Engineering Stimuli-Responsive Polymeric Nanoassemblies: Rational Designs for Intracellular Delivery of Biologics

Kingshuk Dutta
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ENGINEERING STIMULI-RESPONSIVE POLYMERIC NANOASSEMBLIES: RATIONAL DESIGNS FOR INTRACELLULAR DELIVERY OF BIOLOGICS

A Dissertation Presented

by

KINGSHUk DUTTA

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

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Department of Chemistry
ENGINEERING STIMULI-RESPONSIVE POLYMERIC NANOASSEMBLIES: RATIONAL DESIGNS FOR INTRACELLULAR DELIVERY OF BIOLOGICS

A Dissertation Presented

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DEDICATION

To my wife and parents
ACKNOWLEDGMENTS

The path towards a PhD consists of four key elements: motivation, hard work, intelligence, and a great mentor. I believe that the mentor plays a key role that essentially shapes the future of a graduate student. A mentor should be able to grow interest within students about an area of research, to provide enough guidance to ensure complete intellectual nourishment, to make them think critically, accept challenging problems and aspire for success. If these are the requirements to be a successful mentor, Prof. Sankaran Thayumanavan truly deserves to be the best I have ever experienced. I am privileged to have him as my graduate mentor. Thank you, Prof. Thai, for your continued support through thick and thin of my PhD dissertation research.

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15th June 2020
ABSTRACT

ENGINEERING STIMULI-RESPONSIVE POLYMERIC NANOASSEMBLIES: RATIONAL DESIGNS FOR INTRACELLULAR DELIVERY OF BIOLOGICS

SEPTEMBER 2020

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Biologic drugs have gained enormous research attention in recent years as reflected by the development of multiple candidates to the clinical pipelines and an increased percentage of FDA approval. This is reasoned by the fact that biologics have been proven to deliver more predictive and promising benefits for many hard-to-cure diseases by ‘drugging the undruggable’ targets. However, the challenges associated with biologic drug development are multi-fold, viz, poor encapsulation efficacy, systemic instability, low cellular internalization and endosomal escape capability. Thus, it is essential to develop new molecular strategies that can not only address the associated drug delivery challenges, but also help strengthen the fundamental chemical understanding to meet the future need of this rapidly evolving field.

Designing a supramolecular container that is capable of stably holding sensitive active and releasing them at target site upon environmental changes is a promising solution to tackle many complex challenges associated with functional delivery of therapeutics.
Addressing this requires a basic understanding of the structural and functional factors to be engineered into the delivery vehicle. To this end, we have explored the interactions between synthetic polymers with various biologies to form well-defined self-assembled structures, wherein the function of the encapsulated active is only revealed upon specific structural modulation of the polymer surrounding it. In this dissertation, we have discussed the development of three distinct self-assembly strategies to reversibly capture sensitive biologies, viz. protein, nucleic acid and antibody. A covalent self-assembly strategy is employed for proteins irrespective of their isoelectric points (chapter 2). Our second strategy utilizes non-covalent interactions (electrostatic and hydrophobic) for complexation with negatively charged nucleic acids (chapter 3). Finally, we studied a combination of covalent and non-covalent interactions for encapsulating large proteins and antibodies (chapter 4 and 5). This dissertation will focus on the inherent challenges associated with functional delivery of proteins and nucleic acids. It will highlight the advantages of rational designs to control the complex interplay between the structural features of the polymers and their biological outcomes.
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CHAPTER 1
INTRODUCTION

Adapted in parts from the manuscript: Dutta, K; Das, R.; Medeiros, J.; Thayumanavan, S. Charge-Conversion Strategies for Nucleic Acid Encapsulation and Delivery, 2020.

1.1 Biologics as potential therapeutic agents

In the post-genomic era, development of bio-therapeutics has gained an enormous momentum due to their potential in controlling the human disease portfolio. Since the approval of first recombinant human insulin by FDA in 1982, research interests have surged to utilize bio-therapeutics for clinical applications.¹ A tremendous growth in the biotechnology sector have placed over 7000 biopharmaceuticals in the development pipeline, 74% of which are first-in-class medicines.² Although small molecule drugs are still dominating the pharmaceutical sector, Biologics License Approval (BLA) rates are rapidly increasing, with 21-22 approved drugs each year between 2017 and 2019.³ Based on a recent analysis, biotherapeutics are expected to capture more than one fourth of the pharmaceutical industry by 2020.⁴

Protein, peptide, antibody, and nucleic acids (siRNA, micro-RNA, mRNA and DNA) are classical examples of biomacromolecules studied under the class of biologics (Figure 1.1). The recent success of biologic drug products can be attributed to their higher specificity and potency compared to small molecule therapeutics. Biologics can lead to more predictive and promising therapeutic benefits in case of many hard-to-cure complex diseases, with potential to drug the undruggable scenarios. The origin of such selectivity comes from the macromolecular architecture of biologics with multivalent binding sites.
that dictate precise target recognition leading to exclusive binding events. However, owing to the dynamic structural features, biologics are ultra-sensitive to external environment and inherently unstable. Consequently, it is hard to keep these molecules stabilized in pharmaceutical formulations and deliver into the target tissues in biologically active forms. The loss of structural integrity and denaturation during systemic circulation presents some of the major hurdles in functional delivery of biologics. Apart from this, high molecular mass and polar surfaces of biologics contribute to their poor cellular permeability.\(^1\) Moreover, characterization, manufacturing and handling of biologic drugs are rather stringent and complicated compared to the traditional small molecule medicines. All these challenges cumulatively prolong the drug discovery timeline leading to an increased burden for successful development of bio-therapeutics. Thus, it is essential to develop strategies guided by simple design rules to address the fundamental challenges in non-destructive encapsulation and on-demand targeted release for these complex biomacromolecules.

This chapter aims to provide an overview of the challenges associated with the successful delivery of biologic drugs and the current state-of-the-art technologies developed to address these challenges. The first part of this chapter will specifically focus on the hurdles related to systemic stability, cellular uptake, endosomal escape, cytotoxicity and immunogenicity issues of the biologics. The second part will discuss engineered delivery platforms to address these challenges with a particular focus on three key classes of biologics, e.g., protein, antibody and nucleic acid therapeutics.
Figure 1.1 Comparison between small molecule and different forms of biologic drugs (reprinted with permission from reference 5 and Amgen).

1.2 Salient features and key determinants for efficient delivery of biologic drugs

Developing effective strategies for biopharmaceuticals requires understanding the key constraints associated with the delivery of biologic drugs. The following sections will discuss the major challenges associated in dealing with delicate biologics.

1.2.1 Stability of biomacromolecules under in vitro and in vivo conditions

Most biologics suffer from poor stability owing to several modes of degradation pathways. Proteins are prone to denaturation, aggregation, chemical and proteolytic degradation when exposed to normal in vitro and in vivo environmental conditions. Nucleic acids considered to be relatively stable, can also undergo chemical and proteolytic degradation in presence of certain enzymes and low pH, respectively. Owing to these stability challenges, different routes of administrations were investigated, e.g., oral, transdermal, mucosal, pulmonary, nanoparticle-based, and depot injections.
these, oral delivery is the most favored one for the ease of administration and high patient compliance. However, proteolytic and enzymatic degradation of the biopharmaceuticals in stomach and liver, along with low bioavailability due to the hydrophilic macromolecular structures, have plagued the scope of oral administration. Moreover, pre-systemic metabolism or ‘first-pass effect’ in liver greatly diminishes the bioavailability of the drug released into systemic circulation. Finally, rapid clearance through kidney shortens the biological half-life of the drug reducing the possibility to reach its target site to impart desired therapeutic action.

1.2.2 Cytotoxicity mediated by nanoparticle-based systems

To address the stability concerns and intracellular trafficking, various delivery vectors are proposed consisting polymer, lipid, peptide and inorganic nanoparticle based systems. However, a relatively low number of nanoparticle based drug delivery formulations are actually considered for clinical development and finally get cleared by U.S. Food and Drug Administration (FDA). A recent analysis shows that the technology translational efficacy is 5% between global clinical trials to publication numbers and, only 10% of the clinical trials give rise to successful products in market. One of the major...
reasons associated with such limited number of clearance is the cytotoxicity issues of the designed delivery agents.

For nanoparticle-based drug delivery systems, cytotoxicity is majorly governed by four prime factors: (a) material composition; (b) morphological parameters, e.g., size and shape; (c) surface charge; and (d) hydrophobicity (Figure 1.2). Nanoparticles with diverse chemical functionalities can bind to extracellular regions like plasma membrane, ion-channels, cell surface receptors, and either damage or block the machineries from functioning normally. Upon penetration into the cells, nanoparticles can even interfere with the intracellular organelles like mitochondria, lysosome, cytoskeleton and nucleus via changing metabolic pathways, generating reactive oxygen species and inhibiting protein functions. The effect of nanoparticle size on toxicity is dependent on several other factors like the choice of materials, physicochemical properties, and cell types. A consensus in this aspect is to utilize 50-200 nm particle to be able to minimize cytotoxic effect exerted by the delivery system. Shape of nanoparticles also has profound effect on toxicity induced by increased reactivity and exposure in circulation. Generally, spherical particles were reported to be less-toxic compared to non-spherical ones. Hydrophobic particles, with identical surface charges, were found to be more cytotoxic when compared to the hydrophilic particles due to the increased interaction with hydrophobic cellular membrane.

The most influential parameter might be the nanoparticle surface charge mediated cytotoxicity. Several reports had suggested that cationic nanoparticles can change the plasma membrane and mitochondrial membrane potential (MMP), thereby creating punch-holes, increasing the production of ROS and activating caspases and cytochrome C
to induce apoptosis.\textsuperscript{14} Whereas anionic and neutral nanoparticles are considered to relatively non-toxic owing to the diminished interaction with the negatively charged lipid bilayer of the plasma membrane.\textsuperscript{17-18}

1.2.3 Immunological effects of designed nanoparticulate drug delivery vehicles

A growing concern in therapeutic biologics development is the induced immunogenicity mediated by particulate material in the delivery formulation or drug aggregates due to instability under in vivo conditions.\textsuperscript{19} Recognition of nanoparticles as foreign objects by immune system could be deleterious, initiating immunosuppression or immunostimulation leading to inflammatory responses, acute toxicity and loss of therapeutic efficacy of the biologic drug.\textsuperscript{20-21} Immunostimulatory responses can be derived by antigenicity of the biopharmaceuticles, causing the production of neutralizing antibody that is capable of dually recognizing endogenous target and exogenous delivery vector. Also, nanoparticulate systems can invoke inflammatory responses upon recognition as foreign substances and release of cytokines by immune cells to trigger destruction of the substance.\textsuperscript{21} Note that, in vaccine development applications nanoparticulate materials can act as adjuvants which are taken up by dendritic cells and macrophages, leading to favorable immune response.\textsuperscript{22} However, downregulation of immune responses (immunosuppression) can alter the therapeutic effect and promote infection as harmful foreign substances are undetected by the immune system.\textsuperscript{21}

1.2.4 Challenges involving endosomal escape

One of the major bottlenecks in biologics delivery is entrapment of the cargo into a vesicle like structure, endosome or early endosome, upon internalization into the cell through various endocytosis mechanisms. While early endosome matures into late
endosome and lysosome, the pH of the vesicles decreases to \(~6.5\), \(~6.0\) and finally to \(~5.0\), respectively.\(^{23}\) Interestingly, the biologics encapsulated in the nanoparticle cargo should be able to evade from the early or late endosome stage. Entrapped cargo eventually gets degraded inside lysosome losing the therapeutic efficacy.\(^{24}\) As endosomal escape is often considered as the rate-limiting step for the delivery of biologics, several studies have elucidated probable escape pathways with the help of specific molecular designs.\(^{23}\) The key to endosomal escape is dictated by the interaction of the nanoparticle with the endosomal membrane, leading to four reported pathways: (i) membrane fusion; (ii) osmotic rupture, (iii) swelling of nanoparticle; (iv) membrane destabilization (Figure 1.3).\(^{23}\) Fusion of nanoparticles with the endosomal membrane can be guided by hydrophobic compatibility, fusogenicity and pH.\(^{15, 25-26}\) Whereas osmotic pressure effect, also known as ‘proton sponge’ effect, is executed by the influx of protons, counter anions and water molecules into the endosomal vesicle due to buffering effect exerted by the nanoparticle leading to enlargement and lysis of the endosomal membrane.\(^{27-29}\) Crosslinked polymeric

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**Figure 1.3** Various proposed pathways for endosomal escape (reproduced with permission from reference \(^{23}\)).
nanoparticles were also reported to have tunable swelling property introducing mechanical strain beneficial for evading endosome.\textsuperscript{30-31} Similarly, pH sensitive polymers with specific architectures (hyperbranched and dendritic polymers) showed efficient destabilization of endosomal membranes.\textsuperscript{32-33} Although the endosomal escape process is grossly classified into the four mechanisms mentioned above, it is likely that more than one mechanism might be acting simultaneously to impart the overall fate of the endocytosis process.

1.3 Responsive polymers as suitable candidates for therapeutics delivery

To address the above-mentioned critical issues involved in the delivery of biologics, several drug delivery strategies have been proposed. As the primary goal of this thesis is to address the delivery issues with three key biologies, viz., protein, nucleic acid (siRNA) and antibody therapeutics, the following sections are devoted to discuss the state of the art technologies in each specific case.

1.3.1 Protein: non-covalent vs. covalent encapsulation strategies

Protein therapeutics is considered to be one of the most promising areas of recent times in the field of pharmaceutical drug development.\textsuperscript{34-35} Being directly involved in various biochemical pathways, proteins perform the pivotal role to dictate the overall fate of major cellular processes.\textsuperscript{36} Thus, regulating specific protein expressions, by either attenuation or enhancement, should furnish significant control over such cellular signaling mechanisms. Being more specific and possessing less cytotoxicity risks compared to non-targeted small molecule drugs, direct delivery of functional proteins into cells is expected to provide exquisite benefit towards many challenging diseases.

To this end, a wide array of brilliant approaches has been attempted to design delivery agents based on liposome, hydrogels, inorganics like silica, carbon nanotubes and
Figure 1.4 (a) Liposomal drug delivery systems; (b) Lysine head-group containing cationic liposomes for intracellular protein delivery (c) Biocompatible hydrogels for oral delivery of proteins (reproduced with permission from references 37-39).

Quantum dots.6, 40-43 Here, we will majorly focus on examples of liposome and polymer based approaches relying on non-covalent and covalent interactions.

Liposome is considered to be an excellent biocompatible nanocarrier for the delivery of both hydrophobic drugs (in bilayer) and hydrophilic protein (inside aqueous lumen) therapeutics (Figure 1.4).37 β-galactosidase (β-gal, ~465 kDa, with four subunits of 116.3 kDa) enzyme has been efficiently encapsulated with the help of a cationic guanidinium-cholesterol lipid (bis (guanidinium)-tren-cholesterol, BGTC) and helper dioleoyl phosphatidylethanolamine (DOPE) lipid, and found to be active after intracellular
Figure 1.5 (a) Protein PEGylation and subsequent formation of nanoparticles; (b) Recent approaches for modified PEGylation methods (reproduced with permission from references 44-47).

delivery. In another study (Figure 1.4b), a lysine headgroup containing cationic liposome was utilized to deliver bovine serum albumin (BSA, 66 kDa). Several apoptotic mediator enzymes (caspase 3, caspase 8 and granzyme B) were encapsulated in a cationic lipid based formulation consisting trifluoroacetylated lipopolyamine (TFA-DODAPL) and DOPE. Upon intracellular delivery, the enzymes remained functional and induced apoptosis. However, these systems generally suffer from poor encapsulation efficacy due to the lack of driving force for hydrophilic proteins to preferentially encapsulate from bulk media to the interior aqueous pool of liposome. This issue can be partly addressed by hydrogels based on natural (polysaccharides, protein and DNA based) and synthetic (polyesters, polyamides, poly(ethylene oxide), polyorthoester and polyporphazene) polymers for protein encapsulation. Nonetheless, application of hydrogel based systems are limited by the localized delivery of therapeutic biologics.
In this context, covalent protein delivery strategies with polymers would be beneficial for rendering high encapsulation efficacy owing to the covalently attached proteins with the delivery vehicle and for imparting structural and in vivo stability from the polymer backbone. PEGylation is one of the FDA approved techniques heavily used in biopharmaceutics development (Figure 1.5). Attachment of PEG chain shows prolonged circulation half-life, reduced immunogenicity and cytotoxicity. In other scenarios, covalent ligation techniques have been employed via polymeric nanocarriers to effectively improve encapsulation efficacy and in vivo stability (Figure 1.5). However, many of these methods require organic solvents and harsh synthetic conditions that tend to irreversibly modify the protein cargo, resulting in denaturation the sensitive cargo and diminished activity.

To address these issues, reversible covalent strategies (Figure 1.6) are being explored that can not only provide high fidelity protein encapsulation, but also help preserve structure and activity through ‘traceless’ release of protein in native form.
1.3.2 Nucleic acid: state of the art approaches in RNAi

Nucleic acid based gene silencing, specifically RNA interference (RNAi), has created an enormous opportunity in therapeutics development.\textsuperscript{57} Despite many promising advantages, RNAi technology possesses several limitations that have hindered its applicability and therapeutic potential.\textsuperscript{7} Poor cellular internalization and systemic instability of naked siRNA in presence of serum nucleases followed by fast renal clearance are two major biological barriers for efficient intracellular delivery of siRNA.\textsuperscript{58} In addition, undesirable toxicity from chemical modifications of siRNA or transfection agents increases the barrier for its successful implementation.\textsuperscript{59} Ever since its inception, these hurdles are reflected in the commercial development of RNAi based therapeutics with only one recent FDA approval in this drug category.\textsuperscript{60} Thus, designing an efficient and safe siRNA delivery strategy still remains a grand challenge to realize the fullest potential of such powerful technology.

To address the critical pitfalls of siRNA-based therapeutics, several approaches have been proposed which can be classified into two major categories: (a) chemical modification of siRNAs and (b) siRNA carriers.\textsuperscript{58, 61} In the first case, siRNAs are chemically modified to enhance its systemic stability by providing nuclease resistance (e.g. phosphorothioate, boranophosphate, 2’OMe modifications; see Figure 1.7 for a general overview of oligonucleotide modifications) and to increase the biological half-life (PEGylation, hydrophobic modifications with cholesterol, bile or fatty acid).\textsuperscript{58} These approaches bring in complexity within siRNA molecules typically resulting in increased toxicity and reduced silencing ability of the delivered siRNA.\textsuperscript{62} On the other hand, potent
siRNA carriers like viral vectors impart high transfection efficacy; albeit introduces elevated risk of immunogenicity and undesired mutagenesis.\(^7\) In this scenario, non-viral

**Figure 1.7** Chemical modifications on oligonucleotides along with their operating mechanisms (reproduced with permission from reference \(^{61}\)).
delivery vehicles\textsuperscript{63-66} like cationic lipids, peptides and polymers provide rather clean and straight-forward but efficient choices, wherein electrostatic forces between cationic delivery agents and negatively charged siRNA molecules result in a condensed complex (Figure 1.8a). The stability of such complexes in biological milieu is essentially proportional to the electrostatic attractions between the siRNA and the siRNA-binder, which is dictated by (i) the amount of cationic charge in play and also by (ii) the ability of these molecular systems to optimize the interactions (e.g. controlled by factors such as

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1_8.png}
\caption{(a) Non-viral vectors for nucleic acid delivery (reproduced with permission from reference \textsuperscript{67}); (b) Molecular design strategy of guanidinium ion appended telechelic dithiol monomers for siRNA delivery (reproduced with permission from reference \textsuperscript{68}).}
\end{figure}
molecular flexibility, etc.). Typically, a high positive charge, dictated by the high N/P ratio (molar ratio of cationic nitrogen in delivery agent to phosphate groups in siRNA), is found to be critical for complete complexation and protection of siRNA (Figure 1.8b).\textsuperscript{68} Interestingly, complexes with high cationic charge, under in vivo conditions, poses increased toxicity and adverse side effects (plasma membrane damage, release of cytochrome C from mitochondria and alteration in membrane potential).\textsuperscript{14} As a remedy, reduction of positive charge at lower N/P ratio in the complex was investigated to attenuate toxicity.\textsuperscript{69} However, this approach might compromise the complex stability under in vivo conditions and does not address the inherent problem of cationic charge driven toxicity. Moreover, competitive binding with negatively charged serum proteins becomes an important parameter at lower N/P ratio which can even result in the leakage of siRNA impeding the therapeutic efficacy. PEGylation of cationic vectors, another promising strategic solution to this problem, enhances steric crowding in the system diminishing the binding efficacy with cationic vectors and retards efficient cellular uptake of siRNA carriers.\textsuperscript{70} In essence, the paradox with cationic charge vs. toxicity vs. binding affinity and stability of siRNA delivery vectors demands a new potent self-assembly strategy for efficient complexation and delivery of siRNA cargo.

1.3.3 Antibody: emerging opportunity to target intracellular proteins

Probing various cellular processes by specific targeting of proteins is immensely important in developing therapeutic candidates.\textsuperscript{71} Antibodies, being the key biomacromolecule utilized for targeting purposes, have so far served the diagnostic need via targeting extracellular proteins. On the other hand, intracellular targeting of specific proteins has only been possible with techniques like microinjection and electroporation,
owing to the membrane impermeable nature of large hydrophilic antibody.\textsuperscript{71-72} However, given the concerns regarding cell membrane damage, cytotoxicity and low throughput methods, those processes are limited to in vitro applications.\textsuperscript{71}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{(a) Molecular design strategy for the endosomolytic peptide based delivery of antibody; a peptide based fast intracellular delivery strategy for (b) fluorescent IgG antibody; and (b) anti-nuclear pore complex antibody (reproduced with permission from references 73-74).}
\end{figure}

To expand the enormous opportunity associated with intracellular targeting, molecular designs for encapsulation of antibodies have been proposed.\textsuperscript{71, 75-79} An endosomolytic peptide is developed by substituting leucine with glutamic acid of a cationic-lytic peptide, M-lycotoxin (Figure 1.9a).\textsuperscript{73} Cytosolic localization of IgG is observed after rupture of endosomal membrane. On the other hand, liposome made with a combination of aminoglycoside lipid dioleyl succinyl paromomycin (DOSP) and imidazole-based helper lipid MM27 has been identified as superior for the intracellular delivery of anti-cytokeratin8 antibody (anti-K8).\textsuperscript{40} Harnessing the strengths of lipid and peptide based systems, a high-speed antibody delivery strategy has been reported with liposomes modified with cell penetrating peptide, octaarginine (R8) and pH-sensitive
fusogenic peptide, GALA.\(^7^4\) Within 30 min of incubation, ~98% of cells are transfected by IgG containing liposomes with endosomal escape and predominant cytosolic localization (Figure 1.9b).

**Figure 1.10** Formation of PIC micelles and intracellular delivery of antibody (reproduced with permission from reference \(^8^0\)).

Further, delivery of nuclear pore complex (anti-NPC) antibody proved the retention of structural and functional features of the delivery antibody (Figure 1.9c). In another elegant approach, anionic polypeptide fused IgG is encapsulated within cationic liposome to inhibit two intracellular targets - multidrug resistance-associated protein 1 (MRP1) and the transcription factor NF-κB.\(^7^2\) Polymeric nanoparticles based on polyethyleneimine (PEI), poly(lactic-co-glycolic acid) (PLGA), poly(ethylene glycol)-block-poly[N-(N′-2-aminoethyl)-2-aminoethyl]aspartamide] \(\text{PEG-b-PAsp(DET)}\) and \(\text{MePh}_{13-b}\)-dG\(_5\).
polyoxanorbornene diester copolymer (dG: dense guanidinium functionality) have also been explored for intracellular trafficking of antibodies. Polyion complex (PIC) comprising copolymers (PAsp: poly[N-(N’-(2-aminoethyl)-2-aminoethyl)aspartamide]) have been shown to successfully deliver IgG and anti-NPC antibody (Figure 1.10). However, given the enormous possibility of antibody therapeutics, there is still a dearth of design strategies for intracellular targeting of disease relevant proteins.

1.4 Summary & dissertation overview

In this chapter, the importance of biologic drugs and the challenges associated with the delivery via biopharmaceutical products are discussed. Along this line, recent state of the art drug delivery strategies for three key important biologic candidates, viz., protein, nucleic acid (siRNA) and antibody therapeutics, are discussed. For protein therapeutics, non-covalent and covalent approaches comprising lipid and polymeric delivery vehicles have been discussed describing the advantages and pitfalls. Being negatively charged, nucleic acids like siRNAs have been encapsulated via cationic nanoparticles, which in turn, raises toxicity issues in proportion with gene silencing. Recent state of the art techniques and existing issues have been discussed have been discussed. Delivery of antibody drugs for intracellular targets is gaining recent attention and has been discussed. However, a dearth of strategies reveals significant challenges associated with the antibody delivery and a dire need of novel drug delivery vehicles.

Targeting the need for advanced molecular designs of delivery vehicles, this dissertation will discuss the design principles of developed strategies that target the existing challenges in the delivery of protein, siRNA (nucleic acid) and antibody therapeutics. Chapter 2 describes a templated self-assembly strategy with a self-immolative polymer to
shrink-wrap protein cargoes and their intracellular trafficking. For siRNA delivery, a tricomponent symbiotic self-assembly strategy has been described in chapter 3 comprising siRNA, polymer and lipids, that address the toxicity challenges from cationic particles. In chapter 4, a rigorous structure-activity study has been reported to improve the conjugation efficacy of protein with the designed activated ester containing self-immolative polymer. Chapter 5 describes engineering of the developed polymer platform to incorporate an electrostatic handle for boosting conjugation efficacy of larger proteins and antibody therapeutics. Summary is described in chapter 6 along with future directions focusing on some ongoing research efforts and prospective ideas as a corollary of the developed drug delivery strategies.
1.5 References


34. Cobo, I.; Li, M.; Sumerlin, B. S.; Perrier, S. "Smart Hybrid Materials by Conjugation of Responsive Polymers to Biomacromolecules" Nat. Mater. 2015, 14 (2), 143-59.


Functional Foreign Transmembrane Protein within a Silica Nanostructure" J. Am. Chem. Soc. 2009, 131 (40), 14255-14257.


46. Gaertner, H. F.; Offord, R. E. "Site-Specific Attachment of Functionalized Poly(Ethylene Glycol) to the Amino Terminus of Proteins" Bioconjugate chemistry 1996, 7 (1), 38-44.


63. Roy, R.; Jerry, D. J.; Thayumanavan, S. "Virus-Inspired Approach to Nonviral Gene Delivery Vehicles" Biomacromolecules 2009, 10 (8), 2189-2193.


CHAPTER 2

TEMPLATED SELF-ASSEMBLY OF A COVALENT POLYMER NETWORK FOR INTRA-CELLULAR PROTEIN DELIVERY AND TRACELESS RELEASE


2.1 Introduction

Using proteins as a therapeutic is attractive, as this promises to directly address genetic deficiencies and therefore mitigates side-effects that plague many small molecule drugs.¹ Potential side-effects from small molecule binders are understandable, as these molecules must be designed to target a specific protein in our complex biological system, highlighted by the nearly 20,000 protein-encoding genes. On the other hand, proteins can directly compensate for a specific deficiency and therefore the drug development is less heuristic. However, realizing the full potential of protein-based therapeutics has been difficult, mainly due to their in vivo instability and immunogenicity. To overcome these issues, approaches to modify protein surfaces have been taken, starting with PEGylation that has been shown to enhance protein circulation lifetimes.²-⁴ More recently, strategies that allow for attaching other polymers to proteins have been developed in order to endow these conjugates with stimulus-responsive characteristics or to realize new self-assembled structures.⁵-⁸ These reports provide examples of innovative strategies that allow for
enhanced circulation lifetimes and thus have impacted the utility of proteins that function in the extracellular environment.\textsuperscript{9}

The next level of challenge involves the ability to develop systems that can handle intracellular proteins, where trafficking the cargo across a cellular membrane is a major hurdle. Two limiting approaches have been taken to address this need, both of which involve non-covalent self-assembly. First involves electrostatic binding of proteins to complementarily charged polymers and nanoparticles.\textsuperscript{10-12} The second approach includes encapsulating proteins in water-filled compartments, such as liposomes.\textsuperscript{13} A limitation of the former approach comprises non-specific fouling of the complex surfaces due to electrostatic interactions and the associated toxicities.\textsuperscript{14} The latter approach has the potential to address the fouling issues, but is fraught with low loading capacities, especially when charge-neutral lipids are used.

\textbf{Figure 2.1} Schematic representation of the formation of a covalent polymer network using the protein as the template and its traceless and triggered release in a reducing environment.
We envisaged that a covalent self-assembly approach, where the protein cargo itself acts as the template for the polymer to self-assemble around it, has the potential to encapsulate proteins with high fidelity and present charge-neutral surface functionalities (Figure 2.1). The key design hypothesis here is that an initial reaction between the side chain functionalities of a random copolymer and multiple surface-exposed functional groups of a target protein would cause a few polymer chains to organize around the protein. This covalent capture then can act as a template to form a polymer sheath around the protein through a polymer side-chain crosslinking step, as schematically illustrated in Figure 2.1. We envisaged that the high-fidelity protein encapsulation within this sheath would be aided by: (a) convex surface of globular proteins on which the reactive functional groups are presented, and (b) high-yielding and multivalent reactions are presented; and (b) high-yielding and multivalent reactions between the protein and polymer side chains.

2.2 Results and discussion

2.2.1 Design and synthesis of a self-immolative polymer for lysine conjugation

Cysteine and lysine are two popular handles for conjugating polymers with proteins, because of their nucleophilicity.\textsuperscript{15-22} Because of the surface availability in larger number of proteins, lysines are generally preferred. However, this amino acid presents a challenge in that it is more difficult to functionalize them in a form, whereby they can be tracelessly liberated in the intracellular environment. We hypothesized that placing reactive side-chain functionalities in a polymer, with responsive self-immolation characteristics,\textsuperscript{23} would result in a novel and general system that is capable of encapsulating proteins with high fidelity and tracelessly releasing them upon encountering a target microenvironment.
The structure of the target polymer P1 (Scheme 2.1), which satisfies all the design requirements, was synthesized by RAFT polymerization. Reaction of an amine with the p-nitrophenylcarbonate (NPC) moiety in P1 will produce the corresponding carbamate, as shown in Scheme 2.1. The polymer is first treated with the protein, where multiple lysine moieties are reacted with the NPC groups in the polymer chains. The remaining NPCs are reacted with a diamine crosslinker to complete the sheath formation around the protein (Figure 2.1 and Scheme 2.1). Note that a disulfide moiety is placed at the β-position, relative to the carbamate oxygen, which serves to render the polymer responsive to the more reductive intracellular environment, compared to the extracellular space. Reductive cleavage of the disulfide will result in carbamate cleavage to release the original amine. This reaction will

Scheme 2.1 Chemical structures of polymers and the reaction scheme for protein conjugation, crosslinking to generate the nano-assembly and its release in the presence of a reducing agent.
cause both the polymer being uncrosslinked and the protein being tracelessly liberated from the polymer.

**Figure 2.2** (a) Top: Bright Field Cryo-TEM image of NA-CytC$^\text{PEG}$ showing both individual and small clustered particles. Inset shows a cluster of 3 protein nanoassemblies, Bottom: HAADF image of a NA-CytC$^\text{PEG}$ nanoassembly. The bright spots with diameters of 2-3 nm are caused by the Fe-content of Cyt C, inset is NA-Empty$^\text{PEG}$ particle with no detectable Fe from EDS (~50 nm); (b) MALDI-MS analysis of the trypsin digest from encapsulated and naked Cyt C; (c, d) SDS-PAGE of the NA-CytC$^\text{PEG}$ under non-reducing and reducing conditions (10 mM DTT, 37 °C for 4 h).

### 2.2.2 Conjugation studies and characterization of protein-polymer nanoassemblies

To test our design strategy, we chose cytochrome C (CytC, pI 9.6) as a model protein, because of its distinct cellular readout in the form of apoptotic cell death. After initially reacting CytC with P1, the polymer-protein conjugate was further secured by crosslinking with ethylenediamine (ED) or tetraethyleneoxide-bis-amine (PEG) to afford nano-assemblies NA-CytC$^\text{ED}$ and NA-CytC$^\text{PEG}$, respectively. Note that the reaction between the NPC moiety and an amine produces p-nitrophenol as a by-product, the distinct absorption of which is conveniently monitored. Therefore, the protein conjugation step was
quantified using the evolution of the absorption spectrum. Encapsulation efficiency and loading capacity were found to be 64-67% and 5-7%, respectively. Dynamic light scattering (DLS) measurements revealed the hydrodynamic diameters of native CytC and the protein-containing nanoassembly to be ~4 nm and ~8-10 nm, respectively. Moreover, zeta potential measurements revealed that the surface of the complex is charge-neutral, suggesting that the complex surface is dominated by the PEG moieties from P1. Figure 2.2a (top) shows a Cryo-EM image of NA-CytC\textsuperscript{PEG}. The average individual particle size is in the 10-30 nm range which is in agreement with DLS data. To obtain a more detailed insight into the protein distribution within the nano-assemblies we employed High Angle Annular Dark Field Microscopy (HAADF) and Energy Dispersive X-Ray Spectroscopy (EDS) at cryo temperatures. Figure 2.2a (bottom) shows a HAADF image of an individual nano-assembly. The whole assembly has a diameter of about 20 nm and the bright spots in the 2-3 nm range are caused by the Fe-content of discrete Cyt C molecules (compare with the empty assemblies in the inset) and EDS analysis confirmed the presence of Fe.

An important objective of this work is to use the polymer sheath to protect the protein from protease degradation. To rigorously test for this, we subjected the polymer-protein conjugate to protease digest with trypsin and analyzed the products using MALDI mass spectrometry. While the unprotected Cyt C afforded characteristic peptide fragment peaks, the conjugate at the same protein concentration did not afford any discernible fragments (Figure 2.2). These results show that assemblies do indeed protect the protein. The conjugates also seem to be generally stable in serum.

Next, we were interested in testing whether the encapsulated protein can be released in a reducing environment. We first tested this possibility using gel electrophoresis (SDS-
As anticipated, when the protein is conjugated to the protein, no bands corresponding to the protein was found (Figure 2.2). When the same SDS-PAGE gel was run under reducing conditions, appearance of protein bands clearly indicated that the encapsulated protein can be re-leased. This is the first indicator, suggesting that the protein release using the reductive self-immolative linker is feasible. We utilized SDS-PAGE experiments to quantify the amount of proteins inside our nanoassemblies. After treating the nanoassemblies with excess dithiothreitol (DTT), the intensity of the protein band in the gel is compared with native proteins of different concentrations to estimate the amount of proteins present inside the nanoassembly (Figure 2.2).

Figure 2.3 Structure and function of released Cyt C from the NA-Cyt C\textsuperscript{PEG}, evaluated by (a) CD spectroscopy; and (b-c) ABTS activity assay: b- assay kinetics, c- % activity of samples treated with DTT.
2.2.3 Structure and activity studies of released protein

The protein encapsulation and release process would be a futile exercise, if the methodology does not preserve the structure and function of the protein upon release. To this end, the secondary structure of the released protein was examined by circular dichroism (CD) spectroscopy, the spectrum of which was found to be identical to that of the native Cyt C (Figure 2.3). This suggests that the conjugation and release processes did not alter the secondary structure of the protein. We also claim that the strategy leads to a traceless release of the protein in reductive environment. To test this hypothesis, the released protein was analyzed by mass spectrometry. The m/z for the released protein matched the native Cyt C, suggesting that there are no remnants of the polymer after the protein’s reductive release.

Next, we investigated whether the function of the protein is maintained by quantifying the released protein’s activity using an ABTS assay (based on 2,2’-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (Figure 2.3b-c). While the nanoassembly did not exhibit any enzymatic activity, the activity recovery was nearly quantitative when the assembly was treated with 10 mM DTT (compare the activities of native Cyt C and released Cyt C in the presence of 10 mM DTT in Figure 2.3). This activity recovery was also found to be dependent on the concentration of DTT and thus the extent of protein release. These results show that the polymer shell can act to ‘turn-off’ the protein function, until it is released in its target environment. Both structure and function recoveries were found to be independent of the crosslinker length in ED and PEG linkers, as shown in Figure 2.3.
2.2.4 **Intracellular delivery and cellular activity studies**

The ultimate goal of the proposed research is to utilize this polymer coating to traffic the protein across the cellular membrane and release it in the cytosol. It is the higher redox potential of the cytosol that is being targeted for selective release. To track the location of the protein under confocal laser scanning microscopy (CLSM), Cyt C was labeled with rhodamine B and the cell nucleus was stained with hoechst 33342. After 4 h incubation, well-distributed red fluorescence from labeled proteins was observed (Figure 2.4a), while negligible fluorescence was observed from cells that were treated with an identical concentration of naked proteins. These results suggest that the polymer conjugate has better access to the cells, compared to the native protein itself. The time course of

![Figure 2.4](image-url) HeLa cells treated with NA-Cyt C\textsuperscript{PEG} conjugates to study cellular uptake: (a) 4 h post-incubation; (b-c) endosomal co-localization and escape at 4 h and 24 h, respectively; (green: lysotracker; red: rhodamine B; blue: hoechst); (d) cell viability after 72 h (dosage represents amount of NA-Protein conjugate); (e) detection of activated caspase-3/7 after 72 h using the Cy5-tagged assay reagent; scale bar: (a) 50 µm, (b, c, e) 10 µm.
cellular internalization process was also monitored under CLSM for this conjugate. Since the most likely pathway for uptake is endocytosis, we were next interested in evaluating whether the proteins are stuck in the endo-some or they escaped the endosome to get into the cytosol. For a cytosolic delivery, the latter is desired. Accordingly, the endosomes were labeled with lysotracker green. The data clearly indicate that the nanoassemblies enter the cells through the endosomes (see co-localization of lysotracker green and rhodamine-B label after 4 h incubation (Figure 2.4b)), but the proteins escape the endosomes over time as seen by the dominant red color in the cell in the 24 h image (Figure 2.4c).

To evaluate whether the delivered Cyt C is active, we evaluated the apoptotic cell death in response to the protein delivery. Cyt C is known to induce apoptosis through interaction with apoptotic protease activating factor 1 (Apaf-1) in cytosol and activation of pro-caspase-9, which in turn initiates pro-caspase-3 leading to activation of caspase dependent apoptotic pathways. The dose-dependent decrease in cell viability of the nanoassembly (Figure 2.4d), combined with the lack of toxicity for the corresponding concentration of the free nanoassembly or naked Cyt C, indicate that the cytosolically delivered Cyt C is causing apoptosis. Further-more, the mechanism of action of Cyt C allows the direct interrogation whether the caspase-dependent pathway is activated in the cytosol. A fluorimetric immunoassay that causes a caspase product to be intercalated the DNA in the nucleus was utilized to assess this possibility. The co-localization of the caspase-processes, cy5-tagged reagent (red) and the nuclear stain (blue) confirmed the apoptotic nuclei in the cells (Figure 2.4e). Control experiments show that the nanoassembly without the Cyt C does not cause the activation of the cy5-tagged reagent.
2.2.5 Versatility of the protein encapsulation approach

The true testament to the versatility of this strategy is the applicability to a broad range of proteins. To test this, we used two other proteins, viz. lysozyme (Lys) and ribonuclease A (RNase A). Both proteins were found to be success-fully conjugated with the polymer P1. SDS-PAGE, CD, and activity studies also show that the encapsulated protein can be released with high fidelity under reducing conditions with high retention in both structure and function (Figure 2.5a-b). It is interesting to note that the overall kinetics of proteins release was observed to have the order: Cyt C<RNase A<Lys. The release kinetics seems to correlate with the number of sur-face exposed lysines in each protein (Cyt C: 19, RNase A: 10, Lys: 6).27-28 This is understandable, since the higher number of anchoring points requires more sites for the reducing agent to process during release. Finally, the intracellular delivery and activity of these proteins were also evaluated (see Figure 2.5).

Figure 2.5 SDS-PAGE showing protein release (reducing conditions), cellular uptake (4 h) and viability (72 h) in HeLa cells for (a) NA-Lys and (b) NA-RNase A conjugates, scale bar: 50 µm.
Figure 2.5). While lysozyme is expected to be innocuous to cells, RNase A with access to cytosolic RNA can initiate cell death.\textsuperscript{29-30} Although both proteins were taken up by the cells as assessed by CLSM studies, the lysosome-containing nanoassembly did not induce any cell death, whereas the RNase A-bearing nanoassembly had a profound effect on cell viability (Figure 2.5). The proteins studied so far are basic (pI >8.5) with a MW of 12-15 kDa. To further test this system, we also investigated the encapsulation and release properties of eGFP with higher MW and lower pI (MW ~27 kDa, pI 5.5). SDS-PAGE and cellular uptake studies show that this protein, too, can be successfully encapsulated and delivered.

2.3 Summary

In summary, we have developed a versatile strategy for the encapsulation of proteins and their traceless release in response to a specific trigger. The encapsulation is templated by the lysine handles in the protein itself, which are then used to wrap the protein with a polymer sheath in a secondary crosslinking step. The versatility of the approach is highlighted by the fact that: (i) it utilizes a functional handle that is abundantly available on the surface of >85\% the globular proteins, which renders the strategy broadly applicable; (ii) the target protein is encapsulated with high fidelity, i.e. high loading capacity; (iii) the cargo is protected from degradation by proteases; (iv) the protein activity is masked in the encapsulated state; (iv) the polymer sheath is re-moved tracelessly with high efficiency in response to a target intracellular environment; (v) the native structure and function are retained upon release; (vi) the protein can be delivered with high fidelity into the cytosol; and (vii) activity of the protein is regained in the cytosol. Thus, we believe that
this simple and general strategy will serve to produce a potent protein therapeutic delivery platform for a broad range of proteins.

2.4 Experimental

2.4.1 Materials

All chemicals, polyethylene glycol monomethyl ether methacrylate (PEGMA; MW 500), 2,2′-dithiodipyridine, 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (chain transfer agent), D,L-dithiothreitol (DTT), lysozyme, RNase A, cytochrome C and rhodamine B isothiocyanate were obtained from Sigma-Aldrich and were used without further purification unless otherwise mentioned. 2,2′-azobis-(2-methylpropionitrile) (AIBN) was procured from Sigma-Aldrich and purified by recrystallization before usage. Pyridyl disulfide ethyl methacrylate (PDSMA) was synthesized using previously reported procedure.31

2.4.2 Synthesis of p(PEGMA-co-PDSMA), P_CoP

PDSMA (0.511 g, 2 mmol), PEGMA (1 g, 2 mmol) and 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (55.7 mg, 0.2 mmol) were weighed and dissolved with 2 mL THF in a 25 mL Schlenk flask. To the reaction mixture, 1 mL AIBN (6.7 mg, 0.0408 mmol) solution in THF was added and mixed for 5 min. The flask was purged with argon and performed three freeze-pump-thaw cycles. After that the reaction vessel was sealed and transferred to an oil bath preheated at 70 °C. The polymerization was quenched after 24 h by cooling down the reaction flask with cold water and the solvent was evaporated. The viscous reaction product was purified by repeated washing with cold diethyl ether and finally dried in high vacuum at room temperature for 24 h. Yield: 96%, GPC (THF) M_n: 27 K. D: 1.1. 1H NMR (400 MHz, CDCl3): δ 8.47, 7.70, 7.13, 4.21–4.07,
3.64–3.37, 3.03, 1.93-1.82, 1.03–0.87. From $^1$H NMR, integration of the methoxy proton (in PEG unit) and the aromatic proton (in pyridine unit) provided the molar ratio of two monomers to be 1:1 (PEG/PDS).

2.4.3 Modification of synthesized p(PEGMA-co-EDSMA) polymer, $P_{PC}$

PDS polymer (1 g, 1.32 mmol PDS repeat unit) was weighed in a 20 mL glass vial and dissolved in 8 mL DCM. Catalytic amount (100 µL) of glacial AcOH was added to it and stirred for 5 min. Afterwards, 2-mercaptoethanol (0.9 mL, 13 mmol) was added dropwise to the reaction mixture and the solution was stirred for 24 h at room temperature. After that, the modified polymer was purified by dialyzing against methanol using a membrane of MWCO: 3.5 kDa. After dialysis, the solvent was evaporated and the polymer was dried under vacuum for 24 h. Yield: 90%, GPC (THF) $M_n$: 26 K. $\Delta$: 1.2. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 4.24–4.09, 3.87, 3.65–3.53, 3.37, 2.95-2.90, 1.93-1.84, 1.04-0.89.

2.4.4 Synthesis of p(PEGMA-co-NPC) (NPC: p-nitrophenyl carbonate), $P_{1}$

Modified polymer P2 (1 g, 1.39 mmol) and 4-Nitrophenyl chloroformate (325 mg, 1.61 mmol) were dissolved in 5 mL DCM taken in a 20 mL glass vial. The reaction mixture was cooled in ice bath for 10 min. To the cold mixture, pyridine (130 µL, 1.61 mmol) was added dropwise under vigorous stirring. Finally, the reaction mixture was stirred at room temperature for 24 h and the self-immolative polymer was purified by dialyzing against DCM/MeOH 1:1 mixture using a MWCO 3.5 kDa membrane. Yield: 98%, GPC (THF) $M_n$: 32 K. $\Delta$: 1.2. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.29-8.27, 7.41-7.42, 4.54, 4.23-4.08, 3.63-3.37, 3.04-2.94, 1.89-1.80, 1.03–0.88.
2.4.5 \(^1\)H-NMR spectra for polymer samples P_{PCP}, P_{PCE} and P_{1}

\(^1\)H-NMR spectra of the samples were recorded on a 400 MHz Bruker NMR spectrometer using residual proton resonance of the solvent as the internal standard and chemical shifts were reported in parts per million (ppm).

**Figure 2.6** \(^1\)H-NMR spectra of p(PEGMA-co-PDSMA), P_{PCP}

**Figure 2.7** \(^1\)H-NMR spectra of p(PEGMA-co-EDSMA), P_{PCE}
2.4.6 Gel permeation chromatography (GPC) for \(\text{PPcP}, \text{PPcE} \) and P1

Molecular weights of all synthesized polymers were estimated by GPC in THF using poly(methyl methacrylate) (PMMA) standards with a refractive index detector.

Figure 2.9 GPC(THF) for polymers \(\text{PPcP}, \text{PPcE} \) and P1
2.4.7 Preparation of polymer-protein nanoassemblies

2.4.7.1 NA-Empty\textsuperscript{ED} and NA-Empty\textsuperscript{PEG}

The self-immolative polymer P1 (10 mg) was dissolved in 2.5 mL phosphate buffer (adjusted to pH 8.5) at 20 °C. A calculated amount (~0.005 mmol) of ethylenediamine (for NA-Empty\textsuperscript{ED}) or (PEO)\textsubscript{4}-bis-amine (for NA-Empty\textsuperscript{PEG}) was added to the solution and kept stirring for 24 h for cross-linking. The resulting nanoassembly was purified by repeated washing (five times) with phosphate buffer pH 7.4 Amicon Ultra Centrifugal Filters MWCO 30 kDa. The final volume of empty nanoassembly was adjusted to 500 µL with phosphate buffer of pH 7.4.

2.4.7.2 NA-Lys\textsuperscript{ED}, NA-Lys\textsuperscript{PEG}, NA-RNaseA\textsuperscript{ED}, NA-RNaseA\textsuperscript{PEG}, NA-CytC\textsuperscript{ED} and NA-CytC\textsuperscript{PEG}

Initially, polymer P1 (10 mg) was dissolved in 1.5 mL phosphate buffer (adjusted to pH 8.5). To this solution, 1 mL solution of a specific protein (1 mg lysozyme or RNase A or cytochrome C in phosphate buffer, pH 8.5) was added dropwise and stirred for 24 h at 20 °C to generate P2. Then, calculated amount (~0.005 mmol) of ethylenediamine (for NA-Protein\textsuperscript{ED}) or (PEO)\textsubscript{4}-bis-amine (for NA-Protein\textsuperscript{PEG}) was added to each solution for cross-linking and mixed for another 24 h at 20 °C. Finally, the reaction mixture was washed (five times) with phosphate buffer (pH 7.4) using Amicon Ultra Centrifugal Filters MWCO 30 kDa to get purified nanoassemblies (NA) encapsulated with proteins. The final volume of all conjugates was adjusted to 500 µL with phosphate buffer of pH 7.4.
2.4.8 Monitoring protein-polymer conjugation, degree of NPC group modification by protein and crosslinking density

The conjugation process and cross-linking density for the protein-polymer conjugates can be evaluated by UV-Vis spectroscopy. The amount of released 4-nitrophenol was monitored at its $\lambda_{\text{max}}$ 400 nm (measured molar extinction co-efficient $\approx$12.14*10$^3$ LM$^{-1}$cm$^{-1}$ at 400 nm). Absorbance of each solution was measured after the conjugation and cross-linking processes. For each absorbance measurement, 25 µL of sample was withdrawn and diluted to 1 mL with distilled water. Cross-linking density was calculated by assuming that the formation of a single chain crosslinking bond would require cleavage of two NPC units and produce two 4-nitrophenol molecules.

The molar ratio of NPC:PEG in the polymer, P1= 50:50 (x mol : y mol, from NMR)

NPC molecular weight = 387 g/mol, PEG molecular weight = 500 g/mol

So, $x \text{ mol} \times 387 \text{ g/mol} + y \text{ mol} \times 500 \text{ g/mol}= 0.1 \text{ mg}$

$x \text{ mol} = 50/50 \text{ y mol}$

Amount of polymer used = 0.1 mg/mL for each absorbance measurement

Moles of NPC-unit (x mol) in the solution= $(0.1/887)/1000= 1.13 \times 10^{-7}$ mol

Example for NA-Lys$^{ED}$.

From Beer-lamber’s law: $A = \varepsilon . c . l$ and path-length=1 cm

So, concentration of 4-nitrophenol, $[c]= 0.3692/ (12.14 \times 10^3) = 3.04 \times 10^{-5}$ M

Thus, moles of 4-nitrophenol in 1 mL solution= $3.04 \times 10^{-8}$ mol

This represents 27 mol% of total NPC unit. As we assume that two 4-nitrophenol are released per crosslinking bond formation and NPC unit is 50 mol% of total polymer.

Therefore, crosslinking density =27/2 *0.5 = 6.7 %.
Table 2.1 NPC modification and crosslinking density of nanoassemblies

<table>
<thead>
<tr>
<th>Sample</th>
<th>Degree of NPC modification by protein*, %</th>
<th>Max. available Lysine per NPC group#, %</th>
<th>Crosslinking density, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA-Lys&lt;sup&gt;ED&lt;/sup&gt;</td>
<td>0.38</td>
<td>2.0</td>
<td>5.7</td>
</tr>
<tr>
<td>NA-Lys&lt;sup&gt;PEG&lt;/sup&gt;</td>
<td>0.61</td>
<td>2.1</td>
<td>7.0</td>
</tr>
<tr>
<td>NA-RNase&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;ED&lt;/sup&gt;</td>
<td>0.21</td>
<td>3.6</td>
<td>6.0</td>
</tr>
<tr>
<td>NA-RNase&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;PEG&lt;/sup&gt;</td>
<td>0.43</td>
<td>3.6</td>
<td>6.0</td>
</tr>
<tr>
<td>NA-Cyt&lt;sub&gt;E&lt;/sub&gt;&lt;sup&gt;ED&lt;/sup&gt;</td>
<td>3.3</td>
<td>8.7</td>
<td>5.2</td>
</tr>
<tr>
<td>NA-Cyt&lt;sub&gt;E&lt;/sub&gt;&lt;sup&gt;PEG&lt;/sup&gt;</td>
<td>3.5</td>
<td>9.2</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* Amount of reacted NPC group, calculated based on amount of 4-nitrophenol released and original content of the same in the reactant polymer.

# moles of lysine/moles of NPC * 100; calculated based on initial polymer dosage (10 mg) and encapsulated proteins (see encapsulation efficiency)

**Figure 2.10** (a) Time-course of absorbance profile for released 4-nitrophenol as a fate of conjugation of lysozyme with polymer P1; Absorbance spectra of polymer-protein conjugates- before and after crosslinking for (b) lysozyme; (c) RNase A and (d)
cytochrome C. UV–visible absorption spectra were recorded on a PerkinElmer Lambda 35 spectrophotometer.

2.4.9 Encapsulation efficacy and loading capacity

All nanoassemblies were evaluated for amount of protein encapsulation after conjugation and cross-linking process. Protein concentration in each sample was measured from the filtrate after crosslinking reaction and the amount of protein was back-calculated in the conjugate. An absorbance based assay (with Pierce™ 660 nm Protein Assay Reagent) was utilized to quantify the protein amount. The encapsulation efficiency (EE) and loading capacity (LC) were calculated based on the following formulas:

\[
EE, \% = \left(\frac{\text{initial protein loaded} - \text{free “unencapsulated” protein}}{\text{initial protein loaded}}\right) \times 100
\]

\[
LC, \% = \left(\frac{\text{amount of “encapsulated” protein}}{\text{amount of polymer}}\right) \times 100
\]

Table 2.2 Encapsulation efficiency and loading capacity of nanoassemblies

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount of protein encapsulated, µg (Initial dose= 1mg)</th>
<th>Encapsulation efficiency (EE), %</th>
<th>Loading capacity (LC), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA-LysED</td>
<td>550</td>
<td>55</td>
<td>5.5</td>
</tr>
<tr>
<td>NA-LysPEG</td>
<td>560</td>
<td>56</td>
<td>5.6</td>
</tr>
<tr>
<td>NA-RNaseAED</td>
<td>558</td>
<td>56</td>
<td>5.6</td>
</tr>
<tr>
<td>NA-RNaseAPEG</td>
<td>553</td>
<td>55</td>
<td>5.5</td>
</tr>
<tr>
<td>NA-CytCED</td>
<td>637</td>
<td>64</td>
<td>6.4</td>
</tr>
<tr>
<td>NA-CytCPEG</td>
<td>673</td>
<td>67</td>
<td>6.7</td>
</tr>
</tbody>
</table>

2.4.10 DLS and zeta potential plots:

Dynamic light scattering (DLS) and zeta potential measurements were performed using a Malvern Nanozetasizer-ZS. All samples were diluted with phosphate buffer pH 7.4 to adjust final concentration to 1 mg/mL.
**Figure 2.11** Particle size analysis of protein-polymer nanoassemblies from DLS measurements

**Table 2.3** Particle size values for the nanoassemblies

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>RNase A</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>NA-EmptyED</td>
<td>9.7 ± 0.1</td>
</tr>
<tr>
<td>NA-EmptyPEG</td>
<td>9.5 ± 0.1</td>
</tr>
<tr>
<td>NA-LysED</td>
<td>9.7 ± 0.2</td>
</tr>
<tr>
<td>NA-LysPEG</td>
<td>9.4 ± 0.3</td>
</tr>
<tr>
<td>NA-RNaseAED</td>
<td>9.1 ± 0.1</td>
</tr>
<tr>
<td>NA-RNaseAPEG</td>
<td>9.5 ± 0.1</td>
</tr>
<tr>
<td>NA-CytCED</td>
<td>9.2 ± 0.2</td>
</tr>
<tr>
<td>NA-CytCPEG</td>
<td>8.6 ± 0.1</td>
</tr>
</tbody>
</table>
Figure 2.12 Zeta potential plots for protein-polymer nanoassemblies

Table 2.4 Zeta potential values for the nanoassemblies

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zeta potential, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA-EmptyED</td>
<td>-7.3 ± 1.3</td>
</tr>
<tr>
<td>NA-EmptyPEG</td>
<td>-9.2 ± 1.5</td>
</tr>
<tr>
<td>NA-LysED</td>
<td>-7.6 ± 0.7</td>
</tr>
<tr>
<td>NA-LysPEG</td>
<td>-7.1 ± 0.4</td>
</tr>
<tr>
<td>NA-RNaseAED</td>
<td>-8.2 ± 0.7</td>
</tr>
<tr>
<td>NA-RNaseAPEG</td>
<td>-8.7 ± 0.7</td>
</tr>
<tr>
<td>NA-CytCED</td>
<td>-6.4 ± 0.5</td>
</tr>
<tr>
<td>NA-CytCPEG</td>
<td>-2.1 ± 0.5</td>
</tr>
</tbody>
</table>
2.4.11 Cryo-TEM sample preparation and instrumentation

(a top) Cryo-EM sample preparation was done using a FEI Vitrobot MKII applying purified NA-Protein sample solution (20-21 mg/ml) to a c-flat holey carbon grid (hole size 2 µm) after washing the grid in chloroform. The samples were then transferred using a Gatan cryo-transfer holder to a FEI TecnaiT12 TEM operating at 120 kV acceleration voltage. Images were acquired using standard low dose methods at 5 µm under focus. (a bottom) Sample preparation and HAADF imaging conditions are described under Figure 2.15.

![Cryo TEM images](image)

**Figure 2.13** Cryo TEM images: (a) Bright Field image of NA-CytC<sup>PEG</sup> was obtained after allowing a drop of sample solution to dry on a carbon coated grid and subsequent transfer into the TEM at room temperature mounted on a cryo-TEM holder. The sample was then cooled to -182 °C for observation. The bright field image shows particles in the size of 20-30 nm, which corresponds well to the size observed after cryo-transfer sample preparation. The contrast is naturally not as good as after the cryo-EM sample preparation (see main text Figure 2a) due to the carbon support film and most likely particles have undergone some deformation due to drying. This sample was also used for the HAADF and EDS measurements at cryo-temperature (main Text Figure 2.22a bottom and Figure 2.15). Due to relatively high beam currents and long acquisition times necessary for EDS measurements it was only possible to perform these measurements on these samples since samples prepared through cryo-transfer are quickly destroyed during the measurements. The bright field image here, all HAADF images (Main text and SI) and all EDS measurement (SI) were done using a JEOL FEM-2200FS field emission TEM equipped with an Oxford 80mm<sup>2</sup> X-Max EDS-Spectrometer. Acceleration Voltage was 200 kV and camera length for HAADF was 100 cm. Samples were mounted on a Gatan 636 Double Tilt Cryo-Holder with Beryllium cradle to avoid Fe signal typical for standard stainless-
steel holders. **(b)** Bright Field image of NA-CytC\(^{\text{PEG}}\) obtained after allowing a drop of sample solution to dry on a carbon coated grid and subsequently observed in a TEM at RT. No additional staining was performed. Compared to the sample in part (a) observed at cryo temperatures the sample quickly degrades under electron beam exposure and forms ring like structures where the average diameter of the inner dark ring is in the range of 10-30 nm. Interestingly, individual NA-CytC\(^{\text{PEG}}\) particles in aggregates each form a single ring allowing to identify the number of NA-CytC\(^{\text{PEG}}\) particles within an aggregate (see calculation and Figure 2.16 for # of proteins per particle). The imaging was done using a JEOL JEM-2000FX TEM operating at 200kV acceleration voltage.

![Room temperature TEM images](image)

**Figure 2.14** Room temperature TEM images: for NA-Protein samples (samples were drop-casted on a carbon coated grid and dried at room temperature before subjecting room temperature TEM study. For all samples, a mixture of individual as well as clustered particles were observed because of drying on TEM grids. This is also in agreement with the cryo-EM images provided in Figure 2a in manuscript and Figure 2.13.
Figure 2.15 Energy Dispersive X-Ray Spectroscopy (EDS) analyses and High Angle Annular Dark Field Microscopy (HAADF, inset) images at cryo temperature (-178 °C) for NA-CytC\textsuperscript{PEG}. Figure 2.15 insets shows HAADF images of individual nano-assemblies. For HAADF and EDS analysis, samples were transferred using a Gatan 636 Double Tilt Cryo-Holder with Beryllium cradle to avoid any contamination from external metal sources. The whole assembly has a diameter of ~20 nm and the bright spots in the range of ~2-3 nm is caused by the Fe-content of discrete CytC protein molecules.\textsuperscript{32} EDS analysis confirmed the presence of Fe inside (a, c) the nanoassemblies and none in the outer space (c). As expected, we did not observe any bright spots of Fe in NA-Empty\textsuperscript{PEG} particle (d, inset) and EDS confirms the absence of Fe in it(d).

2.4.12 Estimation of approximate number of proteins per particle:

Example for NA-Lys\textsuperscript{ED}:

Diameter of nanoassembly, $D = 9.7$ nm, radius, $R = 4.85$ nm

Volume of each particle, $V_{PPC} = \frac{4}{3}\pi R^3 = \frac{4}{3}\pi (4.85)^3 \text{ nm}^3 = 477.6 \times 10^{-21} \text{ cm}^3$

Again, for lysozyme, diameter of protein, $d = 3.6$ nm, radius, $r = 1.8$ nm

Volume of each protein, $V_{LY} = \frac{4}{3}\pi r^3 = \frac{4}{3}\pi (1.8)^3 \text{ nm}^3 = 24.4 \times 10^{-21} \text{ cm}^3$
Assuming that maximum sphere packing efficiency to be ~74%, number of lysozyme per nanoassembly to be \( \frac{477.6 \times 10^{-21}}{24.4 \times 10^{-21}} \times 0.74 = 14 \)

Based on *Carl Friedrich Gauss* theory on close packing of sphere in space: highest average packing density is given by \( \frac{\pi}{3\sqrt{2}} \approx 0.74 \)

The results for all other nanoassemblies are summarized below:

**Table 2.5** Theoretically calculated number of proteins per nanoassembly based on size

<table>
<thead>
<tr>
<th>Sample</th>
<th># of Protein per PPC particle</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA-Lys\textsuperscript{ED}</td>
<td>14</td>
</tr>
<tr>
<td>NA-Lys\textsuperscript{PEG}</td>
<td>14</td>
</tr>
<tr>
<td>NA-RNase\textsuperscript{AE}</td>
<td>16</td>
</tr>
<tr>
<td>NA-RNase\textsuperscript{PEG}</td>
<td>18</td>
</tr>
<tr>
<td>NA-CytC\textsuperscript{ED}</td>
<td>7</td>
</tr>
<tr>
<td>NA-CytC\textsuperscript{PEG}</td>
<td>6</td>
</tr>
</tbody>
</table>

**Figure 2.16** HAADF images of different NA-CytC\textsuperscript{PEG} particles. The bright spots with a diameter of 2-3 nm are caused by the Fe-content of the Cyt C. This allows to identify and
approximately count number of CytC proteins per particle through Image J software (see discussions in Figure 2.13b), approx. # of proteins are reported in the inset, scale bar: 20 nm.

2.4.13 Enzymatic degradation (trypsin digest) study

Polymer-protein conjugate solutions and native proteins (lysozyme, RNase A and cytochrome C) were subjected to enzymatic degradation study to evaluate the stealth power of polymeric nanoassemblies to encapsulate and protect the sensitive cargos from protease mediated cleavage. Sample solutions were prepared with polymer-protein complexes (with final protein concentration of 0.39 mg/mL based on previous protein analysis) in NaHCO₃ buffer (pH=8.0). The concentrations of native proteins in each control sample were also kept identical for comparison purpose. After that 10% acetonitrile was added to each sample to denature the protein and incubated at 50 ºC for 45 minutes. For RNase A, samples were treated with 15% AcOH and incubated at 90 ºC for 4 h. After hydrolysis, samples were freeze-dried and finally added 10% acetonitrile and 90% NaHCO₃ buffer of pH 8.0. Finally, all samples were digested with trypsin from porcine pancreas at a ratio of 1:25 (trypsin:protein) at 37 ºC for 17 h. After digestion samples were collected by centrifugation and subjected to MALDI-MS analysis. The matrix was prepared with a solvent mixture of acetonitrile, water and trifluoroacetic acid (with a ratio 50:47.5:2.5) containing 10 mg/mL α-cyano-hydroxycinnamic acid. The matrix and digested samples were mixed at 1:1 ratio and spotted on the MALDI target for fragmental analysis.
Figure 2.17 Trypsin digest for ED and PEG-crosslinked polymer-protein nanoassemblies

Table 2.6 Major MS-Digest fragments for Lysozyme, RNase A and Cytochrome C from MALDI-MS analysis

<table>
<thead>
<tr>
<th>m/z</th>
<th>Start</th>
<th>End</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1045</td>
<td>135</td>
<td>143</td>
<td>(K)GTDVQAWIR(G)</td>
</tr>
<tr>
<td>1428</td>
<td>52</td>
<td>63</td>
<td>(K)FESNFNTQATNR(N)</td>
</tr>
<tr>
<td>1676</td>
<td>116</td>
<td>130</td>
<td>(K)IVSDGNGMNAWVAWR(N)</td>
</tr>
<tr>
<td>1753</td>
<td>64</td>
<td>79</td>
<td>(R)NTDGSTDYGILQINSR(W)</td>
</tr>
<tr>
<td>1151</td>
<td>1</td>
<td>10</td>
<td>(-)MPAPATTYER(I)</td>
</tr>
<tr>
<td>1547</td>
<td>85</td>
<td>98</td>
<td>(K)LWSSLTLGSYKGK(N)</td>
</tr>
<tr>
<td>1662</td>
<td>1</td>
<td>14</td>
<td>(-)MPAPATTYERIVYK(N)</td>
</tr>
<tr>
<td>1685</td>
<td>26</td>
<td>41</td>
<td>(R)LEFQDGGVGLTAQFK(Q)</td>
</tr>
<tr>
<td>1168</td>
<td>29</td>
<td>39</td>
<td>(K)TGPNLHGLFGR(K)</td>
</tr>
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<td>1478</td>
<td>89</td>
<td>100</td>
<td>(K)KTEREDIAYLK(K)</td>
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<td>90</td>
<td>101</td>
<td>(K)KTEREDIAYLKK(A)</td>
</tr>
<tr>
<td>1598</td>
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<td>54</td>
<td>(R)KGTQAPGFTYTDANK(N)</td>
</tr>
<tr>
<td>1633</td>
<td>10</td>
<td>23</td>
<td>(K)IFVQKCAQCHTVEK(G)</td>
</tr>
</tbody>
</table>
2.4.14 Assessment of serum stability of NA-Lys\textsuperscript{PEG}, NA-RNase\textsuperscript{PEG} and NA-CytC\textsuperscript{PEG} nanoassemblies

To the stability of nanoassemblies in serum is considered to be an important criterion to perform as an efficient delivery vehicle. The serum stability of the PPCs were performed by monitoring the changes in particle size through DLS.\textsuperscript{33} All samples were incubated with differential amounts of serum (0%, 10%, 25% and 50%) for 6 h at 37 °C before subjecting to DLS measurements. Conjugates were found to quite stable with negligible shifts in the particle sizes confirming no protein adsorption leading to aggregation and biofouling.

![Particle size analysis of nanoassemblies in presence of serum](image)

**Figure 2.18** Particle size analysis of nanoassemblies in presence of serum

2.4.15 Lysine residues in proteins

![Lysine residues in proteins](image)

**Figure 2.19** Lysine residues in Lysozyme (#6), RNase A (#10) and Cytochrome C (#19)
2.4.16 SDS-PAGE for protein-polymer conjugation and release studies:

30 µL of different samples containing NA-Empty<sup>ED</sup>, NA-Empty<sup>PEG</sup>, NA-Lys<sup>ED</sup>, NA-Lys<sup>PEG</sup>, NA-RNaseA<sup>ED</sup>, NA-RNaseA<sup>PEG</sup>, NA-CytC<sup>ED</sup> and NA-CytC<sup>PEG</sup> were mixed with 10 µL of loading buffer (DTT free) and 25 µL of each sample was loaded on acrylamide gel. For release experiment, identical protein-polymer conjugate samples were treated with 10 mM DTT and incubated at 37 ºC for 4 h before subjecting to acrylamide gel electrophoresis. To calculate the amount of released protein from each sample, standard curves were generated from the known concentrations of pure protein samples loaded into the gel lanes. The gel image analysis and quantification were performed with Bio-Rad Image Lab<sup>TM</sup> software.

Figure 2.20 SDS-PAGE for encapsulation analysis with nanoassemblies containing Lys and RNaseA

2.4.17 Release kinetics of proteins from the protein-polymer nanoassemblies:

To monitor the release kinetics of proteins (lysozyme, RNase A and cytochrome C), 30 µL of NA-Lys<sup>ED</sup>, NA-Lys<sup>PEG</sup>, NA-RNaseA<sup>ED</sup>, NA-RNaseA<sup>PEG</sup>, NA-CytC<sup>ED</sup> and NA-CytC<sup>PEG</sup> samples were incubated at 37 ºC with requisite amounts of 10 mM DTT for different time intervals. After each incubation time, samples were collected and
immediately frozen at -20 ºC. Finally, all samples were subjected to SDS-PAGE analysis to quantify the amount of released proteins.

Figure 2.21 Release kinetics of proteins from the nanoassemblies by SDS-PAGE at disulfide of polymer to DTT ratio 1:1.

Figure 2.22 Release kinetics of proteins from the PPCs by SDS-PAGE at disulfide of polymer to DTT ratio 1:10, quantification data provided in the 3D bar graph after 6 h of release.
**Figure 2.23** Release kinetics of proteins from the nanoassemblies: (a, b) Plot of concentration of protein released (µM) vs. time; (c, d) Normalized plot of released/encapsulated protein vs. time. Although the concentration/amount of released proteins are approximately same for both RNase A and Cyt C, the normalized plots (moles of released/encapsulated protein vs. time) differentiates the protein release behavior more clearly and the released protein amounts for each can be correlated with their lysine content.

**2.4.18 Activity assays:**

To measure the activity of released proteins from different polymer-protein conjugates, first samples were treated with 10 mM DTT and incubated at 37 °C for 4 h. Identical samples were subjected to 50 µM DTT mimicking extra-cellular reducing environment and incubated under similar condition. SpectraMax® M5 spectrophotometer (Molecular Devices) was utilized for evaluating all activities through absorbance and fluorescence measurements.
a. For lysozyme:

The EnzChek® Lysozyme Assay Kit (Thermo-Fisher Scientific) was used to check the lysozyme activity on a substrate based on *Micrococcus lysodeikticus* cell walls which was labeled with fluorescein to such an extent that the fluorescence is quenched. Due to lysozyme’s enzymatic activity, the mucopolysaccharide cell walls of the labelled microorganism containing β-(1-4)-glucosidic linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues were hydrolysed releasing proportional amount of fluorescein. To perform the assay, 20 µL sample was mixed with 20 µL prepared substrate solution and subjected to fluorescence measurement (Ex/Em: 494/518 nm) over 1h with SpectraMax® M5 spectrophotometer.

b. For RNase A:

RNaseAlert® activity kit (Thermo-Fisher Scientific) was used to check the activity of released RNase A for all samples. RNase A cleaves the oligonucleotide substrate of the assay consisting a fluorophore and a quencher present at two extreme ends, thus releasing the fluorophore which can be detected and quantified with a fluorometer. For a typical kinetic experiment, the substrate was mixed with 5 µL test buffer, 35 µL nuclease free water and 10 µL sample (diluted X10,000 from DTT experiment). 40 µL of the prepared sample mixture was transferred to a black 96-well plate and immediately measured for fluorescence (Ex/Em: 490/520 nm) with SpectraMax® M5 spectrophotometer over a 30 min time course.

c. For cytochrome C:

The peroxidase activity of cytochrome C was determined by examining the catalytic conversion of 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS).
Cytochrome C catalyzes the reduction of H$_2$O$_2$ to water which is coupled with one-electron oxidation of chromogenic ABTS forming a brilliant blue-green ABTS radical cation. The assay kinetics can be monitored by observing the changes in absorbance spectra of the radical cation at 418 nm. Before subjecting to activity measurement, DTT treated samples were washed thoroughly with PBS buffer of pH 7.4 to remove DTT and other byproducts. The test solution was prepared by mixing 100 µL sample solution with 400 µL H$_2$O$_2$ (25 mM) and 500 µL ABTS (1 mg/mL). Absorbance spectra were recorded for all samples at 418 nm for a time course of 5 min using SpectraMax® M5 spectrophotometer.

Table 2.7 Abbreviations used in activity assay plots

<table>
<thead>
<tr>
<th>Sample</th>
<th>Details</th>
<th>Sample</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein$_{DTT}$ 0.05 mM</td>
<td>Native protein treated with 0.05 mM DTT</td>
<td>NA-Protein$_{ED}$ 0.05 mM</td>
<td>Protein encapsulated PEG-crosslinked nanoassemblies treated with 0.05 mM DTT</td>
</tr>
<tr>
<td>Protein$_{DTT}$ 10 mM</td>
<td>Native protein treated with 10 mM DTT</td>
<td>NA-Protein$_{ED}$ 10 mM</td>
<td>Protein encapsulated ED-crosslinked nanoassemblies treated with 10 mM DTT</td>
</tr>
<tr>
<td>NA-Empty$_{ED}$</td>
<td>Empty ED-crosslinked nanoassemblies treated with 10 mM DTT</td>
<td>NA-Protein$_{PEG}$ 10 mM</td>
<td>Protein encapsulated PEG-crosslinked nanoassemblies treated with 10 mM DTT</td>
</tr>
<tr>
<td>NA-Empty$_{PEG}$</td>
<td>Empty PEG-crosslinked nanoassemblies treated with 10 mM DTT</td>
<td>Blank</td>
<td>Only phosphate buffer, pH 7.4</td>
</tr>
<tr>
<td>NA-Protein$_{ED}$ 0.05 mM</td>
<td>Protein encapsulated ED-crosslinked nanoassemblies treated with 0.05 mM DTT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4.19 Circular dichroism (CD) spectra:

CD spectra of the released and native protein samples were recorded on JASCO J-1500 spectrophotometer. In a typical experiment, NA-protein sample was incubated with requisite amount of DTT for 24 h. After that, the sample was dialyzed against PBS buffer pH 7.4 with a membrane MWCO 20 kDa for 2 days to separate the polymer. Finally, the purified sample was concentrated with Amicon Ultra Centrifugal Filters MWCO 3K and the concentration was measured with Pierce 660 nm Protein Assay Reagent. For recording
the spectra, 200 µL protein solution was injected into a quartz cuvette of 1-mm path length, equilibrated at 25 °C for 10 min and scanned from 190 to 250 nm (scan rate: 20 nm/min, interval: 0.2 nm, average of three spectra).

![Image of CD spectra](image)

**Figure 2.25** CD spectra of native proteins and polymer-protein nanoassemblies.

### 2.4.20 MALDI-MS spectra for the released proteins:

MALDI-MS analyses were performed with Bruker Autoflex III time-of-flight mass spectrometer. All mass spectra were acquired in the reflectron mode with an average of 500 laser shots at ~60% optimized power.
Figure 2.26 Comparison of MALDI-MS spectra of the native proteins and released proteins from nanoassemblies.
2.4.21 Labeling of proteins with Rhodamine B:

To perform the cell-uptake studies, fluorescence-labelled proteins (lysozyme, RNase A and cytochrome C) were prepared with Rhodamine B isothiocyanate (RB). In a typical labelling procedure, proteins (4 mg) were dissolved separately in 2 mL of 0.1 M NaHCO₃ buffer (pH 8.5) and stirred for 15 min at 4 °C. RB (5 eq. of each protein, 10 mg/mL in DMSO) was added dropwise to each protein solution and stirred at 4 °C for 2 h protected from light. The RB-labelled-proteins were purified by extensive dialysis with 50 mM Tris pH 7.4 and 50 mM NaCl mixture to remove excess RB and concentrated using 3 kDa Amicon Ultra Centrifugal Filters. Protein concentrations in each labelled conjugate were calculated using UV-Vis spectroscopy. The molar ratio of RB and labelled lysozyme, RNase A and cytochrome C were estimated to be 0.62, 0.43 and 0.63, respectively. All labelled polymer-protein conjugates were prepared with the RB-labelled proteins following the method described under ‘Synthesis of polymer−protein nanoassemblies’. 
2.4.22 Cell culture:

Human cervical carcinoma (HeLa) cells were cultured in T75 cell culture flask containing Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) in a humidified incubator with 5% CO₂ at 37 °C. Culture media was supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic-antimycotic (100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of Amphotericin B).

2.4.22.1 Cellular uptake studies for protein delivery and endosomal escape:

Cell internalization studies were performed with HeLa cells seeded at 100,000 cells/mL in glass-bottomed petri-dishes and cultured for 1 day at 37°C in a 5% CO₂ incubator. Prior to delivery cells were washed three times with PBS buffer and incubated with 1 mL media containing 6 µL polymer-Rhodamine B-protein conjugate or Rhodamine B-protein conjugate (protein concentration 1 mg/mL) at 37 °C for 4 h. After that, cell nucleus was stained with Hoechst 33342 (8 µM) and finally the media was replaced with fresh stock and incubated for another 1 h before subjecting to CLSM analysis. In addition, to study the endosomal escape of the labelled proteins, HeLa cells were incubated with labelled nanoassemblies for 4 and 24 h. After that it was stained with LysoTracker® Green to label endosomes/lysosomes and studied the co-localization of red and green fluorescence channels. Live cell imaging was performed using Nikon Spectral A1+ confocal microscope.
**Figure 2.28** Negligible uptake for HeLa cells incubated with only proteins, cell nucleus was stained with Hoechst 33342, scale bar: 50 µm.

**Figure 2.29** Cellular internalization with ED-crosslinked nanoassemblies, cell nucleus was stained with Hoechst 33342, scale bar: 50 µm.
**Figure 2.30** (a) Depth profiling for NA-CytC\textsuperscript{PEG} dosed nanoassemblies: pseudo-colored consecutive slices through z-axis and (b) z-stack orthogonal projection from CLSM experiment showing localization of cytochrome C inside HeLa cells.

**Figure 2.31** (a) Time course of uptake for NA-CytC\textsuperscript{PEG}, (b) Fluorescence intensity measurement from the red channel by Image J software at different time points of uptake experiment, expressed as Corrected total cell fluorescence, CTCF = Integrated Density - (Area of selected cell × Mean fluorescence of background).\textsuperscript{34}
Figure 2.32 Endosomal escape studies by co-localization of LysoTracker Green and Rhodamine B tagged protein; co-localization of dyes after 4 h incubation confirms existence in the endosomes and after 24 h distinct red fluorescence confirms release of proteins into the cytosol from endosomes. Scale bar: 10 µm.

2.4.22.2 Cell viability with alamarBlue® assay:

HeLa cells were seeded into 96-well tissue culture plates at a density of 5000 cells/well/100 µL sample and incubated at 37 °C. After 24 h, culture media was replaced and cells were treated with different concentrations of protein-polymer conjugates and control protein samples (0.1 mg/mL to 2 mg/mL NA-Protein<sup>PEG</sup> concentration; for naked protein concentration is matched with the encapsulated one in the NA-Protein sample and can be calculated from encapsulation efficacies, 2 mg/mL of NA-CytC<sup>PEG</sup> refers to 126 µg/mL of cytochrome C) in 100 µL media. All samples were incubated for 6 h at 37 °C, then the media was replaced and incubated for another 66 h at 37 °C. Afterwards media was replaced, washed with PBS buffer for three times and each well was treated with 100 µL 10% alamarBlue in media with serum. Finally, samples were incubated for 1 h and subjected to fluorescence measurement with SpectraMax® M5 at 560 nm excitation/590 nm emission wavelength in a black 96-well flat-bottomed plate.

2.4.22.3 Study of apoptosis with NA-CytC<sup>PEG</sup> nanoassembly:

HeLa cells were seeded at 40,000 cells/mL density in glass-bottomed petri-dishes and cultured for 1 day at 37°C in a 5% CO<sub>2</sub> incubator. Cells were washed three times with
PBS buffer and incubated with 1 mL media containing NA-CytC<sup>PEG</sup> conjugate (2 mg/mL) at 37 °C for 4 h. After that, the media was replaced and cells were incubated for another 68 h. To detect the apoptotic cells, each sample was treated with CellEvent™ Caspase-3/7 Red Detection Reagent (10 µM) and hoechst 33342 (8 µM) to stain the nucleus by incubating for 30 min before subjecting to CLSM analysis. The apoptosis assay reagent consists of a DEVD peptide attached to a nucleic acid-binding cy5-dye. When bound with the peptide, the dye becomes intrinsically non-fluorescent as the DEVD peptide retards the DNA-binding ability of the dye. Once caspase-3/7 enzymes are activated in apoptotic cells by the delivery of cytochrome C, the DEVD peptide is cleaved by those and enable the dye to bind to DNA to produce a bright, fluorogenic response. Co-localization of blue (hoechst) and red (cy5) channels was studied to check the nuclei of the apoptotic cells.

**Figure 2.33** Detection of activated caspase-3/7 after 72 h in control HeLa cells treated with NA-Empty<sup>PEG</sup> sample; scale bar: 10 µm, no co-localization of hoechst and cy5-tagged assay reagent was observed in the nucleus.

### 2.4.22.4 Encapsulation, release and cellular internalization studies with eGFP

To check the robustness of the polymeric delivery system, we investigated the encapsulation and release properties of eGFP that has higher MW and lower pI (MW ~27 kDa, pI 5.5) compared to lysozyme, RNase A and cytochrome C (MW between 12-15 kDa and pI >8.5). eGFP was encapsulated within polymer matrix by the reactive coupling method described in “synthesis of polymer-protein nanoassemblies” section, and was
subsequently released with the aid of redox-stimuli DTT (see section ‘SDS-PAGE for protein-polymer conjugation and release studies’, Figure 2.31). NA-eGFP<sup>PEG</sup> assemblies were subjected to cellular uptake studies with HeLa cells (dosage 2 mg/mL, see previous ‘cellular uptake studies for protein delivery’ section) and imaged using confocal microscope. Evenly distributed green fluorescence of delivered eGFP (Figure 2.35) demonstrates successful delivery of the protein into HeLa cells.<sup>35</sup>

![Figure 2.34 SDS-PAGE for encapsulation and release analysis with nanoassembly containing eGFP.](image)

**Figure 2.34** SDS-PAGE for encapsulation and release analysis with nanoassembly containing eGFP.

![Figure 2.35 Cellular internalization with NA-eGFP<sup>PEG</sup> nanoassemblies, cell nucleus was stained with Hoechst 33342, scale bar: 20 µm.](image)

**Figure 2.35** Cellular internalization with NA-eGFP<sup>PEG</sup> nanoassemblies, cell nucleus was stained with Hoechst 33342, scale bar: 20 µm.
2.5 References


References:


CHAPTER 3
A SYMBIOTIC SELF-ASSEMBLY STRATEGY TOWARDS LIPID-ENCASED CROSSLINKED POLYMER NANOPARTICLE FOR EFFICIENT GENE SILENCING


3.1 Introduction

Self-assembly that relies on non-covalent intermolecular interactions, comprising single or multi-component molecular building blocks, plays a fundamentally important role in many biological processes and in the development of novel functional materials.1-2 However, designing and assembling multiple molecular entities to generate a predictable and controlled supramolecular assembly have considerable challenges; but if achieved, this can propel the design of materials with functional capabilities that are currently not attainable. With this in mind, we have designed a new three-component self-assembling system, where the parts are interdependent in the formation of the nanoassembly. In addition to demonstrating the formation and the characterization of such an assembly, the utility of such a nanostructure in addressing current toxicity and cellular delivery challenges involving small interfering RNA (siRNA) molecules has been investigated.

Although discovery of siRNA, a potential gene silencing agent, has created attractive opportunities targeting a wide array of diseases,3-5 RNAi technology has produced only one drug approved for clinical use.6 Poor cellular internalization, serum instability, rapid clearance, severe cytotoxicity and potential immunological flare-ups have been identified as the critical barriers for
such promising technology.\textsuperscript{7-12} Potential solutions like, chemically modified siRNAs and viral vectors have yet to overcome issues owing to cytotoxicity, stability, immunogenicity and reduced silencing ability upon structural modifications.\textsuperscript{7, 13-14} Interestingly, non-viral approaches, based on electrostatic complexation of nucleic acids using cationic lipids, peptides, nanoparticles, or polymers, have the potential to resolve the major reported issues.\textsuperscript{15-20} However, the key obstacle in these carrier-based delivery systems is the adverse side effects originated from the cationic charge mediated alteration in membrane potentials of cellular organelles and non-specific interactions with negatively charged serum proteins.\textsuperscript{13, 21-22}

To address this, two interesting approaches, viz. charge-masking strategies\textsuperscript{23-24} and spherical nucleic acids\textsuperscript{25} have been reported wherein cationic charges are masked and negatively charged nucleic acids are decorated on surfaces, respectively. In addition to the non-cationic surface display, the degradable cationic blocks offer opportunities to mitigate toxicity issues associated with cationic polymers as well.\textsuperscript{26-29} Mimicking viral mechanism of cellular entry, another polymeric delivery agent, virus-inspired polymer for endosomal release, is developed with a hydrophilic cationic block and an endosomolytic peptide displayed only under acidic pH.\textsuperscript{30} In a significant departure from the conventional approaches, direct decoration of a high density of nucleic acids themselves as surface functionalities on nanoparticles and polymers have produced negatively charged nanostructures with good cellular internalization and gene silencing capabilities.\textsuperscript{25, 31} Natural green tea catechin condensed siRNA-cationic polymer nanoparticles and fluorinated oligoethyleneimine nanocomplexes have also shown encouraging outcomes for siRNA delivery.\textsuperscript{32-33} Despite these advances, there still exists a need for RNAi-based therapeutic approaches that would retain the key advantages of non-viral carriers, while mitigating their shortcomings.
Inspired by this, we envisaged that a possible solution to all these delicate and overwhelming challenges would be a noncationic approach wherein the cationic charge is irreversibly removed after encapsulation of siRNA without compromising stability and activity of the nucleic acid. To this end, we designed a unique and well-defined ‘symbiotic self-assembly’ approach to efficiently self-assemble a polymer, lipids, and the nucleic acid to form stable lipid decorated siRNA-polymer (L-siP) nanoassemblies (Scheme 3.1). We term this as symbiotic self-

**Scheme 3.1** Symbiotic self-assembly strategy to construct L-siP nanoassembly and its key molecular components.

assembly, because none of these three components would form the nanostructures by themselves under the required conditions, but they produce well-defined assemblies when brought together in a sequence. We outline the design hypothesis, self-assembly, and their utility in effectively
delivering siRNA molecules inside cells, using four different siRNAs in different cell lines. The system has been designed such that: (i) the high binding affinity results in efficient capture of siRNAs inside the assemblies; (ii) although electrostatics is utilized to capture the siRNAs, the residual assembly is non-cationic due to an in situ crosslinking protocol that removes the cationic charge on the polymer, yet incarcerates the siRNA; (iii) the surface charge of the assemblies is non-cationic; and (iv) the siRNA can be released using a trigger that corresponds to the operational environment of the cargo.

3.2 Results and discussion

The self-assembly process was envisioned in three key steps. First, a cationic amphiphilic polymer would be utilized to electrostatically capture the siRNA (Scheme 3.1, Figure 3.1a-b). Note that electrostatic interaction energy is governed by Coulomb’s law ($E = q_1q_2/4\pi\varepsilon_0\varepsilon_r$), where $\varepsilon$ represents the dielectric constant of the medium. In this case, the ionic interaction between the polymer and the siRNA can be significantly enhanced in low dielectric media. The hydrophobic alkyl chain in the amphiphilic random copolymer facilitates the complexation between the polymer and the siRNA in an organic-rich solvent medium, where the electrostatic interactions are expected to be strong. However, the medium is not completely non-aqueous, as polyelectrolyte interactions are entropically driven$^{34}$ and it is important to accommodate the critical counterion dissociation that facilitates the interaction between these two macromolecules. Second, while we utilize the cationic charge to bind the siRNA with high affinity, we are interested in eliminating the cationic charge in the system since this has been implicated in many complications in non-viral carriers.$^{13, 21-22}$ Therefore, we utilize a cationic functional group that can be triggered to concurrently self-crosslink and release the cationic functionality (Scheme 3.1, Figure 3.1b-c). Such a process
switches the driving force for retaining the siRNA within the assembly, from an electrostatic one to a combination of physical incarceration (due to crosslinking) and solvophobic forces (due to the

Figure 3.1 (a) Synthesis of p(PDSMA-co-DodecylMA) polymer (P^{PDS/DD}) and post-polymerization modification to install cationic charge yielding ⊕P^{PDS/DD} polymer; (b) Reaction scheme for preparation of non-cationic L-siP nanoparticles via disulfide based crosslinking; (c) Schematic representation of differentially crosslinked polymer-siRNA assemblies; (d) ¹H NMR spectra of ⊕P^{PDS/DD} polymer treated with different amounts of DTT for crosslinking in acetone:water (deuterated)= 70:30.
unfavorable organic-rich medium for the highly charged siRNA). Finally, the resultant complex from this process is relatively apolar with lipophilic alkyl chains on their surface; this complex therefore is not amenable for distribution in aqueous media. We utilize this incompatibility to achieve a hydrophobic force driven coating of charge-neutral lipids in aqueous medium. The concentration of the lipids in this step is such that it does not exhibit self-assembly by itself, but does so on the surface of the existing hydrophobic exterior of the polymer-siRNA complex. This symbiotic self-assembly, where the organization of each of these components into the assembly is dependent on the presence of other components in the system, is thus achieved in three convenient steps.

3.2.1 Synthesis, characterization and crosslinking study of cationic PDS-Dodecyl polymer ($\oplus$P<sub>PDS/DD</sub>)

To achieve the desired self-assembly, a cationic random co-polymer PDS-Dodecyl polymer ($\oplus$P<sub>PDS/DD</sub>) was targeted (Figure 3.1a). This copolymer was synthesized via RAFT polymerization of pyridyl disulfide methacrylate and dodecyl methacrylate monomers (molar ratio 9:1, Figure 3.1) to obtain p(PDSMA-co-DodecylMA) polymer ($\text{P}^\text{PDS/DD}$; MW = 14.5 kDa; Đ: 1.3). The nitrogens in the PDSMA side-chain were quantitatively methylated using methyl triflate to achieve the $\oplus$P<sub>PDS/DD</sub> polymer (Figure 3.1). All polymers were characterized with $^1$H, $^{13}$C & $^1$H-$^{15}$N NMR, GPC and FT-IR spectroscopy. Methylation of the P<sub>PDS/DD</sub> polymer was confirmed from the chemical shift and integration of pyridinium ring protons in the final $\oplus$P<sub>PDS/DD</sub> polymer. To further confirm the synthesis of the $\oplus$P<sub>PDS/DD</sub> polymer, two-dimensional heteronuclear correlation NMR experiments ($^1$H-$^{15}$N HMBC) were performed with the synthesized polymers and 1-methylpyridine-2-thione molecule, released after crosslinking with 1,4-dithiothreitol (DTT, reported in equivalents). A clear shift of $^{15}$N NMR band for $\oplus$P<sub>PDS/DD</sub> polymer, in addition to
appearance of a new correlation band corresponding to methylated nitrogen, confirms successful installation of cationic charge in the $\oplus \text{P}^{\text{PDS/DD}}$ polymer. Further, $1\text{H NMR}$ spectra for differentially crosslinked samples in presence of different amounts of DTT showed the disappearance of the methylated-PDS units with the concurrent appearance of the peaks that correspond to the small molecule byproduct of the crosslinking reaction, N-methylpyridothione (Figure 3.1d). These experiments were carried with the polymer by itself without self-assembling the polymer through electrostatic complexation with siRNA, for characterization purposes. Note that complete crosslinking was achieved at 1 equiv DTT (2 times excess than required) which could be attributed to suppressed reactivity owing to steric barriers.

3.2.2 ‘Symbiotic self-assembly’ to create L-siP nanoassemblies

The first step of proposed self-assembly (Scheme 3.1) involves electrostatic complexation between $\oplus \text{P}^{\text{PDS/DD}}$ polymer with naked siRNA in acetone:water mixture (70:30 v/v). In addition to reducing the dielectric constant of the media to facilitate greater Coulombic interaction, the choice of solvent mixture is also optimized for the solubility of both $\oplus \text{P}^{\text{PDS/DD}}$ polymer & siRNA. The amphiphilicity of $\oplus \text{P}^{\text{PDS/DD}}$ polymer, governed by the ratio of cationic PDS and dodecyl moieties, was also found to affect the solubility of such complexes. We found that 90:10 ratio polymer exhibited better solubility and complexation capability, compared to that of the initially attempted 70:30 ratio polymer. All subsequent complexation experiments were conducted with the 90:10 ratio polymer in the acetone:water co-solvent system. To further analyze whether the observed interaction between the polymer and the siRNA is indeed based on electrostatics, we conducted molecular dynamics (MD) simulations of a coarse-grained (CG) molecular model of this system (see experimental for details about the model) in the acetone:water 70:30 mixture. Simulations show that while the cationic polymers and the oligonucleotides are held together,
removal of cationic charge in the polymer detaches the oligonucleotides from the polymer chain. This supports the idea that the self-assembly process is controlled by electrostatic interactions (see experimental).

The next step in our self-assembly involves structurally reinforcing the complex through a chemical crosslinking reaction, which concurrently also releases the cationic charge from the polymer. This crosslinking step was executed using DTT, where the rapid thiol-disulfide exchange reaction between DTT and the methylated-PDS unit affords a thiol moiety on the polymer side chain, along with a stable small molecule byproduct, viz. \( N \)-methylpyridine-2-thione. The thiol moiety on the polymer chain can subsequently react with other methylated-PDS units within the complex to generate crosslinks. Note that this crosslinking step helps to shed positive charge, while also stitching the polymer chains to cage siRNA, thus preventing the loss of siRNA that is electrostatically encapsulated in the first step. More details on the effect of crosslinking density is provided in the next section.

Note that the complexation and crosslinking steps were conducted in an organic rich (acetone-water) media. In order for using this complex to deliver the siRNA molecule to cells, this complex must be in an aqueous media. To achieve this, the complex was coated with lipids in aqueous phase. Since the crosslinked polymer-siRNA complex was achieved in an organic solvent, we envisaged that the surface of the complex is apolar and would therefore be viable for coating with lipid molecules. Accordingly, we used a combination of a zwitterionic lipid (DOPE) and a PEGylated lipid (DSPE PEG-2000), because of their fusogenic and solubilization abilities respectively.\(^9,36-37\) Briefly, a mixture of DOPE and DSPE PEG-2000 lipids was initially dissolved in water at a concentration much below their critical aggregation concentrations.\(^38\) The crosslinked polymer-siRNA nanoassembly was then added to the aqueous lipid mixture (water/organic=20,
Figure 3.2 Effect of N/P ratio & cross-linking on encapsulation stability & siRNA release: (a) Variation of N/P for uncrosslinked particles; (b) Effect of variation of cross-linking measured by the DTT feed amount at higher N/P ratio on encapsulation & siRNA release; (c) DLS size distribution and (d) correlation diagram for N/P 15 nanoassemblies at different cross-linking; (e) Lipid coated nanoassembly constructed with CG-MD simulation: snapshot of the equilibrated L-siP15 (cut in half on the major diameter to clearly see the interior). Polymer is shown in transparent green, the dsDNA in yellow and the polymer outer layer is transparent grey (inset: exterior of the NA, where lipids are shown in grey); (f) Radial distribution functions $g(r)$ providing the relative probability to find polymer, dsDNA and lipids at various distances from the center of the nanoassembly.
v/v) for the self-assembly of lipids on its surface (see experimental for details) to produce the final lipid-polymer-siRNA (L-siP) nanoassembly (Scheme 3.1). Interestingly, the overall self-assembly process is considered symbiotic, because the lipids are used at concentrations well below their CACs and yet it self-assembles on the surface of the polymer-siRNA complex, which in itself required functional complementarity for its formation.

3.2.3 Effect of N/P and crosslinking on complexation and triggered release of siRNA from L-siP

The relative ratio of complementary charges (N/P) is often used, with number of positively charged nitrogens (N) in the polymer to the number of negatively charged phosphate moieties (P) in the nucleic acid as the measure, to evaluate the complexation efficiency. To understand the effect of this ratio on complexation, we systematically varied the amount of polymer to investigate the construction of L-siP assemblies at different N/P ratios (Figure 3.2). All particles were coated with DOPE and PEG-lipids to make them hydrophilic and stable in aqueous medium. The siRNA encapsulation was evaluated with agarose gel retardation assay (Figure 3.2a-b). First, complexation was studied at different N/P ratios without any crosslinking. At low polymer concentrations (N/P of 2.5 and 5), the complexation was found to be inefficient as noted from the significant presence of free siRNA in the gel. At N/P 7.5 and above, the siRNA encapsulation was found to be efficient (Figure 3.2a). In addition to the encapsulation efficiency, it is also necessary that we release the encapsulated siRNA molecules in the presence of a biologically relevant intracellular trigger.39-40 Therefore, the release of siRNA from these assemblies were assessed in the presence of a redox-stimulus at a concentration that is similar to that found in the cytosol (10 mM glutathione, GSH). In the presence of this stimulus, the siRNA release was found to be significant in the N/P 7.5 complex, but was significantly lower at higher N/P ratios (Figure 3.2a).
However, dynamic light scattering (DLS) measurements revealed non-uniform, bimodal and broad particle size distributions with poor correlation coefficients for all uncrosslinked nanoassemblies (see experimental) suggesting unstable nanoparticle formations.

The study of uncrosslinked complexes above provides an initial insight into the optimal N/P ratios. Note however that in our final complex, the positive charges will be removed through crosslinking. Therefore, the efficiency of siRNA encapsulation in this scenario might be very different as the balance between weakening of the complexation due to charge removal and strengthening the incarceration due to crosslinking would play an important role. To evaluate this balance, we studied the effect of crosslinking towards the formation of L-siP particles at different N/P ratios (Figure 3.2b). Upon increasing crosslinking degree, leakage of siRNA was evident from the assemblies formed at N/P 7.5 and 10. At higher N/P ratios (starting from N/P 15), in combination with higher crosslinking (0.25 equiv DTT onwards), the siRNA encapsulation was found to be stable (Figure 3.2b), as evident from narrow particle size distributions with excellent correlation coefficients. However, the release of siRNA was found to be crosslinking dependent, where the maximum release was obtained between 0.25-1 equiv of DTT crosslinking. This can be attributed to the critical balance of positive charge and crosslinking degree in L-siP nanoassemblies. At lower crosslinking degree (0-0.1 equiv DTT), residual cationic charge left after DTT treatment becomes the dominating factor and is able to hold back the siRNA tightly in the nanoassemblies. While at higher crosslinking degree (2 equiv DTT), siRNAs might be deeply buried and shielded inside the nanoassembly preventing adequate exposure to external reducing release environment. Evidently, at even higher N/P 25, where the polymeric burden was more, negligible release of siRNA was observed for similar reasons (Figure 3.2b). Based on these observations, a threshold of N/P 15 was thus considered as an efficient L-siP system for both
siRNA encapsulation and release (also see later for quantification). As L-siP nanoassemblies contain lipids, the zeta potential values were found to be negative (-30 to -40 mV, see experimental) as an effect of efficient coating of DOPE and PEG- lipids.\textsuperscript{41-42}

To further understand the complexation and release process, we also carried out CG-MD simulations (see experimental). In our models, to mimic the crosslinking process, we introduced -SS- groups in the polymer assembly, while cleaving the positively charged functionalities, assuming that crosslinking has occurred. When we introduced the crosslinking in the equilibrated N/P 7.5 model system, we could clearly observe leakage of the oligos from the polymer (see experimental). Next, we built a molecular model for the more promising N/P 15 L-siP system (Figure 3.2d and 3.2e). We started from an N/P 15 polymer-oligo network that was spontaneously formed in 70:30 acetone:water (v/v) via CG-MD simulation (details are available in the Computational Methods in experimental). Then, we introduced a 100\% crosslinking by adding a suitable number of DTT molecules in the system. To be sure to form a single aggregate in the center of the simulation box (useful for successful steps) following compaction due to crosslinking, we used a recently optimized simulation technique that push the system toward a single aggregation center.\textsuperscript{43} The DTT molecules selectively and strongly interacted with the -SS- groups in the polymer during this CG-MD simulation, while after coordination the cationic groups of the polymer involved in the process have been cleaved. Next, we added DOPE lipid molecules and replaced the solvent consistently with the experiments (water/organic=20, v/v). We then equilibrated the L-siP model nanoparticle via CG-MD simulation, which provided us with an insight into the structure of this assembly at a resolution <5 Å (Figure 3.2d). The lipid layer (transparent grey) covers the interior of the assembly, where the oligos (yellow) appear as trapped
Figure 3.3 (a) CG-MD simulation snapshots (taken after 4 µs) showing how the oligo release proceeds at different degrees of de-crosslinking: no decrosslinking, 90% of crosslinking removed and complete de-crosslinking; (b) Quantification of the different release behaviors as the number of contacts between oligo and polymer as a function of simulation time (initial number of contacts normalized to 1); (c) Ribogreen assay to quantify encapsulated & released siRNA from L-siP_{15/1}; Summary of encapsulation (d) and release profiles (e) at different N/P & crosslinking ratios showing the most promising composition to be N/P 15 at 1 equiv DTT crosslinking.
in the polymer matrix (green). From the equilibrated phase CG-MD trajectory we computed the radial distribution functions of the polymer, lipid and oligonucleotides ($g(r)$: relative probability to find these in space) as a function of the distance from the center of the nanoparticle (Figure 3.2e). The superposition of the cyan and purple curves in Figure 3.2e indicates that the polymer and the oligos are uniformly mixed in the core of the assembly while the lipids cover it and constitute the shell of the nanoassembly, which resulted well stable L-siP in such experimental conditions.

Starting from this equilibrated crosslinked L-siP model, we further probed the nanoassembly to understand the effect of crosslinking on stabilization and release of oligonucleotides. Upon deleting the lipid shell, we explored the release of encapsulated oligonucleotides as a function of the de-crosslinking ratio. The polymer-oligo complex remains stable in 100% DTT, i.e., fully crosslinked condition (Figure 3.3a). However, at 90% de-crosslinking (90% of DTT molecules eliminated from the model) we observed partial release of oligoes from the assembly, which completely disassembled under complete removal of DTT (0% DTT). Figure 3.3b quantifies these observations, showing the number of contacts between the polymer and the guest oligos over time calculated with respect to the initially equilibrated nanoassembly.

We further experimentally confirmed the encapsulation and release of siRNA from the L-siP nanoparticle via RiboGreen assay for the promising N/P 15 case (Figure 3.3c, see experimental). Under the encapsulated state, siRNA is significantly inaccessible to the assay reagent showing no apparent fluorescence signal generation. However, the amount of siRNA, once released from the assembly under reducing conditions, the fluorescent signal generation was found to be dependent on the crosslink density. These observations further support the results obtained
from the agarose gel retardation study above (Figure 3.2a-b). The degree of siRNA encapsulation and release obtained from agarose gel studies is summarized in Figure 3.3d and 3.3e.

To further understand the fate of siRNA during encapsulation and release steps, we also performed $^{31}$P NMR experiments to monitor the processes (see experimental). A phosphorothioate-modified siRNA (PTsi, $\delta \sim 53$ ppm) is utilized to distinguish it from the

![Figure 3.4](image)

**Figure 3.4** Cryo-TEM images of L-siP particles at N/P 15 (a) without crosslinking & (b) with cross-linking (DTT-1 equiv), scale: 50 nm; (c) N-STORM confocal microscopy images of single L-siP$^{15}$ particle, red: cy3-siRNA, green: carboxyfluorescein labelled DSPE-PEG lipid that coats the polymer-siRNA nanoassembly, scale: 100 nm.
phosphorus in phosphate groups of DOPE and DSPE-PEG lipids ($\delta \sim 0$ ppm). Once encapsulated the mobility of the siRNA inside the polymer cage will be significantly impaired compared to its situation in bulk solvent. As a result, the $^{31}$P band at $\delta \sim 54$ ppm (for PTsi) is completely eliminated after the formation of L-siP nanoassembly, whereas it reappears upon subjecting it with the release condition (with 10 mM GSH & Triton X-100, see experimental). This evidence further supports the results obtained from agarose gel retardation and RiboGreen assay studies (Figure 3.2a-b, 3.3c). Evidently, to reduce the quantity of polymer in the L-siP assembly construction with suitable high crosslinking degree (in context of reducing cytotoxicity by shedding cationic charge), we chose the L-siP assembly (L-siP$^{15/1}$) constructed under N/P 15 with crosslinking using 1 equiv of DTT (we choose excess DTT to ensure complete removal of cationic charge as discussed in Figure 3.1d) as the desired candidate for further cellular experiments.

### 3.2.4 Cryo-TEM and N-STORM confocal imaging of L-siP nanoassembly

Prior to evaluating the intracellular delivery of siRNA using these nanoassemblies, we further characterized the nanoassemblies via cryo-TEM and N-STORM confocal fluorescence microscopy (Figure 3.4). Cryo-TEM studies were performed for both the N/P 15 uncrosslinked and crosslinked samples. Images of the uncrosslinked assembly (Figure 3.4a) clearly show a fractal morphology consisting loose aggregates, whereas crosslinked L-siP15/1 nanoassembly (Figure 3.4b) showed uniform spherical assemblies with an average size of $\sim 100$ nm. These results are in accordance with our earlier DLS observations (Figure 3.2b-c). To further characterize the particle construction, we prepared L-siP15/1 nanoassembly utilizing carboxy-fluorescein labelled DSPE PEG-2000 lipid and cy3-labelled siRNA. As per our symbiotic self-assembly hypothesis, polymer complexed siRNA would dwell in the inner core while the shell would comprise lipid mixture
Figure 3.5 siRNA delivery in MDA-MB-231 cell line: (a) Cellular uptake of cy3-labelled siRNA (4 h incubation), scale: 20 µm; (b) Quantification of uptake with flow cytometry (4 h incubation); (c) Mechanism of siRNA transfection in presence and absence of endocytic inhibitors; (d) Endosomal colocalization of red cy3-siRNA and Lysotracker blue (pseudo-colored as green) after 4 h and escape after 24 h incubation, scale: 20 µm; (e) Calcein assay showing efficient escape of calcein from endosome and localization in cytosol in presence of L-siP15/1 NA, scale: 10 µm; (f) Cellular viability and (g) LDH cytotoxicity assay for empty L-siP, L-siP nanoassemblies with different siRNA concentrations (25, 50, 100, 200 & 500 nM) at N/P 15 and Lipofectamine (LF)-siRNA complexes (with fixed 100 nM siRNA) at 2, 5 and 10 µg/mL LF concentrations; Student’s t-test (compared L-siP/siNC sample bearing 100 nM siRNA with L-siP/Empty and LF samples): ***p<0.001, **p<0.01, *p<0.05, ns (non-significant)>0.05.

surrounding the electrostatic polymer-siRNA complex. The N-STORM images in Figure 3.4c clearly demonstrate the L-siP nanoassembly design with red cy3-siRNA comprising the inner part and green lipid layer encapsulating it externally. Interestingly, color-coded intensity profile defined by the electron scattering cross-section across the particles also matches the N-STORM measurements (Figure 3.4c & see experimental).
3.2.5 Cellular uptake and intracellular distribution

To test the ability of L-siP15/1 towards intracellular delivery of siRNA, we prepared these nanoassemblies using cy3-labelled siRNA and investigated their cellular distribution in three different cancer cell lines, viz. mammary gland/breast cancer cell line MDA-MB-231, cervical cancer cell line HeLa and a prostate cancer cell line DU-145 (Figure 3.5, for MDA-MB-231 and see experimental for HeLa & DU-145). As shown in Figure 3.5a, a clear distribution of red fluorescence in the cytosolic region confirms efficient transfection of cy3-siRNA nanoassembly (Figure 3.5b for flow cytometry quantification). The transfection efficacy was also evaluated in HeLa and DU-145 cells though confocal laser scanning microscopy (CLSM) (see experimental) and flow cytometry (see experimental). A quantitative comparison reveals the following order of uptake potency in different cell lines: MDA-MB-231>HeLa>DU-145.

Next, we probed the cellular uptake mechanism via utilizing different inhibitors for endocytic pathways in the above-mentioned cell lines through flow cytometry with cy3-siRNA containing L-siP15/1 nanoassembly (Figure 3.5c & see experimental). EIPA and hyperosmolar sucrose, inhibitors for macropinocytosis and clathrin-dependent endocytosis, respectively were found to have a striking effect on uptake in MDA-MB-231 and HeLa cells, whereas the effect of nystatin, an inhibitor for caveolae-mediated endocytosis, was found to be comparatively reduced.47-49 These results show that the major cellular internalization proceed through macropinocytosis and clathrin-dependent pathways for MDA-MB-231 and HeLa cell lines. In contrary, DU-145 cells only showed a significant decrease in fluorescence intensity when incubated in presence of EIPA suggesting macropinocytosis being the exclusive choice of uptake pathway (see experimental).
To evaluate the intracellular distribution of delivered cy3-siRNA through L-siP\textsuperscript{15/1} assembly over time, we performed CLSM of MDA-MB-231 cells in presence of endo/lysosomal stain, lysotracker blue (pseudo-colored in green in Figure 3.5d). After 4 hours of incubation, the red fluorescence from cy3-siRNA was observed to be co-localized with lysotracker blue (Figure 3.5d), indicating that the nanoassemblies are located in endo/lysosomal compartments. Interestingly, this co-localization diminishes significantly after 24 h incubation, as indicated by clear separation of red (cy3-siRNA) and blue (pseudo-colored in green for lysotracker stain) channels suggesting endosomal disruption and release of siRNA into the cytosol. A quantitative comparison is reflected in the decrease in co-localization ratio from 0.65 (4 h) to a lower value 0.34 (24 h). The probable reason for such facile intracellular release of siRNA cargo could be explained from the fusogenicity of DOPE lipid, employed in decorating the L-siP nanoassembly, through attachment and fusion with anionic endosomal membrane.\textsuperscript{7}

To further investigate whether endosomal disruption is indeed facilitated by L-siP nanoassemblies, we performed calcein green assay (Figure 3.5e and see experimental). Calcein, a membrane-impermeable dye, shows punctate green fluorescence above its self-quenching concentration when entrapped in endo-lysosomal compartments.\textsuperscript{50} However, the green fluorescence changes to a bright diffused pattern (dequenched state), if calcein can be released in cytosol after successful escape from endosomes mediated by delivery agents. As shown in Figure 3.5e & see experimental, the punctate green fluorescence of calcein in control cells confirms the endosomal entrapment, whereas a diffused fluorescence is observed for L-siP\textsuperscript{15/1} nanoassembly treated cells, suggesting efficient endosomolytic activity of the L-siP assemblies.
3.2.6 Evaluation of cytotoxicity and nuclease stability for L-siP<sub>15/1</sub>

Stability of the siRNA-polyion based electrostatic complex is mostly guided by the overall high cationic charge which eventually compromises the safety of the delivery agent increasing cytotoxicity. To evaluate the safety feature of L-siP<sub>15/1</sub> nanoassembly, we evaluated cellular viability and plasma membrane integrity in MDA-MB-231 (Figure 3.5f-g), HeLa and DU-145 (see experimental) cell lines. L-siP/siNC nanoassembly (siNC: negative control siRNA) showed ~86% cellular viability even at 200 nM siRNA concentration (with comparable polymer amount for N/P 15), whereas viability reduces to ~42% for lipofectamine-siRNA sample (LF-siNC) at an identical concentration (Figure 3.5e). Cytotoxicity study in HeLa and DU-145 cells also demonstrate a high cellular viability compared to lipofectamine (see experimental).

Next, we were interested in checking the integrity of the plasma membrane through lactate dehydrogenase (LDH) assay. The compromised cell membrane would release cytosolic LDH enzyme into cell culture media which, in turn, can be quantified through an absorbance-based assay. Figure 3.5g shows a minimal to no membrane damage mediated by L-siP assemblies even at a significantly high dosage (200 or 500 nM siRNA). In comparison, LF-siNC samples showed ~3 to 7-fold increase in membrane damage compared to untreated cells (Figure 3.5g, see experimental). These results demonstrate significantly less cytotoxicity of the designed L-siP nanoassembly desirable for a safe delivery agent. As L-siP nanoassembly comprises biocompatible DOPE/PEG-based lipids and methacrylate-derived polymers, it will be prone to slow hydrolytic and enzymatic degradation under in vivo conditions with significantly less probability of systemic accumulation.

One of the bottlenecks of RNAi based technology is the limited stability of naked siRNA with a plasma half-life of <10 min due to the degradation mediated by serum endonucleases.
Figure 3.6 eGFP silencing with L-siP\textsuperscript{15/1}: Confocal microscopy images (a) for eGFP silencing and flow cytometry data (b) for quantification of eGFP fluorescence in HeLa\textsuperscript{eGFP} cells; scale: 20 µm; Student’s t-test: ***\(p<0.001\), **\(p<0.01\), *\(p<0.05\), ns (non-significant)\(>0.05\).

Thus, a critical requirement for an efficient delivery agent is to provide end-to-end protection till the cargo is delivered in the intracellular space. To this goal, we investigated the stability of the encapsulated siRNA in the presence of RNase A and 10% fetal bovine serum.\textsuperscript{56-57} After incubation at different time interval with RNase A and serum, the L-siP/siNC nanoassemblies were subjected to redox-triggered release condition (10 mM GSH) and evaluated in agarose gel retardation assay. L-siP nanoassembly is efficient in protecting siRNA even after 24 h of incubation, whereas the unprotected naked siRNA is completely degraded within 4 h of incubation in presence of RNase A and serum (see experimental).
3.2.7 Gene silencing efficacy and retrieval of cytotoxicity mediated through PLK1 & MDR1 siRNAs

Finally, we were interested in checking the efficacy of the L-siP\textsuperscript{15/1} assembly in silencing specific gene activity. To this end, HeLa\textsuperscript{eGFP} and DU-145\textsuperscript{eGFP} cells, stably expressing eGFP, were treated with L-siP\textsuperscript{15/1} nanoassembly containing 50, 100 and 200 nM GFP-siRNA. The reduction of green fluorescent intensity was evaluated through CLSM and flow cytometry (Figure 3.6, see experimental). CLSM images, shown in Figure 3.6a (see experimental), reveals a clear decrease in green fluorescence intensity for both HeLa\textsuperscript{eGFP} and DU-145\textsuperscript{eGFP} cells upon treatment of L-siP\textsuperscript{15/1} nanoassembly. Further, the GFP expression (quantified through flow cytometry, Figure 3.6b & see experimental) was decreased to 43% at 200 nM siRNA concentration (L-siP/siGFP) in HeLa\textsuperscript{eGFP} cells in comparison to ~84% for negative control siRNA (L-siP/siNC) treated cells. A similar trend was also observed in DU-145\textsuperscript{eGFP} cells where L-siP/siGFP and L-siP/siNC treated cells showed 50% and 110% eGFP expression, respectively. Lipofectamine RNAiMAX-eGFP siRNA complex (LF-siGFP), evaluated as positive controls, exhibited reduction of eGFP expression up to 61% and 33% for HeLa\textsuperscript{eGFP} and DU-145\textsuperscript{eGFP} cells, respectively at similar siRNA concentrations.

Encouraged by these results, we further tested the gene knock-down efficacy of the L-siP\textsuperscript{15/1} nanoassemblies towards two other gene types, PLK1 and MDR1, through evaluation of mRNA transcription levels by quantitative real-time polymerase chain reaction (qRT-PCR) and protein expressions by western blot analysis (Figure 3.7). PLK1, a critical controller of mitosis, is found to be overexpressed in many cancer cells, leading to faster tumor progression.\textsuperscript{58} On the other hand, MDR1 gene in multi-drug resistant cells upregulates the expression of drug transporter proteins, like P-glycoprotein (P-gp).\textsuperscript{59} Although various small molecule inhibitors for PLK1 and MDR1 are reported in literature, siRNA-based silencing is considered to be advantageous due to
Figure 3.7 Gene silencing studies: qRT-PCR (a, b) and western blot (c, d) analysis for (a, c) PLK1 and (b, d) MDR1; Cellular viability mediated by knock-down of (e) PLK1 gene and (f) MDR1 gene (after treatment of doxorubicin for MDR1). Uptake comparison of doxorubicin via confocal microscopy (g) in untreated and L-siP/siMDR1 treated cells; orthogonal view is for L-siP+Dox sample; scale: 10 µm; Student’s t-test: ***p<0.001, **p<0.01, *p<0.05, ns (non-significant)>0.05.

its specificity, much reduced toxicity and wide applicability in multiple cancer cells. We separately constructed L-siP assemblies based on PLK1- & MDR1-siRNA and evaluated the gene knock-down efficacy in MDA-MB-231 and NCI-ADR/RES cell lines, respectively. qRT-PCR studies showed efficient silencing of both PLK1 and MDR1 genes as evident by the reduced relative gene expression levels of ~12-18% in cells treated with L-siP nanoassemblies containing PLK1 and MDR1 siRNAs (Figure 3.7a and 3.7b, at 50 or 100 nM siRNA concentrations). Moreover, western blot analyses (Figure 3.7c, 3.7d and see experimental) revealed that PLK1 and P-gp protein expressions were reduced to ~25% and ~31% (~24% and 51% for lipofectamine), respectively compared to untreated cells.

To further demonstrate the consequence of L-siP nanoassembly mediated siRNA delivery and gene silencing, cellular viability studies were conducted on both PLK1 and MDR1 transfected
MDA-MB-231 and NCI-ADR/RES cell lines, respectively. For PLK1 compromised cells (Figure 3.7e), viability was reduced to ~47% for L-siP nanoassembly (~51% for lipofectamine positive control). Similarly, when treated with anti-cancer drug -doxorubicin (Dox), MDR1 depleted NCI-ADR/RES cells showed mere ~31% viability compared to ~73% and ~35% for free Dox and lipofectamine transfected positive control cells, respectively (Figure 3.7f). Moreover, CLSM images (Figure 3.7g) confirm significantly higher red fluorescence intensity in cells from Dox suggesting efficient penetration of the drug in L-siP nanoassembly (MDR1-siRNA) treated cells, whereas a rather subdued red fluorescence is observed for only free drug-treated cells.

3.3 Summary

In summary, we report a unique siRNA encapsulation and intracellular delivery approach by developing a new symbiotic self-assembly strategy of a polymer, siRNA and lipid molecules. In this approach, the initial complexation with the siRNA is made possible through classical electrostatic interactions. The key feature here is that this interaction was carried out in a relatively apolar media that not only enhances the binding affinity between the polymer and the siRNA, but also facilitates the retention of siRNA within the *in situ* generated polymer ‘cage’. Note that the electrostatic complex is converted to a physically incarcerating capsule through a crosslinking reaction, which concurrently removes the positive charge in the polymer. As the positive charges are being removed, but before the crosslinking reinforcement is fully in place, the siRNA molecules could escape the complex. However, the siRNA remains stably encapsulated, because the bulk environment of this in situ crosslinking reaction is apolar and incompatible. This structure is then finally camouflaged by the coating of a zwitterionic lipid that also imparts biocompatibility and endosomolytic ability. We call this process symbiotic, because each of these components require the other components in the solution in order to provide the final self-assembled structure.
We have rigorously characterized each of the steps in the self-assembly process, nanoassembly formation, lipid coverage, cellular internalization and cytosolic release using both experimental and computational modeling approaches. Efficient gene-silencing mediated by the designed nanoassembly provides evidence for successful integration and leverage of the built-in molecular features. We have shown that this self-assembly strategy offers several advantages: (i) reduction of cytotoxicity from cationic charge-based delivery vectors; (ii) tunability in crosslinking degree affecting siRNA binding and release efficacy; (iii) a biologically relevant trigger for siRNA release; (iv) efficient cargo protection from degradation by nucleases; and (v) integrated utility of the useful features of lipids (biocompatible surface & tuning endosomal escape) and designer polymers (structural integrity, multivalent interaction & protection). We anticipate that the strategy reported herein could potentially serve as a safe platform and aid in the development of RNAi based therapeutics.

3.4 Experimental

3.4.1 Materials & characterizations

Dodecyl methacrylate, 2,2′-dithiodipyridine, 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid, D, L-dithiothreitol (DTT), Glutathione were obtained from Sigma-Aldrich, USA and were used without further purification unless otherwise mentioned. 2,2′-azobis-(2-methylpropionitrile) (AIBN) was procured from Sigma-Aldrich, USA and purified by recrystallization before usage. Methyl trifluoromethanesulfonate was procured from Matrix Scientific, USA. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (18:1 (Δ9-Cis) PE: DOPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt, DSPE-PEG2000) lipids were procured from Avanti Polar Lipids. Silencer™ Negative Control No. 1 siRNA (Catalog #: AM4611, proprietary sequence not provided),
Silencer™ Cy™3-labeled Negative Control No. 1 siRNA (Catalog #: AM4621, proprietary sequence not provided), Silencer™ GFP (eGFP) siRNA (Catalog #: AM4626, proprietary sequence not provided) and MDR1/ABCB1-siRNA (sense: 5’-GCUUAACACCCGACUUACAtt-3’, antisense: 5’-UGUAAGUCCGGUGUUAAGCtc-3’) were procured from Thermo-Fisher Scientific. PLK1-siRNA was obtained from Qiagen, USA (sense: 5’-CGGGCAAGAUUGUGCCUAATT-3’, antisense: 5’-UUAGGCACAAUCUUUGCCCGC-3’). Lipofectamine RNAiMAX, LysoTracker™ Blue DND-22, were obtained from Thermo-Fisher Scientific. Phosphorothioate siRNA (PTsi) was obtained from Dharmacon (sense: A*U*G*U*A*U*U*G*G*C*C*U*G*U*A*U*U*A*G, antisense: C*U*A*A*U*A*C*A*G*G*C*C*A*A*U*A*C*A*U; * denotes phosphorothioate modifications).

3.4.2 Synthesis of random co-polymer for complexation with siRNA

3.4.2.1 Synthesis of p(PDSMA-co-DodecylMA) polymer (P_{PDS/ND})

Pyridyl disulfide ethyl methacrylate (PDSMA) was synthesized using previously reported procedure. Reversible Addition-Fragmentation chain Transfer (RAFT) polymerization was utilized to synthesize p(PDSMA-co-DodecylMA) Polymer. In a typical procedure, PDSMA (0.903 g, 3.5 mmol), dodecyl methacrylate and chain transfer agent 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid were taken in a 25 mL Schlenk flask and dissolved in 2 mL dry THF. To this mixture, 1.1 mg (0.007 mmol) AIBN, dissolved in 1 mL dry THF, was added. The solution was mixed for 5 min, the flask was subjected to three freeze-pump-thaw cycles and purged with argon. Finally, the sealed flask was transferred to a preheated oil-bath and the polymerization was carried out at 70 °C for 24 h. After that, the Schlenk flask was cooled down to quench the reaction and THF was evaporated. The reaction mixture was purified by precipitating in diethyl ether for three times and finally dried in vacuo for overnight at room temperature. Yield:
78%, GPC (THF) Mₙ: 14.5 kDa, D: 1.3; ¹H NMR (400 MHz, CDCl₃): δ ppm 4.24–4.09, 3.87, 3.65–3.53, 3.37, 2.95-2.90, 1.93-1.84, 1.04-0.89; ¹³C NMR (100 MHz, CDCl₃): δ ppm 177.4, 177.07, 176.34, 159.69, 159.56, 159.5, 149.87, 137.31, 121.07, 120.01, 119.96, 62.84, 54.47, 45.34, 45.28, 45.00, 32.06, 31.71, 29.80, 29.50, 29.18, 25.41, 22.83, 14.25, 11.56.

3.4.2.2 Synthesis of cationic-PDS-dodecyl polymer (⊕P₂DS/DD)

In a 20 mL glass vial, 0.75 g of P₂DS/DD polymer was weighed and dissolved in 5 mL DCM. The solution was cooled in ice for 10 min and after that methyl trifluoromethanesulfonate (0.73 g, 4.4 mmol) was added to it dropwise. The mixture was stirred at 4 °C overnight, the solvent was dried and precipitated in diethyl ether three times to purify. The polymer was dried overnight in vacuo at room temperature. Yield: 75%, calculated Mₙ: ~22 kDa; ¹H NMR (400 MHz, CDCl₃): δ ppm 4.24–4.09, 3.87, 3.65–3.53, 3.37, 2.95-2.90, 1.93-1.84, 1.04-0.89; ¹³C NMR (100 MHz, CDCl₃): δ ppm 177.33, 159.3, 159.13, 147.57, 144.35, 125.51, 124.03, 122.05, 115.7, 62.51, 45.7, 45.13, 44.86, 37.1, 36.97, 31.73, 31.35, 29.43, 22.4, 19.08, 16.95, 13.02.

3.4.3 Characterization of polymers

(a) Gel permeation chromatography (GPC)

GPC of P₂DS/DD polymer was performed in Agilent 1260 LC instrument with a refractive index detector using THF as the eluent and molecular weights were calculated against poly(methyl methacrylate, PMMA) standards.

(b) ¹H, ¹³C, ¹H-¹⁵N & ³¹P NMR

¹H, ¹³C and ³¹P NMR spectra of the samples were recorded on a Bruker NMR spectrometer (400.13 MHz for ¹H, 100.62 MHz for ¹³C & 161.97 MHz for ³¹P). 2D NMR (¹H-¹⁵N Heteronuclear
Figure 3.8 \(^1\)H NMR spectra of (a) P^{PDS/DD} and (b) ΘP^{PDS/DD} polymers. Multiple Bond Correlation, HMBC) correlation spectra were acquired on Bruker NMR spectrometer (500.13 MHz for \(^1\)H, 50.68 MHz for \(^{15}\)N).

Molar ratio between the PDSMA and DodecylMA monomers for P^{PDS/DD} polymer was calculated from the ratio of the integrations of -CH\(_2\) protons adjacent to the methacrylate monomers (PDSMA, δ 4.21 and DocecylMA, δ 3.9 with x:y= 9:1).

(c) FT-IR spectra
FT-IR spectra of the polymers was recorded on a Bruker Alpha FT-IR (ATR) spectrometer from 3500 cm\(^{-1}\) - 400 cm\(^{-1}\) range.

Figure 3.9 \(^1\)H-\(^{15}\)N HMBC spectra of 1-methylpyridine-2-thione, \(\text{P}^{\text{PDS/DD}}\) and \(\oplus \text{P}^{\text{PDS/DD}}\) polymers. The absence of some correlation peaks with ring protons for the \(\oplus \text{P}^{\text{PDS/DD}}\) polymer can be attributed to the short \(T_2\) (spin-spin) relaxation which can be approximately calculated as, \(T_2 \approx 1/(\pi\delta/2)\), where \(\delta\) is peak width at half height. For macromolecules like polymers, signals from protons with short \(T_2\) often do not survive the 2D pulses that keep magnetization along xy plane. This also suggests slower molecular reorientation of cationic pyridine ring protons compared to the N-methyl protons.
Figure 3.10 Gel permeation chromatography of $\text{P}^{\text{PDS/DD}}$ polymer.

Figure 3.11 FT-IR spectra of $\text{P}^{\text{PDS/DD}}$ and $\oplus \text{P}^{\text{PDS/DD}}$ polymers. A band at 1617 cm$^{-1}$ is observed for N-methylated pyridinium polymer ($\oplus \text{P}^{\text{PDS/DD}}$) characteristic of C=N vibration related to quaternary nitrogen atom in the ring. This band is absent in the $\text{P}^{\text{PDS/DD}}$ polymer. The pyridine ring low intensity band ($\text{P}^{\text{PDS/DD}}$ polymer) at 1485 cm$^{-1}$ due to conjugated C=C and C=N bonds become stronger in the $\oplus \text{P}^{\text{PDS/DD}}$ polymer spectra. C=O stretching at 1718/1722 cm$^{-1}$ is used as calibration peak for both polymers.

3.4.4 Study of siRNA encapsulation with varying dosages of $\oplus \text{P}^{\text{PDS/DD}}$ polymer (at different N/P ratios)

3.4.4.1 Preparation of L-siP nanoassembly without crosslinking

To study the effect of N/P ratio on complexation, two different sets of solutions were prepared containing siRNA and $\oplus \text{P}^{\text{PDS/DD}}$ in a mixed solvent system (acetone:water=70:30). In
first set, a fixed amount of siRNA (2 µg) was dosed in acetone/water solvent mixtures (70:30) to get 50 µL identical solutions. In another set, different amounts $\Theta p^{PDS/DD}$ polymer solution (2 mg/mL in acetone:water= 70:30) were diluted with same mixed solvent system to achieve another 50 µL solutions. Afterwards, the polymer solutions were added to the fixed amount siRNA solutions (containing 2 µg siRNA) to finally achieve N/P ratios of 5, 7.5, 10, 15, 20 and 25. All these mixed solutions were incubated for 2h in an orbital shaker at 20 °C to facilitate complexation. Meanwhile, mixed lipid solutions were prepared in 2 mL water containing 20% wt./wt. DOPE & 10 mol% DSPE-PEG (based on $\Theta p^{PDS/DD}$ polymer) and stirred for 30 min. Next, each of 100 µL nanoassembly solutions, after 2 h complexation period, was added to an aqueous pool of lipid mixture and stirred for 3 h at 20 °C. In this step, the glass vials were kept open to facilitate the evaporation of organic solvents and maturation/hardening of the nanoassemblies. Finally, the solutions were filtered through Amicon Ultra Centrifugal Filters MWCO 10 kDa to remove remaining organic solvents, purify and concentrate the solutions. The final volume of L-siP nanoassembly solutions was adjusted to 100 µL with nuclease-free deionized water.

3.4.4.2 Preparation of L-siP nanoassembly with crosslinking

Crosslinking of L-siP nanoassemblies were achieved by introducing DTT solutions after the complexation step. Different amounts of DTT (0.1, 0.25, 0.5, 1 and 2 equivalents with respect to PDS moiety in $\Theta p^{PDS/DD}$ polymer) dissolved in acetone/water (70:30) mix solvent were added to the siRNA-polymer mixtures after 2 h complexation step. Each solution was incubated in an orbital shaker at 20 °C for another 2 h. After that all solutions were subjected to lipid locating step.

3.4.5 Agarose-gel retardation assay for proof of complexation & siRNA release

3.4.5.1 Encapsulation study
20 µL samples (with different N/P ratios and varying crosslinking) were mixed with 4 µL of gel loading buffer and loaded into 2% agarose gel made in TAE buffer containing EtBr. Samples were run in horizontal electrophoresis system at 110 V for 1 h and subjected to imaging analysis with NuGenius gel imager system (Syngene).

3.4.5.2 Release study

20 µL sample was mixed with 1 µL Triton-x (0.1 g/mL) and sonicated for 5 min. Next, 250 mM glutathione solution was added to it and the pH was adjusted to ~7-8 with 1 N NaOH solution (final glutathione concentration was 10 mM). The mixture was incubated for 6 h at 37 °C and subjected to agarose gel electrophoresis and visualization method as described above.

3.4.6 MD-simulations: modelling for encapsulation & release

Construction of coarse-grained (CG) models, simulation parameters, features of N/P 7.5 and 15 systems, DTT crosslinking, Construction of complete L-siP nanoassembly and release simulations of siRNA are described in detail in Supporting Information. Here, we have decided to use dsDNA as our CG models are based on the MARTINI force field, for which reliable dsDNA parameters are already available in literature. However, our choice is justified also by the fact that, at the level of precision of our CG models, the difference between dsDNA and siRNA is likely to be negligible, given the electrostatic nature of self-assembly in this system and the fact that the charges would be the same in molecules of same lengths.

3.4.7 MD-simulation details about modelling for encapsulation & release

CG models – The CG model for the cationic PDS-Dodecyl polymer (⊕P_{PDS/DD}) has been built based on the popular MARTINI force field. The CG structure has been mapped from an all atom (AA) structure of the polymer, while the appropriate CG MARTINI beads have been selected to preserve the different hydrophobicity of the mapped chemical groups. Experiments have been
conducted using double strand dsRNA fragments, which are rigid charged molecules. For convenience, the CG simulations have been conducted using rigid 20-bp dsDNA segments of the same length instead of RNA, given the availability of a reliable MARTINI model for DNA (using dsDNA instead of dsRNA make little difference in our models, as the aggregation is controlled by electrostatic interactions and that can be both thought of as rigid charged cylindrical molecules). DTT has been mapped consistently on an AA model. The DTT CG model is composed of four CG beads – two central SP2 beads plus two terminal SC5 beads. The interaction between the DTT terminal SC5 beads and the first SC5 bead of the PDSMA chains has been then artificially strongly enhanced, to mimic the effect of DTT crosslinking ((i) strong selective spontaneous coordination and (ii) formation of stable bonds).

Figure 3.12 Coarse grained (CG) representation of the cationic-PDS-Dodecyl polymer (⊕p^{PDS/DD}). The CG MARTINI beads used in the model are indicated in the figure. SQ0 beads are charged (+1e). When modelling crosslinking, (i) the interaction of the first SC5 bead of the PDSMA chain with DTT has been artificially augmented to observe the spontaneous formation of stable (not breakable) bonds, and (ii) the side groups coordinated to DTT have been then detached from the rest of the polymer.
Figure 3.13 CG-MD simulations at N/P 7.5 ratio showing the leakage of oligonucleotides from the polymers following to crosslinking (insertion of DTT molecules). (a) The self-assembled system before DTT insertion appears as a quite loose network, kept together mainly by electrostatic interactions. When DTT crosslinking happens, polymers undergo compaction and the guest nucleotides detach from the polymer chains due to the loss of electrostatic interactions. (b) Quantification of the leakage: number of polymer-polymer and polymer-dsDNA contacts as a function of the CG-MD simulation time after the insertion of DTT.

Simulation parameters – All simulations have been performed with the GROMACS molecular dynamics suite, patched with the PLUMED plugin. In production runs, we used the MD integrator with a time step of 20 fs, the v-rescale thermostat with a time constant of 2 ps and the Parrinello-Rahman barostat with a time constant of 8 ps.

Aggregation in N/P 7.5 and N/P 15 systems – We built the two systems by inserting 6 (or 12) dsDNA chains in a simulation box containing the same amount (58) of cationic polymer chains, a
70:30 acetone:water mixture and monovalent counterions to ensure charge neutrality of the system. In both systems, we observed spontaneous aggregation between the dsDNA fragments and the cationic polymer chains, led by the electrostatic interaction between their oppositely charged groups. The final aggregates in 70:30 acetone:water appeared as quite loose and not very dense), consistent with experimental observations of the systems before the insertion of DTT.

**DTT crosslinking** – The effect of crosslinking has been mimicked in two steps: (i) the insertion of DTT molecules that selectively bind to the S-S groups of the polymers and (ii) the cleavage of positively charged groups of the polymers. For (i), we first imposed an artificially strong interaction between the terminal DTT CG beads and the first SC5 beads in the side charged groups of the polymer) – this allowed us to model the spontaneous coordination of DTT to those side groups that are really accessible by DTT molecules. Then (ii), the side charged groups coordinated to DTT have been detached from the rest of the polymer (deletion of the explicit bonds), and the SQ0 bead has been turned neutral, consistent with the experimental process.

**Creation of the L-siP nanoparticle for the N/P 15 system** – To create a model of the L-siP nanoparticle, we started from the self-assembled polymer-dsDNA system at N/P 15 and introduced a 100% crosslinking as described above. We replaced the 70:30 acetone:water mixture with a (more polar) 20:1 water:acetone solvent mixture, coherent with the experiments, and introduced in the system enough DOPE lipids to cover the assembled aggregate surface. In order to obtain a single aggregate at the center of the simulation box, we used a recently optimized simulation technique (involving the use of PLUMED plugin) that drives the spontaneous assembly of self-assembling molecules toward a single aggregation center (instead of forming multiple nuclei, which slow down the aggregation process). The system has been then equilibrated for 4 µs of CG-
MD simulation. The radial distribution functions of Figure 3.2f have been calculated with the \textit{gmx rdf} tool of the GROMACS suite and normalized to have the same integral.

\textit{Release simulations} – To model the release of dsDNA fragments at different crosslinking conditions, we started from the equilibrated L-siP nanoparticle at N/P 15 ratio and 100% of crosslinking (1 equiv DTT). We removed the DOPE lipid molecules and performed 3 different simulations: one keeping all coordinated DTT in the system (100% crosslinking), one removing 90% of the DTT molecules (thus, effectively simulating a residual 10% crosslinking condition) and a last one removing all the DTT (0% residual crosslinking). The dsDNA release has been quantified by monitoring the number of contacts between the polymer and the guest oligos in time during the CG-MD run calculated with \textit{gmx mindist} and normalized dividing by the initial value (corresponding to the equilibrated L-siP nanoparticle).

\textbf{3.4.8 Visualization of L-siP nanoassembly with N-STORM confocal microscope}

For N-STORM imaging, cy3-labelled siRNA loaded L-siP nanoassemblies were prepared and the outer lipid layer of the nanoassembly was incorporated with carboxyfluorescein labelled PEG-lipid (18:0 PEG2000 PE CF). The particles were first deposited on a L-lysine coated 35 mm glass bottomed petri-dish and incubated at room temperature for 15 min. Next, excess solution was withdrawn and gently washed with nuclease free water to remove loosely bound particles. Finally, imaging buffers (5% m/v glucose, 0.1 M MEA, 1 mg/mL glucose oxidase and catalase) were added to the wet petri-dish and transferred to NIKON confocal/N-STORM set-up for imaging (100X TIRF objective, excitation at 488 & 561 nm).

\textbf{3.4.9 Measurement of DLS and Zeta-potentials for L-siP nanoassemblies}

Dynamic light scattering (DLS) and zeta potential measurements were performed using a Malvern Nanozetasizer-ZS. Prepared L-siP nanoassembly and control samples were diluted with Milli-Q
water (50 µL sample added to 850 µL water) before subjected to particle size and zeta potential measurements.

**Figure 3.14** DLS size distribution (a) and correlation diagram (b) of uncrosslinked particles at different N/P ratios.

**Figure 3.15** Zeta potential measurements for (a) uncrosslinked samples at different N/P ratios; (b) for L-siP15/1 with varying crosslinking; (c) lipids and mixture of (lipids + ⊕PDS/DD). At higher N/P ratio and crosslinking density zeta potential decrease up to ~10 mV. Note that the zeta potential is not expected to show a significant change as the lipids layers are surrounding the particles in all cases which shows negative zeta potentials.

### 3.4.10 Encapsulation efficiency and loading capacity

As in N/P 15 with 1 equiv. DTT crosslinking condition, there was no unencapsulated siRNA band observed in agarose gel electrophoresis, the encapsulation efficacy for L-siP15/1 nanoassembly was 100%.

The loading capacity for L-siP15/1 nanoassembly is calculated based on the following formula:
\[ LC, \% = \frac{\text{amount of encapsulated siRNA}}{\text{amount of polymer}} \times 100 \]

LC for L-siP15/1 nanoassembly was found to be \((2 \, \mu g \times 100/27 \, \mu g =) 7.4\%\).

**Figure 3.16** 31P NMR spectra indicating peaks from phosphorothioate-siRNA (PTsi, \(\delta \approx 53.56-55.97 \, ppm\)) and lipids (\(\delta \approx -0 \, ppm\)). For crosslinked \(\oplus P^{\text{PDS/DD}} + \text{PTsi} + \text{final L-siP (with PTsi & Lipid)}\) samples, no 31P peak from siRNA is observed due to complexation, whereas peak from lipid coating is visible. Once PTsi is released from L-siP nanoassembly, it becomes visible again in 31P NMR.

**Figure 3.17** Color-coded intensity profiling of a single L-siP15/1 nanoassembly showing a dense crosslinked polymer core with relatively light external lipid coating, scale: 50 nm.
3.4.11 Cryo-TEM study of L-siP nanoassemblies-N/P 15- crosslinked (1 equiv DTT) & uncrosslinked

Cryo Transmission Electron Microscopy was performed using FEI (Fisher Scientific) Tecnai T12 instrument operated at 120 kV using a Gatan 636 cryo-transfer holder. Imaging was done under low dose conditions. Samples were prepared using a FEI (Fisher Scientific) Vitrobot MKII using liquid ethane. Lacey Carbon on 200 mesh Copper Grids were glow discharged for 30 s prior usage.

3.4.12 Quantification of siRNA encapsulation & release for L-siP nanoassembly through Quant-iT™ RiboGreen assay

To generate a standard curve of free siRNA, different concentrations of negative control siRNA solutions were prepared in nuclease-free water as per the manufacturer’s protocol. L-siP nanoassemblies encapsulated with negative control siRNA with differential crosslinking density were diluted with TE buffer and mixed with RiboGreen reagent in a black 96 well plate. Another set of L-siP nanoassemblies were subjected to glutathione mediated release condition at first; then mixed with buffer and RiboGreen reagent. Samples were incubated at room temperature for 5 min and then subjected to fluorescence measurements in SpectraMax® M5 fluorescence microplate reader (excitation: 480 nm, emission: 520 nm).

3.4.13 Cell culture

HeLa (cervical cancer), DU 145 (prostate cancer), MDA-MB-231 (mammary gland/breast cancer), NCI/ADR-RES (derived from the ovarian cell line OVCAR-8/Adriamycin (Doxorubicin or Dox) resistant, procured from NCI, Frederick), HeLa^{GFP} and DU 145^{GFP} (eGFP transfected) cell lines were cultured in 100 mm cell culture petri-dish containing Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) in a humidified incubator with 5% CO2 at 37 °C.
Culture media was supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic-antimycotic (100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of Amphotericin B).

3.4.14 **Cellular internalization, endosomal escape and L-siP nanoassembly stability studies**

**Figure 3.18** Cellular uptake of cy3-labelled siRNA in HeLa, DU-145 and MDA-MB-231 cell lines, scale: 20 μm.
3.4.14.1 Cellular internalization studies

(a) Confocal microscopy

HeLa, DU 145 and MDA-MB-231 cell lines were seeded at $1 \times 10^5$ cells/mL density (1 mL) in 35 mm glass-bottomed petri-dishes and cultured for 3 days at 37°C and 5% CO₂ incubator for proper adhesion. Afterwards cells were washed three times with PBS buffer and incubated with L-siP nanoassemblies containing 100 nM cyanine-3-siRNA in 1 mL serum-free media at 37 °C for 4 h. Next, the media was removed, washed three times with PBS and incubated with NucBlue™ Live ReadyProbes™ reagent in FBS containing media for 1 h to stain cell nucleus. Live cell imaging was performed using Nikon Spectral A1+ confocal microscope. All images were analyzed with Nikon NIS-Elements software.

(b) Flow cytometry for uptake quantification

HeLa, DU 145 and MDA-MB-231 cell lines were seeded at $1 \times 10^5$ cells/mL density (1 mL) in 12 well plate and cultured for 1 day at 37°C and 5% CO₂ incubator. Next, cells were washed three times with PBS buffer and incubated with L-siP nanoassemblies containing 100 nM cyanine-3-siRNA in 1 mL serum-free media at 37 °C for 4 h. Next, cells were washed with cold PBS and trypsinized (0.25% trypsin-EDTA solution) for 10 min to remove non-internalized samples from cell surface and de-touch from well plate. Cells were pelleted by centrifugation, washed two times with cold PBS and finally re-suspended with 500 µL cold PBS for flow cytometry analysis in BD LSRFortessa™ instrument. Data analyses was performed with FlowJo software to obtain fluorescence intensities of cell samples.

(c) Mechanism of cellular uptake

HeLa, DU 145 and MDA-MB-231 cell lines were seeded at $1.2 \times 10^5$ cells/mL density (1 mL) in 12 well plate and cultured for 1 day at 37°C and 5% CO₂ incubator. Next, cells were washed
Figure 3.19 Quantification of cellular uptake and mechanism of siRNA transfection in presence and absence of endocytic inhibitors for Hela, DU-145 and MDA-MB-231 cell lines through flow cytometry (a, c: bar graph; b, d: histogram plots).

with PBS and pre-incubated with EIPA (100 µM, macro-pinocytosis pathway), Nystatin (30 µM, caveolin pathway) and Hyper-osmolar sucrose (45 mM, clathrine pathway) in serum-free media for 1 h at 37°C. After pre-incubation step, cells were co-incubated for another 1 h with L-siP nanoassemblies loaded with cy3-siRNA (50 nM, 1 mL serum-free media) and different inhibitors with final concentrations mentioned above. Untreated and L-siP-cy3-siRNA treated cells without
any inhibitor were used as negative and positive controls, respectively. Next, cells were harvested as described before for flow cytometry analysis.

### 3.4.14.2  Endosomal escape

MDA-MB-231 cells were seeded at $1.5 \times 10^5$ cells/mL density (1 mL) in 35 mm glass-bottomed petri-dishes and cultured for 1 day at $37^\circ C$ and 5% CO$_2$ incubator.

![Figure 3.20](image)

**Figure 3.20** Calcein assay for MDA-MB-231 cells: z projection (with 2 µm increment) and orthogonal view, scale: 20 µm.

(a) **Colocalization of Lysotracker Blue-cy3 siRNA**

To study the endosomal escape, cells were washed with PBS three times and incubated with 100 nM cy3-siRNA L-siP nanoassemblies for 4 h in serum-free media. Next, one set of samples was subjected to confocal imaging after endosome staining and the other set was incubated for 24 h in complete media before subjecting to endosome staining and confocal microscopy. Lysotracker blue was used to stain endosomes as per manufacturer’s protocol. Live cell imaging was performed using Nikon Spectral A1+ confocal microscope. To improve visibility,
Lysotracker Blue stain in the images was pseud-colored with green and colocalization with red cyan3-siRNA was studied. All images were analyzed with Nikon NIS-Elements 4.0 software.

(b) Calcein assay

Cells were transfected with negative control siRNA loaded L-siP nanoassembly (100 nM siRNA) and 100 µM calcein for 8 h in serum-free media. Next, those were washed three times with PBS and incubated with complete media for another 2 h. A control set of cells was generated with only calcein treatment (without L-siP). Before subjecting to CLSM study, cell nucleus was stained with NucBlue™ Live ReadyProbes™ reagent. Cells were imaged with Nikon Spinning Disk Confocal Microscope (excitation 488 nm laser) and data was analysed with Nikon NIS-Elements software.

3.4.14.3 Stability of L-siP nanoassembly in presence of RNase A and serum

To test the L-siP nanoassemblies stability in presence of RNase A, 15 µL of L-siP nanoassembly solution (N/P 15, 1 equiv DTT crosslinked) was mixed with 1 µL RNase A solution (50 µg/mL) or serum (10% v/v) and incubated at 37 °C for different time intervals. After that, 2 µL of EDTA (0.5 M) and 2 µL SDS (100 mM) were added to it and heated at 60 °C for 5 min. Afterwards, samples were subjected to glutathione (10 mM) based release conditions and finally loaded into 2% agarose gel (EtBr stained) to check the band intensity. For control, equal amount of naked siRNA was subjected to the identical conditions.
Figure 3.21 Stability assessment of siRNA (Naked or L-siP15/1) in presence of RNase A and 10% Serum at 37 °C.

Figure 3.22 Cellular viability of (a) HeLa and (b) DU-145 cells in presence of L-siP nanoassembly at different loaded concentrations of siRNA; L-siP/Empty: crosslinked nanoassembly without siRNA (with same polymer concentrations as in L-siP/siNC); siNC: negative control siRNA; LF: Lipofectamine; Student’s t-test: ***p<0.001, **p<0.01, *p<0.05, ns (non-significant)>0.05.

Figure 3.23 Cytotoxicity measured by LDH assay in (a) HeLa and (b) DU-145 cells in presence of L-siP nanoassembly at different loaded concentrations of siRNA; L-siP/Empty: crosslinked nanoassembly without siRNA (with same polymer concentrations as in L-siP/siNC); siNC:
negative control siRNA; LF: Lipofectamine; Student’s t-test: ***p<0.001, **p<0.01, *p<0.05, ns (non-significant)>0.05.

**Figure 3.24** Comparison of cytotoxicity measured by (a) cellular viability and (b) LDH assay in MDA-MB-231, HeLa and DU-145 cells in presence of L-siP nanoassembly (empty, cross-linked nanoassembly without siRNA) and Lipofectamine RNAiMAX at different concentrations of polymer and lipofectamine (1, 2, 4, 8 and 10 µg/mL, which corresponds to 25, 50, 100, 200 and 500 nM siRNA incorporated L-siP in Figure 5.5f-g).

3.4.15 Toxicity studies of L-siP nanoassemblies in HeLa/DU-145/MDA-MB-231 cell lines

3.4.15.1 AlamarBlue assay for cellular viability

HeLa, DU 145 and MDA-MB-231 cells were seeded (7.5 ×10^3 cells in 0.1 mL per well) into 96-well tissue culture plates and incubated at 37 °C. After 24 h, cell culture media was replaced with serum-free media containing L-siP nanoassemblies bearing different concentrations of negative control siRNA (25, 50, 100, 200 and 500 nM). Identical control crosslinked nanoassemblies devoid of any siRNA, L-siP(empty), were also tested for toxicity. Lipofectamine® RNAiMAX was complexed at different dosages (2, 5 and 10 µg/mL) with negative control siRNA (at 100 nM) and compared with other samples. After 24 h incubation, media was replaced with complete fresh one and incubated for another 2 days (72 h in total). After that cells were washed
with PBS for three times and each well was treated with 100 μL 10% alamarBlue in complete media. Finally, cells were incubated for 1 h at 37 °C and solutions were transferred to black 96-well flat-bottomed plate for fluorescence measurement with SpectraMax® M5 microplate reader (excitation: 560 nm, emission: 590 nm).

### 3.4.15.2 LDH cytotoxicity assay for membrane damage studies

For LDH assay, all cells were incubated with L-siP nanoassemblies for 24 h and then subjected to Pierce LDH cytotoxicity assay.\textsuperscript{61} 50 μL of media was collected from 96 well cell culture plate and transferred to another 96 well plate. To that solution 50 μL LDH reaction mixture was added and incubated at room temperature for 30 min. Next, 50 μL stop solution was added to each well and subjected to absorbance measurements with SpectraMax® M5 microplate reader at 490 nm and 680 nm (cytotoxicity was calculated based on absorbance, A= A\textsubscript{490}-A\textsubscript{680}).

### 3.4.16 Gene silencing studies

![Confocal microscopy images and flow cytometry data for quantification of eGFP fluorescence](image)

**Figure 3.25** eGFP silencing with L-siP15/1 in DU-145eGFP cells: (a) Confocal microscopy images and (b) flow cytometry data for quantification of eGFP fluorescence, respectively; scale: 20 μm; Student’s t-test: ***p<0.001, **p<0.01, *p<0.05, ns (non-significant)>0.05.

#### 3.4.16.1 Knockdown of GFP in GFP-transfected HeLa & DU-145 cell lines through flow cytometry & confocal microscopy
To study the gene silencing, GFP-transfected HeLa and DU-145 cells were plated in 12 well plate (5× 10^4 cells in each well) and incubated for 24 h at 37 °C. After that cells were transfected with GFP-siRNA loaded L-siP nanoassemblies (50, 100 & 200 nM siRNA concentrations) and incubated for 24 h. Next, media was replaced with fresh one and incubated for another 24 h at 37 °C. Finally, cells were trypsinized, pelleted by centrifugation and washed two times with PBS followed by suspension in 500 µL PBS. Flow cytometry was performed with this cell suspension in a BD LSRFortessa™ instrument (excitation wavelength: 488 nm, FITC channel) to check the reduction in GFP fluorescence intensity. FlowJo version 10 software was used to analyze data and obtain fluorescence intensities of the samples.

For confocal microscopy analyses for GFP-silencing, 1×10^5 cells were plated in 35 mm glass-bottomed petri-dishes and incubated for 3 days at 37°C and 5% CO₂ incubator for proper adhesion. Afterwards cells were transfected with L-siP-GFP-siRNA nanoassemblies, incubated for 24 h, washed and subjected to further 24 h incubation before subjecting to washed three times with PBS buffer and incubated with L-siP nanoassemblies containing 100 nM cy3-siRNA in 1 mL serum-free media at 37 °C for 24 h. Next, the media was removed, washed three times with PBS and incubated with NucBlue™ Live ReadyProbes™ reagent in FBS containing media for 1 h to stain cell nucleus. Live cell imaging was performed using Nikon Spinning Disk confocal microscope. All images were analyzed with Nikon NIS-Elements software.

3.4.16.2 Knockdown of PLK1 in MDA-MB-231 and MDR1 in NCI-ADR/RES cell lines through qPCR & western blot studies

1.5 × 10^5 cells were plated in 6 well tissue culture plate, incubated for 24 h at 37 °C- 5% CO₂ atmosphere and then transfected with L-siP nanoassemblies containing PLK1 and MDR1 siRNA (at 50 & 100 nM siRNA concentration) for MDA-MB-231 & NCI-ADR/RES cells,
respectively. Control samples containing L-siP nanoassemblies without siRNA, untreated cells and only siRNA treated cells were subjected to similar conditions. After transfection (24 h), media was changed and incubated at 37 °C- 5% CO2 atmosphere for another 24 h.

**qPCR studies**

Finally, cells were washed and total RNA was isolated by RNA extraction kit (High Pure RNA Isolation Kit, Roche) according to manufacturer’s protocol. Isolated RNAs were checked for purity and concentrations by measuring absorbances at 260/280 nm.

Next, cDNA synthesis was performed with iScript cDNA synthesis kit (Bio-Rad Laboratories) from the isolated RNA. After that, RT-PCR was performed using the synthesized cDNA, PerfeCTa MultiPlex qPCR SuperMix (Low ROX) and Taqman probes for PLK1, MDR1 and control β-actin genes in Mx3005P qPCR System (Stratagene/Agilent). Target gene expression levels were normalized and reported as fold increase compared to β-actin using the ΔΔCT method.

**Western blot analyses**

To isolate total proteins, cells were washed with cold PBS buffer once and scraped to de-touch from the plate and transferred with cold PBS to an eppendorf tube. Afterwards, cells were pelletized by centrifugation, washed with cold PBS twice to remove proteins from media. Next, RIPA lysis buffer containing protease/phosphatase inhibitor mix was added to the cell pellet keeping it in ice and incubated for 15 min, followed by 3×30 s sonication to ensure complete lysis of cells. Finally, lysed cells were centrifuged at 14000 rpm - 4 °C to collect soluble protein extracts and quantified with 660 nm protein assay.

Western blot analyses were performed to identify PLK1 and MDR1/P-gp protein levels in cells. Rabbit monoclonal antibodies (PLK1, MDR1/ABCB1 & β-Actin mAbs, Cell Signaling) were used to detect target proteins and loading control. HRP-linked anti-rabbit IgG was used as secondary
antibody and proteins bands were detected by enhanced chemiluminescence (ECL) reagent (Luminol, coumaric acid and H2O2).

3.4.17 Cellular viability post-PLK1 and MDR1 knockdown

MDA-MB-231 and NCI/ADR-RES cells were seeded at a density of 5×10^3 cells/well/100 µL in 96-well tissue culture plate. After 24 h incubation, media well removed and treated with L-siP nanoassemblies loaded with PLK1 siRNA (for MDA-MB-231 cells) and MDR1 siRNA (for NCI/ADR-RES) at final siRNA concentration of 100 nM in serum-free media. Control sets of samples were also subjected to identical conditions. After 24 h incubation, media was replaced with fresh complete one and incubated for another 48 h.

For PLK1 siRNA treated samples, cells were subjected to alamarBlue assay (as described in manuscript). For MDR1-siRNA treated samples, media was removed, treated with 10 µM doxorubicin in complete media and incubated for another 48 h. After that cells were subjected to alamarBlue assay (as described in manuscript).

3.4.18 Uptake of doxorubicin in NCI/ADR-RES cells after MDR1 knockdown

NCI/ADR-RES cells were seeded at 1×10^5 cells/mL density (1 mL) in 35 mm glass-bottomed petri-dishes and cultured for 3 days at 37°C and 5% CO2 incubator for proper adhesion. Afterwards, media was removed, treated with L-siP nanoassemblies loaded with MDR1-siRNA (final siRNA concentration: 100 nM) in serum-free media and incubated for 24 h. Next, media was replaced with fresh complete one and incubated for another 48 h. After that, cells were treated with 10 µM doxorubicin and incubated for 4 h. Finally, media was removed, cells were washed with PBS and nucleus was stained with NucBlue™ Live ReadyProbes™ reagent before subjecting to confocal microscopy by Nikon Spectral A1+ confocal microscope. All images were analyzed with Nikon NIS-Elements software.
3.5 Abbreviations

‘L-siP’: ‘Lipid decorated siRNA-Polymer’ nano-assembly; siNC: siRNA-Negative Control; LF: Lipofectamine; PLK1: polo-like kinase-1; MDR1: multi drug resistant-1; CLSM: Confocal Laser Scanning Microscopy.
3.6 References


CHAPTER 4

A STRUCTURE-ACTIVITY STUDY TO MODULATE REACTIVE SIDE CHAINS OF A SELF-IMMOLATIVE POLYMER FOR OPTIMAL PROTEIN CONJUGATION

Adapted in parts from the manuscript under review: Dutta, K; Kanjilal, P. Das, R.; Medeiros, J.; Thayumanavan, S. “Synergistic Interplay of Covalent and Non-Covalent Interactions in Reactive Polymer Nanoassembly Facilitates Intracellular Delivery of Antibodies”

4.1 Introduction

Controlled polymerization and post-modification techniques have provided many impressive examples of creating functional polymers for utilization in catalysis, sensing, tissue-engineering and controlled drug delivery.\textsuperscript{1-10} Amongst these, activated ester polymers have gained significant attention for providing enormous flexibility in bioconjugation processes to install a desired functionality, which was otherwise impaired due to the structural instability of sensitive biomolecules under harsh reaction conditions.\textsuperscript{1,11-15} Inspired by this, we had designed a self-immolative polymer containing activated carbonate moieties and reported a covalent self-assembly approach for encapsulation of functional proteins through the reactive side chains.\textsuperscript{16} Lysines, an abundant surface functionality in majority of proteins,\textsuperscript{16-17} have been utilized as conjugation handles for reaction with the activated carbonate moieties to form self-assembled nanostructures. Due to the presence of reactive side-chain functionalities that are responsive to redox stimuli, the encapsulated proteins could be released in a ‘traceless’ manner with retention of its
catalytic activity. Understanding the potential of the newly developed polymer platform, we envisaged the utilization of such reactive covalent self-assembly approach for conjugation of functional antibody through the conveniently available lysines present on the surface of antibodies. However, slow macromolecular reaction kinetics owing to high pKₐ of lysine amines, incomplete reactivity of activated carbonate groups with lysines, and competitive hydrolytic degradation of polymer were found to be major hurdles in the successful extension of this approach for conjugation of larger biomacromolecules, such as antibodies (~150 kDa).

**Figure 4.1** Schematic representation of activated carbonate containing self-immolative polymers tested for lysine conjugation

In this chapter, we have synthesized various activated carbonates containing self-immolative polymers and studied the kinetics of aminolysis vs. hydrolysis in presence of a small molecule lysine analogue (Figure 4.1). Down-selected activated carbonate polymer with higher degree of aminolysis and low hydrolysis is utilized to test encapsulation and stimuli-responsive release of proteins.
4.2 Results and discussion

4.2.1 Synthesis of activated carbonate-containing monomers and polymers for protein conjugation

While the reaction between amines and various activated ester moieties are well-established, including for bioconjugation, such an understanding does not exist for activated carbonates.\textsuperscript{1,11-12,21} Note that utilization of an activated carbonate, instead of the classical activated ester, is critical for reversibility in polymer-protein conjugation through surface lysines. We also envisaged that the resultant carbamate linker would impart hydrolytic stability of the covalent connection, and provide potential biocompatibility due to resemblance to the biologically abundant amide moiety. Stable conjugation with reversible features is critical for a versatile assembly that can translocate the protein across a cellular membrane and release the protein cargo in its native form without any remnants of polymer.\textsuperscript{13,22} To address this, we incorporated a disulfide bond for redox-mediated cleavage at the $\beta$-position of the carbonate moiety in the polymer chain. Upon cleavage of the disulfide owing to the presence of higher intracellular glutathione concentration, the self-immolative mechanism will kick-in to release the attached protein tracelessly in its pristine form (Figure 4.2).\textsuperscript{18} Although we have successfully demonstrated encapsulation of proteins with $p$-nitrophenyl-carbonate, this functionality falls short of the ability to encapsulate larger proteins, such as antibodies, likely due to low reactivity and competitive hydrolysis issues. We surmised that identifying a reactive functionality that is biased towards aminolysis over hydrolysis would address these challenges.
Figure 4.2 (a) Schematic of the designed random copolymers for evaluating protein conjugation; (b) General reaction scheme for the synthesis of activated ester containing monomers; (c) General reaction scheme for polymerization to achieve random copolymers of PEG and activated ester monomers; and (d) Activated carbonate polymer mediated protein conjugation and ‘traceless’ release.
To this end, we synthesized a library of random copolymers containing six potential activated carbonate candidates, viz., nitrophenyl (NPC, previously reported), pentafluorophenyl (PFP), trichlorophenyl (TCL), hexafluoropropanol (CF3), trimethylaminophenyl (NMe3) carbonate moieties (Figure 4.2). The polymers were synthesized via RAFT polymerization technique using carbonate methacrylate and PEG methacrylate monomers (Figure 4.2, see experimental section for detailed synthetic procedures). Post-polymerization modification of a PEG-hydroxyethylene disulfide polymer was utilized to synthesize NHS carbonate ester polymer (see experimental section). All monomers and polymers were characterized by NMR (1H, 13C and 19F NMR (as required)) and gel permeation chromatography. The ratio between the carbonate and PEG groups (~2:8) were evaluated from 1H NMR.

4.2.2 Comparison of aminolysis vs. hydrolysis and efficacy studies

To test the designed polymers for conjugation efficacy and degradation kinetics, we first investigated the aminolysis reaction in the presence of a protected small molecule lysine analogue (Figure 4.3). Kinetic studies of the desired aminolysis and the competitive hydrolytic degradation were performed by reacting polymers with the lysine analog in phosphate buffer (pH 8.5, previously optimized for protein conjugation). For TCL-, CF3-, NMe3-, and NPC-polymers, both aminolysis and hydrolysis rates were found to be very slow. Aminolysis rates for PFP- and NHS-polymers were found to be much faster. However, with the NHS-polymer, this increase in rate of aminolysis was also accompanied by an increase in the rate of hydrolysis. Considering that we require fast aminolysis rates, combined with a significant bias towards aminolysis relative to hydrolysis rates, we concluded that the PFP-polymer would be the best choice for protein conjugation.
Figure 4.3 Kinetics studies with synthesized activated ester containing polymers to examine extent of aminolysis and hydrolysis reactions.

To investigate the translation of our findings with small molecules on to protein-polymer conjugation, we investigated the reactions of NPC-, PFP-, and NHS-polymers with a model protein, horseradish peroxidase (HRP). Percentage encapsulation of proteins, as evident from the SDS-PAGE analysis, was found to follow the following order: PFP (48%) > NHS (18%) > NPC (11%) (Figure 4.4a). Importantly, to ‘shrink-wrap’ the protein cargo after conjugation, a crosslinking reaction is employed to better protect it from degrading environmental conditions. However, residual activated ester group analyses revealed only ~9% remaining groups for NHS-polymer leaving little room for crosslinking reaction (54% and 90% for PFP and NPC-polymers, Figure 4.4b). We also found similar
Figure 4.4 (b) SDS-PAGE analyses for studying HRP encapsulation percentage with the NPC, PFP and NHS-polymers (PFP:48%, NHS: 18% and NPC:11%); (c) Measurements of residual activated carbonate ester moieties left after protein conjugation that can be utilized for crosslinking reaction; (d) SDS-PAGE analyses to show similar encapsulation percentage with Cyt C for the NPC, PFP and NHS-polymers (PFP:54%, NHS: 22% and NPC:18%).

bioconjugation efficacy (PFP:54%, NHS: 22% and NPC:18%) for another protein cytochrome C (Figure 4.4c). These studies further confirm that the PFP-polymer is the appropriate down-selected candidate for protein conjugation.

4.3 Summary

In summary, we demonstrated a versatile strategy for encapsulation of proteins using an activated carbonate polymer platform. Towards developing a fundamental understanding in the activated carbonate chemistry for protein conjugation, we first developed the structure-reactivity relationship by synthesizing and testing several activated carbonate-bearing polymers for their reactivity in aminolysis vs. hydrolysis. PFP-polymer not only provided faster aminolysis rate along with slower hydrolysis, this also enabled to utilize the left-over reactive carbonate functionalities for enforcing secondary-crosslinking to secure the nanoparticle formation. Based on the kinetics studies with small molecule lysine analogue, we chose PFP-carbonate as the preferred functionality for encapsulating
proteins. We tested the protein conjugation efficacy and redox-mediated release of the proteins thereafter via SDS-PAGE analyses. This polymer platform is expected to provide beneficial effect in protein conjugation and would be tested and improved for encapsulation of larger proteins and antibodies.

4.4 Experimental

4.4.1 Materials

2-Hydroxyethyl disulfide, methacryloyl chloride, bis(trichloromethyl) carbonate (triphosgene), 4-nitrophenol, 2,4,5-trichlorophenol, N-hydroxysuccinimide and 1,1,1,3,3,3-hexafluoro-2-propanol were procured from Sigma-Aldrich and were used without further purification unless otherwise mentioned. 4-(Dimethylamino)phenol was purchased from Combi-Blocks, USA. Polyethylene glycol monomethyl ether methacrylate (PEGMA; MW 500), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid, 4-Cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl] pentanoic acid (chain transfer agents), D,L-dithiothreitol (DTT), bis(pentafluorophenyl) carbonate and N,N′-disuccinimidyl carbonate were also procured from Sigma-Aldrich. 2,2′-azobis-(2-methylpropionitrile) (AIBN) was purchased from Sigma-Aldrich, USA and purified by recrystallization in methanol. All protein samples, e.g., peroxidase from horseradish and cytochrome C were purchased from Sigma-Aldrich.

4.4.2 Synthesis of activated ester containing monomers and polymers

4.4.2.1 Synthesis of methacrylate monomer of 2-hydroxyethyl disulfide

4 g (26 mmol, 1 equiv) of 2-hydroxyethyl disulfide was weighed in a 250 mL round-bottom flask and was dissolved in 75 mL dry THF. 5.4 mL (3.9 g, 39 mmol, 1.5 equiv) of triethylamine was added to the solution and stirred for 5 min. The mixture was
cooled in ice bath for 10 min. 2.5 mL (26 mmol, 1 equiv) of methacryloyl chloride dissolved in 75 mL DCM was added to the reaction flask through a dropping funnel over a period of 30 min. After complete addition, the ice bath was removed, and the reaction was continued for another 12 h under argon. After that the solvent was evaporated in a rotary evaporator. The reaction mixture was dissolved in ethyl acetate (50 mL), washed with distilled water (3×50 mL), saturated NaCl solution (1×50 mL) and finally dried over Na₂SO₄. The organic layer was collected and purified by flash column chromatography using hexane/ethyl acetate (75:25 v/v). Yield: 76%. ¹H NMR (400 MHz, CDCl₃) (δ ppm): 6.13-6.14 (dd, 1H), 5.59-5.6 (t, 1H), 4.41-4.43 (t, 2H), 3.87-3.91 (q, 2H), 2.96-2.99 (t, 2H), 2.87-2.90 (t, 2H), 2.06-2.09 (t, 1H), 1.95 (t, 3H). ¹³C NMR (100 MHz, CDCl₃) (δ ppm): 167.24, 136.02, 126.11, 62.61, 60.20, 41.69, 36.99, 18.29. ESI-MS (m/z) for C₈H₁₄O₃S₂ expected [M]+: 222.04, obtained: [M+Na]+: 245.02.

4.4.2.2 General synthesis of methacrylate activated ester monomers

In a typical procedure, 500 mg (2.25 mmol, 3 equiv) of 2-hydroxyethyl disulfide methacrylate was taken in a 20 mL glass vial and dissolved in 7.5 mL of dry THF. 275 mg (2.25 mmol, 3 equiv) of 4-dimethylaminopyridine (DMAP) was added to the solution and stirred for 10 min to dissolve. The reaction vial was stoppered and purged with argon. 227 mg (0.75 mmol, 1 equiv, purity: 98%) of triphosgene dissolved in 5 mL of dry THF was added to the vial in a dropwise manner. The reaction was stirred for 5 h. After that calculated amount of DMAP (see specific examples below) was added to the vial. Finally, phenols/alcohols for corresponding activated esters were added (dissolved in 2.5 mL dry THF, see specific examples below) to the reaction mixture dropwise and stirred for 12 h.
The reaction mixture was filtered to remove the salt, THF was dried in a rotary evaporator and the mixture was purified with column chromatography (DCM: hexane 90:10).

**Nitrophenyl carbonate methacrylate monomer (NPC-monomer):**

![Nitrophenyl carbonate methacrylate monomer](image)

Amount of chemicals used as par the general synthetic procedure described above:

4-nitrophenol: 344 mg (2.47 mmol) and DMAP: 302 mg (2.47 mmol), Yield: 56%. ¹H NMR (400 MHz, CDCl₃) (δ ppm): 8.27-8.30 (m, 2H), 7.26-7.41 (m, 2H), 6.13-6.14 (dd, 1H), 5.59-5.60 (t, 1H), 4.53-4.57 (t, 2H), 4.42-4.45 (t, 2H), 3.00-3.06 (m, 4H), 1.94-1.95 (dd, 3H). ¹³C NMR (100 MHz, CDCl₃) (δ ppm):167.13, 155.40, 152.32, 145.50, 135.99, 126.10, 125.35, 121.79, 66.79, 62.46, 37.23, 36.81, 18.29. ESI-MS (m/z) for C₁₅H₁₁NO₇S₂ expected [M⁺]: 387.04, obtained: [M+Na⁺]: 410.03.

**Pentafluorophenyl carbonate methacrylate monomer (PFP-monomer):**

![Pentafluorophenyl carbonate methacrylate monomer](image)

Amount of chemicals used as par the general synthetic procedure described above:

Pentafluorophenol (PFP): 455 mg (2.47 mmol), and DMAP: 302 mg (2.47 mmol), Yield: 49%. ¹H NMR (400 MHz, CDCl₃) (δ ppm): 6.13-6.14 (d, 1H), 5.59-5.60 (m, 1H), 4.56-4.60 (td, 2H), 4.41-4.45 (td, 2H), 2.99-3.06 (ddd, 4H), 1.95-1.96 (d, 3H). ¹³C NMR (100 MHz, CDCl₃) (δ ppm): 167.13, 151.14, 142.46, 141.14, 140.12, 138.61, 136.65, 136.00, 126.11, 126.08, 68.03, 62.76, 62.40, 42.20, 40.44, 37.41, 37.33, 37.16, 36.46, 30.94, 30.64, 29.71, 25.48, 24.79, 18.30, 18.27. ¹⁹F NMR (376 MHz, CDCl₃) (δ ppm): -152.84-152.79, -
156.94-156.83, -161.63-161.51. ESI-MS (m/z) for C\textsubscript{15}H\textsubscript{13}F\textsubscript{5}O\textsubscript{5}S\textsubscript{2} expected [M]\textsuperscript{+}: 432.01, obtained: [M+Na]\textsuperscript{+}: 455.00.

**Trichlorophenyl carbonate methacrylate monomer (TCL-monomer):**

\[
\text{\begin{array}{c}
\text{\textcolor{black}{\textbf{}}}
\end{array}}
\]

Amount of chemicals used as par the general synthetic procedure described above:

2,4,5-trichlorophenol: 488 mg (2.47 mmol) and DMAP: 302 mg (2.47 mmol), Yield: 89%.

\textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) (\textdelta ppm): 7.56 (s, 1H), 7.4 (s, 1H), 6.13-6.14 (dd, 1H), 5.59-5.60 (t, 1H), 4.53-4.56 (t, 2H), (4.41-4.43 (t, 2H), 2.99-3.05 (dt, 4H), 1.95 (dd, 3H). \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) (\textdelta ppm): 167.12, 151.91, 145.78, 135.99, 131.64, 131.21, 131.09, 126.09, 124.91, 67.16, 62.46, 37.28, 36.70, 18.29. ESI-MS (m/z) for C\textsubscript{15}H\textsubscript{15}Cl\textsubscript{3}O\textsubscript{5}S\textsubscript{2} expected [M]\textsuperscript{+}: 445.75, obtained: [M+Na]\textsuperscript{+}: 468.93.

**Hexafluoropropanol carbonate methacrylate monomer (CF3-monomer):**

\[
\text{\begin{array}{c}
\text{\textcolor{black}{\textbf{}}}
\end{array}}
\]

Amount of chemicals used as par the general synthetic procedure described above:

1,1,1,3,3,3-hexafluoro-2-propanol: 415 mg (2.47 mmol) and DMAP: 302 mg (2.47 mmol), Yield: 48%. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) (\textdelta ppm): 6.13-6.14 (dq, 1H), 5.60 (q, 1H), 5.53-5.60 (m, 1H), 4.52-4.56 (t, 2H), 4.40-4.43 (t, 2H), 2.97-3.02 (td, 4H), 1.95-1.96 (dd, 3H). \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) (\textdelta ppm): 167.13, 152.70, 136.00, 126.08, 121.57, 118.76, 71.10, 70.75, 70.41, 70.06, 69.71, 67.97, 62.38, 37.31, 36.39, 18.26. \textsuperscript{19}F NMR (376 MHz,
(CDCl₃) (δ ppm): -73.49. ESI-MS (m/z) for C₁₂H₁₄F₆Cl₃O₅S₂ expected [M⁺]: 416.02, obtained: [M+Na⁺]: 439.01.

**Dimethylamino carbonate methacrylate monomer (NMe₂-monomer):**

![Dimethylamino carbonate methacrylate monomer](image)

Amount of chemicals used as per the general synthetic procedure described above-
4-(Dimethylamino)phenol: 429 mg (2.47 mmol) and DMAP: 302 mg (2.47 mmol), Yield: 53%. ¹H NMR (400 MHz, DMSO-d₆) (δ ppm): 7.01-7.03 (d, 2H), 6.70-6.72 (d, 2H), 6.05-6.06 (dd, 1H), 5.70-5.71 (t, 1H), 4.32-4.41 (m, 4H), 3.04-3.09 (m, 4H), 2.88 (s, 6H), 1.88-1.89 (d, 3H). ¹³C NMR (100 MHz, DMSO-d₆) (δ ppm): 166.84, 166.82, 154.03, 149.13, 141.93, 136.13, 126.61, 126.58, 121.84, 113.16, 66.27, 65.77, 62.68, 62.65, 40.90, 40.68, 40.63, 40.42, 40.21, 40.01, 36.81, 36.74, 36.73, 18.43, 18.40. ESI-MS (m/z) for C₁₇H₂₃NO₅S₂ expected [M⁺]: 385.1, obtained: [M+Na⁺]: 408.09.

### 4.4.2.3 General synthesis of random copolymers

PEGMA, methacrylate activated ester monomer, 4-Cyano-4-[(dodecylsulfanylthiocarbonylsulfanyl)sulfanyl] pentanoic acid (RAFT agent) were weighed in a 4 mL glass vial (amount of reagents taken are listed below) and dissolved in 200 μL dioxane. Next, AIBN solution in dioxane was added to this solution. Total volume of solvent (dioxane) was 0.3 mL. To prepare for polymerization, the glass vial was purged with argon and stoppered. After that, the vial was subjected to four freeze-pump-thaw cycles, sealed completely and transferred to an oil bath preheated at 80 °C. The polymerization was continued for 48 h and then quenched by cooling down with cold water. The solvent was evaporated, and the product was first precipitated in hexane. The final product was
collected after redissolving in DCM and precipitating in diethyl ether two times. Finally, the polymer was dried overnight under high vacuum.

**NPC polymer:**

Amount of chemicals used as per the general synthetic procedure described above: PEGMA: 258 mg (0.52 mmol), NPC monomer: 50 mg (0.13 mmol), RAFT: 4.1 mg (0.0102 mmol), AIBN: 0.34 mg (0.0021 mmol). Yield: 88%. GPC (THF), $M_n$: 21 kDa, $Đ$: 1.3. $^1$H NMR (400 MHz, CDCl$_3$) ($δ$ ppm): 8.30, 8.28, 7.44, 7.42, 7.41, 4.57, 4.55, 4.54, 4.22, 4.07, 3.65, 3.64, 3.61, 3.55, 3.54, 3.54, 3.53, 3.37, 3.06, 3.04, 2.95, 1.94, 1.82, 1.01, 0.88, 0.87, 0.85. $^{13}$C NMR (100 MHz, CDCl$_3$) ($δ$ ppm): 155.46, 152.30, 145.50, 125.41, 121.97, 71.95, 70.62, 70.58, 70.54, 68.60, 68.47, 66.83, 59.05, 36.65, 31.91, 29.65, 29.34, 22.69, 14.15. From $^1$H NMR, the molar ratio of the repeating units was determined by integrating the methylene protons next to the methacrylate ester groups in the activated carbonate and PEG monomeric units ($x:y ≈ 0.2:0.8$).

**PFP polymer:**

Amount of chemicals used as per the general synthetic procedure described above: PEGMA: 232 mg (0.46 mmol), PFP monomer: 50 mg (0.12 mmol), RAFT: 3.7 mg (0.0092 mmol), AIBN: 0.30 mg (0.0018 mmol). Yield: 96%. GPC (THF), $M_n$: 22 kDa, $Đ$: 1.2. $^1$H NMR (400 MHz, CDCl$_3$) ($δ$ ppm): 4.60, 4.58, 4.23, 4.22, 4.08, 3.81, 3.71, 3.67, 3.67, 3.65, 3.64, 3.64, 3.63, 3.62, 3.59,
3.55, 3.55, 3.54, 3.53, 3.37, 3.06, 2.95, 1.87, 1.76, 1.25, 1.02, 0.89, 0.87, 0.86, 0.86. $^{13}$C NMR (100 MHz, CDCl$_3$) ($\delta$ ppm): 177.25, 151.22, 72.08, 70.75, 70.72, 70.67, 70.58, 68.73, 68.60, 68.27, 63.94, 59.17, 45.29, 44.99, 36.48. $^{19}$F NMR (376 MHz, CDCl$_3$) ($\delta$ ppm): -152.96, -157.12, -161.74. From $^1$H NMR, the molar ratio of the repeating units was determined by integrating the methylene protons next to the methacrylate ester groups in the activated carbonate and PEG monomeric units (x:y $\approx$ 0.2:0.8).

**TCL polymer:**

Amount of chemicals used as par the general synthetic procedure described above: PEGMA: 224 mg (0.45 mmol), TCL monomer: 50 mg (0.11 mmol), RAFT: 3.6 mg (0.0089 mmol), AIBN: 0.29 mg (0.0018 mmol). Yield: 91%. GPC (THF), $M_n$: 22 kDa, $\bar{D}$: 1.3. $^1$H NMR (400 MHz, CDCl$_3$) ($\delta$ ppm): 7.60, 7.46, 7.44, 4.57, 4.56, 4.54, 4.23, 4.23, 4.08, 3.67, 3.66, 3.65, 3.63, 3.62, 3.56, 3.55, 3.55, 3.54, 3.38, 3.06, 2.95, 1.82, 1.81, 1.26, 1.02, 0.89, 0.88, 0.88, 0.86, 0.85. $^{13}$C NMR (100 MHz, CDCl$_3$) ($\delta$ ppm): 152.00, 145.98, 131.72, 131.36, 131.14, 126.24, 125.16, 72.08, 70.75, 70.71, 70.66, 68.73, 68.60, 67.35, 59.17, 36.69, 32.04, 29.76, 22.82. From $^1$H NMR, the molar ratio of the repeating units was determined by integrating the methylene protons next to the methacrylate ester groups in the activated carbonate and PEG monomeric units (x:y $\approx$ 0.2:0.8).
CF3 polymer: 

Amount of chemicals used as per the general synthetic procedure described above: PEGMA: 240 mg (0.48 mmol), NPC monomer: 50 mg (0.12 mmol), RAFT: 3.8 mg (0.0095 mmol), AIBN: 0.31 mg (0.0019 mmol). Yield: 84%. GPC (THF), Mₙ: 28 kDa, Đ: 1.2. ¹H NMR (400 MHz, CDCl₃) (δ ppm): 5.66, 4.57, 4.21, 4.08, 3.65, 3.65, 3.38, 1.89, 1.79, 1.59, 1.26, 1.02, 0.88, 0.86. ¹³C NMR (100 MHz, CDCl₃) (δ ppm): 71.95, 70.61, 70.58, 70.53, 68.47, 68.06, 63.81, 59.04, 44.85, 30.94. ¹⁹F NMR (376 MHz, CDCl₃) (δ ppm): -73.33. From ¹H NMR, the molar ratio of the repeating units was determined by integrating the methylene protons next to the methacrylate ester groups in the activated carbonate and PEG monomeric units (x:y ≈ 0.2:0.8).

NMe2 polymer: 

Amount of chemicals used as per the general synthetic procedure described above: PEGMA: 259 mg (0.52 mmol), NPC monomer: 50 mg (0.13 mmol), RAFT: 4.2 mg (0.0103 mmol), AIBN: 0.34 mg (0.0021 mmol). Yield: 95%. GPC (THF), Mₙ: 31 kDa, Đ: 1.2. ¹H NMR (500 MHz, DMSO-d₆) (δ ppm): 7.06, 7.04, 6.75, 6.73, 4.45, 4.20, 4.05, 4.00, 3.64, 3.95, 3.28, 3.11, 3.03, 2.92, 1.83, 1.28, 1.19, 0.99, 0.89, 0.84. ¹³C NMR (100 MHz, DMSO-d₆) (δ ppm): 152.90, 147.98, 140.86, 120.67, 111.98, 70.69, 69.20, 69.01, 65.22, 57.43. From ¹H NMR, the molar ratio of the repeating units was determined by integrating the methylene
protons next to the methacrylate ester groups in the activated carbonate and PEG monomeric units (x:y ≈ 0.2:0.8).

**NMe3 polymer:**

\[
\text{Scheme 4.1 Methylation of NMe2-polymer to form NMe3-polymer}
\]

For methylation reaction, NMe2 polymer (25 mg, 0.0524 mmol) was first weighed in a 1.5 mL glass vial and dissolved in 400 μL DCM. Next, 296 mg (130 μL, 2.1 mmol) of methyl iodide was added dropwise to the solution. After 12 h of reaction, solvent was evaporated and the polymer was precipitated in diethyl ether three times. Finally, the polymer was dried overnight under high vacuum. Yield: 92%. GPC (TFE), \(M_n\): 32 kDa, \(Đ\): 1.3. \(^1\)H NMR (400 MHz, DMSO-d6) (δ ppm): 8.10, 8.08, 7.58, 7.56, 4.51, 4.19, 4.06, 3.66, 3.63, 3.56, 3.55, 3.47, 3.47, 3.46, 3.45, 3.40, 3.28, 3.14, 2.68, 2.68, 2.67, 2.56, 2.56, 2.40, 2.40, 1.28, 0.99, 0.89, 0.88, 0.84. \(^{13}\)C NMR (100 MHz, DMSO-d6) (δ ppm): 151.75, 150.45, 144.09, 122.04, 121.70, 70.68, 69.19, 69.00, 57.43, 56.01.
Scheme 4.2 Synthesis of random copolymers of NHS

Co-polymer of PEGMA and hydroxyethylene disulfide, was synthesized based on a previously reported procedure (see synthesis of p(PEGMA-co-EDSMA).\textsuperscript{16} To install the NHS carbonate ester group, 300 mg of p(PEGMA-co-EDSMA) polymer was weighed in a glass vial and dissolved in 2 mL DMF. Next, 606 mg (2.83 mmol) of proton-sponge (1,8-Bis(dimethylamino)naphthalene) was added to the solution and stirred for 15 min to dissolve. After that, 724 mg (2.83 mmol) N,N'-disuccinimidyl carbonate was added to the reaction mixture portion-wise over 15 min. The reaction was stirred for 2 days. The product was precipitated with diethyl ether twice and then dialyzed to purify in DCM:MeOH (1:1) solvent system. Finally, the polymer was dried overnight under high vacuum. Yield: 92%.

GPC (THF), $M_n$: 17 kDa, $D$: 1.2.

$^1$H NMR (400 MHz, CDCl\textsubscript{3}) (δ ppm): 4.59, 4.40, 4.22, 3.67, 3.65, 3.65, 3.64, 3.63, 3.62, 3.56, 3.55, 3.54, 3.53, 3.37, 3.05, 2.96, 2.87, 1.88, 1.78, 1.72, 1.25, 1.02, 0.88, 0.88, 0.85.

$^{13}$C NMR (100 MHz, CDCl\textsubscript{3}) (δ ppm): 168.92, 151.60, 77.48, 72.09, 70.76, 70.72, 70.67, 68.60, 63.95, 59.18, 45.01, 25.68. From $^1$H NMR, the molar ratio of the repeating units was determined by integrating the methylene protons next to the methacrylate ester groups in the activated carbonate and PEG monomeric units ($x$:$y \approx 0.2:0.8$).
4.4.3 Kinetics study with activated ester polymers and small molecule lysine analogue

![Scheme 4.3 Kinetics of aminolysis study with small molecule lysine analogue](image)

Scheme 4.3 Kinetics of aminolysis study with small molecule lysine analogue

To compare the kinetics of aminolysis vs. hydrolysis for different activated ester containing polymers in the context of protein encapsulation, we have studied the reaction of a protected small molecule lysine analogue, \( \text{N}_\alpha-\text{Acetyl-L-lysine methyl ester hydrochloride (Lys, Sigma)} \), with all reactive carbonate ester polymers. In a typical procedure, \(~1\, \text{mg} \) of polymer was dissolved in \( 3.5\, \text{mL} \) phosphate buffer (100 mM, pH 8.5). The solution is transferred to three UV-Vis cuvettes and \( 10\, \mu\text{L} \) Lys solution (10 mg/mL) was added. The cuvettes were sealed, stirred with magnetic stirrers and UV-Vis measurements were taken at different time points over a period of 12 h. Only polymer solutions in phosphate buffer (100 mM, pH 8.5) without the addition of Lys reagent were tested to evaluate the degree of hydrolysis. For NHS and CF\(_3\)-polymers, \(^1\text{H}\) NMR measurements were performed for kinetics analysis under identical conditions.

4.4.4 Conjugation of proteins with NPC, PFP and NHS-polymers

In a typical process, the self-immolative polymers were dissolved in phosphate buffer (100 mM, pH 8.5) to make 10 mg/mL solution. Protein stocks (horseradish peroxidase, HRP and cytochrome C, Cyt C) were also prepared at 400 \( \mu\text{g/mL} \) concentration.
(in phosphate buffer, 100 mM, pH 8.5). Finally, 25 μL of protein stock solution was added dropwise to a stirred 50 μL of polymer solution. The reaction was continued for 12 h at room temperature and subjected to SDS-PAGE analyses.

### 4.4.5 SDS-PAGE for protein-polymer conjugation

40 μL of protein-polymer conjugates were mixed with 10 μL of gel loading buffer (DTT free) and incubated at 95 °C for 5 min. After that, 40 μL of each sample was loaded into the acrylamide gel and electrophoresis was continued at constant voltage (130 V) for 45 min. To study the protein release, protein-polymer conjugate samples were treated with 10 mM GSH, incubated at 37 °C for 4 h and subjected to acrylamide gel electrophoresis. The amount of released protein from the nanoassembly was estimated via generating a standard curve from the known amounts of pure proteins loaded into the gel lanes. All gel image analysis and quantification were performed with Bio-Rad Image Lab™ software.

![Figure 4.5](image)

Figure 4.5 $^1$H NMR spectrum (400 MHz) of methacrylate monomer of 2-hydroxyethyl disulfide in CDCl$_3$. 

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Figure 4.6. $^{13}$C NMR spectrum (100 MHz) of methacrylate monomer of 2-hydroxyethyl disulfide in CDCl$_3$.

Figure 4.7 ESI-MS analysis of methacrylate monomer of 2-hydroxyethyl disulfide in methanol.
**Figure 4.8.** $^1$H NMR spectrum (400 MHz) of NPC-monomer in CDCl$_3$.

**Figure 4.9** $^{13}$C NMR spectrum (100 MHz) of NPC-monomer in CDCl$_3$. 
Figure 4.10 ESI-MS analysis of NPC-monomer in methanol.

Figure 4.11 $^1$H NMR spectrum (400 MHz) of PFP-monomer in CDCl$_3$. 
Figure 4.12 $^{13}$C NMR spectrum (100 MHz) of PFP-monomer in CDCl$_3$.

Figure 4.13 $^{19}$F NMR spectrum (376 MHz) of PFP-monomer in CDCl$_3$. 

Figure 4.14 ESI-MS analysis of PFP-monomer in methanol.

Figure 4.15 $^1$H NMR spectrum (400 MHz) of TCL-monomer in CDCl$_3$. 
Figure 4.16 $^{13}$C NMR spectrum (100 MHz) of TCL-monomer in CDCl$_3$.

Figure 4.17 ESI-MS analysis of TCL-monomer in methanol.
**Figure 4.18** $^1$H NMR spectrum (400 MHz) of CF3-monomer in CDCl$_3$.

**Figure 4.19** $^{13}$C NMR spectrum (100 MHz) of CF3-monomer in CDCl$_3$. 
Figure 4.20 $^{19}$F NMR spectrum (376 MHz) of CF$_3$-monomer in CDCl$_3$.

Figure 4.21 ESI-MS analysis of CF$_3$-monomer in methanol.
Figure 4.22 $^1$H NMR spectrum (400 MHz) of NMe2-monomer in CDCl₃.

Figure 4.23 $^{13}$C NMR spectrum (100 MHz) of NMe2-monomer in CDCl₃.
Figure 4.24 ESI-MS analysis of NMe2-monomer in methanol.

Figure 4.25 $^1$H NMR spectrum (400 MHz) of NPC-polymer in CDCl₃.
**Figure 4.26** $^1$H NMR spectrum (400 MHz) of PFP-polymer in CDCl$_3$.

**Figure 4.27** $^{19}$F NMR spectrum (376 MHz) of PFP-polymer in CDCl$_3$. 
Figure 4.28 $^1$H NMR spectrum (400 MHz) of TCL-polymer in CDCl$_3$.

Figure 4.29 $^1$H NMR spectrum (400 MHz) of CF$_3$-polymer in CDCl$_3$. 
Figure 4.30 $^{19}$F NMR spectrum (376 MHz) of CF3-polymer in CDCl3.

Figure 4.31 $^1$H NMR spectrum (400 MHz) of NMe2-polymer in DMSO-d$_6$. 
Figure 4.32 $^1$H NMR spectrum (400 MHz) of NMe3-polymer in DMSO-d$_6$.

Figure 4.33 $^1$H NMR spectrum (400 MHz) of NHS-polymer in CDCl$_3$. 
4.5 References


7. Cobo, I.; Li, M.; Sumerlin, B. S.; Perrier, S. "Smart Hybrid Materials by Conjugation of Responsive Polymers to Biomacromolecules" Nat. Mater. 2015, 14 (2), 143-159.


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CHAPTER 5

HARNESSING ELECTROSTATIC AND COVALENT CONJUGATION STRATEGIES FOR EFFICIENT ENCAPSULATION AND INTRACELLULAR DELIVERY OF ANTIBODY

Adapted in parts from the manuscript under review: Dutta, K; Kanjilal, P. Das, R.; Thayumanavan, S. “Synergistic Interplay of Covalent and Non-Covalent Interactions in Reactive Polymer Nanoassembly Facilitates Intracellular Delivery of Antibodies”

5.1 Introduction

Molecular self-assembly, inspired by recognition processes in nature, has formed the basis for many functional supramolecular architectures. Although these self-assembled structures are mainly governed by weak non-covalent forces, the co-existence of both covalent and non-covalent interactions is also prevalent in many biological processes. For example, covalent modifications of histones through acetylation and methylation of lysines dictate their electrostatic non-covalent binding interactions with negatively charged DNA in the chromatin structure. Similarly, in synthetic chemistry the concept of dynamic covalent bonds, coupled with non-covalent templating, has been utilized to create supramolecular structures and identification of ligands for protein targets. In this article, we report a covalent self-assembly strategy that is templated by non-covalent interactions between the host and the guest molecules to address a key challenge in achieving robust encapsulation of complex and sensitive biomacromolecules.
Intracellular targeting of “undruggable” proteins is an unresolved challenge that impacts many diseases with low life expectancy. Antibodies, long-standing diagnostic candidates in the biologics toolkit, could now serve to address this therapeutic challenge as it is possible to produce them at large scale for many protein targets. Unlike small-molecule drugs, antibodies present very high specificity to its target antigens, thus offering therapeutic benefits with minimal side-effects. Binding to a particular epitope via the Fab region of antibody could turn-off the cellular activity of the protein of interest causing deactivation of relevant biological signaling pathways. In fact, antibody-based therapeutics occupy a large portion of the FDA-approved biologics. But, these biologics target only the extracellular epitopes. When it comes to intracellular targets, its utility has been confined to diagnostics so far and for developing a fundamental understanding of a limited number of cellular processes. This is mainly attributed to the inability of antibodies to penetrate live cell membrane, owing to their large hydrophilic nature and entrapment in endosomal compartment. Although conventional strategies, such as electroporation and microinjection, are able to traffic antibodies inside cells, they are limited because of their poor efficacy and the potential for imparting severe cytotoxicity due to cell membrane damage.

Acknowledging the therapeutics need, two key approaches targeting intracellular delivery of antibody have been taken: (a) physical encapsulation, and (b) electrostatic complexation strategies with the help of peptide, lipid, inorganic and polymer based nanoparticles. However, given the enormous possibility of antibody therapeutics, the dearth of literature for intracellular trafficking of functional antibody points to the
significant challenges associated with such macromolecular cargo delivery without the loss of biological activity.

In this chapter, we have utilized a down-selected activated carbonate polymer with higher degree of aminolysis and low hydrolysis from our previous studies (Chapter 4) and further improved the efficacy for protein and antibody encapsulation (Figure 5.1). With the help of an electrostatics-aided covalent capture strategy, we demonstrate efficient encapsulation of large proteins, such as antibodies. Finally, we investigate the cellular trafficking of functional antibodies to probe cytosolic delivery and evaluated their biological activities in targeting specific intracellular epitopes (Figure 5.1).

**Figure 5.1** Schematic representation of an electrostatic-aided covalent self-assembly of polymer network using protein (antibody) as the template and its transport into the cytosol to deliver the cargo in functional form.
5.2 Results and discussion

5.2.1 Electrostatics-aided covalent self-assembly strategy for the generation of polymer-protein nanoassembly

In addition to identifying PFP-moiety as the optimal functionality for protein conjugation (discussed in Chapter 4), we were interested in tuning the structural features further to boost the encapsulation efficacy. Protein surfaces are composed of diverse arrays of amino acids with different surface charges and hydrophobicity. Charged residues on protein surfaces play an important role in dynamic reversible interactions with other biomacromolecules (in protein-protein and protein-antibody complexes).\textsuperscript{23} For most water-soluble proteins, charges are distributed on the surfaces as a patch with an average size between 1-2 nm.\textsuperscript{24} We hypothesized that introducing a negatively charged group in the activated ester polymer backbone could help recruit protein near the vicinity of polymer \textit{via} electrostatic interaction with positively charged patches, based on amino acid residues such as lysines, arginines and histidines.\textsuperscript{24-26} We hypothesized that once electrostatically drawn to polymer, the proximity-induced reactivity between lysine functionalities from the protein and the activated carbonate esters on polymer backbone should increase. To test this possibility, we synthesized a random copolymer consisting three different monomeric units, bearing PFP carbonate ester, PEG and 3-sulfopropyl functionalities with a compositional ratio of 2:6:2 (\textit{P}	extsubscript{Ab}, Figure 5.2a). Sulfonate moiety, having pK\textsubscript{a} \approx −0.5,\textsuperscript{27} would remain as negatively charged at the conjugation of pH 8.5, for interactions with positively charged groups on protein surface. To test the effect of mere electrostatic interactions in protein conjugation, we also synthesized a control polymer consisting PEG and sulfopropyl moieties (SO3-polymer, Figure 5.2a).
5.2.2 Polymer-protein nanoassembly formation and characterizations

Before pursuing the antibody conjugation, we tested the encapsulation efficacy and the subsequent stimulus-mediated release of proteins with the designed random copolymer, $P_{Ab}$. Two different proteins of very different molecular weights, HRP (~44 kDa) and β-gal (~465 kDa, with four subunits of 116.3 kDa), were chosen to form

![Diagram of polymer-protein nanoassembly](image)

**Figure 5.2** (a) Design of self-immolative polymer, $P_{Ab}$, for protein and antibody conjugation along with control polymers; Polymer-protein nanoassembly characterizations: (b, c) Dynamic light scattering measurements; and (d,e) Transmission electron microscopy images for NA-HRP and NA-β-gal, respectively along with naked proteins, scale bar: 500 nm; (f,g) SDS-PAGE analyses under reducing and non-reducing (with GSH) conditions to show efficient encapsulation and redox-mediated release with the NA-HRP and NA-β-gal nanoassemblies, respectively.
polymer-protein nanoassembly. Whereas the $\zeta$ potential was measured to be similar to the naked protein for NA-HRP (naked HRP: -8 mV; NA-HRP: -9 mV), it was reduced significantly in the case of NA-\(\beta\)-gal (naked $\beta$-gal: -25 mV; NA-$\beta$-gal: -13 mV). Dynamic light scattering (DLS) measurements showed uniform distribution of nanoassemblies with 119 and 162 nm hydrodynamic diameters for NA-HRP and NA-$\beta$-gal, respectively (Figure 5.2b-c), suggesting efficient shrink-wrapping with polymer network. Further analyses with transmission electron microscopy (TEM) revealed discrete nanoassemblies with distribution patterns matching with the DLS studies (Figure 5.2d-e).

Gel electrophoresis (SDS-PAGE) studies under non-reducing conditions showed no protein band, confirming that the proteins are effectively wrapped by the polymer (Figure 5.2f-g). Note that the encapsulation efficacies were much higher, $\sim$91% and $\sim$82% for HRP and $\beta$-gal respectively, for the $P_{Ab}$ polymer, compared to the corresponding PFP-polymer without the sulfonate moieties (see Chapter 4). Interestingly, control SO3-polymer without the PFP units also showed negligible protein encapsulation (<10%), suggesting that electrostatics alone is not sufficient for efficient encapsulation of proteins either. Thus, it is the combination of electrostatic and covalent self-assembly that offer efficient wrapping of proteins by the polymer.

Next, we investigated whether the nanoassembly can release the proteins in a stimuli-responsive manner. The protein bands reappeared in the gel electrophoresis studies, when the nanoassembly was run in the gel under reducing conditions (10 mM glutathione). This concentration corresponds to the typical intracellular GSH concentrations of the cytosol. Retaining the structure and function of the released proteins are critical in developing an effective encapsulation strategy. Towards this goal, we first
investigated the secondary structure of the released HRP and β-gal from the nanoassemblies. Circular dichroism spectra of the released proteins showed no apparent changes suggesting conservation of secondary structure of proteins releasing from the

**Figure 5.3** (a,b) Circular dichroism spectra of native HRP, NA-HRP and β-gal, NA-β-gal, respectively; (c,d) Comparison of protein activity for native, encapsulated and released HRP and β-gal proteins (Encap and Release: NA-Protein nanoassemblies without and with 10 mM GSH treatment, respectively); (e,f) Intracellular uptake of rhodamine tagged HRP and β-gal delivered with NA-HRP<sup>Rhod</sup> and NA-β-gal<sup>Rhod</sup> indicating uniform cellular distribution in HeLa cells, respectively. Scale bar: 20 μm; and (g,h) X-gal staining assay showing cytosolic activity of β-gal delivered through NA-β-gal, whereas naked protein sample remained unstained due to cell membrane impermeability.
nanoassembly upon treatment of glutathione (Figure 5.3a-b). Similarly, in vitro activity studies of the released proteins revealed that the proteins activities were greatly silenced (6% and 5% for HRP and β-gal, respectively; Figure 5.3c-d). However, upon releasing from the shrink-wrapped state, both proteins regained their enzymatic activities (84% and 87% for HRP and β-gal, respectively). Thus, both structure and functional assays show efficacy of the polymer shell in efficiently wrapping the protein and the recovery of activity of the protein upon encountering a specific environmental stimulus.

Finally, cellular internalization of the protein cargoes was tested for nanoassemblies encapsulated with rhodamine-tagged HRP and β-gal via confocal laser scanning microscopy (CLSM, Figure 5.3e-f). Uniform red fluorescence in HeLa and MDA-MB-231 cell lines suggested that the nanoassemblies were efficiently internalized by the cells. Furthermore, we were also interested in investigating whether the delivered protein is active, i.e. if the protein was unwrapped to be activated inside the cells. To this end, intracellular activity of delivered β-gal was tested using the x-gal assay. Generation of intense blue color in the cells, compared to the controls, suggests that the protein was not only transported across the cellular membrane to the cytosol, but that it is active inside the cells (Figure 5.3g-h).

### 5.2.3 Extension of the encapsulation strategy to antibody and cellular delivery

Inspired by the results with globular proteins, we tested the ability of these polymers to encapsulate and deliver antibodies inside cells. A typical immunoglobulin G (IgG) antibody is about ~150 kDa in molecular weight and has ~82 surface lysines. The developed encapsulation strategy with activated ester and negatively charged sulfonate moieties could provide chemical and electrostatic handles for boosting the encapsulation
of a such large antibody. Indeed, we were able to efficiently form polymer-IgG nanoassemblies (NA-IgG), as evident from the absence of IgG band (at ~150 kDa) for

Figure 5.4 (a) SDS-PAGE study to show efficient encapsulation of IgG inside the NA-IgG nanoassembly indicated by the absence of IgG band; (b) Transmission electron microscopy images for NA-IgG, scale bar: 500 nm; and (c) Dynamic light scattering and (d) ζ potential measurements for native IgG and NA-IgG nanoassembly; (e) Intracellular uptake of NA-IgG Rhod in HeLa cells, scale bar: 20 μm; (f) Mechanism of cellular uptake for NA-IgG_Rhod in presence and absence of endocytic inhibitors; (g) Endosomal colocalization and escape studies after incubation with NA-IgG_Rhod in HeLa cells at 4 and 24 h (green: lysotracker; red: rhodamine B-IgG; blue: nuclear stain, scale bar: 10 μm.)
NA-IgG samples in the SDS-PAGE analysis (Figure 5.4a). TEM and DLS studies revealed monomodal distribution of NA-IgG samples with an average size of 94 nm and ζ potential was found to be -12 mV (Figure 5.4b-d).

Our ultimate aim is to traffic the antibody for intracellular targeting of specific proteins. Prior to delivering a functional antibody, we were interested in testing the cellular localization of a fluorophore-labelled antibody. A rhodamine labelled IgG was first encapsulated in the polymer nanoassembly (NA-IgG\textsuperscript{Rhod}) and delivered in HeLa cells. A uniform distribution of red fluorescence, as observed from CLSM studies, confirmed the intracellular access of the delivered IgG (Figure 5.4e). When tested with other cell lines, the transfection efficacies were found to be as follows: HeLa: 69%, MDA-MB-231: 80% and EMT6: 91%. Cellular uptake can be governed by various pathways.\textsuperscript{30} To probe the cellular internalization mechanism for our nanoassemblies, we incubated cells with different endocytosis pathway inhibitors and checked their influence in cellular uptake of nanoparticle via flow cytometry.\textsuperscript{30-32} As evident from Figure 5.4f, the uptake is governed by the clathrin-mediated endocytosis pathway for HeLa cells, since hyperosmolar sucrose is a dominant uptake inhibitor. Upon being endocytosed inside the cells, the next important step for cytosolic access is to escape from the endosome. Time dependent CLSM studies with lysotracker green (an endosome/lysosome marker stain) showed colocalization of red (from rhodamine-labelled IgG) and green (lysotracker) channels after 4 h of incubation with the NA-IgG (Figure 5.4g). escape from endosome and cytosolic accumulation of IgG after 24 h. Also, high cellular viability for NA-IgG samples across three different cell lines suggest that the designed nanoassemblies are non-toxic even at a high dosage of 2 mg/mL.
5.2.4 Intracellular trafficking of functional antibodies: anti-pAkt and anti-NPC antibody

In order to test the potential of polymeric nanoassemblies in trafficking antibody inside cells, it is critical to show that the antibody is able to recognize the targeted epitope inside the cells. To this end, we aimed to test the ability of these nanoassemblies in delivering two functional monoclonal antibodies, viz. anti-nuclear pore complex (anti-

**Figure 5.5** (a) Schematic of cytosolic delivery for anti-NPC and anti-pAkt antibodies in functional forms through the polymer nanoassembly; (b) Immunostained HeLa cells after NA-anti-NPC delivery showing highlighted nuclear pore complex localized in nuclear membrane, scale: 10 μm; (c) Cellular viability study in MCF-7 cells after delivery of NA-anti-pAkt showing dose dependent decrease in cell survival; (d) Detection of caspase 3/7 in MCF7 cells after NA-anti-pAkt delivery (6 h) using a green fluorescent dye labelled caspase substrate, scale bar: 20 μm; and (e) Western blot analysis showing cleavage of PARP owing to the activation of caspase pathway in MCF-7 cells.
NPC) and anti-phospho-Akt (Ser473) antibody (Figure 5.5a). The epitopes for both these antibodies are present in the cytosol and thus analyzing these antibodies would also confirm the endosomal escape that we observed in our fluorescence microscopy studies. When delivered inside cells, anti-NPC antibody bind to the nuclear pore complex located on the nuclear membrane.\textsuperscript{15-16} While naked anti-NPC antibody could not penetrate cellular membrane efficiently and failed to locate on the nuclear membrane, NA-anti-NPC could traffic the antibody and successfully highlighted the nuclear pore complex of the cells. This is evident from the red-colored membrane of the nucleus (stained with a blue dye), due to the binding of the anti-NPC antibody (Figure 5.5b).

Gratified by this observation, we further tested the intracellular activity with another antibody, anti-pAkt. Protein kinase B, also known as Akt, is an intracellular signal transduction protein responsible for activation of nuclear factor-κB (NF-κB) and several other proteins in the Akt signaling pathway that are responsible for cellular growth.\textsuperscript{18, 33} It inhibits the key apoptotic pathway in many cancer cells and therefore blocking this could result in reinstating the apoptosis mechanism.\textsuperscript{33-34} With this goal in mind, we delivered anti-pAkt antibody with our polymer nanoassembly and were gratified to see that the cellular viability had reduced in a dose-dependent manner (Figure 5.5c). In addition to utilizing viability as an assay, we were also interested in showing that this is indeed due to the specific inhibition of the Akt pathway. If these results were indeed due to the reactivation of the targeted apoptosis pathway, we should observe the presence of caspase 3/7 enzymes, one of the key controllers for cellular apoptosis pathways. To this end, we examined the presence of caspase 3/7 via immunofluorescence technique. The assay utilizes a caspase substrate attached to a nucleic acid binding dye that only fluoresces upon
substrate cleavage by active cellular caspases after binding to nuclear DNA. A clear green fluorescence from the detection assay localized on cellular nucleus confirmed the activation of caspase pathways leading to cellular apoptosis (Figure 5d). To further probe the apoptosis process, we studied the cleavage of poly(ADP-ribose) polymerase (PARP) protein via intracellularly activated caspases through western blot analyses (Figure 5.5e). The apoptotic activation led to cleavage of PARP (~116 kDa) into 89 and 24 kDa fragments with the NA-anti-pAkt sample, while naked anti-pAkt control did not show any discernible amount of activity. These studies demonstrate the ability of the developed polymeric nanoassembly system in delivering antibody cargoes into the cytosol with the retention of recognition function.

5.3 Conclusions

In summary, we demonstrated a versatile strategy for encapsulation of large proteins and antibody therapeutics using an activated carbonate polymer platform. Based on the kinetics studies and previous investigation in protein encapsulation experiments (chapter 4), we chose PFP-carbonate as the preferred functionality for further development in encapsulating large proteins, such as antibodies. To further boost the conjugation efficiency, we engineered the polymer structures with electrostatic handles that offered higher degree of encapsulation presumably through proximity-induced reactivity enhancement. We show here that the electrostatics-aided covalent encapsulation strategy provided a robust platform to (i) capture larger antibody molecules with high fidelity that are found difficult to encapsulate otherwise; (ii) protect the structure and silence the cargo activity while in encapsulated state; (iii) enable regaining the functional activity of the payload upon release by the influence of an intracellular stimulus; (iv)
efficiently deliver the encapsulated cargo into the cytosol; and (v) ensure that the desired biological functions of the cargo are retained upon intracellular delivery and cytosolic release. We believe that the developed polymer nanoassembly system would serve as a generalized protein delivery platform, specifically for antibody-based intracellular drug targets, which is so far considered as one of the most challenging goals for the development of antibody therapeutics.

5.4 Experimental

5.4.1 Materials

Reagents used for polymerization- polyethylene glycol monomethyl ether methacrylate (PEGMA; MW 500), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid, 4-Cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl] pentanoic acid (chain transfer agents), D,L-dithiothreitol (DTT), bis(pentafluorophenyl) carbonate and N,N′-disuccinimidyl carbonate were also procured from Sigma-Aldrich. 2,2′-azobis-(2-methylpropionitrile) (AIBN) was purchased from Sigma-Aldrich, USA and purified by recrystallization in methanol. All protein samples, e.g., peroxidase from horseradish and β-galactosidase were purchased from Sigma-Aldrich. Anti-nuclear pore complex proteins antibody (Anti-NPC) and phospho-AKT1 (ser473) recombinant rabbit monoclonal antibody (Anti-pAkt) were purchased from abcam and Thermo Fisher Scientific, respectively.

5.4.2 Synthesis of random copolymer consisting pentafluorophenyl carbonate, PEG and sulfopropyl methacrylate monomers (PFP-SO3-polymer), P_{Ab}
Scheme 5.1 Polymerization reaction scheme for synthesis of P_Ab polymer

PEGMA (180 mg, 0.36 mmol), PFP monomer (50 mg, 0.12 mmol), 3-sulfopropyl methacrylate potassium salt (29 mg, 0.12 mmol) and 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (2.3 mg, 0.0083 mmol, RAFT agent) were weighed in a 4 mL glass vial and dissolved in 1 mL DMF by stirring for 15 min. AIBN solution in DMF was added to this solution. Total volume of solvent (DMF) was 1.2 mL. To prepare for polymerization, the glass vial was purged with argon and stoppered. After that, the vial was subjected to four freeze-pump-thaw cycles, sealed completely and transferred to an oil bath preheated at 80 °C. The polymerization was continued for 48 h and then quenched by cooling down with cold water. The solvent was evaporated, the product was first precipitated in diethyl ether and purified by extensive dialysis in acetone:methanol (1:1). Finally, the polymer was dried overnight under high vacuum. Yield: 79%, GPC (TFE), M_n: 38 kDa, Đ: 1.1. From ^1H NMR, the molar ratio of the repeating units was determined by integrating the methylene protons next to the methacrylate ester groups in the activated carbonate, methyl protons of PEG monomeric unit and the methylene protons next to the sulfonate group in sulfopropyl units (i:j:k ≈ 0.2:0.6:0.2). ^1H NMR (400 MHz, DMSO-d6) (δ ppm): 4.59, 4.15, 4.01, 3.60, 3.51, 3.45,
3.43, 3.43, 3.42, 3.41, 3.33, 3.24, 3.13, 2.99, 2.01, 1.99, 1.75, 1.24, 1.23, 0.95, 0.87, 0.86, 0.85, 0.85, 0.84, 0.80, 0.80, 0.78. \(^{13}\text{C NMR (100 MHz, DMSO-d}_6\) (\(\delta\) ppm): 149.86, 139.20, 133.53, 129.24, 127.55, 125.32, 118.12, 114.61, 107.44, 77.28, 70.67, 69.16, 68.97, 57.43, 47.31, 36.46, 35.15. \(^{19}\text{F NMR (376 MHz, DMSO-d}_6\) (\(\delta\) ppm): -154.01, -157.47, -162.27.

**Figure 5.6** \(^{1}\text{H NMR spectrum (400 MHz) of PFP-SO3-polymer, P}_{\text{Ab}}\) in DMSO-d6.

**Figure 5.7** \(^{19}\text{F NMR spectrum (376 MHz) of PFP-SO3-polymer, P}_{\text{Ab}}\) in DMSO-d6.
5.4.3 Synthesis of control sulfonate polymer, SO3-polymer

**Scheme 5.2** Polymerization reaction scheme for the synthesis of control SO3-polymer

PEGMA (750 mg, 1.5 mmol), 3-sulfopropyl methacrylate potassium salt (92 mg, 0.38 mmol) and 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (7.8 mg, 0.028 mmol, RAFT agent) were weighed in a 20 mL glass vial and dissolved in 2 mL 2,2,2-trifluoroethanol (TFE) by stirring for 15 min. AIBN solution in TFE was added to this solution. Total volume of solvent was 2.5 mL. To prepare for polymerization, the glass vial was purged with argon and stoppered. After that, the vial was subjected to four freeze-pump-thaw cycles, sealed completely and transferred to an oil bath preheated at 70 °C. The polymerization was continued for 24 h and then quenched by cooling down with cold water. The solvent was evaporated, the product was first precipitated in diethyl ether and purified by extensive dialysis in water. Finally, the polymer was lyophilized, and dried overnight under high vacuum. Yield: 92%, GPC (TFE), $M_n$: 28 kDa, $D$: 1.1. From $^1$H NMR, the molar ratio of the repeating units was determined by integrating the methyl protons of PEG monomeric units and methylene protons next to the sulfonate group in sulfopropyl units ($j:k \approx 0.8:0.2$). $^1$H NMR (400 MHz, D$_2$O) (δ ppm): 4.20, 3.81, 3.72, 3.65, 3.65, 3.64, 3.63, 3.55, 3.41, 3.40, 2.95, 2.13, 1.95, 1.60, 1.28, 1.10, 0.93. $^{13}$C NMR (100 MHz, D$_2$O) (δ ppm): 71.04, 69.79, 69.63, 69.48, 68.16, 58.10, 47.98, 45.05, 44.78, 23.60.
5.4.4 Encapsulation of proteins and antibody with activated ester polymer, P_{Ab}

In a typical process, the self-immolative polymer, P_{Ab}, was dissolved in phosphate buffer (100 mM, pH 8.5) to make 10 mg/mL solution. Protein stocks were also prepared in phosphate buffer (100 mM, pH 8.5, [HRP] = 400 μg/mL, [β-gal] and [IgG] = 200 μg/mL). Finally, 25 μL of protein stock solution was added dropwise to a stirred 50 μL of polymer solution. The reaction was continued for 12 h at room temperature. Next, calculated amount of (PEO)$_4$-bis-amine was added and stirred for another 12 h for crosslinking. Finally, the reaction mixture was dialyzed to purify the nanoassemblies encapsulated with proteins.

Figure 5.8 $^1$H NMR spectrum (400 MHz) of SO3-polymer in D$_2$O.

Scheme 5.3 Reaction scheme for covalent protein encapsulation aided by electrostatic interactions
5.4.5 Measurement of DLS and Zeta-potentials

Dynamic light scattering (DLS) and zeta potential measurements were performed using a Malvern Nanozetasizer-ZS instrument. All nanoassemblies and protein samples were diluted with PBS (pH 7.4) to adjust final concentration to 1 mg/mL.

![Zeta potential measurements for NA-HRP and NA-β-gal samples along with the native proteins.](a) (b)

Figure 5.9 ζ potential measurements for NA-HRP and NA-β-gal samples along with the native proteins.

5.4.6 TEM measurements

Bright Field TEM images were captured on a JEOL JEM-2000FX TEM operating at 200kV acceleration voltage. 0.5 μL of prepared Polymer-protein assemblies were drop casted on a carbon coated TEM grid and dried overnight at room temperature. The sample was subjected to TEM analyses without any additional staining.

5.4.7 SDS-PAGE for polymer-protein conjugation and release studies

40 μL of protein-polymer conjugates were mixed with 10 μL of gel loading buffer (DTT free) and incubated at 95 °C for 5 min. After that, 40 μL of each sample was loaded into the acrylamide gel and electrophoresis was continued at constant voltage (130 V) for 45 min. To study the protein release, protein-polymer conjugate samples were treated with 10 mM GSH, incubated at 37 °C for 4 h and subjected to acrylamide gel electrophoresis. The amount of released protein from the nanoassembly was estimated via generating a
standard curve from the known amounts of pure proteins loaded into the gel lanes. All gel image analysis and quantification were performed with Bio-Rad Image Lab\textsuperscript{TM} software.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figures.png}
\caption{SDS-PAGE of control SO3-polymer showing inefficient protein (HRP) conjugation.}
\end{figure}

\subsection*{5.4.8 Activity assays for HRP and \(\beta\)-gal}

To measure the activity of released proteins from the nanoassembly, polymer-protein conjugates were treated with 10 mM glutathione (GSH) for 4 h.

\textit{HRP (ABTS assay):}

The enzymatic activity of HRP and Cyt C can be studied by examining the catalytic conversion of 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). Proteins with peroxidase activity (e.g., HRP, Cyt C) catalyze the reduction of \(\text{H}_2\text{O}_2\) to water in presence of ABTS forming oxidized ABTS radical cation which imparts a brilliant blue-green color. The kinetics of the activity assay can be studied by monitoring the absorbance of the ABTS radical cation at 418 nm. After releasing proteins from the nanoassembly, samples were washed with PBS to remove traces of glutathione and other byproducts that might interfere with the assay reagent. Test solution was prepared in a 96 well plate by mixing 2 \(\mu\text{L}\) of sample solution with 80 \(\mu\text{L}\) \(\text{H}_2\text{O}_2\) (25 mM) and 100 \(\mu\text{L}\) ABTS.
(1 mg/mL). Absorbance spectra were recorded using a SpectraMax® iD5 multiplate reader at 418 nm for a period of 6 min.

\( \beta \)-gal \((ONPG\ assay)\)

The activity of released \( \beta \)-gal protein was measured via the \( \beta \)-Gal Assay Kit reagent (Thermo Fisher Scientific). \( \beta \)-gal catalyzes the hydrolyses of ortho-nitrophenyl-\( \beta \)-D-galactopyranoside (ONPG) substrate producing ONP anion with a bright yellow color (absorbance 420 nm). Test solution was prepared in a 96 well plate by adding 2 \( \mu \)L released protein sample solution, 98 \( \mu \)L PBS buffer and 40 \( \mu \)L ONPG assay reagent. The assay kinetics was immediately recorded over 45 min using a SpectraMax® iD5 multiplate reader at 420 nm.

**Figure 5.11** Kinetic plots for activity assay studies with NA-HRP and NA-\( \beta \)-gal samples along with naked proteins and controls. *Encap* and *Release*: NA-Protein nanoassemblies without and with GSH treatment, respectively.

\( \beta \)-gal \((in\ HeLa\ cells)\)

To check the cellular activity of the \( \beta \)-gal trafficked inside the cytosol via the nanoassemblies, an intracellular activity assay (x-gal assay) was performed. HeLa cells were seeded \((1 \times 10^5\ cells)\) in a 24 well tissue culture plate and incubated for 24 h at 37°C and 5% CO\(_2\) atmosphere to ensure complete adhesion. Next, cells were washed three times with PBS buffer and incubated with NA-\( \beta \)-gal nanoassemblies (protein concentration 10 \( \mu \)g/mL) at 37 °C for 6 h in complete media. Afterwards, media was removed, cells were
washed with PBS three times and stained with the Senescence β-Galactosidase Staining Kit (Cell Signaling) according to the manufacturer’s protocol. Cells were imaged with a light microscope to capture the development of blue color which was indicative of β-gal activity delivered inside cells.

Figure 5.12 X-gal cellular assay for untreated and treated HeLa cells with polymer, β-gal and NA-β-gal showing nanoassembly mediated delivery of β-gal in cells in its active form.

5.4.9 Circular dichroism (CD) spectra measurements

Polymer-protein conjugates were incubated with 10 mM glutathione (GSH) for 6 h to release proteins from the nanoassembly. After that samples were centrifuged in Amicon Ultra Centrifugal Filter (MWCO 50 kDa for HRP, MWCO 200 kDa for β-gal) to remove residual polymers from the proteins. After that protein samples were concentrated in Amicon filters with MWCO 10 kDa and protein concentrations were measured with Pierce 660 nm Protein Assay Reagent (Thermo Fisher Scientific). Next, the protein samples (200 μL) were transferred to a quartz cuvette (path length: 1 mm) and measured the CD spectra with a JASCO J-1500 spectrophotometer (scan rate: 20 nm/min, interval: 0.2 nm).
5.4.10 Cell culture-general information

All cell lines were cultured in 100 mm cell culture petri-dish with Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) in a humidified incubator with 5% CO₂ at 37 °C. DMEM/F12 media was supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic-antimycotic (100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of Amphotericin B).

5.4.11 Cellular uptake of rhodamine tagged HRP and β-gal

HeLa and MDA-MB-231 cells were seeded at 1×10⁵ cells/mL density (1 mL) in 35 mm glass-bottomed petri-dishes. Cells were cultured for 3 days at 37°C and 5% CO₂ atmosphere to ensure complete adhesion. Next, cells were washed three times with PBS buffer and incubated with NA-HRP^{Rhod} and NA-β-gal^{Rhod} nanoassemblies (rhodamine conjugated proteins, concentration 10 μg/mL) at 37 °C for 6 h in complete media. Afterwards, media was removed, washed with PBS three times and incubated with NucBlue™ Live ReadyProbes™ reagent (Thermo Fisher Scientific) in complete media for 1 h to stain the cell nucleus. Live cell imaging was performed using Nikon Spectral A1+ confocal microscope and images were analyzed with Nikon NIS-Elements 4.0 software.

5.4.12 Measurement of DLS and Zeta-potentials for polymer-IgG nanoassembly (NA-IgG)

Dynamic light scattering (DLS) and zeta potential measurements were performed using a Malvern Nanozetasizer-ZS instrument. All nanoassemblies and protein samples were diluted with PBS (pH 7.4) to adjust final concentration to 1 mg/mL.
5.4.13 TEM measurements of NA-IgG

Bright Field TEM images were captured on a JEOL JEM-2000FX TEM operating at 200kV acceleration voltage. 0.5 uL of prepared Polymer-IgG assemblies were drop casted on a carbon coated TEM grid and dried overnight at room temperature. The sample was subjected to TEM analyses without any additional staining.

5.4.14 SDS-PAGE for IgG conjugation and release studies

40 μL of protein-polymer conjugates were mixed with 10 μL of gel loading buffer (DTT free) and incubated at 95 °C for 5 min. After that, 40 μL of each sample was loaded into the acrylamide gel and electrophoresis was continued at constant voltage (130 V) for 45 min. To study the protein release, protein-polymer conjugate samples were treated with 10 mM GSH, incubated at 37 °C for 4 h and subjected to acrylamide gel electrophoresis. The amount of released protein from the nanoassembly was estimated via generating a standard curve from the known amounts of pure proteins loaded into the gel lanes. All gel image analysis and quantification were performed with Bio-Rad Image Lab™ software.

5.4.15 Cellular uptake studies for polymer-IgG nano assembly (NA-IgG)

HeLa, MDA-MB-231 and EMT6 cells were seeded at 1×10^5 cells/mL density (1 mL) in 35 mm glass-bottomed petri-dishes. Cells were cultured for 3 days at 37°C and 5% CO₂ incubator to ensure complete adhesion. Next, cells were washed three times with PBS buffer and incubated with polymer-IgG nanoassemblies (rhodamine conjugated IgG, concentration 10 μg/mL) at 37 °C for 6 h in complete media. Afterwards, media was removed, washed with PBS three times and incubated with NucBlue™ Live ReadyProbes™ reagent (Thermo Fisher Scientific) in complete media for 1 h to stain the
cell nucleus. Live cell imaging was performed using Nikon Spectral A1+ confocal microscope and images were analyzed with Nikon NIS-Elements 4.0 software.

5.4.16 Endocytosis mechanism studies

HeLa, MDA-MB-231 and EMT6 cells were seeded at $1.2 \times 10^5$ cells/mL density (1 mL) in a 24 well tissue culture plate and incubated for 24 h at 37 °C. Afterwards, cells were washed with PBS three times and pre-incubated with EIPA (100 μM, macro-pinocytosis pathway), Nystatin (30 μM, caveolin pathway) and hyper-osmolar sucrose (45 mM, clathrine pathway) in serum-free media for 1 h at 37°C. Next, media was removed and cells were co-incubated for another 1 h in presence of polymer-IgG nanoassemblies (with rhodamine conjugated IgG, concentration 10 μg/mL, in 1 mL serum-free media) and different inhibitors (concentrations same as above). Untreated and polymer-rhodamine-IgG treated cells without any inhibitor were used as negative and positive controls, respectively. Afterwards, cells were harvested by washing with cold PBS followed by trypsinization (0.25% trypsin-EDTA solution) for 5 min. Detached cells were collected in Eppendorf tubes, pelleted by centrifugation, washed two times with cold PBS and finally re-suspended in 400 μL cold PBS. Samples were immediately analyzed in a BD LSRFortessa™ flow cytometer instrument. Data analyses were performed with FlowJo software (version 10) to obtain fluorescence intensities of cell samples.
Figure 5.13 Studies of cellular uptake mechanism (endocytosis) through flow cytometry after NA-IgG\textsuperscript{Rhod} incubation in presence and absence of endocytic inhibitors (a) MDA-MB-231 and (b) EMT6 cell lines.

5.4.17 Endosomal escape study

HeLa, MDA-MB-231 and EMT6 cells were seeded at \(1 \times 10^5\) cells/mL density (1 mL) in 35 mm glass-bottomed petri-dishes. Cells were cultured for 3 days at 37°C and 5% CO\textsubscript{2} incubator to ensure complete adhesion. Next, cells were washed three times with PBS buffer and incubated with polymer-IgG nanoassemblies (rhodamine conjugated IgG, concentration 10 \(\mu\)g/mL) at 37 °C for 4 h in complete media. To study the endosomal escape, one set of samples was subjected to endosomal staining with Lysotracker Green (Thermo Fisher Scientific) for confocal microscopy. The other set was further incubated in complete media for 24 h before subjecting to endosome staining and confocal microscopy. Cell nucleus was stained with NucBlue™ Live ReadyProbes™ reagent (Thermo Fisher Scientific) and live cell imaging was performed using Nikon Spectral A1+ confocal microscope. All images were analyzed with Nikon NIS-Elements 4.0 software.

5.4.18 Cell viability with alamarBlue® assay

HeLa, MDA-MB-231 and EMT6 cells were seeded in a 96 well tissue culture plate (7500 cells/100 \(\mu\)L) and incubated for 24 h at 37 °C. After that, media was replaced, and
cells were treated with polymer-antibody conjugates and empty crosslinked polymers at different concentrations. Samples were incubated for another 24 h at 37 °C. Next, media was removed, cells were washed with PBS and were treated with 100 μL 10% alamarBlue in complete media. The well plate was incubated for 1 h at 37 °C. Finally, the reagent was transferred to a black 96 well flat-bottomed plate and subjected to fluorescence measurement in a SpectraMax® iD5 microplate reader (excitation/emission: 560/590 nm).

Figure 5.14 Cytotoxicity studies in HeLa, MDA-MB-231 and EMT6 cells after incubation with only polymer and NA-IgG complexes, respectively.

5.4.19 Intracellular delivery of anti-NPC antibody

1×10^5 HeLa cells were plated in a 35 mm glass-bottom tissue culture plate and incubated at 37 °C-5% CO₂ atmosphere for 24 h. After that media was removed and cells were transfected with polymer-antibody (Anti-NPC) conjugate (Anti-NPC concentration 20 μg/mL) and naked Anti-NPC antibody for 24 h in complete media. After that, media was removed and cells were washed with PBS three times. 4% paraformaldehyde (Biotium) was used to fix the cells (at room temperature, 10 min). After fixation, celles
were washed again two times with PBS and permeabilized with permeabilization buffer (1 mL, Biotium) for 10 min at room temperature. Next, cells were washed two times with PBS and blocked with 2% BSA solution for 1 h. After washing once with PBS, cells were incubated with secondary antibody (Anti-Mouse IgG H&L, Alexa Fluor® 647 conjugated, abcam) and nuclear stain (NucBlue™ Live ReadyProbes™ reagent, Thermo Fisher Scientific) for 1 h at room temperature. Finally, cells were washed two times with PBS and subjected to confocal laser scanning microscopy using Nikon Spectral A1+ confocal microscope. All images were analyzed with Nikon NIS-Elements 4.0 software.

**Figure 5.15** (a) Additional images for NA-anti-NPC nanoassembly treated HeLa cells showing staining of nuclear pore complex in red; and (b) Naked Anti-NPC antibody treated HeLa cells showing no apparent nuclear pore complex staining. Scale bar: 10 μm.

### 5.4.20 Cellular viability after delivery of pAkt antibody

MCF-7 cells were seeded in a 96 well tissue culture plate (7500 cells/100 μL) and incubated for 24 h at 37 °C. After that, media was replaced, and cells were treated with polymer-pAkt antibody conjugates, empty crosslinked polymers, and pAkt antibody and polymer-igG antibody conjugates at different concentrations. Further, cells were incubated for 24 h at 37 °C. Afterwards, media was removed, cells were washed with PBS
and treated with 100 µL 10% alamarBlue in complete media. The well plate was incubated for 1 h at 37 °C. Finally, the reagent was transferred to a black 96 well flat-bottomed plate and subjected to fluorescence measurement in a SpectraMax® iD5 microplate reader (excitation/emission: 560/590 nm).

5.4.21 Study of apoptosis with polymer-antibody nanoassembly

1×10^5 MCF-7 cells were plated in a 24 well tissue culture plate and incubated at 37 °C-5% CO₂ atmosphere for 24 h. After that media was removed and cells were transfected with polymer-antibody (pAkt) conjugate (pAkt concentration 10 µg/mL), empty crosslinked polymer and naked pAkt antibody for 6 h in complete media. After that media was replaced and cells were treated with CellEvent™ Caspase-3/7 Green Detection Reagent (based on the manufacturer’s protocol) to identify apoptotic cells.

Figure 5.16 Detection of caspase 3/7 for untreated and only crosslinked polymer treated MCF-7 cells. No green fluorescence was observed indicating absence of any apoptosis pathway. Scale bar: 10 µm.

5.4.22 Western blot analysis for detection of PARP cleavage via activation of apoptosis

2×10^5 MCF-7 cells were plated in a 6 well tissue culture plate and incubated at 37 °C-5% CO₂ atmosphere for 24 h. After that media was removed and cells were transfected with polymer-antibody (pAkt) conjugate (pAkt concentration 10 and 20 µg/mL), empty
crosslinked polymer and naked pAkt antibody for 24 h in complete media. Positive control samples were transfected with staurosporine (at 1 and 2 μM concentrations) and incubated for 24 h. After transfection, media was removed, and cells were washed with ice cold PBS two times to prepare for isolation of total protein. Next, cells were scraped to remove from the plate, transferred with cold PBS in an eppendorf tube and centrifuged to pelletize. Two cold PBS washings were performed to remove any proteins from media. Next, RIPA lysis buffer (Thermo Fisher Scientific) containing protease/phosphatase inhibitor mix (Cell Signaling) was added to the centrifuge tubes containing cell pellets and incubated for 15 min at room temperature. To ensure complete lysis, tubes were sonicated (3×30 s) and incubated at room temperature for another 15 min. Finally, lysed cells were centrifuged at 14000 rpm at 4 °C for 30 min and collected the soluble protein fraction from the supernatant. The protein content was quantified with BCA assay. Western blot analysis was performed to quantify the amount of PARP and its cleavage due to activation of caspase enzymes. Target proteins and loading control were detected with rabbit monoclonal antibodies (PARP & β-Actin mAbs, Cell Signaling). AP-linked anti-rabbit IgG was used as secondary antibody and proteins bands were detected via chromogenic technique with 1-Step™ NBT/BCIP substrate (Thermo Fisher Scientific).
5.5 References


CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

6.1. Summary of the dissertation

Biologics are considered to be a safer therapeutic option that can not only minimize toxicity and off-target effects of small molecule drugs, but those can also drug many ‘undruggable’ targets. However, due to hydrophilicity and fragile nature of the biologic drugs, it is difficult to achieve efficient intracellular trafficking of those with retention of structural properties and functional attributes. Acknowledging the challenges, this dissertation discusses design strategies of stimuli-responsive polymeric delivery systems for the encapsulation of biologics.

In chapter 1, we have discussed the opportunities of biologic drugs and challenges associated with their intracellular delivery. Next, we describe specific hurdles associated with three kinds of biomacromolecules, viz., protein, nucleic acid and antibody, and state of the art techniques to address those. By highlighting the promises and pitfalls, we have established the benefits of such existing methodologies as well as the need for new molecular designs to address the drawbacks.

To this end, in chapter 2, we have introduced the design of a self-immolative polymer, containing activated carbonate and disulfide linkages, for covalent conjugation of proteins through their surface exposed lysines. The polymer-protein nanoassemblies are not only able to encapsulate proteins, but those can also efficiently protect the sensitive biomacromolecules from enzymatic degradation and release the cargoes only in presence
of reducing environment. Further, cellular studies have revealed the efficient tracking of proteins into the cytosol with retention of their biological activities.

As nucleic acids are highly negatively charged, a non-covalent self-assembly strategy is employed in chapter 3 comprising protein, siRNA and zwitterionic lipids acting symbiotically to form a stable self-assembled structure guided by electrostatic and hydrophobic interactions. While a synthetic cationic polymer is designed to electrostatically complex siRNA, we hypothesize to employ a crosslinking reaction in the second step to neutralize the cationic charge without any loss of siRNA from the nanoassembly. We have utilized these Lipid-siRNA-Polymer (L-siP) nanoassemblies to deliver multiple siRNAs and have assessed their efficacies in silencing multiple target genes.

In chapter 4, we have studied the structure-property relationship to improve the efficacy of our previously designed polymer for protein delivery, as discussed in chapter 2. By studying a series of activated carbonate polymers for reactivity and protein conjugation efficiency, we have identified a candidate that shows significantly higher aminolysis with lysines of protein compared to undesired competitive hydrolysis. In chapter 5, the down-selected activated carbonate containing polymer is structurally tuned further to boost conjugation efficiency with larger proteins. By employing an electrostatics-aided covalent conjugation strategy, we have showed efficient encapsulation and redox-triggered cytosolic delivery of proteins with retention of cellular activity. We have extended the strategy for encapsulation of antibody drugs and have shown successful cytosolic delivery to recognize the target epitopes that resulted in the perturbation of cellular signaling pathways.
6.2. Future directions

6.2.1. Self-immolative ‘pro-drug’ strategy for antibody-drug-polymer conjugates

The ultimate goal of drug delivery is to package and transport drug without leakage and degradation en route to its target site. However, delivery of hydrophobic drug is challenging due to the aqueous insolubility, short half-life and spill-off from the delivery vehicles. A pro-drug concept, where the actual drug is generated in situ after getting released from a covalently attached molecule or delivery vehicle, can be beneficial to address these issues.

To this end, we hypothesize a covalent self-assembly strategy to encapsulate a suitable hydrophobic drug within a crosslinked polymer network. The self-immolative polymer platform that we have developed for encapsulation of protein and antibody could

![Scheme 6.1](image_url)

Scheme 6.1 A pro-drug approach for encapsulation of hydrophobic drug having a nucleophilic reactive handle for attachment with the polymer and its extension in antibody-drug conjugate strategy.
be utilized to encapsulate and release the drug on demand with the help of redox stimulus (Scheme 6.1). Furthermore, this polymeric nanoparticle can also be decorated with an antibody for targeting purposes. The drug loading capacity can be desirably tuned with this approach which, in turn, can address the current bottleneck of antibody-drug conjugates (ADC) suffering from lower antibody drug ratio (ADR).

6.2.2. Tuning HLB of host-guest assemblies for enhancing encapsulation efficiency

Designing a universal molecular container that can encapsulate small molecule actives with a wide range of logP values is one of the most challenging objectives with significant academic and industrial importance. If achieved, it can create enormous opportunities in formulation development across many technological sectors.

Scheme 6.2 Modulation of hydrophilic-lipophilic balance to generate stable nanoparticles with high encapsulation efficacy of small molecules with a wide range of logP.
Inspired by previous studies from our lab and ongoing research activities, we hypothesize to create polymeric nanogel systems with tunable hydrophilic-lipophilic balance (HLB) that will be able to predictively capture small molecule actives, e.g., therapeutic drugs, actives for home and personal care (skin care products, detergents and beverages) and agricultural crop protection (herbicide, fungicide and insecticide) formulations.

To this end, the homo, random and block copolymer based nanogel systems, developed in our lab, can be further optimized. Based on our previous observations with polymer nanogel based encapsulation approaches, we hypothesize that both microenvironmental and bulk hydrophobicity of the nanogels affect the encapsulation of molecules with varying logP. To test this, we can create polymer nanogels with systematic variation of amphiphilicity by choosing appropriate crosslinker. For example, a homopolymer nanogel system can be stabilized in aqueous media via crosslinking with an oligoethylene glycol based crosslinker (see Scheme 6.2 for details). Thus, by choosing an appropriate combination of polymer (homopolymer, random or block copolymer) and crosslinker (with varying hydrophobicity, e.g., PEG, aromatic, alkyl dithiols) systems, it might be possible to capture actives with a wide range of logP and stabilize in the crosslinked nanogels. A fundamental study, such as this, might help to design custom-made nanogels with high encapsulation efficacy.

6.2.3. AND-gated approach for nucleic acid delivery based on ‘L-siP’ strategy

Delivery of therapeutics to specific diseased cells requires the drug encapsulated vehicle to cross several barriers before it is delivered to the actual place of action of the therapeutic molecule. However, physiological conditions differ drastically across the
gateways in the complex biological milieu. Thus, it is often difficult to design a unique delivery strategy that can adapt the ongoing changes throughout the course of journey of the drug delivery vehicle. This can be addressed via incorporating a stimuli-responsive unit that can response to a specific environmental cue to release the encapsulated drug on target site. However, due to differential physiological conditions in the delivery pathway, a better strategy could be to engineer a delivery vehicle responsive towards dual stimuli where the resulting response from each stimulus would synergize to facilitate the desired therapeutic action.

Building on the symbiotic self-assembly strategy discussed in chapter 3, we hypothesize the design of a 2nd generation ‘L-siP’ nanoassembly with built-in ‘AND’ gated

![Image of L-siP nanoassembly](image)

**Scheme 6.3** Concept of 2nd generation ‘L-siP’ nanoassembly based on AND gate stimuli-response units.
stimuli-responsive units, wherein two stimuli incorporated in the polymer and lipid structures will be sequentially processed to release the nucleic acid cargo. To this end, a pH responsive unit can be incorporated in the lipid structure and a redox-responsive unit will be present in the polymer backbone (Scheme 6.3). In the final tri-component self-assembled nanostructure, both pH and redox-stimuli-responsive units are needed to be processed for an efficient cargo release. In addition, the initial electrostatic complexation efficacy between the polymer and the nucleic acid can also be tested with different cationic moieties incorporated in the polymer.


10. Akishiba, M.; Takeuchi, T.; Kawaguchi, Y.; Sakamoto, K.; Yu, H. H.; Nakase, I.; Takatani-


17. Bochicchio, D.; Pavan, G. M., Effect of Concentration on the Supramolecular Polymerization Mechanism via Implicit-Solvent Coarse-Grained Simulations of Water-Soluble 1,3,5-


33. Cheng, Y. L.; Yumul, R. C.; Pun, S. H., Virus-Inspired Polymer for Efficient In Vitro and In


50. Durymanov, M.; Reineke, J., Non-viral Delivery of Nucleic Acids: Insight into Mechanisms


59. Frohlich, E., The role of surface charge in cellular uptake and cytotoxicity of medical


78. Heyes, J.; Palmer, L.; Bremner, K.; MacLachlan, I., Cationic lipid saturation influences


Ther. 2020, 3 (3).


113. Liechty, W. B.; Kryscio, D. R.; Slaughter, B. V.; Peppas, N. A., Polymers for drug delivery


130. McKinlay, C. J.; Vargas, J. R.; Blake, T. R.; Hardy, J. W.; Kanada, M.; Contag, C. H.; Wender, P. A.; Waymouth, R. M., Charge-altering releasable transporters (CARTs) for the


171. Santra, S.; Kaittanis, C.; Perez, J. M., Cytochrome c Encapsulating Theranostic Nanoparticles: A Novel Bifunctional System for Targeted Delivery of Therapeutic Membrane-


Macromolecules **2009**, *42* (20), 7650-7656.


