38 (s, 12H), 4.01-3.81 (m, 22H), 1.81-1.20 (m, 111H), 0.94-0.87 (m, 23H). \(^{13}\)C-NMR (100 MHz; MeOD): \(\delta 176.2, 176.2, 167.9, 167.4, 167.4, 160.7, 159.1, 158.5, 157.2, 154.9, 143.6, 143.4, 140.1, 139.5, 139.0, 136.1, 132.1, 131.0, 130.6, 130.5, 129.5, 129.4, 128.4, 120.5, 110.1, 107.2, 106.1, 105.9, 105.5, 100.7, 100.7, 72.2, 70.1, 70.0, 69.3, 69.3, 68.5, 67.9, 67.7, 63.5, 63.5, 60.7, 59.8, 59.7, 58.1, 55.2, 38.7, 37.6, 37.1, 33.5, 31.6, 30.2, 29.4, 29.3, 29.3, 29.3, 29.1, 29.1, 29.1, 29.0, 28.8, 28.7, 25.8, 25.8, 25.7, 24.7, 23.5, 22.6, 22.4, 22.3, 22.3, 13.1, 13.1, 13.0, 13.0, 10.0. +ToF MS, \(m/z\) calculated for C\(_{200}\)H\(_{287}\)N\(_{21}\)O\(_{35}\) \([\text{M}^+ + \text{H}_2\text{O}]\): 3268.98 found, 3268.90.

Dynamic Light-Scattering (DLS) experiments

The size distribution of the micelles was determined by Nano series Nano-ZS (Malvern Instrument) Zetasizer. In a typical experiment a stock solution of 25-µM dendrimer was prepared in milli-Q water by sonicating the solution for 2 hours at room temperature. The resultant solution was then filtered through 0.22-µm filters and then the size of the micellar solutions were measured by DLS.

Spectroscopic measurements

Fluorescence emission spectra were recorded on a JASCO (FP-6500) spectrofluorimeter, using 1 mL disposable fluorescence cuvettes. The emission spectra for Nile red were recorded by exciting at 550 nm, with the excitation and emission bandwidth set at 3 and 5 nm respectively.

Determination of critical aggregation constants (CAC)
CACs of the dendrimers were determined by using the emission spectrum of encapsulated hydrophobic guest molecule, Nile red. Excess amount of Nile red was added to the 100 μM stock solutions of G1 and G2 dendrimers in water and these solutions were sonicated at room temperature for 2 h to encapsulate Nile red. The solutions were then filtered into a cuvette and emission spectra were recorded. Fluorescence emission intensity of Nile red was monitored at different dendrimer concentrations. The emission intensity was then plotted against the concentrations of the dendrimers and the CACs were determined to be the inflection point observed in such a plot.

Figure 2.7: CAC calculation for G2 zwitterionic dendrimer
Dye release studies

25 μM solutions of Nile red containing G1 and G2 dendrons were exposed with various proteins, and fluorescence intensity changes of these solutions were monitored at different time intervals. Change in the emission intensity of Nile red was used to calculate the percentage dye release by the following equation.

\[
\% \text{ Dye release} = \frac{I_0 - I}{I_0} \times 100
\]

where \( I_0 \) is the initial emission intensity, \( I \) – emission intensity after adding protein.

TEM measurements were performed using a TEM JEOL 2000F. Samples were prepared by dipping copper EM grids in aqueous solution of 100 μM dendrimer solutions and the excess of solvent was removed by placing the tissue in the bottom of the EM grid.

IR Spectra of G2 Zwitterionic dendrimer and its precursors

![Image of IR Spectra](image)

**Figure 2.8:** IR spectral representation of transformation of G2 propargyl dendrimer, 17 to zwitterionic dendrimer, G2.
NMR spectra for the final zwitterionic dendrimers were presented in the next few pages.

Figure 2.9: HT voltage measurements for PEG dendrimers (left), Zwitterionic dendrimers (right) at higher temperatures.
NMR structures of dendrimers
NMR structures of dendrimers
2.5 References


CHAPTER 3
PROTEIN BINDING INDUCED DISASSEMBLY OF ZWITTERIONIC
DENDRITIC NANOASSEMBLIES

3.1 Introduction

Amphiphilic macromolecules capable of responding to environmental cues i.e, stimuli responsive properties are highly promising materials, because of their ability to retain drug molecules until they are exposed to a specific trigger, for drug delivery applications. However, most of the well studied systems are based on pH\textsuperscript{1}, temperature\textsuperscript{2-7}, and light\textsuperscript{8-10}. Designing macromolecules that respond to biological stimuli such as proteins are of particularly high significance because it opens up the possibility to utilize over-expressed proteins at diseased cell sites as triggers to release drug payloads. Macromolecular assemblies that respond to non-enzymatic actions such as protein-ligand binding interactions are effective because (i) they will be broadly applicable for both enzymatic and non-enzymatic proteins; (ii) overexpression of non-enzymatic proteins are highly relevant to a variety of diseases.

In this chapter, we discuss a new strategy that has been developed to disassemble the amphiphilic assemblies of our dendritic system using protein-ligand binding interactions.

Dendrimer micellar assemblies are interesting for this purpose for two main reasons; (i) their ability to exhibit lower Critical Aggregation Concentrations (CACs)\textsuperscript{2, 11-13} that polymeric micellar assemblies provide, while offering the molecular level information that small molecule surfactants can provide, (ii) systematic control over the size and number of functional groups by dendrimer generation provides an opportunity for differential release rate. In this case, we have utilized our facially amphiphilic biaryl dendrimers that form micelle-like and inverse micelle-like structures in polar and apolar solvents, respectively.\textsuperscript{2, 13} It is also
important to remember that the micellar assemblies of these dendrimers have been shown to sequester hydrophobic guest molecules in their interiors. Since the hydrophilic-lipophilic balance (HLB) dictates the formation of micellar assemblies, perturbing the HLB would result in the disassembly of micellar assemblies. In contrast to classical amphiphilic dendrimers, our facially amphiphilic dendrimers form micellar assemblies by the aggregation of several dendritic molecules due to the orthogonal placement of hydrophilic and lipophilic units in every repeat unit of the dendrimer. Recently, we have developed a new class of amphiphilic dendrimers by incorporating zwitterionic components as hydrophilic groups. Taking advantage of this special characteristic of our dendrimers, we envisaged the possibility of disassembling the micellar assemblies by the HLB disturbed disaggregation. In this chapter, we utilize recently developed zwitterionic dendrimers for protein-ligand induced disassembly studies. The key advantage of zwitterionic dendrimers over peg based systems are (i) zwitterionic species are highly water-soluble and hence enhances the water solubility of the amphiphiles; (ii) zwitterionic hydrophilic functionalities are short in length and hence facilitate specific interactions among specific protein and ligands, while minimizing non-specific interaction induced effects; (iii) Unlike PEG zwitterionic species are resistant harsh biological
degradation conditions and are temperature insensitive (i.e. zwitterionic species do not exhibit any LCST behavior)\textsuperscript{15, 16}. These are highly advantageous features when engineering materials for targeted drug delivery applications. In this chapter we set out to test the scope of zwitterionic species utility in protein binding induced disassembly applications.

3.2 Molecular Design and Synthesis

The molecular structures of dendrons (G\textsubscript{1}-G\textsubscript{2}) with alkyl chain as the lipophilic unit and clickable zwitterionic moiety as the hydrophilic unit, Biotin ligand installed at the core of the dendron are shown in Chart 3.1. Since the zwitterionic components are short in length and are capable of reducing non-specific interactions, we have chosen them as hydrophilic components. Short zwitterionic hydrophilic functionalities facilitate the specific protein-ligand interactions while reducing any non-specific interaction induced disassembly. This also allows us to conveniently compare zwitterionic dendrimers with well-studied structurally similar peg based dendrons. For the synthesis of the targeted dendrons (G\textsubscript{1}-G\textsubscript{2}) we have devised a modular strategy that would allow us to introduce any desired protein-responsive ligand could be installed at the end of the synthesis. For this purpose, we will use

\begin{center}
\textbf{Chart 3.1:} Molecular structures of G\textsubscript{1} and G\textsubscript{2} zwitterionic biotin dendrons
\end{center}
alkyne units at the core of the dendron to install the desired zwitterionic unit using click chemistry\textsuperscript{17} at the end.

3.3 Results and Discussion

Currently, we thoroughly characterized G1 zwitterionic dendrimer and all the results present here correspond to the G1 dendron. G2 zwitterionic dendrimer synthesis, characterization, their self-assembly properties, and interactions with specific proteins are underway in our laboratories.

3.3.1 Self-assembly Characteristics

We have evaluated the self-assembly characteristics of zwitterionic dendrimer with biotin ligand functionality, (Figure 1). Successful guest encapsulation of Nile red, size evolution in DLS data indicates the self-assembled aggregate formation with hydrophobic pockets in them. TEM data further corroborates the micelle-type aggregate formation.

\textbf{Figure 3.1:} a) self-assembled aggregates of zwitterionic dendrimer with ligand confirmed by hydrophobic guest encapsulation, DLS and TEM data.
3.3.2 Testing the Disassembly via Dynamic Light Scattering Studies

After the successful syntheses and self-assembly characterization of G1 dendron, we investigated the extravidin-induced disassembly using DLS studies. The size of 10-µM solution of G1 (above its CAC) in HEPES buffer was found to be 70 nm. Upon addition of 4-µM solution of extravidin, we were gratified to find a systematic increase in the size of G1 dendron assembly with time, finally reaching around 1000 nm after 12 hrs (Figure 3.2). The increase in size with time indicates that the tetravalent extravidin binding to dendritic assembly via multivalent interactions leading to disassembly and turbid solution formation. Additionally, the control proteins with varying pI, i.e. pepsin (pI = 2.8, Mn = 30 kDa), thrombin (pI = 7.2, Mn = 36 kDa), and chymotrypsin (pI = 9.2, Mn = 25 kDa) do not show such disassembly, confirming the specificity of the protein-ligand interaction as the reason for disassembly.

**Figure 3.2:** Size evolution upon interaction of extravidin with zwitterionic dendrimer
3.3.3 Testing the Disassembly via Fluorescence Studies

Based on our hypothesis, the disassembly should accompany the hydrophobic guest release from the interiors of the micellar assemblies. When extravidin was added to the Nile red encapsulated dendron in HEPES buffer, we observed a systematic decrease in fluorescence intensity of Nile red as a function of time (Figure 3.3). The time-dependent decrease in fluorescence intensity indicates that the disassembly is indeed accompanied by the release of guest from the hydrophobic interiors. This was further supported by the addition of control proteins to the nile red encapsulated ZWD where no significant guest release was observed. From Figure the noteworthy points are, the hydrophobic guest release happens in the presence of specific protein, i.e, extravidin and the control proteins do not exhibit such guest release. The guest release does not occur with structurally similar zwitterionic dendrimer (control dendron).

3.4 Summary

In this chapter we presented design and synthesis of zwitterionic dendrimer with biotin functionalities embedded at the core of the dendron and evaluated its self-assembly characteristics, and we studied the interactions of self-assembled aggregates.

![Guest release from zwitterionic dendrimer in the presence of extravidin](image)
with specific protein extravidin. We observed that, guest release and morphological changes in the assemblies occur only in the presence of protein specific to the ligand incorporated and more these changes are more pronounced in case of zwitterionic dendrimers when compared with peg based dendrimers and it is attributed to short zwitterionic components facilitating the interaction between aggregates and protein. Further evaluation of this hypothesis with dendron variations, such as G2, and with different proteins-ligand interactions, such as IgG-Anti-2,4-DNP are underway in our laboratories.

3.5 Experimental Section

3.5.1 Synthesis of Zwitterionic Dendrimer with Biotin

Scheme 3.2: General procedure for the synthesis of propargyl G1-OH (3) dendrimer

General procedure for the synthesis of propargyl G1-OH (3) dendrimer

To a solution of biaryl monomer 1 (1.0 eq) and appropriate bromobenzyl compound (2-3 eq) in dry acetone, was added K$_2$CO$_3$ (3 eq) and 18-crown-6 (0.2 eq). The reaction mixture was refluxed under argon atmosphere for 12 h. The progress of the reaction was monitored by TLC. After completion of the reaction, acetone was evaporated and the crude reaction mixture was partitioned between ethyl acetate and
water. The aqueous layer was extracted twice with ethyl acetate and the combined organic layer was dried over Na$_2$SO$_4$ and evaporated to dryness. The crude product was purified by silica gel column chromatography.

General procedure for N-methylation of triazole dendrimers

A solution of dendritic triazole compound (1.0 eq), Iodomethane (10 eq for 1 triazole ring) in dry acetonitrile was heated in a sealed tube at 60 °C for 24 h. All volatile compounds were removed under vacuum with a rotary evaporator to give crude dendritic triazolium adduct, which then washed with ethyl ether (3 x 10 mL), dried under vacuum afford the crude compound, which was used for next step without further purification.

General procedure for hydrolysis reaction of N-methyl-triazolium ester dendrimers

To a solution of n-methyl-triazolium ester dendrons (1.0 eq) in anhydrous dichloromethane was added TFA. The reaction mixture was allowed to stir for 24 h. The solvent was evaporated using rotary evaporator to afford the crude zwitterionic dendron, which was further purified by using reverse phase column chromatography or by simple precipitation in Acetone/Methanol mixture by trial and error, this precipitation method needs to be repeated to establish a reproducible protocol.

General procedure for click reaction

The mixture of dendritic acetylene compound (1.0 eq), azide (2.5 eq for 1 acetylene group), CuSO$_4$. 5H$_2$O (0.5 equiv.) and sodium ascorbate (0.5 eq.) in DMSO solvent mixture was heated at 50 °C for 36 h. The reaction progress was monitored by TLC. After completion of the reaction, the reaction mixture was partitioned between ethyl acetate and saturated aqueous NH$_4$Cl solution. The aqueous layer was extracted twice with ethyl acetate and the combined organic layer was dried over Na$_2$SO$_4$ and
evaporated to dryness. The crude product was purified by silica gel column chromatography.

Synthesis of compound 3

According to general procedure for hydroxyl dendrimer preparation, compound 1 (0.3 g) was treated with bromo species 2 (0.229 g) to give 0.310 g of 3. 

$^1$H-NMR (400 MHz; CDCl$_3$): $\delta$ 7.73 (s, 2H), 6.66-6.49 (m, 13H), 5.23 (s, 5H), 5.06 (s, 5H), 4.98 (s, 3H), 4.74 (s, 2H), 4.57 (s, 2H), 3.93 (d, $J = 6.5$ Hz, 7H), 1.81-1.74 (m, 6H), 1.66-1.59 (m, 4H), 1.50-1.45 (m, 26H), 1.32-1.22 (m, 52H), 0.91-0.87 (m, 5H). 

MALDI-ToF m/z calculated for C$_{78}$H$_{112}$N$_6$O$_{13}$ [M+H$^+$]: 1340.83 found, 1362.94.

Synthesis of compound 4

According to general procedure for N-methylation of triazole, compound 3 (430 mg) was treated with excess Iodomethane (0.55 mL) to yield 385 mg of crude compound. This crude product was treated with 10 mL DCM/TFA (6mL/4mL) for 48
hours, then solvent was removed by rotary evaporation to obtain compound 4. Compound 4 is used as such for the next click reaction.

Synthesis of zwitterionic Dendron G1-biotin, 5

According to the general procedure for click reaction, G1 zwitterionic species 4 (300 mg) was treated with excess biotin azide (3 equiv) in dichloromethane-water (8 mL) to yield 0.17 g of zwitterionic Dendron with biotin ligand, 5. 1H-NMR (400 MHz; MeOD): δ 8.77-8.72 (m, 1H), 7.81-7.74 (m, 1H), 6.87-6.46 (m, 9H), 5.43-5.36 (m, 2H), 5.24-5.03 (m, 5H), 4.68-4.58 (m, 2H), 4.39-4.28 (m, 3H), 4.10-4.05 (m, 2H), 4.02-3.83 (m, 5H), 3.78-3.70 (m, 1H), 3.40-3.35 (m, 2H), 3.22-3.19 (m, 1H), 1.82-1.69 (m, 4H), 1.67-1.09 (m, 48H), 1.00-0.78 (m, 10H). MALDI-TOF m/z calculated for C_{88}H_{124}N_{12}O_{16}S [M^++H]: 1612.90; found 1625.84.
3.6 References


CHAPTER 4
ZWITTERIONIC AMPHIPHILIC HOMOPOLYMER ASSEMBLIES

4.1 Introduction

Supramolecular nanoassemblies capable of reducing non-specific interactions with complex biological milieu have attracted tremendous research interest because of their potential in targeted nanoparticle based drug delivery applications\(^1\)\(^-\)\(^4\). Hence, recent efforts to design and develop versatile amphiphilic polymers that form stable nano-sized aggregates with effective non-fouling characteristics and hydrophobic guest encapsulation capabilities are on the rise\(^5\)\(^-\)\(^9\). Zwitterionic materials\(^10\), \(^11\) are replacing PEG based materials\(^12\), \(^13\) because of their high water solubility, charge neutrality, and their ability to reduce non-specific interactions with biological media such as serum and blood platelets\(^14\)\(^-\)\(^17\).

Incorporation of zwitterionic moieties as hydrophilic components in amphiphilic macromolecules could lead to a new class of nanoassemblies with anti-biofouling characteristics. In literature, there have been few reports on zwitterionic amphiphilic block copolymers that form self-assembled aggregates because of the mutual immiscibility of the hydrophilic and hydrophobic blocks in the bulk solvent\(^8\), \(^18\)\(^-\)\(^21\). A useful alternate to amphiphilic block copolymers, because of the synthetically and functionally more accessible features, involves random copolymers and homopolymers\(^22\)\(^-\)\(^24\), \(^25\)\(^-\)\(^28\).

In this chapter, we present a one-pot synthesis of acrylamide-based zwitterionic amphiphilic homopolymers with hydrophilic and hydrophobic components placed orthogonally in every repeat unit (Scheme 4.1), where the hydrophilic part of the amphiphilic polymer is based on a zwitterionic moiety. Amphiphilic homopolymers often possess charged functionalities, such as the cationic
tertiary ammonium\textsuperscript{29,30} or the anionic carboxylate\textsuperscript{31,32} moieties. Because of their ionic nature, the formed assemblies are prone to non-specific interactions with biological macromolecules. In fact, we have utilized this feature of the amphiphilic homopolymers in protein sensing and peptide detection\textsuperscript{29,33,34}. In this article, we present a new method to introduce glycinebetaine as charge-neutral zwitterionic, hydrophilic moieties in amphiphilic homopolymers. Further, we evaluate the self-assembly characteristics, pH-sensitivity, and the biocompatibility of the polymers relative to the corresponding polyelectrolyte nanoassemblies.

4.2 Results and Discussion

4.2.1 Design and Synthesis

Scheme 4.1: Schematic representation of solvent dependent self-assembled aggregate formation of zwitterionic amphiphilic polymers

We first synthesized the reactive polymer, poly(pentafluorophenyl acrylate) 1, \textit{via} RAFT polymerization of pentafluorophenylacrylate\textsuperscript{35} using cyanomethyl dodecyl trithiocarbonate, as the chain transfer agent (see experimental section for details). Here, we observed that the polymer molecular weight can be precisely controlled by
varying the monomer to chain transfer agent concentration (Repeating Units of polymer, \( P_n = \frac{[M]}{[CTA]} \)). We prepared polymers with varying molecular weights as shown in figure 4.1. We chose to work with polymer, 1 (Mn = 12 kDa, PD = was then treated with the secondary amine 2 in dry DCM in the presence of Hünig’s base for 24 hours at room temperature. The tertiary amine moiety in the product polymer 3 was then converted to a zwitterionic moiety by treating this polymer with a slight excess of 2-bromoacetic acid 4. When the reaction mixture was precipitated in ethyl acetate, the zwitterionic polymer 5 was obtained as a white powder. The conversion of reactive polymer to zwitterionic polymer was confirmed by NMR and IR (Scheme 4.2). The evolution of peaks corresponding to alkyl and zwitterionic components in

![Retention Time (sec)]

**Figure 4.1:** Molecular weight control in the preparation of pentafluorophenylacrylate polymer

the region of 1 to 5 ppm in \(^1\)H-NMR (Scheme 4.2c), and disappearance of broad \(^{19}\)F-NMR peaks in the corresponding to pentafluorophenyl groups of reactive polymer in
the region of -150 to -160 ppm (Figure 4.6) indicates the conversion of reactive polymer to the intermediate polymer 3. Further, the disappearance of ester C=O stretching band corresponding to reactive polymer at 1780 cm\(^{-1}\) and evolution of amide C=O stretching band at 1640 cm\(^{-1}\) corresponding to polymer 3. The evolution of carboxylate stretching band in the region of 3300 cm\(^{-1}\) (Scheme 4.2b) in polymer 5 confirms the zwitterionic polymer structure.

4.2.2 Self-assembly Characteristics

We investigated the solubility of 5 in aqueous phase. We were gratified to observe higher water solubility for these polymers (> 1 mg/mL or ~ mM) compared to structurally similar cationic or anionic polymers (~0.01 mg/mL or ~ µM). Note that we have previously shown that amphiphilic homopolymers are capable of providing nanoassemblies in both polar and apolar media, i.e. micelle-like assemblies in aqueous phase and inverse micelle-like assemblies in apolar organic solvents\(^{22,36}\). We were interested in investigating whether such possibility exists for the zwitterionic

![Scheme 4.2: a) Conversion of reactive polymer to zwitterionic polymer; b) 1H-NMR data confirms the conversion of reactive polymer to zwitterionic polymer; c) IR spectral data further confirms the zwitterionic polymer formation](image)

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amphiphilic homopolymer as well. Therefore, we studied the solubility of these polymers in apolar solvents and we found these polymers to be quite soluble in apolar solvents as well. We then studied the self-assembly characteristics of this polymers in aqueous solutions by using 0.1 mg/mL solution. To assess whether these polymers self-assemble into nanostructures, we investigated these polymers using dynamic light scattering (DLS) and transmission electron microscopy (TEM).

The sizes of the assemblies were found to be 110 nm and 60 nm for

![Figure 4.2: Self-assembly characteristics of zwitterionic polymers. (a) Emission spectra of hydrophobic, Nile red ($\lambda_{ex} = 550$ nm) and hydrophilic, Rose bengal ($\lambda_{ex} = 530$ nm) encapsulated in micellar and inverse micellar aggregates solutions. On the other hand, no guest encapsulation is observed in the corresponding solvents without polymers; (b) Sizes of aggregates as determined by DLS; (c, d) TEM images of the micellar and inverse micellar assemblies.](image-url)
assemblies formed in water and toluene respectively (Figure 4.2b). The sizes of these assemblies were found to be smaller, when analyzed by TEM. This is understandable, as the solvation of the aggregates vary significantly. Nonetheless, TEM suggests that these aggregates are likely to be spherical in morphology (Figure 2c,d).

If these assemblies were indeed micelle-like or inverse micelle-like from a host-guest perspective, then the hydrophobic interiors of these self-assembled aggregates in the aqueous phase should be able to encapsulate hydrophobic guest molecules. To test this possibility we used hydrophobic small molecule Nile red, as a spectroscopic probe. As such, Nile red is quite insoluble in aqueous solutions, however in the presence of polymer, it became soluble indicating its encapsulation in the hydrophobic pockets of the self-assembled aggregates. We further confirmed the non-covalent encapsulation of Nile red via absorbance (Figure 4.7) and fluorescence spectroscopy (Figure 4.2a). Similarly, the inverse micellar type aggregates should be able to encapsulate hydrophilic molecules such as Rose bengal in organic solvents. Rose bengal is insoluble in apolar solvents, but becomes soluble in apolar polymer solutions, such as 0.1mg/mL polymer-DCM solution indicating the hydrophilic interior of inverse micellar assemblies (Figure 4.2a). Overall, combination of these experiments confirms that amphiphilic zwitterionic homopolymers form micelle-like and inverse micelle-like assemblies depending on the polarity of the solvent.

4.2.3 pH dependent Zeta potential and size variations in zwitterionic polymeric assemblies

Next, we investigated the effect of pH on the surface charge of the polymeric assemblies\(^{37-39}\). In the glycinebetaine moiety, one would expect the carboxylate moiety to be pH-sensitive as this functionality can be reversibly protonated upon
reducing the pH. Although the pKa of the polycarboxylate moiety itself is around 4.5, it is interesting to investigate the effect of the proximal quaternary ammonium ion on the pKa of this functional group in the polymer. Therefore, we studied the variations in the zeta potential of the polymer assembly at different pH. We systematically varied the pH of the solution, using 0.01 M HCl or NaOH, from pH 2 to 10. We observed a gradual change in the zeta potential of the assembly from about +20 mV to about -20 mV, upon going from low pH to high pH. The isoelectric point, i.e. the pH at which the overall surface charge of the assembly is zero, was found to be about 6.5. This is significantly higher than that observed for the carboxylate moiety (6) by itself i.e. pH ~ 2.1 the carboxylate polymer zeta potential is close to zero (at pH below 2, the polymer precipitates out completely, Figure 4.11). This difference is understandable, because the quaternary ammonium moiety should stabilize the carboxylate counterion, thus increasing the pKa of the proximal carboxylate moiety.

On the other hand, this trend was surprising at pH above the isoelectric point. We anticipated that the cationic quaternary ammonium moiety could be considered to be a permanent charge and therefore at high pH (where all the carboxylates are deprotonated), there will be very little impact on the surface charge of the assembly.

Figure 4.3: a) Plot of pH dependent zeta potential variations, IEP = Isoelectric point at pH 6.5; b) pH dependent size variations of the micellar assemblies
However, the surface charge continued to decrease. This is likely due to a stronger association of the tetraalkylammonium species with OH\(^{-}\) ions at high pH. To test this possibility, we investigated the pH dependence of the surface charge of cationic polyelectrolyte 7. We were gratified to observe the gradual decreasing trend in zeta potential from +20 mV to ~ +14 mV indicating that cationic ammonium species indeed form a complex with OH\(^{-}\) ions at high pH (Figure 4.11b).

We further studied the effect of pH on the size of the self-assembled aggregates. Here, we hypothesize that the nature of charge of the hydrophilic component of amphiphilic polymer would play an important on the size of the resulting self-assembled aggregates, attributed to electrostatic attractive or repulsive interactions among the charges. To investigate this possibility we studied the variations in hydrodynamic size of the polymeric assemblies with changes in pH of the solution using dynamic light scattering technique. We observed that the size of the assemblies are smaller at higher and lower pH, but the size significantly increased as we reach the isoelectric point. This is attributed to the high density of charge on the pendent groups at low and high pH, which exhibits electrostatic repulsions between the pendent ionic groups in the polymer chains at microscopic level leading to formation of smaller aggregates. On the other hand, as we reach the isoelectric point, the reduced electrostatic repulsions result in the formation of the larger aggregates. This feature of the zwitterionic polymer could find use in pH-sensitive surface charge

![Chart 4.1: Structures of ionic and charge Neutral PEG based control polymers](image-url)
variations, which have been explored for use in drug delivery and biomedical diagnostics.\textsuperscript{40, 41}

4.2.4 Cytotoxicity Studies

Finally, considering the potential implications of a nanoassembly with charge-neutral and pH-sensitive charge conversion characteristics in biological applications, we evaluated and compared the cytotoxicity of our zwitterionic polymeric assemblies to that of structurally similar ionic (6, 7) and neutral PEG based amphiphilic polymers, 8 (see experimental for the synthesis and characterization). If surface functional groups are primary determinants of cytotoxicity, zwitterionic and the PEG-based polymeric assemblies should possess least cytotoxicity because of their charge neutral characteristics. Similarly, cationic polyelectrolyte assemblies should be more toxic compared to the anionic ones. To test this hypothesis, we evaluated the in vitro cellular viability of all the polymers using Alamar blue assay on healthy HEK-293T

\textbf{Figure 4.4:} \textit{in vitro} cellular viability of polymers on 293T cell line
cell line and cancer HeLa cell line. Indeed, we observed that zwitterionic polymers exhibit concentration independent, high cell viability with both HEK-293 and HeLa cell lines (Figure 4.4, 4.5). On the other hand, cationic polymers are the most toxic followed by anionic and charge-neutral PEG based polymers, and their cytotoxicity was found to be concentration-dependent. We were surprised to find that the PEG-based assemblies were more toxic. The reason for this difference is not clear to us at this time and will be a subject of future investigations.

Figure 4.5: *in vitro* cellular viability of polymers on HeLa cell line

4.3 Summary

We have presented a one-pot synthesis of zwitterionic amphiphilic homopolymers using reactive polymer precursors. These polymers form self-assembled aggregates and stably encapsulate guest molecules both in polar and apolar solvents. Due to the presence of glycine betaine as the zwitterionic hydrophilic component of the polymer, it reversibly switches among cationic, zwitterionic, and
anionic forms depending on the pH of the solution. These variations also afford pH-dependent size variations, which are attributed to electrostatic repulsion typical of polyionic assemblies. Cytotoxicity studies indicate that zwitterionic polymeric assemblies are least cytotoxic among structurally similar polymers. Facile preparation, and robust guest encapsulation capabilities, combined with their biocompatibility and multifunctional conjugation sites make them ideal candidates for conjugation with disease relevant antibodies or ligand functionalities. These advantageous features of zwitterionic amphiphilic polymer have potential applications in development of targeted drug delivery vehicles with efficient anti-fouling characteristics.

4.4 Experimental Section

Materials and Methods:

All chemicals and solvents were purchased from commercial sources and were used as such, unless otherwise mentioned. $^1$H-NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer, while $^{19}$F-NMR spectra were collected on a 300 MHz Bruker NMR Spectrometer using the residual proton resonance of the solvent as the internal standard. Chemical shifts are reported in parts per million (ppm). When peak multiplicities are given, the following abbreviations are used: s, singlet; bs, broad singlet; d, doublet; t, triplet; m, multiplet. $^{13}$C-NMR spectra were proton decoupled and recorded on a 100 MHz Bruker spectrometer using carbon signal of the deuterated solvent as the internal standard. Molecular weight of the polymers was measured by gel permeation chromatography (GPC, THF) using a PMMA standard with a refractive index detector. THF was used as eluent with a flow rate of 1mL/min. Dynamic light scattering (DLS) measurements were performed using a Malvern Nanozetasizer. FTIR spectra were recorded on a Perkin Elmer Spectrometer.
4.4.1 Synthetic Schemes

Synthesis of zwitterionic polymer from the starting materials:

Scheme 4.3: Synthesis of zwitterionic polymer from reactive polymer, 3.

Synthesis of anionic and neutral TEG based amphiphilic polymers:

Scheme 4.4: Schematic of synthesis of control ionic- and peg-based polymers from reactive polymer

All the secondary amines are synthesized following the well known reported procedures\textsuperscript{42} \textsuperscript{43} \textsuperscript{44}, hence there is no further mention of their synthetic details.
Synthesis of secondary amines:

i) Synthesis of tertiary amine species:

\[
\begin{array}{c}
\text{O} \quad \text{Br} \\
\text{H}_2\text{N} \text{N} \\
\text{KOH} \\
\end{array}
\quad \quad 
\quad \begin{array}{c}
\text{H}_2\text{N} \text{N} \\
\text{KOH, 0 °C to RT, 2 h} \\
\end{array}
\]

ii) Synthesis of TEG amine species:

\[
\begin{array}{c}
\text{HO} \quad \text{O} \\
\text{TsO} \\
\text{NaNO}_2, \text{DMF} \\
\text{reflux, 24 h} \\
\end{array}
\quad \quad 
\quad \begin{array}{c}
\text{TsO} \quad \text{O} \\
\text{N}_3 \\
\text{PPh}_3, \text{H}_2\text{O, RT} \\
\text{70 %} \\
\end{array}
\]

iii) Synthesis of t-butyl protected ester amine species:

\[
\begin{array}{c}
\text{Br} \\
\text{H}_2\text{N} \text{N} \\
\text{KOH, 0 °C to RT} \\
\end{array}
\quad \quad 
\quad \begin{array}{c}
\text{H}_2\text{N} \text{O} \\
\text{L AH, 0 °C to RT, 2 h} \\
\end{array}
\]

Synthesis of reactive polymer, 3:

The pentafluorophenylacrylate monomer, 1 was synthesized following the known protocol. The acrylate monomer (500 mg, 2.1 mmol) was added to a 20 mL vial
with dry benzene (1.5 mL), chain transfer agent cyanomethyl dodecyl trithiocarbonate, 2 (14 mg), AIBN (3.4 mg, 0.21 mmol) and the resulting reaction mixture is purged with Argon for 2 min and immediately capped it under argon conditions. This reaction mixture is stirred at 70 °C for 6 hours. This reaction mixture is precipitated in hexanes to obtain pure product (60 % yield). GPC (PMMA/THF): \( M_n = 12 \text{ K Da}, M_w = 14 \text{ K Da}, PD = 1.1; \) \(^1\text{H}-\text{NMR (400 MHz; CDCl}_3\):} \( \delta 3.20-3.00 \) (br s, 1H), 2.20-1.90 (br s, 2H). \(^{19}\text{F}-\text{NMR (300 MHz; CDCl}_3\):} -150.00 (s, 2F), -154.00 (s, 1F), -160.00 (s, 2F).

Synthesis of zwitterionic polymer, 7:

![Zwitterionic Polymer Structure](image)

To the reactive polymer 3, (300 mg, 1.26 mmol) in dry THF (2 mL), secondary amine, 4 (620 mg, 2.52 mmol) was added. To the resulting reaction mixture Hunig’s base

![19F-NMR Data](image)

**Figure 4.6:** \(^{19}\text{F}-\text{NMR peaks corresponding to pentafluorophenyl group disappear upon conversation of reactive polymer to zwitterionic polymer, indicating the efficient post functionalization of reactive polymer.**
(0.46 mL, 2.52 mmol) was added as base and stirred for 48 hr. IR and NMR confirm the conversion of activated ester polymer to intermediate tertiary amine species. This intermediate species is used as such for the next step without any further purification. To this tertiary amine polymer, excess bromoacetic acid was added and stirred for 48, then the reaction was precipitated several times in ethyl acetate to get pure white solid of zwitterionic polymer (60 % yield). \(^1\text{H}-\text{NMR}\) (400 MHz; CDCl\(_3\)): \(\delta\) 4.36 (t, \(J = 35.1\) Hz, 2H), 3.78-3.46 (m, 7H), 3.00 (d, 2H), 1.68 (d 2H), 1.28 (s, 14H), 0.90 (t, \(J = 5.8\) Hz, 3H).

**Figure 4.7:** a). Absorbance spectra of hydrophobic and hydrophilic guest molecules (Nile Red, DiI, and DiO) encapsulated in micellar assemblies; b). hydrophilic guest molecule, Rose Bengal in inverse micellar assemblies.

Dynamic Light-Scattering (DLS) experiments

The size distribution of the micelles was determined by Nano series Nano-ZS (Malvern Instrument) Zetasizer. In a typical experiment a stock solution of 0.1mg/mL polymer solution was prepared in milli-Q water by sonicating the solution for 2 hours at room temperature. The resultant solution was then filtered through 0.22-\(\mu\)m filters and then the size of the micellar solutions were measured by DLS.
Spectroscopic measurements

Fluorescence emission spectra were recorded on a JASCO (FP-6500) spectrofluorimeter, using 1 mL disposable fluorescence cuvettes. The emission spectra for Nile red and Rose Bengal were recorded by exciting at 550 nm and 525 nm respectively, with the excitation and emission bandwidth set at 3 and 5 nm.

Figure 4.8: pH dependent zeta potential for zwitterionic polymer

Figure 4.9: pH dependent size variations for zwitterionic polymer
Cytotoxicity studies

The *in vitro* cellular viability of the amphiphilic polymers were evaluated on healthy 293T and HeLa cancer cell lines. The cells were cultured in T75 cell culture flasks using Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) with 10% fetal bovine serum (FBS) supplement. The cells were seeded at 10,000 cells/well/200 µL in a 96 well plate and allowed to grow for 24 hours under incubation at 37 °C and 5% CO2. These cells were then treated polymers of different concentrations and were incubated for another 24 hours. Cell viability was measured using the Alamar Blue assay with each data point measured in triplicate. Fluorescence

**Figure 4.10:** Size and zeta potential for control polymers
Figure 4.11: Zeta potential changes with the changes in pH of the solution were monitored for ionic polymers: a) Anionic polymers, b) Cationic polymers. Measurements were made using the plate reader SpectraMax M5 by setting the excitation wavelength at 560 nm and monitoring emission at 590 nm on a black well plate.
4.5 References


CHAPTER 5

DEGRADABLE SELF-CROSSLINKED POLYMERIC NANOGELS

5.1 Introduction

Design and development of amphiphilic systems capable of non-covalently encapsulating hydrophobic guest molecules, retaining, and then releasing them under specific trigger are important goals in supramolecular chemistry and such smart systems have clear applications in the research areas of drug delivery and therapeutics.\(^1\)\(^-\)\(^9\) Besides, the ability to control the size and stability of such self-organized hosts are of greater interest because of their potential for passive targeting of tumor tissue through the enhanced permeability and retention effect (EPR).\(^10\)\(^-\)\(^13\)

The stability issues associated with the supramolecular hosts could be circumvented by chemically cross-linking the self-organized supramolecular hosts.\(^6\),\(^\,14\)\(^-\)\(^17\) however, the existing methods to prepare such polymeric nanoparticles or nanogels face certain complications, as they are prepared by microemulsion or inverse microemulsion methods.\(^6\),\(^7\),\(^18\),\(^19\) Microemulsion methods involve oil-in-water emulsion and utilizes hydrophobic monomers as feed to form water insoluble nanogels. Inverse microemulsion (water-in-oil) methods are usually applied to form water-soluble nanogels. However the continuous phase in this protocol is hydrophobic hence it is not possible to encapsulate hydrophobic guest molecules. Besides, these methods involve multiple purification steps to remove the unreacted monomer and the surfactant material used as the emulsion stabilizer.\(^20\),\(^21\)

An attractive alternative to form stable nanoparticular systems is to chemically cross-link the supramolecular assembly in its native self-assembled state. In our group we recently developed a facile method that allows for the design and syntheses of water-soluble polymer nanogels under emulsion-free conditions with stable
hydrophobic guest encapsulation capabilities. The nanogel precursor is acrylamide based random copolymer with PEG and pyridyldisulfide (PDS) moieties as pendent groups. Because of the amphiphilic nature these polymers self-organize to form nanoscale aggregates. The amphiphilic polymer structure and the synthetic approach are shown in scheme 5.4. These aggregates were then covalently locked, to form stable nanogels, by addition of deficient amounts of DTT; nanogels have been further demonstrated to be responsive to biologically relevant triggers, releasing encapsulated guest molecules, such as GSH. However, in this process the end products are non-biodegradable polymers that could be cytotoxic and are harder for safe clearance from the human body.

Figure 5.1: Self-cross-linked polymeric nanogels preparation from degradable random copolymers

An ideal nanoparticular system should be effectively cleared from human body after its intended function of releasing a drug at a specific site for a specific trigger and undergo degradation to GRAS (Generally Recognized As Safe) components; it is indeed a challenging task. In this chapter we introduce degradable amphiphilic polymer platform that has potential to meet few or more of these challenging requirements. The molecular structure of the polymer possesses the degradable carbonate backbone, PDS and PEG pendent chains. For a stimuli-responsive functional groups, we considered a disulfide bond (these bonds are
susceptible to biochemical reductants such as glutathione (GSH), thioredoxin, and peroxiredoxin.\textsuperscript{28, 29} pH-sensitive and esterase active polycarbonate backbones. We have previously reported a synthetic methodology in which a pyridyl disulfide (PDS) side chain functionality as a handle for incorporating thiol-based functional groups onto polymers.\textsuperscript{30, 23} The chemical basis for this is the higher reactivity of the pyridyl disulfide bonds with free thiols over other disulfide bonds, with release of a stable 2-thiopyridone byproduct. This PDS group preference for free thiols could be exploited to affect cross-linking between the polymer chains.

Our hypothesis involves synthesis of reactive polymers (polymers possessing activated ester leaving groups) with degradable backbone and post functionalization of reactive components of the polymer with nucleophiles such as functional alcohols or amines (in this case, hydrophobic PDS-OH and hydrophilic PEG-OH or zwitterionic alcohols). The resulting random copolymer because of its amphiphilicity would form self-organized structures. With the addition of deficient amount of DTT, a corresponding small percentage of PDS groups will be converted to free thiols. These free thiols would then react with an equivalent amount of the remaining PDS functionalities to create disulfide bonds, which would effectively cross-link the polymer chains, independent of whether the process is intra-chain or inter-chain capturing the assembly in its native state. If this indeed were the case, the degradable polymer nanoparticles would be obtained upon treatment of our polymers with deficient amount of DTT, without the need for ultrahigh dilution preparative conditions. We also envisaged that the hydrophobic interior in the aggregate would facilitate the encapsulation of hydrophobic guest molecules prior to cross-linking.
The objective with this project is to incorporate the following advantageous features onto the nanogels (Figure 5.2): (i) high water solubility and non-specific interaction reducing capabilities; (ii) hydrophobic guest encapsulation; (iii) stimuli responsiveness (pH sensitivity and esterase activity); (iv) ability to functionalize the surfaces with targeting species; (v) Size control. These features are highly desirable for drug delivery applications, imaging and tissue engineering.

5.2 Results and Discussion

5.2.1 Design and Synthesis

The polymer nanogel precursor is based on a random copolymer that contains degradable carbonate backbone, oligoethyleneglycol (OEG) units and pyridyldisulfide (PDS) groups as side chain functionalities. The role of the OEG group is to introduce a charge-neutral hydrophilic functional group, which is known to reduce non-specific interactions with biological medium and biocompatibility. The PDS functionality
plays several important roles: (i) this is a hydrophobic functionality and thus plays a critical role in providing a supramolecular amphiphilic assembly in the aqueous phase. (ii) the hydrophobic environment facilitated by the PDS functionality acts as hydrophobic pockets for non covalent hydrophobic guest encapsulation prior to crosslinking. (iii) The PDS functionality is reactive towards thiols and thus provides a mild method for disulfide cross-linking to form the nanogels. (iv) Since the nanogels are based on disulfide cross-linkers this provides a pathway to trigger the release of the stably encapsulated guest molecules in response to an external stimulus. Finally, the degradable polycarbonate backbone provides several advantageous features. The degradable component is known to be sensitive to pH, several esterases, and known to undergo hydrolytic cleavage over the time. Hence pH or esterases could be used as biorelevant triggers to control the release of guest molecules. The polymer itself slowly degrades to biocompatible bis(2,2-methylol) propionic acid (bis-MPA).

5.2.2 Preparation of Random Copolymer, 7

Scheme 5.2: Synthesis of reactive aliphatic polycarbonate, 4.

The reactive cyclic carbonate monomer comprising a reactive pentafluorophenyl ester group 3, is prepared by following the reported protocol31 (scheme 5.1). This monomer can be polymerized by ring opening methods to form aliphatic carbonate polymer comprising reactive pentafluorophenyl ester groups, this reactive polymer forms the basis for all the subsequent polymer synthesis. The cyclic carbonate monomer upon treatment with Triflic acid, opens up the cyclic carbonate ring resulting in the
formation of reactive polycarbonate (Scheme 5.2). The reactive polymer preparation is confirmed by $^1$H-NMR and $^{19}$F-NMR (see experimental section). This reactive polymer could be functionalized with various alcohols in the presence of catalytic amounts of TBAF. Here we utilized this possibility to prepare degradable amphiphilic random copolymers, i.e. by treating the reactive polymer with PDS-OH and PEG-OH in the presence of TBAF. Here TBAF generates the reactive acyl fluoride species, then the nucleophilic attack of alcohols on the acyl fluoride results in the formation of corresponding ester. The functionalization/replacement of reactive pentafluorophenyl (PFP) components with corresponding alcohols are confirmed by monitoring the disappearance of $^{19}$F-NMR peaks in the pure random copolymer, 7. Random copolymer 7, containing 60 % of the PEG and 40 % of the PDS functionalities was prepared (Scheme 5.3).

**5.2.3 Nanogel preparation from random copolymer, 7**

The next step is to convert the polymeric aggregates into chemically cross-linked nanogels. We hypothesized the formation of the nanogel through the following process. The random copolymers were dissolved in aqueous solutions then a deficient amount of dithiothreitol (DTT) was added, this would cause the cleavage of a well-defined percentage of the PDS groups to the corresponding thiol functionalities. These thiol functionalities will then react within the polymeric aggregates with
unreacted PDS functionalities. This would result in covalent cross-links within the polymeric aggregates causing the formation of the nanogels as shown in figure 5.2.

Next, we were interested in identifying whether the obtained particle is indeed a stable cross-linked structure and if the particle is completely controlled by the size of a polymer aggregate obtained prior to the cross-linking reaction. For this purpose, we analyzed the size of the assemblies prior to the DTT addition. DLS studies revealed aggregates of about 100 nm, which changes to 80 nm after the cross-linking reaction. The concentrations of the polymers in aqueous solutions used for this studies are 1 mg/mL. Then we studied the guest encapsulation characteristics of the random copolymers via absorbance and fluorescence studies. We chose hydrophobic guest molecules such as DiI, and DiO (Figure 5.4). These hydrophobic molecules are inherently insoluble in water but could become solubilized in the presence of hydrophobic environments such as the interior of random copolymer assemblies. The absorbance and fluorescence data indicate the encapsulation of these guests in the random copolymer assemblies.

To investigate the possibility of encapsulating hydrophobic guest molecules within the interiors of these nanogels, we carried out the DTT based cross-linking reaction in the presence of Nile red, a hydrophobic dye. The encapsulated Nile red

Figure 5.3: Sizes of the assemblies, a) random copolymer assemblies; b) corresponding nanogels
does not leak out from the assembly even after the dialyzing of the nanogels solutions for three days. This clearly demonstrates that the stable encapsulation of guests inside the assembly (Figure 5.4).

5.3 Triggered release of guest molecules from nanogels

This nanogels to be of importance, they should not only stably encapsulate the guests but should be capable of releasing it upon addition of a specific biologically relevant trigger\textsuperscript{34-38}. These nanogels possess disulfide bonds, which are particularly attractive as stimulus-sensitive functionalities in medicinal chemistry, as they could be cleaved in the presence of reducing agents. Reducing agents, such as glutathione

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure54.png}
\caption{Hydrophobic guest encapsulation in random copolymer solutions, a) DiO; b) DiI.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure55.png}
\caption{Guest encapsulation in random copolymer and its corresponding nanogel}
\end{figure}

5.3 Triggered release of guest molecules from nanogels

This nanogels to be of importance, they should not only stably encapsulate the guests but should be capable of releasing it upon addition of a specific biologically relevant trigger\textsuperscript{34-38}. These nanogels possess disulfide bonds, which are particularly attractive as stimulus-sensitive functionalities in medicinal chemistry, as they could be cleaved in the presence of reducing agents. Reducing agents, such as glutathione
(GSH), thioredoxin, and peroxiredoxin are found at varying concentrations throughout the body. Particularly, GSH is found in millimolar concentrations in the cytosol, but in micromolar concentrations extracellular solutions\textsuperscript{29, 39-41}. Hence, nanogels responsive to GSH are of high significance for targeted drug delivery applications\textsuperscript{42-47}. To test the possibility of triggered release for our nanogels towards GSH, we have added 10 mM of GSH to 1 mg/mL nanogel solution and investigated the release of Nile red by observing the spectral emission intensity changes caused by the release and precipitation of the fluorescent guest molecules. To our gratification, we observed consistent decrease in fluorescence intensity of the nanogels solution indicating the guest release and precipitation over the time period of 72 hours (Figure 5.6).

![Figure 5.6: GSH sensitive release of guest molecules from nanogels over the time period of 72 hours](image)

Next, we were interested in exploring the pH and enzyme-sensitive nature of our nanogels. Aliphatic polycarbonate backbones are one of the attractive material choices because of their biocompatibility, nontoxic degradation products, and absence of autocatalytic degradation processes\textsuperscript{48-51}. Besides polycarbonates are known to
undergo degradation without the increase in the acidity levels of the solution, which reduces the harmful effects of degraded components on the drugs and healthy tissues. To test if our functional polymers undergo degradation we studied the effect of pH on the degradation of polymers. The random copolymer solutions (1 mg/mL in aqueous solution) were stirred at pH 3 and pH 10 for extended periods of time. We monitored the rate of degradation of polymers by monitoring the molecular weight changes from GPC data and by visual inspection (Figure 5.7). In case of pH 10 solutions, degradation was apparent from GPC data where we observed the molecular weight of polymer decreasing from 12 kDa to 3 kDa. On the other hand, we did not observe any significant degradation in case of pH 3 solutions. Degradation studies in the presence of esterases (PLE) are currently under investigation in our laboratories.

5.4 Conclusions

In this chapter, we have presented a degradable amphiphilic random copolymer system that could form degradable nanogels. The amphiphilic polymer assembly could encapsulate hydrophobic guest molecules and retain them stably upon intra/intermolecular disulfide formation of PDS pendent chains present in the polymer. Since our polymers possess aliphatic disulfide bonds both are biodegradable in a stimuli-sensitive environment such as pH, enzymes, and reducing agents; these nanoparticles hold great promise as intracellular drug delivery vehicles. We have demonstrated that these nanogels undergo disaggregation in the presence of GSH, and
undergo degradation at high pH. Preliminary studies indicate that these nanogels are responsive to enzymes such PLE (esterase). Taken together, the degradable polymeric materials with guest encapsulation capabilities establish a new approach for development of nanoparticular systems for a range of biomedical applications such as drug delivery, imaging, biosensing, and tissue engineering.

5.5 Experimental Section

Materials and Methods:

2,2’-Dithiodipyridine, 2-mercaptoethanol, polyethylene glycol (MW 350), D,L-dithiothreitol (DTT), Bis(pentafluorophenyl)carbonate (PFC), 2,2-Bis(hydroxymethyl)propionic acid (bis-MPA), 1,1’-dioctadecyl-3,3,3’,3’-tetramethyl-indocarbocyanine perchlorate (DiI), 3,3’-dioctadecyloxacarbocyanine (DiO), Nile red, Triflic acid, tetrabutylammoniumfluoride (TBAF), CsF and other conventional reagents were obtained from commercial sources and were used as received unless otherwise mentioned. 1H-NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer using the residual proton resonance of the solvent as the internal standard. Molecular weights of the polymers were estimated by gel permeation chromatography (GPC) using PMMA standards with a refractive index detector. Dynamic light scattering (DLS) measurements were performed using a Malvern Nanozetasizer. The fluorescence spectra were obtained from a JASCO FP-6500 spectrofluorimeter. FTIR spectra were recorded on a Perkin Elmer Spectrometer.

5.5.1 Synthetic Schemes

Synthesis of activated cyclic carbonate monomer, 3:

Activated monomer was synthesized following the reported protocol31. A 100 mL round bottom flask was charged with 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) (3.00 g, 22 mmol), bis-(pentafluorophenyl)carbonate (PFC) (21.70 g, 55 mmol,
2.5 eq.), CsF (0.7 g, 4.6 mmol, 0.2 eq.), and 70 mL of anhydrous tetrahydrofuran (THF). Initially the reaction was heterogeneous, but after one hour a clear homogeneous solution was formed that was allowed to stir for 20 hours. The solvent was removed \textit{in vacuo}. The residue was re-dissolved in methylene chloride and, after 10 min, a byproduct precipitated and could be quantitatively recovered. This byproduct was identified as pentafluorophenol by $^{19}$F-NMR and MS (observed m/z: 184). The filtrate was extracted with sodium bicarbonate and water and was dried with MgSO$_4$. The solvent was evaporated \textit{in vacuo} and the product was recrystallized from ethyl acetate/hexane mixture to give activated carbonate monomer, 3 as a white crystalline powder. Yield: 5.20 g (70 % yield). MALDI-MS, calc’d m/z for C12H7F5O5: 326, found: 326.2. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 4.85 (d, J = 10.8 Hz, 2H), 4.36 (d, J = 10.8 Hz, 2H), 1.55 (s, 3H). $^{19}$F-NMR (376 MHz, CDCl$_3$) (CFCl$_3$=0
ppm): δ -154.0~154.1 (m, 2F), -157.3 (t, 1F, J = 2Hz), -162.4~162.5 (m, 2F). $^{13}$C-NMR (100 MHz, CDCl3) δ 167.9, 146.8, 142.2~136.7(m, 5C), 128.0, 72.4(2C), 41.0, 17.5.

Synthesis of activated polycarbonate, 4:

$$\text{The activated polycarbonate, 4 from monomer is prepared by following the reported synthetic protocol}^{32}. \text{An argon-purged small vial was charged with 1-hexanol (R-OH: 0.003 g, 0.01 mmol), 3 (0.357 g, 1.09 mmol), and 1.45 g of dichloromethane (1 M with respect to initial concentration of monomer). The monomer only partially dissolves at this concentration. Trifluoromethanesulfonic acid (Triflic acid) (0.008 g, 0.05 mmol) was added to the stirring solution. As the reaction proceeded, the undissolved monomer slowly became soluble. The reaction was monitored by $^1$H-NMR. Once the reaction was complete (∼12 h at this catalyst loading and degree of polymerization), the polymer was precipitated into hexanes, isolated, and dried to obtain a white solid (yield: 0.356 g, 98.9%). (If excess triflic acid was added, the polymer turned a slight brown color.) $^1$H NMR (CDCl3, 400 MHz): δ = 4.48 (s, 4H), 1.51 (s, 3H). GPC (RI): Mn (PD) = 12 000 Da (1.12).

Synthesis of amphiphilic random copolymer, 7:
A 20-mL glass vial containing a small magnetic stir-bar was charged with activated polymer, 4 (0.1 g), PEG-OH, 5 (0.75 g, 0.6 eq. with respect to pendant pentafluorophenyl ester), PDS-OH, 6 (0.75 g, 0.6 eq. with respect to pendant pentafluorophenyl ester), and DMF (0.50 mL). The mixture was stirred for 3 min to dissolve the polymer. At this point, a solution of 1.0 M TBAF (0.24 mL, 0.75 eq.) was added dropwise from a 1.0 mL syringe. The vial was then capped and the reaction mixture was allowed to stir at room temperature for 24 h, after which a small volume was withdrawn for NMR and GPC analysis. The viscous reaction mixture was dialyzed for 48 hours in MeOH:DCM (75:25, V/V) solution, then the solvent was removed by rotary evaporation, this afforded the transesterified polymer for NMR analysis. The pure product formation is confirmed by $^1$H-NMR and $^{19}$F-NMR analysis. The disappearance of $^{19}$F-NMR peaks in the random copolymer confirmed the efficient and complete of post functionalization. $^1$H-NMR (400 MHz; CDCl$_3$): δ 8.49 (s, 1H), 7.70 (s, 2H), 7.14 (s, 1H), 4.33 (d, $J = 3.8$ Hz, 16H), 3.67-3.56 (m, 29H), 3.39 (s, 3H), 3.10-3.00 (m, 2H), 1.28 (s, 9H). GPC (RI): $M_n$ (PD) = 16 000 Da (1.4).
5.6 References


CHAPTER 6
SUMMARY AND FUTURE DIRECTIONS

6.1 Introduction

This dissertation mainly focused on a unique class of amphiphilic macromolecules designed, developed and thoroughly investigated in our laboratories, i.e. facially amphiphilic macromolecules\(^1\text{-}^5\), amphiphilic random copolymers\(^6\text{-}^{10}\). In chapter 1, we discussed the molecular design, synthesis of facially amphiphilic dendrimers and polymers. As explained in chapter 1, we utilized ionic carboxylate groups\(^11\text{-}^{12}\), quaternary amines\(^13\text{-}^{14}\) or neutral PEG\(^1\text{-}^{2}\) as hydrophilic components to impart water solubility for the facially amphiphilic dendrimers and polymers. Even though, these systems have their unique advantages when it comes to targeted drug delivery applications they could pose some issues such as non-specific interactions or non-availability of targeting ligands for specific interaction induced effects.

In this thesis we set out to study the utility of zwitterionic moieties as hydrophilic components because, it is well established that zwitterionic materials are effective in reducing the non-specific interactions with biological media\(^15\text{-}^{18}\) and this materials are currently under intense study to explore their plausible utility in tissue engineering, anti-fouling coatings, and therapeutics. Even though, zwitterionic materials have been well studied for surface coatings, introduction of zwitterionic species as hydrophilic components in amphiphilic macromolecules is not thoroughly investigated. Introduction of zwitterionic species as hydrophilic groups in amphiphilic systems have some unique advantages: The assemblies formed from zwitterionic amphiphiles self-organize in such a way that zwitterionic components are exposed to external aqueous environment thus rendering the formed assemblies charge neutral and hence do not interact non-specifically with biomaterials such as proteins. Besides
zwitterionic groups provides a functional handle, which is of great importance for the introduction of targeting moieties such as ligands or antibodies.

To explore these afore mentioned possibilities we designed zwitterionic amphiphilic dendrimers. In chapter 2, we introduced a new method for the facile introduction of zwitterionic species at the end of the dendrimer synthesis by Huisgen click chemistry. We thoroughly characterized this new class of clickable zwitterionic dendrimers. These dendrimers spontaneously form self-assembled aggregates and encapsulates wide range of hydrophobic guests. Zeta potential studies confirmed the charge neutral characteristics of the assemblies, which is shown to minimize their non-specific interactions with biological macromolecules such as proteins. Unlike peg based amphiphiles, zwitterionic dendritic assemblies are not temperature sensitive at biologically relevant temperatures (30 – 40 °C). Zwitterionic amphiphilic dendritic assemblies provide a molecular design to develop molecular level understanding of the requirements for biofouling resistant materials, which can be easily extended to a variety of other zwitterionic combinations and for variety of polymeric scaffolds.

In chapter 3, we installed ligand functionality, biotin at the core of the dendron, to investigate the specific interaction based disassembly between dendritic assemblies and extravidin. Here we hypothesize that short, highly hydrophilic zwitterionic functional groups facilitate the specific interactions and hence leading to effective disassembly. Our preliminary studies with G1 dendron showed that biotin-extravidin interactions indeed result in the disassembly of the aggregates with more than 70% guest release within the first 12 hours. In chapter 4, considering the synthetic complexity of zwitterionic dendrimers, we further simplified the molecular design by developing a molecular design for zwitterionic amphiphilic homopolymers. Here we prepared an activated polymer, which is used as a primary scaffold to
prepare zwitterionic homopolymer. The reactive polymer upon reaction with N,N-decyl, dimethylaminoethane, forms a precursor tertiary amine polymer which upon treatment with bromoacetic acid forms a glycinebetaine based zwitterionic amphiphilic polymer. These polymers are highly soluble in aqueous solutions and also in organic solvents forming micellar and inverse micellar solutions respectively. We investigated the zeta potential of these assemblies at various pH and their corresponding size variations, this provided a reproducible and reversible pattern in the zeta potential and sizes. This amphiphilic homopolymer design is of great interest in the development of polymeric excipients for drug delivery applications.

In chapter 5, with the understanding from the previous chapters, we designed a new class of amphiphilic random copolymers with degradable backbones based on aliphatic polycarbonates. This design is particularly inspired from chapter 4, where we treated reactive polymers with functional secondary amines to form zwitterionic polymers. Here we performed ROP of activated cyclic carbonate monomers to form activated carbonate polymers. These polymers upon treatment with functional hydrophilic and hydrophobic alcohols form an amphiphilic random copolymer. The strategic choice of hydrophobic alcohol here is 2-(pyridyldisulfide) ethanol, acts as crosslinking functionality (in the presence of DTT) forming nanogels. These nanogels undergo stimuli sensitive degradation in the presence of stimuli-sensitive environment because of the presence of pH, enzyme sensitive carbonate backbones, redox-sensitive disulfide functionalities. Besides the final degraded components of these polymers are non-toxic small molecules, which could be clearly by reticuloendothelial system. These nanogel materials provide a new platform for the development of degradable smart polymeric materials for tissue engineering, imaging, and controlled drug delivery applications.
6.2 Future Directions

In this dissertation, we mainly focused on the synthesis of amphiphilic macromolecules, their characterization, and explored their ability to encapsulate hydrophobic guests. We are further interested in introduction of zwitterionic hydrophilic groups, exploring their stimuli-sensitive characteristics, such as multi-stimuli-sensitivity of self-assembled aggregates, cytotoxicity, controlled release inside cells, degradation characteristics and guest release kinetics of nanogels with varying cross-link densities. Further these materials to be of any use they should be biocompatible and suitable for disease relevant applications; these are important aspects that will be factored in as we develop better understanding with current platforms and will be of high importance in our future material choices and design of amphiphilic macromolecules.

6.2.1 Zwitterionic Random Copolymers and Corresponding Nanogels

![Scheme 6.1: a) Schematic of cross-linkable zwitterionic random copolymers and their corresponding assemblies; b) Synthetic scheme for zwitterionic random copolymers without cross-linkable units.](image)

In chapter 5, we presented amphiphilic random copolymers with PEG as hydrophilic species; we are interested in further improving on this design by replacing PEG with zwitterionic moieties, to take advantage of zwitterionic features, while still using PDS moieties to cross-link and form the corresponding nanogels. The synthetic
protocols for the synthesis of zwitterionic random copolymers and nanogels are shown in scheme. The objective here is to synthesize nanogels with the knowledge gained from PEG based materials and gain control over the solubility, guest encapsulation, functionalization or coating, size of nanogels.

6.2.2 Multi-Stimuli Sensitivity of Nanogels

Stimuli-responsive polymeric assemblies are promising materials for variety of biomedical applications, particularly for nanoscopic drug delivery applications\textsuperscript{20,21}. In diseased state a combination of environmental changes occur simultaneously leading to behavioral changes in materials such as protein-folding, signaling cascade of events (over-expression of proteins, changes in the pH of solution etc.). This feature could be used to our advantage for targeted release of drugs at disease site. This can be achieved by incorporating the chemical components that responsive to environmental changes into amphiphilic polymer structures. Our polycarbonate materials are designed explicitly to exploit such environmental changes (pH, protein, and/or redox conditions) as cues to disassemble the aggregates with ensued drug release. Polycarbonate backbones are known to be sensitive to pH and enzymes such as esterase, and the disulfide cross-linked moieties are responsive to glutathione (scheme 6.2). In our future studies we will explore the rate of drug release with the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Scheme_6.2.png}
\caption{The polymer structures and cross-linked nanogels with multi-stimuli components embedded into them}
\end{figure}
combination of these stimuli, such as pH + esterase, esterase + GSH, GSH + pH, and finally with all the three stimuli, i.e, pH + esterase + GSH. These self-assembled aggregates are capable of forming self-assembled aggregates in the size ranges of 10-200 nm making them very attractive candidates for nanoparticular drug delivery and imaging applications.

6.3 Summary

In this chapter we summarized the key aspects of all chapters discussed in this thesis and presented the ongoing research in our laboratories on the amphiphilic polycarbonate materials. We hope that the findings of this dissertation on the new class of zwitterionic amphiphilic macromolecules and polycarbonates provide insight and add to the knowledge base of material design considerations and their optimization for effective drug delivery vehicles.
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