Bioreducible Polymer Nanosystems For Gene Delivery

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BIOREDUCTIBLE POLYMER NANOSYSTEMS FOR GENE DELIVERY

by

YI ZOU

DISSERTATION

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**TABLE OF CONTENTS**

ACKNOWLEDGEMENTS .................................................................................................................... ii

Chapter 1 Introduction .......................................................................................................................... 1

Chapter 2 Literature Review .................................................................................................................. 4

2.1 Gene delivery background .............................................................................................................. 4

2.2 Synthetic polymers used in gene delivery ....................................................................................... 5

2.3 Barriers for polymeric gene delivery vectors .................................................................................. 6

2.4 Current development of synthetic gene delivery vectors .............................................................. 8

2.6 AFM imaging of gene delivery systems ......................................................................................... 11

Chapter 3 DNA release dynamics from bioreducible polyplexes ....................................................... 17

3.1 Introduction ..................................................................................................................................... 17

3.2 Materials and Methods .................................................................................................................... 21

3.2.1 Materials .................................................................................................................................... 21

3.2.2 Polyplex preparation ................................................................................................................... 21

3.2.3 AFM imaging .............................................................................................................................. 22

3.3 Results ........................................................................................................................................... 22

3.3.1 Polyplex self-assembly ............................................................................................................... 22

3.3.2 A proposed genetic disassembly route for bioreducible polyplexes ........................................... 24
3.4 Discussion ............................................................................................................... 26
3.5 Conclusion .............................................................................................................. 30

Chapter 4 DNA Release Dynamics from Polyplexes by Interpolyelectrolyte Exchange ......................................................................................................................... 32

4.1 Introduction ............................................................................................................. 32
4.2 Experimental Methods ............................................................................................ 35
  4.2.1 Materials ........................................................................................................... 35
  4.2.2 Synthesis of bioreducible poly(amido amine)s ................................................. 35
  4.2.3 Polyplex preparation ....................................................................................... 36
  4.2.4 AFM characterization ....................................................................................... 36
  4.2.5 Fluoremetry ....................................................................................................... 37

4.3 Results ..................................................................................................................... 37
  4.3.1 Polyplex assembly ............................................................................................ 37
  4.3.2 Critical counter-polyelectrolyte concentration .............................................. 38
  4.3.3 In situ real time AFM study of DNA release dynamics ................................... 41
  4.3.4 Cell transfection ............................................................................................... 46

4.4 Discussion ............................................................................................................... 47
4.5 Conclusion .............................................................................................................. 50
Chapter 5 Engineering Bioreducible Layer-by-layer Films for Sequential and Sustained DNA Delivery .................................................................52

5.1 Introduction ............................................................................................................. 52

5.2 Experimental ........................................................................................................... 56

5.2.1 Materials. ..........................................................................................................56

5.2.2 Synthesis of bioreducible PAAs. .................................................................57

5.2.3 Deposition of the LbL films.................................................................58

5.2.4 Crosslinking of the LbL films.................................................................59

5.2.5 AFM imaging...............................................................................................59

5.2.6 AFM nano-indentation force measurements........................................60

5.2.7 Ellipsometry...............................................................................................60

5.2.8 Fluorescence spectroscopy..........................................................................61

5.2.9 Dynamic light scattering (DLS). ..........................................................61

5.2.10 Transfection activities in vitro. .........................................................62

5.3 Results..................................................................................................................... 63

5.3.1 Structural analysis of the LbL films.......................................................63

5.3.2 The Effect of crosslinking on the LbL films..........................................67
A.3 Results and Discussion

A.3.1 Characterization of polystyrene particles with different surface roughness

A.3.2 Surface roughness effect on surface force profiles in the absence of the
surfactants

A.3.4 Surface roughness effect on surface force profiles in the presence of
surfactants

A.3.5 Surfactant adsorbed layer structure

A.4 Conclusion

References

Abstract

Autobiographical Statement
LIST OF TABLES

Table 5.1: Composition of LbL films and thickness measured by AFM................................. 63

Table A.1: Adsorption layer thickness, elastic modulus and adhesion force between PS colloids.........................................................................................................................99

Table A.2: Surfactant concentration and CMC........................................................................101

Table A.3: Fitting parameters of double exponential model for interaction between PS\textsuperscript{S} and PS\textsuperscript{R}.........................................................................................................................104

Table A.4: Zeta potential, hydrodynamic diameter, and adsorption layer thickness measured by dynamic light scattering.........................................................................................109
LIST OF FIGURES AND SCHEMES

Figure 2.1: Sequence of scanning images of a 3-kbp plasmid at 65% humidity. With each successive scan the plasmid becomes more detached from the substrate..................12

Figure 2.2: In-situ AFM images of the disassembly of recA filament from DNA in water............14

Figure 2.3: In situ real time AFM sequence of DNA release from (a) NLS6⁺ polyplexes and (b) HRP10⁺ polyplexes in 20 mM DTT and 0.4 M NaCl solution........................................16

Figure 3.1: A typical setup for in situ AFM operation. The AFM probe scans over immobilized polypeplexes or LbL films deposited on a 2-D surface at a minimal contact force in simulated physiologic solution...................................................................................18

Scheme 3.1: Bioreducible polymers investigated. a) Polypeptides of histidine-rich peptide (CKHHHKHHHKC) and nuclear localization signal (CGAGPKKKRKVC) peptide. b) Linear poly(amido amine) (PAA). c) Reducible hyperbranched (RHB) PAA. The R groups represent two different amide monomers. ................................................20

Figure 3.2: AFM height images of time dependence in linear PAA polyplex formation (disulfide content 15%, Mw 54500 g/mol, N/P 4). (a) 10 min and (b) 60 min. The images are captured in tapping mode in air.................................................................23

Figure 3.3: Size and morphology distribution. Polyplexes whose volume is in the range of 1–2 ×10⁴ nm³ are represented by (a–c). Polyplexes whose volume is in the range of 2–5×10⁴ nm³ are represented by (d–f).................................................................24

Figure 3.4: Real time AFM images showing (a) morphology transition from rod to toroid, (b) morphology transition from sphere to toroid, (c) particle-particle interaction and aggregation..................................................................................................................26

Figure 4.1: Fluorescence intensity as a function of the N/P ratio for RHB PAA and LPAA........38

Figure 4.2: Fluorescence assay for a) LPAA and RHB PAA/DNA polyplexes (N/P = 12), incubation time 30 min, b) PEI PAA/DNA polyplexes (N/P = 12), incubation time 30 min, with different heparin concentration...............................................................39
Figure 4.3: Time dependence of fluorescence intensity of DNA release from LPAA/DNA polyplex at various heparin concentrations.................................................................40

Figure 4.4: Time elapse in situ AFM images sequence for LPAA/DNA polyplexes (N/P=12) treated by 500μg/mL heparin for 5s then imaged in 30mM pH 4.5 acetate buffer.....42

Figure 4.5: a) Volume change and b) release percentage, during the release shown in Figure 4.4. Each data point represents the average value of the same five monomolecular polyplexes in the field.................................................................................................43

Figure 4.6: Time elapse in situ AFM images sequence for LPAA/DNA (N/P=12) treated by 500 μg/mL heparin for 5s then imaged in 30mM PH4.5 acetate buffer............................44

Figure 4.7: Time elapse in situ AFM images sequence for RHB PAA/DNA polyplexes (N/P=12) treated by 500 μg/mL heparin for 5s then imaged in 30mM pH 4.5 acetate buffer. .........................................................................................................................45

Figure 4.8: Volume change during the release shown in Figure 4.7. Each data point represents the average value of the same five monomolecular polyplexes in the field............45

Figure 4.9: Luciferase transfection of NIH 3T3 cells by RHB PAA and LPAA polyplexes. N/P ratio was 12 for both polyplexes. Cell culture media contained 10% FBS............47

Scheme 5.1: Molecular structure of bioreducible PAAs. Composition of R₁ change represents different reducible monomer content and change of R₂ represents linear or hyperbranched chain architecture.................................................................54

Scheme 5.2: Structure and composition of type A and type B films.................................................................56

Figure 5.1: a) Automated slide dipper based on Carl Zeiss HMS50 slide stainer. b) Customized Teflon substrate baskets made by Dr. Song Xu.................................................................59

Figure 5.2: Film thickness as function of number of bi-layers measured by AFM and ellipsometry. Linear fitting for film B1 shows slop = 4.1 nm per bilayer...............64
Figure 5.3: AFM height image of a) film A1, and b) film B1.................................66

Figure 5.4: RMS roughness as a function of number of bi-layers measured by AFM for film A1 and film B1. Lines are linear fitting..........................................................66

Figure 5.5: Time lapse images obtained by *in situ* AFM showing the thickness change of film A1 after immersing in 20 mM DTT.................................................................69

Figure 5.6: AFM images measured in air showing the thickness change of film B1 after immersing in 20 mM DTT..........................................................71

Figure 5.7: Thickness change as function of time in degradation solution measured by AFM for a) film A1, b) for film B1..........................................................72

Figure 5.8: Thickness change as function of time in degradation solution measured by AFM for a) film A1, b) for film B1..........................................................73

Figure 5.9: Hydrodynamic diameter of degradation product measured by DLS...............74

Figure 5.10: Fluorescence intensity of degradation product for a) film A0; b) film A1, where the bottom half is made of TRITC labeled PAA while top half is made of FITC labeled PAA; c) film B1..........................................................75

Figure 5.11: GFP transfection of HEK 293 cells on film B1 with fibronectin top coating showing improved transfection.........................................................77

Figure 5.12: AFM images of film B1 after culturing with a) NIH 3T3 cells or b) HEK 293 cells for 7 days. The thickness of the film is 29.2 nm for a) and 31.8 nm for b)........77

Figure 5.13: Luciferase transfection of HEK 293 cells with A3 film and A5 film coated suture..........................................................78

Figure 5.14: Optic microscope image for immature dendritic cells growing on A3 film coated suture after 3 days..............................................................79
Figure 5.15: In vivo transfection of mice using Lbl film coated suture and electroporation

Figure A.1: Chemical structures of C12E5 (top), Pluronic F108 (middle), and Joncryl 60 (bottom).

Figure A.2: SEM image of a PS colloidal probe.

Figure A.3: AFM height images of the PS spheres: a–b) untreated colloids (scan size and z range for a) are 15µm and 2µm and for b) are 750 nm and 20 nm), c–d) colloids heated for 4 h (scan size and z range for c) are 20 µm and 2 µm and for d) are 1 µm and 20 nm, and e–f) colloids heated for 12 h (scan size and z range for e) are 20 µm and 5µm and for f) are 1.5 µm and 30 nm).

Figure A.4: Force curves between PS^S (or PS^R) and mica in deionized water and 1 mM NaCl. The experimental data are fitted with the DLVO theory as represented by the solid lines.

Figure A.5: Force versus Separation curves for PS^S and PS^R.

Figure A.6: Surface tension measurement for Joncryl 60.

Figure A.7: Force measurement and double exponential fitting for PS^S in 5% Pluronic F108 solution in logarithm scale.

Figure A.8: a) Indentation vs. Force curves measured by nanoindentation experiment in various surfactant solutions.
Chapter 1

Introduction

The promise of gene therapy to treat a variety of genetic and acquired diseases has fueled research on gene delivery systems. Non-viral gene carriers, especially synthetic polymers, such as poly(ethylenimine) (PEI)\(^1\) and poly(L-lysine) (PLL),\(^2\) are attractive alternatives to viral carriers because they show lower safety risks and can be tailored to specific therapeutic needs. However, a major problem of polymeric gene delivery carriers is their low transfection efficiency. One strategy to enhance gene delivery efficiency while maintain the safety is to use stimuli-responsive polymers, which are sensitive to pH changes or redox gradients. Bioreducible polymers are a series of polymers that contain disulfide bonds, which are bioreducible by reducing agents such as glutathione inside the nucleus or thiol-containing membrane proteins.\(^3\)\(^,\)\(^4\) The disulfide linkers in the bioreducible polymers are cleaved during the thiol-disulfide exchange reaction resulting in the degradation of the polymer and the release of drug or gene.

Bioreducible poly(amido amine)s (PAAs) containing the disulfide bond have demonstrated redox-sensitive behavior, reduced cytotoxicity, and enhanced gene delivery efficiency than the non-reducible polycations.\(^3\)\(^,\)\(^4\) In this dissertation, PAAs were used as the major component of non-viral gene delivery carriers. They were synthesized via Michael addition copolymerization. By varying the reaction condition, a series of PAAs were obtained with different polymer structures, \(e.g.,\) disulfide content, molecular weight, and molecular architecture.

There are two ways that PAAs can be used for gene delivery. Mixing of the polyanion (DNA) and polycation (PAA) in aqueous solution results in a homogeneous
solution containing nano-sized polyplexes. Polyplexes are capable of providing systematic delivery via intravenous injection.\(^5\) On the other hand, alternative dipping of a substrate in PAA and DNA solutions produces Layer-by-Layer (LbL) films. LbL films provide sustained and localized delivery,\(^6\) and they are ideal coatings for biomaterials due to the ease of assembly on a variety of substrates and substrate geometries.

Current research in this field is still dominated by "black-box" strategies that just test reporter gene expression levels of various formulations. The goal of this dissertation is to establish the correlation between the physiochemical property of the polymer, fabrication process of gene delivery carrier, and gene delivery efficiency.

In this dissertation, Chapter 2 presents the background and literature review on the subject in four aspects. The first aspect is about gene delivery background. The second aspect overviews synthetic polymers used for gene delivery. The third aspect is the barriers for polymeric gene delivery vectors. The forth aspect is the current development on synthetic gene delivery vectors, and how other researchers overcome these barriers.

Chapter 3 summarizes the experimental methods used in this dissertation, especially focusing on \textit{in situ} real time AFM and direct force measurements. Other techniques such as dynamic light scattering, fluorimetry, gel electrophoresis, and cell transfection are also included.

Chapter 4 describes the study of DNA release dynamics from biodegradable polyplexes. The release is triggered by a redox gradient and depolymerization of biodegradable polymer. The DNA release process is revealed at single molecule level in simulated physiological solution using \textit{in situ} real time AFM.
Chapter 5 demonstrates DNA release dynamics from polyplexes via interpolyelectrolyte exchange mechanism, which is one of the barriers for gene delivery. *In situ* AFM shows it shares a similar morphological pathway with depolymerization induced DNA release. Different polyplexes were investigated and the *in vitro* transfection results suggest that the one with a higher resistance to inter-polyelectrolyte exchange tends to have higher transfection efficiency.

Chapter 6 describes the research on bioreducible LbL films. It shows that interlayer diffusion is an important factor for the film growth and degradation behavior. By using highly charged and non-diffusible PEI as barrier layers, the DNA release profile was improved. Together with cross-linking and cell interaction ligands, the *in vitro* transfection efficiency and cell adhesion were improved. Attempts were also made to delivery cancer vaccine to mice using LbL film-coated suture.

Appendix illustrates the influence of nanoscale surface roughness on colloidal force measurements. This research used colloidal probe and direct force measurement technique based on AFM. It shows a great potential in pigment dispersant research for industrial application. This work is in part supported by BASF.
Chapter 2

Literature Review

2.1 Gene delivery background

Inspired by viruses, the first attempt of gene therapy was made in 1970’s. Viral gene delivery vectors can be divided into two categories: the one is integrating vector, which is capable of providing life-long expression of the transgene, while the other is non-integrating vector, which provides temporary transfection. Both of the viral vectors requires incorporation of viral genome with transgene sequences. Such recombinant viruses may be toxic to the target cells or have the potential of inducing side effects, including immunological reactions. After decades of research, viral vectors still have several severe problems including delivery capacity, toxicity, immune response, residual pathogenicity, and cause of secondary carcinogenesis. Usually, viral vectors inherit the high efficiency from the virus, but the safety is one of the major concerns. In some cases, the toxicity and immunological reaction could be deadly. For example, a patient enrolled in a gene therapy trial died after received viral vector administration at the University of Pennsylvania, Philadelphia caused by massive immune response.

As a result, the focus of gene delivery research is shifting from viral vectors to non-viral vectors. Non-viral based gene delivery uses DNA alone or complexed to cationic lipids or polymers. DNA itself has very low transfection efficiency and requires as high as $10^6$ DNA copies to transfect a single cell. By employing microneedle or electroporation, naked DNA can be delivered to cells directly. Because extracellular barriers are avoided, the delivery efficiency is greatly enhanced. However, both methods can only deliver DNA to limited number of cells, which limits the application.
2.2 Synthetic polymers used in gene delivery

Due to the drawbacks of viral vectors, non-virus based gene delivery vectors become attractive alternatives. There are two major categories of non-viral gene delivery vectors: cationic lipids and cationic polymers (polycations). The cationic charge provides electrostatic interaction with negatively charged DNA molecule, resulting in the condensation of DNA chain and formation nano-sized particles that can be uptaken by cells. The DNA/cationic lipid complex is also called lipoplex, which shields the DNA by amphiphilic lipid bilayers. Successful transfections using cationic lipids have been reported, and there are commercial products available such as Lipofectamine 2000.

On the other hand, polycation complexes with DNA to form the polyplex, and the polyplex is stabilized by excess cationic charges. Various polymers have been reported as candidates of gene delivery carriers, including PEI, PLL, PAA, et al. The relative low transfection efficiency comparing to viral vectors is still a major challenge for polymeric gene delivery vectors. The use of stimuli-responsive polymers for gene delivery represents a major advance to improve gene delivery efficiency and further reduce cytotoxicity. Polymers containing the disulfide bonds are bioreducible by the reducing agents in sub-cellular compartments. For example, the disulfide bonds are cleavable by glutathione inside the nucleus as well as thiol groups on membrane proteins. Upon the disulfide-thiol exchange reaction the high-molecular-weight polycations are degraded into low-molecular-weight oligocations. The lower binding affinity of oligocations with DNA allows the release of DNA.
2.3 Barriers for polymeric gene delivery vectors

The entire gene delivery pathway faces a number of barriers causing low delivery efficiency. These barriers can be classified as either extracellular barriers or intracellular barriers. Before gene delivery vectors reach the targeting cells, the extracellular barrier is the instability of delivery vectors and DNA in the extracellular space. The poor correlation between \textit{in vitro} and \textit{in vivo} transfection efficiency results suggests that extracellular barriers can greatly hinder gene delivery.\(^{29}\) The interpolyelectrolyte interaction between polycation vectors and polyanions such as serum proteins, and soluble glycosaminoglycans may release DNA from polyplexes before entering into cells. Ruponen \textit{et al.} shows DNA release prior to cell entry occurs in various gene delivery systems, which interferes with transfection.\(^{30}\) Oupicky \textit{et al.} used real-time PCR to follow the DNA concentration in blood showing that foreign DNA was cleared from plasma in several minutes.\(^{31}\) Fluorimetry study of polyplex stability by Dash \textit{et al.} shows protein binding to polyplexes as the main reason for failure of polyplexes to delivery DNA. In another study by Oupicky \textit{et al.} albumin was shown to bind to block copolymer of N-(2-hydroxypropyl)-methacrylamide with 2-(trimethylammonio)ethyl methacrylate polyplexes without fully releasing DNA.\(^{32}\)

For \textit{in vivo} delivery, selective targeting of specific cell or organ is also essential, because it is very likely that gene delivery vectors will be taken up by immune cells or be trapped in liver, spleen or lung through the reticuloendothelial system (RES).\(^{31}\) This may be another reason of the discrepancy between \textit{in vivo} and \textit{in vitro} performance for a particular vector. It is reported that abdominal epidermis showed highest transfection among all five tissues (skin epidermis, dermis, muscle, liver, and pancreas).\(^{33}\) By study
the distribution of cationic liposome-DNA complexes in 29 organs, it is found that endothelial cells, leukocytes, and macrophages have the highest uptake.\textsuperscript{34} Reporter gene expression were generally consistent with the pattern of uptake by endothelial cells, while tumor tissue maybe targeted due to its increased vascular growth.\textsuperscript{34} \textit{In vivo} transfection in the lung can be one to two orders of magnitude higher than in other organs,\textsuperscript{35} and uptake of DNA into the tumor depends on vascularization of the tumor while necrosis and macrophage infiltration may facilitate degradation of the DNA.\textsuperscript{36} Virus-based delivery vectors also showed remote expression in liver and testis that far away from where gene delivery vector was administrated.\textsuperscript{37}

Previous study shows both physiochemical properties\textsuperscript{38, 39} and biological activity of the gene delivery vectors can greatly affect the cellular uptake,\textsuperscript{40} thus determine the gene delivery efficiency.\textsuperscript{40} For example, size is critical for both \textit{in vivo} and \textit{in vitro} delivery, supercoiled pDNA performs better than open circular linear-pDNA counterparts because of its smaller polyplex size. Circular plasmid is more efficient than linearized plasmid DNA formulated by single-site digestion and smaller linear gene cassette generated by PCR.\textsuperscript{41-43} Smaller plasmids also show higher cell uptake and transfection in mesenchymal stem cells and neural stem cells.\textsuperscript{44} On the other hand, charge also determines the bias of biodistribution.\textsuperscript{45} Non-specific electrostatic interaction mediated delivery greatly prefers positively charged vectors. It is found that even with several millivolts of positive surface potential, the cellular uptake is magnitude higher than negatively charged one.\textsuperscript{46} Adding biologically active peptides is shown to be effective to enhance the delivery of PEI/DNA polyplex and expression in lung, liver and spleen.\textsuperscript{47, 48}
Major intracellular barriers include endosomal escape, cytoplasmic transport, and nuclear entry.\textsuperscript{29,49-51} The success of PEI as polymeric gene delivery partially contributes to the proton sponge effect that breaks down the endosome by protonation in acidic environment.\textsuperscript{52} By enhancing the capability of endosomal escape, the gene delivery efficiency can also be improved.\textsuperscript{51} The intracellular trafficking is determined by various factors and is still under extensive investigation.\textsuperscript{53} The use of ligands that have specific biological function is reported to be effective in directing intracellular trafficking.\textsuperscript{22,54}

\textbf{2.4 Current development of synthetic gene delivery vectors}

Considering the complexity of gene delivery process, the synthetic gene delivery vectors are evolving from naked DNA to binary complex with cationic lipids or polymers to multifunctional vectors.

First of all, several strategies have been developed to increase the stability of gene delivery vectors. For example, inspired by the well established drug delivery strategy, hydrogel was used to encapsulate polyplexes.\textsuperscript{55} Even with naked DNA, after hydrogels encapsulation and the delivery efficiency was improved.\textsuperscript{55} Trentin, D et al.\textsuperscript{56} reported that fibrin hydrogel encapsulated PLL/DNA polyplex shows further enhanced gene delivery efficiency. In addition, Lei, Y et al.\textsuperscript{57-59} showed enzymatically degradable PEG, HA acid, PEG, and fibrin hydrogels are effective in promoting polyplex-based gene delivery. This method is also capable of localized delivery similar to the LbL film method. Other hydrogel components reported include collagen\textsuperscript{55}, Pluronic-hyaluronic acid\textsuperscript{60}, PVA\textsuperscript{61}, PEG-poly(lactic acid)-PEG\textsuperscript{62}, alginate\textsuperscript{63}, oligo(polyethylene glycol) fumarate.\textsuperscript{64} Liposomes also could be used as encapsulation agents to protect the polyplex.\textsuperscript{65} This combines the advantages of both polycation based non-viral gene delivery and cationic
liposome based gene delivery systems. In the most recent study of liposome protected PEI/DNA polyplex by Schäfer, J et al. the stability of the polyplex is also improved by coating, which decreases binding with extracellular components such as albumin and increases transfection activity. The stability can also be improved by cross-linking, which links the amine groups of the polycation with cross-linking agents such as dithiobis(succinimidylpropionate) (DSP) and disuccinimidyl suberate (DSS). Cross-linking can also be applied to LbL films. The changes in mechanical strength and hydrophobicity by cross-linking result in better cell adhesion and cell growth.

Cellular uptake is one of the key steps to achieve gene delivery. It can be enhanced by tailoring the physicochemical properties of the gene delivery systems for targeted cells. For example, by increasing the content of linoleic acid, which results in hydrophobicity of polyplex, cellular uptake is increased by more than three folds. In order to promote cellular uptake, another popular strategy is to incorporate biological ligands with gene delivery vectors. It is reported that transferrin targeting peptide B6 can greatly increase the cellular uptake of polyplexes. HA is another widely used ligand that is mediated by the CD44 receptor. Cell-binding ligand transferrin (Tf) and epidermal growth factor (EGF) are also reported to be effective in promoting cellular uptake. For LbL films, triggering ligand-receptor interaction by using galactosylated chitosan is reported to be another effective method for improving the cellular uptake of DNA from the LbL film.

Peptides with biological activity are proven to be effective. For example, nuclear localization signal peptide shows the capability of enhancing access, and melittin promotes vesicular escape and enhanced nuclear entry.
Similar strategies mentioned above can also be used to improve the design of polymer prior to polyplex or LbL film fabrication. HA and PEG are two widely studied modification reagents. HA could be grafted to the PLL chain using NaBH$_3$CN as a reducing agent.\textsuperscript{81} It is also reported that HA could be used to modify PEI using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).\textsuperscript{82} This study confirms that HA receptor mediated endocytosis could improve intracellular gene delivery. PEG modification is also called PEGylation. Basically, there are two groups of PEGylation reagents, alkylating PEGs and acylating PEGs.\textsuperscript{83} All these reagents have the capability to react with functional amido groups with different selectivity and preferential reaction conditions. For Alkylating PEGs, PEG aldehyde prefers to react with $\alpha$-and $\varepsilon$-amino groups in protein at neutral or mild alkaline pHs, while good selectivity for the $\alpha$-amino terminal amino acid at pH 5~6. Tresylated-PEG reacts with amines around pH 8 and low temperature. PEG epoxide prefers to react at high pH between 8.5 and 9.5 and it is preferred for amino conjugation, but still capable to react with hydroxyl, imidazole and thiol groups. For acylating PEGs, hydroxysuccinimidyld esters (OSu) activated alkyl acids are highly reactive towards amino groups, but the reaction rate greatly depends on the structure of the polymer chain; PEG-p-nitrophenylcarbonate and PEG-trichlorophenylcarbonate react much slower than OSu activated carboxylate-PEG; PEG-oxycarbonylimidazole and PEG-benzotriazole carbonate also could yield carbamate linkages. The PEGylation reagent used in our study is mPEG-acrylate, which has been demonstrate as an effective PEGylation agent for PAA\textsuperscript{84}, PEI\textsuperscript{85} and PLL.\textsuperscript{86, 87} Another attractive benefit of PEGylation is that PEG could serve as a universal connector enabling further conjugation with other functional groups and signal sequences.\textsuperscript{88}
2.6 AFM imaging of gene delivery systems

In order to establish the correlation between physiochemical properties of the polymeric vector and its gene delivery performance, AFM was used in this dissertation to reveal the process in simulated physiological condition at nanometer scale. AFM was invented in 1982\textsuperscript{89} at IBM Research -Zurich by G. Bennig and H. Rohrer who earned Nobel Prize in 1986. Because there is no high energy electron involved, AFM is non-destructive and could be performed on nonconductive materials either in air or in solution. Some alternative techniques such as Cryo-TEM are also capable to visualize nucleic acids in time-resolved level.\textsuperscript{90} But, due to the destructive sample preparation process and limitation of TEM operation condition, it is not able to provide intact structure information and in-situ release dynamics.

There are two kinds of basic operation methods--contact mode and tapping mode or so-called AC mode. In the contact mode, AFM is operated at constant deflection so that the interaction force between sample and tip is constant. In the tapping mode the tip is oscillating at resonance frequency and scanning across the surface with a constant damped amplitude. The interaction force in the shear direction can be maintained at a lower magnitude in the tapping mode in solution to make it more suitable for soft biological samples.

The tapping mode in liquid provides the unbeatable advantage for dynamic observation of biological samples at single molecular level. It was first invented by Putman \textit{et al.}\textsuperscript{91} in 1994 and applied to biomembrane study a few months later.\textsuperscript{92} The most attractive feature is the ability to monitor nanoscale features in real time and biologically relevant conditions. Several research groups started to use this feature in nucleic acid
study right after the AFM was commercially available.\textsuperscript{93-96} DNA structure was investigated in the presence of water, which is not possible for SEM and TEM. As shown in Figure 2.2, DNA structure was investigated at high relative humidity (rh). Three sequential images were obtained at 65\% rh. Silicon nitride AFM tip moved DNA molecule from side to side slightly at first Fig. 2.2A and then more pronouncedly (Fig. 2.2B and Fig. 2.2C). By the fourth image the plasmid disappeared. Probably it has been swept aside by the AFM tip. In contrast, DNA molecules could remain on mica surface after repeated AFM scans when humidity is below 45\% rh.

![Figure 2.2](image)

\textbf{Figure 2.2} Sequence of scanning images of a 3-kbp plasmid at 65\% humidity. With each successive scan the plasmid becomes more detached from the substrate. Z range is 4 nm. (Reprinted with permission from \textit{Bustamante, C.; Vesenka, J.; Tang, C. L.; Rees, W.; Guthold, M.; Keller, R., Circular DNA molecules imaged in air by scanning force microscopy. Biochemistry 1992, 31 (1), 22-26. Copyright (1992) American Chemical Society.})

Later, \textit{in situ} real time AFM was extended to the study of DNA condensation dynamics, which has significant meaning on gene therapy.\textsuperscript{97} The result illustrates formation process of polyplexes in real time. Condensates were prepared by mixing 20 µl
of PEG-modified PAA solution (138 µg/ml) with 20 µl of DNA solution (20 µg/ml). Then 20 µl of polyplex solution was deposited onto freshly cleaved mica after 5 min incubation. Imaging was conducted in pH 7.4 phosphate-buffered saline. This is the first time that condensation dynamics of polyplex is revealed in molecular level. One interesting discovery is that the toroid structure is formed by fusion of the ends of a curled plectonemic condensate.

The reverse process--DNA complex dissociation was also studied using real time *in situ* AFM reported by Li, B. S et al.\(^9\) In this case, DNA was bonded with RecA protein in the presence of ATP\(_\gamma\)S, which performed as a cofactor. After removing ATP\(_\gamma\)S from solution, the complex started to disassemble. The dynamics is shown in Figure 2.3. The morphological pathway for this disassembly started from dissociation at multiple sites with gaps formation, and then the gaps expanded while new gaps were formed as well.
Figure 2.3 In-situ AFM images of the disassembly of recA filament from DNA in water. (Reprint with permission from B.S. Li, B.D. Sattin, and M.C. Goh, Direct and Real-time Visualization of the Disassembly of a Single RecA-DNA-ATPyS Complex using AFM Imaging in Fluid. Nano letters, 2006, 6(7), 1474-1478. Copyright (2006) American Chemical Society.)

The microscopic view of DNA release provides a means to correlate molecular and nanoscale attributes of the complexes with \textit{in vitro} and \textit{in vivo} gene delivery results. Our group studied DNA release dynamics on bioreducible polypeptide/DNA polyplexes.
The AFM result shows that there are two different release mechanisms between histidine-rich polypeptide HRP and nuclear localization signal polypeptide NLS. The in situ AFM images were captured continuously in simulated physiologic conditions with DTT as the reducing agent. Figure 2.4 shows the time-lapse DNA release sequences from the two polyplexes under identical release condition (0.2 M NaCl and 20 mM DTT). The difference is attributed to the charge valence of the reduced oligocation fragments (NLS$^{6+}$ vs. HRP$^{10+}$) The NLS polyplexes release DNA abruptly regardless of their initial size and morphology. Meanwhile, HRP polyplexes release DNA gradually and incompletely. In other words, DNA release from NLS polyplexes displays an abrupt and size-independent disassembly mechanism while DNA release from HRP polyplexes displays a cooperative and size dependent disassembly mechanism.
Figure 2.4 In situ real time AFM sequence of DNA release from (a) NLS6\(^+\) polyplexes and (b) HRP\(^{10+}\) polyplexes in 20 mM DTT and 0.4 M NaCl solution. Time zero corresponds to the addition of DTT. The z range is 10 nm. Scan size is 2.6 \(\times\) 2.6 \(\mu\)m\(^2\) for (a) and 2.0 \(\times\) 2.0 \(\mu\)m\(^2\) for (b). (Reprinted with permission from Wan, L.; Manickam, D. S.; Oupicky, D.; Mao, G. Z., DNA Release Dynamics from Reducible Polyplexes by Atomic Force Microscopy. Langmuir 2008, 24, (21), 12474-12482. Copyright (2008) American Chemical Society.)
Chapter 3

DNA release dynamics from bioreducible polyplexes

3.1 Introduction

AFM has become the microscope of choice for the investigation of biological, biophysical, biochemical, and biomimetic processes. AFM is non-destructive and is capable of imaging both dry and wet samples. Furthermore, its application has been extended to investigating physiochemical properties of thin films,\textsuperscript{99,100} lipid bilayers,\textsuperscript{101} live cells,\textsuperscript{103,104} and other biological samples.\textsuperscript{105,106} In contrast to the electron microscopes such as cryo-TEM,\textsuperscript{90} which is another widely used tool to obtain structural understanding at the nanometer scale, biological sample preparation for AFM is relatively straightforward permitting intact nanostructure to be imaged in biologically compatible solution environment. AFM has been used extensively to study morphology, adsorption, and condensation of nucleic acids for more than two decades.\textsuperscript{93-96} \textit{In situ} real-time AFM has also been reported in the studies of DNA condensation\textsuperscript{97} and release\textsuperscript{98} dynamics. AFM has contributed significant knowledge to DNA condensation and release dynamics in nano-systems.

There are two basic AFM operational methods, the Contact Mode and Tapping Mode (also called the AC Mode). In the Contact Mode, AFM operates at a constant deflection so that the interaction force between the sample and probe is constant. In the Tapping Mode, the AFM probe oscillates near its resonant frequency and scans the surface with constant damped amplitude. Due to its oscillatory nature, the Tapping Mode usually provides clearer images for soft biological samples,\textsuperscript{91} especially in solution.\textsuperscript{92} By imaging continuously in solution, it becomes possible to monitor the disassembly of
DNA/polymer assemblies in real time and at the molecular scale. Figure 3.1 shows the *in situ* AFM setup for DNA release studies.

**Figure 3.1** A typical setup for *in situ* AFM operation. The AFM probe scans over immobilized polyplexes or LbL films deposited on a 2-D surface at a minimal contact force in simulated physiologic solution.

The promise of gene therapy to treat a variety of genetic and acquired diseases has fueled research on gene delivery nano-systems. Virus-based gene delivery nano-systems have shortcomings including delivery capacity, toxicity, immune response, residual pathogenicity, and cause of secondary carcinogenesis.\(^7\) Nonviral gene carriers, especially positively charged synthetic polymers, such as poly(ethylenimine) (PEI)\(^1\) and poly(L-lysine) (PLL),\(^2\) are attractive alternatives because they show lower safety risks and can be tailored to specific therapeutic needs. But a major challenge for nonviral gene carriers is their low transfection efficiency. Currently the field is dominated by “black-box” strategies that test reporter gene expression levels of various formulations. A critical question to the overall gene delivery efficiency is how and when plasmid DNA is dissociated from its complexes with polycations.\(^22,107-111\) Tools such as AFM that allow correlating physiochemical properties of gene delivery carriers with *in vitro* and *in vivo* gene delivery results will provide understanding that leads to fine tuning of the gene delivery efficiency.
One strategy to enhance gene delivery efficiency while maintain cytotoxicity is to use stimuli-responsive polymers, \textit{i.e.}, bioreducible polymers.\textsuperscript{3, 4} Scheme 3.1 lists examples of bioreducible polymers that have been used in our previous studies. These polymers contain disulfide bonds that are bioreducible by redox agents such as glutathione inside the nucleus or thiol-containing membrane proteins. The disulfide linkers in the bioreducible polymers are cleaved during the thiol-disulfide exchange reaction. High molecular weight polycations are degraded into low-molecular-weight oligomers with lower binding affinity to the DNA thus allowing it to be released from the polyplexes. This chapter focuses on recent advances in AFM imaging of the DNA release processes from polyplexes containing bioreducible PAA. 1,4-dithiothreitol (DTT) is used to simulate the reducing environment \textit{in vivo}.\textsuperscript{28}
Scheme 3.1 Bioreducible polymers investigated. a) Polypeptides of histidine-rich peptide (CKHHKHKKHHC) and nuclear localization signal (CGAGPKKKRKVC) peptide. b) Linear poly(amido amine) (PAA). c) Reducible hyperbranched (RHB) PAA. The R groups represent two different amide monomers. \(N,N'\)-cystaminebisacrylamide (CBA) contains the disulfide bond while \(N,N'\)-methylenebisacrylamide (MBA) does not. d) Cross-linked poly(2-dimethylaminoethyl methacrylate) (rPDMAEMA).
3.2 Materials and Methods

3.2.1 Materials

Plasmid DNA vectors, gWiz High-Expression GFP plasmid (6.7 kb) and gWiz High-Expression Secreted Alkaline Phosphatase (SEAP) plasmid (5.8 kb), are purchased from Aldevron. Dithiothreitol (DTT, Sigma), 1-(2-aminoethyl)piperazine (AEPZ, Aldrich), 1-methylpiperazine (Aldrich), N,N’-methylenebisacrylamide (MBA, Aldrich), and N,N’-cystaminebisacrylamide (CBA, Polysciences) are purchased in the highest commercially available purity and used without further purification. Hyperbranched and linear bioreducible PAAs are synthesized via Michael addition copolymerization. Bioreducible poly(2-dimethylaminoethyl methacrylate)s are synthesized via reversible addition–fragmentation chain transfer polymerization. 1,5-diiodopentane (DIP) is purchased from Acros Organics and used without further purification. Water is deionized to 18 MΩ×cm resistivity using the Nanopure system from Barnstead. Grade V5 muscovite mica is purchased from Ted Pella. Polished n-type silicon wafers (resistivity 50-75 Ω cm) are purchased from Wafer World.

3.2.2 Polyplex preparation

Concentrated DNA stock (1 g/L) is diluted in 30 mM acetate buffer (pH 4.5 or 5.0). DNA solution containing 20 mg/L DNA is used to prepare all polyplexes at various N/P ratio (amine-to-DNA phosphate molar ratio). The polymer solution is added to the DNA solution and mixed by vortexing at 3200 rpm (Fisher Scientific Vortex Mixer) for 10 s, then the solution is incubated at room temperature for 30 min following previously developed procedures.
3.2.3 AFM imaging

AFM imaging is conducted using Multimode IIIa from digital instrument and Dimension 3100 AFM from VEECO. Polyplexes are immobilized on freshly cleaved mica and extra solute is removed by rinsing with deionized water for three times. Tapping mode in liquid is performed using silicon nitride probes (NP type, Veeco) with a nominal radius of curvature of 20 nm and cantilever spring constant of 0.38 N/m as provided by the manufacturer. Usually, the polyplexes are imaged in 50 µL simulated physiological solution. The surface is imaged continuously at an average rate of 1–2 Hz on a 2×2 or 5×5 µm² area until no significant changes are observed. The ranges of frequency, amplitude, integral, and proportional gains used are 7.5-8.5 kHz, 0.5−1 V, 0.5−2, and 0.75−3 respectively. For LbL films, disassembly is conducted in DTT solution (pH 5–7, salt concentration 0–0.2 M) prior to imaging, and the samples are imaged in air. All AFM images are analyzed using Nanoscope software version 5.12b by Veeco.

3.3 Results

3.3.1 Polyplex self-assembly

As shown in Figure 3.2, AFM images illustrate the morphology evolution with reaction time (incubation time). The result suggests that the self-assembly of polyplexes is kinetics dominated.22, 23 Rods are favorable at the beginning of the reaction while toroids emerge later. This agrees with a previous study, which reported the kinetically dominated polyplex formation phenomenon using transmission electron microscope (TEM).114 In order to maximize characteristic toroid population, 30 min incubation is used for most of the study. For a 6732 bp plasmid DNA, monomolecular polyplex theoretical volume is calculated to be $1.4 \times 10^4$ nm³ by assuming interhexagonal
separation between a neighboring polycation/DNA chain of 2.7 nm. Comparing to the histogram of bearing volume analysis for RHB/DNA polyplex, it can be concluded that most of the polyplexes only contain a single DNA chain with three characteristic morphologies, which are toroid, rod and spheroid (Figure 3.3).

Figure 3.2 AFM height images of time dependence in linear PAA polyplex formation (disulfide content 15%, Mw 54 500 g/mol, N/P 4). (a) 10 min and (b) 60 min. The images are captured in tapping mode in air. The scan size is 2 µm, and the z range is 10 nm. (Reprinted from Wan, L. et al. J. Phys. Chem. B 2009, 113, (42), 13735-13741. Copyright (2009) American Chemical Society.)

This kinetically dominated process is also indicated by invariant size and morphology distribution of polyplex regardless of polycation molecular weight (25 000–130 000 g/mol), chain architecture (linear vs. hyperbranched), and disulfide content (0–100% CBA). At the same incubation time, the average outer diameter of toroids remains to be 100 nm, which is also supported by light scattering data. In addition, colloidal stability of polyplexes benefits from their highly charged surface, which is measured to be larger than +40 mV.
Figure 3.3 Size and morphology distribution. The scan size is 350 nm. The z range is 15 nm for (g–i) and 8 nm for all others. Polymers whose volume is in the range of $1–2 \times 10^4$ nm$^3$ are represented by (a–c). Polymers whose volume is in the range of $2–5\times 10^4$ nm$^3$ are represented by (d–f). (Reprinted from Wan, L. et al. J. Phys. Chem. B 2009, 113, 13735-13741. Copyright (2009) American Chemical Society.)

3.3.2 A proposed genetic disassembly route for bioreducible polyplexes

Another in situ AFM study on DNA release triggered by polycation degradation has been conducted with bioreducible PAA polyplexes. Recently, bioreducible PAAs have become promising DNA delivery vectors because of their low cytotoxicity and high transfection efficiency.

The PAAs are synthesized via Michael addition copolymerization reaction. In addition to their potential pharmaceutical application, by varying the feed ratio of three...
monomers, a series of bioreducible PAAs with several variables are obtained providing a good model to study the correlation between their physiochemical properties and biological activity. Despite of the variation in disulfide content, molecular weight, and polymer chain architecture, a common morphological route of DNA release has been observed.

The genetic morphological pathway of polyplexes is visualized by *in situ* AFM (Figure 3.4). Because of depolymerization induced by thiol-disulfide exchange reaction, high-molecular-weight polycation is converted to low-molecular-weight oligomers. Then, the transition energy is lowered to allow various forms to converge into the lowest energy form, which is toroid structure in this case. As shown in Figure 3.4a,b, DNA release begins with morphological transition from metastable rod and spherical particles to the toroid form. Afterwards, at the intermediate stage as shown by Figure 3.4c, the toroids interact with each other by aggregation and fusion. Depolymerization also weakens the electrostatic interaction thus enables DNA strands to rearrange from kinetically constrained binding sites. In the last stage (Figure 3.4d), DNA worm-like chains gradually unravel from the polyplex resulting in loose loops/tails that are held by a central compact core.
Figure 3.4 Real time AFM images showing (a) morphology transition from rod to toroid, (b) morphology transition from sphere to toroid, (c) particle-particle interaction and aggregation (A, B, and C are three polyplexes groups containing two individual ones at the beginning and fused into each other in the end), and (d) a typical DNA release sequence including all three stages. Time zero corresponds to the injection of the DTT solution. The scan size is 500 nm for (a), 600 nm for (b), 1 µm for (c), and 2 µm for (d). The z range is 7 nm for (a), 8 nm for (b), 10 nm for (c), and 6 nm for (d). (Reprinted from Wan, L. et al. J. Phys. Chem. B 2009, 113, 13735-13741. Copyright (2009) American Chemical Society.)

3.4 Discussion

DNA condensation remains an active topic for experimental and theoretical research, because of its importance in cell biology, virology, polymer physics, and
biotechnology. DNA condensation is driven by an entropy increase associated with the release of counterions upon the polyplex formation. The final condensate structure is determined by a variety of intermolecular forces including forces resisting condensation, such as bending, entropy loss upon demixing of polymer and solvent, and electrostatic repulsion among DNA chains, and favorable forces, such as correlated multivalent counterion fluctuation and cooperative hydration. Manning’s counterion condensation theory predicts that 90% of the DNA charges must be neutralized for condensation to occur. In the reverse process of decondensation, when a sufficient number of binding cations are lost, DNA is released from the polyplex. Our experimental results confirm that the binding affinity dependence on polycation chain length is the main principle behind controlled DNA release by bioreducible polycations. However, the decondensation process does not appear to be instant as predicted for stiff polymers such as DNA, and the system exhibits a great deal of structural heterogeneity.

Various morphologies of DNA condensates have been reported including toroids, rods, spheroids, and less defined ones including rings and flower-like particles depending on condensing conditions, properties of DNA molecules, and condensing agents. The polyplex formation is described by the nucleation and growth process. It starts with the formation of a nucleation loop or rod followed by the intramolecular collapse of the entire DNA molecule to form a monomolecular toroid or rod. Monomolecular polyplexes grow into multimolecular toroids or rods by incorporating free DNA molecules. Molecular simulations of DNA condensation show that the monomolecular toroid is more stable than the rod morphology. Nucleation kinetics favor the rod form, which is the reason why more rods were found at incubation times shorter than 30 min. Spheroids
and flower-like polyplexes are often associated with high-molecular-weight polyelectrolytes and are also considered to be kinetically trapped. Polyplexes are unstable relative to the aggregated phase and they are expected to grow with time because of kinetic and thermodynamic factors. In low salt conditions, polyplex formation is dominated by kinetics and its structure is trapped in far from its equilibrium state. The morphological instability of polyplexes represents one of the major obstacles for successful nonviral gene delivery systems. Higher level aggregation can be a disadvantage for gene delivery because of the difficulty in trafficking large particles and the introduction of too many DNA fragments into one cell.

In our study, we found that the initial polyplex size and morphological distributions are rather insensitive to the polymer structure. For example, we observed toroids with average outer diameter of 100 nm in a wide range of molecular weights (25000–130000 g/mol) and disulfide content (0–100% CBA), as well as different chain architecture (linear vs hyperbranched). Our experimental evidence indicates that the polyplex formation is dominated by kinetic factors. The relative stability among the different forms also indicates that the activation energy from one to another is prohibitively high. The polyplexes were formed in dilute solution during vigorous mixing, which ensured small polyplex size. In addition, N/P ratios greater than 2 plus the highly charged nature of the poly(amiido amine)s resulted in overall positive zeta potential values (data not shown). The positive surface charge further limits particle growth during polyplex formation.

DNA release from the bioreducible polyplexes is triggered by a depolymerization process that converts high-molecular-weight poly(amiido amine)s into low-molecular-
weight oligomers and monomers. The polyplex becomes unstable; that is, the polyanion and polycation dissolve in solution, when the molecular weight of the polycation is greatly reduced. This chain length dependence originates from a loss of entropy as a result of a higher number of shorter chains bound to DNA. The depolymerization rate is much faster than the AFM time period. Therefore, we interpret the polyplex morphologies after DTT injection as those exhibited by polyplexes containing low-molecule-weight cations. The experimental evidence points to the role of depolymerization in driving the transition from frozen states to the lowest energy state. When the high-molecular-weight polycation is converted to low-molecular-weight cations, the transition energy is lowered to allow different forms to converge into the lowest energy form, that is, the toroid structure. Depolymerization weakens the electrostatic interaction and enables rearrangement by freeing counterion from kinetically constrained binding sites. The chain length reduction results in a loss of excess cations thus allowing closer packing as well as the loss of the shell of the excess polycation that provides colloidal stabilization. The role of depolymerization in a sense is similar to that of salt. Our study shows that the toroid is the more stable form in the case of low-molecular-weight counterions for both monomolecular and multimolecular polyplexes. Since all of the in situ AFM experiments were conducted in DNA-free solutions, the structural transitions are accomplished without the free DNA, thus providing a new mechanism for DNA morphological transition and an alternative to the DNA-assisted mechanism.\textsuperscript{136}

Polyplex interaction during disassembly is also interesting. Two neighboring polyplexes are attracted to each other and fuse into one. The interparticle interaction is
facilitated by a reduction in the positive charges or a local charge reversal due to depolymerization. The same local charge reversal mechanism results in intersegment attraction in polyplex formation.\textsuperscript{137} The less compact condensate starts to recover phosphate groups that make part of the chain negatively charged. The negatively charged DNA interacts electrostatically with positively charged condensate surface. The hydrophobic interaction may also play a role if oligocations rearrange to expose the hydrophobic parts as in the ladder structure initially proposed by Kabanov. The interparticle interaction and particle growth in our case are not mediated by free DNA. Instead, the same correlated electrostatic fluctuation and hydrophobic interactions between neighboring particles and chains are responsible. A previous study observed the commensurate reorganization of two DNA strands, which was considered as a precursor to the formation of a larger condensate.\textsuperscript{138} The commensurate association may also play a role in DNA release dynamics, for example, the rod orientation from the radial to the tangential direction with respect to the nearby toroid before fusion.

3.5 Conclusion

The bioreducible poly(amido amine) polyplexes provide an ideal system to study molecular disassembly and DNA decondensation dynamics by real-time AFM. The polyplexes are stable in the oxidizing environment representative of the nonreducing extracellular space. DNA release is triggered by mild DTT and salt concentrations compatible with the physiological environment. The results demonstrate DNA release dynamics from bioreducible polyplexes to consist of three stages that take place at different times. In the first stage, upon depolymerization, polyplexes evolve from metastable structures into the more favorable toroid structure. In the second stage, toroids
interact with each other by aggregation and fusion. In the last stage, DNA gradually unravels from the polyplex resulting in highly decondensed wormlike chains and loops that are held by a central compact core. Our data demonstrate that the DNA release rate can be precisely controlled by the disulfide bond content. The effect of polymer architecture and molecular weight needs further investigation. The polyplex colloidal stability, the intermediate structure, and their interactions impact its delivery efficiency and its effect on cell viability. In order to mimic conditions in cytosol, future experiments will be conducted in conditions more closely resembling cytosol, for example, in glutathione instead of DTT.
Chapter 4

DNA Release Dynamics from Polyplexes by Interpolyelectrolyte Exchange

4.1 Introduction

The promise of gene therapy to treat a variety of genetic and acquired diseases has fueled research on gene delivery systems. Nonviral gene carriers, especially polycations, are attractive alternatives to viral carriers because they show lower safety risks and can be tailored to specific therapeutic needs. A major problem of nonviral gene carriers is low transfection efficiency. Currently the field is dominated by “black-box” strategies that test reporter gene expression levels of various formulations. A critical question to the overall gene delivery efficiency is how and when plasmid DNA is dissociated from its complexes with polycations (polyplexes).\textsuperscript{22, 107-111} Most research on nonviral gene delivery has focused on intracellular barriers including endosomal escape, cytoplasmic transport, and nuclear entry and less focused on extracellular barriers.\textsuperscript{29, 49, 50} The poor correlation between \textit{in vitro} and \textit{in vivo} transfection efficiency results suggests that extracellular barriers can also hinder DNA delivery of polyplexes. The interpolyelectrolyte interaction between polycation vectors and polyanions such as serum proteins, and soluble glycosaminoglycans may release DNA from polyplexes before entering into cells. Ruponen et al. shows DNA release prior to cell entry occurs in various gene delivery systems, which interferes with transfection.\textsuperscript{30} Oupicky et al. used real-time PCR to follow the DNA concentration in blood.\textsuperscript{31} The results show DNA clearance from plasma within a few minutes. Fluorescence study of polyplex stability byDash et al. shows protein binding to polyplexes as the main reason for failure of polyplexes to delivery DNA.\textsuperscript{139} In another study by Oupicky et al. albumin was shown to bind to block
copolymer of N-(2-hydroxypropyl)-methacrylamide with 2-(trimethylammonio)ethyl methacrylate polyplexes without fully releasing DNA.\textsuperscript{4} Highly charged polyanions such as sodium poly(styrenesulfonate) was shown to exert strong DNA release capability on polyplexes.\textsuperscript{140, 141} Polyplex stability can be improved by three kinds of strategies: 1) encapsulating polyplexes with hydrogel system, \textit{e.g.} degradable poly(ethylene glycol) hydrogels.\textsuperscript{57} 2) modifying polyplexes by coating a protection layer such as poly[N-(2-hydroxypropyl)methacrylamide] (pHPMA) \textsuperscript{67}, hyaluronic acid (HA) \textsuperscript{142}, and PEG\textsuperscript{143}. These coatings could decrease albumin binding affinity and increase transfection activity. 3) crosslinking polyplexes. Crosslinking polyplex surface also could increase stability, \textit{e.g.}, reacting amine groups of the polycation with crosslinking agents such as dithiobis(succinimidylpropionate) (DSP) and disuccinimidyl suberate (DSS).\textsuperscript{69,70} It is shown that the crosslinking method was effective in preventing DNA release from polyplexes by heparin.\textsuperscript{70} 4) Modifying the polycation prior to polyplex preparation where HA\textsuperscript{81, 82} and PEG\textsuperscript{83-87} are two widely used polymer blocks.

Recently, we used AFM to visualize plasmid DNA in various decondensed states from bioreducible polyplexes under simulated physiological reducing conditions using dithiothreitol (DTT) as the reducing agent.\textsuperscript{22, 23, 144} Prior to our work, AFM was mainly used to study DNA condensation and self-assembled nanostructure of polyplexes.\textsuperscript{97, 138, 145-153} Our study revealed distinctive stages of polyplex disassembly including the existence of intermediate structures with a high degree of structural heterogeneity, disassembly-induced aggregation, and the dependence of the DNA release rate on the disulfide content, polymer architecture, and solution conditions.
In this paper, in order to illustrate how extracellular barriers interfere with DNA delivery, we will discuss the DNA release dynamics via inter-polyelectrolyte exchange mechanism from macroscopic and microscopic views by fluorescence and AFM, respectively. The DNA delivery vectors used in this study are reducible linear poly(amide amine) LPAA and PEI. Polycations containing the disulfide bond such as LPAA have demonstrated their potential as the next generation of polymeric gene carriers in overcoming the cytotoxicity problem of the first generation carriers such as PEI. The disulfide bond is stable in oxidative extracellular environment but is cleavable in the reductive intracellular space thus allowing DNA release only inside the cell. The reductive intracellular environment is maintained by the glutathione redox buffer. The ratio between glutathione and oxidized glutathione is maintained around (30–100):1 with the total glutathione concentration ~2mM. The degradation of the disulfide-containing polycation proceeds via thiol/disulfide exchange reactions with glutathione.

Polyelectrolytes including extracellular matrix and cytosolic proteins, cell surface glycosaminoglycans (GAGs), cytosolic RNA, and chromosomal DNA can interact and destabilize the polyplex via the interpolyelectrolyte exchange reaction mechanism. This study reports a study of this mechanism on DNA release dynamics using heparin sodium salt, a highly sulfated GAG that has been identified as the main cause of DNA release from polyplexes.
4.2 Experimental Methods

4.2.1 Materials

Plasmid DNA vector, gWiz high-expression luciferase, containing luciferase reporter gene, was purchased from Aldevron. The contour length of DNA with 6732 base pairs is estimated to be 2.3 µm. Dithiothreitol (DTT, Sigma), 1-(2-aminoethyl)piperazine (AEPZ, Aldrich), 1-methylpiperazine (Aldrich), N,N’-methylenebisacrylamide (MBA, Aldrich), and N,N’-cystaminebisacrylamide (CBA, Polysciences) were purchased in the highest commercially available purity and used without further purification. Heparin sodium salt (H4784, Mw 16,000 Da) was purchased from Sigma and used without further purification. Water was deionized to 18 MΩ×cm resistivity using the Nanopure system from Barnstead. Grade V5 muscovite mica was purchased from Ted Pella and was hand cleaved just before use.

4.2.2 Synthesis of bioreducible poly(amido amine)s

The synthesis of hyperbranched and linear bioreducible poly(amido amine)s by Michael addition copolymerization was reported in an earlier paper. The different reactivity of the amines in AEPZ allows synthesis of either linear or hyperbranched polymers by simply changing the ratio of AEPZ-to-bisacrylamide monomers. A 1:2 molar ratio of AEPZ to CBA+MBA yields hyperbranched polymers, while a 1:1 ratio leads to linear polymers. The chemical composition of the hyperbranched polymers is further varied by the CBA to MBA ratio, i.e. the reducible disulfide chain density. Table 1 lists the chemical composition and molecular weight characteristics of all the poly(amido amine)s studied here. The chemical composition was characterized by $^1$H NMR and $^{13}$C NMR using a Varian spectrometer (400 MHz) (see Supplementary
Information). Number-average \((M_n)\) and weight-average \((M_w)\) molecular weight and polydispersity index \((M_n/M_w)\) were determined by size exclusion chromatography (SEC) in 0.03 M sodium acetate (pH 4.5) using Shimadzu LC-10ADVP liquid chromatography equipped with CTO-10ASVP Shimadzu column oven and Polymer Labs PL gel 5 mm mixed C column. SEC data were analyzed using Astra 5.3.1.4 software from Wyatt Technology. Refractive index increments \((dn/dC)\) were determined by an interferometric refractometer and used in SEC analysis.

### 4.2.3 Polyplex preparation

All polyplex solutions contained 42 mg/L DNA in 30 mM sodium acetate buffer (pH 4.5). The condition is set to improve binding ability of PAA. Then, polymer solution is added to DNA solution and mixed by vortexing at 3200 rpm (Fisher Scientific Vortex Mixer) for 10s followed by incubating at room temperature for 30 min following previously developed procedures.\(^{22, 23}\) The N/P ratio (amine-to-DNA phosphate molar ratio) of the polyplexes was 12.

### 4.2.4 AFM characterization

AFM imaging was conducted using a Dimension 3100 AFM from VEECO. 20 \(\mu\)l of polyplexes solution was placed on 1 cm\(^2\) freshly cleaved mica. After 5 min, excess solution was removed and the surface was rinsed with deionized water three times. In order to image the DNA release dynamics from polyplexes, tapping mode was performed in liquid using silicon nitride probes (NP type, VEECO) with a nominal radius of curvature of 20 nm and cantilever spring constant of 0.38 N/m as provided by the manufacturer. Usually, the polyplexes were imaged in 50 \(\mu\)L solution. AFM imaging ensued immediately after solution injection. The surface was imaged continuously at an
average rate of 1–2 Hz on a 2×2 or 5×5 µm² area until no significant changes were observed at the surface. The ranges of frequency, amplitude, integral, and proportional gains used are 8 kHz, 0.5–1 V, 0.5–2, and 0.75–3 respectively. The AFM images were analyzed using Nanoscope software version 5.12b by Veeco.

4.2.5 Fluorometry

The DNA release kinetics was determined by measuring the fluorescence intensity of EtBr-labeled DNA using SpectraMax M5 plate reader (Molecular Devices). Polyplexes prepared as above were incubated at room temperature for 30 min before adding heparin and EtBr. The final 200 µl solution contained 20 mg/L DNA and 5 mg/L EtBr. Release percentage is calculated based on the ratio of current fluorescence intensity to maximum fluorescence intensity.

4.3 Results

4.3.1 Polyplex assembly

First of all, fluorescence assay is used to study both DNA condensation and release processes. The basic idea is to use a fluorescence dye, such as EtBr, to probe the un-condensed DNA. EtBr intercalates into free sites of the DNA double helix chain and produces a large increase in the fluorescence quantum yield. This is used to test the condensing capacity of DNA delivery vectors. The N/P ratio is the molar ratio of amine in polymer to phosphate in DNA. It needs to be greater than 2 in order to cause DNA condensation signaled by the decrease in DNA/EtBr fluorescence. As shown in Figure 4.1, the fluorescence intensity of DNA/Etbr decreases by adding positively charged polymers, indicating fewer free sites. Both RHB PAA and LPAA show the capability to induce DNA condensation. The fluorescence quenching strongly increases at low N/P
ratio smaller than 4, then reaches a plateau, where DNA is in fully condensed status. The RHB PAA reaches a plateau at N/P larger than 2 comparing to 4 for LPAA, which means RHB PAA is more efficient in condensing DNA. This probably is due to the difference in chain flexibility and charge density. Similar phenomenon is also reported by other researchers with branched and linear PEIs. Previous study has concluded that complete condensation of DNA is necessary for efficient DNA delivery. In this study, the N/P ratio is fixed at 12. Dynamic light scattering shows both polyplexes have hydrodynamic diameter of 60 nm and zeta potential of 44 mV.

**Figure 4.1** Fluorescence intensity as a function of the N/P ratio for RHB PAA and LPAA.

### 4.3.2 Critical counter-polyelectrolyte concentration

The interpolyelectrolyte exchange can be understood from both thermodynamic and kinetic aspects. It is found that heparin and PSS behave similarly in this reaction. In order to determine the critical counter polyelectrolyte concentration for the reaction, the same fluorescence method was used. The polyanion was gradually added into the polyplex solution in the presence of EtBr. This causes the chemical equilibrium to shift
and a slight increase in fluorescence intensity was observed. Since the DNA is still in the
condensed state, majority of the binding sites were occupied and the overall fluorescence
intensity is still low. The majority of heparin complexes with free polycation in the
solution. This corresponds to the slow growth regime, where heparin concentration is
lower than 30 µg/ml and PSS concentration is lower than 100 µg/ml.

**Figure 4.2** Fluorescence assay for a) LPAA and RHB PAA/DNA polyplexes (N/P
= 12), incubation time 30 min, b) PEI PAA/DNA polyplexes (N/P = 12), incubation time
30 min, with different heparin concentration. Lines are drawn to guide the eye.
Further increase in the polyanion concentration (heparin or PSS) causes DNA release from the polyplex as signaled by a rapid increase in ETBr fluorescence. Critical concentration here is defined as the polyanion concentration at 50% maximum fluorescence intensity. The critical concentration for heparin was determined to be 50 µg/ml, which is equivalent to 0.3 mM ionic charges, while that of PSS was found to be 180 µg/ml and 0.9 mM ionic charges. The difference between heparin and PSS may be due to difference in chain flexibility. The result agrees with the study done by gel electrophoresis showing that 30–40 µg/ml heparin caused DNA release from the LPAA/DNA polyplex.\(^{174}\)

In addition to the thermodynamic equilibrium, it is also important to investigate the kinetics of the release process. The time dependence of the fluorescence intensity is shown in Figure 4.3. The release showed little variation after the initial increase, and the degree of release was determined by counter electrolyte concentration. The reaction was too fast for fluorescence observation.
Figure 4.3 Time dependence of fluorescence intensity of DNA release from LPAA/DNA polyplex at various heparin concentrations.

4.3.3 In situ real time AFM study of DNA release dynamics.

Previous study has shown that adsorption of oppositely charged polyelectrolyte is very fast, and the rate limiting step of polyelectrolyte exchange reaction is the desorption of exchanged polyelectrolyte. In order to capture the intermediate status of DNA release using AFM without interference from materials in the solution, heparin was only deposited on the surface while the buffer solution used for observing DNA release was free of heparin. As shown in Figure 4.4, heparin deposited on the surface shows similar capability to induce DNA release from the polyplex. The duration of DNA release appears to be similar to the previous case supporting our assumption that DNA release here is dominated by surface-adsorbed heparin molecule interaction with surface-adsorbed polyplex, i.e., surface reaction being the limiting step in the interpolyelectrolyte exchange reaction. More importantly, in physiological environment, the majority of heparin is immobilized on the extracellular matrix. Therefore, this method is also relevant to biological conditions. A dominant intermediate feature is the swelling of polyplexes accompanying the interpolyelectrolyte exchange reaction. Figure 4.5 shows the quantified volume change and release percentage as a function of time based on the analysis of AFM images. There was a gradual increase in particle volume in the first hour followed by a gradual decrease. This swelling is attributed to the osmotic pressure existing between the solution and the semi-impermeable polyelectrolyte particles as well.
as the expansion of polyelectrolyte chains as a result of an overall increase in charge density in the partially disassembled complex.

**Figure 4.4** Time elapse *in situ* AFM images sequence for LPAA/DNA polyplexes (N/P=12) treated by 500µg/mL heparin for 5s then imaged in 30mM pH 4.5 acetate buffer. Scan size is 2.2 µm; Z-range is 8nm.

DNA release percentage was calculated based on measured DNA contour length in comparison to the theoretical length, 2.3µm for 6,732 bps DNA. The measured contour length represents a lower limit due to the spatial resolution of AFM. The appearance of loose DNA strands coincides with maximum degree of swelling at around 30 min. We surmise that swelling and loosening of the polyelectrolyte network is a prerequisite for DNA release in the case of interpolyelectrolyte exchange reaction.
Figure 4.5 a). Volume change and b) release percentage, during the release shown in Figure 4.4. Each data point represents the average value of the same five monomolecular polyplexes in the field.

As shown in Figure 4.6, the release process could be simplified into three stages at the single molecule level. First, polyplexes transit from various morphologies to toroid structure. This morphological convergence occurring near the beginning of the release seems to a universal pathway for DNA release. We reported similar phenomenon in our
previous study about DNA release via depolymerization of reducible polycation\textsuperscript{23}. Secondly, the toroid swells into spheroid with significant increase in volume. Finally single DNA chains are released from the particle.

Figure 4.6 Time elapse \textit{in situ} AFM images sequence for LPAA/DNA (N/P=12) treated by 500 µg/mL heparin for 5s then imaged in 30mM PH4.5 acetate buffer. Scan size is 500 nm; Z-range is 8nm.

In contrast, at the identical condition, RHB PAA/DNA polyplex showed stronger resistance against heparin exchange (Figure 4.7). As shown in Figure 4.8, similar swelling of polyplex particles was observed. However, the volume increase reached a plateau after about 30 min. There was no volume decrease afterwards indicating little loss of DNA from the polyplex.
Figure 4.7 Time elapse *in situ* AFM images sequence for RHB PAA/DNA polyplexes (N/P=12) treated by 500 µg/mL heparin for 5s then imaged in 30mM pH 4.5 acetate buffer. Scan size is 2.2 µm; Z-range is 8nm.

Figure 4.8 Volume change during the release shown in Figure 4.7. Each data point represents the average value of the same five monomolecular polyplexes in the field.
4.3.4 Cell transfection.

The stability of polyplexes in extracellular environment is critical for successful gene delivery.\textsuperscript{39} Better understanding of the correlation of DNA release dynamics at molecular level and the cell transfection efficiency helps to improve the design of polymeric gene delivery vectors. The \textit{in vitro} cell transfection efficiency of both LPAA and RHB PAA polyplexes were evaluated with NIH 3T3 cells in the presence of 10\% FBS, which mimics the extracellular environment \textit{in vivo} (Figure 4.9). The experiment was done by Dr. Jing Li (University of Nebraska Medical Center, Omaha, NE). The cells were cultured for 24-48 hour to 70–80\% confluent for maximal uptake. 150 µL growth medium containing 10\% FBS mixed and polyplex were added to culture plate and incubated for 4 hour. The FBS contains various negatively charged proteins and polyelectrolytes,\textsuperscript{178} which have the potential of inducing DNA release. It is found that in the presence of FBS, the transfection efficiency of RHB PAA polyplex is more than 10-folds higher than that of LPAA. Light scattering and fluorimetry did not show significant difference between the two polyplexes. However, AFM results captured difference in degree of DNA release due to exchange with heparin between the two types of polyplexes. Considering the AFM and cell transfection results together extracellular polyanions may play a significant role in polyplex transfection efficiency and polyplexes that are more resistant to interpolyelectrolyte exchange give rise to higher transfection efficiency.
Figure 4.9 Luciferase transfection of NIH 3T3 cells by RHB PAA and LPAA polyplexes. N/P ratio was 12 for both polyplexes. Cell culture media contained 10% FBS.

4.4. Discussion

DNA release dynamics are determined by both thermodynamic and kinetic factors. We find that DNA release in solution is determined by thermodynamic equations of interpolyelectrolyte exchange reaction, while DNA release from polyplexes immobilized on a surface is also affected by slow kinetics of adsorbed polyelectrolytes.

Binding affinity difference between DNA and heparin towards the polycation is a critical factor in inter-polyelectrolyte exchange. The thermodynamic equilibrium of DNA condensation by polycation has been discussed by Bloomfield et al.\textsuperscript{124, 179, 180} The competition between DNA and polyanion to another polycation is more complicated. The work by Zelikin et al. modeled DNA- poly-L-histidine (PLH)-polyanion system as the adsorption of an oligomer with degree of polymerization \(n\) onto a one-dimensional lattice. The selectivity \(\varphi\) of oligomer binding to polyanion-1 and polyanion-2 can be expressed approximately as: \(\varphi_0 = \left( \frac{K_1}{K_2} \right)^n\), where \(K_i\) is the binding constant of oligomer to...
polyanion-i. The polyanion-1 and polyanion-2 here are assumed to have same degree of polymerization \( (n = n_1 = n_2) \). The normalized fluorescence quenching \( \Theta \) obtained from fluorimetry experiment can be correlated with the selectivity via the following equation.

\[
\left( \frac{K_{11}}{K_{12}} \right)^n = \frac{\Theta^2}{(1-\Theta)^2} \exp \left[ \frac{2(1-\Theta)^2}{\Theta(1-\Theta)} \right]
\]

In realistic situation, when the degree of polymerization is different \( (n_1 \neq n_2) \), the selectivity becomes:

\[
\ln \varphi = \ln \varphi_0 + \ln K_{12}^{n_1-n_2}
\]

Because \( n \) is the degree of polymerization, which is in thousands range, the equilibrium is very sensitive to \( K_{11}/K_{12} \) value. Similar to PSS, the \( K_{11}/K_{12} \) value of heparin is estimated to be 0.5-0.75. This means the equilibrium strongly favors the release of DNA, which explains the fast release.

The critical heparin concentration is determined by the amount of polycation in the solution. In this study, the DNA concentration is 20 \( \mu \)g/ml, which contains \( 6.1 \times 10^{-5} \) M negative charge. The N/P ratio is 12 so the amine group concentration is \( 7.3 \times 10^{-4} \) M. The pKa value for the amine groups in PAA is around 10, so the net positive charge is about \( 1.7 \times 10^{-4} \) M. When heparin is added to the polyplex solution, it complexes with free polycation then form ternary complex with polyplex. The net charge of heparin sodium salt at critical concentration is \( 2.7 \times 10^{-4} \) M, which is slightly higher than the total positive charge.

The charge equilibrium determines the critical heparin concentration, so there is no significant difference between that of LPAA and RHB PAA. But the structural difference between LPAA/DNA polyplex and RHB PAA/DNA polyplex causes deviation...
in reaction kinetics. Murayama et al\textsuperscript{182} investigated spermidine/dye exchange reaction and explained the results from a combined experimental and computational view. It is found that higher condensation degree inhibits polyanion(DNA)-polyanion(Heparin) exchange. In our case, the size of monomolecular polyplex is $1.6 \pm 0.3 \times 10^4 \text{ nm}^3$ for LPAA while $1.2 \pm 0.1 \times 10^4 \text{ nm}^3$ for RHB PAA, indicating RHB PAA polyplex is more densely packed. Polyplexes at more condensed states have lower energy so they are more stable, and entangled chains are also kinetically trapped.

Our results show that DNA release from inter-polyelectrolyte exchange shares a similar morphological pathway with depolymerization induced DNA release from bioreducible polyplexes, where initially various metastable structures formed during fast polyplex formation tend to transit into a common toroid structure. The thermodynamic explanation is that polyelectrolyte exchange and depolymerization lower the transition energy and allow different metastable forms to converge into the lowest energy form, which is the toroid structure. Depolymerization and polyelectrolyte exchange weaken the electrostatic interaction and enable rearrangement by freeing counterion from kinetically constrained binding sites.

When the reaction is carried out on mica, DNA release dynamics is strongly affected by polyelectrolyte adsorption states. First, because of the spatial limit, the surface concentration of heparin is limited. This could explain why we did not observed DNA fully release from RHB PAA polyplex at the same condition. The reason may be that the maximum surface heparin concentration is still below the critical value to induce DNA release from the polyplex. Furthermore, the electrostatic attraction between polyplexes and mica surface stabilizes polyplexes, in other words, raises the activation
energy. Additionally, surface confinement effect leads to strong negative activation entropy, which means a very tight transition state. These two factors make activation energy higher for the surface reaction and it requires much higher heparin concentration.

For reaction kinetics, according to the previous study by Bloomfield and Grosberg, coil-globule transition is abrupt for stiff polymers such as DNA. Thus, in solution, DNA release, just like its reverse process--DNA condensation, is abrupt. The slow increase in release percentage in the second stage probably relates to slow disassembly of central compact core structure, which has been found in DNA release process via different mechanisms. However, the origin of the stability of the core is not clear yet.

When the reaction occurs at the surface, the reaction rate tends to slow down significantly due to adsorbed states of polyelectrolytes. This slow kinetics allow AFM observation of the DNA release morphological pathway including swelling and formation of the ternary polyplex structure prior to DNA chains coming out of the polyplex. We suggest that polyelectrolyte reactions in extracellular environment may be mimicked by the types of AFM experiments conducted here on surfaces.

4.5 Conclusion

In this study, we investigated dynamics of DNA release from LPAA/DNA and RHB PAA/DNA polyplexes in solution and on mica surface. The results show that the release behavior is greatly dependent on polymer structure and polyplex condensation degree as well as spatial status (free or adsorbed). AFM results reveal that the release is a three-stage process including morphological convergence, swelling, and chain expansion/rearrangement. The morphological convergence into the toroid structure is a
common morphological pathway for DNA release by inter-polyelectrolyte exchange and depolymerization. The DNA release dynamics at the molecular level can be used to guide the synthesis and engineering of polymeric gene delivery nanosystems to achieve high transfection efficiency.
Chapter 5

Engineering Bioreducible Layer-by-layer Films for Sequential and Sustained DNA Delivery

5.1 Introduction

The Layer-by-layer (LbL) method of assembling polyelectrolyte multilayers (PEMs) has become one of the most promising methods for the development of biomedical coatings to mimic cellular microenvironments and to release therapeutic drugs and nucleic acids from the surfaces of biomedical devices. The LbL technique is based on alternating depositions of polycations and polyanions on a substrate surface. The LbL method has several advantages over other types of thin films such as the Langmuir-Blodgett (LB) multilayers and self-assembled monolayers (SAMs). The LbL method allows a wide variety of biological molecules including DNA, RNA, proteins, peptides, and polysaccharides to be incorporated into surface coatings with precisely controlled amount and spatial distribution. The LbL films can be deposited on a wide variety of biomedical devices including Ti and porous Ti, stainless steel stents, and micro-needles. The most beneficial aspect of the LbL films is their potential to enable programmable and sustained release of multiple therapeutic molecules from implantable biomedical devices with high degrees of spatiotemporal control. The sequential disassembly of the LbL films can potentially meet the need for programmable gene delivery in tissue/bone regeneration and vaccine delivery. In addition to the capability of localized delivery, LbL films made of synthetic and natural polycations including poly(ethylenimine) (PEI), poly(L-lysine) (PLL), and polysaccharides are less toxic than virus-based gene delivery systems, but the synthetic systems need to
overcome their generally low transfection efficiency in order to meet clinical requirements.

LbL films are highly stable due to the polyvalent nature of the electrostatic interactions. Their disassembly requires conditions incompatible with physiologic environment.\textsuperscript{197} Successful use of DNA films for gene delivery requires film disassembly under physiologic conditions.\textsuperscript{198} Several strategies for LbL film disassembly have been reported that rely on the use of hydrolytically or enzymatically degradable polycations.\textsuperscript{199-201} Our previous work has shown by \textit{in vitro} and \textit{in vivo} experiments that local extracellular reducing microenvironment can also be used as a trigger for the disassembly of LbL films containing bioreducible polycations and DNA.\textsuperscript{193} The high local DNA concentration in the surface-mediated delivery has been linked to higher transfection efficiency than the non-surface-mediated counterpart.\textsuperscript{202-204} Whereas the reducing nature of the intracellular environment is well known and has been widely exploited in drug and gene delivery\textsuperscript{54, 155} and disassembly of LbL films,\textsuperscript{205-207} the reducing microenvironment of the plasma membrane receives less attention. There have been limited studies on utilizing cell membrane thiols to improve gene delivery including our own \textit{in vitro} and \textit{in vivo} work.\textsuperscript{208-210} The presence of reactive oxygen species and absence of a redox buffer means that the extracellular space is predominantly oxidizing.\textsuperscript{211} Despite the oxidizing nature of the extracellular environment, the presence of redox-active thiols in numerous proteins on the cellular plasma membrane suggests that at least the microenvironment of the cell surface can support disulfide reductions.\textsuperscript{212-214} The redox activity of the plasma membrane is closely correlated with the levels of redox enzymes at the membrane.\textsuperscript{215-217} The maintenance of the thiol groups is mediated
by the transfer or shuffling of hydrogens and electrons between the cysteine thiols of these surface proteins. The total levels of redox-active thiols on the surface of cells are in the range of $4\text{–}30\ \text{nmol/10}^6\ \text{cells}$. Relying on cellular exofacial redox activity for disassembly of the bioreducible films offers major advantage over the two alternative methods (hydrolytic and enzymatic).

Scheme 5.1 Molecular structure of bioreducible PAAs. Composition of $R_1$ change represents different reducible monomer content and change of $R_2$ represents linear or hyperbranched chain architecture.

Our ultimate goal is to achieve disassembly of bioreducible DNA films \textit{in vitro} and \textit{in vivo} with nanometer precision, that is, LbL disassembly. This work has been carried out to better understand the relationship between the internal LbL film structure and its disassembly behavior. We continue our research using the bioreducible poly(amide amine)s (PAAs) synthesized in our lab. The PAAs have demonstrated redox-sensitive behavior, reduced cytotoxicity, and enhanced gene delivery efficiency than the non-reducible polycations. The molecular structure of the PAAs is shown in Scheme 1. The PAAs contain disulfide bonds that are cleavable by endogenic reducing agents such as thiol-containing membrane proteins and glutathione following the thiol-disulfide exchange reaction. The PAA are degraded into low-molecular-weight oligocations following the reaction, and the oligocations have lower binding affinity to DNA. This
causes the disassembly of the LbL films and release of DNA to the cellular/film microenvironment.

LbL films are made traditionally by manually dipping the substrate into polyelectrolyte and rinsing solutions, which is a tedious and labor intensive process. Recent improvements include micro capillary, automated dipping machines, and computer-assisted spray-coating and spin-coating. It is also known that LbL film structures are influenced by temperature, pH, salt concentration, and polyelectrolyte concentration. This study focuses on the assembly and disassembly behaviors of two types of bioreducible LbL films. Type A is made of alternating layers of PAA and DNA while Type B is made of PAA/DNA with periodically inserted PEI/DNA bi-layers as illustrated by Scheme 5.2. The film assembly process is studied by AFM and ellipsometry while the film disassembly in a reducible solution is monitored by AFM, fluorescence, and dynamic light scattering (DLS). We show that LbL disassembly and sustained DNA release have been achieved by adding the PEI/DNA bi-layer as a barrier layer into the PAA/DNA multilayer structure. *In vitro* transfection studies are carried out using the Human Embryonic Kidney 293 cell line (HEK293). *In vivo* transfection studies are carried out by implanting LbL-covered suture into the muscle of mice. The results show that incorporation of the PEI barrier layer is effective in promoting surface degradation of PAA/DNA LbL films and sustained DNA release and transfection to the cell/substrate microenvironment.
Scheme 5.2 Structure and composition of type A and type B films.

5.2 Experimental

5.2.1 Materials.

Dithiothreitol (DTT, 99%), 1-(2-aminoethyl)piperazine (AEPZ, 99%), 1-methylpiperazine (MPZ, 99%), N,N’-methylenebisacrylamide (MBA, 99%), and 1,5-Diiodopentane (DIP, 97%), fluorescein isothiocyanate (FITC, 90%), and tetramethyl rhodamine isothiocyanate (TRITC, 90%) were purchased from Sigma-Aldrich and used as received. N,N’-cystaminebisacrylamide (CBA, 98%) was obtained from Sigma-Aldrich and was recrystallized before use. Cy5 nucleic acid labeling kit was purchased from Mirus Bio. Water was deionized to 18 MΩ·cm resistivity using a Nanopure System from Barnstead. Grade V5 muscovite mica and 15 mm glass slides were purchased from Ted Pella. Mica was hand cleaved just before use. Polished n-type silicon wafers (resistivity 50–75 Ω·cm) were purchased from Wafer World. Silicon wafers were cut to 1×1 cm² pieces and cleaned via standard RCA-1 procedure. The RCA-1 solution was prepared by adding 65 ml NH₄OH (27%) into 325 ml deionized water and heating the solution to 70°C followed by adding 65 ml H₂O₂ (30%). The RCA-1 process took 15 min. Glass slides were soaked in the mixture of methanol and HCl (V/V = 1:1) for 30 min, then immersed in 98% H₂SO₄ for another 30 min followed by rinsing with deionized
water. The silk suture was treated in an aqueous solution of 0.1% (w/v) \( \text{Na}_2\text{CO}_3 \) at 98-100°C for 30 min to remove sericin. The sericin removal step is important because it can elicit undesirable immune response after implantation. Copious amount of deionized water was used to remove \( \text{Na}_2\text{CO}_3 \) followed by drying in a \( \text{N}_2 \) stream.

GFP plasmid was prepared using a Qiagen Plasmid Giga kit (Qiagen, Valencia, CA). The plasmid was isolated from bacterial lysate by anion-exchange column chromatography, then concentrated and desalted by isopropanol precipitation, which may be repeated in order to remove any protein residue.

### 5.2.2 Synthesis of bioreducible PAAs.

The synthesis of hyperbranched and linear bioreducible PAAs by Michael addition copolymerization has been reported in an earlier paper.\(^{112}\) The different reactivity of the amines in AEPZ allows synthesis of either linear or hyperbranched polymers by simply changing the molar ratio of AEPZ to bisacrylamide.\(^ {170}\) A 1:2 molar ratio of AEPZ to CBA+MBA yields hyperbranched polymers while a 1:1 ratio leads to linear polymers. Products were fractionated by semi-permeable membranes first with a cut-off molecular weight of 30 kDa followed by one with a cut-off of 10 kDa. The chemical composition of the hyperbranched polymers is further varied by the CBA to MBA ratio in order to vary the reducible disulfide bond content. The chemical composition was characterized by \(^1\text{H} \) NMR and \(^{13}\text{C} \) NMR using a Varian spectrometer (400 MHz) (see Supplementary Information). Number-average (\( M_n \)), weight-average (\( M_w \)) molecular weight, and polydispersity index (\( M_n/M_w \)) were determined by size exclusion chromatography (SEC) in 0.03 M sodium acetate (pH 4.5) using Waters Ultrahydrogel 250 PKGD column on Waters Alliance 2695 HPLC. Molecular weights were calculated
using a calibration curve, which was obtained using the polyethylene glycol/polyethylene oxide ReadyCal standard set (Mp 200–1,200,000) from Sigma-Aldrich. The PAA used in this study is hyperbranched and contains 33% CBA unless otherwise stated. Molecular weight and polydispersity data are listed in the Supporting Information.

5.2.3 Deposition of the LbL films.

The LbL films were deposited on various substrates by the dip coating method using a programmable Carl Zeiss HMS50 slide stainer with a home built substrate holder (Figure 5.1) to improve reproducibility of LbL deposition and data collection. The substrate holder was made from Teflon and holds up to five glass disks. The separation distance between neighboring slots was adjusted to allow maximum packing of the disks while not trapping too much water in between the disks. The bottom edge of the holder is tapered to facilitate water drainage from the disks and holder. The substrate was dipped alternatively in the polycation solution (0.5 g/L with 30 mM pH 5.5 acetate buffer and 0.1 M NaCl) for 150 s and polyanion solution (0.25 g/L with 30 mM pH 5.5 acetate buffer and 0.1 M NaCl) for 150 s. Each polyelectrolyte solution dipping is followed by three rinsing steps of 45 s each in deionized water. The polycation/polyanion deposition procedure was repeated until a desired number of layers were obtained. The dipping solution was refreshed after 8 dipping cycles in order to minimize concentration variation. Some of the LbL films were terminated with the polycation layer while the rest were terminated with a fibronectin layer deposited by placing a 100 μL droplet of fibronectin solution (0.2 g/L) on the substrate for 1 min.
5.2.4 Crosslinking of the LbL films.

The crosslinking of the LbL film was conducted following our prior work. The LbL film was placed in 1 M DIP hexane solution at 50°C for 1 h. Then, the sample was rinsed with ethanol and deionized water and dried with filtered air.

5.2.5 AFM imaging.

AFM imaging was conducted using a Dimension 3100 AFM from VEECO. Tapping mode in air was used to measure thickness and surface morphology. The AFM tapping mode probes in air were silicon probes (VEECO) with a nominal frequency of 150 kHz. The AFM images were analyzed using Nanoscope software version 5.12b by VEECO. In order to measure the film thickness by AFM, the film was scratched with a new razor blade that penetrates the film but stops at the glass surface. The film thickness is the step height between the film and substrate exposed by the scratch. The film surface roughness was the root-mean-squared roughness $RMS=\sqrt{\frac{\sum z_i^2}{N}}$ where $z_i$ is the height value of each measurement point and $N$ is the number of measurement points.
points in AFM height images. All RMS values reported were obtained on an area of $5 \times 5$ µm$^2$. AFM tapping mode in liquid was used to monitor film degradation \textit{in situ} in 50 µL 20 mM DTT in PBS buffer using silicon nitride probes (NP type, VEECO) with a nominal radius of curvature of 20 nm and a nominal cantilever spring constant of 0.38 N/m. AFM imaging ensued immediately after solution injection. The surface was imaged continuously at an average rate of 1–2 Hz until no significant changes were observed at the surface. The ranges of frequency, amplitude, integral, and proportional gains used are 8 kHz, 0.5–1 V, 0.5–2, and 0.75–3, respectively.

5.2.6 AFM nano-indentation force measurements.

The AFM force measurements were conducted in PBS buffer (pH = 7.4) containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 2 mM KH$_2$PO$_4$. The force calibration curves were converted to the force-versus-indentation curves by defining sensitivity, zero force ($F = 0$), and contact point. The sensitivity in nm/V was obtained from the slope of the constant compliance regime of the retraction curve and was multiplied by the raw voltage value to yield the cantilever deflection, $Z_C$. The force is $F = k \cdot Z_C$. $k$ is the spring constant of the cantilever. The AFM probe [] and spring constant is 0.1 N/m as provided by the manufacturer. The contact point, \textit{i.e.}, indentation $\delta = 0$, is defined as the point where the force becomes repulsive. The indentation is defined as: $\delta = \begin{cases} \Delta \text{(piezo position)} - Z_C \quad (Z_C > 0) \\ 0 \quad (Z_C = 0) \end{cases}$.

5.2.7 Ellipsometry.

A phase-modulated ellipsometer (Beaglehole Instruments, New Zealand) fixed at the angle of incidence near the Brewster angle ($\theta_B \approx 70^\circ$) was used to quantify the film
thickness. The thickness was obtained using the Drude equation and the ellipticity, 
\[ \rho = \Im\left(\frac{r_p}{r_s}\right) \mid_{\theta_h} \]
measured by ellipsometer, where \( r_p \) and \( r_s \) are the complex reflection
amplitudes for \( p \) and \( s \).

5.2.8 Fluorescence spectroscopy.

The degradation of the LbL films was monitored by a fluorometer (SpectraMax
M5 Plate Reader, Molecular Devices). The PAA was labeled by either FITC or TRITC.
The FITC labeling was conducted by mixing 1 mg FITC powder and 25 mg PAA in 0.1
M pH 9.0 sodium carbonate/bicarbonate buffer for 24 h.\textsuperscript{228, 229} The unreacted dye was
removed using a semi-permeable membrane with a cut-off molecular weight of 3 kDa.
Because the emission wavelength of FITC (525 nm) is close to that of GFP (509 nm),
TRITC-labeled PAA was used in GFP transfection experiments. TRITC was dissolved in
dimethyl sulfoxide (DMSO) solution (c = 50 g/L) before adding to the PAA solution.
PAAs labeled with the two dyes were used to distinguish PAA released from the top zone
from that released from the bottom zone in the LbL film degradation study. The dye-
labeled LbL films were constructed by mixing labeled PAA with unlabeled PAA in a 1:3
weight ratio in the PAA layer deposition.

The LbL film degradation was carried out by immersing it in 5 ml 20 mM DTT
solution. The solution in contact with the film was periodically analyzed by collecting
and concentrating it to 0.25 ml in order to obtain sufficient fluorescence signals.

5.2.9 Dynamic light scattering (DLS).

The degradation solution was analyzed using a zetasizer (Nanosizer ZS, Malvern
Instrument) in order to determine the size and surface potential of the particulate products
released from the LbL film during film degradation by DTT. The 15 mm glass slide
covered by the LbL films was broken into smaller pieces in order to fit the micro-cuvette (ZEN0040, Malvern Instrument). A stainless steel mesh was inserted in the micro-cuvette with the LbL film coated glass slide placed on top. The micro-cuvette was filled with 1 ml 20 mM DTT solution to completely cover the slide. DLS measurements proceeded immediately to measure the effective hydrodynamic diameter ($D_{H}$) of the products released from the LbL film. The backscattering angle $\Theta$ was fixed at 172° with a laser wavelength $\lambda = 633$ nm. The size measurement range was set between 1 nm and 6 µm. $R_{H}$ is a function of the diffusion coefficient ($D$), temperature ($T$), and viscosity ($\eta$) according to the Stokes-Einstein equation: $R_{H} = \frac{kT}{3\pi\eta D}$. $k$ is Boltzmann constant. $T$ is 25°C, and $D$ is obtained from autocorrelation function via the cumulant fitting.

5.2.10 Transfection activities in vitro.

Transfection experiments were performed with Human neonatal dermal fibroblasts cells and HEK 293 cells using the gWiz GFP plasmid. LbL films deposited on 15mm diameter glass slides were placed at the bottom of 12-well plates. Cells were grown to 80% confluence, trypsinized, washed with PBS, and resuspended in DMEM. Then, $4 \times 10^4$ cells were placed on the film and incubate in 5% CO$_2$ at 37 °C for 1 h, followed by adding fresh DMEM solution supplemented with 10% FBS. The Cell culture medium was replaced every day by carefully washing the substrate with PBS and transferring the sample into new well plates with 2 mL of fresh medium. Cell attachment and proliferation were imaged daily with an inverted optical microscope. The transfection efficiency was evaluated by the number of transfected cells and fluorescence intensity.
5.3 Results

5.3.1 Structural analysis of the LbL films.

We focused on two types of LbL films as shown in Scheme 5.2. Both types contain 16.5 bi-layers of polycation/DNA with the very bottom and very top layers both being the polycation layer. For the polycation layer, PAAs with different chemical compositions were used and in some cases the PAA layer was replaced with a PEI layer. For the polyanion layer, in one case HA is mixed with DNA. Type A contains PAA/DNA bi-layers. Type B contains hybrid PAA/DNA and PEI/DNA bi-layers. The film composition of each sub-type of film used in this study is listed in Table 5.1.

**Table 5.1** Composition of LbL films and thickness measured by AFM.

<table>
<thead>
<tr>
<th>Film</th>
<th>Polycation</th>
<th>Polyanion</th>
<th>No. of bi-layers</th>
<th>Thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0</td>
<td>Hyperbranched PAA (10% CBA)</td>
<td>DNA</td>
<td>16.5</td>
<td>96</td>
</tr>
<tr>
<td>A1</td>
<td>Hyperbranched PAA (33% CBA)</td>
<td>DNA</td>
<td>16.5</td>
<td>104</td>
</tr>
<tr>
<td>A2</td>
<td>Hyperbranched PAA (67% CBA)</td>
<td>DNA</td>
<td>16.5</td>
<td>100</td>
</tr>
<tr>
<td>A3</td>
<td>Hyperbranched PAA (100% CBA)</td>
<td>DNA</td>
<td>16.5</td>
<td>121</td>
</tr>
<tr>
<td>A4</td>
<td>Hyperbranched PAA (33% CBA), PEI, w/w=1:1</td>
<td>DNA</td>
<td>16.5</td>
<td>68</td>
</tr>
<tr>
<td>A5</td>
<td>PEI</td>
<td>DNA</td>
<td>16.5</td>
<td>70</td>
</tr>
<tr>
<td>A6</td>
<td>PEI (first 5 layers), Hyperbranched PAA (33% CBA)</td>
<td>PSS (first 5 layers), DNA</td>
<td>32.5</td>
<td>230</td>
</tr>
<tr>
<td>B1</td>
<td>Hyperbranched PAA (33% CBA)</td>
<td>DNA</td>
<td>16.5</td>
<td>75</td>
</tr>
</tbody>
</table>
The production rate and consistency of LbL film assembly have been improved from our previous operation by utilizing the Carl Zeiss slide stainer and a home-built substrate holder. The film deposition consistency was checked by AFM and ellipsometry. The slide stainer, also used by others,\textsuperscript{221} allows programmable dipping and rinse cycles with consistent immersion times and rates of lifting substrates in and out of the beaker. In order to increase the production rate and create duplicates of LbL films we constructed a substrate holder that fits the slide stainer lift arm. We experimented with several designs and the optimal design is shown in Scheme 5.1. This holder is able to hold five glass disks with all of them in full contact with the solution when immersed. The combination of hydrophobic and inner Teflon as holder material and the tapered bottom allow efficient water drainage to minimize liquid transfer from one beaker to the next beaker.

![Graph](image.png)

**Figure 5.2** Film thickness as function of number of bi-layers measured by AFM and ellipsometry. Linear fitting for film B1 shows slop = 4.1 nm per bilayer.
The film thickness measured in air as a function of the number of bi-layers deposited is shown in Figure 5.2. The AFM result agrees with ellipsometry result very well with error less than 5%. Film A1 displays an exponential growth pattern that deviates from the linear line. For the initial 6 bi-layers, the average layer thickness is 1.9 nm for PAA and 2.1 nm for DNA. For the final 6.5 bi-layers the layer thickness for PAA increased to 3.9 nm and for DNA 4.0 nm. The two-regime growth behavior has been widely reported on LbL films containing DNA.\textsuperscript{72, 224, 230} In contrast, Film B1 film with every third PAA/DNA bi-layer being replaced by the PEI/DNA bi-layer, the film growth is visibly more linear with constant layer thickness of 2.0 nm for the PAA or PEI layer and 2.6 nm for the DNA layer.

In addition to the A1 and B1 films, LbL films with different compositions were also studied and the results can be found in Table 5.1. PAAs with various disulfide-containing CBA contents (10\%, 33\%, 67\%, and 100\%) and chain architecture (linear and hyperbranched) were used to prepare the LbL films. We also experimented with mixing PEI and PAA in the same layer as opposed to putting them in separate layers. Another film consists of five bilayers of PEI/PSS used as non-degrade primer layers. All the thickness and roughness data are listed in Table 5.1. The total film thickness values suggest that exponential growth of PAA/DNA films can be arrested by either inserting PEI/DNA bi-layers periodically throughout the film assembly process or using a mixture of PEI and PAA solution for each of the polycation layer. The significance of the two growth patterns on film disassembly will be discussed later in the paper.

The film morphology and surface roughness were studied by AFM. Representative AFM images of A1 and B1 films are shown in Figure 5.3 and the surface
roughness measurements as a function of the number of bi-layers are shown in Figure 5.4. Both films change from smooth surface with fine network-like appearance to rougher surface covered by particles of tens of nanometers in size similar to our previous results.\textsuperscript{231, 232} There is no significant difference in surface morphology and roughness between A1 and B1 films, which is also consistent with studies by others.\textsuperscript{72, 231, 232}

**Figure 5.3** AFM height image of a) film A1, and b) film B1. Z-range is 200 nm.

**Figure 5.4** RMS roughness as a function of number of bi-layers measured by AFM for film A1 and film B1. Lines are linear fitting.

The total DNA content in each type of films was determined by immersing the film in 20 mM DTT in PBS buffer for 14 days and measuring the DNA concentration in
the degradation solution. A series of DNA solutions with known concentrations were used to construct the calibration curve. 0.5 µg/ml ethidium bromide (EtBr) was as the fluorescent label of DNA. The total DNA content in A1 is 6.6 µg/cm$^2$ with 0.23 µg/cm$^2$ per layer in the initial growth regime and and 0.56 µg/cm$^2$ per layer in the final growth regime. The total DNA content in B1 is ~ 5.0 µg/cm$^2$ with 0.31 µg/cm$^2$ DNA per layer. All these results are within reasonable range reported in literature. The result agrees with reported value using of 0.35 µg/cm$^2$ per DNA layer in type A film with bioreducible poly(2-dimethylaminoethyl methacrylate) and SEAP plasmid DNA.

5.3.2 The Effect of crosslinking on the LbL films.

Our previous work has demonstrated crosslinking of the LbL films improve film rigidity and cell adhesion. DIP was used as crosslinker, which reacts with amine groups on the PAA. AFM results show marked difference in the degree of swelling between crosslinked films and non-crosslinked films. Before crosslinking, A1 film thickness was 104 nm in air and it increased to 600 nm when measured in the PBS buffer. In contrast, the crosslinked A1 film thickness only showed a slight increase from 70 nm in air to 80 nm in the PBS buffer. The overall reduction in the film thickness as a result of crosslinking has also been observed previously.

AFM images of A1 films before and after crosslinking and corresponding AFM nanoindentation data are shown in Figure 5.5. Indentation, $\delta$, and indentation force, $F$, were obtained from AFM force curves as described in the Experimental Section. The Hertz model for a sphere indenting on a flat surface, $F = \frac{4E\sqrt{R}}{3(1-\sigma^2)}\delta^{3/2}$, was used to determine the apparent Young's modulus of the film, $E$. $E$ was calculated from the slope of $F$ versus $\delta^{1.5}$ plot as shown in Figure 5.5b and 5.5d. The Poisson’s ratio, $\sigma$, is
assumed to be 0.5. R is fixed at 10 nm according to the nominal probe radius data provided by manufacturer. In order to minimize the solid substrate contribution the indentation data used for Hertz model fitting are limited to an indentation depth less than 10 nm, which is approximately 2% of the non-crosslinked film thickness and 12% of the crosslinked film thickness. The probe velocity was fixed at 100 nm/s in order to avoid viscoelasticity effect. The nano-indentation measurements were carried out in PBS buffer at 25°C. The E value for non-crosslinked A1 film is 0.43±0.05 MPa. In contrast, the Young’s modulus of the crosslinked A1 film is 3.1±0.4 MPa. We have observed similar results in our previous work.
**Figure 5.** Time lapse images obtained by *in situ* AFM showing the thickness change of film A1 after immersing in 20 mM DTT. Scan size is 30 µm and Z-range is 2 µm.

In addition to the increase of E, the hydrophobicity of the film is increased after crosslinking because DIP adds more hydrophobic alkyl chains to the film. Contact angle measurements show that the A1 film changes its water contact angle from 35° to 65° after crosslinking.

### 5.3.3 Film disassembly in DTT solution.

The advantage of the LbL film is its stratified nanostructure, which makes it theoretically possible to control DNA release one DNA layer at a time. The degradation kinetics of the two types of LbL films in the DTT solution was studied by AFM, fluorometer, and DLS. We found that the periodic insertion of a PEI/DNA bi-layer in between the PAA/DNA bi-layers has a strong effect on the film degradation kinetics.

AFM imaging of the A1 film in 20 mM DTT was conducted and the images captured at different times are shown in Figure 5.6. Patches of micrometer dimensions, 3 µm by 17 µm at 64 min, 9 µm by 15 µm at 72 min, 6 µm by 19 µm at 83 min, were found to leave the substrate after an hour of immersion. The film was completely released from the substrate after 100 min of immersion. In contrast AFM imaging of the B1 film in 20 mM DTT shows a gradual and prolonged release of possibly nanometer sized particles. Figure 5.7 shows the AFM results of the B1 film degraded by the DTT solution. It took 38 h to complete the B1 film degradation. It should be noted that the AFM data on the B1 film were collected *ex situ* due to the long degradation time. The film was taken out of the DTT solution periodically to be imaged by AFM in PBS buffer. In addition we did
not observe micrometer sized patches ever developed during the B1 film degradation, which suggests that the degradation products are likely smaller than the micrometer size. Figure 5.8 plots the film thickness change measured by in situ AFM when it is immersed in the DTT solution. For the A1 film, the film thickness started to decrease significantly after 1 h of the DTT treatment. It was reduced to zero after 90 min. For the B1 film, there is a significant reduction in the first 3 h followed by a gradual decrease in film thickness up to 120 h of the DTT treatment. The total film degradation time was increased from 90 min to 120 h by inserting a PEI/DNA bi-layer after every two PAA/DNA bi-layer depositions.
Figure 5.6 AFM images measured in air showing the thickness change of film B1 after immersing in 20 mM DTT.
Figure 5.7 Thickness change as function of time in degradation solution measured by AFM for a) film A1, b) for film B1.
Figure 5.8 Thickness change as function of time in degradation solution measured by AFM for a) film A1, b) for film B1.

The products released from the LbL films upon the DTT reduction were analyzed by *in situ* DLS. The hydrodynamic diameter of the particles was measured as a function of the film immersion time in the DTT solution and is plotted in Figure 5.9. The average hydrodynamic diameter of the particles released from the A1 film is around 700 nm at the
beginning and the value decreases to 400 nm after 1 h. The value fluctuates in the range of 70–200 nm from 2 to 8 h of immersion time. Since the A1 film completes degradation in 1.5 h we assume that the smaller particles after 1.5 h are the secondary products from the primary particles directly generated by the film. In contrast, the particles released from the B1 film display a constant size range of 300–400 nm. The DLS data are consistent with the AFM data in that the insertion of the PEI layer reduces the size of particles released from the PAA/DNA film. Particle size strongly influences its cellular uptake mechanism and amount.\textsuperscript{38,39} For example, the cellular uptake of 100 nm particles is 250-folds higher than that of 10 µm particles.\textsuperscript{239} As a result, the large degradation product will have very low cell uptake, which leads to low gene delivery efficiency.

**Figure 5.9** Hydrodynamic diameter of degradation product measured by DLS.
Figure 5.10 Fluorescence intensity of degradation product for a) film A0; b) film A1, the bottom half are made of TRITC label PAA while top half are made of FITC labeled PAA; c) film B1.

Because the PAAs do not have strong UV-vis adsorption, it is difficult to measured released amount of polymer directly. Besides, labeled polymer can also be used to probe the location of released film materials in cells, so fluorescence labeled film was
used to study the release profile. The fluorescence intensity is measured using fluorometer in arbitrary unit. Even though we can't get the exact mass of released polymer, because fluorescence intensity is roughly proportional to polymer concentration, the relative released percentage was obtained by comparing to I_0 (the first data point). (As shown in Figure 5.10, the RHB/DNA film releases majority of the film material within one hour. In contrast, RHB/PEI/DNA film keeps releasing for about one week. In figure 5.10a, the top half of the LbL film contains FITC labeled PAA and the bottom portion of the film is made of TRITC labeled PAA. The data show that TRITC labeled PAA is rereleased at the same rate of the FITC labeled PAA consistent with a bulk erosion behavior for the A1 film. We also used PAAs with lower reducible monomer (CBA) content that show slower degradation rates for their polyplexes.\textsuperscript{23} PAA containing 10\% CBA was used to construct the LbL film (A0 film) and its film degradation rate was reduced from that of the A1 film (Figure 5.10b). However, the bulk release behavior remains.

5.3.4 Transfection \textit{in vitro}

Transfection studies of HEK239 cells on A1 and B1 films were carried out. A fibronectin layer was added as the terminal layer in both films to improve cell adhesion. In this study, fibronectin is applied on top of the LbL film. Figure 5.11 shows the result from the B1 film.
Figure 5.11 GFP transfection of HEK 293 cells on film B1 with fibronectin top coating showing improved transfection.

AFM imaging of the film B3 (Figure 5.12) after removed the cells by vigorous rinsing shows that after one week, the film thickness is reduced to 50% of the original value indicating that cellular film degradation occurs at a much slower rate that DTT degradation. This is understood as the extracellular microenvironment is very different from the DTT solution. The total levels of redox-active thiols on the surface of cells are in the range of 4–30 nmol/10^6 cells. The degradation kinetics simulated by using DTT must be scaled accordingly to the actual cellular redox conditions.
Figure 5.12 AFM images of film B1 after culturing with a) NIH 3T3 cells or b) HEK 293 cells for 7 days. Z-range is 150 nm. The thickness of the film is 29.2 nm for a) and 31.8 nm for b).

The PAA here contained 100% CBA and (PEI/DNA)\textsubscript{16.5} film was used for comparison. The effect of fibronectin top layer was also investigated. As shown in Figure 5.13, all four films with were able to transfect HEK293 cells. The films containing reducible PAA showed higher efficiency than the film made of widely used non-reducible PEI. However, the effect of fibronectin coating seems to be insignificant. Immature dendritic cells were also cultured with film A3 (without fibronectin top coating) coated suture. Even though cell attachment was good (Figure 5.14), no transfection was detected.

![Figure 5.13 Luciferase transfection of HEK 293 cells with A3 film and A5 film coated suture. 5mm suture was cut and dispersed in a co-culture with HEK293 cells for 72h. Suture was either coated with (bottom row) or without (top row) fibronectin. Luciferase activity was measured by imaging after addition of luciferin to culture media. The luminescence is inverted; dark spotting within the wells indicates luciferase activity.](image-url)
**Figure 5.14** Optic microscope image for immature dendritic cells growing on A3 film coated suture after 3 days.

### 5.3.5 Transfection *in vivo*.

The silk suture was coated LbL films (A3) containing luciferase-expressing plasmid pGL4 instead of GFP. Mice received 1cm film A3 and A5 coated suture. The DNA content in 1 cm film A3 coated suture was determined to be ~1 µg pGL4 via real time PCR. Electroporation was used for comparison. The transfection result was examined after 10 days. As shown in Figure 5.15, both films failed to induce luciferase activity in the mice, while electroporation showed positive result. The suture was taken out from mice and real-time PCR analysis indicates suture has ~1/10,000 the original DNA quantity remaining at this time point. The discrepancy of transfection efficiency for HEK293 cells and dendritic cells may indicate that the A3 film lacks ability to target immune responding cells.
Figure 5.15 In vivo transfection of mice using Lbl film coated suture and electroporation. After 10 days, mice received D-luciferin substrate and were imaged for 30 minutes to detect luciferase activity. The luminescence image overlays a white light image to identify signal location. Intensity of light detection ranges from red (low) to blue (high).

5.4 Discussion

The layered structure of LbL films has been assumed until recent evidence showing diffusive layer interface with the degree of interlayer diffusion playing an important role in the final structure of the LbL films.\textsuperscript{230, 241-244} For polyelectrolytes capable of interlayer diffusion, there exists a diffusion zone in the LbL films, which acts as a reservoir during layer growth until reaching a kinetic limit.\textsuperscript{72, 243-245} The growing reservoir with increasing number of layers results in the exponential growth of the LbL films.\textsuperscript{243, 244} DNA is considered a non-diffusible polyelectrolyte due to its high charge density, chain length, and chain rigidity.\textsuperscript{245} Our data show that PAA likely undergoes interlayer diffusion that results in exponential growth. The simultaneous release of FITC and TRITC labeled PAAs in the A1 film also points to a high degree of interlayer diffusion. Others have also reported dye labeled polyelectrolytes diffusing through the entire film due to interlayer diffusion.\textsuperscript{230} LbL films with interlayer diffusion do not
exhibit well-defined layered structure and their degradation follows a bulk erosion behavior – not desirable for sequential DNA/drug release. An indicator of degree of interlayer diffusion is the film growth pattern. An LbL film that displays an exponential growth pattern likely contains significant interlayer diffusion. An LbL film undergoes a linear growth likely maintains the layered internal structure. This bulk erosion of the A3 film deposited on the suture is consistent with the in vivo transfection study and real-time PCR analysis indicating only $\sim 1/10^4$ the original DNA quantity remained on suture after 10 days. Attempts are made by adding non-degradable primer layers consisting 5 bi-layer of PEI/PSS. 27.5 bi-layers of PAA/DNA were deposited on top of the primer layer (A6 film). However, in situ AFM results show that primer layers do not prevent bulk release.

Our results show that periodic insertion of the PEI layer changes the film growth from exponential to linear. The hybrid PAA and PEI film shows slower and sequential DNA release with stable release products all of them are desirable attributes for controlled DNA delivery. PEI has a smaller diffusion coefficient and has a higher charge density than PAA. It may play two roles in the LbL film. First, it acts as a barrier that screens the residue charge on the film. Theoretical work has found residual charge is one of the main driving forces for interlayer diffusion. Secondly, the limited diffusion also causes blending with neighboring layers. As a result, slow diffusion species will affect the diffusion coefficient of fast diffusion species. The linear growth and gradual release of type B film indicates that the interlayer diffusion is limited and layered structure is improved in the film.
5.5 Conclusion

In summary, we present the initial study of using non-degradable, non-diffusible polycation as barrier to regulate the internal structure of LbL film. The interlayer diffusion is greatly restrained indicated by film growth model change. The LbL film remains stimuli-responsive. The DNA release dynamics from the LbL film changes from fast and abrupt to sustained and gradual by adding such barrier layers. We also incorporated two cell interaction ligand--fibronectin and HA in the LbL film to promote receptor-mediated cell adhesion and uptake. *In vitro* study shows improved cell proliferation and transfection. This works provides a potential way for better control the LbL film degradation and improving localized gene delivery.
Chapter 6

Conclusions and recommendations for future research

6.1 Conclusions

Bioreducible PAAs showed great potential as non-viral gene delivery vectors that can complex DNA forming polyplexes systemic gene delivery or LbL films for localized gene delivery.

Self-assembled DNA/PAA polyplexes continue to gain strength as viable alternatives to viral vectors. In order to correlate physiochemical attributes with transfection and toxicity data, the DNA release dynamics were investigated. Redox potential gradients and negatively charged polyelectrolyte were used as stimuli to induce DNA release. AFM was employed to study DNA release dynamics in simulated physiologic conditions with DTT or heparin. DTT triggers the depolymerization of high-molecular-weight polycations into low-molecular-weight oligocations via thiol-disulfide exchange reaction, thus causes DNA release. Heparin also shows the capability of inducing DNA release by polyelectrolyte exchange reaction. A DNA release pathway was found to be common. The three-stage pathway begins with morphological change from metastable nanostructures into the more favorable toroid structure. Then toroids interact with each other by aggregation and fusion. Finally, DNA wormlike chains gradually unravel from the polyplex resulting in loose loops/tails that are held by a central compact core. The release dynamics can be tuned by altering the polymer structure, and affects cell transfection performance as a result.

Layer-by-layer (LbL) films containing cationic polyelectrolytes and DNA is a promising vector for localized gene delivery. The degradation of PAA/DNA LbL films
are modulated by insertion of a non-bioreducible polycation, PEI, as a barrier layer and crosslinking of the LbL films using 1,5-diodopentane. The cell transfection is improved by PEI layers, which are found to be effective in lowering the degradation rate of the film. The gene delivery efficiency is further improved by the incorporation of a fibronectin terminal layer and hyaluronic acid. The hybrid bioreducible and non-bioreducible LbL film demonstrates its potential to achieve LbL disassembly and sequential delivery of bioactive molecules down to the molecular scale.

6.2 Recommendations for future research

Polymeric gene delivery vectors must overcome multiple barriers in order to be expressed at the target cell. The stage of the pathway at which DNA is released is critical to gene delivery efficacy. The molecular interactions between gene delivery vectors and cell membrane and subcellular organelles are important for cell entry, intracellular trafficking, and nuclear targeting stages. The design of smart multi-component polymeric vectors for effective and safe gene delivery will be the main direction of future research. Future work will focus on tuning structure of polymers and polyplexes. The goal is to formulate a polyplex that is stable in extracellular environment while remain stimuli-responsive inside cells. The more detailed study of molecular mechanisms of the vector disassembly obtained in closer to physiologic conditions will be critical for the success.
Influence of Nanoscale Surface Roughness on Colloidal Force Measurements

A.1 Introduction

Understanding of colloidal stability is essential for the development and processing of formulations of food, pharmaceuticals, adhesives, coatings, inks, and paints as well as working with aspects of cellular functions and targeted drug delivery. Colloidal forces impact dry powder handling, transport, blending, and fluidization. In colloidal dispersion systems, surfactant dispersants are essential in maintaining dispersion stability. They adsorb to the particle surface to provide electrostatic and steric barriers and reduce colloidal adhesion and aggregation. Dispersion formulations can be guided by a knowledge of interparticle force curves, for example, the use of adhesion minima to predict degrees of particle agglomeration/aggregation and sedimentation rates. The interparticle force curves are commonly calculated according to the Derjaguin–Laudau–Verwey–Overbeek (DLVO) theory, which consists of the attractive Van der Waals interaction and the repulsive electrostatic double-layer interaction. The actual colloidal behavior may deviate from the DLVO prediction due to surface roughness and non-uniform surface charge density. Surface roughness is known to affect particle deposition on surfaces, dispersion stability, flow through porous media, and sedimentation. For example, deposition of carbon black particles on a planar surface can fit the DLVO theory only when the contact point is moved to a separation distance of 50 Å due to protruding surface asperities. The deposition rates of polystyrene latex particles on glass beads in porous media are higher than those predicted by the DLVO theory, which have been attributed to the surface roughness of particles and collectors.
It has been suggested that the total interaction energy may be determined by the radii of curvature of surface asperities rather than the colloidal particle curvature indicating the dominance of surface roughness effects.\textsuperscript{258}

AFM using colloidal probes (colloidal probe microscopy or CPM) is an essential tool for direct measurements of force-versus-distance curves.\textsuperscript{105, 259-261} A variety of microparticles have been used as probes including silica,\textsuperscript{259, 260, 262-264} zirconia,\textsuperscript{265} alumina,\textsuperscript{261} titanium dioxide,\textsuperscript{266} polystyrene,\textsuperscript{238, 267, 268} and cellulose.\textsuperscript{269} While most of the studies have shown agreements between the experiments and the DLVO predictions, deviations from the DLVO theory have been observed when the separation distance is small (< 3 nm),\textsuperscript{259, 260} salt concentration is high (> 1 M),\textsuperscript{261} or surface roughness influence is non-negligible. For example, the CPM measurements between an iron oxide particle and a flat silica surface show that the magnitude of the adhesion is significantly less than the DLVO prediction.\textsuperscript{270} The discrepancy has been attributed to a large effective separation at contact as a result of the surface roughness. In the same study, it has been found that the pull-off force increases with the loading force. In another CPM study between a silica particle and a planar silica surface, adhesion between surfaces with non-negligible double-layer interactions is lower than the theoretical value;\textsuperscript{271} however in this case the difference has been attributed to a short-range repulsive hydration force, ~ 1 nm, and not the surface roughness. The adhesion between smooth silica particles measured by the CPM has been shown to increase with particle radius\textsuperscript{272} consistent with the Johnson, Kendall, and Roberts (JKR)\textsuperscript{273} and Derjaguin, Müller, and Toporaov (DMT)\textsuperscript{274} models. However, the adhesion measured between carbonyl iron powder particles shows no correlation with the particle radius, which has been attributed to the higher surface
roughness of the carbonyl iron powder.\textsuperscript{275} Lower than predicted adhesion values have been measured between particles of titanium carbide, glass, sapphire, germanium, tin, and polystyrene, which has been attributed to surface irregularities.\textsuperscript{253, 276, 277} Theoretical modeling of the surface roughness effect has shown that at large separations, surface roughness has a greater impact on the electrostatic repulsion by reducing the secondary potential energy minimum and moving it to larger separation distances; and at smaller separations, surface roughness has a greater impact on the Van der Waals attraction by lowering the height of the primary barrier to flocculation.\textsuperscript{278} The adhesion force between rough particles may be overestimated due to the reduced area of contact between asperities if using a contact area value calculated from the overall particle radius.\textsuperscript{247}

In this study, well characterized model colloids with well defined nanoscale surface roughness are used to study the surface roughness effect on colloidal forces. Polystyrene latex particles are used because they are widely used in the CPM and are commercially available. In addition, polystyrene surface roughness can be varied at the nanometer scale.\textsuperscript{279, 280} We conduct CPM measurements as well as AFM indentation and dynamic light scattering (DLS) measurements between commercial polystyrene particles with a diameter of 15 µm and root-mean-square (RMS) surface roughness of 30 nm (denoted here as PS\textsuperscript{R}), and heat-treated polystyrene particles with roughness reduced to 1 nm (denoted here as PS\textsuperscript{S}), in order to determine the effect of nanoscale surface roughness on colloidal force curves. Approaching and retracting force curves are measured in various salt and surfactant solutions. The surfactants used are: non-polymeric nonionic surfactant pentaethylene glycol monododecyl ether (C\textsubscript{12}E\textsubscript{5}), polymeric nonionic surfactant poly(ethylene oxide)$_\text{x}$–poly(propylene oxide)$_\text{y}$–poly(ethylene oxide)$_\text{x}$
(Pluronic® F108), and polymeric ionic styrene/acrylic surfactant Joncryl® 60. In the absence of the surfactant, nanometer surface roughness affects colloidal forces only in high salt conditions when the Debye length becomes smaller than the linear dimension of the surface roughness. On the other hand, the adhesion was found to be stronger between rougher colloids. The adhesion reduced to zero by all three surfactants above a critical solution concentration. Under otherwise identical conditions, a higher surfactant concentration is necessary in order to eliminate the adhesion between PS\textsuperscript{R} than PS\textsuperscript{S}. This study demonstrates that surface roughness even at the nanometer scale can affect colloidal forces significantly and should be taken into account in colloidal dispersion formulations. The results suggest that the amount of dispersants necessary to provide colloidal stability can be fine tuned by surface roughness.

A.2 Experimental

A.2.1 Materials

Deionized water with 18 MΩ×cm resistivity (Nanopure system, Barnstead) has been used. Grade 2 muscovite mica has been purchased from Mica New York and hand-cleaved just before use. Polystyrene latex suspensions (0.25 wt%) containing particles of 15 µm in diameter have been purchased from Polyscience. The suspension has been dialyzed in order to remove soluble impurities. GC grade C\textsubscript{12}E\textsubscript{5} (98%) has been purchased from Sigma-Aldrich and used as received. Pluronic F108 and Joncryl 60 have been provided by BASF and used as received. The chemical structures of the three surfactants are given in Figure A.1.
A.2.2 Heat treatment of commercial PS to reduce surface roughness

The surface roughness of polystyrene is reduced by heating above its glass transition temperature (~ 105°C). The colloids are placed in an oven at 120°C in N₂ for different lengths of time. Colloids with the lowest surface roughness are obtained after 4 h in the oven and are used in this study.

A.2.3 AFM imaging

AFM imaging is conducted using VEECO Dimension 3100 with a G scanner. The particle morphology is determined by AFM height, amplitude, and phase images in the tapping mode in ambient air. Uncoated silicon probes (TESP, VEECO) with a factory-specified spring constant of 40 N/m, length of 125 µm, width of 40 µm, and nominal probe radius of curvature less than 10 nm are used. The scan rate used is in the range of 0.1–1.0 Hz with a scan size range of 1–30 µm. Integral and proportional gains are
approximately 0.1–0.4 and 0.2–0.8, respectively. Images have been analyzed using the Nanoscope software from Digital Instruments (Version 5.12). The surface roughness is determined using the root-mean-squared roughness $RMS=\left[\sum(z_i^2/N)\right]^{1/2}$ where $z_i$ is the height value of each measurement point and $N$ is the number of measurement points. All RMS values reported have been obtained on 500×500 nm² sized images.

**A.2.4 CPM**

Colloidal probes are prepared following the literature procedure.\textsuperscript{259, 281} Epoxy glue (Epo-Tek\textsuperscript{377}, Epoxy Technology) is heated in the water bath at 80°C for 30 min in order to reach an appropriate viscosity. A small amount of the glue is transferred to a glass slide. A tip-less AFM cantilever (PNP-TR-TL-20, Nanoandmore) is moved, using the Dimension 3100 automatic stage as a micro-manipulator, first to contact the glue and then a polystyrene particle so that the particle is glued to the end of the cantilever. Only 10 µL or less glue is needed in this operation. The colloidal probe is placed in a desiccator for at least 24 h before use. Figure A.2 shows a typical colloidal probe constructed.

![Figure A.2 SEM image of a PS colloidal probe. The bar length = 10 µm.](image)
The AFM force-versus-distance curves are obtained in the liquid contact mode using the force calibration command between the colloidal probe and the colloids immobilized on mica or glass. The colloids are glued on the solid substrate using epoxy. The probe is brought to rest in a close proximity to the colloidal layer on the substrate and is equilibrated for at least 1 h at 25°C before the measurements. 100 µl of liquid is injected into the liquid cell. The center-to-center alignment of the two colloids is conducted first by a coarse alignment using the integrated optical microscope followed by a fine alignment using the AFM height images. After the coarse alignment we take several AFM height images at different spots close to the center, and the spot that gives the best fit between the top portions of the height image and those of an ideal spherical cap shape is chosen for the subsequent force measurements. The alignment error of this procedure is estimated to be less than 50 nm for 15 µm particles. In one study using 4.8 and 6.9 µm colloids, the force curves show no changes when the center of one colloid is moved 200 nm off the center of the other colloid. Therefore we conclude that our measurements accurately represent colloidal forces between particles aligned along their central axes. Each force curve reported here has been compiled from 20 or more force measurements.

The force calibration curves are typically plotted as the photodiode signal (in volts) versus piezoelectric scanner position (in nanometer). The force calibration curves are converted to the force-versus-distance curves by defining sensitivity, zero force ($F = 0$), and zero separation ($D = 0$). The sensitivity in nm/V is obtained from the slope of the constant compliance regime of the retraction curve and is multiplied by the raw voltage value to yield the cantilever deflection, $Z_C$. The force is $F = k \cdot Z_C$. $k$ is the spring
constant of the cantilever. The nominal spring constant provided by the manufacturer is 0.08 N/m. We have verified the spring constant by the resonance frequency method.\textsuperscript{283} The measured value is 0.082 ± 0.003 N/m (N = 6), and 0.08 N/m is used here. Zero force is determined by identifying a linear region at large separation where the deflection is constant. Zero separation is determined from the constant compliance region at high force where the deflection is linear with the expansion of the piezoelectric crystal. When the separation is zero, it is assumed that the two PS colloids are in hard contact. In the nonlinear regime, the separation distance $D = \Delta(piezo \ position) - Z_C$. Surface roughness contributes uncertainty and error in zero separation determination and there is no simple solution to this problem.\textsuperscript{105}

**A.2.5 AFM nanoindentation**

Nanoindentation experiments are conducted in the surfactant solution on the immobilized colloidal layer in order to determine the adsorbed surfactant layer thickness and apparent elastic modulus. The force calibration curves are converted to the force-versus-indentation curves. The contact point, i.e., indentation $\delta = 0$, is defined as the point where the force becomes repulsive. The indentation is defined as:

$$
\delta = \begin{cases} 
\Delta(piezo \ position) - Z_C & (Z_C > 0) \\
0 & (Z_C = 0) 
\end{cases}
$$

Eqn. 1

The adsorbed layer thickness is estimated at the point of the maximum indentation. It is noted that AFM nanoindentation sometimes underestimates the film thickness due to incomplete penetration by the AFM probe into the adsorbed layer down to the bare substrate; however, AFM nanoindentation is a widely used tool for the study of surfactant and polymer adsorption.\textsuperscript{284-290} There is also uncertainty in the probe/layer contact point
determination. Following the literature, we determine the contact point as follows. In the CPM the contact point is determined as the intersection between the extrapolation of the non-contact regime and constant compliance regime. In nanoindentation, the contact point is the position that the force becomes positive.

**A.2.6 Dynamic light scattering (DLS)**

The polystyrene latex diameter in different surfactant solutions is determined by DLS (Nanotrac NPA250). The effective hydrodynamic radius \( R_H \) is measured. The backscattering angle \( \Theta \) is fixed at 180° with a laser wavelength \( \lambda = 633 \) nm. The size measurement range is set between 1 nm and 6 \( \mu \)m. \( R_H \) is a function of the diffusion coefficient (D), temperature (T), and viscosity (\( \eta \)) according to the Stokes-Einstein equation (Eqn. 2):

\[
R_H = \frac{kT}{3\pi\eta D}.
\]

Eqn. 2

k is Boltzmann constant. T is 25°C and D is obtained from autocorrelation function via the cumulant fitting.

**A.3 Results and Discussion**

**A.3.1 Characterization of polystyrene particles with different surface roughness**

The colloids glued to the mica are imaged by AFM. Figure A.3 shows the AFM images (high and low magnifications) of untreated colloids (a–b) and colloids heated for 4 h (c–d) and 12 h (e–f). The colloids heated for 4 h exhibit the lowest surface roughness with an RMS = 1.0 nm. The untreated colloids have an RMS of 30.0 nm and the colloids heated for 12 h have an RMS of 2.0 nm. The surface asperities are evenly distributed across the surface of the treated and untreated colloids. Small particulates are detected on the colloids heated for 12 h. Similar debris have been found by others and attributed to
small precursor particles formed during the latex synthesis. In the following experiments, two types of colloids are used: smooth colloids after 4 h heating (denoted as PS) and untreated colloids (denoted as PS).

**Figure A.3** AFM height images of the PS spheres: a–b) untreated colloids (scan size and z range for a) are 15μm and 2μm and for b) are 750 nm and 20 nm), c–d) colloids heated for 4 h (scan size and z range for c) are 20 μm and 2 μm and for d) are 1 μm and 20 nm, and e–f) colloids heated for 12 h (scan size and z range for e) are 20 μm and 5μm and for f) are 1.5 μm and 30 nm).
A.3.2 Surface roughness effect on surface force profiles in the absence of the surfactants

First, we compare force curves between a colloidal probe, PS\textsuperscript{R} or PS\textsuperscript{S}, and mica in deionized water with or without 1 mM NaCl.

![Force curves between PS\textsuperscript{S} (or PS\textsuperscript{R}) and mica in deionized water and 1 mM NaCl.](image)

**Figure A.4** Force curves between PS\textsuperscript{S} (or PS\textsuperscript{R}) and mica in deionized water and 1 mM NaCl. The experimental data are fitted with the DLVO theory as represented by the solid lines.

The approaching force divided by the colloidal probe radius $R$ versus separation distance $D$, $F/R$ vs. $D$, curves are plotted in Figure A.4. The curves are fitted using the least squares regression method with the DLVO theory. The equation (Eqn. 3) assumes a non-retarded Van der Waals interaction, the Derjaguin approximation for a sphere (1) with radius $R$ interacting with a surface (2) at small $D$, and a constant surface potential:\textsuperscript{292}

$$F/R = -\frac{A}{6D^2} + \frac{128\pi kT}{\kappa} \frac{\Gamma_1 \Gamma_2}{D} \exp(-\kappa D). \quad \text{Eqn. 3}$$

$R$ is fixed at 7.5 µm. $A$ is the Hamaker constant. $A$ is fixed at $6.1 \times 10^{-21}$ J for mica-water-polystyrene and $9.5 \times 10^{-21}$ J for polystyrene-water-polystyrene.\textsuperscript{293, 294} $k$ is the
Boltzmann constant. \( T \) is temperature (298 K). \( 1/\kappa \) is the Debye length. \( n \) is the bulk electrolyte concentration. \( \Gamma_1 = \tanh \left( \frac{ze\Psi_1}{4kT} \right) \). \( \Gamma_2 = \tanh \left( \frac{ze\Psi_2}{4kT} \right) \). \( z \) is ionic valence. \( e \) is the charge constant of an electron. \( \Psi_1 \) is the surface potential of the colloid. \( \Psi_2 \) is the surface potential of the mica (-6.3 mV). Here the mica surface potential is kept artificially low due to the assumption of \( \kappa D \gg 1 \).

For \( PS^S \) in deionized water, the fitted \( 1/\kappa \) is 30.2 nm corresponding to an ionic strength of 1.0\times10^{-4} \text{ M}. This is the result of atmospheric CO\(_2\) in deionized water. Upon the addition of 1 mM NaCl, the fitted \( 1/\kappa \) decreases to 9.0 nm. This is comparable to the calculated vale of 9.6 nm for 1 mM NaCl. The fitted surface potential of the \( PS^S \) colloid is -6.4 mV in deionized water and -5.7 mV in 1 mM NaCl.

For \( PS^R \) in deionized water, the approaching force curve (Figure A.4) as well as the fitted \( 1/\kappa \) (29.1 nm) and \( \Psi_1 \) (-5.9 mV) are almost identical to those of \( PS^S \) in deionized water. The approaching force curve of \( PS^R \) in 1 mM NaCl is shifted to a longer range than that of \( PS^S \) (Figure A.4). The fitted \( 1/\kappa \) and \( \Psi_1 \) are 8.2 nm and -5.6 mV. Our data suggest that 30 nm surface roughness affects surface forces in high salt conditions in which \( 1/\kappa \) values is smaller than surface roughness.

The jump-in distance, defined by the jump-in point where the force curve becomes discontinuous, for \( PS^S \) is 7.6 ± 1.3 nm in deionized water and 6.0 ± 0.6 nm in 1 mM NaCl, while it increases to 12.5 ± 3.1 nm in deionized water and 12.2 ± 1.0 nm in 1 mM NaCl for \( PS^R \). The standard deviation for jump-in distance is calculated based on 20 force curves. It shows the net attractive force shifts to a longer range with increasing surface roughness.
In the above force curve fittings, the root-mean-squared deviation (RMSD) is $1.0 \times 10^{-3}$ nN and $8.6 \times 10^{-4}$ nN for PS$^S$ in deionized water and 1 mM NaCl, respectively. For PS$^R$ the RMSD is $1.3 \times 10^{-3}$ nN and $7.4 \times 10^{-4}$ nN in deionized water and 1 mM NaCl, respectively. We estimate our fitting to have an error of 2–4%, which is comparable to 5% reported in the literature. Errors in the fitting using Eqn. 3 can also arise due to the assumption of $\kappa D \gg 1$. The actual surface potentials of mica and polystyrene may be much more negative than the values in the fitting.

Next we present approaching force curves measured between a pair of colloids of either the PS$^S$ type or the PS$^R$ type (Figure A.5). The nanometer surface roughness appears to affect force curves in both low salt (deionized water) and high salt (1 mM NaCl) conditions. The jump-in distances, marked by the arrows in Figure A.5, are $6.6 \pm 1.0$ nm in deionized water and $5.9 \pm 1.3$ nm in 1 mM NaCl for PS$^S$ and $12.9 \pm 3.4$ nm in deionized water and $15.1 \pm 2.6$ nm in 1 mM NaCl for PS$^R$. The net attraction increases with surface roughness. The pull-off force corresponding to the maximum adhesive force in the retracting force curve is $3.4 \pm 0.5$ nN for PS$^S$ and $37 \pm 8$ nN for PS$^R$ (Table A.1) showing that adhesion increases with surface roughness. We observe little effect by salt on the adhesion. Our results of increased adhesion with increasing surface roughness is consistent with theoretical predictions of an increase in the jump-in distance and decrease in the potential energy barrier as a result of surface protruding asperities. The most commonly used model to describe adhesion between colloidal particles containing nanoscale roughness is the Rumpf or the modified Rumpf model shown below (Eqn. 4):

$$F_{\text{adhesion}} = \frac{AR}{12H_0^2} \left( \frac{1}{1+R/1.48\text{RMS}} + \frac{1}{(1+1.48\text{RMS}/H_0)^2} \right),$$  
Eqn. 4
Where $H_0$ is the contact distance, which is defined as the minimum distance between the two objects. $H_0$ is close to the interplanar spacing of 0.3–0.4 nm.\textsuperscript{297, 298} For polystyrene, it is reported to be 0.28 nm according to single crystal X-ray diffraction.\textsuperscript{299} Thus, the Rumpf model predicts $F_{\text{adhesion}} = 2.3$ nN for RMS = 1 nm and 0.9 nN for RMS = 30 nm ($A$ is assumed to be $9.5 \times 10^{-21}$ J and $R$ is assumed to be 7.5 $\mu$m in the calculations). The Rumpf prediction matches closely the experimental value between PS$^S$ but not that between PS$^R$, probably because this model does not take into account multi-point contacts,\textsuperscript{298, 300, 301} and physical lodging between surface asperities. The calculated value is only 0.9 nN for RMS roughness of 30 nm; in contrast, the experimental result is 37 ± 8 nN.

**Figure A.5** Force versus Separation curves for PS$^S$ and PS$^R$. Each set of curves includes the approaching part and retraction part. The force minimum in retraction part is considered as adhesion force.
Table A.1 Adsorption layer thickness, elastic modulus and adhesion force between PS colloids.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Adsorption layer thickness $h$(nm)</th>
<th>Elastic modulus (MPa)</th>
<th>Adhesion force (nN) for PS$^S$</th>
<th>Adhesion force (nN) for PS$^R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.4±0.5</td>
<td>37±8</td>
</tr>
<tr>
<td>C$_{12}$E$_5$ 0.2%</td>
<td>2.5±0.3</td>
<td>0.20±0.05</td>
<td>0</td>
<td>2.0±0.3</td>
</tr>
<tr>
<td>C$_{12}$E$_5$ 1%</td>
<td>3.1±0.1</td>
<td>0.25±0.04</td>
<td>0</td>
<td>1.0±0.4</td>
</tr>
<tr>
<td>C$_{12}$E$_5$ 5%</td>
<td>3.0±0.2</td>
<td>0.26±0.04</td>
<td>0</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>F108 0.2%</td>
<td>11±1</td>
<td>0.46±0.01</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F108 1%</td>
<td>12±0.5</td>
<td>0.48±0.01</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F108 5%</td>
<td>16±1</td>
<td>0.47±0.02</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Joncryl 60 0.2%</td>
<td>5.0±0.8</td>
<td>0.66±0.02</td>
<td>0</td>
<td>46±10</td>
</tr>
<tr>
<td>Joncryl 60 1%</td>
<td>8.0±0.5</td>
<td>0.69±0.04</td>
<td>0</td>
<td>0.10±0.03</td>
</tr>
<tr>
<td>Joncryl 60 5%</td>
<td>10±1</td>
<td>0.88±0.08</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

In addition, roughness can induce uneven surface charge distribution and uncertainty of interfacial position.\(^{302}\) It should be noted that in addition to surface roughness (or local curvature at the area of contact) the pull-off force is generally a function of the compressive force during approach and the physical properties of the particles including Young’s modulus, Poisson’s ratio, surface hardness, interfacial energy, and radius of the particle.\(^{247}\) Both JKR and DMT models indicate $F_{adhesion} \propto a \propto E^{-2/3},$ \(^{298}\) where $a$ is contact area and $E$ is Young’s modulus. For rough and relatively soft PS colloids, there may be lock-and-key contact, which results in an increase in $a.$ In an indentation study,\(^{303}\) the apparent Young’s modulus is 0.3 GPa for an outer rough layer and 4 GPa for the inner core material. Thus, PS$^R$ may appear to have a smaller $E$ and
undergoes a higher degree of deformation, which may contribute to a stronger
adhesion.\textsuperscript{304}

The force curves between two polystyrene colloids are fitted using Eqn. 5
assuming a non-retarded Van der Waals force, Derjaguin approximation for two identical
spheres of radius R with small D, and constant and low surface potential of $|\Psi| < 25$
mV:\textsuperscript{292, 305}

$$\begin{align*}
\frac{F}{R} &= -\frac{A}{12D^2} + 2\pi \varepsilon_0 \varepsilon_r \Psi^2 \kappa \frac{\exp(-\kappa D)}{1 + \exp(-\kappa D)}. \\
\end{align*}$$

Eqn. 5

$\varepsilon_0$ is the permittivity in vacuum and $\varepsilon_r$ is the relative permittivity for water.

For PS\textsuperscript{S}, the fitted Debye length, surface potential, and RMSD are 25.7 nm, -24.7
mV, and $8.9 \times 10^{-4}$ nN, in deionized water and 9.3 nm, -24.5 mV, and $7.9 \times 10^{-4}$ nN in 1
mM NaCl. For PS\textsuperscript{R}, the fitted Debye length, surface potential and RMSD are 24.0 nm, -
24.5 mV, and $4.8 \times 10^{-4}$ nN in deionized water and 10.6 nm, -14.4 mV, and $4.1 \times 10^{-4}$ nN in
1 mM NaCl. Because of the symmetric geometry, analytical expression of the interaction
is available as shown in Eqn. 5, which does not require $\kappa D \gg 1$ as in Eqn. 3. As a result,
the fitted zeta potential values agree better with literature values\textsuperscript{306} and our DLS
measurements. The differences between PS\textsuperscript{S} and PS\textsuperscript{R} in fitted Debye length and surface
potential are -0.7 nm (2.7\%) and 0.2 mV (0.8\%) in deionized water and 1.3 nm (14.0\%),
6.4 mV (23.4\%), in 1 mM NaCl. Again we observe nanometer surface roughness to have
a stronger effect in high salt conditions than low salt conditions. More importantly, both
PS-Mica and PS-PS interaction data suggest that the roughness effect becomes
pronounced when the double layer thickness is close to surface roughness.
A.3.4 Surface roughness effect on surface force profiles in the presence of surfactants

Three surfactants are used in this study. C_{12}E_{5} is a nonionic non-polymeric surfactant whose adsorption behavior has been well studied;\textsuperscript{286, 307-309} Pluronic F108 is a nonionic polymeric surfactant, used widely in consumer and industrial products as antifoaming agents, wetting agents, dispersants, thickeners, and emulsifiers.\textsuperscript{310} Joncryl 60 is a polymeric and anionic surfactant, and is used for coating, emulsion, pigment dispersion, and in new flexo inks. The force curves are measured in three application-relevant concentrations: 0.2wt\%, 1wt\%, and 5wt\%. The CMC values at 25°C of the surfactants are listed in Table A.2. Figure A.6 shows the surface tension measurement of Joncryl 60. In the case of C_{12}E_{5} and Joncryl 60, since the concentration range used in this study is above the CMC and the critical aggregation concentration (CAC),\textsuperscript{309, 311, 312} the adsorbed surfactant layer coexists with the micellar phase in solution.\textsuperscript{308} In the case of F108, the concentration range used encompasses its CMC.\textsuperscript{183} The concentrations relative to the CMC of each of the surfactant are listed in Table A.2.

**Tables A.2** Surfactant concentration and CMC

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>CMC (g/ml)</th>
<th>Wt%</th>
<th>Relative concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{12}E_{5}</td>
<td>2.8×10^{-5}, 309, 312</td>
<td>0.20%</td>
<td>71</td>
</tr>
<tr>
<td>Pluronic F108</td>
<td>4.5×10^{-2}, 183</td>
<td>1%</td>
<td>357</td>
</tr>
<tr>
<td>Joncryl 60</td>
<td>2.0×10^{-4}</td>
<td>5%</td>
<td>1786</td>
</tr>
<tr>
<td></td>
<td>0.20%</td>
<td>1%</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>0.22</td>
<td>5%</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>50</td>
<td>5%</td>
</tr>
</tbody>
</table>
Figure A.6 Surface tension measurement for Joncryl 60. The CMC was determined to be 2×10^{-4} g/ml.

The approaching force curves between a pair of colloidal particles, either the PS\textsuperscript{S} or PS\textsuperscript{R} type, in the presence of a surfactant solution contain two repulsive force terms, a long-range electrostatic force term \( F_{\text{elec}} \) and a short-range steric repulsion term \( F_{\text{steric}} \).\textsuperscript{313,314} Figure A.7 is a force curve measured between two PS\textsuperscript{S} particles in 5\% F108 in which the experimental data are fitted with \( F_{\text{elec}} \) and \( F_{\text{steric}} \) terms as described by Eqn. 6–8.\textsuperscript{313,314,315,316}

\[
\frac{F}{R} = \frac{F_{\text{elec}} + F_{\text{steric}}}{R} \quad \text{Eqn. 6}
\]

\[
F_{\text{elec}} = k_{e} \exp(-\kappa D) = 64\pi R n k T \tanh^{2}\left(\frac{ze\Psi}{4kT}\right) \kappa^{-1} \exp(-\kappa D) \quad \text{Eqn. 7}
\]

\[
F_{\text{steric}} = k_{s} \exp(-D/\lambda) \quad \text{Eqn. 8}
\]

The fitting parameters are listed in Table A.3. Eqn. 7 contains assumptions similar to those for Eqn. 3. In Eqn.7, \( k_{s} \) is related to the surfactant layer packing density, and \( \lambda \) is proportional to the radius of gyration of the polymer (\( R_{g} \)).\textsuperscript{314-316} In the case of \( C_{12}E_{5} \), \( k_{s} \) is smaller for PS\textsuperscript{R} indicating more disordered layer due to surface roughness. The force
curves measured in 5% C_{12}E_5 may contain significant contribution from micelles in solution as indicated by the unreasonable high value of the Debye length. There does not seem to be a significant impact by surface roughness in F108 solution. However the smaller $k_s$ values for F108 than C_{12}E_5 indicate that the polymeric surfactant is less densely packed than the monomeric surfactant. Surface roughness has a significant impact on the interactions between the polystyrene colloids in the charged Joncryl 60 solution. The force curves measured in 0.2%, 1%, and 5% are different between pairs of PS^S and PS^R. In the case of 1% and 5% Joncryl 60 $F_{elec}$ drops to zero due to high electrolyte concentrations.

![Figure A.7](image)

**Figure A.7** Force measurement and double exponential fitting for PS^S in 5% Pluronic F108 solution in logarithm scale. Dots represent experiment data and solid line is fitted curve.
The adhesion measured by the pull-off force during retraction of PS\textsuperscript{S} probe and PS\textsuperscript{S} covered substrate is zero in 0.2% surfactant solutions of all three surfactants. But when the same force measurements are conducted between the PS\textsuperscript{R} colloids, we find that only in Pluronic F108 0.2% solution the adhesion is zero. The adhesion values in 0.2%, 1%, and 5% C\textsubscript{12}E\textsubscript{5} are 2.0 ± 0.3 nN, 1.0 ± 0.4 nN, and 0.5 ± 0.1 nN, respectively. The adhesion values in 0.2% and 1% Joncryl 60 solution are 46 ± 10 nN and 0.1 ± 0.03, respectively. The adhesion becomes zero only when 5% Joncryl 60 is used. Therefore we can conclude that dispersant performance in preventing colloidal adhesion could be impacted by surface roughness at the nanoscale. Higher concentrations of dispersants may be necessary to achieve the same degree of colloidal stability when the colloids have

<table>
<thead>
<tr>
<th></th>
<th>PS\textsuperscript{S}</th>
<th></th>
<th>PS\textsuperscript{R}</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$k_s$ (nN)</td>
<td>$\lambda$ (nm)</td>
<td>$k_e$ (nN)</td>
</tr>
<tr>
<td>C\textsubscript{12}E\textsubscript{5} 0.2%</td>
<td>12.0</td>
<td>2.06</td>
<td>0.0755</td>
</tr>
<tr>
<td>C\textsubscript{12}E\textsubscript{5} 1%</td>
<td>12.2</td>
<td>2.04</td>
<td>0.0867</td>
</tr>
<tr>
<td>C\textsubscript{12}E\textsubscript{5} 5%</td>
<td>6.86</td>
<td>3.50</td>
<td>0.218</td>
</tr>
<tr>
<td>F108 0.2%</td>
<td>3.01</td>
<td>2.07</td>
<td>0.525</td>
</tr>
<tr>
<td>F108 1%</td>
<td>2.16</td>
<td>3.08</td>
<td>0.458</td>
</tr>
<tr>
<td>F108 5%</td>
<td>1.40</td>
<td>5.91</td>
<td>0.307</td>
</tr>
<tr>
<td>Joncryl 60 0.2%</td>
<td>0.298</td>
<td>1.52</td>
<td>1.336</td>
</tr>
<tr>
<td>Joncryl 60 1%</td>
<td>12.5</td>
<td>2.04</td>
<td>0</td>
</tr>
<tr>
<td>Joncryl 60 5%</td>
<td>14.1</td>
<td>2.48</td>
<td>0</td>
</tr>
</tbody>
</table>
a rougher surface. The surface roughness effect differs among the three surfactants. It has more significant effect on C\textsubscript{12}E\textsubscript{5} and Joncryl 60 than on F108 as seen from the \(k_s\) and \(k_e\) values given in Table A.3. It is possible that the packing in the F108 layer is unperturbed neither by local topography during adsorption nor by pressure applied by an approaching colloid with surface asperities.\textsuperscript{298, 317, 318} It is also important to consider the molecular weight (\(M_w\)) differences and hence their \(R_g\) value differences between the three materials: F108 (\(M_w = 14,600\)), Joncryl 60 (\(M_w = 8500\)), and C\textsubscript{12}E\textsubscript{5} (\(M_w = 390\)). One possible reason for this observation is the closeness of the \(R_g\) values of single F108 chains to the dimensions of the asperities resulting in possible “masking”. It is also important to note that in all reported experiments, Joncryl 60 and C\textsubscript{12}E\textsubscript{5} systems are at concentrations far above their CMC values (Table A.2) containing association structures (micelles) with much larger dimensions than observed layer thickness values indicating their lack of participation in adsorption in aggregated state.

A.3.5 Surfactant adsorbed layer structure

It is commonly known that thicker and more rigid surfactant layers are more efficient in stabilizing colloid dispersions.\textsuperscript{314, 319, 320} The nanoindentation experiments provide direct measurements of two parameters—thickness (\(\delta\)) and Young’s modulus (\(E\)) of the adsorbed layer, and quantitatively evaluation of both quantities can predict dispersant performance. Nanoindentation experiments are conducted to determine the thickness and density of the adsorbed surfactant layer using AFM probes with a nominal radius of curvature of 10 nm and a spring constant of 0.1 N/m. Hertz model (Eqn. 9) is commonly used in nanoindentation experiments to determine the Young's modulus:\textsuperscript{236}

\[
F = \frac{4E\sqrt{R}}{3(1-\sigma^2)} \delta^{3/2}, \tag{Eqn. 9}
\]
where $F$ is applied force, $E$ is Young’s modulus, $R$ is probe radius, $\sigma$ is Poisson’s ratio, and $\delta$ is the indentation distance. Eqn. 8 is only valid for thick films. For thin physisorbed films a modified Hertz model (Eqn. 10 and 11) containing a correction term, $\beta$, is used here:\textsuperscript{237}

$$F = \frac{16E}{9} R^{1/2} \delta^{3/2} \beta.$$ \hspace{1cm} \text{Eqn. 10}

$$\beta = 1 + 0.884 \chi + 0.781 \chi^2 + 0.386 \chi^3 + 0.0048 \chi^4 \quad \text{with} \quad \chi = \sqrt{R \delta / h}.$$ \hspace{1cm} \text{Eqn. 11}

$h$ is the adsorbed layer thickness. The elastic modulus, $E$, is calculated from the slope of $F$ versus $16R^{1/2} \delta^{3/2} \beta / 9$ plot. We assume a Poisson’s ratio of 0.5. An example of the data fitting is given in Figure A.8b by fitting the nanoindentation curve measured in 0.2% Pluronic F108 with the modified Hertz model. The fitted $E$ values are listed in Table A.1. The standard deviation is as high as 0.05 MPa, as a result of the small thickness and low modulus, and is also reported by other researchers.\textsuperscript{237, 321}
Figure A.8. a) Indentation vs. Force curves measured by nanoindentation experiment in various surfactant solutions. The dotted line is drawn to guide eyes showing how thickness is determined. b) The fitting curves for 0.2% Pluronic F108 as an example for linear fitting based on Eqn. 10. The slope (0.46 MPa) equals Young's modulus.

The thickness of the adsorbed layer is determined by the maximum indentation as shown by the arrow in Figure A.8a in the case of 5% F108. The adsorbed layer thickness values are given in Table A.1. The C12E5 adsorbed layer thickness is determined to be 2.5–3.1 nm in the concentration range of 0.2–5%, which is slightly less than the
adsorption layer thickness reported by others. The elastic modulus of the C\textsubscript{12}E\textsubscript{5} layer is in the range of 0.20–0.26 MPa. Since all the measurements are conducted in solutions with concentrations above the CMC of C\textsubscript{12}E\textsubscript{5} the adsorbed layer structure of C\textsubscript{12}E\textsubscript{5} does not vary with concentration.

F108 shows a film thickness around 13 nm and a slight trend of thickness increase with concentration. The Young's modulus of the adsorbed F108 layer also shows a weak dependence on concentration consistent with pseudo adsorption plateau associated with polymer adsorption. In comparison, the moduli of adsorbed poly(N-isopropylacrylamide), polyethylene glycol (PEG, 20 kDa), and PEG (35 kDa) are reported to be 0.12–0.15 MPa, 0.09 MPa, and 0.15 MPa, respectively.

In the case of Joncryl 60 both the thickness and Young's modulus of the adsorbed layer increase with increasing concentration. The Young’s modulus of Joncryl 60 is higher than those of C\textsubscript{12}E\textsubscript{5} and F108 due to it being ionic. The increase of Joncryl 60 concentration causes an increase in the ionic strength, which results in an increase in chain flexibility of Joncryl 60. This allows more molecules to be adsorbed at the interface due to closer molecular packing. The increased adsorption and packing density result in an increased rigidity of the Joncryl 60 layer. Our values are close to 1.2 MPa reported by others for surface-grafted acrylic acid layer.
Table A.4 Zeta potential, hydrodynamic diameter, and adsorption layer thickness measured by dynamic light scattering. Adsorption layer thickness by AFM is listed for comparison.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Zeta potential (mV)</th>
<th>$R_H$ (nm)</th>
<th>$\delta_H$ (nm)</th>
<th>$\delta_{h}$ by AFM (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-44.5</td>
<td>54.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C$_{12}$E$_5$ 0.2%</td>
<td>-23</td>
<td>56.9</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>C$_{12}$E$_5$ 1%</td>
<td>-10.4</td>
<td>57</td>
<td>2.6</td>
<td>3.1</td>
</tr>
<tr>
<td>C$_{12}$E$_5$ 5%</td>
<td>-0.4</td>
<td>57.8</td>
<td>3.4</td>
<td>3.0</td>
</tr>
<tr>
<td>F108 0.2%</td>
<td>-21</td>
<td>62.2</td>
<td>7.8</td>
<td>11</td>
</tr>
<tr>
<td>F108 1%</td>
<td>-13.7</td>
<td>78.6</td>
<td>24.2</td>
<td>12</td>
</tr>
<tr>
<td>F108 5%</td>
<td>-4</td>
<td>100.9</td>
<td>46.5</td>
<td>16</td>
</tr>
<tr>
<td>Joncryl 60 0.2%</td>
<td>-23.4</td>
<td>62.9</td>
<td>8.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Joncryl 60 1%</td>
<td>-31.6</td>
<td>64.8</td>
<td>10.4</td>
<td>8.0</td>
</tr>
<tr>
<td>Joncryl 60 5%</td>
<td>-58.6</td>
<td>66</td>
<td>11.6</td>
<td>10</td>
</tr>
</tbody>
</table>

The adsorbed layer thickness is also measured by the DLS.$^{329, 330}$ For the DLS measurements we use 100 nm in diameter polystyrene particles instead of the 15 µm ones used in AFM force measurements. The hydrodynamic thickness, $\delta_H$, of the adsorbed layer is calculated by subtracting the hydrodynamic diameter of the bare latex particle from that of the same particle in the presence of surfactant solution.$^{331}$ Table A.4 summarizes the hydrodynamic diameter and zeta potential of 100 nm polystyrene in various surfactant solutions. The hydrodynamic thickness results agree with the AFM measurements.$^{332}$ The difference is likely due to the underestimation by AFM nanoindentation the water layer and weakly adsorbed surfactant layer.$^{332, 333}$ The zeta potentials measured agree with the literature values.$^{306}$ The decrease in the zeta potential with concentration
indicates that nonionic C\textsubscript{12}E\textsubscript{5} and Pluronic F108 layers screen the surface charge while the adsorption of ionic Joncryl 60 increases the surface charge of the polystyrene colloid.

### A.4 Conclusion

CPM is used to study the surface roughness effect on colloidal forces and colloidal stabilization by surfactant adsorption. We conducted CPM measurements as well as AFM indentation and DLS measurements between commercial polystyrene particles with a diameter of 15 µm and root-mean-square (RMS) surface roughness of 30 nm and heat-treated polystyrene particles with roughness reduced to 1 nm. Approaching and retracting force curves are measured in various salt and surfactant solutions. The surfactants used are: non-polymeric nonionic surfactant pentaethylene glycol monododecyl ether (C\textsubscript{12}E\textsubscript{5}), polymeric nonionic surfactant poly(ethylene oxide)\textsubscript{x}–poly(propylene oxide)\textsubscript{y}–poly(ethylene oxide)\textsubscript{x} (Pluronic\textsuperscript{®} F108), and polymeric ionic styrene/acrylic surfactant Joncryl\textsuperscript{®} 60. In the absence of the surfactant, nanometer surface roughness affects colloidal forces only in high salt conditions when the Debye length becomes relatively small. The adhesion is stronger between rougher colloids. The adhesion between the smoother colloids is reduced to zero in 0.2 wt% of all three surfactant solutions. However a higher amount of surfactants is necessary to eliminate adhesion between the rougher colloids and the degree of adhesion reduction varies sensitively with the surfactant structure and molecular weight. This study demonstrates that surface roughness even at the nanometer scale can affect colloidal forces significantly and should be taken into account in developing colloidal dispersion formulations. The results suggest that the amount of dispersants necessary to provide colloidal stability depends strongly on surface roughness even in nm scale.
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ABSTRACT

BIOREDUCTIBLE POLYMERS FOR GENE DELIVERY

by

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This dissertation describes the research of bioreducible polymers for gene delivery. A series of bioreducible poly(amido amine)s (PAAs) were synthesized. They complex with DNA forming polyplex nanoparticles and layer-by-layer (LbL) thin films as gene delivery vectors. Atomic force microscope (AFM), especially in situ real time AFM, provides a microscopic view of DNA release dynamics. It is shown that the depolymerization of bioreducible polymer triggers DNA release via disulfide-thiol exchange reaction. The AFM images revealed a three-stage pathway beginning with a morphological change from metastable nanostructures into the more favorable toroid structure. Then toroids interact with each other by aggregation and fusion. Finally, DNA wormlike chains gradually unravel from the polyplex resulting in loose loops/tails that are held by a central compact core. Polyelectrolyte exchange induced DNA release shares a similar morphological pathway. The transfection efficiency difference could be correlated with DNA release dynamics and polymer structure. On the other hand, the degradation kinetics of PAA/DNA LbL films are modulated by insertion of a non-bioreducible polycation, poly(ethylenimine) (PEI), as a barrier layer and crosslinking of
the LbL films using 1,5-diiodopentane. The PEI barrier layer is found to be effective in lowering the degradation rate of the film. Without the PEI barrier layer, the PAA/DNA films undergo fast bulk degradation with micrometer size particles released to the solution. The periodic insertion of the PEI layer changes the PAA/DNA degradation behavior to prolonged surface erosion. Transfection studies on PAA/DNA films with and without the PEI barrier layer are carried out *in vitro* and *in vivo*. The cell transfection is further improved by the incorporation of a fibronectin terminal layer and hyaluronic acid. The study of bioreducible polymers and DNA release at molecular level provides strategies for developing non-viral gene delivery vectors.
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