3-26-2018

Understanding the Functional Group-dependent Self-assembly and Cellular Entry of Cationic Conjugated Polymer Nanoparticles

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DOI: 10.25148/etd.FIDC004087

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

UNDERSTANDING THE FUNCTIONAL GROUP-DEPENDENT SELF-ASSEMBLY AND
CELLULAR ENTRY OF CATIONIC CONJUGATED POLYMER NANOPARTICLES

A dissertation submitted in partial fulfillment of the

requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

Prakash Manandhar

2018

To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Prakash Manandhar, and entitled Understanding the Functional Group-dependent Self-assembly and Cellular Entry of Cationic Conjugated Polymer Nanoparticles, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2018

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DEDICATION

I dedicate this dissertation to my family for all of their love and support. My life wouldn't be beautiful without you and I wouldn't be here without you. I am blessed to have you in my life.

I love you all!

ACKNOWLEDGMENTS

First of all, I would like to thank my advisor Dr. Joong Ho Moon for his continuous support and guidance during graduate school. Your mentorship made my graduate life successful; you were there to help and guide me when I was lost. Thank you for trusting me and encouraging me to be a better scientist. I would like to thank my committee members Dr. Kevin O'Shea, Dr. Bruce McCord, Dr. Jeffrey Joens, and Dr. Juan Liuzzi for continuous support and always providing new depths to my research. I would like to thank Dr. Jin He for his continuous support and guidance for the SICM paper. I would like to thank Dr. Timothy Allen lab for allowing me to use confocal microscope. I would like to thank Dr. Manuel Barbieri lab for allowing me to use flow cytometry facility. I would like to thank Dr. Jeremy Chambers lab for allowing me to use microplate reader. I would like to thank the department of Chemistry for providing me funding through teaching assistantships.

I would like to thank all the post doctorate scholars, graduate students and undergraduate students that I met during my graduate school experience. I want to thank Dr. Eladio Mendez, for your support during the early stages of my research. I want to thank Dr. Tereza Vokata for being a mentor and teaching me all the lab techniques. Finally, I will be able to wear that gown you sent me on my birthday. I can't forget Dr. Megan Twomey, for your continuous support during my entire graduate life. You made my life easier in lab professionally as well as personally. You really are a doctor when it comes to solving the problems. I also like to thank Salauddin Ahmed for his assistance providing polymers for my cellular study. I would like to thank Ana Katarina Huba, for reading all my papers and presentations. Thank you for being an extra pair of eyes for my entire graduate school experience. I would like to thank Feng Chen from physics department for all the SICM work. Thank you for being a patient collaborator. Now, we only know, how painful it was to work on weekend to get better results when people were not around in the lab. I would like to thank all

my friends who are in Nepal and USA. Special thanks to my roommates Bibek Chand and Maria Gabryszewska. Thank you for listening to all my frustrations and encouraging me all the time. Thank you for making a home away from home. You guys rock!

I would like to give special thanks to my Mom and Dad. Both of you taught me to be a strong person no matter how bad life throws you obstacles. “Being weak is not an option, be strong and fight for the right thing” is that what you taught me entire life. Here I am now; you made this possible. Without your love and support I wouldn’t be able to hold this degree. Thank you for being there whenever I needed.

My deepest gratitude goes to my wife Biva Shrestha who helped and encouraged me in every aspect of graduate school. You made my life easier in a million ways; I know you are there for the ups and downs of my life. I can’t even imagine writing this dissertation without your love, care and support. Thank you for making my life beautiful and colorful. Finally, I would like to thank all my family members for your unconditional love and support. I am blessed to have you all in my life, and your contribution to make me a refined person will not be forgotten. I love you all!

ABSTRACT OF THE DISSERTATION

UNDERSTANDING THE FUNCTIONAL GROUP-DEPENDENT SELF-ASSEMBLY AND
CELLULAR ENTRY OF CATIONIC CONJUGATED POLYMER NANOPARTICLES

by

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Florida International University, 2018

Miami, Florida

Professor Joong Ho Moon, Major Professor

Highly fluorescent conjugated polymers (CPs) are an important class of biomaterials used for various biological applications including labelling, sensing, and delivery of biological substances. Synthetic versatility and tunable emission make CPs a superior class of biomaterials. Understanding the structure function relationship of CPs plays a vital role in designing high performing biomaterials. The cationic CPs are self-assembled to conjugated polymer nanoparticles (CPNs) in aqueous environment due to their amphiphilicity. The physical and biophysical properties of CPNs are highly dependent on the chemical functionality and backbone structure of CPs. Modulation of the surface property and backbone structure of CPNs play an important role for efficient internalization of CPNs into cells. The goal of this dissertation is to understand the structure function relationship of CPNs in an aqueous environment and the change in their photo physical properties upon the self-assembly of CPNs with different backbone structure upon complexation with biologically significant polysaccharides and cell membrane.

This work presents the self-assembly of a set of four cationic CPs with different connectivity and backbone structure upon complexation with a linear polyanion hyaluronic acid (HA). The study of photo physical properties changes upon the complexation with series of Glycosaminoglycans (GAGs) provides more insight about how the self-assembly behavior of cationic CPs changes upon the exposure to negatively charged polysaccharides. The understanding of the self-assembly of

CPNs with negatively charged biologically important macromolecules under *in vitro* conditions can give us an idea of photophysical property changes of CPNs during the treatment of CPNs in the cellular environment. The study of the interaction of CPNs with cell membranes using scanning ion conductance microscopy (SICM)-based topography, potential mapping, and confocal microscopy imaging is presented. CPNs are able to induce transient pore like feature formation on the cell membrane during the cellular internalization process. A comparative study of cellular labelling and delivery of siRNA of five CPNs with guanidine motif is presented. The subcellular localization and delivery of siRNA were dependent on the side chain hydrophilicity. The CPNs fabricated with hydrophilic aminoethoxyethanol possesses excellent cellular imaging with higher siRNA delivery.

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ABBREVIATIONS AND ACRONYMS

AA	acetic acid
AFM	atomic force microscopy
APTES	(3-Aminopropyl) triethoxysilane
ARS	Approach-Retract Scanning
ATCC	American Tissue Culture Center
ATR	attenuated total reflection
Boc	<i>t</i> -butyl carbamate
BSA	bovine serum albumin
CD	circular dichroism
CD44	Cluster Determinant 44 receptor
CMC	carboxymethyl cellulose
CMA	carboxymethyl amylose
CME	clathrin-mediated endocytosis
CvME	caveolae-mediated endocytosis
CP	conjugated polymer
CPE	conjugated polymer electrolyte
CPN	conjugated polymer nanoparticle
CS	chondroitin sulfate
DCM	dichloromethane
DIPA	<i>N,N</i> -diisopropylamine
DLS	dynamic light scattering
dppf	1,1'-bis(diphenylphosphino)ferrocene
DMF	dimethylformamide
DMSO	dimethylsulfoxide

DMEM	Dulbecco's modified eagle medium
DPB	1,4-diphenylbutadiyne
DS	dermatan sulfate
EA	ethyl acetate
EtOH	ethanol
FEM	finite element methods
FT-IR	Fourier transform infrared spectroscopy
GPC	gel permeation chromatography
HA	hyaluronic acid
HD	hydrodynamic diameter
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HeLa	human cervical carcinoma
HS	heparin sulfate
HWHM	half width at half maximum
HPLC	high performance liquid chromatography
ICD	induced circular dichroism
IRF	instrument response function
L	linker
LDA	linear discriminant analysis
MeCN	acetonitrile
MeOH	methanol
M_n	number average molecular weight
MPC	micropinocytosis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
M_w	weight average molecular weight

NaN ₃	sodium azide
NIR	near infrared
NMR	nuclear magnetic resonance
NP	nanoparticle
NTA	nanoparticle tracking analysis
OD	optical density
PCC	Pearson's correlation coefficient
PDMS	Polydimethylsiloxane
PEDOT	polyethylene-doxythiophene
PEG	polyethylene glycol
PDI	polydispersity index
Pd[Cl ₂ (PPh ₃) ₂]	bis(triphenylphosphine)palladium(II) dichloride
Pd(OAc) ₂	palladium(II) acetate
Pd[(PPh ₃) ₄]	tetrakis(triphenylphosphine)palladium(0)
PNP	Poisson-Nernst-Planck
PPB	poly(<i>p</i> -phenylenebutadiynylene)
PPB-L	poly(<i>p</i> -phenylenebutadiynylene) with non-conjugated Linker (L)
PPE	poly(<i>p</i> -phenyleneethynylene)
PPP	poly(<i>p</i> -phenylene)
PPV	poly(<i>p</i> -phenylenevinylene)
PT	poly(thiophene)
PTFE	polytetrafluoroethylene
PMDETA	(N,N,N',N'',N'''-pentamethyldiethylenetriamine)
QY	quantum yield
R	substituent

RBF	round bottom flask
SiC	silicon carbide
SICM	scanning ionic conductance microscopy
SPG	schizophyllan
rt	room temperature
TCSPC	time-correlated single-photon counting
τ_F	fluorescence lifetime
TEA	trimethylamine
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TPP	triphenylphosphonium
TMSA	trimethylsilylacetylene
TLC	thin layer chromatography
UV-Vis	ultraviolet-visible
VT-CD	variable temperature circular dichroism

CHAPTER I

Introduction

1.1 Development of polymer as a biomaterial for the cellular studies

Polymers have been known for many years for their effective delivery of different biomaterials including drugs, protein, and nucleic acids into cells.[1-5] Polymers act as a scaffold system for biomaterial delivery and this system has benefits such as improving bioavailability, solubility, and retention time of therapeutic cargo while delving biomaterial into the cells. The structure and function of polymers can be modulated for specific applications through chemical structure modulation. Polymers can be designed in a smart way to deliver biomaterial into the target organelles with specific therapeutic abilities. Smart polymers include a class of polymeric materials which are specially designed for the delivery of therapeutic cargo into the target organelles. These deliveries are made by responding to a biological phenomenon inside the cells, such as changes in environmental pH, enzymatic condition, and temperature.[6, 7] Smart polymers can be designed in such a way that they contain pH, enzyme or temperature sensitive functionalities, a hydrophobic unit, an organelle targeting group, versatile conjugation, and a site for the incorporation of desired biomaterial.[6, 7] For this reason, conjugated polymers (CPs) can be an excellent choice of polymers for the development of smart polymers. Conjugated polymers can be designed as smart polymers because they can meet all the desired criteria for smart polymer by careful incorporation of desired criteria during the synthesis.

1.2 Conjugated polymers (CPs)

Highly fluorescent CPs are well known for their excellent photophysical properties. Conjugated polymers have the capability of modulation of their structure by the simple modification of synthetic procedures. Conjugated polymers can be modified as required application by modification of their monomer structure. The modification can lead to formation of completely different functional CPs with dramatically different chemical properties. Conjugated polymers have high fluorescence with reasonable quantum yield so they can be used for varieties of

optoelectronic, sensing, and biomedical applications.[2, 8-12] Conjugated polymers are chosen for many biological applications because they possess desirable properties such as a high quantum yield, lower cytotoxicity, higher photostability, and synthetic flexibility.[4] Chemical structure of CPs are very diverse; for instance, CPs can be designed as a smart polymer with a rational incorporation of different desired units in the polymer structure. CPs contain phenylene groups attached to conjugated triple bond unit within the backbone making them hydrophobic, which is one of the requirements for the designing polymer as a smart polymer. The hydrophobic nature of CPs' backbone can be tuned by changing the backbone structure as shown in Figure 1.1. Varieties of CPs have been reported depending upon their backbone structure for numerous applications. The key strategy to develop a variety of CPs is: careful incorporation of different functional monomers depending on the requirement of photophysical properties and biological applications. Besides backbone structure, the side chain of CPs can easily be tuned to incorporate a desired conjugation site for further modification or introduction of functional group for specific targeting or labelling purposes. (Detail explained in section 1.3). The possibility of organelle targeting, and conjugation of desired biomaterials is another requirement for the designing polymer as a smart polymer.

Figure 1.1. Common examples of CPs with different backbone structure.

1.3 Synthesis of CPs and design strategies

1.3.1 Designing strategies for different color emissive CPs

Synthesis of CPs are straightforward. CPs can be synthesized by using various coupling reactions (e.g., Sonogashira coupling, Glaser coupling, Suzuki coupling, Heck coupling and oxidative coupling).[13] One of the enticing features of using CPs as a biomaterial is their controllability to

make different colored fluorescent material using same polymerization condition by simply changing the monomer structure. By adjusting the co-monomer composition, the emission color of CPs can be tuned from visible spectrum to the near infrared (NIR).[14] For example, a Palladium catalyzed Sonogashira reaction involves the coupling of aryl halides with a terminal alkyne in the presence of catalytic amount of copper and base. The Sonogashira coupling condition can be employed to synthesize blue, green and red fluorescent CPs simply by changing the monomer structure as shown in Figure 1.2.[15]

Figure 1.2: Synthetic examples of CPs showing synthesis of different colored fluorescent CPs.

1.3.2 Designing strategies for surface charge modulation of CPs

Another benefit of using CPs is the unlimited possibilities of side chain modification with different functional groups. The enticing possibility of side chain modification generally carries two important benefits. First, the solubility of CPs is always a concern during synthesis because of their hydrophobic backbone structure. Modification of side chain with oxygen rich moiety could enhance the solubility of CPs leading to increased hydrophilicity. Secondly, side chain is the main functional

Incorporation of desired functional or targeting group in the side chain is always important. It determines the potential application of CPNs for various applications such sensing, imaging, and delivery. Post polymerization modification of functional group is an easy approach for the synthesis of synthetically challenging polymers. Braeken et al. has successfully shown a nearly quantitative click functionalization reaction on an azide functionalized poly(*para*-phenyleneethynylene) (PPE) scaffold using a simple post polymerization technique Figure 1.4).[17] In the reaction azide-functionalized copolymer was reacted with phenylacetylene in the presence of copper bromide (CuBr) and N,N,N',N'',N'''-pentamethyldiethylenetriamine (PMDETA).

Figure 1.4. An example of post polymerization reaction scheme showing possibility of side chain i.e. azide functional group of PPE scaffold using click chemistry. Adapted from Braeken et al.[17]

1.3.3 Designing strategies for backbone structure modulation of CPs

Highly versatile CPs can be synthesized by using several reported synthetic reactions. Our research group especially focuses on synthesis of two classes of CPs with two different backbones i.e., poly(*para*-phenyleneethynylene) (PPE) and poly(*para*-phenylbutadiynylene) (PPB). The reasons for choosing these two backbones structures in CPs are because of their facile synthetic protocols with various applications including cellular imaging, delivery, and sensing. Poly(*para*-phenyleneethynylene) and poly(*para*-phenylbutadiynylene) also provide two different classes of CPs with completely different backbone structures, hence leading to an entirely different aggregation behavior with different photophysical properties. Figure 1.5 is the reaction condition depicted for the synthesis of PPE and PPB in our lab.

Figure1.5. Sonogashira and Glaser coupling reaction scheme for PPE and PPB synthesis, respectively.

The difference between these two-reaction conditions are the monomers feeding and reaction conditions. The Sonogashira coupling reaction is air sensitive and uses an air free nitrogen environment with a palladium as a catalyst, catalytic amount of copper, and an appropriate base. The Sonogashira reaction requires both terminal halides and the terminal alkyne group for the cross-coupling reaction to occur, whereas the Glaser coupling reaction only requires terminal alkyne for the homo coupling reaction to occur. Glaser coupling requires copper as a catalyst and an oxygen rich environment. Glaser coupling results in a formation of CPs with extended conjugation length between the aromatic phenylene rings.

1.4 Nanoparticle formation of CPs

The hydrophobic conjugated backbone in CPs is the main reason for the poor solubility of the CPs. The introduction of hydrophilic units or charged ionic groups in the side chain of CPs enhance the solubility of CPs in organic solvents. Even though increased solubility of the CPs tends to dissipate as soon as CPs are introduced to excess aqueous solvent or poor solvent. When well solvated CPs in good organic solvent (e.g., THF, DMF, DMSO) are mixed to an excess amount of aqueous solvent or poor solvent, the hydrophobic backbone of CPs self-assembles leading to formation of Conjugated Polymer Nanoparticles (CPNs). The hydrophobic backbone of CPs plays a vital role during the process of converting CPs to into the nanoparticles where hydrophobic backbone tries to squeeze within the CPs backbone causing intrachain collapse or comes closer to another CPs chain causing inter-chain collapse. While the hydrophobic conjugated backbone tries to avoid contact with poor solvents they aggregate into spherical shape leading to formation of nanoparticles. The method of nanoparticles formation is called the nano-precipitation method as depicted in Figure 1.6 and is an easier way to make nanoparticles than other way of nanoparticles formation protocols where extra surfactant or additives are not required. It is important to understand the self-assembly behavior of CPs in aqueous solvent because for many biological applications, CPs must be compatible with negatively charged biological aqueous environments. It

is well known that CPNs can also be prepared by the complexation with oppositely charged macromolecules like carbohydrates.[18] For example, cationic CPs in aqueous solvent can interact with negatively charged functional group of biomolecules to generate nanoparticles as shown in Figure 1.6.

Figure 1.6. Schematic representation of different preparation methods of conjugated polymer nanoparticles. Adapted from Feng et al.[19]

The electrostatic interaction of opposite charges of CPs and macromolecules drives to create a blended CPNs/macromolecules complex with retained properties from CPs as well as macromolecules. Our lab has already reported the preparation of core shell like nanoparticles by simple mixing of hyaluronic acid (HA) with positively charged CPs.[18] Another way to prepare CPNs by the mini-emulsion method. In mini-emulsion method CPs are first dissolved in an organic solvent (generally immiscible with water) with an emulsifier or stabilizer. The mixture of CPs and

stabilizer is now added to aqueous solvent under ultra-sonication and vigorous stirring. The process creates a small organic droplet stabilized by the emulsifier. After evaporation of organic solvent, it creates polymer encapsulated nanoparticles in aqueous solvent as shown in Figure 1.6.

1.5 Characterization of CPs and Conjugated polymers nanoparticles (CPNs)

Various characterization techniques can be utilized for the complete characterization of synthesized polymers in the lab. After intensive purification by the re-precipitation method, the polymers are dried in a high vacuum and further characterization was done in the lab. The molecular weight of polymers is determined by using gel permeation chromatography (GPC). Gel permeation chromatography is a kind of size exclusion chromatography (SEC) where polymers with high molecular weight will be eluted faster whereas smaller molecular weight are retained within the pores of column and will be eluted slowly. Molecular weight of synthesized polymer is determined by using relative standard (i.e., polystyrene). From the chromatogram, we can easily determine weight average molecular weight (M_w), number average molecular weight (M_n), and polydispersity index (PDI) as shown in the Figure 7. PDI of polymer is determined by using the equation $PDI = M_w/M_n$.

Figure 1.7. Example of GPC chromatogram illustrating molecular weight distribution.

Similar to other organic compounds the chemical structure of a polymer can be quantitatively determined using nuclear magnetic resonance (NMR) spectroscopy. It provides chemical structural information of the polymer. Fourier Transform-Infrared (FT-IR) is a commonly used analytical technique to determine the functional group present in the polymers. When polymer sample is irradiated with IR radiation, it is absorbed and transmitted on the basis of frequencies of vibrations between the bonds of atoms present in the sample. Conjugated polymer nanoparticles' size can be determined by using dynamic light scattering (DLS). It can also determine the zeta potential of the particles. Most recently, we started using nanoparticles tracking analysis (NTA) to determine the size of nanoparticles which offers a better representation of the hydrodynamic diameters of particles with the size distributions than the widely used dynamic light scattering (DLS).[20]

1.6 CPs to supramolecular self-assembly

Self-assembly of CPs is a well-known behavior because of their hydrophobic backbone structure. Self-assembly behavior of CPs can be induced by two different ways. First, by changing the solvents from good organic solvents to aqueous solvents; CPs are well solvated when they are dissolved in good organic solvents like DMSO, DMF, and THF. As soon as CPs are introduced into aqueous solvents like methanol or water they tend to self-assemble to minimize the repulsive hydrophilic versus hydrophobic interaction leading to formation of CPNs. Second, self-assembly behavior of CPs can be induced by interacting CPs with oppositely charged small molecules or macromolecules (i.e., protein, carbohydrates, and DNA). When oppositely charged molecules are introduced to CPs, electrostatic interaction between CPs and macromolecules will direct the formation of self-assembled CPs and oppositely charged molecules complex.

Understanding the self-assembly behavior of CPs is important; the behavior will eventually directly or indirectly relate with the cellular interaction when introducing CPs into the cell during the

cellular study. When CPs are treated with the cells, they come into contact with various biological environments. So, understanding of self-assembly behavior of CPs could be helpful to understand the cellular response upon introduction of CPs.

Photoluminescence property of CPs is one of crucial characteristics for using CPs as a biomaterial for many applications. Photoluminescence properties of CPs can easily be altered because of to the high probability of aggregation of CPs backbone during the self-assembly process. Depending upon the scope of application, aromatic π interaction is a good thing when it comes to applications such as where CPs backbone acts as a molecular wire. The intermolecular interaction through π -stacking is important for designing optoelectronic devices and interaction helps to increase efficient exciton and charge transport. However, very strong interaction of π -stacking will cause decreased photoluminescence causing low quantum yield. The effect of decreased quantum yield is problematic when designing biomaterial for cellular imaging and delivery purpose. The understanding of photophysical properties changes because of the interaction of backbone is crucial for designing CPs for the various electronic and cellular applications.

1.7 CPNs self-assembly characterization

Conjugated polymers can self-assemble to give nanoparticles. One easy way to determine the self-assembly behavior of CPNs is to measure the photophysical parameters. Understanding self-assembly behavior before introducing CPNs in the cells plays a vital role for designing CPNs for delivery or bioimaging or bio sensing purposes. Biological systems always involve different kinds of macromolecules like protein, carbohydrates, and serum, so it is always important to know how CPNs will behave with different macromolecules. Preservation of photostability and quantum yield of CPNs are always a concern for the biological application. One reason for choosing CPNs over other reported delivery materials is because of its excellent photoluminescence. The fluorescent property of CPNs makes it easier to track CPNs translocation inside the cell once they are

introduced inside the cells. Depending on the nature of self-assembly behavior, the fluorescence quantum yield of CPs can be increased or decreased. Numata et al. have reported that PPE type of CPs with negatively charged sulfite as a side chain can self-assemble in presence of polysaccharide schizophyllan (SPG) where PPE backbone is encapsulated by a protective sheath of SPG.[21] Poly(*para*-phenyleneethynylene) on the molecular level avoided the interpolymer aggregation forming chiral insulated PPE wire. As a consequence of increased conjugation length, more planar and rigid conformation of the PPE is increased. The increased planar and rigid conformation increases the fluorescent quantum yield dramatically after complexation with SPG.[21] Formation of a chiral complex is further supported by appearance of characteristic induced circular dichroism (ICD) pattern in the π - π^* transition region. Recently Nilsson et al. reported complexation of negatively charged conjugated polythiophene (PT) polymers with positively charged synthetic peptide also created well-ordered super molecules with ICD pattern in the π - π^* transition region with increased emission intensity.[22] These reported works have successfully shown that careful complexation of CPNs with negatively charged macromolecules can help to preserve the fluorescent quantum yield of the CPNs. The idea of preservation of fluorescent quantum yield of CPNs in presence of negatively charged macromolecules is important because during the cellular internalization process CPNs are going to encounter abundant negatively charged macromolecules inside the cell or in the cell membrane. Understanding self-assembly of CPNs with negatively charged macromolecules *in vitro* condition can give us idea in advance what to expect in terms of photophysical properties when introducing CPNs to target cells.

1.8 Cellular interaction of CPNs and their characterization

It is always important to understand cellular interaction of CPNs when they are introduced to the cell. The cell membrane consists of negatively charged phospholipids bilayer with hydrophilic head group of bilayers on the cell surface. The eukaryotic cell membrane is a tight biological barrier

which only allows uptake of molecules selectively into the cell.[23] When designing of biomaterial as a nanocarrier considerably importance is given for the smooth internalization of biomaterial inside the cells. For this reason, cationic biomaterials are designed keeping in mind that electrostatic attraction with anionic phospholipids bilayer. Hydrophobicity is another important parameter because it has been shown that it plays an important role in the complex formation during the interaction of biomaterial with the cell membrane.[14, 24]

Figure 1.8. Schematic diagram showing possible cellular uptake mechanisms. Adapted from Wexselblatt et al.[25]

Nanocarriers can transverse the cell membrane using energy dependent uptake mechanism i.e., endocytosis. Endocytosis mechanism further sub categorized into two pathways. 1. Endocytic pathway: phagocytosis (i.e., “cell-eating”) is the process used for uptake of larger particles such as bacteria, pinocytosis (i.e., “cell-drinking”) is the process used for internalized fluids surrounding the cell, macropinocytosis (MPC), 2. Receptor-mediated endocytic pathway: clathrin-mediated (CME) and caveolae-mediated (CvME).[25, 26] After nanocarriers are internalized inside the cells

they are trafficked into endosome. Where these nanocarriers are either transported into another cellular compartment or they are thrown out to the cell surface. Figure 1.8 shows possible cellular mechanism for the internalization of nanocarriers.

1.9 Factors affecting cellular uptake of nanoparticles

Conjugated polymer nanoparticles are a novel class of nanoparticles with their own distinct emission profiles making material visually trackable inside the cells. One of the reasons for increasing use of CPNs as cellular imaging and delivery material is their controllable synthetic versatility and easy modulation of luminescent properties for desired application. There is always a room for improving nanoparticles properties and during the synthesis process we can easily control the size, charge, and emission color as described in section 1.3. Though we design and synthesize nanoparticles utmost care and precautions but their cellular uptake and fate is always determined by the complex cellular environment. Parameters that hugely determine the fate of nanocarriers are described as:

1.9.1 Nanoparticles size

One of the reasons for choosing CPNs as a delivery vector in the therapeutic field is their tunable particle size. Nanoparticles encapsulated with targeted delivery biomaterial could have size range of 100-1000 nm.[27] It has been shown that nanoparticles with the size of <200 nm have ability to carry and deliver encapsulated cargo into the cells. Smaller nanoparticles tend to have higher surface area with higher cellular membrane interactions so they are expected to have better cellular internalization. While designing nanoparticles as an encapsulating agent for delivery of biomaterial they should be able to have reasonable size so that they could hold biomaterial perfectly.[28] It is also worth noting that very small size nanoparticles can readily penetrate cells and translocate different cells, tissues, and organs that are not supposed to take place leading to adverse effects in human health. [26]

1.9.2 Nanoparticles shape

The internalization of nanoparticles also depends on the shape of nanoparticles. Depending upon the shape of nanoparticles the surface area that comes to contact with cell membrane could vary significantly as shown in Figure 1.9A. The internalization of nanoparticles is expected to be increased when there is higher contact of nanoparticles' surface area with the cell membrane. For example elongated nanoparticles could have higher surface area in contact with cell membrane compare to spherical one. For this reason elongated nanoparticles expected to interact more efficiently with the cell membrane. [28]

Figure 1.9. The effect of shape and surface charge on the nanoparticle-cell membrane interaction. Adapted from Salatin et al.[28]

1.9.3 Surface charges and functionalities of nanoparticles

Surface charge or functionality modification is a smart way to improve CPNs for desired cellular application. Surface functionalites modification can allow to incorporate different fucntional groups for different biological recognition such as peptide, glycan, antibodies. Negatively charged cell membrane interacts differently with positively or negatively surface charged CPNs as shown in Figure 1.9B. Electrostatic interactions between CPNs and negatively charged cell membrane play vital role for the CPNs internalization so having positive charge at the nanoparticles surface makes material as a better candidate for the improved internalization.[29] Varities of CPNs can be created with different charge groups for example nanoparticles with cationic amine surface charge has better cellular interlization as compared to particles with sulfate, hydroxyl and carboxylate

group. Other than modifying CPNs surface charges there is also a way to incorporate cell surface receptor specific ligands in the nanoparticles so that nanoparticles can interact specific receptor that is overexpressed in the cell surface. In receptor mediated internalization target ligands are fabricated in nanoparticles surface and ligands specifically interact with the receptor present in the cell surface. As a consequence of higher binding affinity and target specific ligand, the receptor mediated internalization of nanoparticles internalization is shown to have better efficacy in cellular internalization. For example: Hyaluronic acid (HA) is an important constituent of extracellular matrix with specific binding to cell surface receptors overexpressed in many types of cancer cells. It is possible to design surface of nanoparticles covalently modified with HA or electrostatically complexed with HA so that they specifically interact with HA receptor present in the cell membrane.[30] Another possible example of receptor mediated internalization could be using the folate group functionalized nanoparticles. Then, the folate modified nanoparticles can be applied to target cancer cells where they bind to overexpressed folate receptors in the cell surface.[5]

1.9.4 Amphiphilicity of nanoparticles

The term amphiphilicity defines as a chemical compound with both hydrophilic and hydrophobic properties together. Conjugated polymer nanoparticles are an excellent examples of compound with amphiphilicity because they can be synthesized with both hydrophilic and hydrophobic properties together. Oxygen rich moieties with charged side chain can clearly make CPNs more hydrophilic where as conjugated backbone with structure make CPNs hydrophobic. The amphiphilicity of CPNs can make CPNs as a superior material while interacting with phospholipid bilayer of cell membrane. Positively charged CPNs can easily interact with polar phosphate group at the beginning of translocation process and later hydrophobic fatty acid bilayer can interact with hydrophobic moiety of CPNs.

1.10 Characterization of cellular interactions

Several biological methods are used to determine the efficacy and efficiency of CPNs interaction with the cell. Before introducing CPNs to cellular environment it is always important to understand the physical properties of CPNs. After careful characterization of CPNs from physical method including molecular weight, chemical structure, size, and photophysical properties. Conjugated polymer nanoparticles are carefully dialyzed in the water for several days or if CPs are readily soluble in organic solvent CPs are dissolved in the biological grade DMSO and mixed well with excess of deionized water and filtered through 0.45 μm syringe filter prior to any further biological experiment. Here are some important biological aspects that we use in our lab to characterize the cellular interactions of CPNs.

1.10.1 Determination of cytotoxicity of CPNs

After synthesis of CPNs it is important to know the cytotoxicity of the CPNs before performing any further cellular studies. Several toxicity assays are available using commercially available reagent. Our lab normally uses the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay to determine the cytotoxicity of CPNs. This cell proliferation assay involves the conversion of soluble MTT into formazan (Figure 1.10) because of mitochondrial metabolic activity of cells and thereby data are correlated with to cell viability with the CPNs untreated control cell sample.

Figure 1.10. A chemical reaction showing conversion of MTT into Formazan by mitochondrial reductase enzyme.

1.10.2 Determination of cellular uptake of CPNs

Flow cytometry is a commonly used method to quantitatively determine amount of CPNs uptake by the cells. The method takes advantage of fluorescence properties of the CPNs and the principle of the method relies on measuring the fluorescence intensity of samples while passing through beam of laser light. Live cells were treated with CPNs for the desired time, washed with PBS multiple times, and treated with Trypan blue in order to quench the fluorescence of CPNs attached in the cell membrane. Then, stream of cell samples was passed through a beam of light in flow cytometer and fluorescence intensity was obtained from the CPNs localized inside the cells measured along with the scattered light. A comparative data of fluorescence intensity of cells sample with and without CPNs give a quantitative number for the CPNs uptake inside the cells. The method can be used to determine verities of CPNs with different absorbance wavelength.

1.10.3 High resolution fluorescence microscopic imaging

In order to provide a clear visual picture of CPNs distribution in different organelles inside the cells, high resolution microscopy is an excellent choice of instrument. The live cells are incubated with CPNs for the desired time and fluorescent images were taken. Microscopic imaging is a visual technique to determine subcellular localization of the CPNs in cells. To give clear visual images about CPNs trafficking and subcellular colocalization, cells are treated with CPNs along with commercially available standard organelle specific dyes. A maximum overlapping of two dyes indicates co-distribution of the dyes and CPNs in the dye specific organelles. The Pearson's Correlation Coefficient (PCC) value is also calculated to quantitatively determine co-localization. Where value +1 means 100 % correlation, value 0 means no correlation and value -1 means perfect anti-correlation. Results can be analyzed as shown in Figure 1.11.[16] Live Hela cells were incubated with two CPNs; CPN-1 showed a PCC value of 0.89 meaning maximum overlapping of green fluorescence of CPN with red fluorescence of mitochondrial dye, whereas CPN-2 showed a

PCC value of 0.26 meaning minimum overlapping of CPNs' green fluorescence with red fluorescence of mitochondrial dye indicating that CPN-1 is highly localized in the mitochondria of the cells.

Figure 1.11: High resolution fluorescence microscopic images of images of HeLa cells incubated with CPNs for 18 h followed by mitochondrial (red) and nuclear (blue) staining. Adapted from Twomey et al.[16]

1.11 Summary

Conjugated polymer nanoparticles are well-known nanocarriers for the delivery of important biological substances such as siRNA, drugs, nucleic acid into cells. The understanding of structure-function relationships of CPs from the beginning of synthesis of polymer to development of ideal nanoparticles/biomaterial is crucial for efficient application. Development of ideal biomaterials always requires extensive planning, careful designing, fabrication, and incorporation of desired components during the synthesis. It is always important to understand how these CPNs interact in *vitro* and in *vivo* experimental conditions. The interaction of CPNs with different biological and chemical environmental condition can affect their structure and chemical behavior changes resulting in completely altered properties of CPs. For the development of successful and effective

nanocarriers, various cellular environment must be considered. That is why, it is also important to understand how subtle change in chemical structure can affect the cellular interactions. The aim of my dissertation work is to shed light on how fundamental structural properties changes can affect *in vitro* study of CPNs and to provide more detail *in vivo* study for developing CPNs as a delivery biomaterial.

Chapter II of dissertation details how four different backbone structures and connectivity of CPs can interact with biologically important anionic polysaccharides (i.e., hyaluronic acid - HA). A detail study of photophysical properties changes upon mixing CPs with HA will be provided. The subtle chemical alteration in backbone creates a huge effect in self-assembly behavior of CPs, and controlled incorporation of non-conjugated flexible linkers can help to enhance the photophysical properties of CPNs with improved solubility of the material. A set of four positively charged CPs are complexed with HA and their possible formation of helical structure will be studied. Chapter II will be further extended to study of complexation of CPs with three different anionic polysaccharides present in cell membrane i.e., glycosaminoglycans (GAGs). Chapter III elaborates the interaction of two different backbone structures of CPNs with (GAGs) and their detail photophysical properties will be provided. We report self-assembly behavior of CPNs is highly dependent on the type and strength of charge present in the GAGs.

Chapter IV details interaction of CPNs with cell membranes with same backbone structure with different functional groups on the side chain. It will provide more detailed understanding of structure function relationships of CPs for the cellular interaction. A detailed study of interaction of cellular membrane with positively charged CPNs is provided using scanning ion conductance microscope (SICM). We report pore like feature formation in the cell membrane upon the treatment of CPNs. The formation of pore like features and recovery of cell membrane is a time dependent process where CPNs play a significant role.

Finally, Chapter V details synthesis of a set of five different CPs with different side chain functionalities. Chapter also details study of cellular uptake of CPNs and delivery of siRNA. Here, we will provide detailed study of guanidine modified CPNs in terms of their cellular uptake and their comparative study with complexation with siRNA. Here, we report that CPNs modified with aminoethoxyethanol is an excellent choice of biomaterial among five class Guanidine modified CPNs for the cellular imaging and delivery of siRNA into cells.

1.12 References

- [1] J.H. Moon, E. Mendez, Y. Kim, A. Kaur, Conjugated polymer nanoparticles for small interfering RNA delivery, *Chem. Commun.* 47(29) (2011) 8370-2.
- [2] K.-Y. Pu, B. Liu, Fluorescent Conjugated Polyelectrolytes for Bioimaging, *Adv. Funct. Mater.* 21(18) (2011) 3408-3423.
- [3] A. Kumari, S.K. Yadav, S.C. Yadav, Biodegradable polymeric nanoparticles based drug delivery systems, *Colloids Surf. B Biointerfaces* 75(1) (2010) 1-18.
- [4] J. Nicolas, S. Mura, D. Brambilla, N. Mackiewicz, P. Couvreur, Design, functionalization strategies and biomedical applications of targeted biodegradable/biocompatible polymer-based nanocarriers for drug delivery, *Chem. Soc. Rev.* 42(3) (2013) 1147-235.
- [5] A.J.C. Kuehne, Conjugated Polymer Nanoparticles toward In Vivo Theranostics - Focus on Targeting, Imaging, Therapy, and the Importance of Clearance, *Adv. Biosys.* (2017) 1700100.
- [6] A.S.H. Mohamed EH El-Sayed, Patrick S Stayton, Smart polymeric carriers for enhanced intracellular delivery of therapeutic macromolecules, *Expert Opin. Biol. Ther.* 5(1) (2005) 23-32.
- [7] R. Tong, L. Tang, L. Ma, C. Tu, R. Baumgartner, J. Cheng, Smart chemistry in polymeric nanomedicine, *Chem. Soc. Rev.* 43(20) (2014) 6982-7012.
- [8] M.R. Pinto, K.S. Schanze, Amplified fluorescence sensing of protease activity with conjugated polyelectrolytes, *Proc. Natl. Acad. Sci. U.S.A.* 101(20) (2004) 7505-10.
- [9] S. Wang, F. Lv, Fluorescence Imaging Applications of Functionalized Conjugated Polyelectrolytes, *Functionalized Conjugated Polyelectrolytes: Design and Biomedical Applications*, Springer Berlin, 2013, pp. 43-55.
- [10] X. Zhao, Y. Liu, K.S. Schanze, A conjugated polyelectrolyte-based fluorescence sensor for pyrophosphate, *Chem. Commun.* (28) (2007) 2914-6.

- [11] C. Zhu, L. Liu, Q. Yang, F. Lv, S. Wang, Water-soluble conjugated polymers for imaging, diagnosis, and therapy, *Chem. Rev.* 112(8) (2012) 4687-735.
- [12] K. Lee, J. Lee, E.J. Jeong, A. Kronk, K.S. Elenitoba-Johnson, M.S. Lim, J. Kim, Conjugated polyelectrolyte-antibody hybrid materials for highly fluorescent live cell-imaging, *Adv. Mater.* 24(18) (2012) 2479-84.
- [13] K. Li, B. Liu, Polymer-encapsulated organic nanoparticles for fluorescence and photoacoustic imaging, *Chem. Soc. Rev.* 43(18) (2014) 6570-97.
- [14] T. Repenko, A. Rix, S. Ludwanowski, D. Go, F. Kiessling, W. Lederle, A.J.C. Kuehne, Bio-degradable highly fluorescent conjugated polymer nanoparticles for bio-medical imaging applications, *Nat. Commun.* 8(1) (2017) 470.
- [15] U.H. Bunz, Poly(aryleneethynylene)s, *Macromol Rapid Commun* 30(9-10) (2009) 772-805.
- [16] M. Twomey, E. Mendez, R.K. Manian, S. Lee, J.H. Moon, Mitochondria-specific conjugated polymer nanoparticles, *Chem. Commun.* 52(27) (2016) 4910-3.
- [17] Y. Braeken, P. Verstappen, L. Lutsen, D. Vanderzande, W. Maes, Synthesis of a multifunctional poly(p-phenylene ethynylene) scaffold with clickable azide-containing side chains for (bio)sensor applications, *Polym. Chem.* 6(37) (2015) 6720-6731.
- [18] M. Twomey, Y. Na, Z. Roche, E. Mendez, N. Panday, J. He, J.H. Moon, Fabrication of core-shell nanoparticles via controlled aggregation of semi-flexible conjugated polymer and hyaluronic acid, *Macromolecules* 46(15) (2013) 6374-6378.
- [19] L. Feng, C. Zhu, H. Yuan, L. Liu, F. Lv, S. Wang, Conjugated polymer nanoparticles: preparation, properties, functionalization and biological applications, *Chem. Soc. Rev.* 42(16) (2013) 6620-33.
- [20] V. Filipe, A. Hawe, W. Jiskoot, Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates, *Pharm. Res.* 27(5) (2010) 796-810.
- [21] M. Numata, T. Fujisawa, C. Li, S. Haraguchi, M. Ikeda, K. Sakurai, S. Shinkai, β -1,3-Glucan (Schizophyllan) Can Act as a One-Dimensional Host for Creating Chirally Twisted Poly(p-phenylene Ethynylene), *Supramol. Chem.* 19(1-2) (2007) 107-113.
- [22] K.P. Nilsson, J. Rydberg, L. Baltzer, O. Inganäs, Twisting macromolecular chains: self-assembly of a chiral supermolecule from nonchiral polythiophene polyanions and random-coil synthetic peptides, *Proc. Natl. Acad. Sci. U.S.A.* 101(31) (2004) 11197-202.
- [23] R. Rothe, L. Liguori, A. Villegas-Mendez, B. Marques, D. Grunwald, E. Drouet, J.L. Lenormand, Characterization of the cell-penetrating properties of the Epstein-Barr virus ZEBRA trans-activator, *J. Biol. Chem.* 285(26) (2010) 20224-33.
- [24] Q. Cui, X. Wang, Y. Yang, S. Li, L. Li, S. Wang, Binding-Directed Energy Transfer of Conjugated Polymer Materials for Dual-Color Imaging of Cell Membrane, *Chem. Mater.* 28(13) (2016) 4661-4669.

- [25] E. Wexselblatt, J.D. Esko, Y. Tor, On guanidinium and cellular uptake, *J. Org. Chem.* 79(15) (2014) 6766-74.
- [26] F. Zhao, Y. Zhao, Y. Liu, X. Chang, C. Chen, Y. Zhao, Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials, *Small* 7(10) (2011) 1322-37.
- [27] M.D. Chavanpatil, A. Khdair, J. Panyam, Nanoparticles for Cellular Drug Delivery: Mechanisms and Factors Influencing Delivery, *J. Nanosci. Nanotechnol.* 6(9) (2006) 2651-2663.
- [28] S. Salatin, A. Yari Khosroushahi, Overviews on the cellular uptake mechanism of polysaccharide colloidal nanoparticles, *J. Cell Mol. Med.* 21(9) (2017) 1668-1686.
- [29] F. Alexis, E. Pridgen, L.K. Molnar, O.C. Farokhzad, Factors Affecting the Clearance and Biodistribution of Polymeric Nanoparticles, *Molecular Pharmaceutics* 5(4) (2008) 505-515.
- [30] S. Misra, V.C. Hascall, R.R. Markwald, S. Ghatak, Interactions between Hyaluronan and Its Receptors (CD44, RHAMM) Regulate the Activities of Inflammation and Cancer, *Front Immunol.* 6 (2015) 201.

Chapter II

Spectroscopic investigation of backbone-dependent ionic complexation of positively charged phenylene-based conjugated polymers and negatively charged hyaluronic acid

Manuscript submitted and under review

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2.1 Graphical abstract

A series of four conjugated polymers (CPs) with different backbone structures were synthesized and complexed with hyaluronic acid. The self-assembly behaviors of these cationic amine charged phenylene-based conjugated polymers were studied by protophysical methods and induced circular dichroism (ICD). The four polymers show remarkably different protophysical properties upon hyaluronic acid (HA) complexation. Out of the four conjugated polymers, PPB-L and PPE with entirely different backbone structure are able to make highly ordered helical structures upon complexation with HA.

Figure 2.1. A schematic presentation of structural reorganization of PPE and PPB-L upon HA complexation resulting induced helix formation.

2.2 Abstract

Understanding the role of polymer backbone structure on aggregation of conjugated polymers (CPs) in an aqueous environment is of prime interest as the physical, photophysical, and biophysical properties required for many applications are closely related to the nature of aggregation structures. We report unique and ordered aggregation behaviors of four positively charged CPs, which differ in the backbone chemical structure and connectivity, upon complexation with a linear polyanion hyaluronic acid (HA). Opposite signals of induced circular dichroism (ICD)

were observed from achiral poly(phenyleneethynylene) (PPE) and flexible linker (L) containing poly(phenylenebutadiynylene) (PPB) (PPB-L) with the same side chain structure, respectively, implying the formation of oppositely handed helical ionic complexes. Intrinsic difference in the interpolymer interaction between PPE and PPB is likely the contributing factor for different aggregation structures upon complexation with HA. The structure-property relationship can be useful to design and fabricate highly ordered macromolecular materials exhibiting unique electrical, photophysical, or biophysical properties.

2.3 Introduction

Aromatic π -electron conjugated polymers (CPs) exhibit unique electronic and luminescent properties used for a variety of electronic, photonic, and biological applications.[1, 2] For biological applications, aqueous compatibility of CPs can be achieved through introduction of positively (e.g., amine) or negatively (e.g., phosphate, carboxylate) charged side-chains.[3] Copolymerization of additional monomers with side-chains containing functional units also allows tailoring of these materials for specific applications including cellular imaging,[4-8] sensing,[9-13] and delivery of biological substances.[14-16] Despite the hydrophilic side chains, most CPs are water insoluble due to the hydrophobic and rigid aromatic backbones that are prone to π - π stack in an aqueous environment. Aggregation generally decreases the physical and photophysical merits of CPs, resulting in poor performance. Therefore, efforts to understand or control the aggregation to achieve necessary properties of CPs are critical.

Among CPs, highly rigid and linear poly(phenyleneethynylene)s (PPEs) and their derivatives have received much attention for various electronic and biological applications because of their excellent photophysical properties and straightforward synthesis via palladium-mediated coupling chemistry. In contrast, reports of the use of poly(phenylenebutadiynylene)s (PPBs), which exhibit similar structural and photophysical properties to those of PPEs, for similar purposes are scarce as

a result of poor solubility in common organic solvents.[17, 18] Previously, we reported a synthetic approach to obtain soluble PPBs by incorporating a flexible linker (L) along the backbone (i.e., PPB-L)[19] and demonstrated that these materials self-assembled into core-shell nanoparticles upon complexation with hyaluronic acid (HA),[20] a linear anionic polysaccharide with specific binding to cell surface receptors overexpressed in many types of cancer cells.[21] The backbone of PPB-L differs from widely studied PPEs in two ways: the phenyleneethynylene (PE)-conjugated bridge is substituted with the longer phenylenebutadiynylene (PB) bridge and the backbone contains a non-conjugated aliphatic linker in small proportions (~10%). The PB backbone is responsible for poor solubility of PPBs in common organic solvents due to high interchain interaction. By introducing the aliphatic linker along the rigid PB backbone, provides additional degrees of freedom to the PPB backbone reducing the interpolymer interaction. The reorganization of PPB-L upon HA complexation was a result of the flexibility of the backbone, thus decreased interpolymer interaction, stemming from the presence of the linker in small amounts. Previous studies of a series of conjugated poly(phenylenevinylene)s (PPVs) with variable flexible linker proportions also concluded that the added flexibility contributes to more ordered polymer conformations and thus a more efficient self-assembly of the conjugated segments.[22] The effect was diminished with higher flexible contents as the conjugated segments become shorter and their interchain interaction and alignment become progressively less favorable.[23, 24]

To investigate the effects of backbone chemical structures on aggregation, we designed and synthesized a set of four positively charged phenylene-based CPs, which differ in the backbone chemical structure (i.e., PE versus PB) and connectivity (i.e., with or without flexible linkers along the backbone), and studied their dramatically different aggregation structures upon complexation with HA. Various electronic spectroscopic data indicate that helical structures were formed when achiral PPE and PPB-L, respectively, complexed with chiral HA. While the presence of flexible linkers in the PPB backbone contributes to a highly ordered structure, the same linker in the PPE

backbone induces no ordered structure, suggesting that an optimal interchain interaction strength plays an important role in forming ordered ionic complexes. The relationship between the backbone structure and the photophysical properties presented in this contribution will serve as a foundation for developing future functional materials exhibiting unique electronic, optical, and biological properties.

2.4 Result and discussion

2.4.1 Polymer synthesis and characterization

In order to examine the effects of the backbone structures on aggregation behaviors, four phenylene-based polymers were designed and synthesized (Figure 2.2). The side chains of all four polymers were tert-butyloxycarbonyl (Boc)-protected amine-containing short ethylene oxides. By deprotecting the Boc groups, the positively charged CPs soluble in common organic solvents such as DMF and DMSO were obtained. The flexible linker was synthesized by reacting dithiopropionic acid with 4-ethynylaniline. Poly(phenyleneethynylene)s were obtained in good yields under the microwave assisted Sonogashira cross-coupling reaction. Similar to other examples of microwave assisted polymerizations of PPEs,[26] high molecular weight PPEs were obtained in a relatively short reaction time (<40 min) with good yields. Incorporation of a small percentage of a non-conjugated linker along the PPE backbone was also successful under the same microwave reaction conditions. However, attempts to synthesize PPB and PPB-L were not successful under the microwave conditions, as low molecular weight oligomers often formed. Instead, we synthesized high molecular weight PPB and PPB-L using our previously published polymerization conditions.[19, 27]

supplementary data Figure S2.4 and S2.8). The hydrodynamic diameters of all four CPs in water were measured using NTA, which offers a better representation of the hydrodynamic diameters of particles with the size distributions than the widely used dynamic light scattering (DLS) (Supplementary data Figure S2.19, S2.20, S2.20, and S22).[28] All four CPs exhibit the range of hydrodynamic diameters of 130–160 nm. Poly(phenylenebutadiynylene)s exhibit slightly larger diameters than PPEs. The hydrodynamic diameters of the CPs/HA complex are smaller compared to CPs. The ionic interactions between the positively charged CPs and the negatively charged HA contribute to the formation of compact complexes due to increased intermolecular π - π interaction of CPs backbone by the oppositely charged polyanion.

Table 2.1 Comparison of average physical and photophysical properties of CPs.

CPs	Mn ^a (g/mol)	HD ^b (nm)		Φ_F ^c (%)		Fluorescence lifetime (τ_F) ^d		k_{nr}		k_r	
		CP	CP/HA Complex	CP	CP/HA Complex	CP	CP/HA Complex	CP	CP/HA Complex	CP	CP/HA Complex
PPB	9,000	160	140	0.4	0.3	-	-	-	-	-	-
PPB-	20,800	154	140	4.3	2.6	0.99	0.03	9.6×10^8	3.2×10^{10}	4.3×10^7	8.5×10^8
PPE	14,300	140	119	4.0	5.7	0.03	1.30	2.8×10^{10}	7.2×10^8	1.2×10^9	4.4×10^7
PPE-L	16,200	133	126	2.3	2.5	0.12	0.05	8.2×10^9	1.9×10^{10}	1.9×10^8	4.8×10^8

^a Number average molecular weight. Determined by gel permeation chromatography in THF; ^b Hydrodynamic Diameter (HD) measured by NTA at 25°C in water; ^c Fluorescence Quantum yield (Φ_F) in H₂O measured relative to diphenylanthracene standard; ^d Average lifetimes measured using fluorescence spectroscopy; k_{nr} - Nonradiative decay rate; k_r - Radiative decay rate.

2.4.2 Absorption and emission spectroscopy

All four positively charged CPs we tested are water insoluble, mixing CPs in DMSO with water (a poor solvent) induces loose aggregation of the hydrophobic polymer chains. The polymer chains are loosely aggregated and stabilized by the positive charges in the side chain, and the shapes of the absorption and emission spectra of CPs in both DMSO and water are similar, except for slightly red-shifted absorption maxima and decreased quantum yields (Supplementary data Figure S2.12 and S2.13).

Upon complexation with HA, all four CPs in water exhibit distinct changes in both absorption and emission spectra (Figure 2.3 and 2.4). Absorption spectra of both PPB/HA and PPB-L/HA were red-shifted with a shoulder and a distinct peak, respectively, at the longer wavelength (Figure 2.3a and 2.3b). The changes in absorption and emission behavior can be explained by either an increased planarization along the conjugated backbone or increased interpolymer π - π stacking.[29] While the absorption spectroscopy has no discriminatory power on the contributing factors for the red shifts, the emission spectra suggest that the degree of π - π stacking in both PPB and PPB-L was increased as substantially evidenced by the decreased emission quantum yields (Figure 2.4a and 2.4b). The decreased emission intensity of the complexes results from the π - π stacked aromatic segments, which promotes self-quenching.[29-31] The distinct peak at the longer wavelength of PPB-L/HA indicates that the added flexibility in small proportions contributes to more efficient polymer chain interaction and reorganization resulting in more ordered structures,[23, 24] while less chain reorganization upon ionic complexation was observed from PPB.

Poly(phenyleneethynylene)s exhibit more distinct changes in both absorption and emission profiles upon HA complexation. The positively charged PPE exhibits ~50 nm red-shifted absorption maximum upon HA complexation (Figure 2.3c), suggesting extended effective π -conjugation length. On the other hand, the PPE-L polymer contains multiple shorter conjugated segments within the polymer chain, which are isolated through the flexible, non-conjugated functionality. The

incorporation of the flexible linker in the PPE backbone influences the aggregation behavior of PPE-L/HA. The red shifted aggregation peak for the complex is not that prominent, indicating that the effective extension of the π -conjugation length of the backbone is not significant as seen in the fully conjugated PPE (Figure 2.3d).

Figure 2.3. Absorption spectra of (a) **PPB**, (b) **PPB-L**, (c) **PPE** and (d) **PPE-L** before (dotted lines) and after (solid lines) complexation with HA.

Figure 2.4. Emission spectra of (a) **PPB**, (b) **PPB-L**, (c) **PPE** and (d) **PPE-L** before (dotted lines) and after (solid lines) complexation with HA.

The emission spectra of PPEs were somewhat different from those of PPBs. The shape of the emission spectra of PPEs upon HA complexation was very similar to those of PPEs in water, although shifted to a longer wavelength (Figure 2.4c). Interestingly, the QYs of PPEs/HA were increased after complexation, contrary to those of PPBs (Table 1). While the sharp absorbance peaks at longer wavelength were observed from both PPE and PPE-L, the unchanged spectral shape and increased emission intensity suggest that PPE chains interacted differently with the same polyanion HA than the PPBs with the same side chains. We speculate that PPE chains experienced better solvation and somewhat decreased extent of interpolymer π - π stacking upon complexation, which contributed to the sharp spectral shape and increased quantum yields.[32, 33]

The flexible linker presented in the PPE backbone decreased the above-mentioned contributing factors, resulting in an absorption shoulder at a longer wavelength. However, the slightly increased (or maintained) emission QYs of PPE-L/HA also suggests that similar solvation effects are at play to decrease the π - π stacking of the PPE-L backbones. The self-assembly difference between PPE

and PPB can be attributed to the different aggregation status of CPs in a good solvent. As we observed previously, PPBs with elongated butadiynylene conjugation are prone to chain-chain aggregation (i.e., interpolymer interactions) even in DMSO, therefore lowering the solubility of PPBs, which has intrinsically high π - π interaction among the PPBs backbone. By adding a small amount of flexible units along the PPB backbone, which improves the polymer solubility in DMSO, the chain-chain stacking was more favorable upon HA complexation.[20]

To further understand the factors contributing to QY changes, fluorescence lifetime measurements were performed. Quantum yield is directly related to the two competing decay processes after excitation: the radiative and non-radiative decay rates.[34] When the excited electrons relax mainly through radiative decay processes, increased QYs will be observed. If the contribution of non-radiative processes is significant, the opposite will be expected.[34] Using the relationship between the fluorescence lifetime (τ_F) and quantum yield (Φ_F), $k_{nr} = (1-\Phi_F)/\tau_F$, the radiative and nonradiative decay rates of PPEs and PPBs were obtained before and after complexation (Table 1). The nonradiative decay rates of PPB-L and PPE were dramatically changed upon complexation with HA: ~33 times increase in PPB-L versus ~39 times decrease in PPE. Although the radiative decay rate of PPB-L/HA was also increased ~20 times, the overall QY of PPB-L/HA was decreased by a higher contribution from the increased nonradiative decay rate. In the case of the PPE, the decreased radiative decay constant upon HA complexation was compensated by the substantially larger decrease in the nonradiative decay rate, resulting in increased QYs. The similar magnitude of increased of both rate constants in PPE-L/HA coincides well with a slightly increased QY.

On the basis of the observed differences in the spectroscopic analyses, we conclude that the main contributing factor affecting the aggregation nature of these four phenylene-based polymers upon polyanion complexation is the backbone structure. The longer PB bridge in the PPB-L backbone gives rise to a dramatic backbone reorganization upon polyanion complexation, favoring stronger interpolymer π - π interactions. On the other hand, the PE backbone in PPE, with red-shifted

absorption maximum and an increased fluorescence quantum yield upon polyanion complexation, is due to the increased effective conjugation length forcing their backbone to be more planar and rigid. We speculate that the flexibility inserted in the PPE backbone (PPE-L) contributes to the decrease in the degree of planarization.

2.4.3 Helical self-assembly monitored by induced circular dichroism spectroscopy

Prompted by the previous reports on induced helix formation of achiral oligo(phenyleneethynylene)s (OPEs) and polythiophenes upon complexation with anionic polysaccharides, we measured the circular dichroism (CD) of CPs/HA complexes in water.[32, 35-37] Hyaluronic acid is an optically active polysaccharides containing a repeating disaccharide subunit of D-glucuronic acid and N-acetyl-D-glucosamine. It has been reported that HA exhibits three- or fourfold left-handed duplex helical conformation.[38] The helical conformation of HA highly depends on the nature of counter ions and pH.[39] Conjugated polymers are optically inactive, so no characteristic CD pattern is expected in the π - π^* transition region. Upon complexation with HA, PPE and PPB-L exhibit a strong induced CD (ICD) peak at the red-shifted absorption maximum, implying the formation of induced helical conformation due to the template effect of HA. A strong ICD was observed from PPE/HA and PPB-L/HA, while very weak or no ICD was observed from PPE-L/HA and PPB/HA (Figure 2.5a and 2.5d). Interestingly, PPB-L/HA exhibits a positive Cotton effect, while PPE/HA exhibits a negative Cotton effect, although the same HA was complexed to structurally similar CPs. The opposite trend of the Cotton effect clearly indicates that the handedness of the ordered complexes is opposite each other. The ICD intensity was increased until at or near the molar equivalency between CPs and HA. Using the Job plot method,[40] we found that PPB-L/HA and PPE/HA form 2:3 and 3:2 complexes, respectively (Supplementary data Figure S2.17 and S2.18). The addition of a small fraction of a non-conjugated flexible linker unit in PPB showed the ICD effect, assuming that the improved solubility originating

from the flexible units allows the chain reorganization upon complexation. We believe that the intrinsic interchain interactions among the PPB backbones are so strong that the complexation with HA could not induce sufficient chain reorganization to form the helical conformation. In the PPB, the interchain interaction of the backbone is the main factor for the decreased solubility and quantum yield of complex upon complexation with HA. In addition to electrostatic interaction, the hydrophobic interaction also plays substantial roles for the efficient complexations producing ICD effect but increased interchain interaction in aggregated PPB lowers the access to hydrophobic moiety with charged amine side chain facing the outside of the aggregate.[19, 20, 41] The decreased hydrophobic interaction site for HA is also a reason for not showing ICD effect in the PPB/HA complex. The result is further supported by the smaller hydrodynamic diameter of the complex due to stronger interchain interaction leading to the formation of compact PPB/HA complex (Table 1).[37] Hasegawa et al. reported that polysaccharide (schizophyllan, SPG) acted as a 1D host to accommodate 1,4-diphenylbutadiyne derivatives (DPBs) within helical superstructures of SPG, which the ordered structure was further polymerized to poly(DPBs)-nanofibers.[41] It shows that the PB unit has a tendency to stack to helical conformation with polysaccharides depending on the hydrophobic interaction, conformational orientation, and steric hindrance.[41] Meanwhile, the insertion of the same flexible linker along the PPE backbone reduced the interpolymer interaction needed to form an ordered helical structure, resulting in very weak ICD.

Figure 2.5. The circular dichroism and absorption spectra of (a) **PPB**, (b) **PPB-L**, (c) **PPE** and (d) **PPE-L** before (dotted lines) and after (solid lines) complexation with HA.

To examine the thermal stability of the complexes, ICD intensity was monitored as a function of temperature (Supplementary data Figure S2.15 and S2.16). Induced circular dichroism intensity of both PPB-L/HA and PPE/HA was decreased as temperature increased, and the ICD intensity was partially recovered after the subsequent cooling. The absorbance spectra of the complexes also blue shifted and decreased gradually as temperature increased. These observations suggest that the complexes are dissociated into individual entities or rearranged to non-helical motif by the applied thermal energy (Supplementary data Figure S2.15). After the thermal treatment (i.e., annealing), CPs/HA complexes did not produce intense ICD, which is in agreement with observation that

thermally stabilized CP chains exhibit more difficulty in adopting the helical structure upon ionic interaction.[36, 37]

2.4.4 Transmission Electron Microscopy

To further characterize the nature of polymer aggregation and the appearance of the corresponding CP/HA complexes, we subjected the CPs and their HA complexes to TEM imaging. The conventional TEM sample preparation technique, which involves the air-drying of a sample droplet on a TEM grid, resulted in the formation of large polymer aggregates with no distinct structural features. We also tried the cryo-ultramicrotome technique to prepare TEM samples in the hope of preserving the aggregation structures in water. Regardless of these efforts, the TEM images were fairly similar to those obtained from the conventional air-drying method. However, some general observations can be drawn from the multiple replicates of the TEM experiments. For instance, the CPs adopt a globular shape, as exemplified by the PPB-L in Figure 2.6a. Upon complexation of PPB-L with HA, we often observed the formation of more ordered microcrystallites structures as depicted in Figure 2.6b. These structural features, which allude to a possible formation of aggregates through increased π - π stacking, were largely absent from the PPE-type polymer images.

Figure 2.6. TEM images of (a) **PPB-L** and (b) **PPB-L/HA** complex (scale bar = 200 nm).

2.5 Conclusion

In summary, we synthesized and tested the aggregation behaviors of a set of phenylene-based CPs with different backbone structures upon ionic complexation with HA. We modulated the interpolymer interaction by introducing a non-conjugated flexible linker at small proportions. The four CPs exhibit a distinctly different aggregation behavior upon HA complexation, as evidenced by their absorption, emission, and ICD profiles. The PPB versus PPE backbone connectivity is the main driving force for this behavior, with the presence of the non-conjugated linker in the backbone further enhance the effect in the PPB series because of the increased backbone flexibility. In conclusion, we discovered and provided spectroscopic evidences that the extent of interpolymer interaction among positively charged phenylene-based CPs is a crucial factor for controlling aggregation structures. Highly ordered helical ionic complexes were obtained when positively charged CPs with appropriate backbone structures (i.e., PPB-L and PPE) interact with negatively charged polysaccharide.

2.6 Outlook

This chapter presented an easy approach to create an ordered helical complex of CPs and HA. Depending on backbone structure and flexibility of CPs, they can create an ordered helical complex upon the complexation with polysaccharides (i.e., HA in this chapter). We discovered PPE and PPB-L are an exciting material with different backbone structure and photophysical properties with ability to form helical complex. So, it would be interesting to see if similar ICD effect is observed for the other class of polysaccharides. For this reason, the project has been extended to see effect complexation with other class of polysaccharides i.e., glycosaminoglycans (GAGs). A detail photophysical properties changes and CD effect are presented in chapter II.

2.7 Experimental

2.5.1 General information

Chemicals, including solvents, were purchased from Fisher Scientific, and used as received. Sodium hyaluronate (HA) was purchased from Lifecore (MW 40 KDa) and used as received. Deuterated solvents were purchased from Cambridge Isotope Laboratories (Cambridge, MA). A microwave reactor (Monowave Edu, Anton Paar) was used for synthesis of PPEs using a silicon carbide vessel. Deprotection of all Boc-protected polymers was conducted in a glass vessel under the microwave. The number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index ($PDI = M_w/M_n$) of CPs were determined by gel permeation chromatography (GPC) against polystyrene standards using a Shimadzu high performance liquid chromatography (HPLC) system fitted with PLgel 5 μ m MIXED-D columns and SPD-20A ultraviolet-visible (UV-vis) detector at a flow rate of 1.0 mL/min. Samples for GPC, small amount (~100 μ L) of polymer in dimethylformamide (DMF) was diluted with 1 mL of HPLC grade THF and then filtered through a 0.45 μ m polytetrafluoroethylene (PTFE) syringe filter prior for injection. The UV-vis spectra were recorded using a Varian Cary 50 Bio spectrophotometer. Fluorescence spectra were obtained using a FluoroLog-3 Spectrofluorometer (Jobin Yvon/Horiba). 9,10-diphenylanthracene (QY = 0.9) in cyclohexane was used as a fluorescence standard. Circular dichroism (CD) were obtained in a Jasco J-815 spectrometer equipped with a Peltier cell holder Jasco CDF-426S by using a glass cuvette with a path length of 1 cm. Fourier transform infrared (FTIR) spectra were recorded on a PerkinElmer Spectrum 100 FTIR Spectrometer. Fine polymer powders were directly mounted on an attenuated total reflection (ATR) cell of the spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz Avance Bruker NMR spectrometer. Chemical shifts were reported in parts per million (ppm) for ^1H NMR on the δ scale based on the middle peak ($\delta = 2.50$ ppm) of the dimethylsulfoxide (DMSO- d_6) solvent as an internal standard. Nanoparticle tracking analysis (NTA) measurements were performed using a

LM10 HS (NanoSight Amesbury, United Kingdom) equipped with a sCMOS camera, a sample chamber, and a 488 nm blue laser.

2.5.2 Synthesis of polymers: Monomers M1, M2 and M3 were synthesized according to literature procedures.[19, 25]

Figure S2.1. Chemical structures of monomers **M1**, **M2** and **M3**.

PPBs. Poly(phenylenebutadiynylene)s were synthesized by a modified Glaser coupling reaction using palladium and copper catalyst.[19, 25] A Schlenk flask was charged with M1 (10.0 mg, 0.0188 mmol), bis(triphenylphosphine) palladium (II) dichloride ($\text{Pd}[\text{Cl}_2(\text{PPh}_3)_2]$) (2.6 mg, 0.00375 mmol), and copper iodide (CuI) (3.4 mg, 0.0178 mmol). The Schlenk flask was evacuated and filled with nitrogen (N_2) gas. A mixed solution of DMF (4 mL) and triethylamine (TEA) (1 mL) was degassed with N_2 gas, and then 2 mL of the mixed solvent was transferred to the Schlenk flask via a cannula. The reaction was heated to 70°C for 18 h. After cooling the reaction solution to room temperature, precipitates were obtained by transferring the solution dropwise to cold diethyl ether. After settling down the polymer precipitates for 5 to 10 min, the supernatant was decanted. The precipitates were re-dissolved in DMF (1 mL), and the same purification method was repeated three more times. The precipitates were dried under high vacuum (6.8 mg, 68%) for at least 8 h prior to FTIR and ^1H NMR characterization. The precipitates were air dried. Deprotection of the Boc group was carried out by heating a mixed solution of Boc-PPB (6.8 mg) in dimethylsulfoxide (DMSO)

(2 mL) and trifluoroacetic acid (TFA) (2 mL) to 100 °C by microwave irradiation for 30 min. The solution was cooled to room temperature and transferred dropwise to cold ethyl acetate (EA) while stirring, producing precipitates. After decantation, the precipitates were dissolved in DMF (1 mL), and the same precipitation procedure was repeated three times and dried thoroughly under high vacuum for two days.

Scheme S2.1. Synthetic route to **PPB**.

Boc-PPB. ^1H NMR (400 MHz, DMSO-d_6 , ppm): δ 7.29 (s, 0.7H), 6.71 (s, 1.0H), 4.19 (br, 2.0H), 3.73 (br, 2.2H), 3.5 (br, 2.9H), 3.09 (br, 2.7H), 1.34 (s, 14.8H). GPC: $M_w = 16,900\text{ g/mol}$, $M_n = 9,000\text{ g/mol}$, $\text{PDI} = 1.87$. UV-Vis (DMF) $\lambda_{\text{max}} = 452\text{ nm}$, Fluo $\lambda_{\text{max}} = 479\text{ nm}$ (ex = 440 nm), QY = 21%.

Figure S2.2. ^1H NMR spectrum of **Boc-PPB** in DMSO-d_6 .

PPB. ^1H NMR (400 MHz, DMSO-d_6 , ppm): δ 7.90 (br, 3.0H), 7.32 (s, 0.8H), 4.23 (br, 2.0H), 3.81 (br, 3.0H), 3.71 (br, 3.4H), 3.01 (s, 3.0H). FT-IR (neat): 3338, 2923, 1674, 1496, 1417, 1186, 1124, 1018, 943, 836, 796, 725 cm^{-1} . UV-Vis (H_2O) $\lambda_{\text{max}} = 434$ nm, Fluo $\lambda_{\text{max}} = 468$ nm (excitation = 440 nm), QY = 0.42%.

Figure S2.3. ^1H NMR spectrum of **PPB** in DMSO-d_6 .

PPB-L. Similarly, PPB-L was synthesized by polymerizing M1 (20.0 mg, 0.0375 mmol) and M2 (1.53 mg, 0.00375 mmol) in the presence of Pd[Cl₂(PPh₃)₂] (5.26 mg, 0.0075 mmol) and CuI (6.7 mg, 0.0356 mmol) in a mixed solvent (2 mL, DMF:TEA=4:1). Purification and Boc deprotection were carried out as described above. (17 mg, 85%).

Scheme S2.2. Synthetic route to **PPB-L**.

Boc-PPB-L. ¹H NMR (400 MHz, DMSO-d₆, ppm): δ 10.31(s, 0.1H), 7.62-7.56 (br, 0.4H), 7.29 (br, 1.0H), 6.73 (s, 1.0H), 4.18 (br, 2.0H), 3.74 (br, 2.0 H), 3.49 (br, 2.2H), 3.11(br, 2.3H) 1.34 (s, 10.4H). GPC: M_w = 40,400 g/mol, M_n = 20,800 g/mol, PDI = 1.94. UV-Vis (DMF) λ_{max} = 448 nm, Fluo λ_{max} = 478 nm (ex = 440 nm), QY = 22%.

Figure S2.4. ^1H NMR spectrum of **Boc-PPB-L** in DMSO-d_6 . Proton “a” integration: Predicted value 1H ($1 \times 1\text{H}$), observed value 1H, Proton “b” integration: Predicted value 2H ($1 \times 2\text{H}$), observed value 2H, Proton “c” integration: Predicted value 1H ($1 \times 1\text{H}$), observed value 0.98H, “Boc” Proton integration: Predicted value 9H ($1 \times 9\text{H}$), observed value 10.1H, Proton “d, e” integration: Predicted value 0.4H ($0.1 \times 4\text{H}$), observed value 0.41H, Proton “f” integration: Predicted value 0.1H ($0.1 \times 1\text{H}$), observed value 0.06H. Value 0.98 H, “Boc” Proton integration: Predicted value 9H ($1 \times 9\text{H}$), observed value 10.1H, Proton “d, e” integration: Predicted value 0.4H ($0.1 \times 4\text{H}$), observed value 0.41H, Proton “f” integration: Predicted value 0.1H ($0.1 \times 1\text{H}$), observed value 0.06H.

PPB-L. ^1H NMR (400 MHz, DMSO-d_6 , ppm): δ 10.38 (s, 0.1H), 7.89 (br, 3.2H), 7.68 (br, 0.2H), 7.53 (br, 0.3H), 7.31 (s, 1.1H), 4.23 (br, 2.0H), 3.81 (br, 2.0H), 3.72 (br, 2.4H), 3.02 (br, 3H). FT-IR (neat): 3401, 2905, 1674, 1501, 1412, 1275, 1195, 1120, 1027, 832, 796, 721 cm^{-1} . UV-Vis (H_2O) $\lambda_{\text{max}} = 440 \text{ nm}$, Fluo $\lambda_{\text{max}} = 468 \text{ nm}$ (ex= 440 nm), QY = 4.3%.

Figure S2.5. ^1H NMR spectrum of **PPB-L** in DMSO-d_6 .

PPEs. Boc-protected primary amine-containing PPEs (Boc-PPEs) were synthesized by microwave-assisted Sonogashira cross-coupling reaction. A silicon carbide (SiC) microwave tube was charged with M1 (20 mg, 0.036 mmol), M3 (26.4 mg, 0.036 mmol), $\text{Pd}[\text{Cl}_2(\text{PPh}_3)_2]$ (5.0 mg, 0.0072 mmol), and CuI (0.34 mg, 0.0018 mmol) and was purged with N_2 gas. A mixed solution of DMF (4 mL) and TEA (1 mL) was prepared and degassed with nitrogen gas in a vial, and then 2 mL of the mixed solution was transferred to the reaction tube via a cannula. The reaction tube was heated to 100 $^\circ\text{C}$

Figure S2.6. ¹H NMR spectrum of **Boc-PPE** in DMSO-D₆.

PPE. ¹H NMR (400 MHz, DMSO-d₆, ppm): δ 8.04 (br, 3.0H), 7.18 (s, 0.8H), 4.23 (br, 2.0H), 3.84 (br, 2.2H), 3.74 (br, 2.3H), 2.98 (s, 2.3H). FT-IR (neat): 3406, 2910, 1674, 1501, 1421, 1270, 1195, 1018, 947, 827, 796, 717 cm⁻¹. UV-Vis (H₂O) λ_{max} = 420 nm, Fluo λ_{max} = 462 nm (ex = 420 nm), QY = 4%.

Figure S2.7. ^1H NMR spectrum of **PPE** in DMSO-d_6 .

PPE-L. A SiC tube was charged with monomers M1 (32.5 mg, 0.0612 mmol), M3 (50.0 mg, 0.068 mmol), and M2 (2.77 mg, 0.0068 mmol) in the presence of palladium (II) acetate $\text{Pd}(\text{OAc})_2$ (3.05 mg, 0.0136 mmol), 1,1'-Bis(diphenylphosphino)ferrocene (dppf) (18.8 mg, 0.034 mmol), and CuI (0.64 mg, 0.0034 mmol). The SiC tube was evacuated and filled with N_2 gas. A mixed solvent of DMF (3 mL), THF (1 mL), and diisopropylamine (DIPA) (1 mL) was prepared and degassed with N_2 gas, and then 4 mL of the mixed solvent was transferred to the SiC tube via a cannula. The reaction was heated to $100\text{ }^\circ\text{C}$ under microwave irradiation for 40 min. The reaction solution was cooled to room temperature and transferred dropwise to cold ether while stirring, resulting in precipitation. After decantation of the supernatant, precipitates were dissolved in 1 mL of DMF. The same precipitation and decantation procedures were repeated three times, yielding yellow powder of Boc-PPE-L (52.2 mg, 75%). Purification and Boc deprotection were carried out as described above.

Scheme S2.4. Synthetic route to **PPE-L**

Boc-PPE-L. ^1H NMR (400 MHz, DMSO-d_6 , ppm): δ 10.22 (s, 0.1H), 7.53 (br, 0.5H), 7.15 (m, 1.0H), 6.70 (s, 1.0H), 4.19 (br, 2.0H), 3.78 (br, 2.3H), 3.51 (m, 2.5H), 3.08 (m, 2.4H), 1.35 (s, 10.05H). GPC: $M_w = 27,200$ g/mol, $M_n = 16,200$ g/mol, $\text{PDI} = 1.67$. UV-Vis (DMF) $\lambda_{\text{max}} = 428$ nm, Fluo $\lambda_{\text{max}} = 470$ nm (ex=420 nm), QY =9.3%.

Figure S2.8. ^1H NMR spectrum of **Boc-PPE-L** in DMSO-d_6 . Proton “a” integration: Predicted value 1H (1 x 2H), observed value 0.98H, Proton “b” integration: Predicted value 2H (1 x 2H), observed value 2, Proton “c” integration: Predicted value 1H (1 x 1H), observed value 0.83H, “Boc” Proton integration: Predicted value 9H (1 x 9H), observed value 9.9H, Proton “d, e” integration: Predicted value 0.4H (0.1 x 4H), observed value 0.54H, Proton “f” integration: Predicted value 0.1H (0.1 x 1H), observed value 0.07H.

PPE-L. ^1H NMR (400 MHz, DMSO-d_6 , ppm): δ 10.32 (br, 0.2H), 8.0 (br, 3.2H), 7.69 (br, 0.2H), 7.45 (br, 0.3H), 7.18 (s, 0.8H), 4.24 (br, 2.0H), 3.84 (br, 1.8H), 3.74 (br, 2.0H), 2.98 (br, 2.3H).

FT-IR (neat): 3401, 2914, 1674, 1514, 1417, 1191, 1018, 947, 832, 796, 717 cm^{-1} . UV-Vis (H_2O)

$\lambda_{\text{max}} = 417 \text{ nm}$, Fluo $\lambda_{\text{max}} = 461 \text{ nm}$ (ex = 420 nm), QY = 2.3%.

Figure S2.9. ^1H NMR spectrum of **PPE-L** in DMSO-d_6 . Proton “a” integration: Predicted value 1H ($1 \times 1\text{H}$), observed value 0.98H, Proton “b” integration: Predicted value 2H ($1 \times 2\text{H}$), observed value 2, Proton “c” integration: Predicted value 1H ($1 \times 1\text{H}$), observed value 0.83H, “Boc” Proton integration: Predicted value 9H ($1 \times 9\text{H}$), observed value 9.9H, Proton “d, e” integration: Predicted value 0.4H ($0.1 \times 4\text{H}$), observed value 0.54H, Proton “f” integration: Predicted value 0.1H ($0.1 \times 1\text{H}$), observed value 0.07H.

2.5.3 Absorption and emission study: Spectroscopic samples were prepared by adding 10 μL of the corresponding CPs in DMSO (2.0 mM) to 490 μL of DI water, which was further diluted by

mixing 100 μL of the CPs in water with 300 μL of DI water. For CPs/HA complexation studies, 100 μL of aqueous CPs solutions were mixed with 270 μL , 230 μL , and 110 μL of DI water, respectively, and then 20 μL , 60 μL , 180 μL of HA solution (200 μM) was added, respectively, and mixed by gentle pipetting to have the final HA concentration of 10, 30, and 90 μM , respectively (Supplementary data Figure S10 and S11). The total volume of DMSO (0.5%, v/v) was kept constant in all the samples. Fluorescence lifetimes were measured using a time-correlated single-photon counting (TCSPC) system that consisted of a picosecond diode laser (Edinburgh Instruments), a SP-130 PCI TCSPC board (Becker & Hickl GmbH), a monochromator (Dongwoo Optron), and an avalanche photodiode detector (ID Quantique). All CPs were excited with a 405-nm ps laser and detected at their fluorescence maxima. Lifetimes were calculated by nonlinear regression fitting of the data using triple-exponential decay convoluted with the instrument response function (IRF) time components, which was ~ 20 ps. Due to the inherent molecular weight inhomogeneity of polymers, which contributes to variation in the lifetimes depending on the excitation wavelengths, the average values were used as a characteristic fluorescence lifetime (τ_F).

Figure S2.10. Absorption spectra of (a) **PPB** (b) **PPB-L** (c) **PPE** and (d) **PPE-L** upon complexation with different concentrations of HA.

Figure S2.11. Emission spectra of (a) **PPB** (b) **PPB-L** (c) **PPE** and (d) **PPE-L** upon complexation with different concentrations of HA.

Figure S2.12. Absorption spectra of (a) **PPB** (b) **PPB-L** (c) **PPE** and (d) **PPE-L** with various volume % (v/v) of DMSO in water.

Figure S2.13. Emission spectra of (a) **PPB** (a) **PPB-L** (a) **PPE** and (a) **PPE-L** with various volume % (v/v) of DMSO in water.

Figure S2.14. Fluorescence life time measurement Red- **PPB-L**, Blue- **PPE-L** and Green- **PPE**.

2.5.4 Circular dichroism (CD) study: Samples for CD measurements were prepared by adding 10 μL of the corresponding CPs in DMSO (20 mM) to 1990 μL of DI water. In order to see how the HA complexation induces CD, samples were prepared by mixing 10 μL of CPs in DMSO (20 mM) with 1890 μL of DI water followed by addition of 100 μL HA (133 μM). The resulting

solutions were mixed by gentle pipetting and the CD spectra were recorded immediately. The total volume of DMSO (0.5%, v/v) was kept constant in all samples. All measurements were performed at 20 °C and three scans were accumulated within the scan range of 550-350 nm at the scan rate of 50 nm/s.

Figure S2.15. Variable Temperature Circular Dichroism (VT-CD) spectra (Heating cycle) of **PPB-L** (left) and **PPE** (right) with HA in a 1:1 molar ratio.

Figure S2.16. Variable Temperature Circular Dichroism (VT-CD) spectra (Cooling cycle) of **PPB-L** (left) and **PPE** (right) with HA in a 1:1 molar ratio

2.5.5 Job plot: In order to determine the binding stoichiometry of the complexation, a job plot was performed in CD spectroscopy. Stock solution (5 mM) of HA was prepared in DI water and CP stock solutions (5 mM) were prepared by diluting concentrated CPs in water. The volume % of DMSO (11%) in solution was adjusted by adding DMSO into each sample. Samples were prepared with different proportions of the HA and CPs solution so that the total concentration of [HA]+ [CPs] for each sample was 250 μ M for PPE and 130 μ M for PPB-L (table S1 and S2). The plot for the ICD intensity at 470 nm gave a maximum at [CPs]/ ([HA] + [CPs]) = 0.6 for PPE (Figure S2.17) and 0.4 for PPB-L (Figure S2.18).

Table S2.1 Data for the job plot performed for the complexation of PPE and HA.

Sample	HA (μ L)	PPE (μ L)	H ₂ O (μ L)	DMSO (μ L)	Total (μ L)	[HA] (μ M)	[PPE] (μ M)	$\frac{[PPE]}{([HA] + [PPE])}$	CD (mdeg)
1	10	90	1900	0	2000	25	225	0.9	3.37
2	20	80	1898.9	1.1	2000	50	200	0.8	6.59
3	25	75	1898.3	1.6	2000	62.5	187.5	0.75	10.41
4	30	70	1897.8	2.2	2000	75	175	0.7	26.30
5	40	60	1896.7	3.3	2000	100	150	0.6	27.31
6	50	50	1895.6	4.4	2000	125	125	0.5	24.46
7	55	45	1895.0	4.9	2000	137.5	112.5	0.45	18.79
8	60	40	1894.5	5.5	2000	150	100	0.4	14.50
9	70	30	1893.4	6.6	2000	175	75	0.3	10.48
10	80	20	1892.3	7.7	2000	200	50	0.2	1.62
11	90	10	1891.2	8.8	2000	225	25	0.1	2.57

Figure S2.17. Job Plot of **PPE** with HA.

Table S2.2. Data for the job plot performed for complexation of PPB-L and HA.

Sample	HA (μL)	PPB-L (μL)	H ₂ O (μL)	DMSO (μL)	Total (μL)	HA (μM)	PPB-L (μM)	$\frac{[\text{PPB} - \text{L}]}{([\text{HA}] + [\text{PPB} - \text{L}])}$	CD (mdeg)
1	6	46	1948	0	2000	15	115	0.884615	4.79
2	12	40	1947.3	0.6	2000	30	100	0.769231	7.19
3	16	36	1946.9	1.1	2000	40	90	0.692308	22.89
4	20	32	1946.4	1.5	2000	50	80	0.615385	15.26
5	22	30	1946.2	1.7	2000	55	75	0.576923	23.47
6	24	28	1946.0	1.9	2000	60	70	0.538462	32.51
7	26	26	1945.8	2.2	2000	65	65	0.5	36.85
8	28	24	1945.5	2.4	2000	70	60	0.461538	49.75
9	30	22	1945.3	2.6	2000	75	55	0.423077	70.60
10	32	20	1945.1	2.8	2000	80	50	0.384615	59.67
11	34	18	1944.9	3.0	2000	85	45	0.346154	30.12
12	36	16	1944.7	3.3	2000	90	40	0.307692	26.81
13	38	14	1944.4	3.5	2000	95	35	0.269231	18.60
14	40	12	1944.2	3.4	2000	100	30	0.230769	9.37
15	42	10	1944.0	3.6	2000	105	25	0.192308	11.50
16	46	6	1943.6	4.4	2000	115	15	0.115385	8.14

Figure S2.18. Job Plot of **PPB-L** with HA.

2.5.6 Determination of hydrodynamics diameters of CPs and CPs/HA complexes

The concentration of NTA samples was approximately 10 μM containing 0.5% DMSO (v/v). Filtered sample solution (0.2 μm syringe filter) was injected into the sample chamber using a 1 mL syringe (Restek Corporation, Pennsylvania, USA) until a few drops of the solution dripped out of the outlet of the chamber. All measurements were performed at 25°C using a LM14C temperature controller (NanoSight, Amesbury, United Kingdom). Each sample was measured three times.

Figure S2.19. NTA for **PPB** (right) and **PPB/HA** complex (left).

Figure S2.20. NTA for **PPB-L** (right) and **PPB-L/HA complex** (left).

Figure S2.21. NTA for **PPE** (right) and **PPE/HA complex** (left).

Figure S2.22. NTA for **PPE-L** (right) and **PPE-L/HA complex** (left).

2.5.7 Sample preparation for transmission electron microscopy (TEM)

To examine the morphology of CPs and CP/HA complexes in water, the aqueous sample solutions in centrifugal tubes were rapidly cooled by submerging into liquid nitrogen. To cut frozen sections, the sample was mounted directly on the microtome holder and sections were obtained using a diamond-trimming knife, which was cooled in a cryo-chamber. The cryo-ultramicrotome was carried out using the RMC PTPC&CRX. The freeze-drying procedure of these samples on Cu-grid was carried out for 3 days under a vacuum, and then imaged using a JEOL JEM-2100 transmission electron microscope operated at 200 kV. Unfortunately, TEM images of the samples prepared by the cryo-ultramicrotome technique were very similar to those obtained from the conventionally air-dried or freeze-dried aqueous sample droplet on a Cu-grid.

2.5.8 FT-IR spectra of polymers

Figure S2.23. FT-IR of PPB (neat).

Figure S2.24. FT-IR of PPB-L (neat).

Figure S2.25. FT-IR of PPE (neat).

Figure S2.26. FT-IR of **PPE-L** (neat).

2.8 References:

- [1] A.C. Grimsdale, K.L. Chan, R.E. Martin, P.G. Jokisz, A.B. Holmes, Synthesis of light-emitting conjugated polymers for applications in electroluminescent devices, *Chem. Rev.* 109(3) (2009) 897-1091.
- [2] D.T. McQuade, A.E. Pullen, T.M. Swager, Conjugated polymer-based chemical sensors, *Chem. Rev.* 100(7) (2000) 2537-2574.
- [3] C. Zhu, L. Liu, Q. Yang, F. Lv, S. Wang, Water-soluble conjugated polymers for imaging, diagnosis, and therapy, *Chem. Rev.* 112(8) (2012) 4687-735.
- [4] J.H. Moon, W. McDaniel, P. MacLean, L.E. Hancock, Live-cell-permeable poly (p-phenylene ethynylene), *Angew. Chemie. Int. Ed* 46(43) (2007) 8223-8225.
- [5] I.B. Kim, H. Shin, A.J. Garcia, U.H. Bunz, Use of a folate-PPE conjugate to image cancer cells in vitro, *Bioconj. Chem.* 18(3) (2007) 815-20.
- [6] K.-Y. Pu, B. Liu, Fluorescent Conjugated Polyelectrolytes for Bioimaging, *Adv. Funct. Mater.* 21(18) (2011) 3408-3423.
- [7] K. Lee, J. Lee, E.J. Jeong, A. Kronk, K.S. Elenitoba-Johnson, M.S. Lim, J. Kim, Conjugated polyelectrolyte-antibody hybrid materials for highly fluorescent live cell-imaging, *Adv. Mater.* 24(18) (2012) 2479-84.
- [8] G. Feng, D. Ding, B. Liu, Fluorescence bioimaging with conjugated polyelectrolytes, *Nanoscale* 4(20) (2012) 6150-65.
- [9] Y. Liu, K. Ogawa, K.S. Schanze, Conjugated polyelectrolytes as fluorescent sensors, *J. Photochem. Photobiol.* 10(4) (2009) 173-190.

- [10] X. Zhao, Y. Liu, K.S. Schanze, A conjugated polyelectrolyte-based fluorescence sensor for pyrophosphate, *Chem. Commun. (Cambridge, England)* (28) (2007) 2914-6.
- [11] J. You, T. Park, J. Kim, J.S. Heo, H.S. Kim, H.O. Kim, E. Kim, Highly fluorescent conjugated polyelectrolyte for protein sensing and cell-compatible chemosensing applications, *ACS Appl. Mater. Interfaces* 6(5) (2014) 3305-11.
- [12] M.D. Disney, J. Zheng, T.M. Swager, P.H. Seeberger, Detection of bacteria with carbohydrate-functionalized fluorescent polymers, *J. Am. Chem. Soc.* 126(41) (2004) 13343-6.
- [13] H.A. Ho, K. Dore, M. Boissinot, M.G. Bergeron, R.M. Tanguay, D. Boudreau, M. Leclerc, Direct molecular detection of nucleic acids by fluorescence signal amplification, *J. Am. Chem. Soc.* 127(36) (2005) 12673-6.
- [14] J.H. Moon, E. Mendez, Y. Kim, A. Kaur, Conjugated polymer nanoparticles for small interfering RNA delivery, *Chem. Commun. (Cambridge, England)* 47(29) (2011) 8370-2.
- [15] A.T. Silva, A. Nguyen, C. Ye, J. Verchot, J.H. Moon, Conjugated polymer nanoparticles for effective siRNA delivery to tobacco BY-2 protoplasts, *BMC Plant Biol.* 10 (2010) 291.
- [16] X. Feng, F. Lv, L. Liu, Q. Yang, S. Wang, G.C. Bazan, A highly emissive conjugated polyelectrolyte vector for gene delivery and transfection, *Adv. Mater* 24(40) (2012) 5428-32.
- [17] K.L. Lo, H.F. Sleiman, Synthesis and molecular recognition of conjugated polymer with DNA-mimetic properties, *Macromolecules* 41(15) (2008) 5590-5603.
- [18] R. Sakai, A. Nagai, Y. Tago, S.-i. Sato, Y. Nishimura, T. Arai, T. Satoh, T. Kakuchi, Fluorescence Turn-On Sensing of Anions Based on Disassembly Process of Urea-Functionalized Poly(phenylenebutadiynylene) Aggregates, *Macromolecules* 45(10) (2012) 4122-4127.
- [19] T. Vokata, J.H. Moon, Synthesis of phenyleneethynylene-doped poly(pphenylenebutadiynylene) s for live cell imaging, *Macromolecules* 46(3) (2013) 1253-1259.
- [20] M. Twomey, Y. Na, Z. Roche, E. Mendez, N. Panday, J. He, J.H. Moon, Fabrication of core-shell nanoparticles via controlled aggregation of semi-flexible conjugated polymer and hyaluronic acid, *Macromolecules* 46(15) (2013) 6374-6378.
- [21] B.P. Toole, Hyaluronan: from extracellular glue to pericellular cue, *Nature Rev. Cancer* 4(7) (2004) 528-39.
- [22] M. Hay, F.L. Klavetter, Aliphatic phenylene vinylene copolymers: Tuning the color of luminescence through co-monomer feed ratios, *J. Am. Chem. Soc.* 117(27) (1995) 7112-7118.
- [23] G. Bounos, S. Ghosh, A.K. Lee, K.N. Plunkett, K.H. DuBay, J.C. Bolinger, R. Zhang, R.A. Friesner, C. Nuckolls, D.R. Reichman, P.F. Barbara, Controlling chain conformation in conjugated polymers using defect inclusion strategies, *J. Am. Chem. Soc.* 133(26) (2011) 10155-60.
- [24] M.C. Traub, K.H. DuBay, S.E. Ingle, X. Zhu, K.N. Plunkett, D.R. Reichman, D.A. Vanden Bout, Chromophore-Controlled Self-Assembly of Highly Ordered Polymer Nanostructures, *J. Phys. Chem. Lett.* 4(15) (2013) 2520-2524.

- [25] M. Twomey, E. Mendez, R.K. Manian, S. Lee, J.H. Moon, Mitochondria-specific conjugated polymer nanoparticles, *Chem. Commun.* 52(27) (2016) 4910-3.
- [26] V.P. Mehta, E.V. Van der Eycken, Microwave-assisted C-C bond forming cross-coupling reactions: an overview, *Chem. Soc. Rev.* 40(10) (2011) 4925-36.
- [27] T. Vokatá, M. Twomey, E. Mendez, J.H. Moon, Synthesis of biodegradable conjugated polymers with controlled backbone flexibility, *J. Polym. Sci. A* 53(11) (2015) 1403-1412.
- [28] V. Filipe, A. Hawe, W. Jiskoot, Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates, *Pharm. Res.* 27(5) (2010) 796-810.
- [29] J. Kim, T.M. Swager, Control of conformational and interpolymer effects in conjugated polymers, *Nature* 411(6841) (2001) 1030-4.
- [30] D. Xie, A. Parthasarathy, K.S. Schanze, Aggregation-induced amplified quenching in conjugated polyelectrolytes with interrupted conjugation, *Langmuir* 27(19) (2011) 11732-6.
- [31] Y. Wang, E.M. Jones, Y. Tang, E. Ji, G.P. Lopez, E.Y. Chi, K.S. Schanze, D.G. Whitten, Effect of polymer chain length on membrane perturbation activity of cationic phenylene ethynylene oligomers and polymers, *Langmuir* 27(17) (2011) 10770-5.
- [32] C. Li, M. Numata, A.H. Bae, K. Sakurai, S. Shinkai, Self-assembly of supramolecular chiral insulated molecular wire, *J. Am. Chem. Soc.* 127(13) (2005) 4548-9.
- [33] M. Numata, T. Fujisawa, C. Li, S. Haraguchi, M. Ikeda, K. Sakurai, S. Shinkai, β -1,3-Glucan (Schizophyllan) Can Act as a One-Dimensional Host for Creating Chirally Twisted Poly(p-phenylene Ethynylene), *Supramol. Chem.* 19(1-2) (2007) 107-113.
- [34] L. O'Neill, P. Lynch, M. McNamara, H.J. Byrne, Correlation of vibrational intensity with fluorescence lifetimes in π conjugated polymers, *Polymer* 49(19) (2008) 4109-4114.
- [35] Y. Tang, Z. Zhou, K. Ogawa, G.P. Lopez, K.S. Schanze, D.G. Whitten, Synthesis, self-assembly, and photophysical behavior of oligo phenylene ethynylenes: from molecular to supramolecular properties, *Langmuir* 25(1) (2009) 21-5.
- [36] T. Shiraki, A. Dawn, Y. Tsuchiya, S. Shinkai, Thermo- and solvent-responsive polymer complex created from supramolecular complexation between a helix-forming polysaccharide and a cationic polythiophene, *J. Am. Chem. Soc.* 132(39) (2010) 13928-35.
- [37] Z. Yao, G. Li, C. Fau - Shi, G. Shi, Optically active supramolecular complexes of water-soluble achiral polythiophenes and folic acid: spectroscopic studies and sensing applications, *Langmuir* 24 (2008) 12829-12835.
- [38] H. Tobata, T. Sagawa, Specific excitonic interactions in the aggregates of hyaluronic acid and cyanine dyes with different lengths of methine group, *Photochem. Photobiol. Sci.* 15(3) (2016) 329-33.

- [39] K.C. Haudek, R.J. Patterson, J.L. Wang, Insights on the conformational properties of hyaluronic acid by using NMR residual dipolar couplings and MD simulations, *Glycobiology* 20(10) (2010) 1199-207.
- [40] E.J. Olson, P. Buhlmann, Getting more out of a Job plot: determination of reactant to product stoichiometry in cases of displacement reactions and n:n complex formation, *J. Org. Chem.* 76(20) (2011) 8406-12.
- [41] T. Hasegawa, S. Haraguchi, M. Numata, C. Li, A.H. Bae, T. Fujisawa, K. Kaneko, K. Sakurai, S. Shinkai, Poly(diacetylene)-nanofibers can be fabricated through photo-irradiation using natural polysaccharide schizophyllan as a one-dimensional mold, *Org. Biomol. Chem.* 3(24) (2005) 4321-8.

Chapter III

Glycosaminoglycan-assisted self-assembly of conjugated polymers

In preparation

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3.1 Abstract

The electrostatic interaction of achiral cationic conjugated polymers (CPs) and chiral polyanionic glycosaminoglycans (GAGs) can self-assemble to create a chiral helical complex, where GAGs act as a chiral polymeric template for the complex. The self-assembly behavior of CPs is highly dependent on their backbone structure. Thus, understanding the structure-property relationship of CPs in terms of their backbone structure is important for designing ordered helical complexes. In this note, we report the self-assembly behaviors of two CPs with different backbone structures but bearing the same side chain structures, upon complexation with four GAGs. Depending on the backbone structure of CPs [i.e., poly(phenyleneethynylene) (**PPE**) vs. Poly(phenylenebutadiynylene) (PPB) with non-conjugated linkers (L), (**PPB-L**)] and charge density of GAGs, the complexes exhibit unique photophysical properties and induced circular dichroism (ICD) effect. Upon complexation, **PPB-L** with four GAGs exhibits characteristic π - π stacking, evident by a ~30 nm red-shifted absorption maximum and decreased emission intensity with spectral broadening. Meanwhile, **PPE** exhibits a similar effect as **PPB-L** when complexed with three sulfated GAGs, where **PPE** upon complexation showed a red shifted absorption maximum (~20 nm) and decreased emission intensity with spectral broadening. But, the effect is remarkably different in the case of unsulfated GAG (i.e., hyaluronic acid, HA) where **PPE** shows a ~50 nm red-shifted absorption maximum and increased emission intensity without spectral broadening. The photophysical properties changed upon complexation with HA is caused by the extended conjugation of the backbone and solvation effect upon complexation. Interestingly, three GAGs (i.e., less sulfated chondroitin sulfate (CS), and dermatan sulfate (DS) along with non-sulfated HA) are able to show ICD effect whereas highly sulfated heparin sulfate (HS) is unable to show a similar effect. These characteristic alternations in photophysical properties and selective ICD effect clearly indicate the favorable conformation change of CPs leading to formation of ordered helical complex. It is expected that the retained optoelectronic properties from CPs and

biological properties from GAGs in the complex can create a new class of highly ordered biomaterial with potential biological application.

3.2 Introduction

Highly emissive light harvesting π -conjugated polymers (CPs) are versatile synthetic polymers with potential applications in sensing, bioimaging, biomaterial delivery, and optoelectronic devices.[1-6] Conjugated polymers are a promising biomaterial for various biological applications due to the ease of modification of the side chain with different functionalities, and in the modulation of the backbone structure to enhance photophysical properties. The introduction of charged groups into the side-chains of CPs can increase the aqueous compatibility of the material, while at the same time can provide added conjugation sites for the modification of functionalities. The charged side chains can also provide interacting sites for the oppositely charged biologically important biomaterials (e.g., peptides, DNA, polysaccharides, small analytes).[7, 8] The development of helical ordered complexes from CPs combined with simple mixing with biological macromolecules is well known in macromolecular chemistry, and has been used for chiral sensing and chiral catalysis.[9-11] The conformational structure and photophysical property changes upon ionic interaction of negatively charged polysaccharides with positively charged amine CPs can result in an ordered helical complexes, with the added advantage of chirality in the system. It has been reported, that the chiral effect in achiral CPs can be induced by using various chiral polysaccharides and biomacromolecules, such as curdan (Cur), schizophyllan (SPG), carboxymethylcellulose (CMC), carboxymethylamylose (CMA), amylose, DNA, and synthetic peptides.[8, 12-17] The development of the hybrid material with biological and physical properties together, has gained much attention due to the potential in optoelectronic and biological applications. Considerable interest has been shown for the synthesis of CPs and their ability to form varieties of the supramolecular chiral complex in aqueous solution.

In chapter II, we have shown that depending upon the backbone structure of CPs, they can selectively form an ordered helix with one of the members of the glycosaminoglycans (GAGs) class of polysaccharides, i.e., hyaluronic acid (HA). We also found that out of four different backbone structures, poly(phenyleneethynylene) (**PPE**) and poly(phenylenebutadiynylene) (**PPB**) with non-conjugated linker (**L**) along the backbone were able to selectively form an ordered helical complex with HA. The backbone of **PPB-L** differs from the **PPE** in two ways: firstly, the phenyleneethynylene (PE)-conjugated bridge is substituted with the longer phenylenebutadiynylene (PB) bridge, and secondly, the polymer contains a non-conjugated aliphatic linker in small proportions (~10%). Both **PPE** and **PPB-L** form a highly ordered helical complex upon complexation with HA, evident by the appearance of a strong red-shifted absorption maximum, changes in the emission intensity and spectral shapes, and strong induced circular dichroism (ICD) peaks at the absorption maxima of CP/HA complexes. In chapter II of the dissertation we have shown that characteristic the ICD effect with opposite signal indicates that preferential handedness of the helix depends on the template effect caused by the chiral HA. We have previously shown that the self-assembly behavior of CPs that differ by the side chain and backbone structure can dramatically change the absorption spectra, emission spectra, and complex size/size distributions in response to three other classes, i.e., chondroitin sulfate (CS), dermatan sulfate (DS), and heparin sulfate (HS).[18] Systematic linear discriminant analysis (LDA) investigation of the fluorescence intensity of CPs with GAGs provided differential interactions of CPs to GAGs in the complex biological medium (synthetic urine), where LDA can be used to establish a pattern for a “chemical nose” type array sensor.[18] GAGs are unbranched chain polymers containing an *N*-acetylglycosamine and uronic acid, and they are covalently attached to cell membrane proteins such as syndecan and glypican forming HS proteoglycan (HSPG) and CS proteoglycan (CSPS).[19] The presence of GAGs in the cell membrane hugely impacts the endocytosis mechanism during the cellular uptake, because most of the delivery vectors, including

CPs, use the electrostatic interaction of vectors with cell surface receptors, such as HS and CS, for delivery of cargo into the cells.[20] It has been reported that the secondary structure of arginine in cell penetrating peptides (CPP) upon binding with HS, also dictates the cellular penetration efficiency. The arginine rich peptides or polymers capable of forming α -helical conformation upon binding with HS, enhance the cell membrane penetration ability.[21, 22] Understanding of *in vitro* photophysical property changes upon complexation of CPs with GAGs can explain how CPs will interact with GAGs present in the cell membrane, while using CPs as a delivery material.

On the basis of these observations and reported literature on possible helix formation from CPs and polysaccharides, we hypothesized that the self-assembly of CPs with other members of GAGs can also form ordered helical conformations, depending on the differences in ionic strength of the GAGs. The ionic strength of GAGs depends on the degree of acetylation and sulfonation in the repeating disaccharides units. For this reason, it is interesting to see how photophysical properties of **PPE** and **PPB-L** change upon complexation with three sulfated GAGs (i.e., CS, DS, and HS). Here, we report a comparative study of the self-assembly of **PPE** and **PPB-L** upon complexation with four GAGs in terms of their photophysical properties and ICD. In this report, we also present that self-assembly of two achiral **PPE** and **PPB-L** leads to the formation of chiral aggregates caused by the chiral template effect of the GAGs, which was confirmed by the appearance of conformational chirality in ICD spectroscopy. The ionic strength of GAGs, backbone structure of CPs, and temperature can influence the induced chirality of the ordered complex. The strong ICD signals for three GAGs (i.e., DS, CS, HA) with both CPs, indicate preferential binding modes for helix formation leading to ordered helical complexes. In this report, we have explored **PPE** and **PPB-L** as emerging candidate CPs for developing optically active helical complexes upon complexation with GAGs.

3.3 Results and discussion

Figure 3.1. Chemical structures of cationic CPs: **PPE** and **PPB-L**.

3.3.1 Polymer Synthesis and characterization

Both **PPE** and **PPB-L** contain aromatic phenylene units with pendant tert-butyloxycarbonyl (Boc) protected amine side chains. The Boc groups are deprotected by trifluoroacetic acid using microwave irradiation, to synthesize positively charged amine groups in the side chains of the CPs (Figure 3.1). The detailed synthesis and characterization of both polymers before and after deprotection are described in the chapter II of the dissertation. Both CP stock solutions were prepared in dimethyl sulfoxide (DMSO) and further diluted in water for the photophysical properties and circular dichroism (CD) studies. The strong absorption maximum for **PPE** was observed at 420 nm and the emission maximum at 462 nm, whereas **PPB-L** shows an absorption maximum at 440 nm and an emission maximum at 470 nm in water. The hydrodynamic diameters of CPs and CPs/GAGs complexes were measured using the nanoparticle tracking analysis (NTA). Both **PPE** and **PPB-L** upon complexation with GAGs showed distinct particles size differences. After the complexation of CPs with GAGs resulted in the formation of small aggregates or microcrystallites due to increased interchain interaction, which is confirmed by the results of the NTA observations (Table 1). Upon the complexation with GAGs, it clearly shows the hydrodynamic diameter of complexes is much smaller compared to CPs, indicating formation of compact complex.

Table 3.1. Comparison of average physical and photophysical properties of CPs

CPs	Mn (g/mol) ^a	Quantum yield (%) ^b					Hydrodynamic Diameter (nm) ^c				
		CPs	CPs/ HS	CPs/ DS	CPs/ CS	CPs/ HA	CPs	CPs/ HS	CPs/ DS	CPs/ CS	CPs/ HA
PPB-L	20800	4.3	1.1	2.1	1.5	2.6	154	118	133	140	140
PPE	14300	4.0	0.8	2.1	1.3	5.7	143	99	86	87	129

^aNumber average molecular weight. Determined by gel permeation chromatography in THF.

^bQuantum yield (QY) in H₂O measured relative to the diphenylanthracene standard.

^cHydrodynamic Diameter (HD) measured by NTA at 25°C in water.

3.3.2 Absorption and emission spectroscopy

Glycosaminoglycans are negatively charged flexible linear bio-polysaccharides with sulfate and carboxylate groups. The ionic strength of different GAGs varies due to differences in degrees of acetylation and sulfonation in the repeating disaccharides units. Along with the hydrophobic backbone of GAGs, the anionic charges originating from the carboxylate and sulfate groups are expected to play an important role in the self-assembly when interacting with CPs. The charge interaction of charged functional groups could significantly influence the preferred conformation of GAGs.[23] In HA, the carboxylate is the only ionic interacting site, but in CS, DS, and HS both carboxylate and sulfate groups are responsible ionic sites for ionic interaction with CPs.[24] The self-assembly behavior of CPs is heavily dependent on π - π interactions of the polymer backbone, and planarization of the backbone. The strength of electrostatic interactions between the negative charge of GAGs and the positive charge of CPs side chains is also expected to contribute to the self-assembly behavior.

Self-assembly behavior study of PPB-L with GAGs by absorption spectroscopy: Figure 3.2 compares the absorption spectra of **PPB-L** upon complexation with four GAGs. The absorption maximum at 440 nm is attributed to a random spaghetti like conformation of **PPB-L**. The absorption maximum of the complex is red shifted from 440 to 470 nm upon complexation with

GAGs. In the case of HA (Figure 3.2d) the red shifted broad peak was observed with a shoulder and a distinct peak at the longer wavelength, this effect is less pronounced in the case of HS, CS and DS. The distinct shift is associated with increased π - π interactions of the polymer backbone of **PPB-L**, demonstrating that the interaction between **PPB-L** and GAGs is forcing the **PPB-L** backbone to adopt a more aggregated π - π stacked conformation.

Figure 3.2. The absorption spectra of **PPB-L** upon complexation with GAGs: (a) HS, (b) DS, (c) CS, and (d) HA.

Self-assembly behavior study of PPE with GAGs by absorption spectroscopy: Complexation of **PPE** with GAGs also exhibited distinct changes in absorption spectra (Figure 3.3). The absorption maximum of **PPE** itself is observed at 420 nm, indicating a random coiled like conformation. The absorption maximum is shifted to 470 nm upon addition of GAGs into the **PPE** solution. In the case of HA (Figure 3.3d) the red shifted broad peak was observed with a shoulder and a distinct peak at the longer wavelength, this effect is not prominent in the case of HS, CS and

DS. This finding can be explained by either an increased planarization along the conjugated backbone, or increased interpolymer π - π stacking.[25]

Figure 3.3. The absorption spectra of **PPE** upon complexation with GAGs: (a) HS, (b) DS, (c) CS, and (d) HA.

Self-assembly behavior study of PPB-L with GAGs by emission spectroscopy: Emission spectra of CPs can provide more insight into conformational transition changes and interchain interactions of CPs backbones. The strong π - π interaction of the **PPB-L** backbone caused by GAGs can be further supported by the emission spectra of the complex. The fluorescence intensity of **PPB-L** is strongly quenched upon complexation with four GAGs (Figure 3.4). In the case of highly sulfated HS, the quantum yield of **PPB-L** is quenched by 75 %, while in the case of unsulfated HA, the quantum yield of **PPB-L** is only quenched by 50 %. These data clearly indicate that strong

electrostatic interactions between sulfate and amine groups highly influence the complex formation process, leading to different conformational structure changes.

Figure 3.4. The emission spectra of **PPB-L** upon complexation with GAGs: (a) HS, (b) DS, (c) CS, and (d) HA.

Self-assembly behavior study of PPE with GAGs by emission spectroscopy: The emission spectra of **PPE** upon complexation with sulfated vs. unsulfated GAGs showed completely different trends. The quantum yield of **PPE** is quenched upon the complexation with all three sulfated GAGs. On the other hand, the quantum yield of the complex is increased significantly upon complexation with unsulfated HA (Figure 3.5). The quantum yield of **PPE** is quenched by 80 % in the case of highly sulfated GAGs (i.e., HS), whereas the quantum yield of **PPE** is quenched by ~50 to 60 % in the case of less-sulfated GAGs (i.e., DS and CS). It is understandable that quantum yield of **PPE**

polymer is quenched upon the complexation of sulfated GAGs because of increased charge density forcing the polymeric backbone to be more π - π stacked. This increased charge density of GAGs is the driving force for the π - π stacking so that interpolymer backbone interactions are a dominant factor causing the reduced quantum yield of the complex. But in the case of complexation with unsulfated GAGs (i.e., HA), the fluorescence intensity is increased to nearly double with a red shifted emission maximum (Figure 3.5d). This result indicates that the **PPE** backbones become more planar, more isolated, solvated, and the insulated molecular wire may be formed during the complexation with HA.[12]

Figure 3.5. The emission spectra of **PPE** upon complexation with GAGs: (a) HS, (b) DS, (c) CS, and (d) HA.

3.3.3 ICD Spectroscopy

Self-assembly behavior study of helical complexes by ICD spectroscopy: To understand the helical complex formation upon complexation with GAGs, circular dichroism of the complexes was measured. Figures 3.6 and 3.7 compare the CD spectra of **PPB-L** and **PPE** before and after complexation with four GAGs. CPs have no chiral center; thus, they are intrinsically optically inactive, and no CD pattern in the π - π^* transition region can be detected. Interestingly, the introduction of GAGs into an aqueous CPs solution resulted in the dramatic difference in ICD spectra. **PPE** and **PPB-L** form selective helical conformation when complexed with three GAGs (i.e., DS, CS, and HA) whereas HS is unable to show ICD effect. It is clear that all GAGs are able to induce the change in absorption maximum of CPs to longer wavelength, whereas only DS, CS, HA are able to show ICD effect. The bisignate cotton effect observed for DS and CS is consistent for the both **PPE** and **PPB-L** indicating that all of them follow same handedness in the ordered complex. But in case of HA the cotton effect observed is in opposite trend, conforming the opposite handedness in the ordered complex. These results indicate that a conformational change and a strong intermolecular stacking are induced upon electrostatic interaction with DS, CS, and HA, whereas no chiral helical conformation is formed from HS and CPs complexation. The inability to form helical complex from CPs and HS complex could be explained by strong interchain interactions between the sulfate group of HS and the amine group of CPs. The ICD effect observed for the DS, CS, and HA suggests that the anionic carboxylic acid in polysaccharides is indispensable for the formation of π -stacked interaction between CPs and GAGs. In the case of highly sulfated HS, the electrostatic interactions between sulfate and amine are very strong; these interactions couldn't keep the helical conformation from the polysaccharides template, hence collapsing the helical conformation and leading to the formation of an aggregated mass of CPs/HS complexes, without any ordered structure. The helical conformation has different helical orientation, depending upon the types of GAGs we used, and the backbone structure of CPs.

Figure 3.6. The circular dichroism spectra of **PPB-L** upon complexation with GAGs: (a) HS, (b) DS, (c) CS, and (d) HA.

Figure 3.7. The circular dichroism spectra of **PPE** upon complexation with GAGs: (a) HS, (b) DS, (c) CS, and (d) HA.

Temperature dependent absorption and CD spectroscopy of chiral helical complexes:

To further elucidate the effect of thermal energy on the formation of helical complexes and their stability, temperature dependent absorption and CD spectra of CPs/GAGs complexes were recorded. As we reported in Chapter II, the ICD effect of both **PPE** and **PPB-L**/HA complexes is decreased with increased temperature, which demonstrates that increasing thermal energy has a direct effect on the stability of the complexes, causing a disturbance in the electrostatic attraction of the CPs/HA complex. The disturbance ultimately caused a structural fluctuation in the complex leading it to disassemble into individual chains.[26, 27] The nominal ICD intensity is regained after cooling the heated complex of PPE/HA complex where as significant ICD intensity is regain after cooling the heated complex of PPB-L/HA complex (Supporting information Figures S3.9 and S3.10). On the other hand, the ICD intensity of the complexes formed by the sulfated GAGs increased at a higher temperature. In the case of **PPB-L** increasing temperature increased the ICD intensity (Figure 3.8) reaching the maximum at a certain temperature ($\sim 70^\circ\text{C}$); a further increase in temperature caused decreased ICD intensity (Figure 3.10). This result clearly indicates that the induced chirality in the CPs/GAGs complexes is thermodynamically controlled, and the fluctuation in ICD effect shows that the formation of complexes could be more ordered in case of sulfated GAGs, and disorder in case of unsulfated HA.

An interesting trend is observed in ICD intensity for **PPE** and sulfated GAGs complexation upon increasing temperature. As temperature increased, the ICD intensity increased for all three sulfated GAGs (Figure 3.9). In case of **PPE**, the trend continued even after cooling the heated sample (70°C) (Figure 3.10). These results clearly indicate that increasing temperature stabilizes the complex and structural conformation, which is caused by stronger electrostatic interactions between the sulfate groups of DS, and CS, and the amine group of CPs, making them stable a conformation. Hyaluronic acid is the only GAGs where increasing temperature decreased the CD effect, which is

caused by the decrease in the integrity of the complex due to poor electrostatic interaction leading to the separation of the polymer chain and GAGs chain

Figure 3.8. Variable-Temperature Circular Dichroism (VT-CD) of the **PPB-L**/GAGs complex in a 1:1 Molar ratio for (a) HS, (b) DS, (c) CS, and (d) HA.

Figure 3.9. Variable-Temperature Circular Dichroism (VT-CD) of the **PPE/GAGs** complex in a 1:1 Molar ratio for (a) HS, (b) DS, (c) CS, and (d) HA.

Figure 3.10. Relative ICD intensity changes of 1:1 Molar ratio of **PPE/GAGs** (left) and **PPB-L/GAGs** (right) as a function of temperature. Data for PPB-L/HS complex was not included since significant CD effect wasn't observed for the complex.

3.4 Conclusion

In summary, we have shown that the self-assembly between CPs and GAGs in aqueous solution highly depends on the charge density of GAGs, and that it can highly affect the electrostatic interaction of complexes and π - π interactions. We have shown that out of four GAGs, three GAGs can form helical complexes, and that the stability of the complex is highly dependent on the charge density of the GAGs and temperature. The present paper has provided a simple way of preparing highly ordered supramolecular complexes of CPs by simple mixing. It has clearly shown that attractive interactions between GAGs and CPs can allow a fine tuning of the overall conformation of CPs/GAGs complexes. The temperature dependence of the ICD effect indicates that the conformation of ordered helical CPs/GAGs complexes is thermodynamically controlled; therefore, depending upon the association and dissociation of the complex, the ICD effect could increase or decrease, which is dictated by the strength of the electrostatic interaction. All these results shed some light on the structure-property relationships in CPs and contributes to the rational design of novel materials with optimized electrical, optical, and photophysical properties.

3.5 Out look

The chapter presented a strategy for creating ordered helical complex using ionic complexation approach. Depending on the charge density and type GAGs are able to induce helical conformation in PPE and PPB-L, it would be interesting to see if similar characteristics ICD effect will be observed for PPE and PPB-L with the other class of polysaccharides like amylose, CMC, CMA and SPG. Since these polysaccharides are also reported in literature for their ability to induce helical conformation with other class of CPs. Also, it would be interesting to understand the subcellular localization and cellular entry mechanism of these ordered helical CPs/GAGs complex because it has been reported that some of the cellular internalization and entry mechanism required formation of helical conformation between GAGs present in the cell membrane and delivery vector during

the cellular internalization process. A new avenue of cellular interaction with ordered helical complex would provide better understanding of designing CPs as a delivery vector in future.

3.6 Experimental

3.6.1 General information: Chemicals, including solvents, were purchased from Fisher Scientific, and used as received. Sodium hyaluronate (HA) was purchased from Lifecore (MW 40 KDa) and used as received. Heparin sodium was purchased from Acros Organics and used as received. Chondroitin sulfate A (CS) and chondroitin sulfate B (dermatan sulfate, DS) were purchased from Sigma Aldrich and used as received. Deuterated solvents were purchased from Cambridge Isotope Laboratories (Cambridge, MA). A microwave reactor (Monowave Edu, Anton Paar) was used for synthesis of PPEs using a silicon carbide vessel. Deprotection of all Boc-protected polymers was conducted in a glass vessel under the microwave. The number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index ($PDI = M_w/M_n$) of CPs were determined by gel permeation chromatography (GPC) against polystyrene standards using a Shimadzu high performance liquid chromatography (HPLC) system fitted with PLgel 5 μ m MIXED-D columns and SPD-20A ultraviolet-visible (UV-vis) detector at a flow rate of 1.0 mL/min. Samples for GPC, small amount (~100 μ L) of polymer in DMF was diluted with 1 mL of HPLC grade THF and then filtered through a 0.45 μ m polytetrafluoroethylene (PTFE) syringe filter prior for injection. The UV-vis spectra were recorded using a Varian Cary 50 Bio spectrophotometer. Fluorescence spectra were obtained using a FluoroLog-3 Spectrofluorometer (Jobin Yvon/Horiba). 9,10-diphenylanthracene ($QY = 0.9$) in cyclohexane was used as a fluorescence standard. Circular dichroism (CD) were obtained in a Jasco J-815 spectrometer equipped with a Peltier cell holder Jasco CDF-426S by using a glass cuvette with a path length of 1 cm. Fourier transform infrared (FTIR) spectra were recorded on a PerkinElmer Spectrum 100 FTIR Spectrometer. Fine polymer powders were directly mounted on an attenuated total reflection

(ATR) cell of the spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz Avance Bruker NMR spectrometer. Chemical shifts were reported in parts per million (ppm) for ^1H NMR on the δ scale based on the middle peak ($\delta = 2.50$ ppm) of the dimethylsulfoxide (DMSO- d_6) solvent as an internal standard. Nanoparticle tracking analysis (NTA) measurements were performed using a LM10 HS (NanoSight Amesbury, United Kingdom) equipped with a sCMOS camera, a sample chamber, and a 488 nm blue laser and Viton fluoroelastomer o-ring. The samples were prepared in similar manner for absorption and emission measurements using water (18 Ω) filtered through 0.45 μm PTFE syringe filters. The samples were injected into the sample chamber with 1 mL sterile syringes (Restek Corporation, Pennsylvania, USA) until the liquid reached the tip of the nozzle. All measurements were performed at 25°C using a LM14C temperature controller (NanoSight, Amesbury, United Kingdom). Each sample was measured three times.

3.6.2 GAGs Structure

Figure S3.1. Chemical structure of GAGs.

3.6.3 Monomer Synthesis: As mentioned in chapter II the Monomers M1, M2 and M3 were synthesized according to literature procedures.^{19, 25}

Figure S3.2. Chemical structures of Monomers M1, M2, and M3.

3.6.4 Polymer synthesis: The detail synthesis and characterization of **PPE** and **PPB-L** has been reported in previous chapter II of the dissertation.

3.6.5 Complexation study of CPs and GAGs by absorption and emission spectroscopy: A stock solution of HA was prepared by dissolving 5.09 mg of HA in 5 mL of deionized water (Concentration 2680 μ M, MW 379.45 g/mol). A master stock solution was prepared by dissolving 5.5 mg of HS in 4 mL of deionized water (Concentration 2067.6 μ M, MW 665 g/mol). A master stock solution of CS was prepared by dissolving 2.2 mg in 2 mL of deionized water (Concentration = 1847 μ M, MW 568.45 g/mol). A master stock solution of DS was prepared by dissolving 2.12 mg in 2 mL of deionized water (Concentration 2229 μ M, MW 475.38 g/mol). Spectroscopic samples were prepared by adding 10 μ L of the corresponding CPs in DMSO (2.0 mM) to 490 μ L of DI water, which was further diluted by mixing 100 μ L of the CPs in water with 300 μ L of DI water. For CPs/HA complexation studies, 100 μ L of aqueous CPs solutions were mixed with 270 μ L, 230 μ L, and 110 μ L of DI water, respectively, and then 20 μ L, 60 μ L, 180 μ L of GAGs solution (200 μ M) was added, respectively, and mixed by gentle pipetting to have the final GAGs concentration of 10, 30, and 90 μ M, respectively. The total volume of DMSO (0.5%, v/v) was kept constant in all the samples.

Figure S3.3. Absorption spectra of **PPB-L** upon complexation with different concentrations of (a) HS (b) DS (c) CS, and (d) HA.

Figure S3.4. Emission spectra of **PPB-L** upon complexation with different concentrations of (a) HS (b) DS (c) CS and (d) HA.

Figure S3.5. Absorption spectra of **PPE** upon complexation with different concentrations of (a) HS (b) DS (c) CS and (d) HA.

Figure S3.6. Emission spectra of **PPB-L** upon complexation with different concentrations of (a) HS (b) DS (c) CS and (d) HA.

Figure S3.7. Variable-temperature absorption spectra of **PPB-L**/ GAGs complex in a 1:1 Molar ratio for (a) HS, (b) DS, (c) CS and (d) HA.

Figure S3.8. Variable-temperature absorption spectra of **PPE** / GAGs complex in a 1:1 Molar ratio for (a) HS, (b) DS, (c) CS and (d) HA.

3.6.6 ICD spectroscopy: Samples for CD measurements were prepared by adding 10 μL of the corresponding CPs in DMSO (20 mM) to 1990 μL of DI water. In order to see how the GAGs complexation induces CD, samples were prepared by mixing 10 μL of CPs in DMSO (20 mM) with 1890 μL of DI water followed by addition of 100 μL GAGs (2000 μM), resulting 1:1 molar ratio of CPs and GAGs concentration. The resulting solutions were mixed by gentle pipetting and the CD spectra were recorded immediately. The total volume of DMSO (0.5%, v/v) was kept constant in all samples. All measurements were performed at 20 $^{\circ}\text{C}$ and three scans were accumulated within the scan range of 550-350 nm at the scan rate of 50 nm/s. For variable temperature CD (VT-CD) experiment, the samples were heated from 20 $^{\circ}\text{C}$ to 70 $^{\circ}\text{C}$ using Jasco CDF-426S Peltier thermostated cell holder attached to CD spectrometer.

Figure S3.9. Variable Temperature circular dichroism (VT-CD) spectra (cooling cycle) of (a) **PPB-L/DS** (b) **PPB-L/CS**, and (c) **PPB-L/HA** complex in a 1:1 molar ratio. Cooling cycle for **PPB-L/HS** complex was not performed because of their inability to cause ICD effect.

Figure S3.10. VT-CD spectra (cooling cycle) of (a) **PPE/HS** (b) **PPE/DS** (c) **PPE/CS**, and (d) **PPE/HA** complex in a 1:1 molar ratio.

3.6.7 Determination of hydrodynamics diameters of CPs and CPs/GAGs complexes

Figure S3.11. NTA data for PPB-L (a) **PPB-L/HS**, (b) **PPB-L/DS**, (c) **PPB-L/CS**, and (d) **PPB-L/HA** complex in aqueous solution.

Figure S3.12. NTA data for a) **PPE/HS**, b) **PPE/DS**, c) **PPE/CS**, and **PPE/HA** complex in aqueous solution.

3.7 References

- [1] X. Feng, F. Lv, L. Liu, H. Tang, C. Xing, Q. Yang, S. Wang, Conjugated polymer nanoparticles for drug delivery and imaging, *ACS Appl. Mater. Interfaces* 2(8) (2010) 2429-35.
- [2] X. Feng, F. Lv, L. Liu, Q. Yang, S. Wang, G.C. Bazan, A highly emissive conjugated polyelectrolyte vector for gene delivery and transfection, *Adv. Mater.* 24(40) (2012) 5428-32.
- [3] K. Lee, J. Lee, E.J. Jeong, A. Kronk, K.S. Elenitoba-Johnson, M.S. Lim, J. Kim, Conjugated polyelectrolyte-antibody hybrid materials for highly fluorescent live cell-imaging, *Adv. Mater.* 24(18) (2012) 2479-84.
- [4] J.H. Moon, E. Mendez, Y. Kim, A. Kaur, Conjugated polymer nanoparticles for small interfering RNA delivery, *Chem. Commun.* 47(29) (2011) 8370-2.
- [5] D.T. McQuade, A.E. Pullen, T.M. Swager, Conjugated polymer-based chemical sensors, *Chem. Rev.* 100(7) (2000) 2537-2574.

- [6] Y. Liu, K. Ogawa, K.S. Schanze, Conjugated polyelectrolytes as fluorescent sensors, *J. Photochem. Photobiol. C* 10(4) (2009) 173-190.
- [7] K.P. Nilsson, J. Rydberg, L. Baltzer, O. Inganäs, Twisting macromolecular chains: self-assembly of a chiral supermolecule from nonchiral polythiophene polyanions and random-coil synthetic peptides, *Proc. Natl. Acad. Sci. U.S.A.* 101(31) (2004) 11197-202.
- [8] J. Rubio-Magnieto, E.G. Azene, J. Knoops, S. Knippenberg, C. Delcourt, A. Thomas, S. Richeter, A. Mehdi, P. Dubois, R. Lazzaroni, D. Beljonne, S. Clement, M. Surin, Self-assembly and hybridization mechanisms of DNA with cationic polythiophene, *Soft Matter* 11(32) (2015) 6460-71.
- [9] A.P. Schenning, E.W. Meijer, Supramolecular electronics; nanowires from self-assembled pi-conjugated systems, *Chem. Commun.* (26) (2005) 3245-58.
- [10] E. Yashima, K. Maeda, H. Iida, Y. Furusho, K. Nagai, Helical Polymers: Synthesis, Structures, and Functions, *Chem. Rev.* 109(11) (2009) 6102-6211.
- [11] E. Yashima, N. Ousaka, D. Taura, K. Shimomura, T. Ikai, K. Maeda, Supramolecular Helical Systems: Helical Assemblies of Small Molecules, Foldamers, and Polymers with Chiral Amplification and Their Functions, *Chem. Rev.* 116(22) (2016) 13752-13990.
- [12] C. Li, M. Numata, A.H. Bae, K. Sakurai, S. Shinkai, Self-assembly of supramolecular chiral insulated molecular wire, *J. Am. Chem. Soc.* 127(13) (2005) 4548-9.
- [13] K. Sakurai, K. Uezu, M. Numata, T. Hasegawa, C. Li, K. Kaneko, S. Shinkai, Beta-1,3-glucan polysaccharides as novel one-dimensional hosts for DNA/RNA, conjugated polymers and nanoparticles, *Chem. Commun.* (35) (2005) 4383-98.
- [14] K.P.R. Nilsson, J. Rydberg, L. Baltzer, O. Inganäs, Twisting macromolecular chains: Self-assembly of a chiral supermolecule from nonchiral polythiophene polyanions and random-coil synthetic peptides, *Proc. Natl. Acad. Sci. U.S.A.* 101(31) (2004) 11197.
- [15] M.J. Frampton, T.D. Claridge, G. Latini, S. Brovelli, F. Cacialli, H.L. Anderson, Amylose-wrapped luminescent conjugated polymers, *Chem. Commun.* (24) (2008) 2797-9.
- [16] Y. Tang, E.H. Hill, Z. Zhou, D.G. Evans, K.S. Schanze, D.G. Whitten, Synthesis, self-assembly, and photophysical properties of cationic oligo(p-phenyleneethynylene)s, *Langmuir* 27(8) (2011) 4945-55.
- [17] M. Numata, S. Shinkai, 'Supramolecular wrapping chemistry' by helix-forming polysaccharides: a powerful strategy for generating diverse polymeric nano-architectures, *Chem. Commun.* 47(7) (2011) 1961-75.
- [18] M. Twomey, T. Vokata, M.R. Kumar, J.H. Moon, Differential interactions of conjugated polymer nanoparticles with glycosaminoglycans in synthetic urine, *Chem. Commun. (Cambridge, England)* 51(19) (2015) 4065-8.

- [19] A. Kim, T.H. Shin, S.M. Shin, C.D. Pham, D.K. Choi, M.H. Kwon, Y.S. Kim, Cellular internalization mechanism and intracellular trafficking of filamentous M13 phages displaying a cell-penetrating transbody and TAT peptide, *PLoS One* 7(12) (2012) e51813.
- [20] F. Zhao, Y. Zhao, Y. Liu, X. Chang, C. Chen, Y. Zhao, Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials, *Small* 7(10) (2011) 1322-37.
- [21] Y. Takechi-Haraya, R. Nadai, H. Kimura, K. Nishitsuji, K. Uchimura, K. Sakai-Kato, K. Kawakami, A. Shigenaga, T. Kawakami, A. Otaka, H. Hojo, N. Sakashita, H. Saito, Enthalpy-driven interactions with sulfated glycosaminoglycans promote cell membrane penetration of arginine peptides, *Biochim. Biophys. Acta* 1858(6) (2016) 1339-1349.
- [22] Y. Takechi, H. Yoshii, M. Tanaka, T. Kawakami, S. Aimoto, H. Saito, Physicochemical mechanism for the enhanced ability of lipid membrane penetration of polyarginine, *Langmuir* 27(11) (2011) 7099-107.
- [23] M. Zsiška, B. Meyer, Influence of sulfate and carboxylate groups on the conformation of chondroitin sulfate related disaccharides, *Carbohydr. Res.* 243(2) (1993) 225-258
- [24] H. Sahoo, P. Schuille, Influence of glycosaminoglycans on lipid dynamics in supported phospholipid bilayers, *Soft Matter* 9(14) (2013).
- [25] M. Numata, T. Fujisawa, C. Li, S. Haraguchi, M. Ikeda, K. Sakurai, S. Shinkai, β -1,3-Glucan (Schizophyllan) Can Act as a One-Dimensional Host for Creating Chirally Twisted Poly(p-phenylene Ethynylene), *Supramol. Chem.* 19(1-2) (2007) 107-113.
- [26] T. Shiraki, A. Dawn, Y. Tsuchiya, S. Shinkai, Thermo- and solvent-responsive polymer complex created from supramolecular complexation between a helix-forming polysaccharide and a cationic polythiophene, *J. Am. Chem. Soc.* 132(39) (2010) 13928-35.
- [27] S. Haraguchi, Y. Tsuchiya, T. Shiraki, K. Sugikawa, K. Sada, S. Shinkai, On the helical motif of the complexes created by association of helix-forming schizophyllan (SPG) and helix-forming polythiophene derivatives, *Chemistry* 15(42) (2009) 11221-8.

Chapter IV

Extracellular potential mapping of live cells interacting positively charged conjugated polymers reveals formation of transient pores on the membrane

Manuscript submitted and under review

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4.1 Graphical abstract

Figure 4.1. Graphical representation SICM set up and surface potential change image on cell membrane upon the interaction with G-CPNs.

4.2 Abstract

In-depth understanding of the cell membrane at the nano-bio interface is highly challenging but fundamentally important for basic cellular physiology and designing smart functional materials. Here, we used a novel scanning ion conductance microscopy (SICM)-based topography and electric potential imaging method to study the live cell membrane interacting with positively charged conjugated polymers (CPs). Two structurally similar CPs, which have different functional groups (i.e., primary amine versus guanidine) at the end of the side chain, were used to investigate the functional group dependent cellular interaction and entry. High spatial resolution topography and extracellular potential images of living cells incubated with CPs for relatively short time (e.g., ~1 h) reveal the induction of dramatic membrane topographic changes and transient pores in the membrane. Surprisingly the pores were not observed at longer incubation time (e.g., ~6 h) despite

the presence of abundant CPs for prolonged interactions with the membranes, and the time needed for maximum pore formation was dependent on the functional group. Non-endocytic entry pathways including translocation via pores are suggested for CPs at a relatively short incubation time, while conventional endocytosis entries are predominant with longer incubation time. This study provides an important observation on how the live cells interact with the charged hydrophobic polymeric materials, suggesting that the pore formation may be one of initial responses of the live cell membrane interacting/interfacing with extracellular materials. The observations and structure-function relationship will significantly contribute to designing smart functional materials for efficient intracellular delivery of sensors or therapeutic substances.

4.3 Introduction

Understanding cellular interaction of nanomaterials is pivotal for designing highly efficient functional biomaterials for cellular labeling, sensing, and delivery of biological/biomedical substances.[1, 2] The initial cellular interaction closely guides the subsequent cellular entry pathways and the following subcellular localizations of the functional materials, which ultimately determines the overall efficiency of the functional materials.[3, 4] Among many influencing factors, the surface chemical functionality and the hydrophobicity play crucial roles in the initial dynamic and static interaction of various components in biological fluids (i.e., ions, proteins, glycans, and lipids).[5-8] While various techniques have provided the chemical and physical information of the biological layer interfacing the surface of nanomaterials,[9-11] a limited knowledge is available about how the live cell membrane interacts with or responds to extracellular nanomaterials.[12, 13]

Despite the importance of the initial interaction between nanomaterials and the cell membrane, *in situ* microscopic elucidation of the membrane-interacting nanomaterials is still challenging due to lack of suitable experimental techniques. Scanning ion conductance microscopy (SICM) is a newly

emerged scanning probe microscopy technique that is highly suitable for live cell membrane imaging and analysis.[14, 15] SICM regulates the sample-tip distance by monitoring the ionic current through an electrolyte-filled glass (or quartz) pipette with a submicroscale to nanoscale opening at the orifice. This method does not require a direct physical contact between the probe and the sample surface, thus minimizing the disturbance to the imaged cell.[16, 17] The SICM can reveal static and dynamic topographical changes of the live cell membrane with nanometer-scale resolution. For example, SICM has been successfully used to study the dynamic changes of the live cell membrane during the endocytosis of virus nanoparticles.[18, 19] In recent years, there have been exciting developments to make SICM a multifunctional microscope for live cell imaging.[20] For example, the surface charge of artificial lipid bilayer membranes[21] and live PC-12 cell surface[22] was mapped by the multifunctional SICM techniques. Here, we apply a multifunctional SICM to investigate the interactions between a cell membrane and extracellular nanomaterials through mapping the surface potential changes of the live cell membrane. To achieve the aim, we develop a new potentiometric SICM method, which was modified from the method reported by Baker's group,[23] by using a high impedance potential amplifier to measure the open-circuit potential near the tip of a dual-barrel nanopipette. We have demonstrated that the potential sensing probe can detect the surface potential/charge of individual nanoparticles.[24]

Previously, we have studied how positively charged conjugated polymers (CPs) interact with live cells by monitoring the cell surface topographical changes using SICM.[25] CPs are intrinsic fluorescent materials that have been used for labeling, sensing, and delivery of biological interests owing to their excellent photophysical and biophysical properties.[26-31] By introducing flexible, hydrophilic charged side chains to the rigid hydrophobic backbone, we have demonstrated that non-aqueous soluble CPs form a nanometer sized self-assembly in an aqueous solution. This is often referred as conjugated polymer nanoparticles (CPNs), and the chemical functionality and composition of CPNs influence the interaction with the live cell membrane and the subsequent

entry pathways.[32-34] Using SICM, we have shown that the hydrophobicity of CPNs plays an important role in adsorption and induction of the cell surface topography changes, as highly charged hydrophilic polyethyleneimine (PEI) induces no detectable changes on the cell membranes.[25] Because the cell surface topography changes are associated with certain live cell activities responding to extracellular materials, detailed understanding on the relationship between CP chemical structures and the membrane properties could lead to development of the next generation of highly efficient cellular delivery materials. However, the soft natures of CPNs made it difficult to differentiate adsorbed CPs from the cell membrane structural features (*e.g.*, protrusions) by using the topographical images alone.

In this report, the detailed cellular interactions of positively charged CPNs were revealed by the new multifunctional SICM technique. Two structurally similar CPs that have different functional groups (*i.e.*, primary amine versus guanidine) at the end of the side chain were used to study the functional-group-dependent cellular interaction and entry of CPNs. SICM topographic studies indicate that substantial topographical changes in the membrane of live human cervical carcinoma (HeLa) cells incubated with the positively charged CPNs. Using the potential-sensing probe of multifunctional SICM, we further confirmed the presence of large topographic features exhibiting either positive potentials due to adsorbed/embedded CPs on the membranes or negative potentials due to the pores in the membranes. Interestingly the structural features were formed differently depending on the functional group and were observed at a relatively short interaction time with CPNs. By combining results from the fluorescence-based cellular entry studies, the direct entry of guanidine-containing CPs is favorable at the initial (~1 h) interaction, while conventional energy-dependent endocytoses become predominate when CPs are abundant for a prolonged interaction (~6 h). This study provides an important observation on how the live cells interact with the charged hydrophobic polymeric materials, and the structure-function relationship will significantly contribute to designing smart extracellular functional materials for labeling, sensing, and delivery.

4.4 Result and discussion

4.4.1 Synthesis and cellular behaviors of CPNs

Our group has paid attention to the unique structural features of CPs: rigid hydrophobic backbones and flexible hydrophilic side chains. Because of the hydrophobic aromatic backbone, which makes CPs insoluble in aqueous solutions, CPs modified with various charged side chains form nanometer-sized particles (i.e., CPNs) in an aqueous solution. By introducing positive charges at the side chains, we have demonstrated non-toxic labeling of live cells,[28, 30] small interfering RNA delivery,[29, 31] and intracellular organelle labeling.[32-34]

Scheme 4.1. Chemical structures of CPNs (i.e., **A-CPNs** and **G-CPNs**).

In this study, we designed CPs containing a primary amine and a guanidine group at the end of flexible ethylene oxide side chains of rigid hydrophobic phenyleneethynylene backbone to study the functional-group-dependent cellular interaction and entry (Scheme 4.1). Along with the effort of developing highly efficient intracellular therapeutic delivery materials, we are interested in using guanidine functional groups in the side chains of CPs. We hypothesized that guanidine-containing CPs will have better cellular entry than the primary amine containing counterpart because of the high pKa value and hydrogen-bonding (H-bonding) ability of guanidine. In arginine-rich transactivator of transcription (TAT) peptides and their synthetic mimics, the charge density from

the guanidine group, hydrophobicity, and rigidity play crucial roles in efficient translocation via multidentated ionic interaction and H-bonding followed by hydrophobic interaction/insertion in the membrane.[35-38] t-Butyloxycarbonyl (Boc)-protected amine- and guanidine-containing CPs, respectively, were successfully synthesized via the palladium-mediated cross-coupling reaction in an organic solvent. De-protection of the Boc group yielded positively charged CPs soluble in DMSO, and further dilution of the CPs in DMSO with an excess amount of water resulted in a clear homogeneous solution of the corresponding CPNs. The hydrodynamic diameter (HD) and zeta potential of the amine containing CPNs (i.e., A-CPNs) in water obtained by the dynamic light scattering method were 141 ± 5.0 nm and 38.4 ± 4.4 mV, respectively. The HD determination of guanidine containing CPNs (i.e., G-CPNs) in water was not successful even at very high concentration (e.g., > 1 mM). The positively charged guanidine containing CPs in water were less aggregated (i.e., better solvated, but not soluble in water) than the primary amine counterparts due to the high pKa value of guanidine, resulting in very weak light scattering. Water insoluble guanidine-containing CPs formed nanoparticles (229 ± 16 nm) in phosphate buffered saline (PBS) when salts screened the positive charges to induce the hydrophobic chain interaction. Cell viability assessment using MTT indicates no viability inhibitions from both CPNs up to $40 \mu\text{M}$ (Supporting Information Figure S4.12).

Figure 4.2. Simultaneously recorded topography and potential images of a living HeLa cell on the collagen treated surface. (A) An illustration of SICM setup for simultaneous topography and potential images of a cell. (B) A 3D topography image. (C) An enhanced color topography image. (D) A potential difference (ΔV) image. (E) Simultaneously recorded time traces of piezo-Z movement and potential changes near sites 1 and 2 indicated in image (D). The blue, red, and green shaded regions in Z-t trace indicate an approach, retract, and pixel moves of the probe. (F) A histogram of ΔV of the potential change image in (D).

4.4.2 SICM topography and potential imaging of the cell surface

The SICM experimental setup is illustrated in Figure 4.2A. The SICM imaging is operated in an approach-retract scan (ARS) mode. [39-40] A nanopipette repeatedly approaches and retracts from the surface at each pixel of the acquired image. To achieve topography and potential dual imaging mode, nanopipettes with dual barrels were fabricated and used. The details of the potential imaging technique setup are given in the methods and the potential sensing mechanism is discussed elsewhere.[24] We have validated the potential imaging method on various controlled samples. The potential change (ΔV) can be correlated to the surface potential and surface charge (see the below sections for detailed explanation). In general, ΔV is more negative on the negatively charged surface than that of the positively charged surface. The topography and ΔV images of a flat

polydimethylsiloxane (PDMS) surface that was sequentially modified with negatively charged bovine serum albumin (BSA) proteins and positively charged G-CPNs are showed in Figure S4.15. We have observed that the mean ΔV of the G-CPNs modified PDMS surface is relatively positive to the BSA surface (i.e., shifted to the positive direction). This control experiment also confirms that we can sense the positive charges of CPNs immobilized on a soft and negative surface in PBS buffer by the SICM-based potential imaging mode.

We then imaged the membrane surfaces of live HeLa cells. HeLa cells were cultured on a collagen hydrogel matrix-modified PDMS surface, which is transparent so that bright field optical microscope images can be used to guide the SICM experiments. The collagen matrix provides a favorable local environment for HeLa cell growth.[41] Figures 4.2B and 4.2C show a 3D topography and enhanced color topography, respectively, of the HeLa cell surface on a supporting collagen matrix. The 3D topography image reveals the large surface contour of a HeLa cell. The enhanced color topography image was obtained by using the height difference of adjacent pixels to enhance the height contrast of the topography image. While the darker color represents the larger height change, the relative height differences were colored as blue (lower surface region such as the collagen matrix) and orange (higher surface region such as the cell surface), respectively. Microvilli features on the cell surface and the porous fiber network on the collagen-supporting matrix are clearly seen in the enhanced color topography image. Therefore, the ARS mode of SICM is capable of resolving high spatial resolution topography of highly contoured living cellular surface, as well as soft and porous surface.

To illustrate the principle of ΔV imaging, time traces of Z-piezo displacement (Z), and potential (V) at two representative sites, marked as 1 (on the cell surface) and 2 (on the collagen matrix surface) in the potential image (Figure 4.2D), are presented in Figure 4.2E. Each pixel of the surface potential image shown in Figure 4.2D was obtained after completing a cycle (i.e., an approach-retract-pixel move) of the probe movement. In each cycle, the probe gradually approached ($\sim 6 \mu\text{m}$)

to the surface at a constant distance from the surface (determined by the set-point current, 1% current decrease), and then quickly retracted to the original position followed by moving laterally (~117 nm here, 78 nm if not mentioned) to the next pixel (see the black color time traces in Figure 4.2E). During the controlled probe movement, the ionic current change was recorded and used as the feedback to control the probe position near the surface. During the approach, the current was reduced to reach the set point of 1% current decrease. Meanwhile, the potential change (blue lines) is also recorded. The ΔV of each pixel point is defined as the potential difference between the closest point and the farthest point from the cell surface in each cycle. It is also obvious in the time traces that the ΔV is much smaller at site 2 than at site 1. Therefore, the ΔV is relatively more negative at the cell surface than at the collagen matrix surface. This is clearly demonstrated by the large color contrast in the ΔV image (Figure 4.2D). Furthermore, two well-separated peaks are evident in the histogram of ΔV (Figure 4.1F) based on the recorded ΔV values of all the pixels (128 x 128) in the ΔV image (Figure 4.2D). In the histogram, the average values of HeLa cell and collagen surface are -1.89 mV and -0.76 mV, respectively, supporting that the HeLa cell surface is relatively more negative than the collagen matrix surface. This result confirms that the SICM-based potential imaging method works well both on the live cell surface and on the soft and complex hydrogel surface.

To better understand the extracellular potential of live cell surface, we compared the topography and ΔV images of live HeLa cells at different temperatures (i.e., 37 °C versus 4 °C). At low temperature, the lipid bilayer of the cell membrane becomes more rigid. This also decreases the overall fluidity of the membrane, decreasing its permeability and restricting entry of small molecules such as oxygen and glucose into the cell.[42-43] While the topography images of cells at different temperatures look similar (Figure S4.16), the surface roughness parameter (i.e., surface area ratio),[25] which quantifies the relative topographical changes, becomes slightly larger (87.0 ± 3.9) at 4 °C compared to control at 37 °C (78.1 ± 4.2), suggesting that the cell membrane is

slightly roughened at the lower temperature. The averaged ΔV mean value from the potential images of three cells is slightly less negative (control: -1.74 ± 0.39 mV; low temperature: -1.61 ± 0.26 mV) after the low temperature treatment. In addition, the cell-to-cell variation of ΔV mean value or the distribution of ΔV over a single cell surface are smaller after the low temperature treatment. These are likely induced by the suppressed cell activities and rigidified cell membrane after low temperature treatment. This result also suggests that the surface potential change measured by the potential probe can be used as a promising new parameter to measure the cell surface activities and the health condition.

Figure 4.3. Pore features appeared in the cell membrane after 1 h G-CPN treatment. (A) A living control cell without G-CPN treatment. (B) A living cell after 1 h G-CPN treatment. (C) A fixed control cell without G-CPN treatment. (D) A fixed cell after 1 h G-CPN treatment. (E) Height profiles through the white lines in Figures A-D. (F) A histogram of nanocrater size measured from similar samples of (B).

4.4.3 Topographic images of HeLa cells incubated with CPNs.

Similar to our previous observation, the surface topography of live cells treated with G-CPNs for 1 h changed dramatically, while relatively smooth surface topography of control cells was observed (Figure 4.3A and B). Many nanometer-sized craters (nanocraters) were observed on the cell surface

treated with G-CPNs. The height profiles along the line drawn in the 3D topography image (line B of Figure 4.3E) further clearly indicate the presence of various sizes of nanocraters. Some of the nanocraters extend over ~micron size height from the relatively flat surface of the membrane. As shown in the size histogram (Figure 4.3F) based on analysis of 82 nanocraters, the lateral size of these nanocraters is 221 ± 50 nm. Interestingly, the large height variations of the nanocraters observed from live cells were substantially reduced after fixation using paraformaldehyde (PFA). The height profile was drastically smoothened at the fixed cells (Figure 4.3E, line D), implying that the somewhat spiky and dynamic height profile may be related to activities of live cells responding dynamically to externally introduced CPNs. By losing the cellular activities due to the membrane crosslinking, the lateral size and height profiles became substantially smoothed and shallowed. It should be noted that the live cell membrane adsorbed with CPNs were not static but highly dynamic. The nanocraters were observed to appear and disappear at the same location when the live cell membrane was scanned repeatedly (Figure S4.17).

Figure 4.4. Correlation between the topography features and the potential changes. **(A-B)** An enhanced color topography image (A) and potential difference (ΔV) image (B) of a live HeLa cell after 1 h G-CPNs treatment. **(C)** The time traces of piezo-Z displacement and potential at sites 1-4. **(D)** A proposed scheme for sites 1-4: 1) The normal cell membrane without CPN attachment; 2) The G-CPNs attached cell membrane; 3) The G-CPNs imbedded cell membrane; 4) A cross-membrane hole with increased ion flux. (D) A scheme to illustrate the possible situations at sites 1-4. For illustration purpose, the drawing is not to scale.

4.4.4 Potential change measurements at the nanocraters

To gain structural information of the nanocraters, we simultaneously measured the topography and potential changes of live cells after treating with G-CPNs for 1 h. Nanocraters are clearly visible in the enhanced color topography image (Figure 4.4A). Interestingly, the corresponding potential image (Figure 4.4B) exhibited significantly more negative mean ΔV with a wide variation (-4.64 ± 1.44 mV), while relatively small ΔV and a narrow distribution (-1.74 ± 0.39 mV) were obtained

from untreated live cells (see Figures 4.3 and S4.16). To understand why the surface potential is more negative on the G-CPNs-treated live cells, we measured the surface potential change of cells incubated with G-CPNs for 1 h and 4h, respectively, followed by PFA fixation. The mean ΔV value of the fixed cells is ~ -0.79 mV (Figure S4.18), which is significantly more positive (positive shift) than that of the live G-CPN treated cells; and even more positive than that of the live control cells (i.e., no CPN-treated live cells). The dramatic positive shift of the surface potential of the G-CPN adsorbed fixed cells can be attributed to the positively charged G-CPNs on the membrane surface. Based on the observation of fixed cells, we speculate that the more negative ΔV of the live cells treated with G-CPNs may be associated with the substantial biochemical and biophysical changes happening at the live cell membrane induced by G-CPNs. The adsorbed positively charged G-CPNs on the membranes likely induce the reorganization of membrane molecules and/or structural changes of lipid membrane, resulting in the recruitment of negatively charged molecules, transient pore formation, and the increased membrane permeability.[38, 44-46] Because the inner content of the cell and the inner surface of the cell membrane are relatively negative, these changes lead to the dramatic negative shift of the measured ΔV . Finite element methods (FEM) based numerical simulations by COMSOL software also confirmed that nanopores on an impermeable thin film with negative potential across the membrane could dramatically reduce the potential probed by the nearby nanopipette with a floating nanoelectrode (see Figure S4.19).

We further investigated the potential changes in the nanocraters by comparing four sites on the cell surface, which are marked in both the topography image (Figure 4.4A) and the ΔV image (Figure 4.4B). For the ΔV image, we set the mean ΔV value as green color marked by a line at the midpoint of the color-coded scale bar. The positive or negative shifts of the ΔV at a specific location from the mean value can be visualized as red or blue color changes in the ΔV map. To help understand the potential change, the corresponding time traces of Z displacement (gray color dash lines) and potential (blue color lines) at these sites are also shown in Figure 4.4C. The black solid lines display

the topographic height change on the cell surface. The ΔV defined by the difference between two red dashed lines reveals the spatial change of the ΔV on the cell surface. Reference site 1 is smooth in the topography image without substantial topographic changes and shows a uniform green-yellow color (corresponding to -5.77 mV) in the potential change image. This is also evident in the stable Z-t and ΔV -t time traces of site 1 in Figure 4.4C. At the small protrusion site 2, the height slightly increases (see the solid gray line in trace for the site 2), while the potential change was relatively more positive (-2.21 mV). The positive shift of potential change at site 2 can be attributed to the presence of positively charged CPs at site 2 of the membrane, as illustrated in Figure 4.4D. Adsorption of positively charged CPs on the negatively charged membrane would increase the topographic height and induce positive shift of the potential change. Sites 3 and 4 are located at the nanocraters. Interestingly, the ΔV at the site 3 was also positive shifted (-2.58 mV), while the topographic height at site 3 is lower than the neighboring membrane. Based on the relative changes of topographic height and ΔV , we assumed that the membrane was in the process of bringing in either adsorbed or membrane embedded CPNs. In contrast, site 4 exhibits a relatively large negative shift of ΔV (-10.67 mV). The significant negative shift of ΔV can be attributed to the pores formed in the membrane. Therefore, the blue spots in the ΔV image likely reveal the pore locations. These blue spots normally only appear in one to two pixels in the nanocraters, which is much smaller than the nanocrater size shown in the topography image. The actual pore size should be even smaller than 78 nm (the size of one pixel) by considering the nanopipette size and the ions diffusion. Limited by the spatial resolution, there may be one or a few pores clustered in the nanocraters. The sites 1-4 could be a sequential snap-shots of G-CPN-cellular interaction and internalization of G-CPNs, as illustrated in Figure 4.4D. Similar to the cell penetration mechanism of Arginine rich peptides,[45] the adsorption of positively charged G-CPN (2) deforms the cell membrane, leads to the formation of nanocraters (3) and eventually transient pores (4). The local surface potential becomes positive at 2 and 3 but very negative at 4.

Figure 4.5. The topography and surface potential changes of cells treated with **G-CPNs** as a function of time. **(A-B)** Topography (A), potential change (ΔV , B) images as a function of **G-CPNs** incubation time. **(C)** Confocal images of HeLa cell treated with **G-CPNs** as a function of time. **(D)** Change of the surface area ratios of HeLa cells with different **G-CPNs** treatment times. **(E)** Histograms of potential (ΔV) of HeLa cells with different **G-CPNs** treatment time. **(F)** The relative fluorescent intensity of **G-CPNs** treated HeLa cells as a function of increasing time measured by flow cytometry. The relative fluorescence values were normalized to control cell and average values are reported ($n = 3$) with standard deviations represented in error bars.

4.4.5 Changes in the cell membrane surface under different treatment times

Based on the observation, we assumed that similar cellular interactions and entry events would be maintained under a constant G-CPN concentration regardless of incubation times because the amounts of G-CPNs (i.e., 20 μM) are greater than needed to interact or cover the initial participating membrane portions and newly available membrane portions due to internalization. Fluorescent

confocal microscopy and flow cytometry experiments indicate that the total amounts of G-CPNs increased proportionally as incubation time increased (see below). In order to examine the relationship between the cell surface topography and potential changes and the incubation time, we prepared a series of cells treated with the same amount of G-CPNs for different incubation times. After incubation for a given time, cells were rinsed and placed in a fresh (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (i.e., no additional CPNs in the buffer) for SICM imaging. Figure 4.5 shows topography (A) and corresponding ΔV (B) images of live HeLa cells incubated with G-CPNs for different times. The trend of topography changes can be summarized as the time course changes of cell surface area ratios (Figure 4.5D). The time course trend in ΔV can be summarized by the ΔV histograms of each potential image at different time points as shown in Figure 4.5E. As the topography trend indicates, the cell membranes responded significantly to G-CPNs at the initial time point (1 h) by exhibiting a significant number of nanocraters. As mentioned in the previous section, the mean ΔV at this time point is evidently negatively shifted to about -5.63 mV with many highly negative ΔV points (greater than -9 mV) located inside the nanocraters. Surprisingly, as incubation time increased to 2 h, the number of nanocraters in the topography image, the surface area ratio, and the level of negative shift of ΔV (-2.65 ± 0.51 mV) all decreased, indicating that the pore formation reached the maximum around the initial incubation (i.e., ~1 h). Similar trends of topographic and potential changes were observed from live cells incubated with A-CPNs under the same experimental condition, except that the maximum topography and potential changes were observed around ~4 h incubation (Supporting Information). From the ΔV histogram in Figure 4.5E, we also noticed that the mean value of ΔV of cell surface becomes more positive (-0.88 ± 0.14 mV) than the normal live cell as the incubation time further increases to 4 h. Considering the fixed cell results as we discussed earlier (Figure S4.18), we attributed this positive potential shift to the presence of many adsorbed G-CPNs on the cell membrane that were not involved in pore formation. The ΔV image in Figure 4.5B indeed revealed

many highly positive spots uniformly distributed on the cell surface. After 6 h treatment, the ΔV (-3.31 ± 0.27 mV) and the topography of the cell become close to those of the normal live cells. At this time point, the adsorbed G-CNPs on the cell membrane surface seems much less, likely it is because internalized to the cells, which is also supported by fluorescence image in Figure 4.5C (see discussion below).

4.4.6 Fluorescence microscopy and flow cytometry studies

Confocal microscopic studies were conducted under the same treatment conditions mentioned in the previous section. Within 1 h incubation, green fluorescent G-CNPs enter live cells and locate relatively evenly throughout the cytosol along with a few green dots located around the perinuclear regions (Figure 4.5C). The existence of pores in nanocraters suggests that G-CNPs may enter the cell through the pores (i.e., non-energy dependent entry, see the mechanism discussion in the next paragraph for details). Based on the relatively uniform fluorescence intensities throughout the cell, we concluded that the amounts of membrane adsorbed G-CNPs within ~ 1 h are either similar or less than those found inside the cells, implying faster cellular entry of G-CNPs. As G-CNPs incubation time increased to 2 h, the intensity of green signals on or near membrane substantially increased along with more green dots in the cytosolic compartments. If the entry mechanism were similar between the two-time points, the internalized fluorescent signal patterns after longer incubation (2 h) would have been similar to those from 1 h incubation without having strong outer membrane staining. As incubation time further increases to 4 h or 6 h, most of G-CNPs were found intracellularly with increased amounts of G-CNPs around the perinuclear regions. Meanwhile, A-CNPs were mostly adsorbed on the membranes after 1 h incubation, and the subsequently increased cellular entry was monitored as incubation time increased (Figure S21C). It is noteworthy that the amounts of CPNs adsorbed on the membrane during the incubation time spans are unclear, as the possibility of similar amounts of membrane-adsorbed CPNs cannot be ruled out, because the fluorescence signals from the increased internal CPNs may overwhelm the signals from the

membrane. The flow cytometry experiment indicates that both types of CPNs exhibit gradual intensity increase as the incubation time increases (Figures 4.5F and S21F). The slopes of fluorescent intensity increase as a function of incubation time are very similar for both CPNs, indicating that the adsorption and/or entry kinetics of CPNs may be similar. Time course confocal microscopy images also support the gradual increase of both CPNs as incubation time increases. Overall, a certain proportion of adsorbed CPNs is involved in the dramatic changes in cell surface topography, potentials, and the membrane pores, while the time needed to induce the changes in the live cell membrane is functional group dependent.

4.4.7 Inhibition assay

In order to evaluate endocytosis pathways of CPNs, live HeLa cells were pre-incubated with pharmacological inhibitors known to block clathrin, caveolae, and macropinocytosis endocytosis, respectively, for 30 min prior to introduction of CPNs. Using flow cytometry, the relative fluorescence intensity of cells under an endocytosis inhibition condition was compared to that of control cells incubated with only CPNs. Although the inhibitors are not highly specific to the pathways, this assay can conveniently provide a clue to the cellular entry pathways of fluorescent materials.[47-48] In order to measure the fluorescence intensity of internalized CPNs by reducing the contributions from the membrane adsorbed CPNs, cells incubated with CPNs under the inhibitory conditions were post-treated with Trypan blue (TB). TB is an azo dye that quenches fluorescence of CPNs via dynamic/static interactions. Although TB may not quench all CPNs adsorbed on the membrane due to limited accessibility, the TB treatment method can provide qualitative cellular entry information based on the proportion of internalized CPNs. As shown in Figure 4.6, entry of both CPNs was not significantly inhibited under the tested inhibition conditions, except for Cytochalasin (macropinocytosis, energy dependent) and sodium azide [i.e., adenosine triphosphate (ATP) depletion condition, energy independent] treatments, implying that both CPNs may enter HeLa cell using similar energy dependent and independent pathways. When

confocal microscopic images of HeLa cells treated with CPNs for 1 h (Figures 4.5C, S21C, and S4.22), it is obvious that G-CPNs rather internalized to the cytosol with low membrane binding and A-CPNs exhibit very high membrane staining. Because the contribution of unquenched A-CPNs to the overall fluorescence intensity is relatively high, the entry mechanisms of A-CPNs studied by the TB treatment are somewhat inconclusive. Meanwhile, both flow cytometry and confocal microscopic data clearly indicate that G-CPNs use various energy-dependent (i.e., micropinocytosis and unidentified mechanisms) and energy-independent (i.e., direct entry via pores) entry mechanisms during the relatively short (~1 h) interaction time.

Figure S4.6. The relative fluorescence intensity of **G-CPNs** (left) and **A-CPNs** (right) in the presence of endocytosis pathway inhibitors such as chlorpromazine, genistein, methyl-β-cyclodextrin, LY294002, cytochalasin D, and sodium azide. Inhibitors were incubated 30 min before CPNs treatment. The relative fluorescence values were normalized to control CPNs and average values are reported (n = 3) with standard deviation represented in error bars.

4.5 Conclusion

The simultaneous monitoring of the topography and surface potential change is highly advantageous for confirming the presence of pores on highly dynamic and complicated live cell membranes. From the dramatic changes in the cell surface topography, potentials, and pore formation, we provide some evidence that CPNs are highly unique and promising biomaterials for various biological applications. It is not clear yet how the live cells precisely respond to CPNs, but the pore formation seems to be one of the initial responses of live cells exposed to extracellular amphiphilic soft materials. In addition, the peak time for pore formation is dependent on the functional groups, and the topography and potential changes in the cell membranes are not directly correlated with the increased availability of CPNs from longer incubation. Even though excess amounts of G-CPNs are always present, the early entry to cell is likely preferred through the pores only formed at a certain time point, while conventional endocytosis related entry seems to be more predominant for longer incubation time. By considering the highly dynamic and complicated cellular entry mechanism, it is again very interesting to observe that the pore formation happens only a certain time point and that the timing of pore formation is functional-group dependent.

4.6 Outlook

This chapter presented a unique strategy to understand the interaction of cell membrane with delivery vector like CPNs using novel SICM technique with capacity of potential mapping of cell surface. Similar to guanidine rich cell penetrating peptides (CPP), we have presented that the guanidine rich CPNs were also able to create transient pores like features in the cell membrane during the process of internalization. Since the writing of this chapter, a series of CPNs with guanidine motif has been synthesized (detail presented in the chapter V of this dissertation). It would be interesting to know that CPNs modified with different organic amine base with guanidine motif are able to show similar characteristics transient pore formation during the process of internalization.

4.7 Experimental

Scanning ion conductance microscopy (SICM) experiments were performed in collaboration with Dr. Jin He lab, Department of Physics (Nano Bio Research Lab). The experiments were performed and analyzed by visiting PhD student, Feng Chen from Nanjing University of Aeronautics and Astronautics. The synthesis and characterization of G-CPNs were performed by graduate student, Md Salauddin Ahmed.

4.7.1 General information

Chemicals: All chemicals and solvents were purchased and used without further purification. CPNs were synthesized as described in supporting information.[49] All other chemicals were purchased from Sigma Aldrich, unless mentioned otherwise. All solutions were prepared using deionized (DI) water ($\sim 18\text{M}\Omega$) from water purification system (Ultra Purelab system, ELGA/Siemens). Phosphate buffered saline (PBS) at pH 7.2 was prepared with the following composition in mM: NaCl 137, KCl 2.7, KH_2PO_4 1.5, and Na_2HPO_4 4.3. We diluted the 1 $\times$ PBS 15 times to get 10 mM PBS. Pharmacological endocytosis inhibitors such as chlorpromazine hydrochloride (#ALX-270-171-G005, Enzo Life Sciences, Inc.), genistein (#AC32827-1000, Acros Organics), methyl- β -cyclodextrin (#377110050, Acros Organics), LY2994002 (#70920, Caymen Chemical), and cytochalasin D (#BML-T109-0001, Enzo Life Sciences, Inc.) were purchased from Fisher Scientific.

Collagen coated PDMS substrate: For live cell imaging, Rat tail collagen I gels were coated on the surface of PDMS substrates using a procedure similar to Pelham and Wang.[50] Before use, the PDMS substrate was sequentially cleaned by ultrasonication in ethanol and deionized (DI) water for 5 min each, and then dried in Ar flow. For surface modification, the cleaned PDMS

substrate was first treated by oxygen plasma for 5 min and then immersed in 1% (3-Aminopropyl) triethoxysilane (APTES) for 1 h. After rinsing with ethanol and DI water, the APTES-modified PDMS substrate was incubated in 0.5% glutaraldehyde in 1× PBS for 1 h, then rinsed with DI water and dried in Ar flow. Collagen gel (4.88 mg/ml) was prepared by mixing purified rat collagen (6.1 mg/ml) on ice with 10× PBS, 0.1 M NaOH, and DI water at a ratio of 32:4:3:1. Collagen gel was pipetted onto the functionalized PDMS substrate and uniformly spread over the substrate with the pipette tip without inducing air bubbles. The gel coated substrate was then incubated for 3 h at 37 °C in a 5% CO₂ incubator and then was kept in 1× PBS outside.

Cell culture: HeLa cells (human cervical carcinoma) purchased from the American Tissue Culture Center (ATCC) were cultured in a complete media (mixture of Dulbecco's minimum essential medium (DMEM)/high glucose (10 mL hyClone, SH3024301) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Thermo Scientific)) at 37 °C in a 5% CO₂ incubator. Then, cells were sub cultured every 48 h.

Dual-barrel nanopipette fabrication and characterization: Dual-barrel nanopipettes were fabricated from quartz theta capillary tubes (FG-G QT120-90-7.5, Sutter Instruments, Novato, CA) using a CO₂-laser-based micropipette puller (P-2000, Sutter Instruments, Novato, CA) with the following parameters: HEAT=835, FIL=3, VEL=40, DEL=220, PUL=165. The pore opening size was obtained by I-V measurements. The diameter of each nanopore is about 170 nm (see details in supporting information).

SICM setup and imaging conditions: The details of the conventional SICM can be found elsewhere.[25] In brief, the SICM (Park Systems Inc., Santa Clara, CA) microscope was mounted on a Nikon Eclipse Ti-U inverted microscope. Approach-Retract Scanning (ARS) mode (or so-called hopping mode[39]) was used to control the nanopipette position during SICM imaging. The nanopipette barrels were filled with the same electrolyte as the bath solution. One barrel of the dual-barrel nanopipette, in which the Ag/AgCl electrode was connected to a low noise current meter

(Axon 200B, Molecular Devices), measured the distance-dependent ionic current for topography imaging and positioning of the pipette in similar fashion to a single barrel nanopipette. The second barrel of the pipette, in which the Ag/AgCl electrode was connected to a high impedance differential amplifier, measured the potential changes that occurred in the vicinity of the nanopipette apex. The maximum instrument lateral (x-y) resolution was about 80 nm, which is close to radius of the nanopipette inner pore size.[51] For SICM imaging, HeLa cells (~20,000 per well) were seeded on the collagen-modified PDMS substrate in a 40 mm cell culture petri dish with 2 ml of complete DMEM media. After 24 h of culture at 37 °C, HeLa cells on the collagen modified PDMS substrate were treated with 20 μ M of CPNs for different times. After washing with 1 $\times$ PBS for six times, the sample was imaged in HEPES buffer, which was kept at 37 °C during SICM imaging. The HEPES buffer solution contained (in mM): NaCl 145; KCl 3; CaCl₂ 2.5; MgCl₂ 1.2; HEPES 10; Glucose 10. The live cells were imaged for no more than 2 h.

CPNs incubation: Solutions with various concentrations of CPNs were prepared. For example, 20 μ L aqueous solution containing 1 mM CPNs was added into the cell culture wells to get the final concentration of 20 μ M. The CPNs were incubated with cells for different time at 37 °C and subsequently washed with PBS to remove the excess CPNs that did not interact with cells. For fixed cell experiments, the cells were fixed with 4% (w/v) PFA for 10 min and washed three times with PBS. The samples were imaged on glass coverslips, supported by a Petri dish, in an appropriate medium. The bath solution used for fixed cell imaging was PBS. For live cell experiments, the cells were cultured on collagen-modified PDMS, and the bath solution used for imaging was HEPES buffer solution.

Cell uptake studies by microscopy: HeLa cells were seeded into a 12-well plate (~20,000/well) with glass coverslip (#1254584, Fisher Scientific) one day prior to CPNs treatment and cultured in a complete media for 24 h under 5% CO₂ at 37 °C. 20 μ M CPNs were prepared in fresh complete

media (500 μ L), and added to cells after washing three times with 1 $\times$ PBS and removing media, then cultured for 1, 2, 4, and 6 h. After incubation, cells were washed three times with PBS and fixed with 4% PFA for 10 min. Cells were then washed three times with 1 $\times$ PBS and coverslips were mounted on microscope slides (#1125441, Fisher Scientific) using a 1:1 glycerol/PBS mounting medium. Fluorescent images of the cells were obtained using an Olympus Fluorview FV1200 confocal microscope (Melville, NY USA) equipped with a bandpass filter for green (513-556 nm) using 60X oil immersion lens (NA 1.35) and n = 1.519 immersion oil. Image J software (Version 1.50b, U.S. National Institute of Health, Bethesda, Maryland, USA) was used to process the image.

Cell uptake studies by flow cytometry: HeLa cells were seeded into a 12-well plate (~50,000/well) one day prior to CPNs treatment. Cells were then incubated with 20 μ M of CPNs for 1, 2, 4, and 6 h. Cells were then washed three times with 1x PBS. After that cells were detached with TrypLE Express and resuspended with 1 $\times$ PBS. After centrifugation (2000 RCF, 3 min), cells pellets were collected and fixed with 4% PFA for 10 min, then resuspended in 1 $\times$ PBS. 10,000 events per measurement were recorded within the gate of control cells, which was selected based on forward and side scattering of control cells to eliminate data collection from dead cells and artifacts. Mean fluorescence intensity of CPNs (FL1 channel, 590-620 nm wavelength range) was obtained using three independent sample sets and accounting for variation in cell autofluorescence. For uptake plots, values were normalized to control cells.

Cell uptake studies under ATP depletion condition by microscopy: Cells were treated with 0.05% of sodium azide (NaN_3) in presence of 2-deoxyglucose ($25 \times 10^{-3}\text{M}$) for 15 min before CPN treatment. 20 μ M of CPNs were treated and incubated for 1 h. After incubation, cells were washed three times with 1 $\times$ PBS and fixed with 4% PFA for 10 min. Cells were then washed three times

with 1× PBS, and coverslips were mounted on microscope slides using a 1:1 glycerol/PBS mounting medium. Fluorescent images of the cells were obtained.

Finite Element Methods (FEM) simulation: We used FEM simulation to solve coupled Poisson-Nernst-Planck (PNP) partial differential equation. To simplify the simulation, fluidic flow term was not included, and the system was assumed to be at a steady state. COMSOL Multiphysics 5.2 with AD/DC and Chemical Reaction Engineering modules were used for FEM simulation. Details of the simulation are given in supporting info.

Data collection and analysis: The time traces of piezo Z movement, current, and potential signals were collected by an oscilloscope through a signal accessing module (Park system). The data analysis was carried out by XEI (Park Systems), Gwyddion, Labview and Origin Pro (OriginLab Corp.) software. The potential change images were constructed by a home-built labview program. The 3D topography images and enhanced color images were analyzed by XEI. The surface area ratio was analyzed by Gwyddion. To avoid variations from cell to cell and batch to batch, we collected the data from at least three cells of each batch and repeated at least three batches.

4.7.2 Synthesis and characterization of CPNs.

Monomer Synthesis: Monomers M1, M2, and M3 were synthesized according to literature procedures.[52,53] Monomer M4 was synthesized as following reaction scheme S1.

Scheme S4.1: Synthesis route to Monomer M4

Synthesis of trimethylsilyl-protected intermediate. A round bottom flask (RBF) was charged with compound **M3** (2.00 g, 1.96 mmol), trimethylsilylacetylene (TMSA) (0.77 g, 7.84 mmol), bis(triphenylphosphine)palladium(II) dichloride ($\text{Pd}[\text{Cl}_2(\text{PPh}_3)_2]$) (137.6 mg, 0.20 mmol), and copper(I) iodide (CuI) (18.6 mg, 0.10 mmol). The RBF was evacuated and filled with N_2 . A solution of tetrahydrofuran (THF) and diisopropylamine (DIPA) was mixed in a 4:1 ratio (v/v) and degassed with N_2 for 10 min, and 50 mL of the solution was transferred to the RBF *via* a cannula. The reaction was stirred at room temperature for 3 h. The yellow reaction mixture was filtered and THF was distilled out under the reduced pressure. The reaction mixture was dissolved in dichloromethane (DCM) and washed with 1M ammonium chloride (NH_4Cl) two times followed by washing with a brine solution. The crude compound mixture was purified by column chromatography under 30% ethyl acetate (EA) in hexane and solid white trimethylsilyl-protected intermediate was collected and dried (1.22 g, 65% yield).

^1H NMR (400 MHz, CDCl_3) δ (ppm): 11.46 (s, 0.95H), 8.67 (s, 0.96H), 7.21 (s, 0.98H), 4.1 (t, $J = 4.4$ Hz, 2H), 3.88 (t, $J = 4.8$ Hz, 2H), 3.78 (t, $J = 4.8$ Hz, 2H), 3.66 (q, $J = 5.2$ Hz, 2H), 1.50 (s, 9.1H), 1.45 (s, 9.21H), 0.10 (s, 18.2H).

Deprotection of trimethylsilyl-protected intermediate. In a RBF, trimethylsilyl-protected intermediate (1.00 g, 1.04 mmol) and potassium carbonate (K_2CO_3) (0.36 g, 2.60 mmol) were mixed in methanol (40 mL). The mixture was stirred for 20 min in room temperature. The completion of reaction was confirmed by thin layer chromatography (TLC). The solvent was distilled by using vacuum distillation. The reaction mixture was then purified by column chromatography under 35% EA in hexane and yellowish solid powder of **M4** was collected (0.51 g, 60% yield).

^1H NMR (400 MHz, CDCl_3) δ (ppm): 11.45 (s, 1.04H), 8.67 (s, 1.00H), 6.98 (s, 1.03H), 4.15 (t, $J = 4.4$ Hz, 2.00H), 3.86 (t, $J = 4.8$ Hz, 2.02H), 3.75 (t, $J = 4.8$ Hz, 2.00H), 3.65 (q, $J = 5.2$ Hz, 2.04H), 3.35 (s, 0.89H), 1.50 (s, 9.32H), 1.45 (s, 9.14H). ^{13}C NMR (400 MHz, CDCl_3) δ (ppm): 156.4,

154.2, 153.1, 118.3, 113.8, 83.2, 83.1, 79.6, 79.4, 70.0, 69.8, 69.4, 40.9, 28.4, 28.2. FT-IR (neat): 3330.9, 3281.4, 2975.3, 2930.5, 1720.2, 1636.1, 1613.1, 1568.2, 1495.8, 1410.3, 1319.7, 1222.7, 1129.0, 1049.9 cm^{-1} .

Figure S4.1. ¹H NMR spectrum of monomer **M4** in CDCl₃.

Figure S4.2. ¹³C NMR spectrum of monomer **M4** in CDCl₃.

Polymer Synthesis:

Scheme S4.2. Synthesis route to A-CPNs

Synthesis of A-CPNs. Boc-protected primary amine containing CPs (Boc-A-CPs) was synthesized by microwave assisted Sonogashira cross-coupling reaction. A silicon carbide (SiC) microwave tube was charged with **M1** (20 mg, 0.036 mmol), **M2** (26.4 mg, 0.036 mmol), Pd[Cl₂(PPh₃)₂] (5.0 mg, 0.0072 mmol), and CuI (0.34 mg, 0.0018 mmol) and was purged with nitrogen (N₂) gas. A mixed solution of dimethylformamide (DMF) (4ml) and triethylamine (TEA) (1ml) was prepared and degassed with N₂ gas in a vial, and then 2ml of the mixed solution was transferred to the reaction tube via a cannula. The reaction tube was heated to 100 °C under microwave irradiation for 40 min. The reaction solution was cooled to room temperature, and then transferred dropwise to cold diethyl ether while stirring, resulting in precipitates. After decantation of the supernatant, the precipitates were dissolved in 1 ml of DMF. The same precipitation/decantation procedure was repeated for three times. The precipitates were then dried under high vacuum for at least 8 h in order to determine yields (30.7 mg, 80%), FTIR, and ¹H NMR characterization.

Boc deprotection. Deprotection of Boc group was carried out by heating a mixed solution of Boc-A-CPs (6.8 mg) in dimethyl sulfoxide (DMSO) (2 mL) and trifluoroacetic acid (TFA) (2 mL) to 100 °C by microwave irradiation for 30 min. The solution was cooled to room temperature and

transferred dropwise to cold EA while stirring, producing precipitates of **A-CPNs**. After decantation, the precipitates were dissolved in DMF (1ml) and the same precipitation procedure was repeated for three times. The precipitates were dried under high vacuum for at least 8 h. The deprotection of boc group was confirmed by ^1H NMR.

Boc-A-CPs: ^1H NMR (400 MHz, DMSO- D_6 , ppm): δ 7.15 (s, 0.9H), 6.71 (s, 1.1H), 4.2 (br, 2.0H), 3.78 (br, 1.9H), 3.51 (br, 1.9H), 3.09 (m, 2.1H), 1.34 (s, 9.4H). GPC: M_w 26,500 g/mol, M_n = 14,300 g/mol, PDI = 1.59. UV-Vis (DMF) λ_{max} = 435 nm, Fluo λ_{max} = 471 nm (ex = 435 nm), QY = 17%.

A-CPNs: ^1H NMR (400 MHz, DMSO- d_6 , ppm): δ 8.04 (br, 3.0H), 7.18 (s, 0.8H), 4.23 (br, 2.0H), 3.84 (br, 2.2H), 3.74 (br, 2.3H), 2.98 (s, 2.3H). FT-IR (neat): 3406, 2910, 1674, 1501, 1421, 1270, 1195, 1018, 947, 827, 796, 717 cm^{-1} . UV-Vis (H_2O) λ_{max} = 420 nm, Fluo λ_{max} = 462 nm (ex = 420 nm), QY = 4%.

Figure S4.3. ^1H NMR spectrum of **Boc-A-CPs** in DMSO- D_6 .

Figure S4.4. ^1H NMR spectrum of A-CPNs in DMSO-d_6 .

Scheme S4.3. Synthesis route to G-CPNs

Synthesis of G-CPNs. Boc-protected guanidine amine containing CPs (Boc-G-CPs) were synthesized using Sonogashira cross-coupling reaction in room temperature. A Schlenk flask was

charged with monomer **M3** (50 mg, 0.04 mmol), **M4** (40.02 mg, 0.04 mmol), PdCl₂(PPh₃)₂ (3.43 mg, 0.0048 mmol), CuI (0.47 mg, 0.0024 mmol). The Schlenk flask was evacuated and filled with N₂. A solution of THF and DIPA mixed in 4:1 ratio and degassed with N₂, and 2 mL was transferred to the Schlenk flask via a cannula. The reaction was stirred at room temperature for 16 h. The solution was then filtered through a glass wool filter process and transferred dropwise to methanol, resulting in precipitation. The supernatant was decanted, and the precipitates were re-dissolved in DCM (0.5 mL) and precipitation/decantation process was repeated two more times in methanol. The precipitates were collected and dried in high vacuum for 16 h (26.9 mg, 65%). The resulting CPs were characterized by gel permeation chromatography (GPC), ¹H NMR, and absorption/emission spectroscopy.

Boc deprotection: A mixture of Boc-G-CPs dissolved in DCM (1mL) and TFA (1mL) were prepared and stirred at room temperature for 48 h. TFA and DCM removed by azeotrope distillation and deprotected polymers were purified by re-precipitating in cold EA (3x) and precipitates of **G-CPNs** were collected after decantation. The precipitates were dried in high vacuum for at least 8 h. The deprotection of boc group was confirmed by ¹H NMR.

Boc-G-CPs: ¹H NMR (400 MHz, CDCl₃, ppm): δ 11.46 (s, 1.09H), 8.62 (s, 1.03H), 7.05 (s, 1.03H), 4.24 (s, 2H), 3.91 (s, 2.17H), 3.74 (s, 2.14H), 3.62 (s, 2.16H), 1.48 (s, 9.23H), 1.45 (s, 9.2H). FT-IR (neat): 3329.4, 3131.7, 2975.3, 2931.3, 1720.1, 1635.2, 1614.0, 1567.7, 1503.9, 1411.8, 1364.5, 1319.8, 1280.5, 1249.7, 1131.0, 1048.8 cm⁻¹. GPC: M_n = 13500 g/mol, M_w = 18000 g/mol, PDI = 1.30, UV-Vis (THF) λ_{max} = 442 nm, Fluo λ_{max} = 469 nm.

G-CPNs: ¹H NMR (400 MHz, DMSO-d₆, ppm): δ 8.13 (1H), 7.92 (2H), 7.27 (2H), 6.87 (H), 5.77 (0.3H), 5.03 (1H), 4.30 (2H), 3.98 (2H), 3.59 (4H), 3.50 (6H), 2.98 (10H), 2.87 (H), 2.01 (6H). FT-IR (neat): 3360.36, 2160.37, 1736.79, 1681.18 cm⁻¹. UV-Vis (DMSO) λ_{max} = 434 nm, Fluo λ_{max} = 490 nm, QY = 8%.

Figure S4.5. ^1H NMR spectrum of **Boc-G-CPs** in CDCl_3 .

Figure S4.6. ^1H NMR spectrum of **G-CPNs** in DMSO-d_6 .

4.7.3 Physical and photophysical properties of CPNs

Determination of zeta potentials of CPNs: Zeta potential measurements were measured by Zetasizer nano-ZS (Zen 3600, Malvern Instruments Ltd.) using a folded capillary cell (Catalog DTS1060), at room temperature. CPNs samples were prepared from concentrated stock solutions

of CPNs dissolved in DMSO, at approximately 0.5 mM in a 5% DMSO (v/v) solution using deionized water (18 Ω) filtered through 0.22 μm polytetrafluoroethylene (PTFE) syringe filter. Each sample was measured three times.

Figure S4.7. Zeta potential of **A-CPNs** in water.

Figure S4.8. Zeta potential of **G-CPNs** in water.

Determination of hydrodynamic diameters (HD) of CPNs

Nanoparticles tracking analysis (NTA) was used to characterize the HD of nanoparticles. Light scattering measurement were performed with a LM10 HS (NanoSight, Amesbury, United

Kingdom), equipped with sCMOS camera, sample chamber with a 488 nm blue laser and Viton fluoroelastomer o-ring. A-CPNs and G-CPNs samples were prepared by diluting the stock CPNs in water (18 Ω) and PBS, respectively, and filtered through 0.2 μm PTFE syringe filter. The concentration of NTA samples was 10 μM containing 0.5% DMSO (v/v). The filtered sample solution was injected into the sample chamber using a 1 mL syringe (Restek Corporation, Pennsylvania, USA) until a few drops of the solution dripped out of the outlet of the chamber. All measurements were performed at 25°C using a LM14C temperature controller (NanoSight, Amesbury, United Kingdom). Each sample was measured three times.

Figure 4.9. NTA of G-CPNs (left) and A-CPNs (right) in water.

Absorption and emission spectra of CPNs

Figure S4.10. UV-vis and emission spectra of A-CPNs in water.

Figure S4.11. UV-vis and emission spectra of **G-CPNs** in water.

Table S4.1. Comparison of average physical and photophysical properties of CPNs.

CPNs	Mn (g/mol) ^a	PDI ^b	$\lambda_{\text{max, abs}}$ (nm) ^c	$\lambda_{\text{max, em}}$ (nm) ^{c,d}	QY (%) ^e	HD (nm) ^f	Zeta potential (mV)
G-CPNs	13,500	1.34	434	490	8	229 ^g	12.0 ± 8.9
A-CPNs	14,300	1.84	420	462	4	140 ^c	38.4 ± 4.4

^a Number average molecular weight determined by gel permeation chromatography in THF

^b PDI (polydispersity index) = M_w/M_n

^c Measured in H₂O

^d Excitation wavelength 400 nm in H₂O

^e Quantum yield (QY) in H₂O measured relative to diphenylanthracene standard.

^f Measured by NTA at 25°C.^g Measured in phosphate-buffered saline (PBS).

4.7.4 Cellular toxicity study

HeLa cells (~10,000 cells per well), in 200 μL of a complete medium, were seeded into a 96-well plate and allowed to attach for one day at 37 °C under a humidified atmosphere of 5% CO₂ / 95% air. A stock solution of CPNs was added into a complete media and diluted to the required amount of CPNs. Final concentrations of 40 μM , 20 μM , 10 μM , 5 μM , and 1 μM of CPNs were added into the complete media by dilution with CPNs stock solutions. After addition of CPNs, the cells were incubated for another 18 h. Cells were treated with 10 μL of methylthiazole tetrazolium (MTT) (5 mg mL⁻¹ in PBS, CALBIOCHEM, Germany) and incubated for 4 h at 37 °C. 200 μL of

medium was removed gently by using pipette and biological grade DMSO (100 μ L) was added to solubilize the purple formazan crystals formed by proliferating cells. Absorbance was measured by a microplate well reader (infinite M1000 PRO, TECAN, Switzerland) at 570 nm. Relative cell viability (%) as a function of CPNs concentration was expressed as a percentage relative to the untreated control cells. All measurements were performed in triplicate and standard deviation was included in the error bar.

Figure S4.12: Toxicity of A-CPNs and G-CPNs towards HeLa cells. Cell viability against varying concentration of CPNs is plotted as columns.

Figure S4.13: Bright field microscopic images of HeLa cells incubated with G-CPNs and A-CPNs (1 μ M, 5 μ M, 10 μ M, 20 μ M and 40 μ M), respectively, for 18 h before the treatment of MTT reagent. The amount of DMSO in the final solution was 0.8% (v/v).

4.7.5 Nanopipette characterization

The optical microscope image of the fabricated dual-barrel nanopipette tip is shown in Figure S4.14 A. The pore opening size was estimated by using ionic current measurement in 10 mM PBS (see Figure S4.14C). [54,55] Based on the distribution of measured pore resistance R_p (Figure S4.14 D), the mean R_p of each nanopore was $0.80 \pm 0.06 \text{ G}\Omega$. The half-cone angle θ of the nanopipette was measured to be about 4° based on SEM image. Figure S4.14 E shows the IV curve measured in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, which was used for live cell imaging. During SICM imaging, 0.1V sample bias was typically used with ionic current around 2.6 nA in HEPES buffer.

Figure S4.14. Characterization of nanopipette.

4.7.6 Potential change of G-CPNs modified PDMS substrate

The glutaraldehyde/(3-Aminopropyl)triethoxysilane (APTES) modified polydimethylsiloxane (PDMS) substrates were first modified with negatively charged bovine serum albumin (BSA) protein (at pH=7.4) by incubation in 5% BSA solution for 1 h. To introduce G-CPNs to the substrate, the BSA modified substrate was washed and then immersed in G-CPNs (20 μ M) for 5 h. Due to possible hydrogen bonding and ion pairing of guanidine group with phosphate group of lipid bilayer, G-CPNs can be adsorbed to the substrate through electrostatic interactions.[56] The topography and potential change (ΔV) images of BSA and CPN modified substrates, respectively, were imaged by SICM. The ΔV histograms of ΔV images are shown in Figure S4.15. The positive shifts of the ΔV histogram of CPNs modified substrates are evident. Therefore, the SICM based potential detection is sensitive to the positive charge of adsorbed G-CPNs on the PDMS substrate.

Figure S4.15: Schematic diagram of surface modification and ΔV histograms (with Gaussian fits) of BSA and G-CPNs modified PDMS substrates.

4.7.7 Topography and potential change images of control group live HeLa cells at 37 °C and 4 °C

For SICM imaging, HeLa cells were seeded into a 40-mm cell culture petri dish (~20,000/dish) with coverslips containing collagen gel coated PDMS and cultured in a complete medium (2000 μ L) for 24 h under 5% CO₂ at 37 °C. For 37 °C sample, cells were taken for SICM imaging

immediately after overnight incubation. For 4 °C sample, overnight incubated cell samples were first cooled to room temperature for 10 min and incubated in 4 °C refrigerator for 1h. Then, cells were washed six times with 1x PBS. For the control, cells were treated with same volume of DMSO as CPNs samples. No notable differences were observed in the topography images at different temperature. The surface area ratio (Figure S4.16C) revealed slightly higher value from cells incubated at 4 °C, suggesting the cell membrane surface is likely stiffened and roughened at lower temperature. Both potential change (ΔV) images are relatively uniform without particularly negative spots. The mean value of ΔV is evidently reduced from -1.74 mV at 37 °C to -1.61 mV at 4 °C.

Figure S4.16: The representative topography (A) and surface potential change (B) images of control group live HeLa cells at 37 °C and 4 °C. (C) Surface area ratio of live HeLa cells at 37 °C and 4 °C. (D) Three ΔV histograms with Gaussian fits (solid lines) from three cell samples at 37 °C and 4 °C. The error is the half width at half maximum (HWHM) of the Gaussian fit.

4.7.8 Dynamic topography changes of live cell membrane treated with G-CPNs

As shown in Figure S4.17, we can observe the appearance and disappearance of nanocraters on the G-CPNs treated live cell membranes. There are adsorbed G-CPNs on the cell membrane but no free CNPs in the HEPES buffer. These dynamic topography changes were never observed on controlled live cell surfaces without CNPs treatments.

Figure S4.17. Sequential topography images reveal the dynamic changes of live cell membrane in HEPES buffer (no **G-CPNs** in the buffer). The images were taken at the same site with 10 min apart. The cell was treated with **G-CPNs** for about 50 minutes before SICM imaging.

4.7.9 Topography and potential change images of fixed HeLa Cells

The topography and surface potential changes were also investigated after fixing the HeLa cells. The mean ΔV values of fixed control cell and live control cell are similar. Some shallow nanocraters

appeared on the fixed control cell. The topography of fixed cells was also investigated with and without G-CPNs treatment. In topography image, more shallow nanocraters appeared on the cell membrane of 1 h G-CPNs treated samples. As shown in ΔV histograms, the peak position of ΔV distribution is shifted to positive direction for the fixed Hela cells after 1 h or 4 h G-CPNs treatment. The positive shift of ΔV can be explained by the adsorption of positive charged G-CPNs without generating pores.

Figure S4.18: The topography and potential change images of fixed cells (i) without, (ii) with 1 h and (iii) with 4 h G-CPNs treatments. The ΔV histograms of all the ΔV images are shown in the right.

4.7.10 Simulation of nanopore with different potential across the membrane

Here, we simulated the effect of a potential drop across the cell membrane. The details of the Finite element methods (FEM) based simulation using COMSOL software package can be found in our previous reports.[54,57] Figures S4.19 A and B show the simulation geometry with 2D axial symmetry. The nanopore at the membrane (with thickness 18 nm) is 85 nm in radius. The pore size of the nanopipette is the same. Negative surface charge was applied both on the surface of the nanopipette and on the membrane surface. To simplify the simulation, only two ions, sodium and chloride ions, are used to fill the solution chambers at 150 mM concentration. A 0.1 V sample bias (V_s) was applied at the top chamber. A V in was applied at the bottom chamber to provide cross membrane potential. A floating potential boundary condition was applied in the potential probe.

Figure S19 D shows the potential change (ΔV) as a function of tip-membrane distance. The ΔV was obtained from the difference between V_m ($Z=900$ nm) and V_m ($Z=85$ nm). The potential differences (ΔV) are -21.01 mV, -16.88 mV and -12.75 mV when V_{in} are -25 mV, 0 and +25 mV, respectively.

Figure S4.19: FEM simulation results. (A) and (B) 2D axial symmetric geometry of the nanopipette and nanopore on a membrane used for the FEM simulation. (C) The potential changes measured by the potential probe as a function of tip-membrane distance in Z direction at three different V_{in} . (D) The electric potential distribution near the nanopipette tip with the following conditions: $V_s = 0.1 \text{ V}$, $V_{in} = -25 \text{ mV}$, $Z = 85 \text{ nm}$.

4.7.11 Topography and potential changes of 1 h CPNs treated live HeLa cells at 4 °C

At 4 °C, the nanocraters are less and mean ΔV is close to that of control cells. It seems like the pore formation process and adsorption of CPNs to the cell membrane was limited by the low temperature.

Figure S4.20: The topography and potential change images of live HeLa cells after 1 h (i) **G-CPNs** and (ii) **A-CPNs** treatment at 4 °C. The ΔV histograms from both ΔV images are shown in the right.

4.7.12 Cell membrane surface evolution with the A-CPNs treatment time.

Similar to Figure 5 in the main text, we also monitored the cell surface topographical, surface potential changes, and the fluorescence intensity distribution as a function of A-CPNs incubation time, as shown in Figure S21. In the topography images, nanocraters began to appear after 1 h treatment, and the area density reached maximum after 4 h treatment and back to normal with longer time treatment. It is obvious in the ΔV histograms that the mean ΔV is negatively shifted (-4.65 ± 0.51 mV) after 1 h A-CPNs treatment. Interestingly, after 2 h treatment, the ΔV becomes less negative (-1.93 ± 0.27 mV) and returns to the mean value close to that of control cell surface. As more nanocraters appeared compared with 1 h sample, these suggested that A-CPNs began to accumulate and attach to the cell outer surface and induce topographical changes of cell surface. At this time point, the outside edge of the cells is much brighter in green fluorescence intensity than that of the cell after 1 h treatment. After 4 h treatment, the cell surface becomes very negative ($-$

6.85 ± 0.94 mV). Therefore, the A-CPNs likely also generate cross-membrane pores after 4 h treatment. After 6 h treatment, the ΔV of the cell surface (-0.49 ± 0.03 mV) is even more positive than the control cell (Figure S4.16), and surface roughness is close to the control cell. At this time points, the nanocraters and pores disappeared and only the positive charge of adsorbed A-CPNs affect the ΔV measurement.

Figure S4.21. (A-B) Topography (A), potential change (B) images as a function of A-CPNs incubation time. (C) Confocal fluorescence images of HeLa cell treated with A-CPNs as a function of time. (D) The change of surface area ratio of HeLa cells with different A-CPNs treatment time. (E) ΔV histograms of ΔV of HeLa cell surface with different A-CPNs treatment time. (F) The relative fluorescent intensity of A-CPNs treated HeLa cells as a function of increasing time measured by flow cytometry. The relative fluorescence values were normalized to control Cells and average values are reported ($n = 3$) with standard deviation represented in error bars.

4.7.13 Inhibition assays.

For endocytosis pathway studies, HeLa cells were seeded into a 12-well plate (~50,000/well) one day prior to CPNs treatment. Cells were then treated without (control) or in the presence of pharmaceutical inhibitors chlorpromazine (24 mM) for clathrin mediated endocytosis (CME), genistein (210 μM) for caveolae mediated endocytosis (CvME), methyl-β-cyclodextrin (1000 μM) for CME and macropinocytosis (CvME/MPC), LYS294002 (120 μM) for macropinocytosis (MPC1), or cytochalasin D (0.04 mM) for macropinocytosis (MPC2) for 30 min before CPNs treatment.[58] For ATP depletion condition, cells were treated with NaN₃ (0.05 %) in the presence of 2-deoxyglucose (25×10^{-3} M). To maximize the blocking any potential endocytosis pathways, cells were treated with excess inhibitors and condition for 30 min before CPNs treatment. After 1 h CPNs (20 μM) treatment, cells were washed three times with 1x PBS and treated with 200 μM of TB and incubated for 10 min. Treatment with TB helps to evaluate the internalized CPNs by quenching the adsorbed CPNs on the cell membrane. After TB treatment, cells were then detached with TrypLE Express and resuspend with 1x PBS. After centrifugation (2000 RCF, 3 min), cells pellet was collected and fixed with 4% PFA for 10 min, then resuspended in 1x PBS. Experimental set ups for the flow cytometry experiment were set as discussed in general section of experimental section.

4.7.14 Cell uptake studies under ATP depletion condition by microscopy.

Figure S4.22. Fluorescence microscopic images of HeLa cells incubated with 20 μ M of **G-CPNs** (left) and **A-CPNs** (right) for 1 h under the ATP depletion condition.

4.8 Reference

- [1] A.E. Nel, L. Madler, D. Velegol, T. Xia, E.M.V. Hoek, P. Somasundaran, F. Klaessig, V. Castranova, M. Thompson, Understanding biophysicochemical interactions at the nano-bio interface, *Nat. Mater.* 8(7) (2009) 543-557.
- [2] V. Mailander, K. Landfester, Interaction of Nanoparticles with Cells, *Biomacromolecules* 10(9) (2009) 2379-2400.
- [3] S. Tenzer, D. Docter, J. Kuharev, A. Musyanovych, V. Fetz, R. Hecht, F. Schlenk, D. Fischer, K. Kiouptsi, C. Reinhardt, K. Landfester, H. Schild, M. Maskos, S.K. Knauer, R.H. Stauber, Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology, *Nat. Nanotechnol.* 8(10) (2013) 772-U1000.
- [4] F. Zhao, Y. Zhao, Y. Liu, X.L. Chang, C.Y. Chen, Y.L. Zhao, Cellular Uptake, Intracellular Trafficking, and Cytotoxicity of Nanomaterials, *Small* 7(10) (2011) 1322-1337.
- [5] P. Del Pino, B. Pelaz, Q. Zhang, P. Maffre, G.U. Nienhaus, W.J. Parak, Protein corona formation around nanoparticles - from the past to the future, *Mater. Horizons* 1(3) (2014) 301-313.
- [6] A. Albanese, P.S. Tang, W.C.W. Chan, The Effect of Nanoparticle Size, Shape, and Surface Chemistry on Biological Systems, in: M.L. Yarmush (Ed.), *Annu. Rev. Biomed. Eng.* Vol 14 2012, pp. 1-16.

- [7] M.J. Ernsting, M. Murakami, A. Roy, S.D. Li, Factors controlling the pharmacokinetics, biodistribution and intratumoral penetration of nanoparticles, *J. Control. Release* 172(3) (2013) 782-794.
- [8] K. Saha, M. Rahimi, M. Yazdani, S.T. Kim, D.F. Moyano, S. Hou, R. Das, R. Mout, F. Rezaee, M. Mahmoudi, V.M. Rotello, Regulation of Macrophage Recognition through the Interplay of Nanoparticle Surface Functionality and Protein Corona, *Acs Nano* 10(4) (2016) 4421-4430.
- [9] T. Cedervall, I. Lynch, S. Lindman, T. Berggard, E. Thulin, H. Nilsson, K.A. Dawson, S. Linse, Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles, *Proc. Natl. Acad. Sci. U.S.A.* 104(7) (2007) 2050-2055.
- [10] S. Schottler, G. Becker, S. Winzen, T. Steinbach, K. Mohr, K. Landfester, V. Mailander, F.R. Wurm, Protein adsorption is required for stealth effect of poly(ethylene glycol)- and poly(phosphoester)-coated nanocarriers, *Nat. Nanotechnol.* 11(4) (2016) 372-377.
- [11] P.M. Kelly, C. Aberg, E. Polo, A. O'Connell, J. Cookman, J. Fallon, Z. Krpetic, K.A. Dawson, Mapping protein binding sites on the biomolecular corona of nanoparticles, *Nat. Nanotechnol.* 10(5) (2015) 472-479.
- [12] E. Salonen, S. Lin, M.L. Reid, M. Allegood, X. Wang, A.M. Rao, I. Vattulainen, P.C. Ke, Real-Time Translocation of Fullerene Reveals Cell Contraction, *Small* 4(11) (2008) 1986-1992.
- [13] L. Rodriguez-Fernandez, R. Valiente, J. Gonzalez, J.C. Villegas, M.L. Fanarraga, Multiwalled Carbon Nanotubes Display Microtubule Biomimetic Properties in Vivo, Enhancing Microtubule Assembly and Stabilization, *Acs Nano* 6(8) (2012) 6614-6625.
- [14] Y. Korchev, C. Bashford, M. Milovanovic, I. Vodyanoy, M. Lab, Scanning ion conductance microscopy of living cells, *Biophys. J.* 73(2) (1997) 653 - 8.
- [15] P. Hansma, B. Drake, O. Marti, S. Gould, C. Prater, The scanning ion-conductance microscope, *Science* 243(4891) (1989) 641 - 3.
- [16] M. Nakajima, Y. Mizutani, F. Iwata, T. Ushiki, Scanning ion conductance microscopy for visualizing the three-dimensional surface topography of cells and tissues, *Sem. Cell Dev. Biol.* 73(Supplement C) (2018) 125-131.
- [17] J. Seifert, J. Rheinlaender, P. Novak, Y.E. Korchev, T.E. Schäffer, Comparison of Atomic Force Microscopy and Scanning Ion Conductance Microscopy for Live Cell Imaging, *Langmuir* 31(24) (2015) 6807-6813.
- [18] A.I. Shevchuk, P. Hobson, M.J. Lab, D. Klenerman, N. Krauzewicz, Y.E. Korchev, Imaging Single Virus Particles on the Surface of Cell Membranes by High-Resolution Scanning Surface Confocal Microscopy, *Biophys. J.* 94(10) (2008) 4089-4094.
- [19] J. Gorelik, A. Shevchuk, M. Ramalho, M. Elliott, C. Lei, C.F. Higgins, M.J. Lab, D. Klenerman, N. Krauzewicz, Y. Korchev, Scanning surface confocal microscopy for simultaneous topographical and fluorescence imaging: Application to single virus-like particle entry into a cell, *Proc. Natl. Acad. Sci.* 99(25) (2002) 16018-16023.

- [20] A. Page, D. Perry, P.R. Unwin, Multifunctional scanning ion conductance microscopy, *Proceedings of the Royal Society A: Mathematical, Physical and Engineering Science* 473(2200) (2017).
- [21] L.H. Klausen, T. Fuhs, M. Dong, Mapping surface charge density of lipid bilayers by quantitative surface conductivity microscopy, *Nature Commun.* 7 (2016) 12447.
- [22] A. Page, D. Perry, P. Young, D. Mitchell, B.G. Frenguelli, P.R. Unwin, Fast Nanoscale Surface Charge Mapping with Pulsed-Potential Scanning Ion Conductance Microscopy, *Anal. Chem.* 88(22) (2016) 10854-10859.
- [23] Y. Zhou, C.-C. Chen, A.E. Weber, L. Zhou, L.A. Baker, Potentiometric-Scanning Ion Conductance Microscopy, *Langmuir* 30(19) (2014) 5669-5675.
- [24] N. Panday, G. Qian, X. Wang, S. Chang, P. Pandey, J. He, Simultaneous Ionic Current and Potential Detection of Nanoparticles by a Multifunctional Nanopipette, *ACS Nano* 10(12) (2016) 11237-11248.
- [25] Y. Shan, N. Panday, Y. Myoung, M. Twomey, X. Wang, W. Li, E. Celik, V. Moy, H. Wang, J.H. Moon, J. He, Scanning Ion Conductance Microscopic Study for Cellular Uptake of Cationic Conjugated Polymer Nanoparticles, *Macromol. Biosci.* 16(4) (2016) 599-607.
- [26] C. Wu, B. Bull, C. Szymanski, K. Christensen, J. McNeill, Multicolor Conjugated Polymer Dots for Biological Fluorescence Imaging, *Acs Nano* 2(11) (2008) 2415-2423.
- [27] Y. Liu, K. Ogawa, K.S. Schanze, Conjugated polyelectrolytes as fluorescent sensors, *J. Photochem. Photobiol. C-Photochem. Rev.* 10(4) (2009) 173-190.
- [28] J.H. Moon, W. McDaniel, P. MacLean, L.E. Hancock, Live-cell-permeable poly (p-phenylene ethynylene), *Angew. Chem. Int. Ed.* 46(43) (2007) 8223-8225.
- [29] J.H. Moon, E. Mendez, Y. Kim, A. Kaur, Conjugated polymer nanoparticles for small interfering RNA delivery, *Chem. Commun.* 47(29) (2011) 8370-8372.
- [30] N.A.A. Rahim, W. McDaniel, K. Bardon, S. Srinivasan, V. Vickerman, P.T.C. So, J.H. Moon, Conjugated Polymer Nanoparticles for Two-Photon Imaging of Endothelial Cells in a Tissue Model, *Adv. Mater.* 21(34) (2009) 3492.
- [31] A.T. Silva, N. Alien, C.M. Ye, J. Verchot, J.H. Moon, Conjugated polymer nanoparticles for effective siRNA delivery to tobacco BY-2 protoplasts, *Bmc Plant Biol.* 10 (2010).
- [32] J.H. Lee, M. Twomey, C. Machado, G. Gomez, M. Doshi, A.J. Gesquiere, J.H. Moon, Caveolae-Mediated Endocytosis of Conjugated Polymer Nanoparticles, *Macromol. Biosci.* 13(7) (2013) 913-920.
- [33] E. Mendez, J.H. Moon, Side chain and backbone structure-dependent subcellular localization and toxicity of conjugated polymer nanoparticles, *Chem. Commun.* 49(54) (2013) 6048-6050.
- [34] M. Twomey, E. Mendez, R.K. Manian, S. Lee, J.H. Moon, Mitochondria-specific conjugated polymer nanoparticles, *Chem. Commun.* 52(27) (2016) 4910-4913.

- [35] H.D. Herce, A.E. Garcia, M.C. Cardoso, Fundamental Molecular Mechanism for the Cellular Uptake of Guanidinium-Rich Molecules, *J. Am. Chem. Soc.* 136(50) (2014) 17459-17467.
- [36] P.A. Wender, D.J. Mitchell, K. Pattabiraman, E.T. Pelkey, L. Steinman, J.B. Rothbard, The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: Peptoid molecular transporters, *Proc. Natl. Acad. Sci. U.S.A.* 97(24) (2000) 13003-13008.
- [37] G. Lattig-Tunnemann, M. Prinz, D. Hoffmann, J. Behlke, C. Palm-Apergi, I. Morano, H.D. Herce, M.C. Cardoso, Backbone rigidity and static presentation of guanidinium groups increases cellular uptake of arginine-rich cell-penetrating peptides, *Nat. Commun.* 2 (2011).
- [38] H.D. Herce, A.E. Garcia, Cell penetrating peptides: how do they do it?, *J. Biol. Phys.* 33(5-6) (2007) 345-56.
- [39] P. Novak, C. Li, A. Shevchuk, R. Stepanyan, M. Caldwell, S. Hughes, T. Smart, J. Gorelik, V. Ostanin, M. Lab, G. Moss, G. Frolenkov, D. Klenerman, Y. Korchev, Nanoscale live-cell imaging using hopping probe ion conductance microscopy, *Nature Methods* 6(4) (2009) 279 - 81.
- [40] T. Takami, F. Iwata, K. Yamazaki, J.W. Son, J.-K. Lee, B.H. Park, T. Kawai, Direct observation of potassium ions in HeLa cell with ion-selective nano-pipette probe, *J. Appl. Phys.* 111(4) (2012) 044702.
- [41] J.R. Staunton, B.L. Doss, S. Lindsay, R. Ros, Correlating confocal microscopy and atomic force indentation reveals metastatic cancer cells stiffen during invasion into collagen I matrices, *Sci. Rep.* 6 (2016) 19686.
- [42] W. Fan, R.M. Evans, Turning up the heat on membrane fluidity, *Cell* 161(5) (2015) 962-963.
- [43] D.A. Los, N. Murata, Membrane fluidity and its roles in the perception of environmental signals, *Biochim. Biophys. Acta.* 1666(1-2) (2004) 142-57.
- [44] F. Sgolastra, B.M. Deronde, J.M. Sarapas, A. Som, G.N. Tew, Designing Mimics of Membrane Active Proteins, *Acc. Chem. Res.* 46(12) (2013) 2977-2987.
- [45] H.D. Herce, A.E. Garcia, Cell Penetrating Peptides: How Do They Do It?, *J. Biol. Phys.* 33(5-6) (2007) 345-356.
- [46] I. Vorobyov, B. Bekker, T.W. Allen, Electrostatics of deformable lipid membranes, *Biophys. J.* 98(12) (2010) 2904-13.
- [47] T.G. Iversen, T. Skotland, K. Sandvig, Endocytosis and intracellular transport of nanoparticles: Present knowledge and need for future studies, *Nano Today* 6(2) (2011) 176-185.
- [48] D. Vercauteren, R.E. Vandenbroucke, A.T. Jones, J. Rejman, J. Demeester, S.C. De Smedt, N.N. Sanders, K. Braeckmans, The Use of Inhibitors to Study Endocytic Pathways of Gene Carriers: Optimization and Pitfalls, *Mol. Ther.* 18(3) (2010) 561-569.
- [49] Y.-J. Ko, E. Mendez, J.H. Moon, Controlled Aggregation in Conjugated Polymer Nanoparticles via Organic Acid Treatments, *Macromolecules* 44(13) (2011) 5527-5530.

- [50] Y.L. Wang, R.J. Pelham, Preparation of a flexible, porous polyacrylamide substrate for mechanical studies of cultured cells, *Methods Enzymol.* 298 (1998) 489-96.
- [51] A.I. Shevchuk, G.I. Frolenkov, D. Sánchez, P.S. James, N. Freedman, M.J. Lab, R. Jones, D. Klenerman, Y.E. Korchev, Imaging Proteins in Membranes of Living Cells by High-Resolution Scanning Ion Conductance Microscopy, *Angew. Chem. Int. Ed.* 45(14) (2006) 2212-2216.
- [52] T. Vokata, J.H. Moon, Synthesis of phenyleneethynylene-doped poly(pphenylenebutadiynylene)s for live cell imaging, *Macromolecules* 46(3) (2013) 1253-1259.
- [53] T. Vokatá, M. Twomey, E. Mendez, J.H. Moon, Synthesis of biodegradable conjugated polymers with controlled backbone flexibility, *J. Polym. Sci. A* 53(11) (2015) 1403-1412.
- [54] Y. Shan, N. Panday, Y. Myoung, M. Twomey, X. Wang, W. Li, E. Celik, V. Moy, H. Wang, J.H. Moon, J. He, Scanning Ion Conductance Microscopic Study for Cellular Uptake of Cationic Conjugated Polymer Nanoparticles, *Macromol. Biosci.* 16(4) (2016) 599-607.
- [55] P.B. Tiwari, L. Astudillo, J. Miksovska, X. Wang, W. Li, Y. Darici, J. He, Quantitative Study of Protein-Protein Interaction by Quartz Nanopipettes, *Nanoscale* 6 (2014) 10255-10263.
- [56] T. Murayama, T. Masuda, S. Afonin, K. Kawano, T. Takatani-Nakase, H. Ida, Y. Takahashi, T. Fukuma, A.S. Ulrich, S. Futaki, Loosening of Lipid Packing Promotes Oligoarginine Entry into Cells, *Angew. Chem. Int. Ed* 56(26) (2017) 7644-7647.
- [57] N. Panday, G. Qian, X. Wang, S. Chang, P. Pandey, J. He, Simultaneous Ionic Current and Potential Detection of Nanoparticles by a Multifunctional Nanopipette, *ACS Nano* 10(12) (2016) 11237-11248.
- [58] J. Lee, M. Twomey, C. Machado, G. Gomez, M. Doshi, A.J. Gesquiere, J.H. Moon, Caveolae-mediated endocytosis of conjugated polymer nanoparticles, *Macromol. Biosci.* 13(7) (2013) 913-20.

CHAPTER V

Modified guanidine functionalized conjugated polymer nanoparticles for small interfering RNA
delivery

Manuscript in preparation.

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5.1 Graphical abstract

Figure 5.1: Schematic diagram showing siRNA delivery by guanidine CPNs modified with hydrophilic group at the terminal end.

5.2 Abstract

The cationic guanidine functional group is known for its capacity to interact with the cell membrane because of its efficient cellular permeability. Guanidine-functionalized, highly emissive conjugated polymers (CPs) were synthesized. For better cellular labelling and delivery purpose, the terminal guanidine-functionalized CPs are further fabricated with four common organic amine bases using post polymerization modification approach. After the self-assembly of CPs in an aqueous solvent, a series of modified guanidine containing conjugated polymer nanoparticles (CPNs) were prepared. The series of five CPNs was tested for improving targeting and labelling of HeLa cells using

confocal microscopy and flow cytometry. The series of CPNs was also tested for improving delivery of small interfering RNA (siRNA) in HeLa cells by complexing CPNs with siRNA. The uptake mechanism and amount of internalized CPNs and siRNA were measured by flow cytometry and visualized by fluorescence microscopy. Our study has shown that modification of guanidine with hydrophilic group rich amine base (i.e. aminoethoxyethanol) increased the solubility and fluorescence (~2.5 times) of CPNs. The hydrophilic, aminoethoxyethanol-modified CPNs (A-CPNs) had better cellular internalization and improved small interfering RNA (siRNA) delivery but modification of guanidine group with hydrophobic group rich amine bases (i.e., di-isopropyl amine and piperidine) had poor CPNs labelling and siRNA delivery.

5.3 Introduction

Conjugated polymers (CPs) are a class of synthetic polymers known for their excellent photophysical properties, facile synthesis, and easy to process. CPs are widely used for sensing, cellular imaging, and delivery of biological substances.[1-10] The ability of CPs to efficiently deliver biological substances across cell membranes without causing cytotoxicity makes for a desirable vector for the delivery of many biological active substances. CPs have been used successfully to deliver a broad range of biologically active molecules intracellularly such as small interfering RNA (siRNA), DNA, and drugs[1, 9, 11-14] The simple concept of electrostatically held complex of cationic CPs and biologically active molecules can be employed for delivery of cargo into live cells. The varieties of positively charged CPs with different functional group and targeting strategies has been reported (e.g., protonated amines, phosphonium, and guanidine).[6] Positively charged guanidine is a naturally occurring functional group present in amino acid called arginine and this arginine rich cell penetrating peptides (CPPs) are well known for their cell permeability in mammalian cells. It has been reported that cationic guanidine group of CPPs is the main molecular factor responsible for their excellent translocation of biological substances with

transient pore formation mechanism.[15-19] In addition, considering that Y shaped guanidine has high pKa $\sim$ 13, it can be protonated over a wide pH range, meaning CPs containing the guanidine group can provide an excellent biomaterial for cellular study.[8] The cationic Y shaped guanidine group can easily form divalent hydrogen bonds with the polar heads of phospholipids bilayer and the various sulfate, carboxylate groups of glycosaminoglycans (GAGs) present in the cell membrane.[16, 18, 20] The guanidine functionalized CPNs can form an electrostatic complex by interacting with negatively charged biologically important molecules. The highly negatively charged siRNA are double-strand RNA with 21-23 base pairs, known for efficient gene knockdown of target cells.[16, 21] The delivery of siRNA into target cells and intracellular organelles has gained much attention for therapeutic applications. However, the efficient delivery of siRNA faces difficulty because of its repulsive interaction between the negatively charged cell membrane and negatively charged phosphodiester group on the siRNA backbone.[22-24] For this reason, the concept of camouflaging siRNA in the highly positively charged aggregated CPs could be advantageous. Moreover, the delivery of siRNA by itself faces the problem of enzymatic degradation before reaching the target intracellular organelles.[22-24] The problem of degradation could be addressed by the complexing siRNA with CPs.

We report herein an easy post-polymerization modification of guanidine terminal functional group leading to a series of CPs with same hydrophobic poly(*p*-phenyleneethynylene) (PPE) backbone. The synthesis of CPs with different functional and targeting groups at the terminal end often involves reconstruction and synthesis of corresponding monomers. The series of guanidine modified CPs reported here can be synthesized by post-polymerization modification from same parent boc protected homo coupled guanidine CPs (Boc-PG-H), hence this design idea will tremendously decrease the synthetic effort for synthesizing high molecular weight CPs using different monomers. The post polymerization modification helped us to get high molecular weight CPs with desired functional group at the terminal end with same backbone structure. The

hydrophobic PPE backbone of CPs causes polymer chains to collapse in poor solvents and tend to self-assemble due to strong interpolymer π - π interaction causing self-quenching with decrease fluorescence quantum yield of CPs.[25, 26] The decreased fluorescence quantum yield clearly doesn't help when using CPs for the labelling and targeting purposes in cellular studies.[4, 27, 28] It is expected that modification of the terminal end of CPs with comparative bulky organic amine bases will reduce the interpolymer π - π interaction and help to improve the fluorescence quantum yield and solubility of CPs.[29-30] We have developed guanidine-modified CPs with different terminal functional groups to enhance the cellular interaction for improved labelling, bioimaging, and delivery. In our approach, parent homo coupled guanidine CPs (PG-H) were post polymerized with four different amine bases (i.e., diisopropylamine (DIPA), morpholine, piperidine and aminoethoxyethanol).

In this investigation, we have successfully designed and modified a series of CPs with a guanidine motif and effectiveness of these CPs for cellular labelling and targeting were tested in HeLa cells. The increased hydrophilicity provided CPs with improved solubility and quantum yield. We have demonstrated that hydrophilic aminoethoxyethanol modified CPs showed improved labelling, imaging, and delivery of siRNA compared to PG-H and other hydrophobic group modified CPs. In this study, we combined synthesis of modulated guanidine functional group CPs, comparative confocal microscopy analysis and flow cytometry analysis of cellular uptake of CPs. Furthermore, to get clear understanding of delivery of cargo from these synthesized CPs for biological important materials, CPN and siRNA complex were prepared and its delivery inside the cells were studied by confocal microscopy and flow cytometry analysis.

5.4 Result and discussion.

5.4.1 Polymer design and synthesis

The parent tert-butyloxycarbonyl (Boc) protected homo coupled guanidine polymers (Boc-PG-H) were synthesized by the palladium/copper mediated sonogashira cross coupling in presence of DIPA as a base at room temperature. A detail synthesis of Boc-PG-H is reported in chapter IV of dissertation. Boc protected intermediate of DIPA modified CPs were synthesized at higher temperature (80 °C) in situ condition during the polymerization process where DIPA plays dual role. In the reaction, DIPA acts as a base for the sonogashira coupling reaction and same time it also reacts with Boc group of guanidine when heated 80 °C as shown in general reaction condition Figure 5.2. Three other intermediate Boc protected base modified CPs were synthesized by simple post polymerization modification of parent Boc-PG-H with three organic amine bases (morpholine, piperidine and aminoethoxyethanol) as shown in general post polymerization reaction condition Figure 5.2 step A. The deprotection of Boc group was carried out by treating trifluoro acetic acid (TFA) as shown in Figure 5.2 step B. After deprotection of Boc group a series of positively charged guanidine modified CPs were obtained. These CPs are readily soluble in common organic solvents like tetrahydrofuran (THF), dimethyl formamide (DMF), and dimethylsulfoxide (DMSO).

Figure 5.2. General post polymerization reaction condition of Boc-PG-H with amine bases (step A) and their Boc derportection (step B) condition (**R** substituent groups represents the side chain alkyl groups attached to amine bases and green solid bar representing the CPs rigid hydrophobic backbone).

Figure 5.3. Chemical structure of a homo coupled guanidine CPNs (i.e., **G-CPNs**) and a series organic amine base modified guanidine CPNs (i.e., **A-CPNs**, **D-CPNs**, **M-CPNs**, and **P-CPNs**).

All five CPs have the same rigid PPE hydrophobic backbone and extended flexible alkoxy side chain attached to aromatic rings with positively charged guanidine or base modified functionalities in the terminal position as shown in Figure 5.3. The rigid aromatic π electron conjugated PPE

backbone makes CPs hydrophobic in nature, but by introducing positive charges at the end of terminal groups makes CPs more hydrophilic. This means that the amphiphilic, well-solvated CPs in an organic solvent self-assemble due to interpolymer π - π interaction to form CPNs when dissolved in an excess of aqueous solvent. Hence, five self-assembled nanoparticles in aqueous solvent are named as G-CPNs, D-CPNs, P-CPNs, M-CPNs and A-CPNs with abbreviation from their terminal base head groups as shown in Figure 5.3. Their physical and photophysical properties were summarized in Table 5.1. The absorbance and emission profiles for five CPs remained same even after the modification with bases. The quantum yield of A-CPNs is increased ~ 2.5 times compare to parent G-CPNs. But the quantum yield of other four CPs practically remained same after the modification.

Table 5.1. Physical and photophysical properties of guanidine modified CPNs

CPNs	Mn ^a (g/mol)	%Yield ^b	$\lambda_{\text{max, abs}}$ (nm) ^c	$\lambda_{\text{max, em}}$ (nm) ^{c,d}	QY ^e	HD ^f
G-CPNs	13,500	65	434	490	8.3	229 $\pm$ 15.7
D-CPNs	13,000	62	418	490	8.1	215 $\pm$ 9.4
M-CPNs	12,400	76	435	488	8.2	246 $\pm$ 10
P-CPNs	12,500	53	435	490	8.4	291 $\pm$ 7.0
A-CPNs	14,000	79	434	494	20	174 $\pm$ 4.7

^aDetermined by gel permeable chromatography in THF

^byield per repeating unit after repeated purification by precipitation

^cMeasured in DMSO

^dExcitation wavelength for G-CPNs, M-CPNs, P-CPNs and A-CPNs were 435 nm and for D-CPNs was 418 nm

^eQuantum yield in DMSO measured relative to diphenylanthracene standard

^fHD- Hydrodynamic diameter measured in PBS

5.4.2 Visualization of *in vitro* uptake of CPNs by fluorescence microscopy

To better understand the cellular uptake and their distribution after the internalization of CPNs, fluorescence microscopy images were collected using confocal laser scanning microscopy (CLSM). The fixed concentration of CPNs (10 μ M) were incubated for different time to optimize the incubation time. Cells were then washed with PBS (3 times), fixed with 4% paraformaldehyde (PFA), and washed again with PBS (3 times). Coverslips with cells were mounted on glass slides with drop of 1:1 mixture of glycerol/PBS solution and were observed under a high-resolution microscope. Most of the CPNs are accumulated in the cell membrane and some CPNs were internalized in the perinuclear area after 1 h incubation. The effect of internalization of CPNs as a function of increasing time were also studied. As shown in Figure 5.4, the internalization of CPNs increased as a function of increasing incubation time for all five CPNs and maximum fluorescent intensity was observed after 20 h incubation. The staining pattern of CPNs appears different for each of the five CPNs. For examples G-CPNs, D-CPNs and A-CPNs are mostly labelled in cytosolic region with punctate pattern. But P-CPNs and M-CPNs are poorly internalized upon 1 h incubation. Their staining pattern is more diffused in cytosolic region of the cells for 1 and 4 h incubation. However, 20 h incubation displayed marked diffused cytosolic labelling together with some punctate signals. It is interesting to note that G-CPNs and A-CPNs are also internalized inside the nucleus after 5 h incubation (supporting information Figure S5.20). It could be because of localization of low molecular weight blue fluorescent CPNs easily passes through the nuclear membrane. Also, the internalization and distribution pattern of CPNs were also studied for different concentration of CPNs for fixed time. After the incubation of CPNs for fixed time (i.e., 1 h), the fluorescence intensity increased as a function of increasing concentration and maximum fluorescent intensity was observed for 20 μ M (supporting information Figure S5.21).

Figure 5.4. Fluorescence microscopic images of HeLa cells incubated with CPNs (10 μ M) for 1, 4, and 6 h. The scale bar is 20 μ m.

Figure 5.5. Relative green fluorescence intensity of series of CPNs (20 μ M) treated HeLa cells as a function of increasing time measured by flow cytometry. The relative fluorescence intensity values were normalized to control cell (cells are not treated with trypan blue (TB)). The relative fluorescence values were normalized to control cell and average values are reported ($n = 3$) with standard deviations represented in error bars.

5.4.3 In vitro cellular uptake of CPNs studies by flow cytometry

To support the visual data obtained from the confocal imaging and to test the effectiveness of internalization of the CPNs a time course experiment was performed using green channel of flow cytometry. It was expected that internalization of CPNs would increase as a function of increasing incubation time resulting in an increased relative fluorescence intensity. Figure 5.5 shows the relative fluorescent intensity of CPNs were increased as a function of increasing time. As we observed in the confocal imaging, the maximum fluorescent intensity is observed for the 20 h CPNs incubation. The fluorescent intensity of M-CPNs and P-CPNs is comparatively weaker than other three CPNs indicating that the amount of CPNs internalized inside the cells is lower compare to three other CPNs. Confocal image showed that the fluorescent intensity of A-CPNs is maximum followed by the D-CPNs and G-CPNs. The flow cytometry data and confocal image clearly indicate that the hydrophilic aminoethoxyethanol plays major role during the cellular internalization process. The hydrophilic aminoethoxyethanol moiety of A-CPNs enhance the solubility of CPNs and it plays significantly role during the internalization of CPNs. Moreover, hydrophobic group (DIPA, morpholine and piperdine) modified CPNs has weak internalization because of increased aggregation of CPNs in medium and causing more non-specific binding in the cell membrane. We believe those nonspecifically bound aggregated CPNs in the cell membrane are washed away during the washing process of cells.

5.4.4 Visualization of *in vitro* uptake of siRNA by high resolution fluorescence microscopy

The efficient delivery and internalization of siRNA were studied by treating commercially available red fluorescent dye labelled siRNA called siGLO (red). The CPNs/siGLO complex was prepared by the mixing CPNs (20 μ M) in Rnase free water and siGLO (100 nM) and the complex was diluted with a culture medium. HeLa cells were then incubated with CPNs/siGLO complx for 1 h. Similar cleaning, fixing and mounting processes were repeated as described above before observing cells

under a high-resolution microscope as shown in Figure 5.6, the overlay image obtained from the green and red channels for CPNs and siGLO show that distinct distribution of siGLO. The red fluorescence with diffused staining pattern from siGLO were distributed in cytosolic region in the case of G-CPN. The red punctate dot for siGLO was observed for D-CPNs which was similar to staining pattern observed for CPNs itself indicating that siGLO are stained in the cytosolic region. Similar to G-CPNs staining pattern M-CPNs also has faint red diffused pattern in the cytosolic region. However, P-CPNs has very poor red fluorescent signal from the siGLO, indicating P-CPNs couldn't delivery of siGLO inside the cells. G-CPNs showed diffused red fluorescence signal from siGLO in the cytoplasm and inside the nucleus, which is similar to staining pattern observed for CPNs itself in the green channel. The staining pattern of siGLO in confocal image indicates that some of the siGLO were dissociated from G-CPNs/siGLO complex and translocated to nucleus but significant amount of siGLO are still remain in the cytosolic region. Interestingly in the case of A-CPNs, the red fluorescence intensity of siGLO is increased significantly inside the nucleus than cytosol compared to the G-CPNs, as shown in the overlay image in Figure 5.6. We speculate that siGLO is dissociated efficiently from A-CPNs/siGLO complex and translocated to the nucleus alone. Microscopic image of A-CPNs/siGLO complex shows all the dissociated siGLO are translocated to nucleus there are no, or very low cytosolic translocation as shown in Figure 5.6. The different localizations of these CPNs were further verified by the quantitative luminescence intensity profiles, where maximum fluorescent intensity was observed in nucleus region in case of A-CPNs (Figure 5.7). The maximum fluorescent intensity observed in nucleus region of A-CPNs is ~3 times higher compare to G-CPNs. The comparative fluorescent intensity traces for green and red channel for A-CPNs/siGLO complex follow completely different traces indicating that siGLO was released from the complex after the internalization as shown in Figure 5.8.

Figure 5.6. Fluorescence microscopic images of HeLa cells incubated with CPNs (20 μ M) upon complexation with siGLO red (100 nM) for 1 h. Top row green channel and fluorescent from CPNs, middle row for red channel and fluorescent from siGLO red and bottom row for channel overlay. The scale bar is 20 μ m.

Figure 5.7. Relative fluorescence intensity profiles along the yellow lines as shown in microscopic images (red channel) of HeLa cells incubated with CPNs (20 μ M)/siGLO red (100 nM) complex for 1 h.

Figure 5.8. Relative fluorescence intensity of red and green channel profiles along the yellow lines as shown in microscopic images of HeLa cells incubated with **A-CPNs** (20 μ M)/siGLO red (100 nM) complex for 1 h.

5.4.5 *In vitro* cellular uptake of siRNA studies by flow cytometry

The relative fluorescence intensity of siGLO translocated in the cells are measured by using flow cytometry. HeLa cells treated with CPNs (20 μ M)/siGLO (100 nM) complex and incubated for different time. The relative fluorescence intensity observed for A-CPNs is $\sim$ 2 times higher than G-CPNs and D-CPNs for 1 h and 20 h incubation indicating efficient delivery of siGLO by A-CPNs (Figure 5.9). But relative fluorescence intensity for both G-CPNs and D-CPNs practically remain same. As shown in Fluorescence microscopic images (Figure 5.6) and relative fluorescent intensity profiles (Figure 5.7), most of siGLO are translocated in cytosolic region in case of D-CPNs, and in case of G-CPNs, they are translocated in both cytosolic and nucleus region. The relative fluorescence intensity of M-CPNs and P-CPNs is very low compare to three CPNs indicating poor delivery of siRNA by these CPNs. The increased fluorescence intensity of siGLO as a function time can also be observed in microscopic image of CPNs (20 μ M) upon complexation with siGLO red (100 nM) for 20 h (supporting information Figure S5.22).

Figure 5.9. Relative red fluorescence intensity of series of CPNs (20 μ M)/siGLO (100 nM) complex treated HeLa cells as a function of increasing time measured by flow cytometry. The relative fluorescence intensity values were normalized to control cell treated with only siGLO (cells are not treated with TB). The relative fluorescence values were normalized to control cell. The fluorescence intensity was measured for three times for same sample and average values are reported with standard deviations represented in error bars.

5.4.6 Cellular uptake mechanism studies

ATP Depletion Condition. The cellular internalization mechanism of CPNs are mostly dependent on endocytic pathways. To understand the ATP dependent cellular uptake mechanism, the HeLa cells were treated ATP depletion condition i.e. sodium azide (NaN_3) and 2-deoxy-D-glucose. Confocal microscope images showed that G-CPNs, M-CPNs and P-CPNs are nonspecifically accumulated in cell membrane with some CPNs are internalized as shown in Figure 5.10. But the D-CPNs and A-CPNs most of them are nonspecifically accumulated in the cell membrane. This observation is further supported by 20% decrease in cell fluorescence intensity in the flow cytometry indicating that the uptake of CPNs is decreased in ATP depletion condition (Figure 5.11).

Figure 5.10. Fluorescence microscopic images of HeLa cells incubated with CPNs (20 μ M) under ATP depletion condition for 1 h. The scale bar is 20 μ m.

Inhibition assay. To understand CPNs internalization mechanism, we explored the effect of several pharmacological drugs that specifically inhibits different endocytosis pathways i.e. chlorpromazine for clathrin mediated endocytosis (CME), genistein for caveolae mediated endocytosis (CvME), methyl- β -cyclodextrin (1000 μ M) for CME and macropinocytosis (CvME/MPC), cytochalasin D for macropinocytosis (MPC). HeLa cells were pretreated (30 min) with pharmacological drugs with standard concentration required to inhibit particular endocytosis pathway and then cotreated with CPNs (5 μ M). After the incubation for 1 h, cells were treated with trypan blue (200 μ M) for 10 min in order to quench the fluorescence from the CPNs adsorbed in the cells membrane. The intracellular fluorescence from CPNs were measured by using flow cytometry. The fluorescence intensity observed for drugs treated CPNs were compared and normalized with the only CPNs treated control cells. The decreased fluorescence intensity for drugs treated CPNs indicates that the endocytosis uptake mechanism is controlled by that drugs of treatment. The inhibition studies indicate that all five CPNs follows the typical endocytosis pathways. The fluorescence intensity of five CPNs are decreased marginally ($\sim$ 20 to 30%) within the error range when treated with these pharmacological drugs indicating that the treatment of inhibitor doesn't affect the cellular uptake process. It also indicates that CPNs can be up taken by multiples endocytosis pathways.

Figure 5.11. The relative fluorescence intensity of series of CPNs (5 μ M) in the presence of endocytosis pathway inhibitors such as chlorpromazine, genistein, methyl- β -cyclodextrin, cytochalasin D, and sodium azide. Inhibitors were incubated 30 min before CPNs treatment. The relative fluorescence values were normalized to control CPNs and average values are reported ($n = 3$) with standard deviation represented in error bars.

5.5 Conclusion.

We have developed a series of highly fluorescent guanidine modified CPNs and the synthetic challenge and time required for monomer synthesis has been minimized by post polymerization modification approach. The post polymerization approach can synthesize various class of amine-based guanidine CPNs with same backbone structure which helps to control the photophysical properties of CPNs. The modification of aminoethoxyethanol base not only helped to increase the

solubility and fluorescence quantum yield of CPNs, it also helped to deliver siRNA efficiently inside the nucleus which is confirmed by high resolution fluorescence microscope. The results show clear difference in cellular labelling and delivery because of hydrophilic vs hydrophobic moiety in the terminal end of side chains. We believe that introducing hydrophilic aminoethoxyethanol group at the terminal group not only helps to improve hydrophilicity and improve siRNA delivery it also adds additional conjugation site for targeted biologically active molecules because of free hydroxyl (-OH) group at the terminal end. It makes A-CPNs as a versatile and superior class of CPNs for the delivery purpose with ample room for improvement because of reactive -OH side chain. The resulting data demonstrate that aminoethoxyethanol functional terminal end provide a platform to conjugate CPNs to biologically important molecules.

5.6 Outlook

The post polymerization synthetic methodology presented in the chapter could be beneficial for the synthesis of wide range of CPNs in lab using common amine containing bases. Same conjugated backbone but different side chain modified CPNs with guanidine motif can provide better understanding of role of side chains over backbone for optoelectronic and cellular application. Since we have presented, A-CPNs had better cellular labelling and delivery properties so alcohol functional group of aminoethoxyethanol can be further modified or conjugated with biologically important biomaterial to get more functional materials for future applications. For instance, folic acid binding folate receptors are overexpressed in tumor cells so folic acid functionalized CPNs can be synthesized by reaction of alcohol functional group A-CPNs with Folic acid using simple N,N'-Dicyclohexylcarbodiimide (DCC) and 4-N, N-dimethylaminopyridine (DMAP) coupling reaction as shown in the reaction scheme 5.1. The covalently attached folic acid are expected to interact selectively with folate receptor present in the tumor cells for improved targeting and labelling purpose.

Scheme 5.1. Reaction scheme for the synthesis of folic acid functionalized A-CPNs.

5.7 Experimental.

A series of five CPNs were obtained from in-house collaboration. The synthesis and characterization of CPNs were performed by graduate student, Md Salauddin Ahmed.

5.7.1 General information.

Chemicals, including solvents, were purchased from Fisher Scientific, and used as received. Deuterated solvents were purchased from Cambridge Isotope Laboratories (Cambridge, MA). All chemicals and solvents were purchased and used without further purification. All solutions were prepared using deionized (DI) water (~18MΩ) from water purification system (Ultra Purelab system, ELGA/Siemens). Pharmacological endocytosis inhibitors such as chlorpromazine hydrochloride (#ALX-270-171-G005, Enzo Life Sciences, Inc.), genistein (#AC32827-1000, Acros Organics), methyl-β-cyclodextrin (#377110050, Acros Organics), LY2994002 (#70920, Caymen Chemical), and cytochalasin D (#BML-T109-0001, Enzo Life Sciences, Inc.) were purchased from Fisher Scientific. The number average molecular weight (M_n), weight average

molecular weight (M_w), and polydispersity index ($PDI = M_w/M_n$) of CPs were determined by gel permeation chromatography (GPC) against polystyrene standards using a Shimadzu high performance liquid chromatography (HPLC) system fitted with PLgel 5 μ m MIXED-D columns and SPD-20A ultraviolet-visible (UV-vis) detector at a flow rate of 1.0 mL/min. Samples for GPC, small amount (~100 μ L) of polymer in dimethylformamide (DMF) or dichloromethane (DCM) was diluted with 1 mL of HPLC grade tetrahydrofuran (THF) and then filtered through a 0.45 μ m polytetrafluoroethylene (PTFE) syringe filter prior for injection. UV-vis spectra were recorded using a Varian Cary 50 Bio spectrophotometer. Fluorescence spectra were obtained using a FluoroLog-3 Spectrofluorometer (Jobin Yvon/Horiba). 9, 10-diphenylanthracene (QY = 0.9) in cyclohexane was used as a fluorescence standard. Fourier transform infrared (FTIR) spectra were recorded on a PerkinElmer Spectrum 100 FTIR Spectrometer. Fine polymer powders were directly mounted on an attenuated total reflection (ATR) cell of the spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz Avance Bruker NMR spectrometer. Chemical shifts were reported in parts per million (ppm) for ^1H NMR on the δ scale based on the middle peak ($\delta = 2.50$ ppm) of the dimethylsulfoxide (DMSO- d_6) solvent as an internal standard. Nanoparticle tracking analysis (NTA) measurements were performed using a LM10 HS (NanoSight Amesbury, United Kingdom) equipped with a sCMOS camera, a sample chamber, and a 488 nm blue laser.

5.7.2 Synthesis and characterizations of CPNs

Monomer synthesis: Monomer M1 was synthesized according to literature procedure.[31] A detail synthesis procedure of monomer M2 was described in experimental section of chapter IV of this dissertation.

Polymer synthesis:

General synthetic protocol for Boc deprotection: Boc-protected polymer dissolved in DCM (1 mL) was mixed with TFA (1 mL) and stirred at room temperature for 24 h. The solvent was evaporated using azeotrope distillation under reduced pressure and the crude CPs were redissolved in 1 mL of DMF. The CPs in DMF were purified by re-precipitation in diethyl ether (1x) and the yellowish fibers were collected after decantation process. The fibers were then redissolved in DMF and precipitation and decantation process were repeated in ethyl acetate (EA) (2x). The precipitates were dried in high vacuum for 24 h. The deprotection of boc group was confirmed by ^1H NMR.

Scheme S5.1. Synthesis route to G-CPNs

Synthesis of Boc-PG-H and G-CPNs: A detail of synthesis and complete characterization of Boc-PG-H and deprotection of Boc group to synthesize G-CPNs are explained in the chapter IV of this dissertation. The reaction scheme involved for the synthesis of G-CPNs is as shown in scheme 5.1.

Scheme S5.2. Synthesis route to D-CPNs

Synthesis of Boc-PG-D: DIPA modified Boc-protected guanidine containing CPs (Boc-PG-D) were synthesized using Sonogashira cross-coupling reaction at 80 °C. A Schlenk flask was charged with monomers M1 (50 mg, 0.04 mmol), M2 (40.02 mg, 0.04 mmol) in the presence of $\text{PdCl}_2(\text{PPh}_3)_2$ (3.43 mg, 0.0048 mmol), CuI (0.47 mg, 0.0024 mmol, THF & DIPA (4:1) were heated at 80 °C for 16 h (26.9 mg, 63%). The resulting CPs in DCM was characterized by GPC and its absorption/emission profiles were measured. The final polymer was allowed to dry under high vacuum for 16 h before ^1H NMR characterization.

^1H NMR (400 MHz, CDCl_3 , ppm): δ 12.42 (s, 0.9H), 8.21 (s, 0.9H), 7.05 (s, 0.9H), 4.23 (s, 2.0H), 3.90 (s, 2H), 3.75 (s, 3.5H), 3.60 (s, 0.6H), 3.54 (s, 1.9H), 1.42 (s, 9.2H), 1.23 (s, 12.2H) FT-IR (neat): 3338.6, 3139.9, 2964.5, 2828.5, 2763.8, 2712.9, 2454.9, 2398.4, 1714.2, 1666.8, 1628.9, 1574.1, 1432.9, 1395.8, 1337.0. GPC: M_n = 13200 g/mol, M_w = 22400 g/mol, PDI = 1.70, UV-Vis (THF) λ_{max} = 434 nm, Fluo λ_{max} = 472 nm.

Synthesis of D-CPNs: The deprotection of Boc-PG-D was carried out as described in general deprotection protocol and final a yellow colored CPs were collected and dried (72.5 % yield).

^1H NMR (400 MHz, DMSO-d_6 , ppm): δ 9.73 (s, 1H), 9.27 (s, 1H), 8.65 (s, 2.0H), 7.5 (s, 2.0H), 4.62 (s, 2.0H), 4.4 (s, 2.0H), 4.03 (s, 1.3H), 3.76 (s, 4.0H), 3.56 (s, 2.3H), 3.50 (m, 6H), 1.17 (m, 13.0H). FT-IR (neat): 3512, 2994.8, 2992.6, 2159.87, 1733.97, 1679.4 cm^{-1} . UV-Vis (DMSO) λ_{max} = 418 nm, Fluo λ_{max} = 490 nm, QY = 8.14.

Figure S5.1. ^1H NMR of **Boc-PG-D** in CDCl_3 .

Figure S5.2. ^1H NMR of **D-CPNs** in DMSO-D_6 .

Scheme S5.3. Synthesis route to **M-CPNs**

Synthesis of Boc-PG-M: A Schlenk flask was charged with Boc-PG-H (10.0 mg, 0.012 mmol) and morpholine (2.57 mg, 0.03 mmol), evacuated and filled with N₂ gas. Anhydrous THF (1.5 mL) was transferred to the Schlenk flask via a cannula. The reaction mixture was stirred at 80 °C for 16 h. A viscous polymer solution was filtered through the glass wool filled in the glass pipette. The polymer precipitates were collected by transferring the solution dropwise to cold diethyl ether (1x). After settling down the polymer precipitates, the supernatant was decanted. The precipitates were redissolved in DMF (1 mL), and the same purification method was repeated for three more times using methanol as a purifying solvent. The yellow gel like precipitate were dried under high vacuum (7.72 mg with 76% yield).

¹H NMR (400 MHz, CDCl₃, ppm) δ: 12.2 (s, 1.0H), 8.3 (s, 1.0H), 7.04 (s, 1.0H), 4.22 (s, 2.0H), 3.90 (s, 2.0H), 3.74 (s, 4.0H), 3.60 (s, 4.0H), 3.52 (s, 4.0H), 1.42 (s, 10.0H). FT-IR (neat): 3331.3, 2925.8, 2857.0, 1716.2, 1669.7, 1629.8, 1585.9, 1562.7, 1507.5, 1475.2, 1454.3, 1409.6, 1366.4, 1348.9, GPC: M_n = 12400 g/mol, M_w = 18600 g/mol, PDI = 1.50. UV-Vis (THF) λ_{max} = 428 nm, Fluo λ_{max} = 469 nm.

Synthesis of M-CPNs: The deprotection of Boc-PG-M was carried out as described in general deprotection protocol and the final yellow gel like polymers were collected and dried (62% yield). ^1H NMR (400 MHz, DMSO- d_6 , ppm): δ 10.17 (s, 1.0H), 9.20 (s, 1.5H), 8.59 (br s, 2.3H), 7.54 (s, 3.0H), 4.38 (br s, 3.3H), 4.04 (s, 2.0H), 3.79 (br s, 4.3H), 3.53 (br m, 11.5H). FT-IR (neat): 3280.78, 2872.4, 1670.82, 1440.13, 1303.92, 1251.30, 1198.86 cm^{-1} . UV-Vis (DMSO) λ_{max} = 435 nm, Fluo λ_{max} = 488 nm, QY = 8.20.

Figure S5.3. ^1H NMR of **Boc-PG-M** in CDCl_3 .

Figure S5.4. ^1H NMR of M-CPNs in DMSO-d_6 .

Scheme S5.4. Synthesis route to P-CPNs

Synthesis of Boc-PG-P: A Schlenk flask was charged with Boc-PG-H (10.0 mg, 0.012 mmol) and piperidine (2.51 mg, 0.03 mmol), evacuated and filled with N_2 gas. Anhydrous THF (1.5 mL) was transferred to the Schlenk flask via a cannula. The reaction mixture was stirred at $80\text{ }^\circ\text{C}$ for 16 h. A viscous polymer solution was filtered through the glass wool filled in the glass pipette. The polymer precipitates were collected by transferring the solution dropwise to cold diethyl ether (1x). After settling down the polymer precipitates the supernatant was decanted. The precipitates were redissolved in DMF (1 mL), and the same purification method was repeated for three more times.

using methanol as purifying solvent. The yellow gel like precipitate were dried under high vacuum (5.36 mg, 53% yield).

^1H NMR (400 MHz), CDCl_3 , ppm): δ 12.33 (s, 1.0H), 8.22 (s, 1.0H), 7.04 (s, 1.0H), 4.22 (s, 2.0H), 3.90 (s, 2.0H), 3.74 (s, 3.0H), 3.72 (s, 2.0H), 3.64 (s, 1.0H), 3.59 (s, 1.5H), 3.58 (s, 2.6H) 1.42 (s, 12.2H). FT-IR (neat): 3332.9, 2929.9, 2853.7, 1714.7, 1666.8, 1630.8, 1583.8, 1560.5, 1475.5, 1423.6, 1365.9, 1253.2. GPC: $M_n = 14600$ g/mol, $M_w = 27800$ g/mol, PDI = 1.90. UV-Vis (THF) $\lambda_{\text{max}} = 436$ nm, Fluo $\lambda_{\text{max}} = 461$ nm.

Synthesis of P-CPNs: The deprotection of Boc-PG-P was carried out as described in general deprotection protocol and the final yellow gel like polymers were collected and dried (66.4% yield).

^1H NMR (400 MHz, DMSO-d_6 , ppm): δ 10.03 (s, 1.3H), 9.20 (s, 1.5H), 8.60 (s, 3.5H), 7.57-7.38 (s, 4.8H), 4.38 (s, 2.0H), 4.03 (s, 2.2H), 3.80 (s, 3.8H), 3.71 (s, 4.0), 3.56 (s, 1.0).. FT-IR (neat): 3305.85, 2940.75, 1667.98, 1444.67, 1252.02, 1198.52 cm^{-1} . UV-Vis (DMSO) $\lambda_{\text{max}} = 435$ nm, Fluo $\lambda_{\text{max}} = 490$ nm, QY = 8.37.

Figure S5.5. ^1H NMR Boc-PG-P in CDCl_3 .

Figure S5.6. ^1H NMR P-CPNs in DMSO- D_6 .

Scheme S5.5. Synthesis route to A-CPNs

Synthesis of Boc-PG-A: A Schlenk flask was charged with Boc-PG-H (10.0 mg, 0.012 mmol) and aminoethoxyethanol (3.10 mg, 0.03 mmol), evacuated and filled with N_2 gas. Anhydrous THF (1.5 mL) was transferred to the Schlenk flask via a cannula. The reaction mixture was stirred at 80°C for 16 h. A viscous polymer solution was filtered through the glass wool filled in the glass pipette. The polymer precipitates were collected by transferring the solution dropwise to cold diethyl ether (1x). After settling down the polymer precipitates the supernatant was decanted. The precipitates were redissolved in DMF (1 mL), and the same purification method was repeated for three more

times using methanol as purifying solvent. The yellow gel like precipitate were dried under high vacuum (8.20 mg, 79% yield).

^1H NMR (400 MHz, CDCl_3 , ppm): δ 12.05 (s, 0.7H), 8.27 (s, 0.6H), 7.04 (s, 0.9H), 6.02 (s, 0.6H), 4.22 (s, 2.0H), 3.89 (s, 2.4H), 3.72 (s, 5.4H), 3.53 (s, 8.3H), 3.36 (s, 2.3H), 1.42 (s, 7.2H). FT-IR (neat): 3323.5, 2929.8, 2870.9, 1716.7, 1633.2, 1597.8, 1507.7, 1453.7, 1411.0, 1347.9, 1311.7, 1277.2, 1227.2. GPC: M_n = 13800 g/mol, M_w = 19600 g/mol, PDI = 1.40. UV-Vis (THF) λ_{max} = 435 nm, Fluo λ_{max} = 465 nm.

Synthesis of A-CPNs: The deprotection of Boc-PG-P was carried out as described in general deprotection protocol and the final yellow gel like polymers were collected and dried (79% yield).

^1H NMR (400 MHz, DMSO-d_6 , ppm): 9.20 (s, 0.9H), 8.51 (s, 2.0H), 7.54-7.15 (s, 3.3H), 4.47 (s, 2.0H), 4.21 (s, 2.0H), 4.03 (s, 1.4H), 3.79 (s, 3.6H), 3.69 (s, 3.4H), 3.23 (s, 4.5H). FT-IR (neat): 3267.35, 2872.85, 1788.01, 1670.04, 1547.08, 1447.00, 1346.70, 1199.44. UV-Vis (DMSO) λ_{max} = 434 nm, Fluo λ_{max} = 494 nm, QY = 19.81.

Figure S5.7. ^1H NMR Boc-PG-A CDCl_3 .

Figure S5.8. ¹H NMR A-CPNs in DMSO-D₆.

5.7.3 Physical and photophysical properties of CPNs

Absorption and emission spectra of CPNs

Figure S5.9. UV-vis and emission spectra of G-CPNs.

Figure S5.10. UV-vis and emission spectra of **D-CPNs**

Figure S5.11. UV-vis and emission spectra of **M-CPNs**

Figure S5.12. UV-vis and emission spectra of **P-CPNs**

Figure S5.13. UV-vis and emission spectra of A-CPNs

Determination of hydrodynamic (HD) of CPNs: The single particle light scattering analysis i.e., nanoparticle tracking analysis (NTA) was used to measure the hydrodynamic diameter of the CPNs. Light scattering measurement were performed with a LM10 HS (NanoSight, Amesbury, United Kingdom), equipped with sCMOS camera, sample chamber with a 488 nm blue laser and Viton fluoroelastomer o-ring. CPNs samples were prepared by diluting the stock CPNs in PBS (pH 7.4) and filtered through 0.2 μm PTFE syringe filter. The final concentration of the CPNs was 0.25-0.50 μM in a total 1 mL solution (2% DMSO and 98% PBS). The filtered sample solution was injected into the sample chamber using a 1 mL syringe (Restek Corporation, Pennsylvania, USA) until a few drops of the solution dripped out of the outlet of the chamber. The individual particles were identified by the in-built software and tracked its motion throughout the duration of the recorded video. The displacement of the particles is a function of Brownian motion, which is related to the hydrodynamic diameters of particles using Stokes-Einstein equation. All measurements were performed at 25°C using a LM14C temperature controller (NanoSight, Amesbury, United Kingdom). Each sample was measured three times.

Figure S5.14. NTA of G-CPNs

Figure S5.15. NTA of D-CPNs

Figure S5.16. NTA of M-CPNs

Figure S5.17. NTA of P-CPNs

Figure S5.18. NTA of A-CPNs

5.7.4 Cell Culture

HeLa cells (human cervical carcinoma) purchased from the American Tissue Culture Center (ATCC) were cultured in a complete media (mixture of Dulbecco's minimum essential medium (DMEM)/high glucose (10 mL hyClone, SH3024301) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Thermo Scientific)) at 37 °C in a 5% CO₂ incubator. Then, cells were sub cultured every 48 h.

5.7.5 Cellular toxicity study

The calorimetric methylthiazole tetrazolium (MTT) assay was performed to determine the cytotoxicity of CPNs. The conversion of soluble MTT into formazan is directly related to metabolic activity of cells and thereby to cell viability. 10,000 HeLa cells were seeded in 96 well plate with 200 µL day before the CPNs treatment. Various concentration (1-40 µM) of CPNs were treated with complete media and incubated for 18 h. After the treatment of MTT reagent (10 µL) and incubated for 4 h at 37 °C. 200 µL of media was removed gently using the pipette and 100 µL of DMSO was added to dissolve the purple formazan crystals. The cytotoxicity was evaluated measuring the absorbance at 570 nm using microplate reader. Cell viability of CPNs was plotted relative cell variability (%) as a function of CPNs concentration as shown in Figure S5.19 and showed limited cytotoxicity from all five CPNs after 18 h of incubation up to 40 µM of CPNs treatment. All the measurements were performed in triplicate and standard deviation was included in the error bar.

Figure S5.19. Toxicity of CPNs toward HeLa cells. Cell viability against varying concentration of CPNs is plotted as columns (n = 3).

5.7.6 Flow cytometry study

Accuri™ C6 Flow cytometry (BD Bioscience, San Jose, CA) equipped with a fluorescence detector was used to analyze the fluorescence intensity of cells incubated with CPNs and CPNs/siRNA complex. HeLa cells were seeded into a 12-well plate (~50,000/well) one day prior to CPNs treatment. To give comparative quantitative number of CPNs and siRNA uptake, two sets of flow cytometry experiments were performed. First set of experiment were performed by treating of cells only with CPNs and other set of experiment performed by treating CPNs/siRNA complex. For consistency, first set of experiment is performed by treating cells with 20 μM of CPNs for different time 10 min, 1 h, 5 h and 20 h and relative fluorescence intensity of CPNs were measured by using flow cytometry in the green channel. The second set of experiment is performed by treating cells were treated with 20 μM of CPNs and 100 nM siRNA for 10 min, 1 h, 5 h and 20 h and relative fluorescence intensity of siRNA were measured by using flow cytometry in the red channel. After incubation, cells were washed with 1x PBS (3 times) followed by detachment of

cells using TrypLE Express and resuspended with 1× PBS. After centrifugation (2000 RCF, 3 min), cells pellets were collected and fixed with 4% PFA for 10 min, then resuspended in 1× PBS. 10,000 events per measurement were recorded within the gate of control cells, which was selected based on forward and side scattering of control cells to eliminate data collection from dead cells and artifacts. The mean fluorescence intensities (average of three replicates) of cells treated with CPNs (FL1 channel, green channel), and CPNs/siRNA complex (FL3 channel, red channel), were normalized to that of untreated control cells. All measurements were triplicated and standard deviation was included in the error bar.

5.7.7 Cell uptake studies by microscopy imaging

HeLa cells were seeded into a 12-well plate (~20,000/well) with glass coverslip (#1254584, Fisher Scientific) one day prior to CPNs and CPNs/siRNA complex treatment and cultured in a complete media for 24 h under 5% CO₂ at 37 °C. The required concentration of CPNs or CPNs/siRNA complex were prepared in fresh complete media (500 µL), and added to cells after washing three times with 1× PBS and removing media, then cultured for required time. After incubation for desired time, cells were washed three times with PBS and fixed with 4% PFA for 10 min. Cells were then washed three times with 1× PBS and coverslips were mounted on microscope slides (#1125441, Fisher Scientific) using a 1:1 glycerol/PBS mounting medium. Fluorescent images of the cells were obtained using an Olympus Fluoview FV1200 confocal microscope (Melville, NY USA) equipped with a bandpass filter for green (513-556 nm) using 60X oil immersion lens (NA 1.35) and n = 1.519 immersion oil. Image J software (Version 1.50b, U.S. National Institute of Health, Bethesda, Maryland, USA) was used to process the image.

Figure S5.20. Fluorescence microscopic images of HeLa cells incubated with CPNs (20 μ M) for 5 h. The scale bar is 20 μ m.

Figure S5.21. Fluorescence microscopic images of HeLa cells incubated with different concentrations of CPNs (5, 10, and 20 μ M) for 1 h. The scale bar is 20 μ m.

Figure S5.22. Fluorescence microscopic images of HeLa cells incubated with CPNs (20 μ M) upon complexation with siGLO red (100 nM) for 6 h. The scale bar is 20 μ m.

5.7.8 Cell uptake studies under ATP depletion condition by microscopy

Cells were treated with 0.05% of sodium azide (NaN_3) in presence of 2-deoxyglucose ($25 \times 10^{-3}\text{M}$) for 15 min before CPN treatment. 20 μ M of CPNs were treated and incubated for 1 h. After incubation, cells were washed three times with $1\times$ PBS and fixed with 4% PFA for 10 min. Cells were then washed three times with $1\times$ PBS, and coverslips were mounted on microscope slides using a 1:1 glycerol/PBS mounting medium then fluorescent images of the cells were obtained.

5.8 References

- [1] A.J.C. Kuehne, Conjugated Polymer Nanoparticles toward In Vivo Theranostics - Focus on Targeting, Imaging, Therapy, and the Importance of Clearance, *Adv. Biosys.* 1(11) (2017).
- [2] K. Li, B. Liu, Water-soluble conjugated polymers as the platform for protein sensors, *Polym. Chem.* 1(3) (2010) 252-259.
- [3] U.H. Bunz, K. Seehafer, M. Bender, M. Porz, Poly(aryleneethynylene)s (PAE) as paradigmatic sensor cores, *Chem. Soc. Rev.* 44(13) (2015) 4322-36.
- [4] Y. Braeken, S. Cheruku, A. Ethirajan, W. Maes, Conjugated Polymer Nanoparticles for Bioimaging, *Materials* 10(12) (2017).
- [5] X. Feng, F. Lv, L. Liu, H. Tang, C. Xing, Q. Yang, S. Wang, Conjugated polymer nanoparticles for drug delivery and imaging, *ACS Appl. Mater. Interfaces* 2(8) (2010) 2429-35.
- [6] M. Twomey, E. Mendez, R.K. Manian, S. Lee, J.H. Moon, Mitochondria-specific conjugated polymer nanoparticles, *Chem. Commun.* 52(27) (2016) 4910-3.
- [7] M. Twomey, T. Vokata, M.R. Kumar, J.H. Moon, Differential interactions of conjugated polymer nanoparticles with glycosaminoglycans in synthetic urine, *Chem. Commun.* 51(19) (2015) 4065-8.
- [8] X. Zhu, J. Yang, K.S. Schanze, Conjugated polyelectrolytes with guanidinium side groups. Synthesis, photophysics and pyrophosphate sensing, *Photochem. Photobiol. Sci.* 13(2) (2014) 293-300.
- [9] X. Wang, F. He, L. Li, H. Wang, R. Yan, L. Li, Conjugated oligomer-based fluorescent nanoparticles as functional nanocarriers for nucleic acids delivery, *ACS Appl. Mater. Interfaces* 5(12) (2013) 5700-8.
- [10] Y. Liu, K. Ogawa, K.S. Schanze, Conjugated polyelectrolytes as fluorescent sensors, *J. Photochem. Photobiol. C* 10(4) (2009) 173-190.
- [11] J.H. Moon, E. Mendez, Y. Kim, A. Kaur, Conjugated polymer nanoparticles for small interfering RNA delivery, *Chem. Commun.* 47(29) (2011) 8370-2.
- [12] K. Krukiewicz, J.K. Zak, Conjugated polymers as robust carriers for controlled delivery of anti-inflammatory drugs, *J. Mater. Sci.* 49(16) (2014) 5738-5745.
- [13] A.T. Silva, A. Nguyen, C. Ye, J. Verchot, J.H. Moon, Conjugated polymer nanoparticles for effective siRNA delivery to tobacco BY-2 protoplasts, *BMC Plant Biol.* 10 (2010) 291.
- [14] X. Feng, F. Lv, L. Liu, Q. Yang, S. Wang, G.C. Bazan, A highly emissive conjugated polyelectrolyte vector for gene delivery and transfection, *Adv. Mater.* 24(40) (2012) 5428-32.

- [15] H. Traboulsi, H. Larkin, M.A. Bonin, L. Volkov, C.L. Lavoie, E. Marsault, Macrocyclic cell penetrating peptides: a study of structure-penetration properties, *Bioconjug. Chem.* 26(3) (2015) 405-11.
- [16] I. Nakase, G. Tanaka, S. Futaki, Cell-penetrating peptides (CPPs) as a vector for the delivery of siRNAs into cells, *Mol. Biosyst.* 9(5) (2013) 855-61.
- [17] Y. Takechi, H. Yoshii, M. Tanaka, T. Kawakami, S. Aimoto, H. Saito, Physicochemical mechanism for the enhanced ability of lipid membrane penetration of polyarginine, *Langmuir* 27(11) (2011) 7099-107.
- [18] Y. Takechi-Haraya, R. Nadai, H. Kimura, K. Nishitsuji, K. Uchimura, K. Sakai-Kato, K. Kawakami, A. Shigenaga, T. Kawakami, A. Otaka, H. Hojo, N. Sakashita, H. Saito, Enthalpy-driven interactions with sulfated glycosaminoglycans promote cell membrane penetration of arginine peptides, *Biochim. Biophys. Acta* 1858(6) (2016) 1339-49.
- [19] W.B. Kauffman, T. Fuselier, J. He, W.C. Wimley, Mechanism Matters: A Taxonomy of Cell Penetrating Peptides, *Trends Biochem. Sci.* 40(12) (2015) 749-64.
- [20] A. Kim, T.H. Shin, S.M. Shin, C.D. Pham, D.K. Choi, M.H. Kwon, Y.S. Kim, Cellular internalization mechanism and intracellular trafficking of filamentous M13 phages displaying a cell-penetrating transbody and TAT peptide, *PLoS One* 7(12) (2012) 51813.
- [21] Y. Jiang, R. Tang, B. Duncan, Z. Jiang, B. Yan, R. Mout, V.M. Rotello, Direct cytosolic delivery of siRNA using nanoparticle-stabilized nanocapsules, *Angew. Chem. Int. Ed Engl.* 54(2) (2015) 506-10.
- [22] H.J. Kim, A. Kim, K. Miyata, K. Kataoka, Recent progress in development of siRNA delivery vehicles for cancer therapy, *Adv. Drug Deliv. Rev.* 104 (2016) 61-77.
- [23] S.J. Lee, M.J. Kim, I.C. Kwon, T.M. Roberts, Delivery strategies and potential targets for siRNA in major cancer types, *Adv. Drug Deliv. Rev.* 104 (2016) 2-15.
- [24] K. Tatiparti, S. Sau, S.K. Kashaw, A.K. Iyer, siRNA Delivery Strategies: A Comprehensive Review of Recent Developments, *Nanomaterials* 7(4) (2017).
- [25] Y.J. Ko, E. Mendez, J.H. Moon, Controlled aggregation in conjugated polymer nanoparticles via organic acid treatments, *Macromolecules* 44(13) (2011) 5527-5530.
- [26] J. Kim, T.M. Swager, Control of conformational and interpolymer effects in conjugated polymers, *Nature* 411(6841) (2001) 1030-4.
- [27] C. Wu, C. B. Bull, C. Szymanski, K. Christensen, J. McNeill, Multicolor conjugated polymer dots for biological fluorescence imaging, *ACS Nano* 2(11) (2008) 2415-2423.
- [28] L.P. Fernando, P.K. Kandel, J. Yu, J. McNeill, P.C. Ackroyd, K.A. Christensen, Mechanism of Cellular Uptake of Highly Fluorescent Conjugated Polymer Nanoparticles, *Biomacromolecules* 11(10) (2010) 2675-2682.

- [29] T. Lei, J. Y. Wang, J. Pei, Roles of Flexible Chains in Organic Semiconducting Materials, *Chem. Mater.* 26(1) (2013) 594-603.
- [30] K.Y. Peng, S.A. Chen, W.S. Fann, S.H. Chen, A.C. Su, Well-packed chains and aggregates in the emission mechanism of conjugated polymers, *J. Phys. Chem. B* 109 (19) (2005) 9368-9373.
- [31] T. Vokatá, M. Twomey, E. Mendez, J.H. Moon, Synthesis of biodegradable conjugated polymers with controlled backbone flexibility, *J. Polym. Sci. A* 53(11) (2015) 1403-1412.

CHAPTER VI

General Conclusions

The facile synthetic approach with tunable photophysical properties make CPs as a superior biomaterial. CPs possess most of the desired properties for the optoelectronics and biological application such as lower cytotoxicity, excellent luminescence, biocompatibility, and easy to process. The fine tuning of CPs at the molecular level could control the structure property relationship of CPs and it can ultimately provide a better insight into the function of CPs in terms of their electronics and biomedical applications. The backbone and side chain structure of CPs are two major factors which decide the final properties of CPs. Moreover, backbone and side chain structures are the major factors we can alter during the synthesis of CPs to achieve the desired functional biomaterial. The dissertation provided an insight into how backbone modulation could affect the most essential parameter of nanoparticle formation i.e., their self-assembly (aggregation). It also provided an insight into the possible ways of side chain modulation of CPNs and their changes in cellular interaction and internalization behavior depending on the functionalities. The dissertation has been sub- categorized into two parts: The first part of the dissertation (chapters II and III) solely focused on the synthesis and understanding of the photophysical properties and their self-assembly behavior and the second part (chapters IV and V) of the dissertation focused more on the application of CPNs with different side chain functionalities for cellular studies.

Chapter II presented a successful synthesis and backbone modification of a set of four positively charged phenylene-based CPs with four different types of connectivity and backbone structures. Additionally, the chapter provided the photophysical properties and ICD effect changes of these CPs upon the complexation with HA. The preservation of the solubility and quantum yield of the CPs are always concerns when designing CPs for optoelectronics and biomedical applications. The chapter provided an easy approach to tune fluorescent properties of CPs by modulating backbone

structure and aggregation behavior. The strong interpolymer π - π interaction caused an issue with the solubility of PPB. The incorporation of small fractions of a linker within the backbone can solve the problem of insolubility of CPs. The photo-physical properties and ICD effect changes upon complexation with HA proved that these CPs (i.e., PPE and PPB-L) are capable of forming ordered helical structures depending upon the backbone structure. The idea of forming ordered helical conformation from PPE and PPB-L could be useful for designing highly ordered materials for optoelectronics and biomedical application.

The dramatic differences in photophysical properties and ICD effect of PPE and PPB-L upon complexation with HA led to extension of chapter II. So, Chapter III provided an extended idea investigation into how two different backbone structures of CPs can interact with other biologically important polysaccharides, i.e., GAGs. Depending upon the backbone structure of the CPs, the ionic strength, and the type of anionic functional group, PPE and PPB-L can selectively form helical conformation with GAGs. Out of four GAGs, three GAGs (i.e., HA, DS and CS) can successfully form helical conformation. The chapter III also detailed the thermal stability of helical complex. We found that highly sulfated GAGs (i.e., HS) is unable to induce CD effect so ordered helical structure is not observed. But less sulfated GAGs (i.e., DS and CS) and unsulfated GAGs (i.e., HA) are able to ICD and they were able to form helical structure. The helical conformation formed from with GAGs (i.e., HA, DS, and CS) with CPs (PPE and PPB-L) has different thermal stability and it depends on the type of charge present in the GAGs. This clearly showed that charge density and type of charge present on the GAGs can alter the ionic complex and their helical structure.

Chapter IV detailed the cellular study of CPNs with different side chain structure using SICM. The chapter compared the interaction of two positively charged (i.e., amine vs guanidine) CPNs with cell membrane. Their dramatically different cellular interaction behavior was described depending on the side chain functionalities. We successfully revealed that these CPNs are capable of inducing

transient pore like feature formation in the cell membrane during the internalization process. The data of pore formation are corroborated by the topographical images and surface potential changes using SICM. We examined the cellular internalization pathway and their localization inside the cells using inhibition assay and confocal microscope images. The amount of CPNs internalized inside the cell were quantified as a function of increasing time using flow cytometry and microscope images. It was found that the amount of CPNs internalized inside the cells are directly proportional to time of incubation of the CPNs.

Chapter V detailed the synthesis of a series of CPNs with guanidine motif resulting from the post polymerization modification of guanidine side chain from organic amine bases (i.e., diisopropyl amine, morpholine, piperidine, and aminoethoxyethanol). It showcased how side chain structure modification affects the cellular interaction and delivery of important biological material (e.g., siRNA for this case). Post polymerization approach is an easy and time efficient approach for the synthesis of varieties of CPNs with same backbone but different targeting functionalities in the side chain. We found that out of five CPNs with guanidine motif, CPNs modified with a hydrophilic base, i.e., aminoethoxyethanol (A-CPNs), increased the solubility and luminescent properties of CPNs and also showed better cellular internalization, cellular imaging, and high siRNA delivery capacity. The chapter included the comparative subcellular localization and cellular imaging study for a series of CPNs with guanidine motif.

Thus, the fate of CPNs are more dependent on the side chain functionalities during the cellular internalization process and backbone structures plays major role in change in photophysical properties and aggregation behavior of CPNs.

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PUBLICATIONS AND PRESENTATIONS

MANANDHAR, P.; Vokata, T.; Lee, Y.; Lee, S.; Jung, H.M.; Moon J.H., “Spectroscopic investigation of backbone-dependent ionic complexation of positively charged phenylene-based conjugated polymers and negatively charged hyaluronic acid”, Manuscript submitted under review.

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MANANDHAR, P.; Ahmed, M.S.; Moon J.H., “Modified guanidine functionalized conjugated MANANDHAR, P.; Moon J.H., “Glycosaminoglycan-assisted self-assembly of conjugated polymers”, in preparation.

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