Safe and Effective RNA-based Nanomedicines for Cancer Management

by

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ABSTRACT

Small interfering ribonucleic acids (siRNA) effectively downregulate the specific gene expression from cleaving the associated messenger RNA (mRNA) in the cytoplasm, which is a promising strategy in cancer management as one of the origins of cancer is the unregulated expression of the oncogene. Over decades, nanotechnological research has focused on enhancing siRNA delivery to the cytoplasm of the target cell since the large molecular weight and anionic nature of siRNA impede the siRNA from permeating the cellular membrane, and the blood circulation time of free siRNA is really short. However, even after a tremendous amount of research on cationic polymers, a safe and effective non-viral vector is not yet available. Obstacles, such as opsonization, non-specific delivery, low cellular uptake, endosomal trap, intracellular release of siRNA and cytotoxicity, further make the development of gene therapy in cancer management more challenging. In this thesis, we report the development of three low toxic, nano-sized polymeric siRNA carriers with cytoplasmic triggered siRNA release capability. We also investigated their efficiency in mediating the knockdown of epidermal growth factor receptor (EGFR) in HeLa cells to determine their potential advantages in siRNA delivery.

First, several acid degradable, galactose-based, cationic, and hyperbranched polymers with varying molecular weights (10 to 20 kDa) (MW) and compositions with 2-lactobioamidoethyl methacrylamide [LAEMA] and 2-aminoethyl methacrylamide hydrochloride [AEMA] at different ratios (2.0, 1.0, and 0.5) were prepared via reversible addition-fragmentation chain-transfer (RAFT) polymerization. Subsequently, the polymers were used to form polyplexes with siRNA, and the EGFR knockdown efficiency in cervical carcinoma was determined. By quantifying the EGFR expression

for each treatment group by Western blot assays, 10 kDa polymer, which has a LAEMA:AEMA (L/A) ratio of 2.0, demonstrated a superior EGFR knockdown efficiency (~60%) than the others, and low toxicity levels. In addition, the polyplexes demonstrated to have excellent stability under physiological conditions for up to 2 days.

Next, novel thermo-responsive and cationic hyperbranched polymers were prepared from (AEMA) and di(ethylene glycol) methyl ether methacrylate (DEGMA) via the RAFT polymerization for siRNA delivery. Thermo-responsiveness of polymers allows the formation of stable polyplexes in the human body environment. Non-degradable and acid-degradable hyperbranched polymers were synthesized using *N,N′*-methylene bis(acrylamide) (MBAm) and 2,2-dimethacroyloxy-1-ethoxypropane (DEP) cross-linkers, respectively. Both types of polymers were capable of forming stable nanosized polyplexes with siRNA. Epidermal growth factor receptor (EGFR) silencing of 95% was achieved with a cationic hyperbranched polymer that incorporated an acid-trigger release strategy, and no significant cytotoxicity was observed. Our results confirmed the high potency of using such hyperbranched polymers for the efficient protection and delivery of siRNA.

Finally, redox-responsive galactose-based hyperbranched polymers (HRRP) were synthesized via RAFT polymerization from redox-responsive cross-linker *N,N′*-bis(methacryloyl)cystamine, LAEMA(L), and AEMA (A) with molecular weights of 10 and 20 kDa and L/A ratios of 1.5 and 1.0 were prepared. Disulfide-based cross-linker was chosen to selectively release the siRNA into the cytoplasm of the cancer cells by the trigger of elevating the level of glutathione (GSH) concentration in the tumor environment. 85% EGFR knockdown efficiency was achieved using 10 kDa HRRP with

L/A ratio of 1.5 without triggering significant cellular death (around 95% cell viability).

All proposed polymers showed enhanced EGFR knockdown efficiency (60% with the least efficient one) while maintaining a low toxicity level with cancer (HeLa) and normal (MRC-5) cell lines *in vitro* studies. All the polymers were capable of forming stable nano-sized polyplexes in the growth medium, even in the presence of serum. By employing passive targeting strategy, enhanced permeation and retention (EPR) effect, and extending the blood circulation time from having stealth property to escape reticuloendothelial system and renal clearance, the potential for success *in vivo* studies is noteworthy high. Before studying these materials in clinical trials, more *in vivo* studies will be required. Furthermore, we intend to use these polymers to deliver other kinds of therapeutic nucleic acid to treat additional cancer types, such as hepatocytes, which would potentially unfold the potential of these polymers as a new nanotechnological platform for nucleic acid delivery.

PREFACE

This thesis contains three publicated works from Yi-Yang Peng and his colleagues under the supervision of Dr. Ravin Narain (Department of Chemical and Material Engineering) and Dr. Piyush Kumar (Oncology Department) in the University of Alberta.

Part of **chapter 2** of this thesis is accepted for publication as a book chapter authored by Diaz-Dussan, Peng, Y.-Y.; Narain, R. Novel Polymer Systems for Gene Therapy, *Macromolecular Engineering: From Precise Synthesis to Macroscopic Materials and Applications, second edition*, Wiley. The part includes in this thesis is my original work.

Chapter 3 of this thesis, "Acid Degradable Cationic Galactose-Based Hyperbranched Polymers as Nanotherapeutic Vehicles for Epidermal Growth Factor Receptor (EGFR) Knockdown in Cervical Carcinoma", has been published by Peng, Y.-Y.; Diaz-Dussan, D.; Kumar, P.; Narain, R. on Biomacromolecules in 2018. I was responsible for experimental design and works, data analysis, and manuscript writing. D. Diaz-Dussan conducted the bio-evaluation in this work and assisted in manuscript preparation. R. Narain and P. Kumar supervised the whole process, provided expert insights and edited on the manuscript.

Chapter 4 of this thesis was submitted to ACS Applied Bio Material and published as "Achieving Safe and Highly Efficient Epidermal Growth Factor Receptor Silencing in Cervical Carcinoma by Cationic Degradable Hyperbranched Polymers" in 2018 by Peng, Y.-Y.; Diaz-Dussan, D.; Vani, J.; Hao, X.; Kumar, P.; Narain, R. I designed and conducted the experimental process, prepared and characterized the polymers, analyzed the experimental data, and wrote the manuscript. J. Vani and X. Hao assisted in polymer preparation and polymer characterization, respectively. D. Diaz-Dussan was responsible for biological characterization and shared the work of manuscript writing. P. Kumar and R. Narain supervised the study and edited the manuscript.

Chapter 5 of this thesis has been published in Bioconjugate Chemistry in the year 2019

as “Tumor Microenvironment-Regulated Redox Responsive Cationic Galactose-Based Hyperbranched Polymers for siRNA Delivery” by Peng, Y.-Y.; Diaz-Dussan, D.; Kumar, P.; Narain, R. First authorship and manuscript preparation were shared between D. Diaz-Dussan and me. Experimental design, polymers synthesis and characterization, and data analysis were done by me. P. Kumar and R. Narain contributed to the supervision and manuscript editing.

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Before moving to the research work, I first greatly thank Dr. Narain and Dr. Kumar for providing me this opportunity to work and investigate the development of polymeric and siRNA-based nanomedicine for cervical cancer treatment. Furthermore, I would like to thank again for their supervision and advice during my research career, which significantly improved and accelerated my progress in obtaining the degree. Second, I am delighted to work with my colleagues, Diana Diaz-Dussan, Jianyang Zhao, Wenda Wang, Yangjun Chen, Anika Benozir Asha, and all current and previous members of Dr. Narain's research group who provided lab training and the awareness about specialized equipment, helped me with experiments, and provided thoughtful discussion to improve the quality of my work.

Last but not the least, a lot of thanks and gratitude goes to my parents and all my family members for their unlimited love and contribution. In the past 30 years, I received endless support, care, and encouragement from them, which enabled me to achieve my work goals and complete this work. I am sincerely thankful for the financial support from the Natural Sciences and Engineering Research Council of Canada (NSERC), Alberta Innovates Health Solutions (AIHS) and the Canadian Foundation for Innovation (CFI). Lastly, I hope everyone stays healthy during the pandemic, and the lifestyle returns to normal soon.

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List of Abbreviations

AB	Azidomethyl benzene
ACVA	4,'4-Azobis (cyanovaleric acid)
AD	Adamantane
AEMA	2-Aminoethyl methacrylamide hydrochloride
AEPP	N-(aminoethyl)piperazine
APMA	2-Aminopropyl methacrylamide hydrochloride
APMBA	(3-Azido-propyl)-(4-methoxy-benzylidene)-amine
APP	N-(3-amino-propyl)-2-pyrrolidinone
Antisense ODNs	Antisense oligonucleotide
ATNRC	Atom transfer nitroxide radical coupling
ATRC	Atom transfer radical coupling
ATRP	Atom transfer radical polymerization
BA	Butyl acrylate
BMAC	*N,N'*-bis(methacryloyl) cystamine
bPEI	Branched Polyethylenimine
BSA	Bovine serum albumin
CD	Cyclodextrin
CDEP	CD-embedding polymers
CDPP	CD-pendants polymers
CMA	4-methylcourmarin
CO2	Carbon Dioxide
COVID19	Coronavirus disease 2019
CPADN	4-cyanopentanoic acid dithionaphthalenoate
CPPs	Cell penetrating peptides
CRISPR	Clustered regularly interspaced short palindromic repeats
CTA	Chain transfer agent
CTP	4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid
CuAAC	copper(I)-catalyzed alkyne-azide cycloaddition reaction
D/A	DEGMA/AEMA ratio

DAPI	4’,6-diamidino-2-phenylindole dihydrochloride
DMAPMA	2-(dimethylamino propyl) methacrylate
DEA	2-(diethylamino) ethyl methacrylate
DEGMA	Di(ethylene glycol) methacrylate
DEP	2,2-Dimethacroyloxy-1-ethoxypropane
DH	Hydrodynamic diameter
DLS	Dynamic Light Scattering
DMAEA	2-dimethylaminoethyl acrylate
DMAEMA	2-dimethylaminoethyl methacrylate
DMAP	4-Dimethylamino-pyridine
DMEM	Dulbecco’s Modified Eagle’s Medium
DMF	Dimethylformamide
DP	Degree of polymerization
dsRNAs	Double-stranded RNAs
EC	European Comission
ECM	Extracellular matrix
EDC	Carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
EGDMA	ethylene glycol dimethacrylate
EGF	Epidermal growth factor
EGFR	Epidermal Growth Factor Receptor
EPR Effect	Enhanced Permeability and Retention Effect
ErbB	Erythroblastic Leukemia Viral Oncogene Homologue
FA	Folic acid
FBS	Fetal Bovine Serum
FDA	Food and Drug Adminstration
FITC	Fluorescein isothiocyanate
GMA	Glycidyl methacrylate
GGT	γ-glutamyltranspeptidase
GPC	Gel Permeation Chromatography

GSH	Reduced Glutathione
GSSH	Oxidized glutathione
hATTR	Hereditary transthyretin-mediated amyloidosis
HGA	Acid degradable galactose-based hyperbranched cationic Polymer
HBP2/1	Acid degradable/Non-acid degradable DEGMA-based hyperbranched cationic polymer
HOBT	Hydroxtbenzotrizole
HPMA	*N*-(2-Hydroxypropyl) methacrylamide
HRP	Streptavidin Horse Radish Peroxidase
HRRP	Redox-responsive Galactose-based and Cationic Hyperbranched Polymers
IC50	Half Maximal Inhibitory Concentration
IgG	Immunoglobulin G
LAEMA	2-Lactobionamidoethyl Methacrylamide
L/A ratio	LAEMA:AEMA ratio
LCST	Lower Critical Solution Temperature
lmPAA	*N*-(3-(1H-imidazol-1-yl)propyl)acrylamide
LnMI	lignin-based macroinitiator
LnPDMAEMA	P(DMAEMA) copolymer graft to the lignin
LRP	Living radical polymerization
MAG	2-dexoy-2-methacrylamide glucopyranose
MBAm	*N, N'*-methylenebiscacrylamide
mRNA	Messenger RNA
miRNA	Micro RNA
MW	Molecular Weight
MWCO	Molecular Weight Cutoff
NIPAM	*N*-Isopropylacrylamide
NMR	Nuclear Magnetic Resonance
NPHC	N-phthaloyl
NPHOC	N-phthaloyl oligosaccharide

OEGMA	(ethylene glycol) methyl ether methacrylate
OMEM	Opti-Modified Eagle's Medium
PAA	Poly(acrylic acid)
PAMAM	Poly(amidoamine)
PBS	Phosphate-buffered Saline
P(DMAEA)	Poly[(2-dimethylamino) ethyl methacrylate]
P(DMAEMA)	Poly[(2-dimethylamino) ethyl methacrylate]
pDNA	Plasmid DNA
PEG	Poly(ethylene glycol)
PEGMA	Poly(ethylene glycol) methacrylate
PEI	Polyethylenimine
PEO	Poly(ethylene oxide)
PHEMA	Poly(2-hydroxyl methacrylate)
PLL	Poly(L-lysine)
PP	Piperazine
PPA-CI	propargyl ester of carbonyl imidazole
PSE	Proton sponge effect
PSMA	Prostate-specific membrane atigen
RAFT	Reversible addition-fragmentation chain transfer
RES	Reticuloendothelial system
RIPA	Radioimmunoprecipitation
RISC	RNA-induced silencing complex
PMA	Propargyl methacrylate
PMBA	Poly[(4-methoxy-benzylidene)-amine]
RNAi	RNA interference
RNS	Reactive Nitrogen Species
RNase	Ribonucleases
ROS	Reactive oxygen species
SDS-PAGE	Polyacrylamide Gel Electrophoresis
shRNA	Short-hairpin RNA

siRNA	small Interfering ribonucleic acid
TAE	Tris Acetate/EDTA
Tf	Human transferrin
TME	Tumor microenvironment
TMS-PMA	(Trimethylsilyl)propargyl methacrylate]
WGA-Rhodamine	Wheat Germ agglutinin and tetramethylrhodamine
w/w	weight/weight

List of Publications

Peer Reviewed Journals:

1. Peng, Y.-Y.; Cheng, Q.; Wang, W.; Wu, M.; Diaz-Dussan, D.; Kumar, P.; Narain, R. Multi-responsive, Injectable, and Self-healing Hydrogels based on Benzoxaborole-tannic acid Complexation. *Polym. Chem.* **2021**, 12, 5623-5630. https://doi.org/10.1039/D1PY00692D **First listed author**
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CHAPTER 1. GENERAL INTRODUCTION

1.1 General Introduction

In 2020, Cervical cancer is still the fourth most commonly diagnosed cancer (estimated 604,000 new cases in 2020) in women and the fourth leading cause of cancer related deaths (estimated 342,000 deaths in 2020) in the World.[1] To address this public global health problem, a more efficient strategy is needed, which can manage cervical cancer, lower the death rate and cure the patients. Overexpression of epidermal growth factor receptor (EGFR) is found in 70-90% of cervical cancer patients that indicates a poor prognosis.[2-5] EGFR, a 170 kDa transmembrane glycoprotein receptor and a family member of erythroblastic leukemia viral oncogene homologue (ErbB) receptors, to activate a tyrosine kinase domain via ligand (Epidermal growth factor, EGF)-binding triggered EGFR dimerization.[6-8] Elevation of cell survival, upregulation of cell growth, and evasion of apoptosis are the responses to the signal transduction cascade produced by EGFR-activated tyrosine kinase domain.[9-12] Because of these facts, EGFR was investigated to be a potential therapeutic target in curing cervical cancer. The tumor regression was induced by increasing the chemotherapeutic sensitivity by lowering the expression of the EGFR gene.[13-14] Small interfering ribonucleic acids (siRNA) is an innovative technique that can silence the specific gene expression from cleaving the messenger RNA (mRNA) in the cytoplasm with the assistance of RNA-induced silencing complex (RISC).[15-16] As siRNAs detect and bind with the target mRNA via complementary base pairing, the silenced gene expression is specific. Therefore, eliminating or reducing the problem-causing gene expression using siRNA therapy has been considered a promising method for treating many genetic diseases and cancers. However, the challenges such as insufficient blood circulation time, cellular membrane impermeability, endosomal trap, and off-target transportation need to be addressed for delivering siRNA to target cells before moving them from the lab to the medical setting.[17-20] There is still a need for a safe and

efficient non-viral vector that overcomes the challenges mentioned above and meets the expectations for pursuing clinical studies. Thus, the exploitation of siRNA in treating cervical cancer at the clinical level needs more work.[21]

1.2 Key Achievments

With a key objective to develop suitable siRNA carriers for siRNA therapy, this thesis focused on preparing either acid or redox-responsive hyperbranched cationic polymeric siRNA carriers that efficiently knockdown EGFR in cervical cancer in a safe manner. All polymers were synthesized by reversible addition-fragmentation chain-transfer (RAFT) polymerization to achieve polymers of controlled dimensions. Galactose-based (Chapters 3 and 5) and poly(ethylene glycol) (Chapter 4) -based monomers were chosen to potentially lower the toxicity of the resulted polymers. For responsive hyperbranched polymers, ketal (Chapter 3 and 4) or disulfide-based (Chapter 5) cross-linker was used, which was effectively cleaved in mild acidic and higher concentration of glutathione (GSH) environment, respectively. This strategy was chosen to efficiently release the siRNA in the cytoplasm and to lower the associated cytotoxicity of the polymer by reducing the molecular weight of the polymer as a result of the hyperbranched polymers getting cleaved into shorter fragments via degradation inside cytoplasm. EGFR silencing efficiency was increased by using these non-viral carriers without compromising the cell viability in comparison to a commercial standard carrier from ThermoFisher Scientific, lipofectamine 3000. Furthermore, the stability of the nano-sized polyplexes in the growth medium in the presence of serum was found to be excellent up to 48 hours, which suggests the particles have a low tendency to accumulate in the physiological environment, thus would retain the desired properties. This is expected to extend the blood circulation time of the particles from escaping the reticuloendothelial system. In addition, the passive targeting strategy enhanced permeation and retention (EPR) effect is

expected to be employed because of the nano-size of the polyplexes. Our efforts following this strategy demonstrated a simple one-step preparation of non-viral vector and facile nanomedicine formulations. Finally, even though exciting *in vitro* results were obtained with our proposed polymers in delivering siRNA to knockdown the EGFR in HeLa cells, pre-clinical *in vivo* studies, including toxicity determination, would be needed before translating these candidates to the clinical level.A step in this direction pursued by me is optimization of the design considerations from smart-responsive RAFT-made non-viral vectors, which is described in the thesis.

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CHAPTER 2. siRNA DELIVERY AND ADVANCED POLYMERIC NON-VIRAL VECTORS

A portion of this chapter is published as a book chapter in Macromolecular Engineering: From Precise Synthesis to Macroscopic Materials and Applications.

2.1 Gene Therapy, RNA Interference, and Small Interfering RNA (siRNA) Delivery

2.1.1 *Gene therapy*

Gene therapy provides a promising therapeutic outcome in curing and preventing various inherited and acquired human diseases such as cancers, infectious diseases, neurological disorders, cardiovascular diseases, and many others.[1-4] In 2020 and 2021, gene therapy has established its global role after Pfizer and Moderna successfully developed mRNA-based vaccines for the treating pandemic, Coronavirus disease 2019 (COVID 19).[5-6] Gene therapy cures or alleviates the symptoms of diseases by adding/reducing missing/abnormal gene expression by delivering exogenous genetic material to the targeted cells.[7] The exogenous genetic materials are nucleic acid-base materials that include plasmid DNA (pDNA),[8] messenger RNA (mRNA),[5,6,9] short-hairpin RNA (shRNA),[10] microRNA (miRNA),[11] antisense oligonucleotide (antisense ODNs),[12] small-interfering RNA (siRNA),[13-14] and clustered regularly interspaced short palindromic repeats (CRISPR).[15] The effect of the genetic materials can either be transient or permanent, depending on whether the original DNA sequence of the host is modified by the introduced genetic materials. Without changing the host genome, the altered gene expression is temporary. More therapeutic nucleic acid is required to be administrated to continue maintaining the effect. If the genetic materials merge with the host genome, the altered gene expression will be sustained without a further supply of the therapeutic nucleic acid, and the foreign DNA sequence is also included in the replicate.[16] Because of the anionic nature of nucleic acid, a “vector” is needed to transport the therapeutic nucleic acid across the negatively-charged cell membrane to reach the site of action. For increasing the efficacy of gene therapy, two types of vectors were used: viral and non-viral. In general, viral vectors, such as adenovirus, adeno-associated virus, and lentiviruses, offer significantly higher transfection efficiency than non-viral vectors, because

delivering the genetic material of the virus to the recognized specific cell is the nature of the virus. However, immunogenicity, toxicity, and oncogenicity are the drawbacks of employing viral vectors in gene therapy in clinical trials.[17-18] Thus, non-viral vectors turn to be the focus for gene delivery as they are safer, economical, and easier to make and handle substitution to viral vectors.[19] **Figure 2-1** and **Figure 2-2** summarize the examples of lipid-based and polymeric non-viral vectors used for gene therapy, respectively.[20] Lipid-based carriers for gene delivery are classified into two categories: cationic lipids and helpers lipids. Cationic lipids usually contain an end with one or multiple cationic groups, a linking spacer, and the other end with either one or two hydrophobic carbon chains or a hydrophobic molecule (DOTMA, DOSPA, DOTAP, DMRIE, and DC-cholesterol). Cationic groups of cationic lipids interact with negatively charged nucleic acid via electrostatic force to form lipoplex.[20-21] Helper lipids are neutral or zwitterionic (Cholesterol, DSPC, and DOPE) that increase the stability of resulted nanoparticles and transfection efficiency.[22] However, poor efficacy, as a

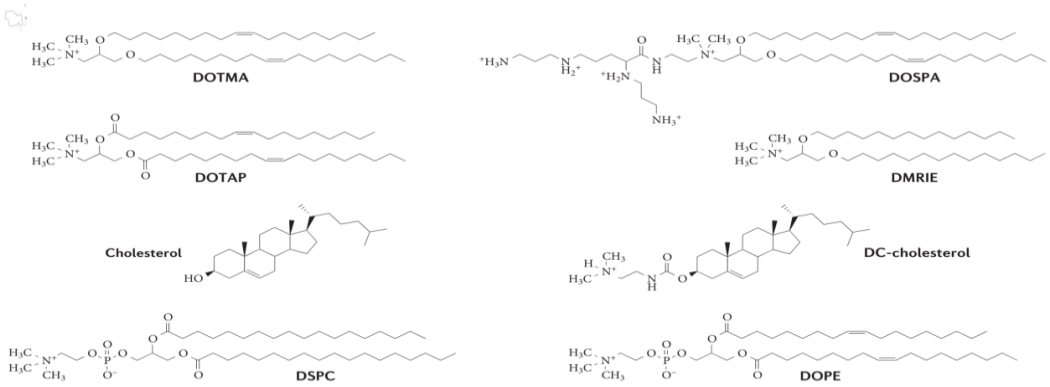


Figure 2-1. Chemical structure of lipid-based gene carriers. Reprinted from reference 20 with permission from the Springer Nature.

result of rapid clearance and poor stability,[23] and associated inflammatory or anti-inflammatory responses induced by some lipid vectors[24] are the concerns with cationic

lipids. Cationic polymers are attractive materials for gene therapy as they are easily made and modified to address the barriers in each treatment, cost-effective, and demonstrate safe toxic profile.[25] Cationic moieties, such as primary, secondary and tertiary amines, among the polymers bind with the negative-charged phosphate backbone of nucleic acid via electrostatic force to form polyplex. The oldest candidates are linear/branched polyethyleneimine (PEI) and poly(L-lysine) (PLL). However, an efficient delivery system with a safe cytotoxicity profile that used these polymers has not been developed yet. Thus, other candidates, such as poly[(2-dimethylamino) ethyl methacrylate] pDMAEMA, polyamidoamine (PAMAM) dendrimers, biodegradable poly(β-amino ester), and cationic glycopolymer (chitosan and β-cyclodextrin-containing polycations), were investigated to determine their potency in gene delivery.[20] However, these candidates cannot provide satisfactory results in pre-clinical or clinical trials; therefore, the development of advanced non-viral vectors is an urgent and legitimate need to exploit full potential of gene therapy at the clinical level.

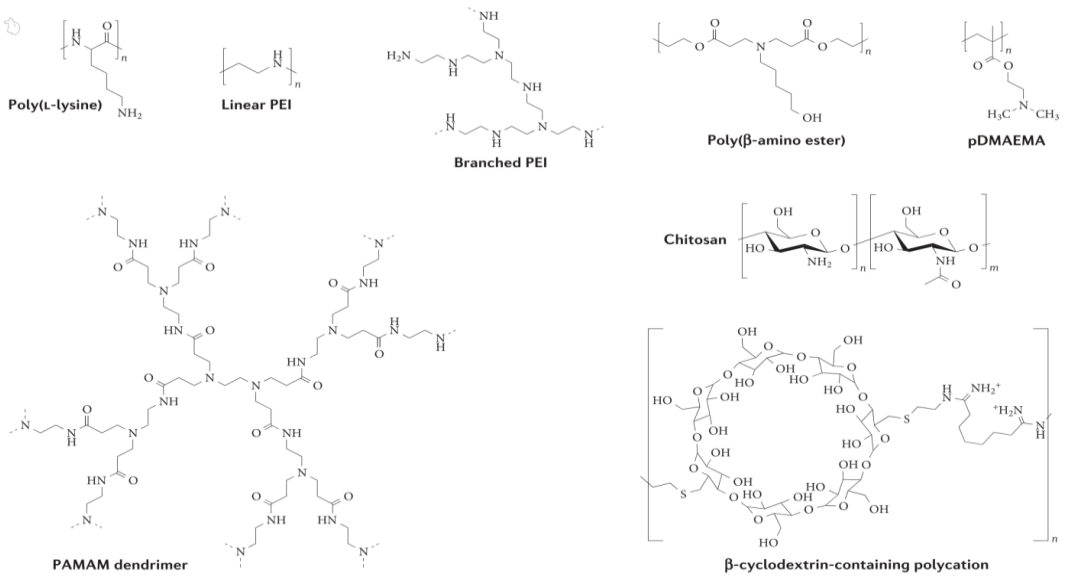


Figure 2-2. Chemical structure of polymeric gene carriers. Reprinted from reference 20 with permission from the Springer Nature.

2.1.2 *RNA interference and small interfering RNA*

The discovery of RNA interference (RNAi) in *Caenorhabditis elegans*[26] and siRNA in plants[27] moved biotechnology to the next era as the process of specific gene regulation becomes more engineerable, feasible, and straightforward. RNA interference (RNAi) is a natural cellular defense mechanism in eukaryotes against viral infection. Because of its significance, the 2006 Nobel Prize for Physiology or Medicine was awarded for this discovery. RNAi induces post-transcriptional gene silencing to control gene expression,[28-29] and it needs to go through multi-steps to achieve the silencing (**Figure 2-3**). At the early stage, double-stranded RNAs (dsRNAs) were used to silencing specific gene expressions from inducing RNAi pathway.[30-31] However, the associated antiviral immune response raised concerns.[32-33] Thus, siRNAs, an analogous form of dsRNAs with less number of nucleotide (20-25 nucleotide with a molecular weight of around 13 kDa),[34] was used to substitute dsRNAs to regulate the gene expression. By using siRNA, antiviral immune responses were significantly lowered or not induced.[33, 35] For utilizing RNAi, siRNA can be introduced to cells by various precursors include pDNA, shRNA, dsRNA, and itself. If pDNA or shRNA is used, dsRNA will be the intermediate product after been transcribed and processed. Subsequently, dsRNA will be cleaved by an endoribonuclease (Dicer), and the resulted fragments are siRNAs.[36-37] Next, RNA-induced silencing complex (RISC), a multiprotein component complex, interacts with siRNA and turns the siRNA into single strands from unwinding double-stranded siRNA.[38-41] The antisense single strand of siRNA within the RISC is used as a guide to detecting the target mRNA via complementary base-pairing.[42-44] After binding with the target mRNA, mRNA is cleaved by a catalytic RISC protein, a member of the argonaute family (Ago2).[45-49] Since RNAi provides specific degradation of post-transcriptional mRNA, RNAi is a promising technique to regulate the gene expression,

thus, possesses high potential and use in treatments, especially in cancer treatment by downregulating the overexpressed oncogene. Furthermore, siRNA therapy is an ideal technique for personalized drug[50] and addresses tumor heterogeneity as it theoretically can target any disease-causing gene from engineering the sequences of siRNAs. Because of the advantages of siRNA mentioned above, siRNA has a lower probability of inducing adverse effects in comparison to the dsRNA and shRNA;[51-52] hence siRNA is the focus of this thesis. However, even siRNA efficiently degrades the targeted mRNA, satisfactory therapeutic outcomes cannot be achieved if siRNA is not localized in the cytoplasm of the targeted cells. Because of the phosphate groups on the backbone of siRNA and its large molecular weight, siRNA is hard to internalize into the cell via passive diffusion through the cellular membrane.[53-55] In addition, unlike DNA, RNA possesses an additional hydroxyl group on the pentose backbone, making siRNA more

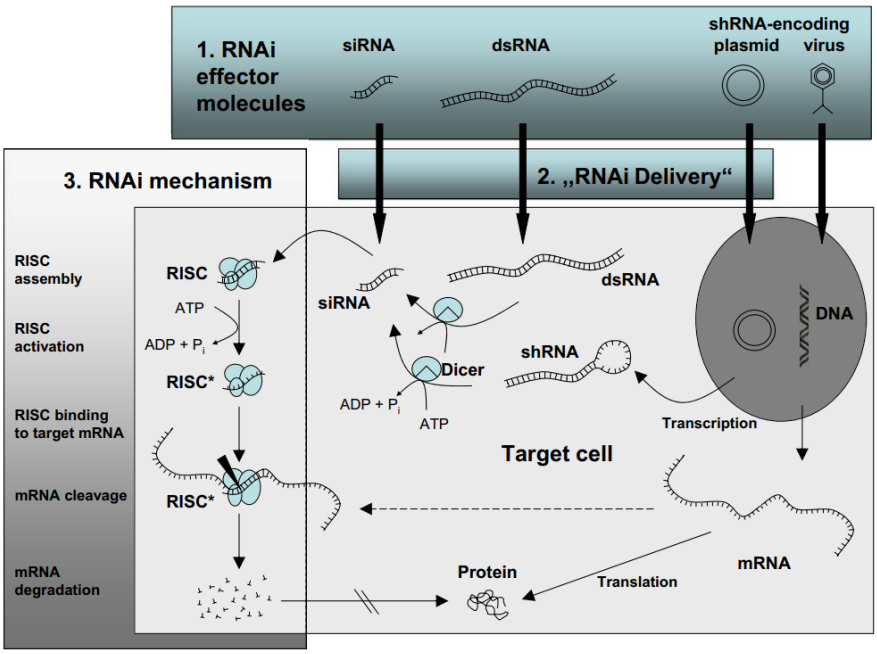


Figure 2-3. Illustration of the choice of siRNA precursors and a RNAi steps. Reprinted from reference 29 with permission from Springer Nature.

prone to hydrolytic degradation by RNases.[56-57] Also, siRNA cannot escape renal filtration because of its small size, resulting in rapid siRNA clearance. All these factors lead to an inadequate blood-circulation time of naked siRNA (between 5-10 min).[58] For increasing the bioavailability of siRNA in the cytoplasm of the targeted cell, non-viral vectors such as proteins,[59-61] lipids,[61-63] polymers,[61,64 & 65] and siRNA modification[60, 66] were evaluated for delivering siRNA. These non-viral vectors were utilized to improve the knockdown efficiency by protecting and increasing the cytoplasmic accumulation of siRNA without inducing significant cell death. After a tremendous amount of studies, many siRNA therapies entered into preclinical and clinical trials.[61, 67] These siRNA-based therapies for treating Adrenal Cortical Carcinoma, Hepatocellular Carcinoma, Metastatic Cancer, Pancreatic Cancer, and Head and Neck cancer are undergoing phase I, II, or III clinical trials, and many others are under pre-clinical trial.[61, 67] Recently in the year 2018, the first RNAi drug, ONPATTRO, has been approved by U.S. Food and Drug Adminstration (FDA) and European Comission (EC) to treat adult patients with peripheral nerve disease (polyneuropathy) that is caused by hereditary transthyretin-mediated amyloidosis (hATTR).[56, 68] Later around the end of 2019, both organizations also approved second RNAi drug, Givlaari, a siRNA drug for treating acute hepatic porphyria.[69] These successes further encouraged us and strengthened the idea of applying siRNA in the medical treatment. Despite these successes, there is no undergoing preclinical siRNA treatment for cervical cancer that exists yet. Thus, developing a non-viral vector for siRNA delivery to treat cervical cancer is the focus of this thesis.

2.2 Considerations for achieving efficient systemic administrated siRNA delivery

A suitable non-viral vector for systemic siRNA delivery needs to fulfill the following requirements: bind/release efficiently with negatively-charged siRNA in the physiological environment/cytoplasm, extend the blood circulation time of siRNA in the physiological

environment, avoid the non-specific protein/serum interaction, guide the siRNA to the cytoplasm of the target cell, escape the endosomal trap and not induce the cell death.

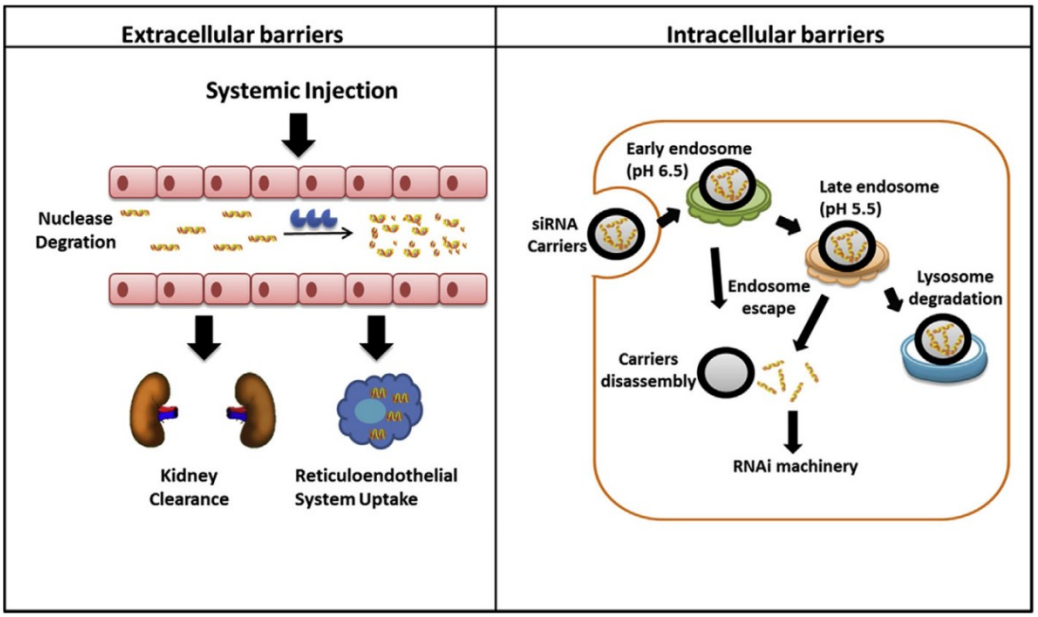


Figure 2-4. Extracellular and intracellular barriers for systemic administration of siRNA. Reprinted from reference 62.

By introducing siRNA via systemic administration, both extracellular and intracellular barriers must be overcome to obtain the desired and safe therapeutic outcome (**Figure 2-4**).

2.2.1 *Extracellular barriers*

While traveling from the administration site to the tumor, instability of siRNA, renal clearance, and reticuloendothelial system (RES) are the major barriers leading to inefficient knockdown efficiency. In the presence of RNases, siRNA protection is an essential requirement to obtain a successful systemic *in vivo* delivery of siRNA since naked siRNAs are degraded easily.[19] Also, the size of siRNA is 7.5 nm in length and 2 nm in diameter[64] which is too large to cross cellular membrane, but not large enough to avoid the clearance from glomeruli.[70] Molecules have to be larger than 8 nm to avoid be filtered into the urine.[71]

Loading siRNA into particles larger than 20 nm is a common strategy to prevent renal clearance.[72] Using cationic polymer to protect siRNA extends their retention time in blood, which was 100 times longer than using naked siRNA.[73] Thus, utilizing non-viral vectors to condensate siRNA to form nano-sized siRNA complexes is a rational and common strategy in addressing enzymatic degradation and renal clearance. However, the size of the polyplexes has to be less than 100 nm to escape the entrapment from the RES in the liver, bone marrow, spleen, and lung. If the polyplexes are trapped by RES, activated monocytes and macrophages degrade the trapped siRNA complexes.[74] Moreover, the cationic polymers usually have nonspecific interactions with negative-charged serum protein in the systemic circulation, a process called opsonization.[75] RES filtration recognizes the opsonized molecules as foreign subjects and removes them by the macrophages.[76] To avoid opsonization, PEGylation (decorating surface of the carrier with polyethyleneglycol [PEG])[77-78] or surface decoration with hydrophilic polymers[79] is a common strategy to have stealth (escape the detection from inhibiting or lowering non-specific interaction with serum protein) property. As explored in Narain's group, siRNA-based polyplexes prepared by cationic galactose-based polymer demonstrated excellent serum stability in an *in vitro* study,[80] which offers an alternative option to circumvent opsonization by decorating galactose-based polymer on the surface of nanoparticles.

If the carriers survive the risks of degradation, phagocytosis, and renal filtration, reaching the site of the tumor is the next challenge. In order to reach the cytoplasm of tumor cells, extravasation and passing through the extracellular matrix (ECM) are needed to facilitate the accumulation of the siRNA-loaded carriers in the tumor microenvironment (TME). Enhanced permeability and retention (EPR) effect resolve this problem by passively guiding the nano-sized carrier to TME.[81-83](**Figure 2-5**) 10-200 nm nanoparticles tend to enter the tumor

site as huge gaps exist between endothelial cells in tumor blood vessels in comparison to normal tissue.[84-86] Once entered into the tumor tissue, the nanoparticles stay in the tumor site because of impaired lymphatic drainage.[87-89] The large spaces between cell-cell junctions of tumor vasculature result from unregulated growth of blood vessels that is induced by tumor angiogenesis. This "leaky" structure facilitates the accumulation of nanoparticles at the tumor site as the gaps are too large to block the nanoparticles. For normal tissue, the cell-cell junctions are tight, which act as blockers to impede the extravasation of nanoparticles, that are larger than 10 nm, to the normal tissue. Lymphatic drainage in the normal tissue recovers the entered foreign macromolecular to the blood vessel effectively. With inefficient lymphatic drainage, nanoparticles are "trapped" in the tumor tissue.[90-91] By designing the polyplexes of the size ranging from 10 to 200 nm the EPR effect is incorporated, the accumulation of polyplexes at the tumor tissue is facilitateed, and enhancement of knockdown efficiency is anticipated if the intracellular barriers of cellular internalization, endosomal trap, and intracellular release of siRNA are overcome.

2.2.2 *Intracellular barriers*

After reaching the tumor tissue, membrane impermeability needs to be addressed to increase cellular uptake of siRNA. Repulsion forces between the siRNA and cellular membrane are reduced from masking the net negative charged siRNA with cationic polymers. Furthermore, polyplexes, with net cationic surface charge and no targeting ligand, interact with the negatively charged proteoglycan on the cellular membrane to form endocytic vesicles and lead to cellular internalization via endocytosis.[92-94] However, the surface charge of polyplexes has to be designed carefully to enhance the internalization of siRNA and minimize their toxicity. Membrane disruption can be induced if the zeta potential of the polyplexes is high enough which destabilizes the cell membrane and results in cell death.[95] Further,

negatively-charged serum in the blood circulation can neutralize the surface charge of nanoparticles and lead to ineffective treatment from low cellular uptake of siRNA complexation.[96] Majority of the siRNA delivery in cells relies on endocytosis. Endocytosis is classified into two kinds: phagocytosis and pinocytosis. Pinocytosis can be further categorized into clathrin-mediated endocytosis, caveolin-mediated endocytosis, clathrin/caveolin-independent endocytosis, and macropinocytosis.[97] Internalized by a specific endocytosis pathway in a certain cell type triggers the initiation of RNAi and silencing.[98] However, the relationship between initiation of RNAi, endocytosis pathway, and cell type is not well understood yet. Thus, designing the non-viral vector that induces a

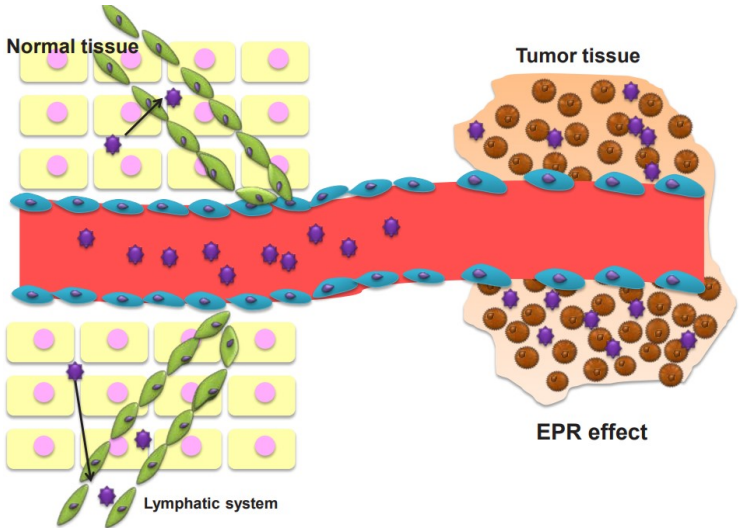


Figure 2-5. Schematic illustration of the enhanced permeability and retention (EPR) effect. Reprinted from reference 81 with permission from JSciMed Central.

specific endocytotic path can be a strategy to enhance transfection efficiency after the relationship becomes clear. Cellular uptake enhancement and specificity can be further ameliorated by triggering receptor-mediated endocytosis (most go through clathrin-mediated endocytosis, caveolin-mediated endocytosis, and clathrin/caveolin-independent endocytosis), which is achieved by decorating the surface of carriers or conjugating siRNA with targeting

ligands.[61, 99] Examples of active targeting ligands used to enhance siRNA delivery are summarized in **Table 2-1**. However, undesired protein adsorption to the nanoparticles reduces the interaction between the ligands and targeting moieties.[107] Beside using targeting ligands, cellular uptake of siRNA can be further improved by engineering the size of the nanoparticle, since this is one of the critical determining factors of the endocytic pathway,[108] but, the optimal size varies with the studies and cell types; thus, more investigations are needed to elucidate the relationship. For a few to several hundred nanometers, and 120 nm to 200 nm,

Table 2-1. Examples of active targeting ligands and their targeted moieties used in siRNA delivery.

Targeting ligand	Type	Targeted moieties	References
Folic acid	Small molecule	Folate receptor	100
Galactose	Small molecule	Asialoglycoprotein receptor	101-102
Transferrin	Protein	Transferrin receptor	103
Trastuzumab	Antibody	Human epidermal growth factor receptor 2	104
RGD (Arginine-GlycineAspartic acid)	Peptide	Integrin receptors	105
PSMA-specific aptamers	Aptamer	Prostate-specific membrane antigen	106

and 250 nm to 3μm, are internalized via pinocytosis, clathrin-or caveolin-mediated endocytosis, and phagocytosis, respectively.[109] For cancer therapy, the size that leads to the phagocytic pathway should be avoided as the phagocytosis mostly happen in monocytes/macrophages, neutrophils, and dendritic cells; this pathway does not lead to cancer cells.[110]

After being taken up by the cell, the endosomal trap is the next challenge. Initially, siRNA complexes-loaded membrane-bound endocytic vesicles fuse with early endosomes (pH 6.5). Subsequently, early endosomes mature to form late endosomes (pH 5.5) from acidifying the endosomes by utilizing membrane-bound proton-pump ATPases. Fusion with lysosomes (pH 4.5-5), which contain digestive enzymes, is the final destination.[111-112] As polyplexes are

degraded in the lysosomes, which leads to low knockdown efficiency, an endosomal escape needs to be achieved before trafficking polyplexes toward the lysosomes. "Proton sponge effect" (PSE) hypothesizes that endosomal escape can be achieved by inducing osmotic endosome swelling that elevates the concentration of ions (H^+ and Cl^-) but not pH in the endosome by utilizing auto-programmed endosomal acidification (proton-pump ATPases) and

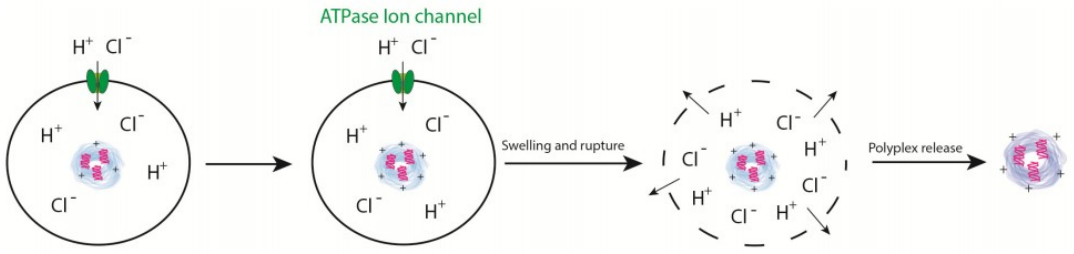


Figure 2-6. Mechanism of the endosome rupture and polyplex release by using "Proton sponge effect". Reptinted from reference 116. https://doi.org/10.3390/polym6061727

weakly basic, protonable, or buffering molecules, to disrupt endosome before degradation (**Figure 2-6**).[113-116] PSE is used to explain why a strongly buffering polyamine, PEI, has higher transfection efficiency than a non-buffering polyamine, polylysine.[117-118] Besides PSE, incorporation of fusogenic proteins/lipids, such as cell-penetrating peptides (CPPs)/DOPE into a delivery system, is also utilized to enhance the endosomal polyplexes release.[119] In addition, damaging the endosomal membrane with reactive oxygen species by exposing endocytosed photosensitizers to the light is also an alternative option to release the polyplexes from the endosome.[120]

After localizing in the cytoplasm, free siRNAs have to be released from polyplexes to join the RNAi pathway.[121] To efficiently release siRNA in the cytoplasm, utilizing cytoplasmic triggering biodegradable polymers (pH and redox-degradable polymers) provides great promise since it lowers the interactions (van der Waals force, hydrogen bonding, and

electrostatic force) between non-viral vectors and siRNA. As endosomes are mildly acidic (pH5-6), pH-degradable polyplexes are stable in blood (pH 7.4), but become small fragments and free siRNA after release from the endosomes.[122-124] The disulfide groups of bio-reducible polyplexes are cleaved when they reach the cytoplasm, resulting in the intracellular release of siRNA.[125-126] The cleavage is induced by a reduction in high cytoplasmic concentration of glutathione (GSH), a tri-peptide (made by glutamate, cysteine, and glycine) that has a higher concentration in the cytoplasm (0.5-10.0 mM) than outside of the cell (~4.5μM). Furthermore, tumor tissues have higher GSH concentration than normal tissues, thus redox-responsive polymers can also be used to enhance the selective release of therapeutic agents to the tumor.[127]

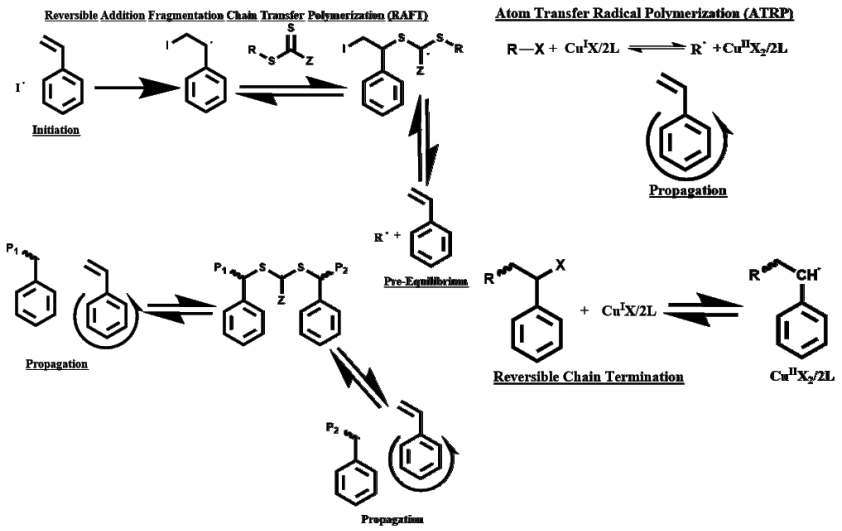
2.2.3 Cytotoxicity

Lastly, cytotoxicity of the delivery system is the major hurdle that limits the application of many efficient delivery systems at the clinical level. The abovementioned biodegradable polymers are the one strategy to lower the cytotoxicity by reducing the molecular weight of non-viral vector after the intracellular release of siRNA; the resulted small fragments can be quickly cleared away via renal filtration.[128] Masking the cationic moieties via pegylation or glycosylation is another potential strategy to reduce cytotoxicity.[129-132] However, the degree of modification needs to be adjusted to the best to obtain a safe and efficient delivery system. Designing cationic non-viral vectors into dendrimer or hyperbranched polymer can also be used to address the cytotoxicity issue since the branched polymers have higher transfection efficiency than their linear analogues generally, which would reduce the required amount of vector to attain the targeted therapeutic goal.[133-136]

2.3 Preparation of advanced well-defined cationic polymers

After the living radical polymerization (LRP) was introduced in 1980,[137] numerous studies have been conducted to synthesize well-defined cationic polymers and investigate their efficiency as gene delivery vectors. LRP maximizes the propagation reaction and minimizes the termination reaction,[138] which allows the preparation of cationic polymers of targeted molecular weights (MW), structure, and composition.[139-140] Thus, LRP is commonly used to produce low polydispersity and multifunctional cationic polymers for gene delivery systems. Because of the surface properties,[141-142] the chemical properties,[143-145] the shape of the resulted polyplexes[146-148] and toxic profiles[149-150], these polymers can be easily tuned by the monomer or cross-linker of choice, the composition of the polymer, the degree of polymerization (DP), and further post-synthesis modifications. The most widely used LRP techniques for making non-viral gene carriers are atom transfer radical polymerization (ATRP) and reversible addition-fragmentation chain-transfer (RAFT) polymerization. As LRP is more tolerant to various functional groups in comparison to free radical polymerization, diverse vinyl-based monomers are polymerized via LRP, which indicates its feasibility in preparing polymer chains with multiple and different types of functional groups.[151] Moreover, even some functional groups are not suitable for LRP, utilization of post-modification can break this restriction. Post-modification of ATRP is through reacting with a terminal halogen.[152] For RAFT polymerization, post-modification can be achieved either through α- or ω-end of RAFT agent.[153-154] Modification of ω-end is introduced through reaction with thiol group, which can be obtained via aminolysis or hydrolysis of thiocarbonylthio group on the RAFT agent.[153-154] In addition, post-modification can be achieved by making polymer chains with functional groups that are reactive but tolerable with the LRP, such as protonated amine,[155] epoxy,[156] aldehyde,[157] and alkyne[158] groups. Therefore, modifying the polymer with the targeted functional group to obtain the desired

physical or chemical properties becomes practicable. In this section, we will focus on describing the novel cationic polymers that were prepared via ATRP and RAFT and the related post-modification. The general process of RAFT and ATRP is shown in **scheme 2-1**.

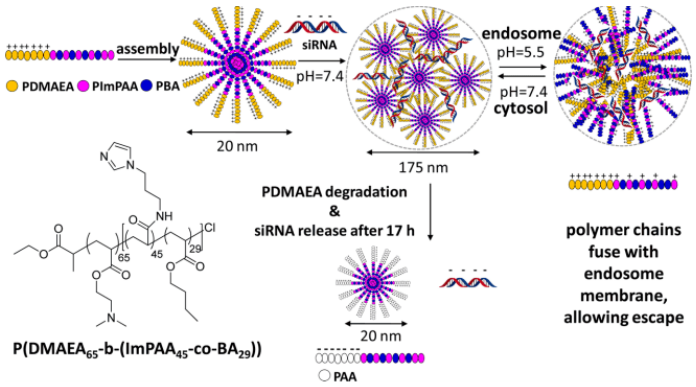


Scheme 2-1. Mechanism of ATRP and RAFT polymerization. (Reproduced from reference 151 with permission from the American Chemical Society).

2.3.1 *Advanced gene carriers prepared via ATRP*.

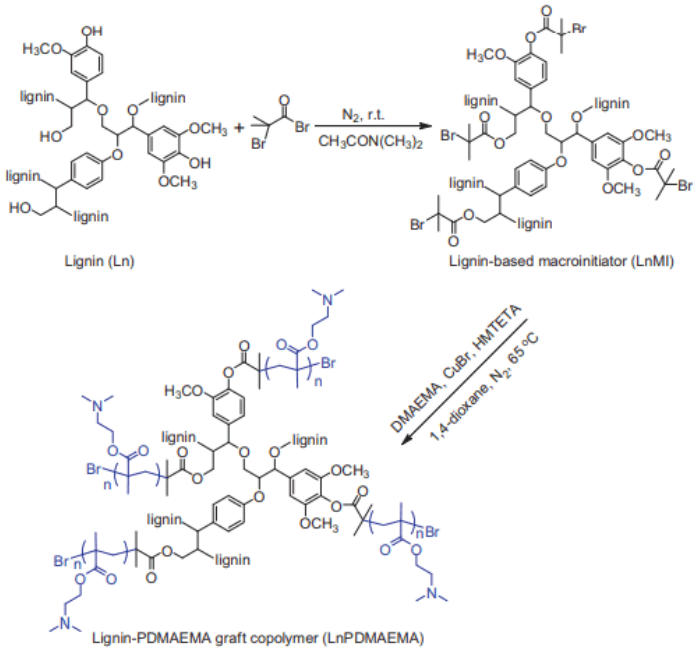
ATRP is a copper-mediated polymerization process which controls the polymerization by the activation or deactivation of the growing polymer. The activation or deactivation is regulated by the equilibrium between the oxidation state of the copper complex.[152] Because of its skewed equilibrium, which tends to deactivate the propagating radicals and transfer them into the dormant state, the controlled polymerization can be achieved. As ATRP allows reversible activation of propagating polymers, preparation of block copolymers such as AB, ABA, or ABC type copolymer can be easily accomplished. Gu *et al*. (**Scheme 2-2**) demonstrated the utilization of influenza virus-inspired self-assembled amphiphilic block copolymers in inducing efficient siRNA delivery.[159] Poly[(2-dimethylaminoethyl

acrylate)]-b-poly[*N*-(3-(1H-imidazol-1-yl)propyl)acrylamide-st-(butyl acrylate)]} (P[DMAEA-b-(lmPAA-st-BA)]) was prepared via ATRP and the resulted nano-sized and siRNA-based polyplexes effectively accumulated in the cytoplasm. DMAEA was used to enhance siRNA-complexation for forming stable polyplexes. The success of cell cytoplasm localization was attributed to the effective endosomal escape mechanism. According to their previous work, protonation of imidazole moieties in the endosome leads to the bilayer separation from the charge interaction, which results in the endosomal escape mechanism.[160] After reaching the cytosol, P(DMAEA) was degraded into PAA, which induced disassembly of the polyplexes and release of siRNA as the anionic charge of PAA block polyplexes have repulsion forces with the negatively charged siRNA.



Scheme 2-2. Illustration of chemical structure of poly[(2-dimethylainoethyl acrylate)]-b-poly[*N*-(3-(1H-imidazol-1-yl)propyl)acrylamide-st-(butyl acrylate)]} that was synthesized via ATRP and the mechanism of polymer self-assembly, complexation with siRNA, fusion with endosomal membrane to enhance the endosomal escape, siRNA release via degradation of PDMAEA. Reprinted from reference 159 with permission from the American Chemical Society.

Despite using linear polymer, branched polymers demonstrated higher gene transfection efficiency and a lower toxic profile in comparison to their linear analogues in general.[150, 161] Preparation of branched polymers was facilitated by utilizing ATRP. Newland *et al*. demonstrated the preparation of hyperbranched poly(2-(dimethylamino)ethyl methacrylate (DMAEMA) from using cross-linker, ethylene glycol dimethacrylate (EGDMA) via a one-batch ATRP.[161] The resulted hyperbranched P(DMAEMA) exhibited superior



Scheme 2-3. Preparation of lignin-based macroinitiator (LnMI) and hyperbranched P(DMAEMA) copolymer, which grafts to the lignin (LnPDMAEMA) via ATRP. Reprinted from reference 162 with permission from the Elsevier.

transfection ability and lower cytotoxicity in comparison to its linear analogue and bPEI, respectively. Besides using cross-linkers, the hyperbranched polymer could also be obtained from using macroinitiators. Liu *et al*. (**Scheme 2-3**) esterified the alcohol and phenol functional groups on lignin with 2-bromo-isobutyric bromide to make lignin-based

macroinitiators (Ln MI) and subsequently conducted ATRP with DMAEMA to prepare a series of hyperbranched polymer (LnPDMAEMA).[162]

Liu *et al*. found that LnPMAEMA demonstrated lower cytotoxicity in comparison to 25 kDa PEI, and *in vitro* gene transfection efficiency of LnPDMAEMA was associated with the chain length of PDMAEMA arms. Zhang *et al*. reported the synthesis of P(DMAEMA)s grafted to a bovine serum albumin (BSA) protein via modification of BSA with 2-bromoisobutyryl bromide and subsequently conducting polymerization of DMAEMA via ATRP. This protein-polymer can form 50 nm of polyplexes with pDNA and possessed similar or better transfection efficiencies than bPEI. The study demonstrated a useful strategy to add biofunctionality to the non-viral system via the conjugation of a protein.[163] Star polymers, which have several polymeric chains attached to a central core, are a kind of hyperbranched polymers that can be prepared easily via ATRP.

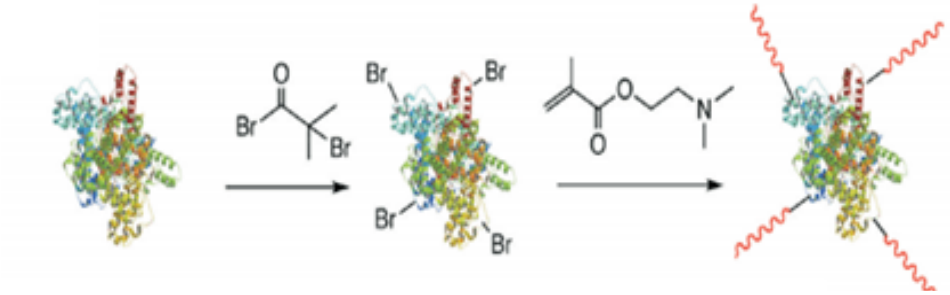


Figure 2-7. Procedure to prepare protein-polymer conjugate nanoparticle from grafting P(DMAEMA) to BSA. Reprinted from reference 163 with permission from the American Chemistry Society.

Cho *et al*. prepared PEG-based star copolymers with a redox-responsive cationic core via the "arm-first" ATRP method.[164] 2 kDa poly(ethylene glycol) methacrylate (PEGMA) was chosen as the arm and polymerized with cationic monomers and disulfide-based cross-linker to form a star copolymer. The polymers were found to be biocompatible carriers and

demonstrated efficient cellular uptake as suggested by the results of confocal microscope and flow cytometry.

Post-modification of ATRP-made polymer is also a common way to prepare branched polymers and ameliorate their efficiency in gene delivery. Jiang *et al*. combined ATRP and 'click' chemistry approach to prepare degradable-brushed polymer.[165] DMAEMA was firstly polymerized with N_3-functionalized initiators via ATRP to prepare PDMAEMA with azido terminal (PDMAEMA-N_3). Poly(2-hydroxy methacrylate) (PHEMA) acted as a backbone of the brushed polymer and esterified with propargyl ester of carbonyl imidazole (PPA-CI) to graft azide-based hydrolyzable linker on the P(HEMA) to create alkyne-functional polymer p(HEMA-st-HEMA-PPA). Subsequently, the PDMAEMA-N_3 were grafted to pHEMA by the reaction between azide and alkyne via copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) reaction. The degradation of carbonate ester linker allowed the detachment of PDMAEMA, which resulted in low molecular weight fragments that can be cleaned via renal filtration and lower the cytotoxicity in comparison to non-degradable high molecular weight PDMAEMA. On the other hand, post-modification with the terminal halogen on ATRP-made polymers is an alternative approach to add physical or chemical functionality or construct different structures of the polymers. Boyer *et al*. summarized several direct and indirect post-modification methods for ATRP prepared polymers (**Figure 2-8**).[138] By utilizing these methods, the targeting ligands can be easily conjugated to the polymers and improve their performance. Furthermore, novel structure polymers can be prepared via this modification.

Even ATRP facilitates the process of preparing various advance non-viral systems, concerns still exist with utilizing ATRP for making gene carriers as ATRP involves toxic metallic components which need to be removed prior to the biological applications. Thus, RAFT polymerization is an alternative and less "toxic" technique for preparing gene non-viral

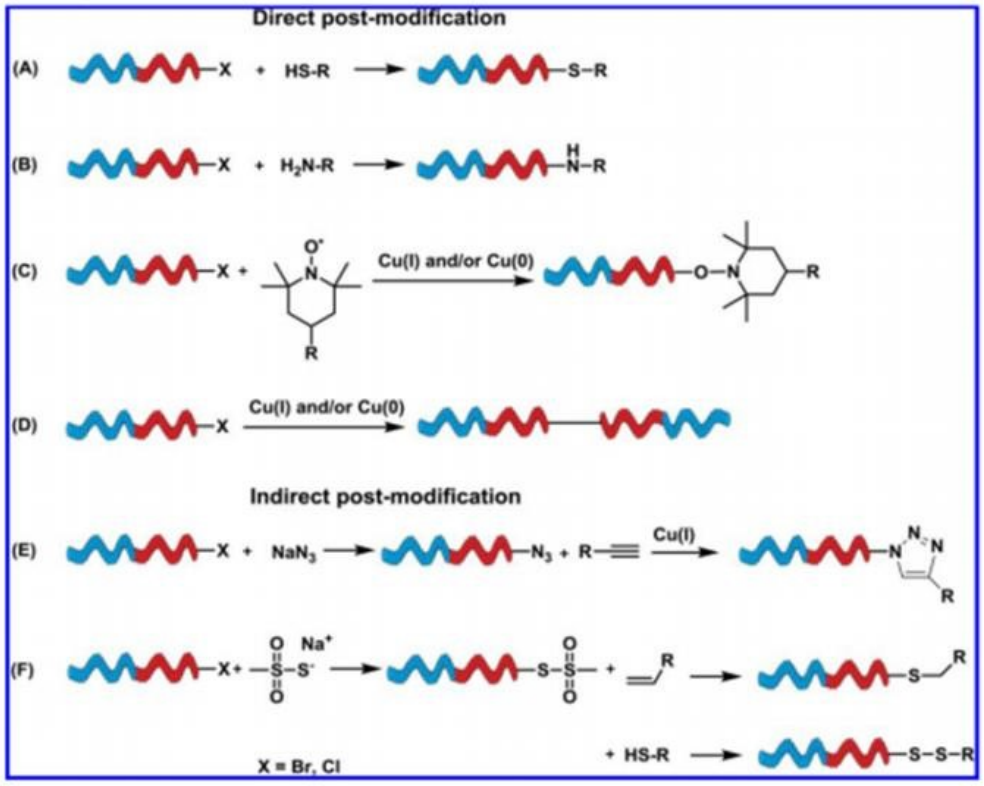


Figure 2-8. Post-modification method via reacting terminal halogen on the ATRP-synthesized polymers. (A) Thio-bromo coupling, (B) bromo-amine coupling, (C) atom transfer nitroxide radical coupling (ATNRC), (D) atom transfer radical coupling (ATRC), (E) CuAAC click chemistry, (F) methanethiosulfonate mediated thiol-ene and thiol-disulfide exchange reactions. Reprinted from reference 138 with permission from the American Chemistry Society. https://pubs.acs.org/doi/10.1021/acs.chemrev.5b00396 Note: Further permissions related to the material excerpted should be directed to the ACS.

vectors as the toxic component, thiocarbonylthio group, can be removed easily via aminolysis or hydrolysis and dialysis.

2.3.2 *Advanced gene carriers prepared via RAFT polymerization*.

RAFT polymerization was first developed by the Commonwealth Scientific and Industrial Organization (CSIRO) in 1998.[166] Definition of RAFT polymerization is conducting radical

polymerization in the existence of chain transfer agent (CTA), thiocarbonylthio compound.[167] CTA controls the polymerization from the rapid degenerate chain transfer process, and the mechanism of RAFT polymerization is illustrated in **Scheme 2-1**. As the structure and molecular weight of the polymers are the significant factors in determining the transfection efficacy, facile and convenient steps for preparing well-defined polymers are some of the major advantages of RAFT polymerization in the synthesis of gene delivery vectors. RAFT polymerization facilitates the preparation of various types of polymers such as linear block copolymer, hyperbranched polymer, nanogel, etc. Block copolymers can be easily prepared from synthesizing macro-CTA first.

Wang *et al*. (**Figure 2-9**) designed a RAFT-made block copolymer, poly(ethylene oxide)-block-poly[*N*-isopropylacrylamide-stat-7-(2-methacryloyloxyethoxy)-4-methylcourmarin-stat-2-(diethylamino)ethyl methacrylate] [(PEO)-b-P(NIPAM-st-CMA-st-DEA)], which self-assembled into a dually gated polymersome via hydrophobic interaction between CMA moieties.[168] The hydrophilic PEO acted as the shell to stabilize the polymersome, and the switches of the gate are temperature (NIPAM) and pH (DEA). The nucleic acid was loaded to the polymersome at 20 ^{o}C where the gate was opened, and subsequently closed the gate by increasing the temperature to 40 ^{o}C. The intracellular release of the load was triggered by the acidic environment in the endosome via protonation of DEA. The polymersome showed efficient gene transfection *in vitro*. Post-modification to the RAFT-made linear cationic polymers is also used to add function for enhancing the efficacy. York *et al*. (**Scheme 2-4**) prepared folate-conjugated block conjugates from post-modification of RAFT-made cationic polymers to incorporate cell specificity into the system.[169] Macro-CTA, [*N*-(2-Hydroxypropyl)methacryamide-stat-*N*-((3-aminopropyl)methacrylamide)]

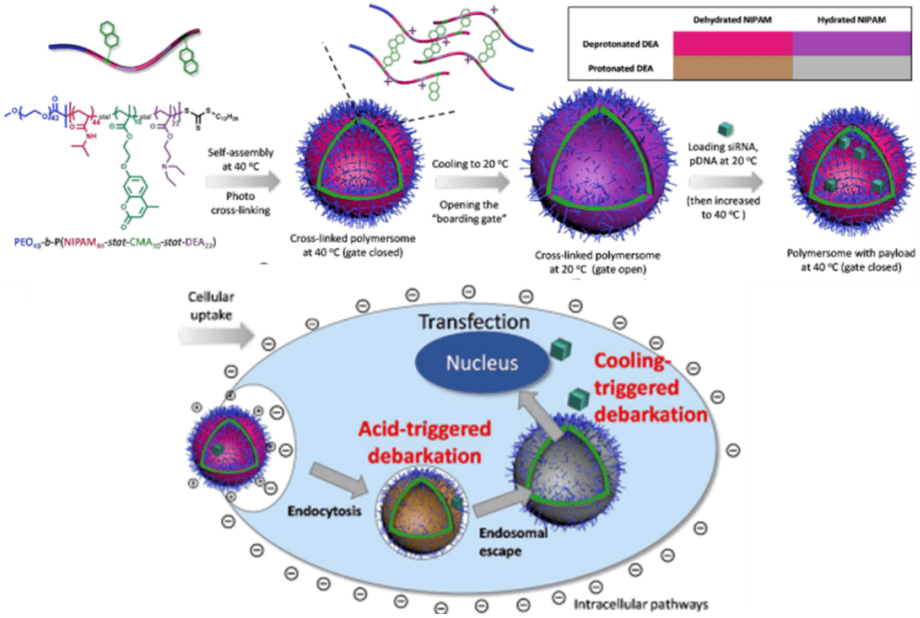
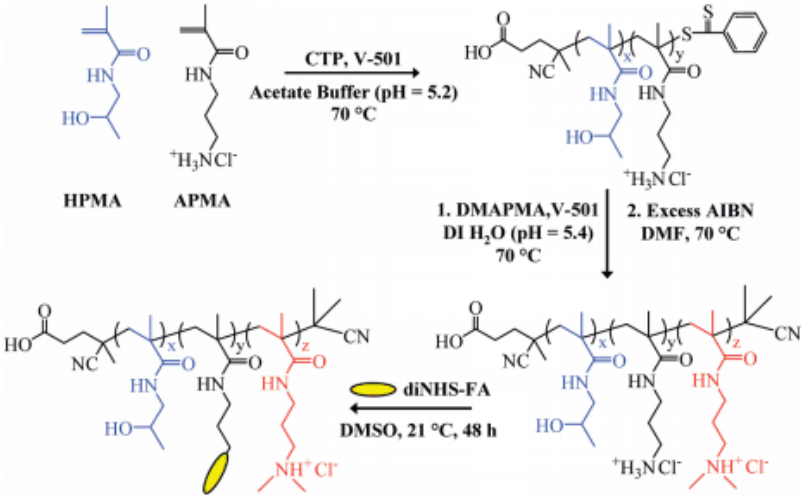


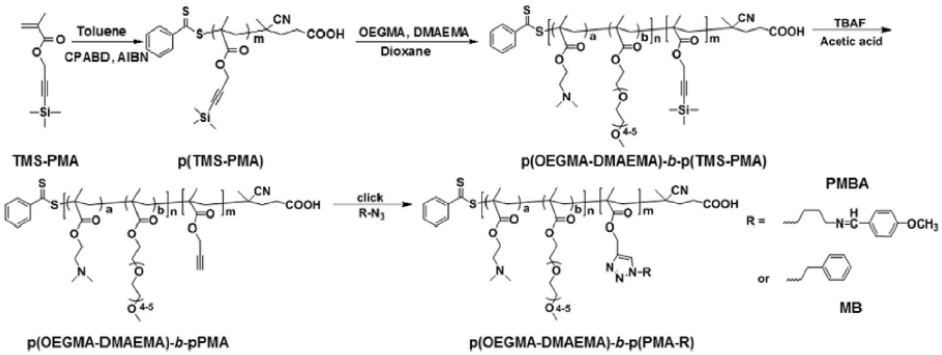
Figure 2-9. Dually gated polymersome formed from self-assembled (PEO-b-P(NIPAM-st-CMA-st-DEA). The encapsulation of siRNA and pDNA was conducted by opening the gate of polymersome at 20 ^{o}C and traping them at 40 ^{o}C by closing the gate. Intracellular release of the load was achieved via protonation of DEA. Reprinted from reference 168 with permission from the American Chemistry Society.

[(HPMA-stat-APMA)], was made first via RAFT polymerization and subsequently created the second block with *N*-(3-dimethylaminopropyl)methacrylamide (DMAPMA) to have [*N*-(2-Hydroxypropyl)methacryamide-stat-*N*-(3-aminopropyl)methacrylamide)-block-*N*-((3-dimethylaminopropyl)methacrylamide)] [(HPMA-stat-APMA)-b-DMAPMA]. The primary amine from APMA on the polymer was modified with NHS-activated Folate (FA), and the resulted polymers accumulated in the cancer cells that overexpress folate receptors, as illustrated by fluorescence microscopy. Cheng *et al*. (**Scheme 2-5**) designed a polymeric system that addresses the dilemma of polyplexes stability and release of nucleic acid from it by making an amphiphilic block copolymer, which formed stable polyplexes in the



Scheme 2-4. Preparation of [(HPMA-stat-APMA)-b-DMAPMA] copolymers and conjugation with Folic Acid (FA) for enhancing the cell specific siRNA delivery. Reprinted from reference 169 with permission from American Chemistry Society.

physiological environment; hydrophobic interactions and its hydrophobic component transformation to hydrophilic via the acid-induced cleavage of benzoic imines facilitates the intracellular release of nucleic acids.[170] P[(trimethylsilyl)propargyl methacrylate]-block-P[2-(dimethylamino)ethyl methacrylate-stat-poly(ethylene glycol) methyl ether methacrylate] [P(OEGMA-DMAEMA)-b-P(TMS-PMA)] was made via RAFT polymerization and subsequently deprotected and reacted with either azidomethyl benzene (AB) or (3-Azido-propyl)-(4-methoxy-benzylidene)-amine (APMBA) via CuAAC. Polymers became pH-sensitive after modification with APMBA because of the presence of acid liable imine bond. The design proved to be a good strategy as the pH-sensitive polymer demonstrated higher plasmid delivery efficiency with HeLa cells in comparison to the pH-insensitive polymers, which was attributed to the elevation of nucleic acid release rate via the cleavage of benzoic imine bonds.



Scheme 2-5. Procedure for synthesizing p(PMA-PMBA)-b-p(OEGMA-DMAEMA) and p(PMA-MB)-b-p(OEGMA-DMAEMA). Reprinted from reference 170 with permission from Elsevier.

Prior to the RAFT polymerization, modification with chain transfer agent (CTA) or RAFT agent is an alternative way to prepare novel non-viral gene carriers. Zhu *et al.*[148] (**Figure 2-10**) modified CTA to prepare bioreducible ABA triblock copolymers. Poly(ethylene glycol) (PEG) was modified to have cystamine groups at both terminals (Cys-PEG-Cys). Two RAFT agents, 4-cyanopentanoic acid dithionaphthalenoate (CPADN), were conjugated by using Cys-PEG-Cys to form CPADN-SS-PEG-SS-CPADN. RAFT polymerization of DMAEMA was conducted subsequently. The resulted triblock copolymers had 28 times higher transfection efficacies as compared to the non-bioreducible polymers which used modified RAFT agent without disulfide bond. Then superior results were attributed to the release of pDNA via the unshielding of polyplexes from cleavage of the disulfide bonds in the cytoplasm.

Post-modification of Ω-end of RAFT-made polymers is also a choice to design innovative non-viral vectors. After the aminolysis or hydrolysis, RAFT-made polymers become thiol-functionalized at Ω-end, which can react with many chemical groups such as alkene,

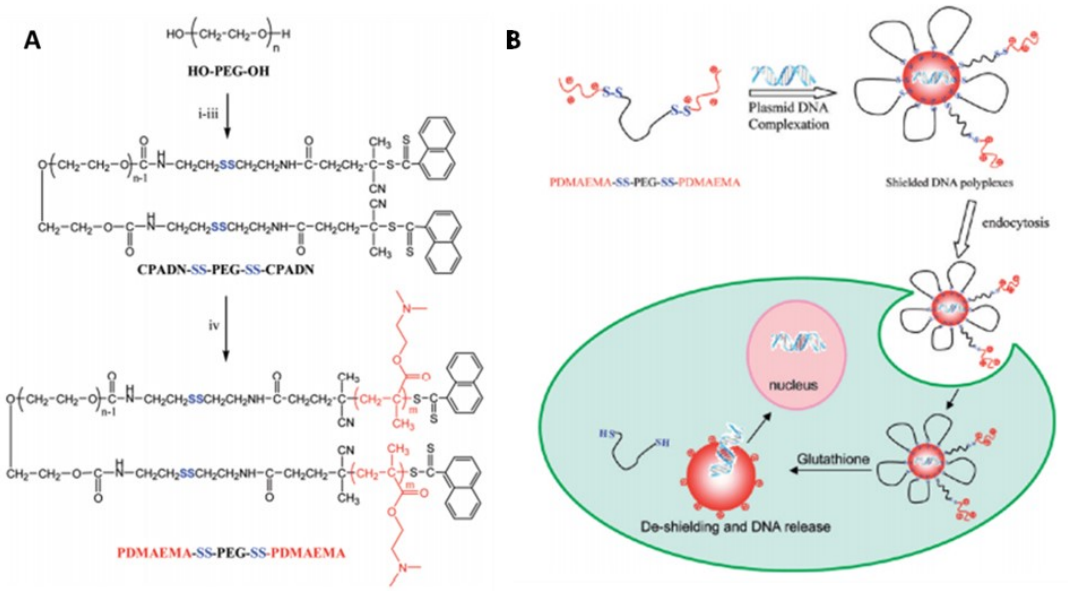


Figure 2-10. Illustration of bioreducible ABA triblock copolymer (PDMAEMA-ss-PEG-ss-PDAEMA) preparation (A). The polyplexes formation and their unshielding in the cytoplasm which results in DNA release (B). Reprinted from reference 148 with permission from the American Chemistry Society.

alkyne, alkyl halide, epoxide, among others—allowing the preparation of a variety of polymer-biomolecules conjugates. Narain *et al*. functionalized the RAFT-made cationic copolymers with a benzoxaborole group via thiol-ene click chemistry.[172] The resulted polymers were incubated with siRNA to form polyplexes. Subsequently, the polyplexes were conjugated to the galactose-based polymers via boron-carbohydrate interaction to create a macromolecule that can release the polyplexes in a mild acidic environment. This strategy enhanced the EGFR knockdown in HeLa cells without elevating the *in vitro* cytotoxicity.

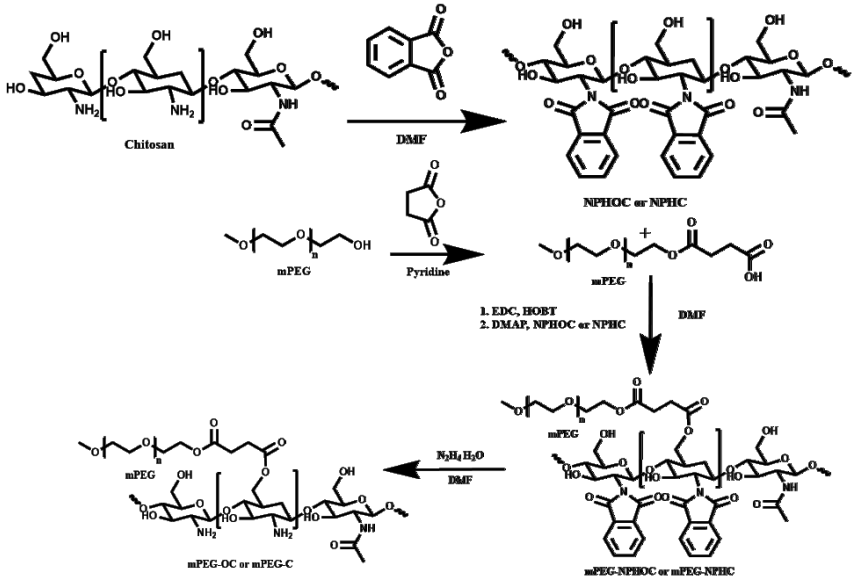
2.4 Advanced cationic glycopolymers in gene delivery

Carbohydrates act as major components in the biological system, which include sources of bio-energy, structural support,[173-175] and cellular recognition[176-178]: Carbohydrates are generally considered low or non-toxic even at really high concentrations. Cationic

glycopolymers are a class of macromolecules that have a repeat unit of carbohydrates and cationic moieties, and can be obtained from natural resources or artificial preparation. Because of the cationic moieties and abundant hydroxyl groups from the carbohydrate units, cationic glycopolymers condense therapeutic nucleic acid by electrostatic interaction and hydrogen bondings.[179] As cytotoxicity and transfection efficiency of cationic glycopolymers are tunable via engineering the ratio between glyco-units and cationic units, shape, structure, and DP of the glycopolymers,[132, 142, 150 & 180-183] these biomaterials possess great potential in drug delivery and gene delivery. In the section below, the developed advance cationic glycopolymers via modifying biopolymers (Chitosan and Cyclodextrins [CD]) or polymerization and/or post-modifications for gene delivery in the literature are introduced.

2.4.1 Chitosan

Chitosan is chosen to be investigated in biomedical applications because of its proven biocompatibility, biodegradability, safety profile, and low immunogenicity.[184-186] However, its poor solubility at pH 7.4, low cell specificity, and low transfection ability restrict its application.[187] PEGylation proves to be an excellent technique to stabilize the chitosan-formed polyplexes in the physiological environment by decreasing opsonization from incorporating "stealth property" (**Scheme 2-6**).[188] The problem of cell specificity can be solved by conjugating chitosan with targeting ligands such as mannose, transferrin, folate, and galactose.[187] Kim *et al*. prepared mannosylated chitosan for inducing receptor-mediated endocytosis to target dendritic cells, which have mannose receptors.[189] The modification elevated the gene transfer efficiency without compromising the cell viability. Slow endosome escape rate was considered to be a factor that led to low transfection efficiency since chitosan does not have buffering capacities in comparison to PEI.[190] Chang *et al*. conjugated histidine to chitosan via disulfide bond-based linker by using 2-iminothiolane and cysteine.[191] The



Scheme 2-6. Preparation of Pegylated Chitosan. DMAP, 4-dimethylamino-pyridine. HOBT, hydroxtbenzotrizole. EDC, Carbodiimide hydrochloride. NPHOC, N-phthaloyl oligosaccharide. NPHC, N-phthaloyl. Reproduced from reference 192 with permission from American Chemistry Society.

improvement of the transfection efficiency after the modification was observed, and more pDNAs were localized in the cytosol as revealed by confocal microscopy.

2.4.2 *Cyclodextrins (CDs)*

CDs, cyclic oligosaccharide composed of 6-8 glucose units, were firstly used in the gene therapy area in 1999.[193-194] CDs are considered attractive to the field as they did not induce an immune response, showed insignificant toxicity in animal studies, can serve as host molecules to form inclusion complexes by entrapping small molecules, possess membrane absorption enhancing properties, and are approved by the FDA as solubilizing agents in pharmaceutical formulations.[194-196]

However, original CDs form unstable polyplexes with nucleic acids; therefore, chemical

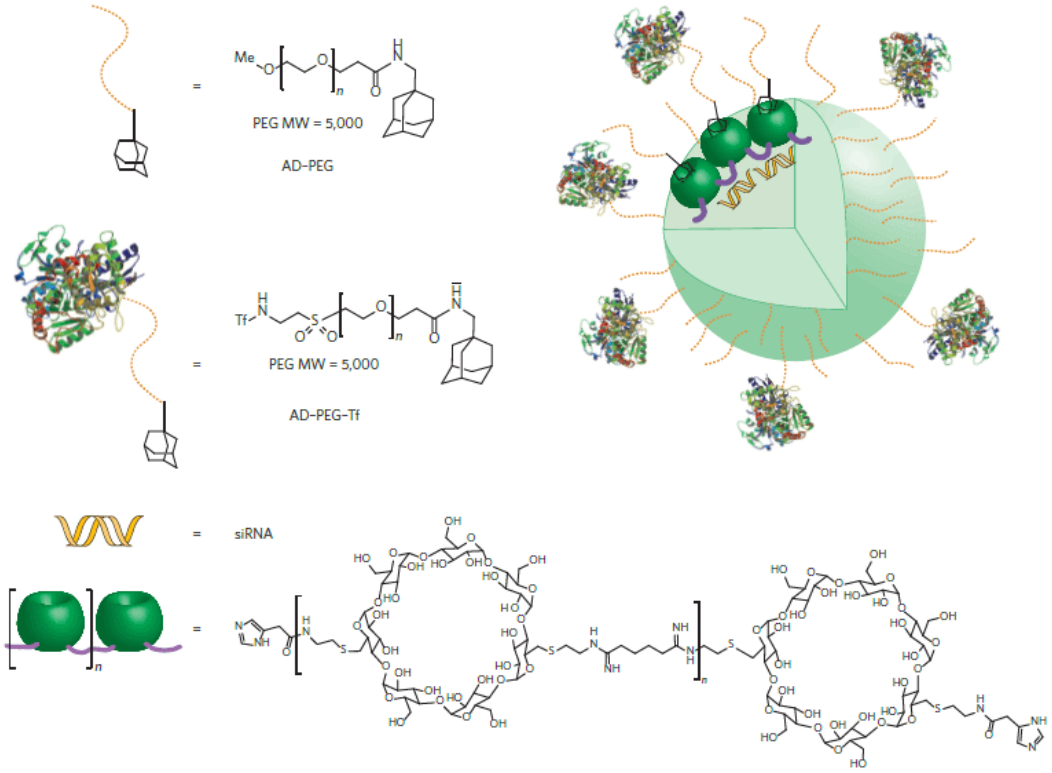


Figure 2-11. Cyclodextrin-based nanoparticles. MW, molecular weight. AD, adamantane, for forming inclusion complexes with CDs. Reprinted from reference 199 with permission from Springer Nature.

modifications to CDs are necessary to apply CDs into gene delivery.[197] The modifications with CDs usually involved the design of CD-embedding polymers (backbone of a polymer) or CD-pendant polymers (CDs grafted with polymers).[187] Davis *et al*. introduced the first CD-embedding polymers (CDEP) for pDNA delivery in 1999 (**Fig. 2-11**) and it was the first targeted siRNA delivery system that translated into the clinics for cancer treatment.[198] CDs were modified to make cyclodextrin-containing polycations and formed the polyplexes with pDNA as the core of the nanoparticle. 5 kDa PEG was conjugated with adamantine and human transferrin (Tf) to make adamantine-PEG (AD-PEG) and adamantine-PEG-transferrin

(AD-PEG-Tf). The modified PEG chains were used to decorate the surface of polyplexes via the formation of inclusion complexes with CDEP and incorporated targeting properties to the polyplexes, which improved the delivery efficacy.

As CDs have a cyclic shape, CDs can act as a core to form star-shaped polymers, which can be easily prepared by designing CD-pendants polymers (CDPP). Li *et al*. prepared a series of cyclodextrin-cored star-shaped pDNA carriers by grafted CDs with epoxide-based polymer chains via conducting atom transfer radical polymerization (ATRP) with glycidyl methacrylate (GMA) and subsequently modified the polymer chains with cationic molecules,

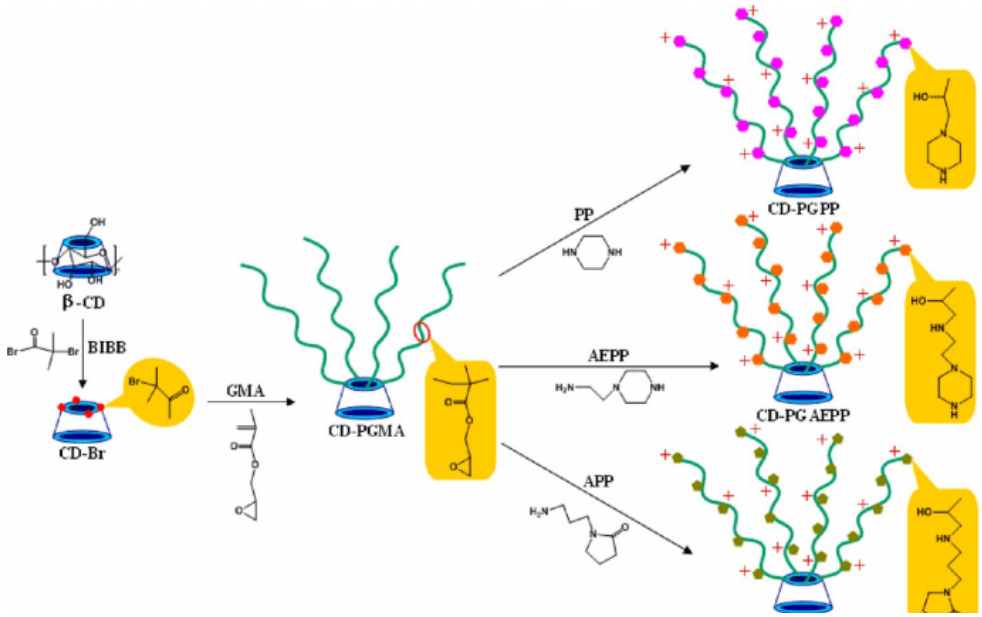


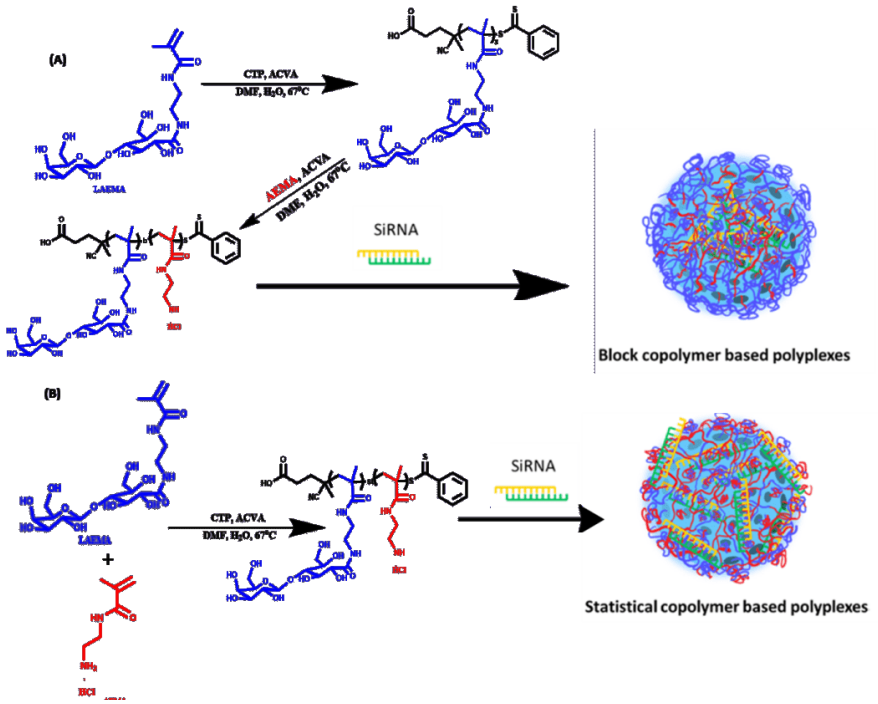
Figure 2-12. Schematic diagram revealing the steps in the preparation of PP, AEPP or APP modified cyclodextrin-cored star-shaped gene carriers. Reprinted from reference 200 with permission from American Chemistry Society.

piperazine (PP), N-(aminoethyl)piperazine(AEPP) or N-(3-amino-propyl)-2-pyrrolidinone (APP) via amine-glycidyl ether reaction (**Figure 2-12**).[200] All modified CDs were excellent in condensing pDNA into 100-200 nm nanoparticles and induced significantly lower

cytotoxicity in HEK 293, COS 7, and HepG2 cell lines in comparison to control 25 kDa PEI.

2.4.3 Synthetic cationic glycopolymers

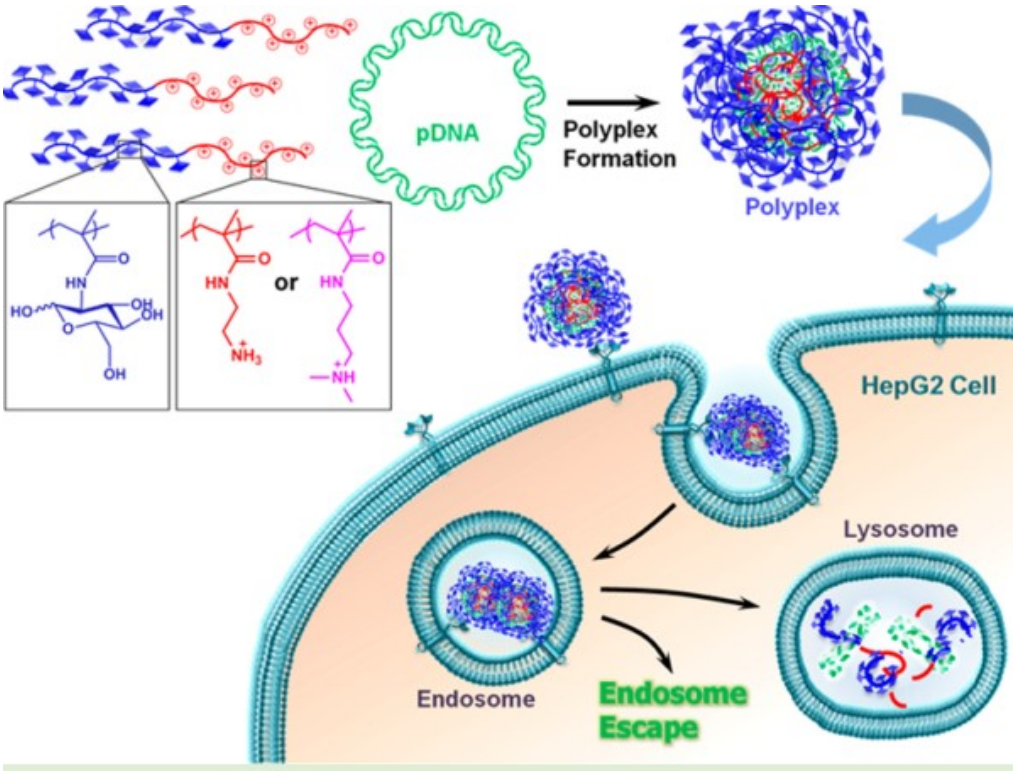
Narain and Reineke groups contributed and invested a lot of effort in developing RAFT-made synthetic cationic glycopolymers for gene delivery. RAFT polymerization facilitates the process of preparing well-defined cationic glycopolymer, and statistical linear or hyperbranched ones can be easily synthesized via *one-pot* method, followed by easy purification steps.[142, 150, 182 & 201] By preparing sugar-based or cationic macroCTA, the



Scheme 2-7. Schematic illustration of block (A) and statistical (B) galactose-based cationic polymers synthesis via RAFT polymerization. Reproduced from reference 171 with permission from the American Chemistry Society.

polymer can further be designed into block linear copolymer or nanogel, and surface properties of the resulted polyplexes are tunable from engineering the content of macroCTA.[142, 147, 171, 181-182, & 202-204] Capability of intracellular delivery of nucleic acid can be

easily added to the hyperbranched cationic glycopolymers or glyconanogel by using acid-degradable cross-linker.[202-203] Narain *et al*. prepared galactose-based cationic statistical and block copolymers from polymerizing 2-aminoethyl methacrylamide hydrochloride (AEMA) and 2-lactobionamidoethyl methacrylamide (LAEMA) via RAFT polymerization (**Scheme 2-7**) and studied their efficacy in siRNA delivery.[168] Galactose-based monomer, LAEMA, was incorporated into the system to enhance biocompatibility. Diblock copolymers were found to have high epidermal growth factor receptor (EGFR) knockdown efficiency in



Scheme 2-8. Formation of polyplexes from using diblock cationic glycopolymers with pDNA and associate intracellular barriers in HepG2 cell. Reprinted from reference 204 with permission from the American Chemistry Society. https://pubs.acs.org/doi/10.1021/bm5001229 Note: Further permissions related to the material excerpted should be directed to the ACS.

HeLa cells but were associated with higher cytotoxicity. The ratio between sugar and cationic moieties needs to be optimized to obtain the desired results. Series of glucose-derived and galactose-based hyperbranched glycopolymers were also prepared by Narain group to investigate the effect of molecular weight, shape, active targeting and contents of carbohydrate of the polymers on transfection efficiency with HepG2 and HEK 293 cell lines.[150] Series of diblock cationic glycopolymers with various DP in glyco and cationic segments were prepared by using glycomonomer (2-deoxy-2-methacrylamide glucopyranose [MAG]) with cationic monomer (either a primary amine-containing monomer (AEMA) or a tertiarty amine-containing monomer (*N*-[3-{*N,N*-dimethylamino} propyl] methacrylamide [DMAPMA]) via RAFT polymerization, by Reineke group to condensate pDNA and studies transfection efficiency in HeLa and HepG2 cell lines (**Scheme 2-8)**.[204] By having glyco-moieties on the surface (PMAG block), the stability of polyplexes in physiological salt and serum environment were significantly improved,[147, 204] and glycopolymers with PAEMA segment offer superior transfection efficiency than glycopolymers with PDMAPMA segment.[204]

2.5 Conclusions

To move siRNA-based cancer treatment from bench to bedside, the development of a safe non-viral vector that circumvents the intercellular and intracellular barriers is a necessity. Numerous strategies, such as nanotechnology, pegylation, glycosylation, ligand conjugation, EPR effect, proton sponge effect, pH degradable covalent bond, bioreducible bond, pH-sensitive groups, and LRP were utilized to address the challenges of blood circulation time of siRNA, opsonization, accumulation at the tumor site, membrane impermeability, endosomal trap, bioavailability of siRNA in the cytoplasm, and cytotoxicity. Hallmarks (tunable polymer composition, uncomplicated pre and post-modifications, and adjustable

polymer structure) of LRP make the fabrication of advanced non-viral vectors with multifunction achievable and lowering the cytotoxic concern that associate with a large size distribution of polyplexes because of the high polydispersity of polymer.[205] Developing a siRNA delivery system based on cationic glycopolymers is also a rational approach because of their biocompatibility, biodegradability, safety profile, low immunogenicity, and a potential method to avoid opsonization. As proved by the studies conducted in Narain and Reineke group, well-defined primary amine-containing glycopolymer demonstrated to be a good candidate for gene delivery as high transfection efficiency were obtained with sacrificed cell viability in vitro studies. Thus, investing more work to ameliorate these sugar-based gene delivery systems on delivering siRNA for cervical cancer management will be worthy.

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CHAPTER 3. ACID DEGRADABLE CATIONIC GALACTOSE-BASED HYPERBRANCHED POLYMERS AS NANOTHERAPEUTIC VEHICLES FOR EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) KNOCKDOWN IN CERVICAL CARCINOMA

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3.1 Introduction

The role of growth factors in driven strong cascade signaling in the development and maintenance of cancer has been known since 1985, where Sporn and Roberts demonstrated that cancer exhibited a lower requirement of exogenous growth factors to maintain high proliferation rates.[1] Since then, several studies have been done that confirm a direct correlation of growth factors as oncogenes.[2,3] Different families of growth factors like the EGF-family have a role in the pathogenesis of certain carcinomas; overexpression and aberrant activation of EGFR (epidermal growth factor receptors). This is a hallmark of various cancers such as lung, breast, and ovarian carcinomas, and leads to uncontrolled cell proliferation and evasion of apoptotic pathways.[4−7] Thus, it is desired to regulate this key protein expression. It has been reported that chemotherapeutic sensitivity has been increased by silencing the EGFR gene, resulting in tumor regression.[8,9] After the discovery of the efficient gene silencing ability of small interfering ribonucleic acids (siRNAs) in 1998, two decades of intensive investigation on gene therapy has demonstrated that a number of malignant and gene-related diseases can be treated by using external gene sources to regulate oncogene expressions.[10−12] Small interfering RNAs (siRNA) are 21−30 nucleotides duplexes which have the capability of cancer suppression through inhibition of gene expression of the causing oncogenes and/or activation of tumor suppressor genes.[13,14] This provides an alternative to cancer treatment from conventional surgery, radiotherapy, and chemotherapy.[15−17] However, many limitations still exist that prevent its clinical application. Due to the presence of ribonucleases, siRNA is vulnerable to degradation in the bloodstream if there is no protection.[18] The small size of siRNAs (less than 5 nm) allows the rapid removal of siRNAs from the bloodstream through renal filtration and clearance,[19] making their delivery in the targeted cells quite a challenge. Naked siRNA cannot easily penetrate through

the cell membrane to reach the cytoplasm due to repulsive forces which leads to low cellular uptake and results in ineffective transfection efficiencies.[20] Prior research has confirmed that these deficiencies can be addressed and the outcomes of treatment can be greatly improved by using nonviral vectors such as lipids,[21,22] proteins,[23,24] polymers,[25,26] and carbohydrate-based polymers.[27−30] Combination therapies have also been used that provided a better therapeutic efficacy than a single therapy.[42] Thus, several studies have been conducted with the hope to develop a versatile nanocarrier that can effectively deliver and release siRNA into the cytoplasm of the targeted cells to enhance the therapeutic efficacy of this technology, for instance toward the treatment of malignant tumors.

Furthermore, this strategy should not lead to adverse effects on the cells as the cell viability would not be affected significantly by the knockdown of overexpressed EGFR on the cellular surface.[28, 30-31] However, siRNA treatment with no toxicity and high transfection efficiency is not yet achievable because of drawbacks in the delivery carrier.[32]

Synthetic carbohydrate-based polymers, also known as glycopolymers, have the potential of overcoming some of the challenges associated with siRNA delivery. Glycopolymers have many unique properties, such as high stability in physiological environment[28,33] enhanced blood biocompatibility,[34] and induced carbohydrate-specific recognition in cell−cell communications[35] that support their suitability to become a leading nanocarrier. Previous studies have shown that cationic glycopolymers could form stable polyplexes with siRNA and high internalization in cells leading to high EGFR knockdown in cervical carcinoma as compared to using siRNA alone.[36,37]

However, these findings have not been clinically viable, as the required high knockdown efficiency could not be achieved without sacrificing cell viability since more cationic moieties are needed to achieve the target outcome. In this study, we aimed to reduce or

eliminate the cytotoxicity of these gene carriers by incorporating acid degradability into the system while maintaining high gene knockdown. Once degraded, the small polymer fragments are expected to be less toxic as compared to the hyperbranched polymers.

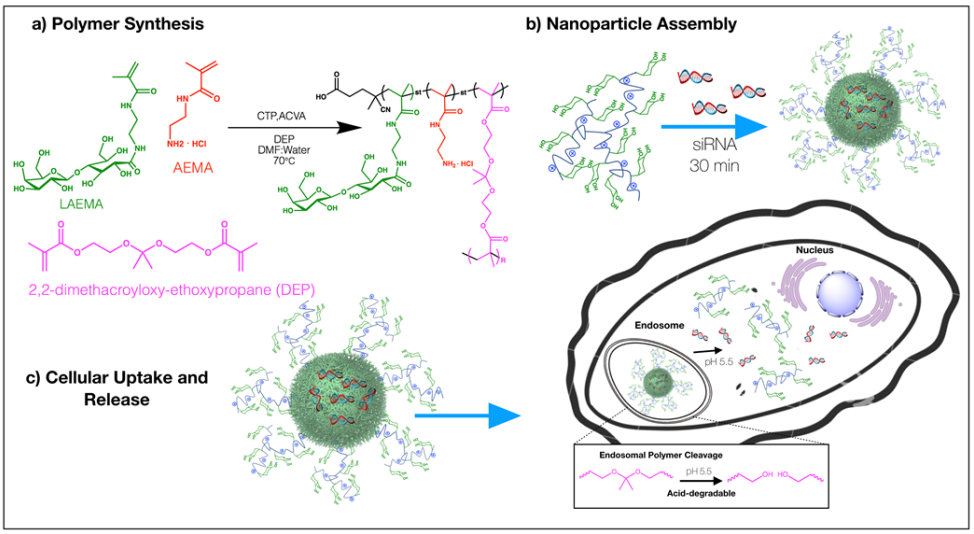
Further, low molecular weight fragments are expected to clear from the body much faster and hence the risk of their accumulation and retention in the body tissues is minimized.[38] Moreover, using this strategy, we can expect a faster release of the siRNA after the polyplexes are internalized in the cells via the endosomes which has a mildly acidic environment (pH 5−6) that can trigger the degradation of the polymer matrix (see **Scheme 1c**). Several design parameters (molecular weight [MW] and composition) were considered to engineer an innovative, safe, and effective siRNA carrier to knockdown the expression of EGFR in cervical carcinoma, which will finally lead to cancer suppression.

3.2 Materals and Methods

3.2.1 Materials

2-Aminoethyl methacrylamide hydrochloride (AEMA),[39] 2-lactobionamidoethyl methacrylamide (LAEMA),[40] 2,2-dimethacroyloxy-1-ethoxypropane (DEP),[41] and 4-cyano-4-(phenylcarbonothuoylthio)pentanoic acid (CTP)[42] were prepared according to the previously reported method. DMF and methanol were purchased from Caledon Laboratories Ltd. (Georgetown, Canada). 4,4′-Azobis(cyanovaleric acid) (ACVA), the initiator, was ordered from Sigma-Aldrich (Oakville, Canada). Opti-MEM(OMEM), DMEM media, 0.225% trypsin-EDTA, Fetal Bovine Serum (FBS), and Streptomycin (5000 µg/mL), Penicillin (5000 U/mL) were obtained from Gibco. Lipofectamine 3000 was purchased from Invitrogen. Control siRNA, human EGFR-specific small interfering RNA (EGFR siRNA), and primary antibody (rabbit polyclonal EGFR specific IgG) and FITC-conjugated control siRNA were ordered from Santa Cruz Biotechnology. (HRP)-conjugated secondary antibody

(Antirabbit IgG) was purchased from Promega Corporation.



Scheme 3-1. (a) Synthesis of acid degradable and cationic galactose-based hyperbranched polymers (P[LAEMA-st-AEMA-st-DMP]) via RAFT polymerization; (b) Polyplexes formation by the electrostatic interactions between siRNA and cationic glycopolymers; (c) siRNAs were released into the cytoplasm via polyplex degradation from the hydrolysis of ketal bond in the acidic environment of the endosome.

3.2.2 Synthesis of acid degradable and cationic galactose-based hyperbranched polymers.

Acid degradable and cationic galactose-based hyperbranched polymers were prepared by modifying a procedure reported earlier.[43] In a typical procedure, 335 mg of LAEMA (0.716 mmol) and 59 mg of AEMA (0.358 mmol) were dissolved into 1 mL of double distilled water and mixed with 0.7 mof methanol solution that predissolved 12 mg of DEP (39.7 µmole, acid degradable cross-linker), 10 mg of CTP (35.8 µmole, the chain transfer agent), and 2.5 mg of ACVA (8.9 µmole, the initiator). After degassing with nitrogen for 30 min, the polymerization process was carried out at 70 °C for 24 h. The reaction was stopped in liquid nitrogen, and the resulting polymer was purified by precipitating in acetone followed by three

washes with methanol to remove residual monomer. The yield of the reaction was 66.1%. Gel permeation chromatography (GPC) and nuclear magnetic resonance (NMR) spectroscopy were used to determine the molecular weight and composition of the acid degradable glycopolymer.

3.2.3 Acid degradability of polymer.

The acid degradable glycopolymer was incubated in acetic buffer (pH5.5) for 1 day. The resulting polymer was analyzed by using GPC.

3.2.4 Preparation of polyplexes.

EGFR-siRNA/Control-siRNA (25 μg) was diluted in 250 μL of OMEM and complexed with acid degradable and cationic galactose-based hyperbranched polymers (in OMEM media) at a weight/weight (w/w) ratio of 50. The mixture was incubated at 23 °C for 30 min. Fluorescent-labeled polyplexes were prepared using the same method except for the incubation with fluorescein isothiocyanate (FITC) control-siRNA.

3.2.5 Characterization of polymers and polyplexes.

The hydrodynamic diameter and charge of polyplexes and polymers were obtained by using ZetaPlus-Zeta Potential Analyzer (Brookhaven Instruments Corporation). The polyplexes were formulated at w/w ratio of 50 in water and OMEM media. The stability of the polyplexes was evaluated in the presence of serum proteins in OMEM after 48 h. To determine the stability of the polyplexes under acidic condition, the polyplexes solution was prepared as mentioned above and pH was adjusted to 6.0 and 5.0, respectively. The hydrodynamic diameters of the polyplexes were measured under various incubation times.

3.2.6 Agarose gel electrophoresis.

The polyplexes were formulated in OMEM at various polymer/siRNA ratios, following the abovementioned method. The polyplexes were loaded onto 1% agarose gel that contained 1

μg/mL ethidium bromide in 1 X Tris Acetate/EDTA (TAE) buffer. The gel was run for 45 min at 130 V and visualized by UV light, and DNA bands were visualized by using UV transilluminator.

3.2.7 Cell culture.

MRC-5 and HeLa cells (cervical cancer) were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and 1% antibiotic (50 units of penicillin, 50 μg streptomycin) in a humidified atmosphere at 37 °C under 5% CO2. The cells were subcultured by detaching with 0.25% trypsin-EDTA at about 80% confluency and were cultured twice a week.

3.2.8 Cytotoxicity of the polymers.

Proliferation (MTT) assay was conducted to obtain the cytotoxicity of polymer in HeLa cells or MRC-5 cells by staining with dimethyl thiazol dyes for metabolically viable cells. IC50 was calculated by using GraphPad Prism Software. Post-transfection cytotoxicity was determined by conducting MTT assay which was performed 48 h after the transfection study.

3.2.9 Transfection of EGFR-siRNA.

100,000 HeLa cells were seeded into 60 mm plates. The polyplexes were prepared in OMEM (in the presence of serum proteins) and 500 μL of the polyplexes/lipofectamine mixture (EGFR siRNA/control siRNA) was added per plate. After 6 h of incubation, the media was removed and replaced with 2 mL of DMEM media containing 10% FBS. The EFGR knockdown efficiency was evaluated after 48 h of cell growth.

3.2.10 Western Blot.

Cells were harvested in RIPA buffer supplemented with a protease inhibitor, and the protein concentrations were determined using a Bradford protein assay kit (Bio-Rad). Eluates were then run on an SDS-PAGE denaturing gel, transferred to a nitrocellulose membrane (0.45 μm), and visualized by probing with EGFR-1005 sc-03 antibody (Santa Cruz) and a

streptavidin horse radish peroxidase (HRP) antirabbit conjugate. The amount of EGFR protein expression was quantified using ImageJ image analysis.

3.2.11 Cellular uptake of polyplexes.

Confocal Fluorescence. HeLa cells were cultured as mentioned above, trypsinized, and seeded onto glass coverslips in a 10 mm plate at a density of 1000 cells per plate. The next day, the medium was removed and replaced with FITC control siRNA polyplexes at a w/w ratio of 50 in OMEM and subsequently incubated for 6 h in a humidified atmosphere at 37 °C under 5% CO2. Medium was then removed, cells were washed with 1×PBS three times, and stained with (1:10000) DAPI dye that was dissolved in PBS for 1 h and fixed with 4% paraformaldehyde for 15 min at 37 °C. Visualization of FITC was performed by using confocal microscopy at 490 nm.

Flow Cytometry. 1,000,000 HeLa cells were trypsinized and subsequently seeded onto a 60 mm plate. After overnight incubation, the cells were treated with FITC−siRNA polyplexes for 6 h at a w/w ratio of 50 in OMEM. Afterward, the medium was removed and the cells were washed with 1×PBS three times and then trypsinized and centrifuged at 1200 rpm. The pellet was resuspended in 1×PBS and cells were characterized by using a BD FACS dual laser flow cytometer (Cross Cancer Institute).

3.3 Results and Discussions

3.3.1 Synthesis of acid degradable galactose-based hyperbranched cationic polymers (HGA).

The acid degradable galactose-based hyperbranched cationic polymers, termed as HGA #, were prepared according to a previous one-pot synthesis process (see **Scheme 3-1a**).[34] 2-Aminoethyl methacrylamide hydrochloride (AEMA) was used since it has a primary amine function which facilitates the polyplex formation with siRNA via electrostatic interaction. 2-Lactobioamidoethyl methacrylamide (LAEMA) was used as a carbohydrate unit to enhance

Table 3-1. Characterization of acid degradable glycopolymers by Gel Permeation Chromatography, Zeta Potential Analysis, and MTT Assay.

Polymer	GPC M_n (kDa)	M_w/M_n	% Cross-lnker (mol%)	Polymer Composition	Zeta Potential (mV)	IC_{50} (mg/mL)	Hydrodynamic Diameter (nm)
HGA 1	21.6	1.26	3.5	($LAEMA_{27}$-st-$AEMA_{54}$-st-DEP)	53.8±2.79	<0.001	142.5±21.5
HGA 2	19.6	1.30	3.5	($LAEMA_{32}$-st-$AEMA_{29}$-st-DEP)	37.75±4.85	0.19	199.9±1.6
HGA 3	17.2	1.29	3.5	($LAEMA_{30}$-st-$AEMA_{17}$-st-DEP)	33.47±0.27	0.60	88.5±12.6
HGA 4	9.6	1.28	3.5	($LAEMA_{11}$-st-$AEMA_{27}$-st-DEP)	52.68±0.27	0.12	200.1±14.2
HGA 5	12.4	1.29	3.5	($LAEMA_{19}$-st-$AEMA_{20}$-st-DEP)	36.86±0.11	0.47	194.3±19.5
HGA 6	8.4	1.25	3.5	($LAEMA_{15}$-st-$AEMA_{8}$-st-DEP)	30.8±4.7	0.98	108.8±2.4

the biocompatibility of the polymer, thereby reducing the cytotoxicity and increasing the stability of polyplexes in the physiological environment. To make polymers acid degradable, an acid degradable crosslinker, 2,2-dimethacroyloxypropane (DEP), was incorporated to allow the breakdown of the polymer into smaller fragments in an acidic environment for lower cytotoxicity. DEP possesses a ketal group which tends to be hydrolyzed into two separate hydroxyl groups in low or mild pH environment (see **Scheme 3-1c**). In addition, the cross-linker also increases the branching degree of the polymer, which is known to improve transfection efficiencies.[43]

HGA 1−6 were prepared with various MW (10 kDa and 20 kDa) and compositions (LAEMA:AEMA ratio [L/A] = 2.0, 1.0, and 0.5), and their characterization was summarized in **Table 3-1**. Polymer compositions were evaluated by ^{1}H NMR spectroscopy (see **Figure 3S1**). Their MWs and compositions were designed and chosen carefully to validate the relationship between these parameters and EGFR knockdown efficiencies for acid degradable hyperbranched cationic glycopolymers. The acid degradability of HGA 6 was proved by analyzing the spectrum by gel permeation chromatography before and after the incubation in acetic buffer (pH 5.5) (see **Figure 3S2**), and the stability of polyplexes under acidic environment (see **Figure 3S3**).

3.3.2 Cell Viability/Cytotoxicity

Before conducting transfection studies, cytotoxicity (MTT) assays (see **Figure 3-1**) were carried out for each polymer in HeLa cell line and IC_{50} values were determined. Based on the data, suitable candidates for bioapplication were selected to minimize carrier toxicity. The correlation between IC_{50} and composition of the polymers were as expected; the IC_{50} was found to be higher in the low MW group (HGA 4−6, 10 kDa) in comparison to the high MW group (HGA 1−3, 20 kDa) and an increment of L/A also made the polymer less toxic.[36,39] As the L/A increased from HGA 4 (L/A: 0.5) to HGA 6 (L/A: 2.0), the IC_{50} became higher. Based on the cytotoxicity of the polymers, only HGA 2, 3, 5, and 6 were moved to the next stage as HGA 1 and 4 were found to be overly toxic for their use in medical applications.

3.3.3 Polyplexes Formation.

To estimate the ability of the resulting cationic glycopolymers to form complexes with siRNA, a gel electrophoresis retardation assay was carried out at different polymer/siRNA (w/w) ratios using 25 kDa branched polyethyleneimine (PEI) as a positive control and free siRNA as a negative control. The polyplexes formation was produced by incubating HGA

polymers with siRNA at various polymer/siRNA weight ratios (w/w) for 30 min at 22 °C (see **Scheme 3-1b**), which were examined by agarose gel electrophoresis. All HGA polymers were able to completely condense with siRNA at a w/w of 25 as evidenced by the agarose gel electrophoresis image (see **Figure 3S4**).

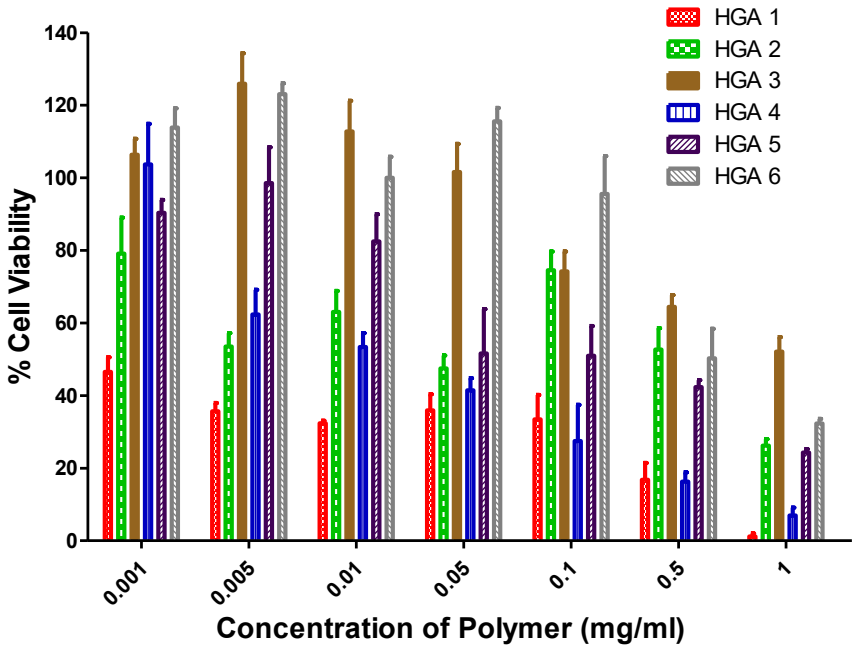


Figure 3-1. Cytotoxicity of polymers in HeLa cells as determined by MTT assay.

Forming stable polyplexes with siRNA is an essential requirement for the gene carrier to protect siRNA in the bloodstream and extend its blood circulation time; however, the knockdown efficiency would not be favorable if the polyplexes are too stable to release siRNA in the cytoplasm.[44] Therefore, after cellular internalization of polyplexes, it is desirable to have a "smart" release strategy to deliver siRNA selectively to the cytosol of the target cells. Since the endosome exhibits a mildly acidic environment, using ketal based cross-linkers in the hyperbranched polymers would be an advantage. This will allow cleavage of the linker, which in turn would lead to the efficient release of siRNA, and hence resulting

in higher EGFR-knockdown efficiencies.

3.3.4 Transfection efficiency of acid degradable galactose-based polyplexes and post-transfection toxicity.

To determine whether these polyplexes are suitable carriers of EGFR siRNA, the EGFR knockdown efficiencies were evaluated by immunoblotting, and effective silencing of EGFR was observed with HGA 6 polyplexes (see **Figure 3-2a−c**) in the presence of serum with no significant off-target knockdown as compared to the commercial nonviral vector Lipofectamine 3000. HGA 3 and 6 had higher EGFR knockdown efficiencies in comparison to HGA 2 and 5 which may suggest that the polymer with an L/A of 1 formed highly stable polyplexes (HGA 2 and 5), insufficiently releasing the siRNA in the cytoplasm.

To further evaluate the potential of HGA 3 and 6 as carriers for siRNA delivery in the treatment of cervical carcinoma, post-transfection cell viability was assessed by MTT assay. HeLa cells were incubated with control siRNA formed polyplexes for 48 h, and then the cell viability was determined. Polyplexes of HGA 3 showed around ∼80% cell viability and HGA 6 was around ∼100%; significantly better in comparison to the standard lipid-based carrier, lipofectamine 3000 (∼30%) (**Figure 3-2d**), thus confirming the greater potential of HGA 6 as a delivery carrier.

To further investigate the potential of the carriers in the EGFR silencing treatment, the MRC-5 cell line was used as model cell line to evaluate the cytotoxicity that associates with HGA 3 and HGA 6 on the normal cell line (see **Figure 3S5**). No significant cytotoxicity was observed in treating noncancerous cell line, MRC-5 cell, with HGA 3 and 6, which gave another supportive evidence to study the material further as both polymers do not lead to significant cellular death on the normal cell line.

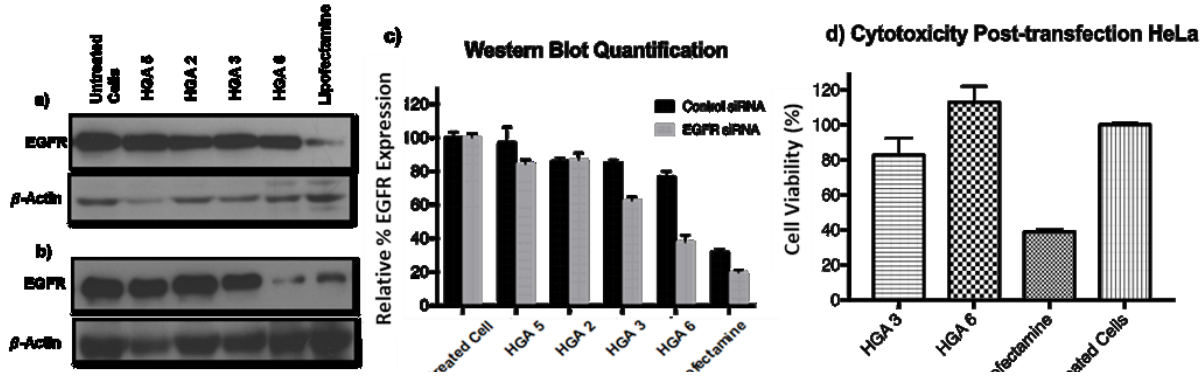


Figure 3-2. EGFR knockdown by Western Blot analysis with HGA 2, 3, 5 & 6 polyplexes at w/w 50 in HeLa cells in the presence of serum. Control siRNA (a) and EGFR siRNA (b) treated group. HeLa cells were transfected with either control or EGFR siRNA for 48 h, and cell lysates were subjected to immunoblot analysis using indicated antibodies; (c) Western Blot quantification was analyzed by ImageJ; (d) Cell viability was determined by MTT assay after 48 h of transfection treatment (treated with control siRNA).

3.3.5 Hydrodynamic Size and Zeta Potential of Polyplexes

To understand the cause of the different behaviors of HGA 3 and 6; the polyplexes were characterized by using dynamic light scattering (DLS) and Zeta Potential Instruments (**Figure 3-3a**). The size difference between HGA 3 and 6 matches with the previous study that polymers with higher MW form smaller size polyplexes.[45,46] Both polyplexes demonstrated similar hydrodynamic diameters (HGA 3 = 40 nm and HGA 6 = 50 nm) and Zeta potentials (~ −5 mV). Thus no direct correlation could be derived from these data. In addition, both polyplexes demonstrated good stability in DMEM medium in the presence of serum (size stayed ~40−50 nm for up to 24 h at 37 °C, **Figure 3-3b**) which is in agreement with our previous reports that suggest that sugar moieties enhance the stabilization of nanoparticles in the presence of serum.[28]

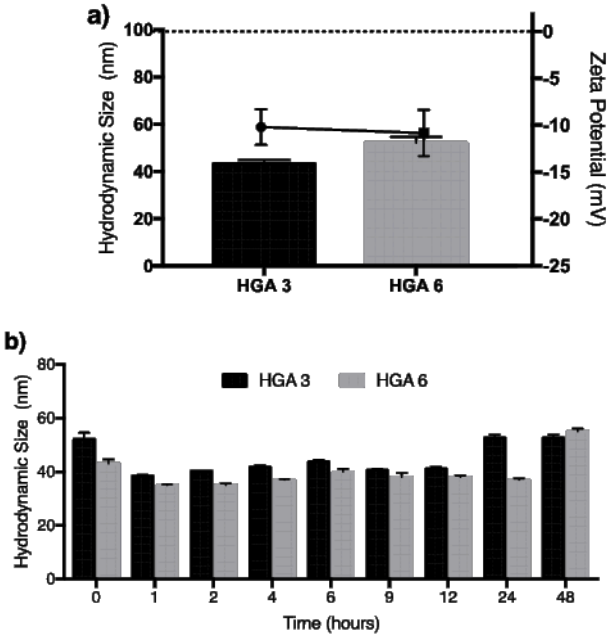


Figure 3-3. Polyplexes characterization. (a)Hydrodynamic Diameter (DLS) and Zeta Potential analysis. (b) Hydrodynamic Diameter and stability of polyplexes, as evidenced by DLS analysis in DMEM media with the presence of serum.

3.3.6 Cellular uptake of polyplexes

Cellular uptake studies were conducted to determine cellular internalization by using confocal microscopy; the internalization of HGA 6 polyplexes, complexed with FITC-control siRNA, was observed in the cytoplasm after 6 h of incubation (see **Figure 3-4b**) in comparison to the control (see **Figure 3-4a**).

Because no significant difference was observed between the characteristics and stability of

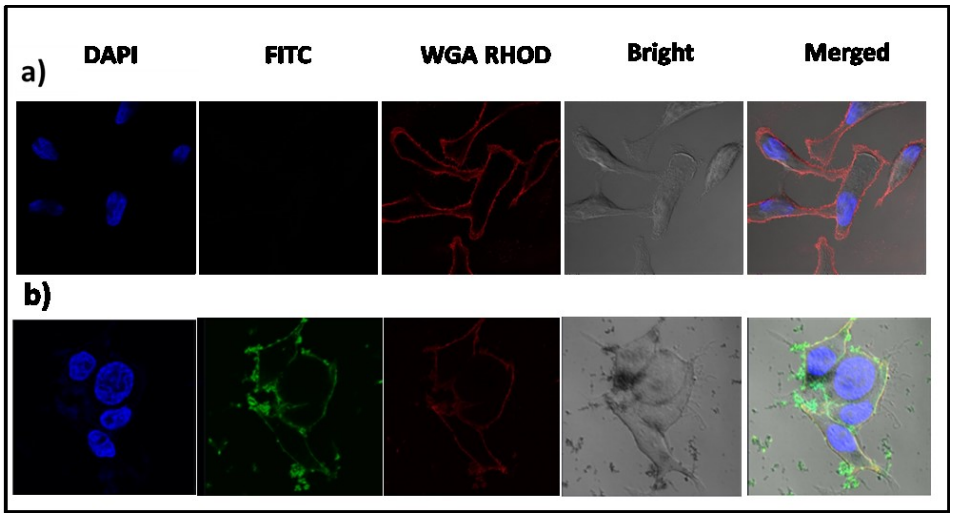


Figure 3-4. Cellular uptake in HeLa (cervical cancer cells) of HGA 6 complexed with FITC-control siRNA at w/w of 50 after 6 h incubation (B); Untreated cells (A). Imaged using confocal fluorescence microscopy. The cellular membrane was stained with wheat germ agglutinin, tetramethylrhodamine (WGA-Rhodamine) dye and the nucleus of cells were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI).

both HGA 3 and 6 polyplexes, flow cytometry was used to further understand the cellular retention and uptake of siRNA. The fluorescence intensity of FITC-control siRNA (see **Figure 3S6**) between HGA 3 and 6 was not significantly different, suggesting that the higher knockdown efficiency of HGA 6 than HGA 3 could depend upon the release of the siRNAs into the cytoplasm. This hypothesis is based on the original molecular weight of each HGA polymer. Since both HGA 3 and 6 were designed with the same cross-linker percentage, the molecular weight of the resulting fragments of HGA 6 (10 kDa) should be smaller than HGA 3 (20 kDa); with smaller molecular weight, the stability of the complexation between siRNAs and degraded HGA 6 should be less in comparison to HGA 3 leading to more effective release of free siRNA into the cytosol. The observed fluorescence intensity signals for both

HGA 3 and 6 polymers were stronger than the untreated group, corroborating its cellular uptake.

3.4 Conclusions

This novel acid degradable galactose-based cationic hyperbranched polymer has proved to be an effective vehicle for siRNA delivery by enriching gene knockdown in the presence of serum proteins. Moreover, biocompatibility was achieved by incorporating acid degradability into the delivery system. Higher molar ratios of the LAEMA residues in the glycopolymers have been shown to contribute to an elevated EGFR silencing and lower cellular toxicity as compared to lower LAEMA ratios. The higher capability of EGFR knockdown may be due to selective release of siRNA into the cytoplasm after the polyplex internalization and degradation. Further studies will focus on applying this system to other potential oncogenes and examining the biological response *in vivo* for their applicability into the clinical level for polymer-assisted siRNA silencing.

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CHAPTER 4. ACHIEVING SAFE AND HIGHLY EFFICIENT EPIDERMAL GROWTH FACTOR RECEPTOR SILENCING IN CERVICAL CARCINOMA BY CATIONIC DEGRADABLE HYPERBRANCHED POLYMERS

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4.1 Introduction

A common trait of various cancers is the unusual activation or overexpression of epidermal growth factor receptors (EGFR), which results in unregulated cell growth and evasion of apoptotic pathways.[1–3] As EGFR is one of the driving factors of cancer development and maintenance, regulation of its expression is an excellent strategy to manage the EGFR related malignant disease; thus, gene therapeutic, which is capable of disrupting gene expression, holds great promise in this application.[4–6] Small interfering RNA (siRNA) is a double-stranded RNA (21−30 nucleotides) molecule that has the capability to silence the expression of specific genes by binding and cleaving the targeted mRNA (messenger RNA) with the assistance of the RNA-induced silencing complex (RISC).[7] Thus, delivery of siRNA to cells is a promising solution to control gene overexpression by the post-transcriptional regulation of gene expression. Studies have revealed that by silencing EGFR expression, chemotherapeutic sensitivity can be enhanced, and this has led to tumor regression.[8] However, administration of naked siRNA during treatment is ineffective because of the degradation of siRNA in the presence of ribonucleases (RNase), due to short half-life (≈ 30 min)[4] in the bloodstream, and poor cellular uptake due to repulsive forces between siRNA and the cellular membrane.[9] Thus, tremendous efforts have been focused toward the development of versatile and advanced siRNA nanocarriers that can successfully deliver and release siRNA inside the targeted cells.[10] In addition, to apply this system in clinics, the vector should not induce any antagonistic effects on normal cells as previous studies have shown that the cell viability of normal cells can be significantly compromised by the knockdown of EGFR receptors on the cell surface.[11–13] Most siRNA carriers are developed from cationic lipid[14] or polymers[15,16] as these materials spontaneously bind to siRNA via electrostatic interactions and formed stable polyplexes, resulting in siRNA protection against degradation and extension of blood

circulation time thus improving the cellular uptake of the polyplexes. However, using cationic polymers still owns some limitations as high knockdown efficiencies cannot be achieved without sacrificing the cell viability as the knockdown efficiency increases with the polymer concentration while cell viability decreases.[17] Polymers based on ethylene glycol methacrylate derivatives (PEG-methacrylates) have great potential in the biomedical field due to their biocompatible characteristics.[18] For instance, cationic copolymers containing (ethylene glycol) (PEG) are less cytotoxic as compared to the cationic homopolymers.[19,20] Moreover, by using cationic ethylene glycol based polymers, an increased blood circulation time of the siRNA−polymer complexes can be achieved as aggregation of the nanoparticles and the interactions to serum proteins can be prevented,[18,19] and also clearance from the reticuloendothelial system is evaded.[21] Moreover, di(ethylene glycol) methacrylate (DEGMA) is temperature responsive, which provides additional stability of the polyplexes (smaller and tighter particles) under physiological temperature after the siRNA complexes with the cationic groups in the polymer,[21] which further increases the lifetime of siRNA in the physiological environment and possibly results in better performance.

To further enhance the biocompatibility, it is desirable to design the siRNA carriers with degradability properties. After delivery of the siRNA cargo, the polymer system is degraded into small fragments, and hence, toxicity, accumulation, and aggregation are prevented and the smaller fragments can be easily cleared via renal filtration.[22−24] Ketal-based cross-linkers, such as 2,2-dimethacroyloxy-1-ethoxypropane (DEP), are promising in biomedical applications because of their pH sensitivity under the mild acidic environment (pH 5−6). This results in a molecular weight (MW) reduction via polymer degradation or cleavage from the hydrolysis of the ketal bond.[25,26] Because of the mild acidic environment in the endosome, acid degradation of the polyplexes is prompted after cellular uptake and this degradation

triggers the release of siRNA into the cytoplasm from the resulting unstable polyplexes.[27] The controlled release of the uncomplexed siRNA into the intracellular site possibly leads to better gene knockdown efficiencies. Additionally, by using a cross-linker, a hyperbranched polymer structure is achieved by RAFT process, which should work better in protecting the nucleic acids, and hence, higher gene knockdown efficiency can be achieved as compared to their linear analogs.[28−30]

The aim of this study is to prepare a biocompatible, nontoxic, and efficient siRNA carrier via the reversible addition−fragmentation chain transfer (RAFT) polymerization and examining the potency of the resulting polymers in protecting and delivery of siRNA in vitro.

4.2 Materials and Methods

4.2.1 Materials.

2-aminoethyl methacrylamide hydrochloride (AEMA),[31] 2,2-dimethacroyloxy-1-ethoxypropane (DEP)[26] and 4-cyano-4-(phenylcarbonothuoylthio)pentanoic acid (CTP)[32] were prepared according to the previously reported method. DMF & methanol were purchased from Caledon Laboratories Ltd. (Georgetown, Canada). Di(ethylene glycol) methyl ether methacrylate (DEGMA), N,N'-methylenebisacrylamide (MBAm), and 4,4'-azobis (cyanovaleric acid) (ACVA), the initiator, was ordered from Sigma-Aldrich (Oakville, Canada). Opti-MEM (OMEM), DMEM media, 0.225% trypsin-EDTA, fetal bovine serum (FBS) and Streptomycin (5000µg/ml), Penicillin (5000 U/ml) were obtained from Gibco. Lipofectamine 3000 was purchased from Invitrogen. Control siRNA, human EGFR-specific small interfering RNA (EGFR siRNA), and primary antibody (rabbit polyclonal EGFR specific IgG) and FITC-conjugated control siRNA were ordered from Santa Cruz Biotechnology. (HRP)-conjugated secondary antibody (Anti-rabbit IgG) was purchased from Promega Corporation.

4.2.2 Synthesis of acid degradable DEGMA-based hyperbranched cationic polymers.

Acid degradable DEGMA-based cationic hyperbranched polymers were prepared by adjusted the previously reported procedure.[30] In a typical procedure, AEMA was dissolved into double distilled water and mixed with 2-propanol solution that dissolved DEGMA and DEP (acid degradable cross-linker), CTP (the chain transfer agent), and ACVA (the initiator). After degassing with nitrogen for 30 min, the polymerization was carried out at 70 ^{o}C for 24 h. The reaction was stopped in liquid nitrogen and the resulting polymer was purified by dialysis with (MWCO 6500-8000 Da). DMF-based Gel permeation chromatography (GPC) and Nuclear magnetic resonance (NMR) was used to determine the molecular weight and composition of the polymer.

4.2.3 Synthesis of non-acid degradable DEGMA-based hyperbranched cationic polymers.

Non-acid degradable DEGMA-based cationic hyperbranched polymers were prepared by following the same method. In a typical procedure, AEMA was dissolved into double distilled water and mixed with a 2-propanol solution that dissolved DEGMA and MBAm, CTP, and ACVA, and the rest procedure was same as the acid degradable polymers.

4.2.4Preparation of polyplexes.

EGFR-siRNA/Control-siRNA (25µg) was diluted in 250 µL of OMEM and complexed with acid degradable and cationic hyperbranched polymers (in OMEM media) at a polymer weight/ siRNA weight (w/w) ratio of 30. The mixture was incubated at 4 ^{o}C for 30 min followed by 37 ^{o}C for 30 min. Fluorescent labeled polyplexes were prepared in the same method except for the incubation with fluorescein isothiocyanate (FITC) control-siRNA.

4.2.5 Characterization of polymers and polyplexes.

The lower critical solution temperature of HBP 2 was characterized based on the turbidity of solution (10mg/ml in PBS) and measured using Jasco ETC-505T temperature controller. The

hydrodynamic diameter and charge of polyplexes, and charge of polymers were obtained by using ZetaPlus-Zeta Potential Analyzer (Brookhaven Instruments Corporation). The polyplexes were formulated at w/w ratio of 90 and 30 for HBP1 and HBP2, respectively, in water and OMEM media. The stability of the polyplexes was evaluated in the presence of serum proteins in OMEM up to 48h. To evaluate the acid degradability of the polyplexes under acidic condition, the polyplexes solution was prepared as mentioned above and pH was adjusted to 5.5 and 4.5, respectively. The hydrodynamic diameter of the polyplexes were measured under various incubation time.

4.2.6 Agarose Gel Electrophoresis.

The polyplexes were formulated in OMEM at various polymer/siRNA ratios, as the abovementioned method. The polyplexes were loaded into 1% agarose gel that containing 1 µg/ml ethidium bromide in 1X Tris Acetate/EDTA (TAE) buffer. The gel was run for 30 min at 130 V and visualized by UV light and DNA bands were visualized by using UV transilluminator.

4.2.7 Cell Culture.

HeLa cells (cervical cancer) and MRC-5 (fibroblast) were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and 1% antibiotic (50 units of penicillin, 50 µg streptomycin) in a humidified atmosphere at 37^{o} C with 5% CO2. The cells were subcultured by detaching with 0.25% trypsin-EDTA at about 80% confluency and were cultured twice a week.

4.2.8 Cytotoxicity of the polymers.

Proliferation (MTT) assay was conducted to obtain the toxicity of polymer in HeLa cells by staining with dimethyl thiazol dyes for metabolically viable cells. IC_{50} was calculated by using GraphPad Prism software. Post-transfection cytotoxicity was determined by conducting

MTT assay which was performed 48 h after the transfection study.

4.2.9 Transfection of EGFR-siRNA.

100,000 HeLa cells were seeded into 60mm plates. The polyplexes were prepared in OMEM (in the presence of serum proteins), and 500µL of the polyplexes/lipofectamine mixture (EGFR siRNA/control siRNA) was added per plate. After 6 h of incubation, the media was removed and replaced with 2ml of DMEM media containing with 10% FBS. The EFGR knockdown efficiency was evaluated after 48 h of cell growth.

4.2.10 Western Blot. Harvesting of cells was done in RiPa buffer supplemented with a protease inhibitor and the protein concentrations were determined using a Bradford protein assay kit (Bio-Rad). Elutates were then run on an SDS-PAGE denaturing gel. Transferred to a nitrocellulose membrane (0.45 µm), and visualized by probing with the EGFR-1005 sc-03 antibody (Santa Cruz) and a streptavidin horse radish peroxidase (HRP) anti-rabbit conjugate. The amount of EGFR protein expression was quantified using ImageJ image analysis.

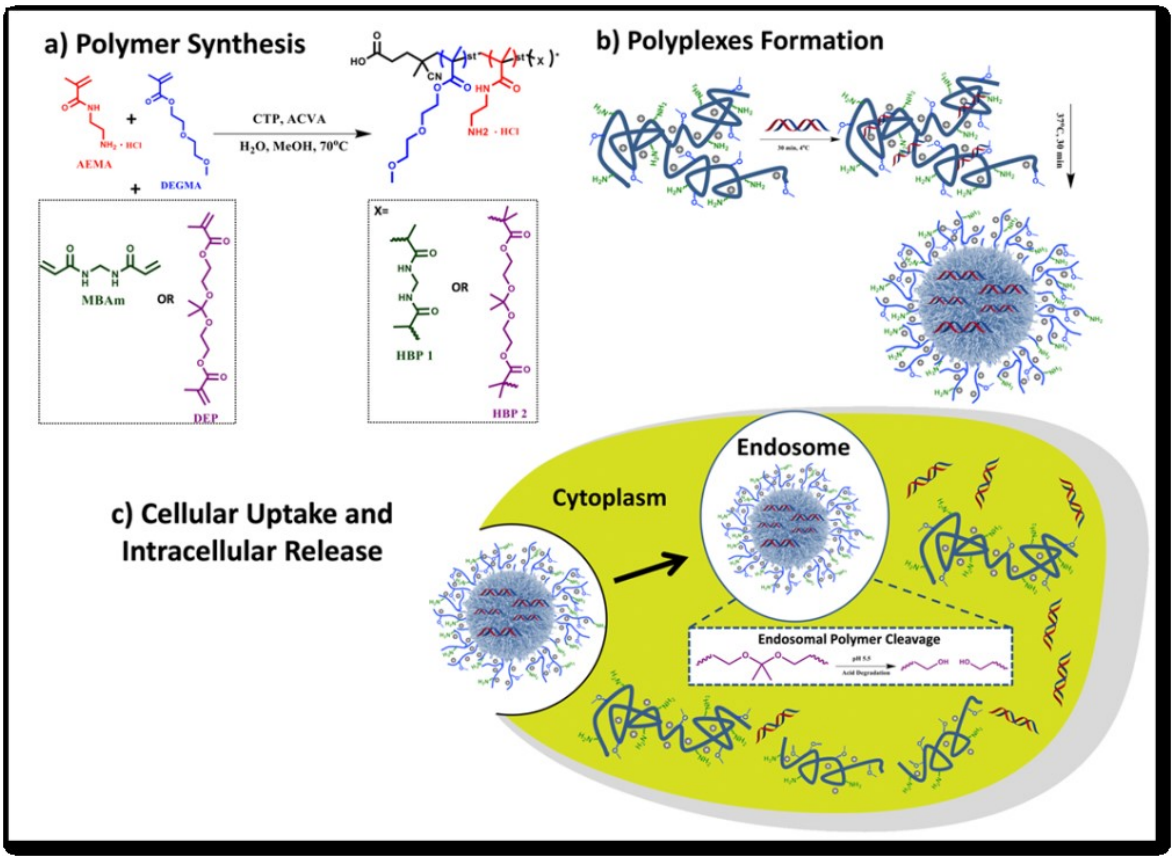
4.2.11 Cellular uptake of polyplexes

Confocal Fluorescence.

HeLa cells were cultured as mentioned above, trypsinized, and seeded onto glass coverslips in 10mm plate at a density of 1000 cells per plate. Next day, the media was removed and replaced with FITC-control siRNA polyplexes at a w/w ratio of 30 in OMEM and subsequently incubated for 6 h in a humidified atmosphere at 37 ^{o}C and 5% CO_2. Media was then removed and washed with 1xPBS three times and then the cells were stained with (1:10000) DAPI dye that dissolved in PBS for 1 h and fixed with 4% Paraformaldehyde for 15 min at 37 ^{o}C. Visualization of FITC was performed by using a confocal microscope at 490nm.

Flow Cytometry.

1,000,000 HeLa cells were trypsinized and subsequently seeded into 60 mm. After incubation one night, the cells were treated with FITC –siRNA polyplexes for 6 h at a w/w ratio of 30 in OMEM. After the media was removed and washed with 1x PBS three times, the cells were trypsinized and centrifuged at 1200 rpm. The pellet was re-suspended in 1xPBS and cells were characterized by using a BD FACS dual laser flow cytometer (Cross Cancer Institute).



Scheme 4-1. Illustration of three steps involved in knockdown of EGFR by acid degradable cationic, hyperbranched DEGMA based polymers. (a) Synthesis of [P(DEGMA-st-AEMA-st-DMP) and P(DEGMA-st-AEMA-st-MBAM)] via RAFT polymerization; (b) polyplexes assembly via electrostatic interactions between the negatively charged siRNA and the cationic DEGMA-based polymers; (c) cellular uptake and endosomal endosomal acidic cleavage of the polymer−siRNAs complexes.

4.3 Results & Discussions

DEGMA-based cationic hyperbranched polymers were prepared from the RAFT copolymerization of 2-aminoethyl methacrylate (AEMA), DEGMA, and cross-linker (see **Scheme 4-1a**). To enhance the intracellular release of the siRNA cargo in the acidic environment, DEP was used as a cross-linker to synthesize a cleavable polymer (HBP 2) and to compare the efficacy of the siRNA release intracellularly with a non-acid cleavable polymer that was also prepared using the nondegradable cross-linker MBAm (HBP 1). The DEGMA residues in the polymer are expected to reduce or eliminate the cytotoxicity by lowering the cationic charge distribution in the resulting polyplexes. Because of the temperature responsiveness of DEGMA, by incubating siRNA with the polymer at a temperature below the lower critical solution temperature (LCST), complexation of siRNA with the cationic moieties can be achieved easily, and subsequent increase in the temperature above the LCST, more stable and compact polyplexes nanoparticles can be achieved (see **Scheme 4-1b**). Both polymers were designed with the same DEGMA/AEMA ratio (D/A) at 4:1 and degree of polymerization (DP) of 100 for comparison purpose and to establish the advantage of incorporating acid degradability. **Table 4-1** summarizes the characterization of the synthesized polymers. ^{1}H NMR spectroscopy was used to determine the polymer structure (see **Figure 4S1**) and gel permeation chromatography data. Temperature responsiveness of HBP 2 was confirmed by UV (see **Figure 4S2**). Zeta potential of both polymers was similar as expected as they have similar cationic charge distribution due to the same D/A ratios.

According to the metabolic activity assay, MTT (see **Figure 4-1**), HBP 2 revealed to be less toxicity as compared to HBP 1. The higher cell viability of HBP2 may partially be attributed to the acid degradability as previously reported.[13] To examine the potency of the system for

Table 4-1. Molecular weight distribution, polymer compositions and zeta potential of the acid degradable DEGMA-based polymers and the half maximal inhibitory concentration (IC_{50}).

Polymer	Acid Degradability	MW (kDa)	PDI	Polymer Composition	Zeta Potential (mV)	IC_{50} (mg/ml)
HBP 1	No	30.0	2.66	P(DEGMA$_{127}$-st-AEMA$_{36}$-st-MBAm)	19.02±3.47	0.832
HBP 2	Yes	16.3	1.23	P(DEGMA$_{69}$-st-AEMA$_{19}$-st-DEP)	18.02±0.00	>1

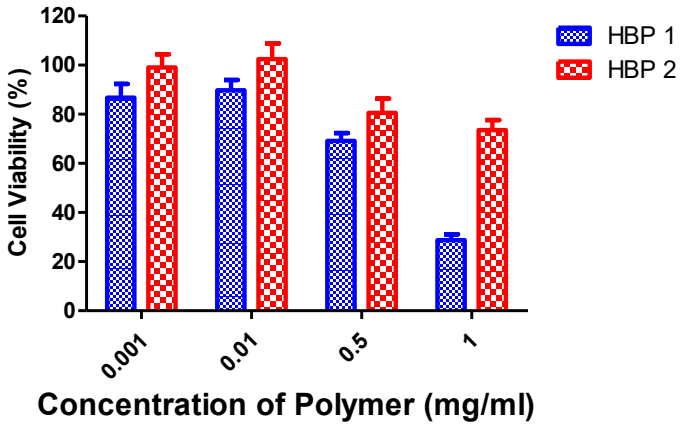


Figure 4-1. Cytotoxicity of polymers in HeLa cells as determined by MTT assay.

siRNA condensation, an agarose gel electrophoresis was conducted. HBP 1 and HBP 2 were found to efficiently complex with siRNA at the polymer/siRNA (w/w) ratios of 75 and 30, respectively (see **Figure 4S3**). To investigate the potential of these polymers for knockdown of EGFR, a Western Blot assay was done, and the EGFR knockdown efficiencies were evaluated. Higher gene silencing was observed with HBP 2 (see **Figure 4-2 a–c**) as compared to HBP 1 and Lipofectamine 3000 (used as a positive control). In siRNA, transfection (even the control) can lead to systemic alterations starting with a protein-level

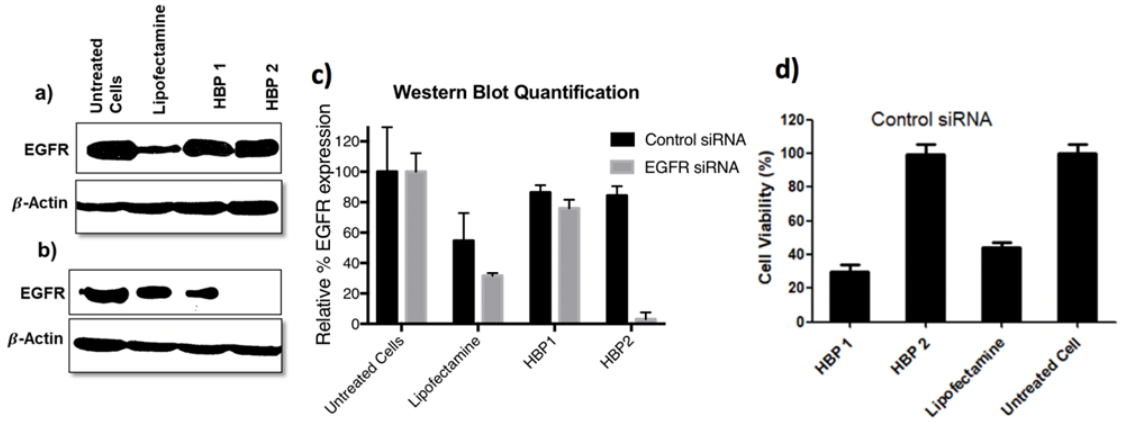


Figure 4-2. Regulation of EGFR expresion in HeLa cells by knockdown analysis using Immunoblot. Lipofectamine 5 wt/wt % was used as a positive control and HBP 1, and two polyplexes at w/w 90 and 30, respectively, in the presence of serum. Transfection was done for 48 h with (a) negative control with unspecific control siRNA and (b) EGFR specific knockdown with EGFR siRNA. (c) Quantification of the protein expression was done with ImageJ normalized againts the housekeeping gene β-actin; (d) cell viability 48 h post-transfection transfection was conducted by MTT assay.

perturbation and end with cell morphology changes. Ideally, the use of a commercial, "nontargeting" control siRNAs as we used in this study prevents these changes to occur. In the case of HBP 1 and HBP 2, the quantifications of EGFR done after the transfection with the control siRNA are very similar to the normalized untreated control (86% and 84%, respectively); for lipofectamine, this value is lower (close to 55% compared to the normalized untreated cells) causing nonspecific knockdown of EGFR in HeLa cells. We hypothesized that this nonspecific knockdown is due to the toxicity of lipofectamine, as has been evidenced in previous studies.[11,15] Notably, the presence of serum proteins does not trigger nonselective knockdown of EGFR when using control siRNA, which was observed in other studies.[12,20] Thus, the degradable HBP 2 polymer holds great potential for *in vivo* studies as its

effectiveness and selective silencing of EGFR was not hampered by the presence of serum proteins. As hypothesized previously, higher cell viability should be observed with HBP 2 polyplexes in comparison with the other polymers, and the results of the MTT assay evidenced this (**Figure 4-2 d**). To further support the potency of HBP 2, MRC-5 (fibroblast), a noncancerous cell line, was used to evaluate the cytotoxicity of HBP 2 and no significant cytotoxicity was observed (see **Figure 4S4**).

To further understand the factors that lead to these differences in EGFR knockdown efficiencies and cell viability between HBP 1 and 2, the polyplexes hydrodynamic size and charge were determined (see **Figure 4-3 a**). Both polymers were capable of forming nanosized particles after complexation with EGFR siRNA in DMEM medium in the presence of fetal bovine serum (FBS). The hydrodynamic diameters of HBP 1 and HBP 2 polyplexes were 90.3 ± 2.7 and 35.9 ± 0.3 nm, respectively. We hypothesized that the larger size of HBP 1 polyplexes could possibly be attributed to its larger polymeric molecular weight and higher w/w ratio requirement for complexation as the necessary amount of polymer to form a polyplex with HBP 1 is higher (33 polymers per siRNA) in comparison to HBP 2 polyplexes (25 polymers per siRNA). However, the factor that led to a better EGFR knockdown efficiency was not attained by comparing the characteristics between the two polyplexes.

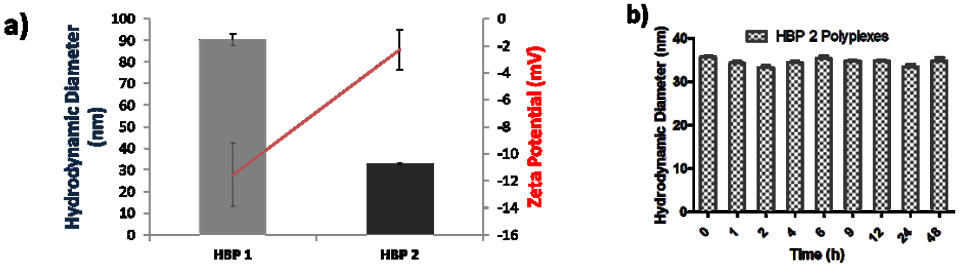


Figure 4-3. DEGMA-based polyplexes. (a) Hydrodynamic diameter (DLS) and zeta potential and (b) stability of the polyplexes after 48 h evidenced by hydrodynamic size variation in DMEM media in the presence of serum.

To better understand the difference in EGFR knockdown efficiency between HBP 1 and HBP 2 polyplexes, quantification of FITC-labeled siRNA polyplexes was achieved by flow cytometry. The results illustrated that both polymers enhanced cellular internalization as the fluorescence strength of FITC-control siRNA polyplexes were significantly higher than the untreated cells. However, the fluorescence values of FITC-control siRNA polyplexes (**Figure 4S5**) between HBP 1 and HBP 2 polyplexes were not significantly different. Thus, the controlled release of siRNA into the cytosol of HeLa cells triggered by acid degradation of the hyperbranched polymers in the endosomes is the most plausible explanation for the higher EGFR knockdown with the HBP 2 polymer.

As HBP 2 polyplexes showed great potential for siRNA delivery and EGFR knockdown in the *in vitro* evaluations, their long-term stability under physiological conditions warrants their potential for *in vivo* studies. Therefore, a stability test of HBP 2 polyplexes in the DMEM media in the presence of serum was conducted to simulate the physiological environment (**Figure 4-3 b**). The study indicated that HBP 2 polyplexes were stable in the serum up to 2 days, which was consistent with previously reported results from the literature that PEGbased polymer possessed "stealth property" preventing aggregating over time.[18] The acid degradability of HBP 2 was confirmed by examining the change in hydrodynamic diameter of the polyplexes under acidic environment pH 4.5 and 5.5 (see **Figure 4S6**).

To further support the internalization enhancement of HBP 2, fluorescently labeled fluorescein isothiocyanate (FITC)-control siRNA was imaged inside HeLa cells using confocal microscopy. The localization of HBP 2 polyplexes in the cytoplasm was confirmed (**Figure 4-4**) and the results of this study provided strong evidence for the cellular uptake enhancement of our cationic acid cleavable DEGMA-based hyperbranched polymers.

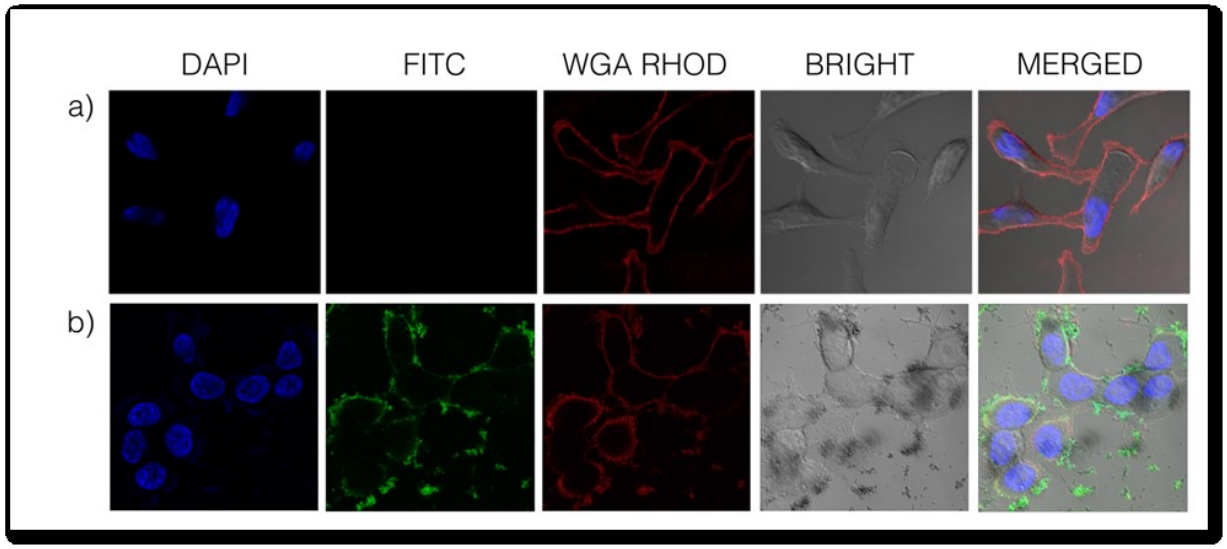


Figure 4-4. Confocal fluorescence microscopy images to confirm cellular internalization of the nanocomplex HBP 2 with fluorescently labeled control siRNA at w/w of 30 in HeLa (cervical cancer cells) after incubation for 6 h (B); untreated cells (A). Wheat germ agglutinin and tetramethylrhodamine (WGA-Rhodamine) dye were used to stain the plasma membrane, and the nuclear staining was done with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI).

4.4 Conclusions

In summary, we demonstrated a simple and effective strategy for the preparation of a nontoxic and efficient siRNA delivery vector. Cationic acid cleavable DEGMA-based hyperbranched polymers condensed and formed nanoparticles that were stable under physiological environment for up to 2 days. The resulting polyplexes have great capabilities in silencing EGFR in HeLa cells without triggering significant cellular death. In addition, incorporation of the acid-cleavable linkages reduced the cytotoxicity of the polymer system as compared to the nondegradable one. Great potential of this new polymer system warrants further *in vivo* evaluations.

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CHAPTER 5. TUMOR MICROENVIRONMENT-REGULATED REDOX-RESPONSIVE CATIONIC GALACTOSE-BASED HYPERBRANCHED POLYMERS FOR siRNA DELIVERY

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5.1 Introduction

Tumor microenvironment-responsive nanoparticles have gained considerable interest for the delivery of anticancer drugs. They primarily target the tumor microenvironment and/or intracellular components to enhance intratumoral accumulation and promote drug release at the target site.[1] Cancer development is associated with an aberrant disturbance of the microenvironment homeostasis, and several elements are known to contribute to its etiology and progression. Glutathione (GSH) is involved in numerous cellular processes, including cell differentiation, proliferation, and apoptosis; and protects cells against exogenous and endogenous toxins, including reactive oxygen (ROS) and nitrogen (RNS) species. Disturbances in GSH balance are observed in various types of tumors, increasing the antioxidant capacity and the resistance to oxidative stress making the neoplastic tissues more resistant to chemotherapy.[2] According to Gamcsik et al., GSH tends to be elevated in breast, ovarian, head and neck, and lung cancer and lower in brain and liver tumors compared to disease-free tissue. Cervical, colorectal, gastric, and esophageal cancers show both higher and lower levels of tumor GSH depending on the response to therapy of the tumors. With a few noteworthy exceptions, tumor GSH tends to be higher than in disease-free tissue.[3]

GSH is a tripeptide that is formed by glutamic acid, cysteine, and glycine. Two forms of GSH are found in the cells: the reduced form (GSH) which is the predominant form and exists in millimolar concentrations (0.5−10 mM) in comparison to the low concentration in the extracellular environment (~4.5 μM), and the oxidized form (GSSG) that is estimated to be less than 1% of the total GSH. The peptide bond linking glutamate and cysteine of GSH is through the γ-carboxyl group of glutamate which is subject to hydrolysis by γ-glutamyltranspeptidase (GGT) enzyme that is present on the external surfaces of certain cell types. As a consequence, GSH is resistant to intracellular degradation, and is only

metabolized extracellularly by organs with GGT.[4] Extracellular compartments are maintained at stable oxidizing potentials, and controlled by the changes in cytoplasmic GSH/GSSG redox potential which are associated with functional state, proliferation, differentiation, and apoptosis.[5] Thus, cellular redox biology is governed by GSH concentration modulation.[6] Redox-modulating strategies to target cancer cells are a promising therapeutic approach that may enable therapeutic selectivity and overcome drug resistance.[7]

Gene therapy has been proposed as an innovative strategy for cancer treatment by delivering genetic materials, either RNA or DNA, into specific human tissues or cells to replace faulty genes, silence malfunctioning gene expression, or introduce new genes to restore cellular functions. Tumor heterogeneity of cancerous cells is the main obstacle for effective cancer treatment, therefore targeting genomic alterations which drive tumor formation, such as tumor suppressor genes, and oncogenes, such as EGFR, is a promising strategy to target this disease.[8] Despite significant development in the field of gene therapy, further optimization is still required to achieve effective therapeutic results. Vector designing is one of the key factors for future successful outcomes. Suitable vectors are required for optimized gene delivery and expression, but current vectors have some limitations, such as associated cytotoxicity, off-target effects, and poor biocompatibility.[9]

Cationic glycopolymers have a superior capability to be used as gene carriers since the protection of siRNA in the physiological environment can be achieved by forming stable polyplexes via electrostatic interactions, and the cytotoxicity of the polymer system can be reduced by sugar residues.[10,11] The serum stability of these polyplexes has also been enhanced[12,13] as the glyco-unit has a stealth property, which extends the lifetime of polyplexes by preventing aggregation. Furthermore, the target gene silencing efficiency can be increased by designing branched polymer structures as the latter is known to have better

transfection efficiencies in comparison to their linear analogs.[14] Another advantage of having a hyperbranched structure is towards the facile preparation of "smart" and responsive siRNA delivery systems. Biocompatible and efficient *in vitro* siRNA carriers were easily prepared by utilizing an acid cleavable cross-linker.[12,15] Redox-responsive carriers had been prepared for gene therapy;[16] thus, in this study, we optimized, modulated, and prepared a redox-responsive galactose-based system and investigated its potential in siRNA delivery.

Galactose-based hyperbranched polymers composed of 2-lactobionamidoethylmethacrylamide (LAEMA), 2-aminoethyl methacrylate (AEMA), and redox-responsive cross-linker *N,N′*-bis(methacryloyl)cystamine (BMAC) with redox-responsive disulfide bonds were designed and synthesized for the rapid release of payloads in the cytosol facilitated by the reduction of the disulfide bond. The formation of polyplexes with siRNA allows the targeted release of these nucleotides intracellularly in cancer cells. Protein tyrosine kinase receptors like EGFR governed diverse signaling networks involved in critical cellular processes, such as cell proliferation, cell differentiation, cell survival, cell metabolism, cell migration, and cell cycle that contribute to tumor carcinogenesis.[17] The most common alteration is the overexpression and constitutive activation of EGFR, which induces activation of various signaling pathways, including Ras-MAPK, PI3K-AKT, Src, and STAT3. These alterations are important for EGF-mediated cell proliferation, differentiation, cell motility, and cell survival.[18]

From our previous study, 60% EGFR silencing in HeLa cells was achieved safely *in vitro* by using 10 kDa acid degradable cationic galactose-based hyperbranched polymer.[12] Based on those promising results, we decided to study and improve further our hyperbranched glycopolymer for siRNA delivery. By using a quick and sensitive redox-responsive strategy, we hypothesized that the EGFR silencing can be further enhanced without compromising the

cytotoxicity of the polymer. In this study, we report on the design and development of an efficient, non-toxic, and redox-responsive nanocarrier for EGFR siRNA delivery in cervical carcinoma via a simple and convenient one-pot method.

5.2 Materials & Methods

5.2.1 Materials.

Dimethylformamide was purchased from Caledon Laboratories Ltd. (Georgetown, Canada). The chain transfer agent, cyanopentanoic acid dithiobenzoate (CTP),[19] LAEMA,[20] and AEMA[21] were prepared in-house via the previously reported methods. Cystamine dihydrochloride[22] and BMAC[23] were synthesized via the methods reported in the literature. Glutathione (GSH), 2-aminoethanethiol hydrochloride, and the initiator, 4,4′-azobis (cyanovaleric acid), were ordered from Sigma-Aldrich (Oakville, Canada). Opti-MEM (OMEM), DMEM media, 0.25% trypsin-EDTA, fetal bovine serum (FBS), and Streptomycin (5000 μg/mL), Penicillin (5000 U/mL) were purchased from Gibco. Lipofectamine 3000 was obtained from Invitrogen. EGFR-specific small interfering RNA (EGFR siRNA), Control siRNA, FITC-conjugated control siRNA, and primary antibody (rabbit polyclonal EGFR specific IgG) were obtained from Santa Cruz Biotechnology. (HRP)-conjugated secondary antibody (Anti-rabbit IgG) was purchased from Promega Corporation.

5.2.2 Preparation of redox-responsive hyperbranched and cationic glycopolymers.

The redox-responsive hyperbranched and cationic glycopolymers were synthesized by minor adjustments of our previously reported methods.[12,15,24] In brief, 0.5 g LAEMA (1.06 mmol) and 88 mg AEMA (0.53 mmol) were dissolved into 1.5 mL doubly-distilled water (DI H2O) and mixed with 1.65 mL methanol solution which contained pre-dissolved 23 mg BMAC (79.74 μmol), 12 mg CTP (43.01 μmol), and 4 mg ACVA (14.28 μmol). The mixture was degassed for 30 minutes and then polymerization was conducted at 67 °C for 24 h. The

reaction was quenched by using liquid nitrogen and the polymers were precipitated in acetone and purified by washing three times with methanol. The yield of the reaction was determined to be 59.52%. Molecular weight and chemical composition of the redox-responsive hyperbranched cationic glycopolymers were characterized by gel permeation chromatography (GPC) and nuclear magnetic resonance (NMR) spectroscopy. The composition of the polymers were determined by obtaining LAEMA:AEMA (L/A) ratio from NMR spectra, calculated by comparing the integration value of sugar peak (δ 4.54) and methyl group on carbon backbone of the polymers (δ 0.6−1.3). ZetaPlus−Zeta Potential Analyzer (Brookhaven Instruments Corporation) was used to evaluate the hydrodynamic diameter and zeta potential of polymers and polyplexes at a scattering angle θ = 90° at 37 °C.

*5.2.3 Gel permeation chromatography***.**

Conventional gel permeation chromatography (GPC) system with a TSK-Gel G5000PWxl column from Tosoh Bioscience was used for the average molecular weights (Mn) and polydispersity of the synthesized polymers. The eluent of the system was 0.5 M sodium acetate/0.5 M acetic acid buffer at a flow rate of 0.5 mL/min. A calibration curve was constructed by using monodisperse Pullulan standards (Mw = 5900−404 000 g/mol).

5.2.4 Redox-responsiveness of polymer.

The redox-responsive polymer was incubated in 10 mM GSH solution for 2 h and the resulted polymer was analyzed by GPC.

5.2.5 Agarose gel electrophoresis.

The polyplexes were formulated in OMEM at varying polymer/siRNA based on the relationship between the ratios of weight of polymers to the weight of siRNA (w/w ratio). W/w ratios of 1, 5, and 25 were evaluated. The polyplexes were loaded in 1% agarose gel containing 1 μg/mL ethidium bromide in 1× Tris Acetate/EDTA (TAE) buffer. The gel was

run for 45 min at 130 V and illuminated with UV light and the RNA bands were visualized using UV transilluminator.

5.2.6 Formation of redox-responsive polyplexes.

25 μg EGFR-siRNA/Control-siRNA were diluted with 250 μL of OMEM and incubated with redox-responsive galactose-based cationic hyperbranched polymers at 10 and 40 w/w ratios for HRRP 1, 3, and 4 and HRRP 2, respectively. The incubation time was 30 min to allow sufficient time for complexation.

5.2.7 Characterization of polyplexes.

The hydrodynamic diameter and zeta potential of the formed polyplexes were determined by ZetaPlus−Zeta Potential Analyzer (Brookhaven Instrument Corporation). The w/w ratios used for polyplexes formation is mentioned above. The serum stability of the polyplexes was evaluated by tracking the change in hydrodynamic diameter at various incubation times in the presence of serum proteins in OMEM up to 3 days.

5.2.8 Cell Culture.

HeLa cells (cervical cancer) and MRC-5 cells (lung fibroblasts) were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and 1% antibiotic (50 units of penicillin, 50 μg streptomycin) in a humidified atmosphere at 37 °C and 5% CO_2. At about 80% confluency, the cells were subcultured by detaching with 0.25% trypsin-EDTA and were cultured twice per week.

5.2.9 Cytotoxicity of the redox-responsive galactose-based and cationic hyperbranched polymers.

Metabolic activity assay (MTT) was performed to determine the inherent toxicity of the complexes in HeLa cells and MRC-5 cells (for HRRP 2), by staining with dimethyl thiazol dyes for metabolically viable cells. IC_{50} values were calculated with GraphPad Prism

software. Post-transfection cytotoxicity was carried out by incubating the polyplexes with HeLa cells for 6 h followed by media change and an additional 48 h of incubation and evaluated by MTT assay.

5.2.10 Transfection of EGFR-siRNA.

HeLa cells were seeded into 60 mm plates at a cell density of 10^5 cells per plate. The polyplexes were prepared in OMEM in the presence of serum proteins at a w/w ratio of 10 and 40 for HRRP 1, 3, and 4 and HRRP 2, respectively. 500 μL of the polyplexes/lipofectamine mixture (0.25 μg EGFR siRNA/control siRNA and w/w 1:1 of lipofectamine) was added per plate. After incubation for 6 h, the media was removed and replaced with 2 mL of DMEM media containing 10% fetal bovine serum (FBS). The EGFR knockdown efficacy was characterized after 48 h of cell growth.

5.2.11 EGFR Knockdown Western Blot evaluation.

Harvesting of cells was done in Radioimmunoprecipitation assay buffer (RiPa) supplemented with protease inhibitor and the protein concentrations were determined using a Bradford protein assay kit (Bio-Rad). Eluates were then run on an SDS-PAGE denaturing gel, transferred to a nitrocellulose membrane (0.45 um), and visualized by probing with EGFR-1005 sc-03 antibody (Santa Cruz) and a streptavidin horseradish peroxidase (HRP) anti-rabbit conjugate. The amount of EGFR protein expression was quantified using ImageJ image software analysis.

5.2.12 Fluorescent labeling of redox-responsive galactose hyperbranched polyplexes.

After overnight incubation of HeLa cells, the media was removed and replaced with fluorescently labeled polyplexes prepared with fluorescein isothiocyanate (FITC) control-siRNA (0.25 μg) in OMEM and 10% FBS and subsequently incubated for 6 h in a humidified atmosphere at 37 °C and 5% CO2.

5.2.13 Cellular uptake of polyplexes.

Confocal Fluorescence Microscopy and Flow Cytometry.

HeLa cells were cultured as mentioned above, trypsinized, and seeded onto glass coverslips in 10 mm plate at a density of 1000 cells per plate. After overnight incubation, the media was removed and replaced, FITC-control siRNA polyplexes in OMEM and subsequently incubated for 6 h in a humidified atmosphere at 37 °C and 5% CO2. After removal of the media and washing with 1× PBS (three times), the cells were stained with (1:10 000) Hoechst dye dissolved in PBS for 1 h and fixed with 4% paraformaldehyde for 15 min at 37 °C. The cell membrane was stained with WGA-Rhodamine dye. Visualization using a confocal microscope at 490 nm emission spectra for FITC was done. For flow cytometry, HeLa cells were trypsinized and subsequently seeded into 60 mm well plate at a density of 1×10^4 cells per plate. After incubation overnight, the cells were treated with the redox-responsive FITC-labeled control siRNA complexes for 6 h at in OMEM. Subsequently, the media was removed and washed with 1 × PBS at pH 7.4 (three times); the cells were trypsinized and centrifuged at 1200 rpm. The pellet was resuspended in 1× PBS buffer and cells were characterized using a BD FACS dual laser flow cytometer (Cross Cancer Institute).

5.3 Results & Discussion

5.3.1 Synthesis of redox-responsive galactose-based and cationic hyperbranched polymers (HRRP).

Various redox-responsive galactose-based cationic hyperbranched polymers (HRRP) with different LAEMA:AEMA feed ratios and targeted molecular weights were synthesized (HRRP 1, 2, 3, and 4) via reversible addition−fragmentation chain transfer (RAFT) polymerization. The synthesis was carried out according to **Scheme 5-1a** and the polymers were characterized as shown in **Table 5-1**. RAFT polymerization was used to prepare

polymers with controlled molecular weights (MW) and narrow polydispersities. Polymers with low polydispersity are desired in this application, as high polydispersity could lead to large size distribution of the resulted polyplexes which can potentially impact the cytotoxicity of the polymer system.[25] On the other hand, monodisperse nanoparticles provide more accurate information to correlate polymer characteristics with the gene knockdown efficiency, which can also offer useful considerations in the future design of the gene delivery system. LAEMA was chosen as a carbohydrate unit to lower the cytotoxicity of the cationic polymer system and to enhance the stability of the polyplexes in the physiological environment due to their stealth property, whereby the galactose sugar residues can help in preventing non-specific protein adsorption.[26,27] Moreover, these sugar units allow us to engineer a gene delivery system whereby the hydrophilic sugar units form a hydrophilic shell to stabilize the resulting particles in the physiological environment.[12,28] The use of AEMA as a cationic unit allows the efficient condensation of siRNA via electrostatic interactions. The presence of primary amines in a methacrylate-based polyplex system is expected to result in higher transfection efficiencies in comparison with secondary and tertiary amine motifs, as previously reported.[29] To make our system more effective and less toxic, we designed a redox-responsive gene carrier by using a disulfide-based cross-linker (BMAC). As such the polymer can break down in the cytoplasm, triggered by the high concentration of glutathione (GSH) in cancer cells. By incorporating redox-responsiveness into the galactose-based cationic hyperbranched polymers, the efficacy of EGFR silencing and biocompatibility of the polyplexes is expected to be enhanced, leading to more EGFR siRNA release into the cytoplasm and as well smaller fragments of the polymer, that can be cleared from the body easily by the kidney.[30]

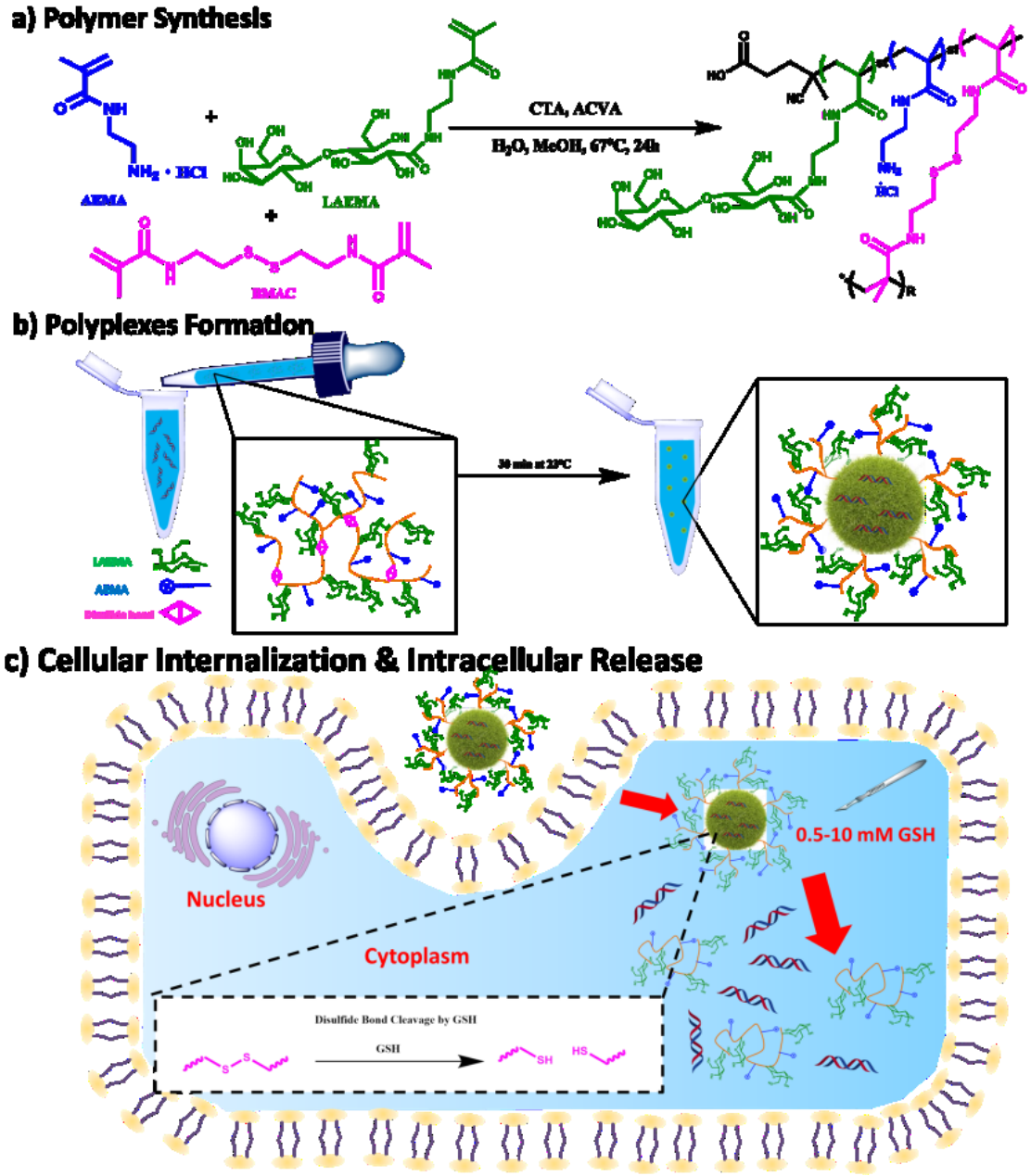
5.3.2 Characterization of the redox-responsive galactose-based and cationic hyperbranched polymers (HRRP).

The polymers were characterized by gel permeation chromatography (GPC) and nuclear magnetic resonance (NMR) spectroscopy (see **Figure 5S2**). Redox-responsiveness of HRRP 2 was confirmed by incubating the polymer in 10 mM GSH environment and analyzed by GPC (see **Figure 5S3**). By confirming the redox-responsiveness, the formed polyplexes are expected to selectively release siRNA into the cytoplasm and lead to high EGFR silencing efficiencies (see **Scheme 5-1c**).

Table 5-1. Molecular weight, polydispersity, zeta potential and polymer composition of prepared redox-responsive galactose-based and cationic hyperbranched polymers.

* HRRP 4 could not be analyzed by GPC as the polymer solution could not pass through 0.45 μm filter. Its composition was estimated by its NMR spectrum and targeted MW which was 22 kDa.

Polymer	GPC M_n (kDa)	M_w/M_n	Polymer Composition	Cross-linker %	Zeta Potential (mV)
HRRP1	8.9	1.14	P(LAEMA$_{13}$-st-AEMA$_{17}$-st-BMAC)	15	21.21± 0.79
HRRP2	9.8	1.27	P(LAEMA$_{16}$-st-AEMA$_{13}$-st-BMAC)	15	18.07± 1.67
HRRP3	18.0	1.86	P(LAEMA$_{27}$-st-AEMA$_{31}$-st-BMAC)	15	42.08± 0.37
HRRP4*	[redacted]	[redacted]	P(LAEMA$_{43}$-st-AEMA$_{26}$-st-BMAC)	15	28.16± 2.78



Scheme 5-1. (a) Preparation of redox-responsive galactose-based and cationic hyperbranched polymers (P(LAEMA-st-AEMA-st-BMAC) via RAFT polymerizatiom; (b) Formation of polyplexes via electrostatic force between redox-responsive glactose-based and cationic hyperbranched poymers and siRNA. (c) Intracellular release of siRNA via degradation of polyplexes due to the breakage of disulfide in the presence of GSH in cytoplasm.

As RAFT polymerization was employed, the MWs of the resulting polymers (HRRP 1, 2, and 3) were close to the targeted MW (10 kDa for HRRP 1 and 2 and 20 kDa for HRRP 3). Zeta potential of the polymers was, as expected, dependent on the LAEMA:AEMA ratio (L/A ratio). The polymers with higher L/A ratios possessed lower zeta potential in comparison to the polymers with lower L/A ratios, as lower L/A polymers have higher cationic charge distribution along the polymers chains. Interestingly, narrow polydispersity was obtained with the polymers that were targeted with 10 kDa (HRRP 1 and 2). 20 kDa targeted polymer (HRRP 3) had a broad PDI and HRRP 4 was not determined by GPC due to aggregation issues of the polymer in solution. Hyperbranched polymers, prepared by the RAFT polymerization, generally have broader molecular weight distributions.[31–33] The synthesized hyperbranched polymers by RAFT with a targeted MW of 10 kDa have narrow Mw/Mn (∼1.2) and similar results were obtained in our previous work.[24] More studies are needed to investigate further this phenomenon in order to prepare low polydispersity hyperbranched polymer from RAFT polymerization, but this work is beyond the focus of this study.

5.3.3 Cytototoxicity associated with the redox-responsive galactose-based cationic hyperbranched polymers.

Polymer architectures, molecular weights, and functional group ratios (carbohydrate to cationic segment) are shown to largely affect the toxicity of polymeric gene carriers. **Figure 5-1a** shows the *in vitro* cell viabilities of HRRP 1,2,3,4 in the HeLa cells. All of the cationic polymers exhibited a low cytotoxicity up to 0.5 mg/mL. Furthermore, the sugar content within the polymers played a predominant role in determining the polymer toxicity. The cytotoxic effect seems to be related to the decrease in the sugar content of the polymer chains, indicating that the galactose decorated cationic HRRP polymers could increase biocompatibility, as has been previously reported.[20] Likewise, the cytotoxicity evaluation of

HRRP2 in a fibroblast normal cell line (MRC-5) (see **Figure 5-1b**) provided the assurance that HRRP 2, being non-toxic, offers great potential to be used in gene therapy treatments. The composition of the carbohydrate segment and the number of carbohydrate residues in the polymer chain contributed to lower the toxicity of these polymers. We hypothesized that this could be due to the favorable interactions between the cell and the polymer surface. For *in vivo* studies, the use of glycopolymers with pendant galactose moieties could perhaps inhibit the activation of macrophages and modulate the innate immunity by the passivation of complement[34] but in vivo studies have not been conducted yet with this nanosystem.

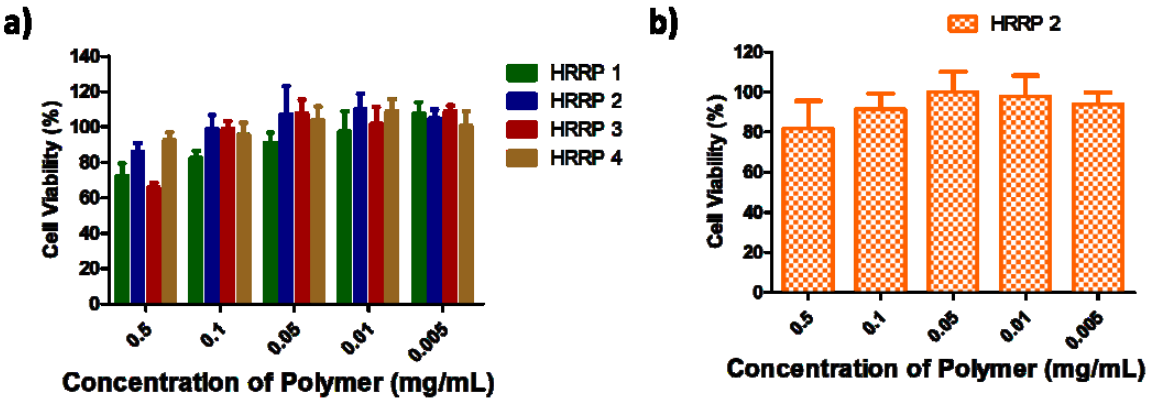


Figure 5-1. Cell viability of the HRRP 1-4 in HeLa cells (a) and HRRP 2 in MRC-5 cells (b) that evaluated by MTT assay.

5.3.4 Characterization of redox-responsive polyplexes by electrophoretic mobility assay.

The successful condensation and protection of the siRNA by the cationic polymers is a primary requirement for the effectiveness of the gene delivery system. Polyplexes were formed by incubating siRNA with HRRP at various w/w ratio (1, 5, and 25) for 30 min at 23 °C. The capabilities of the different functionalized HRRP polymers to condense small interfering RNA (siRNA) were confirmed by agarose gel electrophoresis. The negatively charged siRNA is prevented from migration to the positive cathode due to its complexation in the cationic redox responsive galactose-based polymers. **Figure 5S4** shows the gel

retardation results of the various polymer/siRNA complexes with increasing w/w ratios in comparison to uncomplexed siRNA. The minimum w/w ratio for complete complexation with siRNA for HRRP 1, 2, 3, and 4 were 5, 25, 5 and 5, respectively. The higher w/w ratio required for HRRP 2 may be attributed to its lower molecular weight and higher L/A ratio, as higher molecular weight polymers needed less polymer to completely condense RNA.[35,36]

5.3.5 In vitro gene transfection assay.

The in vitro gene transfection efficiencies of the galactose decorated HRRP/siRNA complexes were assessed using EGFR protein quantification by Western blot in the HeLa cell line. **Figure 5-2c** shows the relative percentage of EGFR expression mediated by the HRRP 1, 2, 3, and 4 vectors at a w/w ratio of 10, 40, 10, and 10, respectively, in the presence of serum in comparison with that of the gold standard, Lipofectamine, at a w/w of 1. HRRP 2 and HRRP 4 exhibited higher knockdown efficiencies than HRRP 1, HRRP 3, and Lipofectamine. RNA condensation ability and gene delivery efficacy of these polymers appear to be dependent on the L/A ratios. Higher L/A ratios resulted in better EGFR silencing efficiencies as previously reported.[12] The gene expression of EGFR for the HRRP 2 and HRRP 4 appears to be lower and almost 30% non-specific silencing was evident with the control siRNA polyplexes. HRRP 2 and 4 polyplexes achieved more specific silencing of EGFR than lipofectamine, but around 20% nonspecific silencing was observed with HRRP 4. The non-specific silencing of HRRP 4 complexes may be attributed to its post-transfection cytotoxicity which was observed in **Figure 5-2d** (around 80% cell viability). The results observed with HRRP 2 polyplexes were quite remarkable, considering the lower toxicity of these HRRP polymers as compared to Lipofectamine. Furthermore, HRRP 2 demonstrated to be a superior siRNA carrier (85% EGFR silencing) in comparison to our previously reported acid degradable cationic hyperbranched galactose-based polymer (60% EGFR silencing) with

similar cell viability.[15]

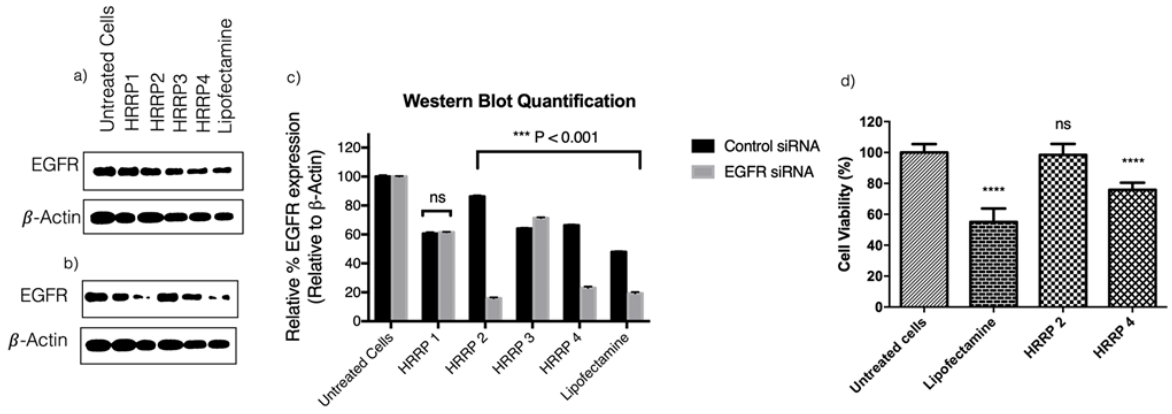


Figure 5-2. *In Vitro* EGFR knockdown in HeLa cell in the presence of serum with HRRP 1, 2, 3, and 4 polyplexes at w/w 10, 40, 10 and 10 respectively. Image of Western Blot Gel for Control siRNA group (a) and EGFR siRNA group (b). HeLa cells were transfected by incubating with either control siRNA or EGFR siRNA polyplexes for 6 h and grow for 48 h. Indicated antibodies was used to cell lysate or immunoblot analysis (c). Post-transfection Cytotoxicity of the polyplexes (using control siRNA) were evaluated by MTT assay after 48h of transfection treatment (d). The statistical analysis was done by multiple t-tests in comparison to the untreated cells; (ns) no significance;(***) $P < 0.001$; (****)$P \leq 0.0001$.

5.3.6 Characterization of the polyplexes.

To further understand the superior efficiency of HRRP 2 and HRRP 4, hydrodynamic diameter and surface charges of the polyplexes were determined (see **Figure 5-3a**). Both HRRP 2 (20.4 ± 0.3 nm) and HRRP 4 (28.0 ± 3.5 nm) formed nanosized polyplexes in the presence of serum protein. These results are attractive as the passive targeting strategy can be employed with 50 nm particles *in vivo* by exploiting the enhanced permeability and retention (EPR) effect.[37,38] Surface charges for HRRP 2 and HRRP 4 polyplexes were 7.42 ± 3.61 mV

and 11.59 ± 2.40 mV, respectively. The post-transfection cytotoxicity, observed with HRRP 4 polyplexes, may be due to the more positive zeta potential, causing membrane disruption and cellular death.[39] On the other hand, opsonization is an important property for a gene carrier as protein adsorption leads to aggregation of polyplexes resulting in reduced circulation time because of clearance by macrophages. [40,41] Excellent stability of up to 24 h at 37 °C was observed with both polyplexes which matches with our previous reports and indicates that aggregation of the polyplexes in the presence of serum was prevented by incorporating the galactose units.[12,13] (see **Figure 5-3b**)

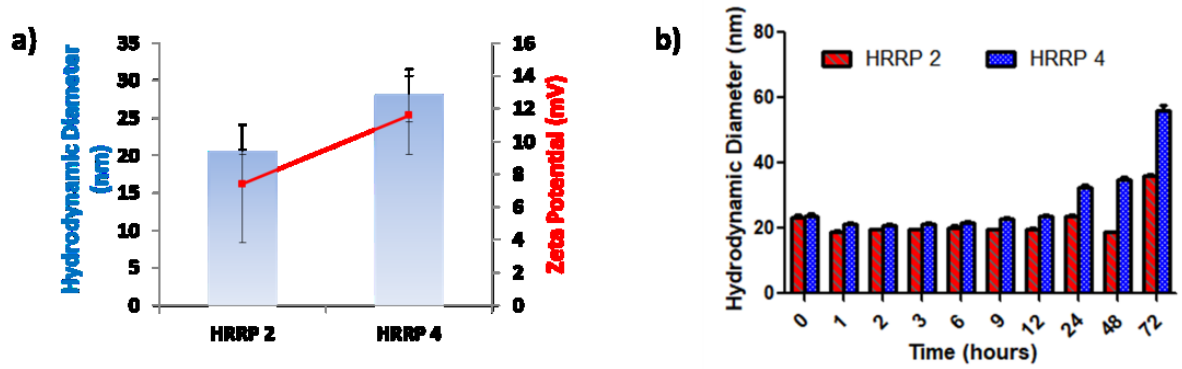


Figure 5-3. Hydrodynamic diameter and zeta potential of polyplexes that formed by HRRP2 and HRRP 4 (a). Serum stability evaluation by tracking the change in hydrodynamic diameter of polyplexes that was formed by HRRP 2 and 4 in the DMEM media in the presence of serum (b).

5.3.7 Cellular uptake of HRRP polyplexes.

One of the main obstacles is the delivery of the nucleotides in the cytoplasm, avoiding challenges such as phagocytic uptake, enzymatic degradation by nucleases, triggering of the immune response, and the hydrophobic plasma membrane barrier. Due to the high metabolic demands stimulated by rapid proliferation, cancer cells overexpressed sugar receptors, potentially enhancing targeted uptake of nanoparticles decorated with sugar moieties such as

glucose and galactose. Likewise, efficient escape from endosomes is one of the most important factors to be considered for the design of gene delivery vehicles. L/A ratio of 1.5 had more effective EGFR silencing capability in comparison to L/A ratio of 1. This is hypothesized to be due to the efficient siRNA release after degradation in the cytoplasm as lower cationic distribution among the polymers make polyplexes less stable and the release of the loaded siRNA can be enhanced, resulting in better transfection efficiencies.[42]

Fluorescently labeled control siRNA have revealed the localization of these complexes within the cytoplasm (**Figure 5-4**) and flow cytometry analysis (**Figure 5S5**) corroborated the successful delivery of the nucleic acids inside the cervical cancer cells. Surprisingly, HRRP 4 polyplexes demonstrate better cellular internalization than HRRP 2 polyplexes which may be attributed to its slightly more positive surface charge. Furthermore, the higher cellular uptake of HRRP 4 polyplexes may be caused by the higher hydrophobicity of the polyplexes.[43]

5.4 Conclusions

In this study, we investigated the potential of redox-responsive galactose-based cationic hyperbranched polymers as siRNA delivery systems. Our results indicated that safe and efficient EGFR silencing in HeLa cells can be achieved by using polyplexes that were 10 kDa and with L/A ratios of 1.5. Furthermore, the resulting nanosized polyplexes have passive targeting strategies (EPR effect), redox-modulating release ability, excellent serum stability, and high cellular internalization as evidenced by the polyplexes sizes in the presence of serum protein and cellular uptake studies evaluated via confocal microscopy and flow cytometry. Taking into account that the EPR effect is dependent on many tumor-specific factors *in vivo*,[44] due to the tumor heterogeneity, with the results obtained and the desired properties of the non-viral vectors, we are convinced to further investigate the potential of this nanosytem for gene therapeutic management at the in vivo level.

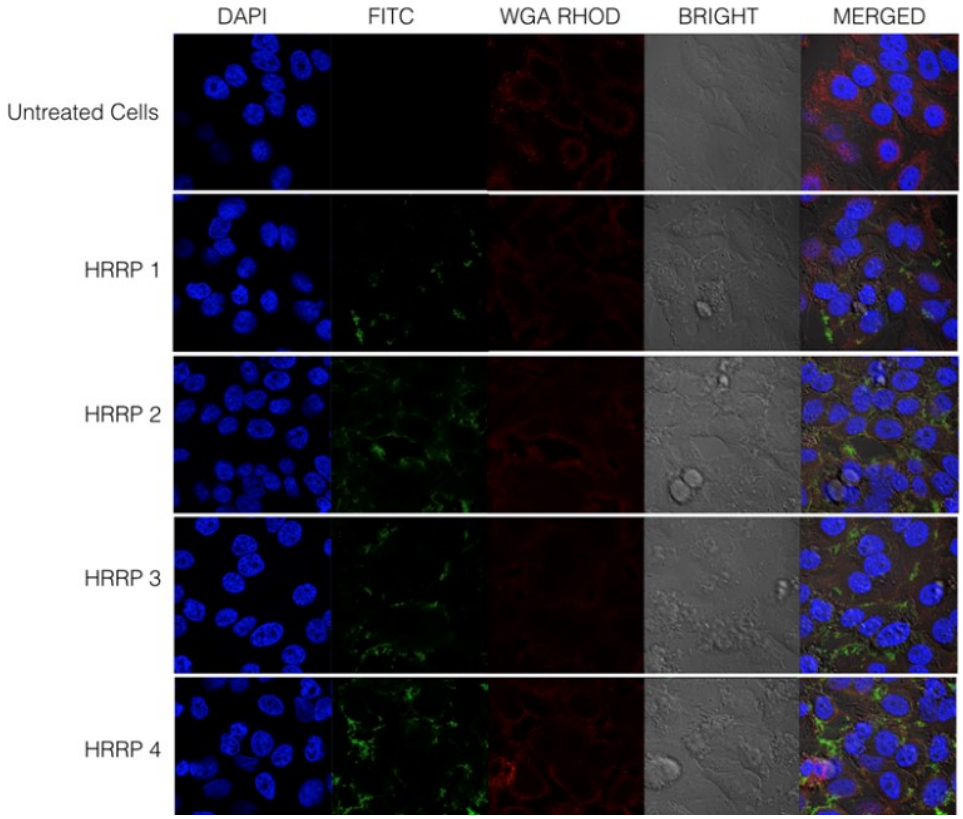


Figure 5-4. Fluorescently-labelled siRNA polyplexes internalization in HeLa cells as evidenced by confocal fluorescence microscopy. Plasma membrane was stained with wheat germ agglutinin and tetramethylrhodamine (WGA-Rhodamine) dye and nucleus was stained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI).

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CHAPTER 6. THESIS CONCLUSIONS AND FUTURE PLANS

6.1 Thesis Conclusions

Nanotechnology provides promising positive results in mediating the siRNA delivery from forming low or non-toxic nano-sized complexes, protecting the siRNAs and prolong their blood circulation time in the physiological environment, guiding the therapeutic agents to the tumor site via EPR effect or active targeting, elevating the cellular uptake, evading the endosomal trap, and releasing the loaded agents from recognizing the cytoplasmic environment.[1-3] Attributing to the invention of RAFT polymerization, the cytotoxicity and physical and chemical properties of siRNA complexes can be tuned easily from the choice of monomer and cross-linker, targeted DP and structure of the polymer and weight ratio between the polymer and siRNA. In addition, by incorporating the carbohydrate units into the cationic polymers, the cytotoxicity of the polymers can be lowered, and serum stability can be significantly enhanced.

In **Chapter 3**, a series of acid degradable, galactose-based, and cationic hyperbranched polymers were prepared via RAFT polymerization by using LAEMA, AEMA, and DEP (ketal based acid degradable cross-linker) at different LAEMA: AEMA (L/A) ratio (2.0, 1.0, and 0.5) and targeted molecular weights (MW) (10 and 20 kDa). The associated cytotoxicity of the cationic glycopolymers decreases as L/A ratio of polymers increases; cationic glycopolymers with L/A ratio at 2.0 has the lowest cytotoxicity compared to other ratios, and polymers with 10 kDa targeted molecular weight are less toxic than the 20 kDa polymers. The zeta potential of the polymers decreases as L/A ratio decreases which is logical as polymers with a lower L/A ratio have higher cationic distribution among the polymers. Because of the low IC_{50} of polymers with an L/A ratio of 0.5, these polymers were not investigated in EGFR knockdown studies. The rest of cationic polymers completely condense siRNA at 50 w/w ratio (minimum ratio is at 25) and the resulted polyplexes were used to

silence EGFR in HeLa cell. HGA 6 (L/A ratio at 2.0 and targeted MW at 10 kDa) was demonstrated to be the superior candidate as the polyplexes prepared from HGA 6 induced 60% *in vitro* EGFR knockdown in the HeLa cell line with around ~100% post-transfection cell viability. The performance of HGA 6 was significantly better than other acid degradable cationic glycopolymers and standard lipid-based carrier, lipofectamine 3000. HGA 6 (10 kDa and L/A ratio at 2.0) triggered more EGFR knockdown than HGA 3 (20 kDa and L/A ratio at 2.0). This phenomenon may be due to the higher intracellular release of siRNA of HGA 6. The MW of degraded fragments of HGA 6 is expected to be lower than HGA 3, resulting in the higher cytoplasmic release of siRNA because of weaker interaction between fragments of degraded HGA 6 and siRNA. The cytotoxicity of HGA 6 with normal cell line (MRC-5) was insignificant, which provided corroboration to investigate the material further. Cellular uptake enhancement of HGA 6 was proved by confocal fluorescence microscopy and flow cytometry. The size and serum stability of HGA 6-made polyplexes were determined to confirm the potency of HGA 6 in animal studies. HGA 6-made polyplexes potentially employ the EPR effect as the size was 50 nm and were stable in DMEM media with the presence of serum for up to 24 hours. These results provided more convincing supports to further investigate HGA 6 in siRNA delivery.

Thermo-responsive and acid degradable cationic DEGMA-based hyperbranched polymer (HBP 2) exhibited to be another potential outstanding siRNA carrier (**Chapter 4**). HBP 2 was prepared via RAFT polymerization by using DEGMA, AEMA, and DEP; DEGMA/AEMA ratio was set at 4.0. Cytotoxicity of HBP 2 was insignificant with HeLa and MRC-5 cell lines as evaluated by metabolic activity assay, MTT. The low cytotoxicity of HBP 2 is potentially originated from the abundant biocompatible PEG units.[4] Because IC_{50} of HBP 2 with HeLa (cancer) and MRC-5 (normal) cell lines were both larger than 1 mg/mL, *in vitro* EGFR

knockdown efficiency of HBP 2 in HeLa cell was subsequently conducted. Cationic moieties of HBP 2 completely condense siRNA at 30 w/w ratio at 4 ^{o}C and formed stable and compact 36 nm polyplexes at 37 ^{o}C (LCST of HBP 2 is at around 36 ^{o}C). The thermo-responsive of HBP 2 was used to trap siRNA for protecting it under a physiological environment. *In vitro* knockdown efficiency of HBP 2-made polyplexes was 95 % with insignificant post-transfection cytotoxicity. We hypothesized that the stable and compact polyplexes were collapsed and released free siRNA after localizing in the cytoplasm because of cleavage of ketal bonds in a mild acidic environment and resulting in high EGFR knockdown efficiency. Because of its excellent achievement on EGFR knockdown, passive targeting capability (36 nm), long serum stability (up to 48 h), cellular uptake enhancement (evidenced by confocal fluorescence microscopy and flow cytometry), HBP 2 possesses potential in siRNA delivery and should have *in vivo* evaluations.

Besides acid degradability, the higher concentration of GSH in the cytoplasm and tumor tissue is targeted to induce the selective intracellular release of siRNA from using redox-responsive polymer. In **chapter 5**, RAFT polymerization was utilized to synthesize four redox-responsive galactose-based and cationic hyperbranched polymers (HRRP 1-4) with different L/A ratios (1.5 and 1.0) and targeting MW (10 and 20 kDa) from using LAEMA, AEMA, and BMAC (disulfide-based redox-responsive cross-linker). As affirmed by MTT assay, IC_{50} of all HRRP was larger than 0.5 mg/mL with HeLa cell line; thus, the *in vitro* EGFR knockdown efficiency in HeLa cell line of all HRRP was examined. Both polymers with a L/A ratio at 1.5 (HRRP 2 [10kDa] and HRRP 4 [20kDa]) led to 85 % and 65 % *in vitro* EGFR knockdown in HeLa and 95 % and 85 % post-transfection cell viability, respectively. Accordingly to the results from confocal fluorescence microscopy and flow cytometry, HRRP 4 had higher cellular uptake than HRRP 2. Therefore, we hypothesized that

the MW of fragments from degraded HRRP needs to be small enough to induce cytoplasmic siRNA release. HRRP 4 had lower EGFR knockdown efficiency and higher post-transfection cytotoxicity than HRRP 2 was attributed to the larger MW of degraded HRRP, which still effectively complexes with siRNA and does not release it and induce more cellular death than the smaller MW fragments from HRRP 2. Incorporation of galactose units into cationic polymers increased the serum stability of resulting polyplexes, matching the results in **chapter 3**. HRRP 2 had excellent *in vitro* serum stability up to 48 h. Because of these promising *in vitro* results, studying this nanosystem at the *in vivo* level is needed before applying this material to treat patients with cervical cancer at the clinical stage.

From these studies, we learned the prime factors that can be used in the future design of RAFT-made non-viral vectors include: utilize acid or redox-triggered polymer degradation, design the MW of the biodegradable and hyperbranched polymer in the range between 10 to 15 kDa, limit the molar ratio between non-toxic moieties and cationic moieties to be more than 1.0. The first criterion is lowering the cytotoxicity and facilitating the cytoplasmic release of siRNA. The second and third factors aim to guarantee the release of siRNA after the degradation of the polymer. If the degraded polymer is still complexed to the siRNA, the efficacy of the treatment will be poor because of the bioavailability of siRNA in the cytoplasm.[3] Besides these considerations, galactose-based polymers also proved to be excellent non-viral vectors for siRNA delivery as these polymers have outstanding serum stability (up to 2 days according to *in vitro* studies) and lowering the associated cytotoxicity of cationic polymers. The associated cytotoxicity of galactose-based cationic polymers relates to the degree decoration of galactose moieties. Elevating the degree decoration of galactose moieties reduces the associate cytotoxicity. Both PEG and galactose moieties demonstrated excellent potential in gene delivery, however galactose moieties possessed an additional

advantage over PEG in terms of their ability to target asialoglycoprotein receptor which enhanced delivery of nucleic acid.

6.2 Future Plans

Our polyplexes have excellent serum stability, potentially employ the EPR effect, are easy to make via the one-pot method and handle. *In vivo* studies will be conducted in the near future to determine whether these candidates can silence EGFR to elevate chemosensitivity of cervical cancer and assist chemotherapy-induced repression of cervical tumors, which would be crucial prior to advancing them in the clinic. Biodistribution studies with the polyplexes will be performed to ascertain the *in vivo* serum stability of the polyplexes and accumulation site of the polyplexes, which would inform whether *in vivo* tumor suppression studies are needed. In addition, as inspired by the success *in vitro studies* of HRRP 2, a company is currently supporting our group to conduct *in vivo studies* on HRRP 2.

Furthermore, because of the abundant galactose groups, utilizing the cationic glycopolymers to deliver nucleic acid to treat liver cancer will be the next focus. Asialoglycoprotein receptor (ASGPR) is primarily expressed on hepatocytes, therefore galactose units can be used as active targeting ligands because of the associated ligand-receptor interaction which is expected to enhance the cellular uptake of the polyplexes and lead to excellent transfection efficiency.[5-6] To determine the potential of these polymers as a new platform for gene delivery, delivery of various kind of therapeutic nucleic acids to other type of cancers will be attempted.

Lastly, benzoxaborole groups form pH-cleavable covalent bonds with galactose groups via boron-carbohydrate interaction at physiogical environment, which offers a promising method to prepare acid degradable polymer for siRNA delivery.[7-8] A cationic polymer with a benzoxaborole-functionalized end group via RAFT polymerization and thiol-ene click

chemistry was recently developed. The polymer was conjugated with a linear galactose-based polymer to create a pH-sensitive siRNA delivery system with 70% EGFR knockdown efficiency in HeLa cells and 80% post-transfection cell viability.[9] As modifying polymer into hyperbranched structure is expected to ameliorate the performance of transfection, our group aim to design a degradable hyperbranched polymer based on this system.[10-11] Several galactose-based and redox-responsive hyperbranched polymer with variety of targeted DP will be prepared from using LAEMA and BMAC via RAFT polymerization. Conjugation between the galactose-based hyperbranched polymer and benzoxanorole-end functionalized cationic polymer will be conducted by mixing both polymer at pH 7.4 environment to prepare dual-responsive (pH and GSH) hyperbranched cationic polymers. *In vitro* studies and physical and chemical characteristic of the resulted polyplexes, will be evaluated to investigate the potential of this strategy in gene delivery.

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APPENDIX

Chapter 3

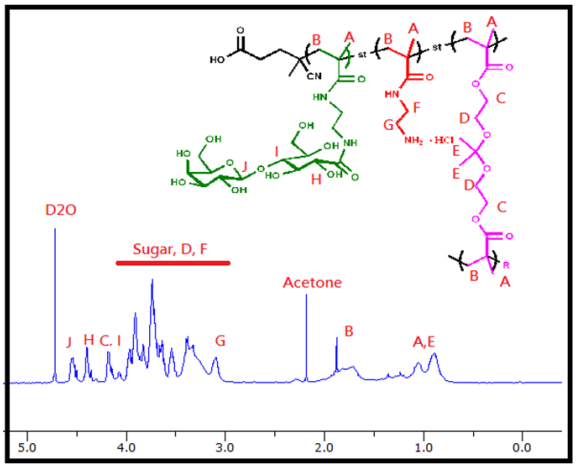


Figure 3S1. ^{1}H NMR spectra of HGA 6 in D_2O.

The NMR spectrum was used to determine the composition of hyperbranched polymer via the following equations:

$$\text{LAEMA containing group} = \text{integral of J}$$

$$\text{LAEMA} + \text{AEMA containg group} = \text{integral of B}/2$$

The LAEMA:AEMA ratio was determined according to the above equations, and the compoisition of the hyperbranched polymers were determined by using the ratio and molecular weight of the polymers.

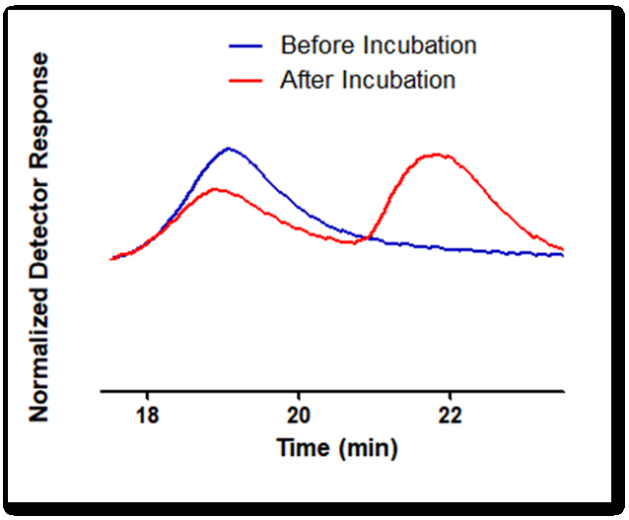


Figure 3S2: GPC spectrum of HGA 6, before and after incubation in pH 5.5 acetate buffer.

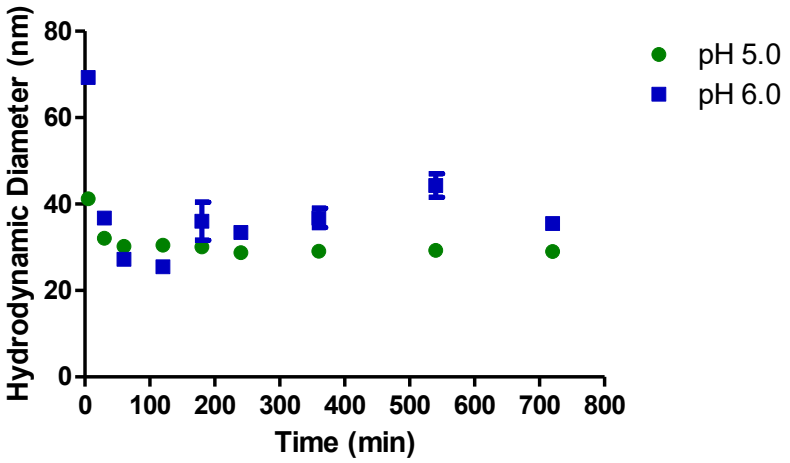


Figure 3S3. Hydrodynamic Diameter and stability of polyplexes (HGA 6) under acidic environment (pH 5.0 & 6.0), as evidenced by DLS analysis in DMEM media with the presence of serum.

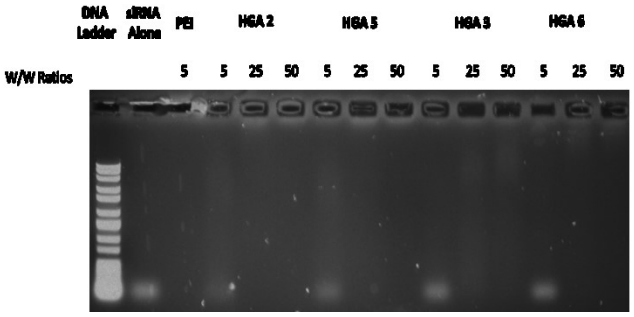


Figure 3S4. Agarose gel electrophoresis showing polyplexes formation at various weight/weight ratios of acid degradable and cationic galactose-based hyperbranched polymers with control siRNA (133ng).

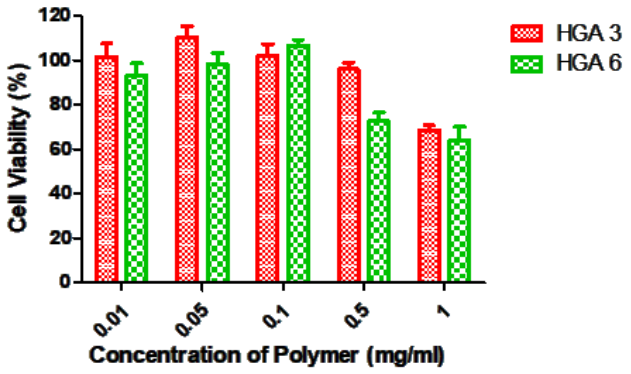


Figure 3S5. Cytotoxicity of polymers in MRC-5 cells as determined by MTT assay.

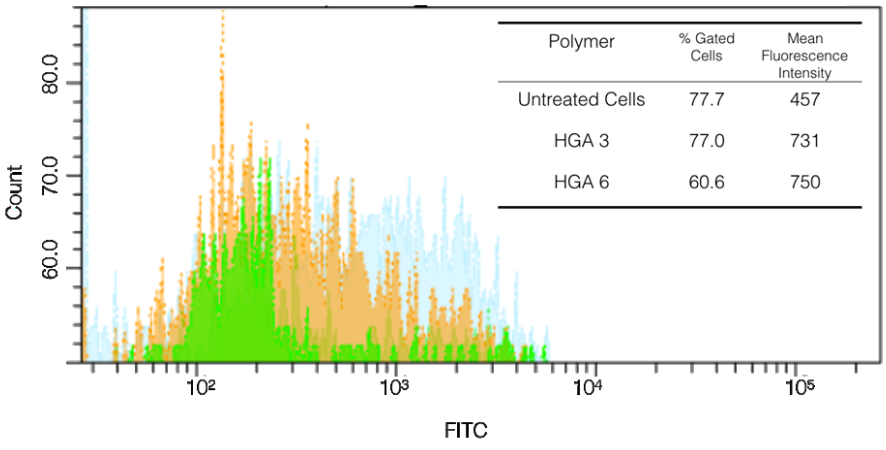


Figure 3S6. Flow cytometry analysis of cellular uptake of FITC-labeled polyplexes as compared to untreated cells (green); HGA 3 (Pale Blue); HGA 6 (Orange).

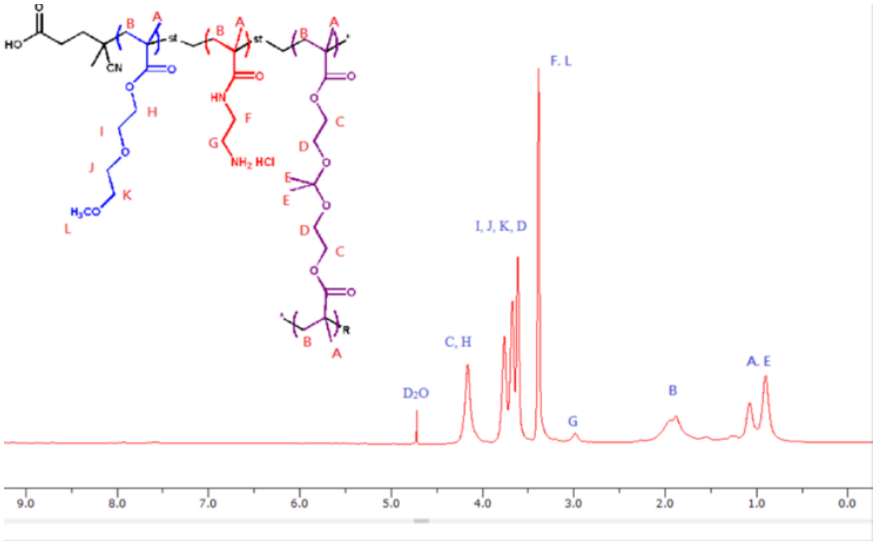


Figure 4S1.[1]H NMR spectra of HBP2 in D_2O.

The NMR spectrum was used to determine the composition of hyperbranched polymer via the following equations:

$$\text{AEMA containing group} = \text{integral of } G/2$$

$$\text{DEGMA} + \text{AEMA containg group} = \text{integral of } B/2$$

The DEGMA:AEMA ratio was determined according to the above equations, and the compoisition of the hyperbranched polymers were determined by using the ratio and molecular weight of the polymers.

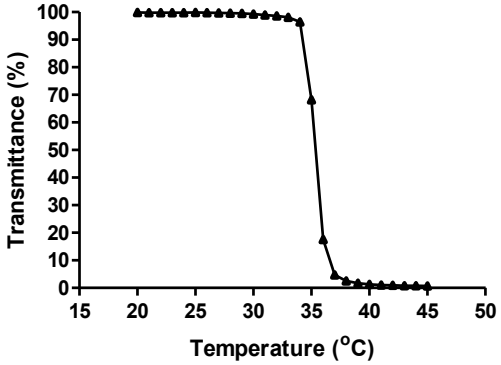


Figure 4S2. Lower Critical Solution Temperature of HBP 2.

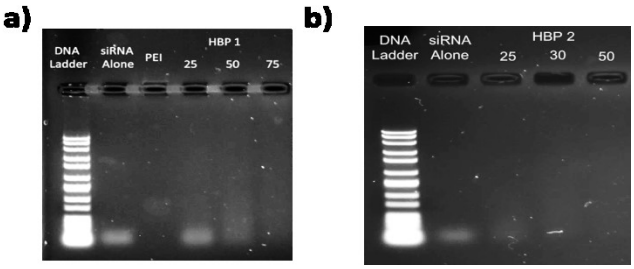


Figure 4S3.Formation of polyplexes at various weight/weight ratio of acid degradable and cationic hyperbranched polymers with control siRNA (133ng) as illustrated by agarose gel electrophoresis: HBP 1(a) & HBP 2 (b).

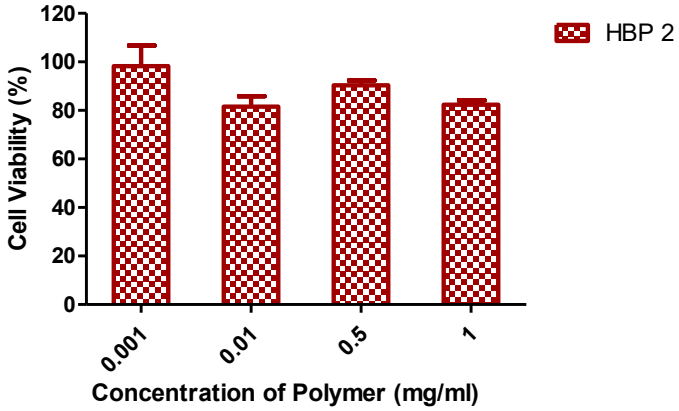


Figure 4S4. Cytotoxicity of polymers in MRC-5 cells as determined by MTT assay.

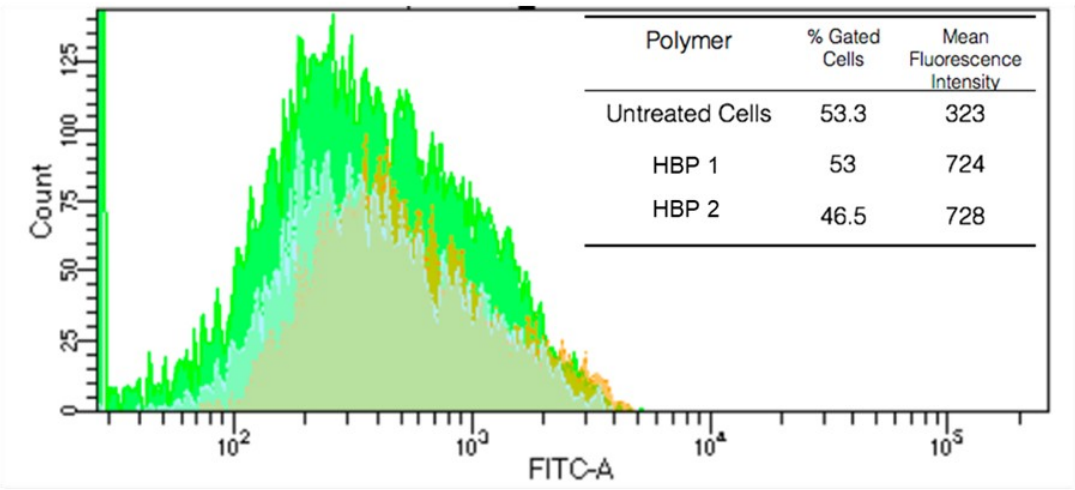


Figure 4S5. Flow cytometry analysis of cellular uptake of FITC-labeled polyplexes in comparison with untreated cells (Pale Blue); HBP 1 (Orange); HBP 2 (Green).

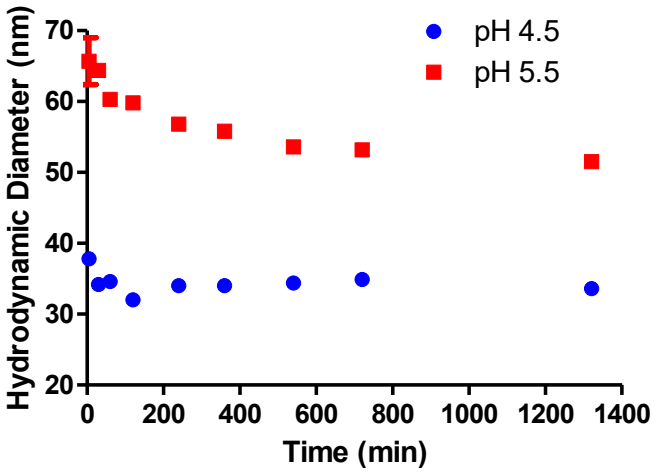


Figure 4S6. Hydrodynamic Diameter and stability of polyplexes (HBP 2) under acidic environment (pH 4.5 & 5.5), as evidenced by DLS analysis in DMEM media with the presence of serum.

Chapter 5

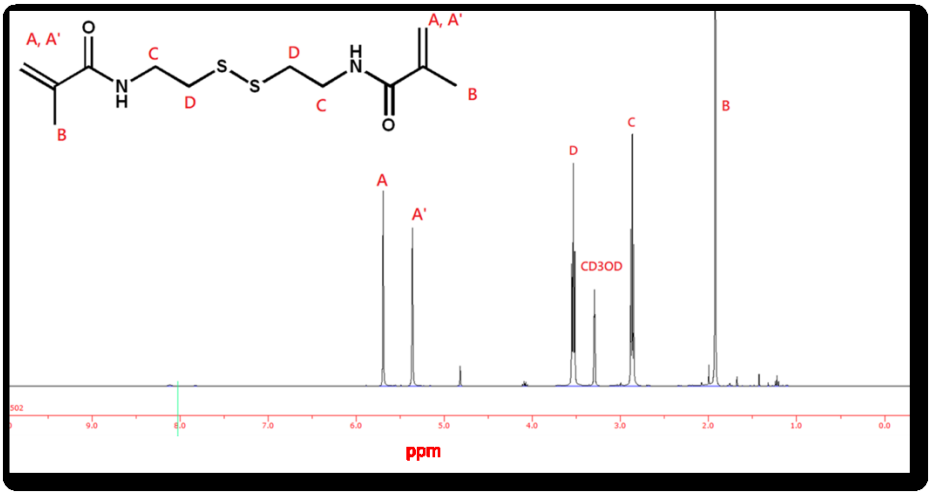


Figure 5S1. ^{1}H NMR spectrum of *N,N*'-bis(methacryloyl)cystamine (BMAC) in CD_3OD.

The NMR spectrum was used to determine the composition of hyperbranched polymer via the following equations:

$$\text{LAEMA containing group} = \text{integral of J}$$

$$\text{LAEMA} + \text{AEMA containg group} = \text{integral of B}/2$$

The LAEMA:AEMA ratio was determined according to the above equations, and the compoisition of the hyperbranched polymers were determined by using the ratio and molecular weight of the polymers.

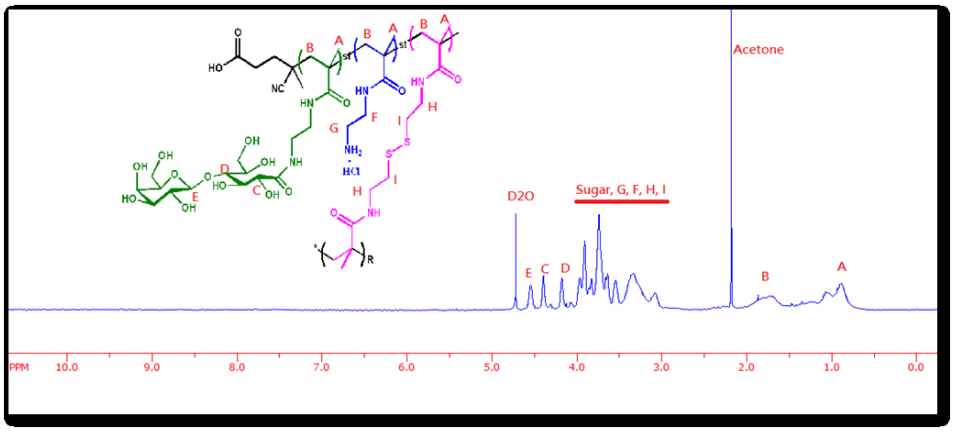


Figure 5S2.^{1}H NMR spectrum of HRRP 2 in D_2O.

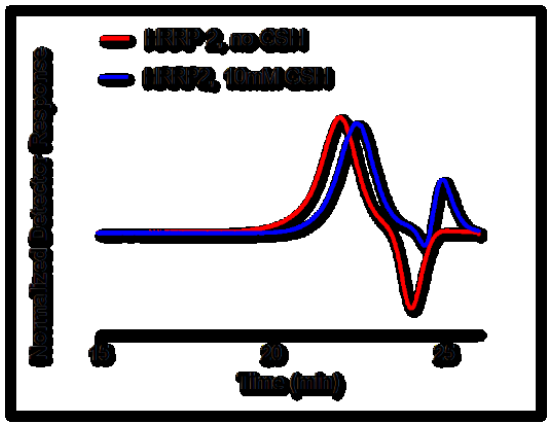


Figure 5S3. GPC spectrum of HRRP 2 before and after incubation with 10mM GSH.

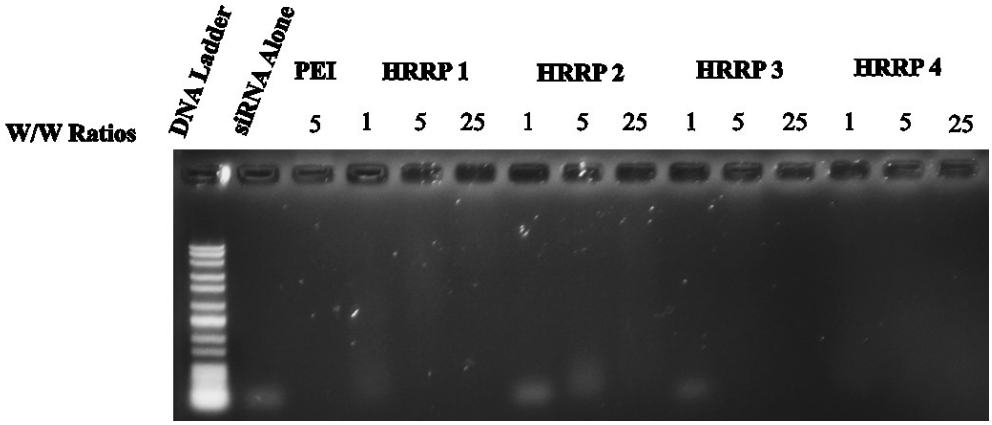
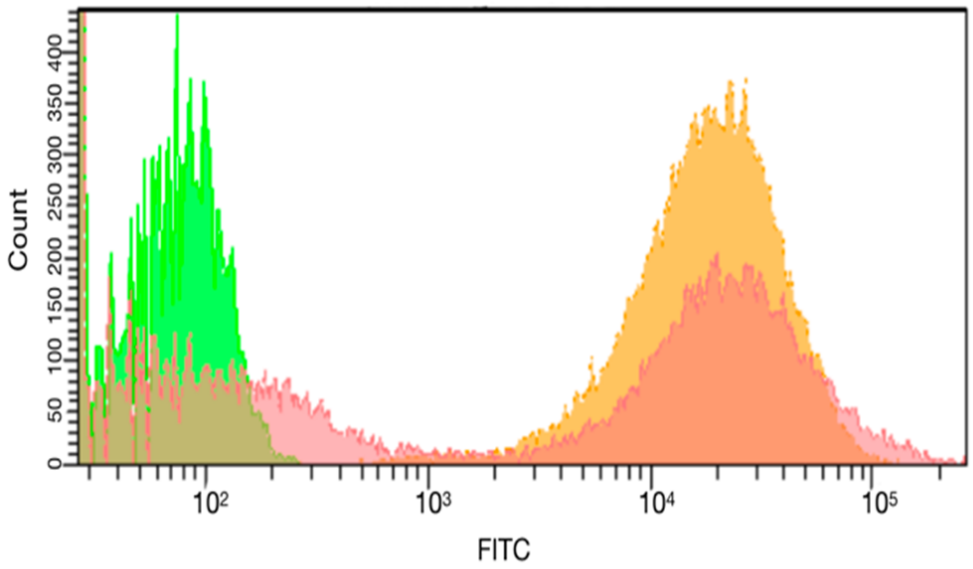


Figure 5S4. Agarose gel electrophoresis illustrating siRNA complexation at different weight/weight ratios (w/w ratio) of redox-responsive and cationic galactose-based hyperbranched polymers with 133ng control siRNA.



Polymer	% Gated Cells	Mean Fluorescence intensity
Untreated Cells (Green)	**58.9**	**10, 671**
HRRP 2 (Red)	**74**	**16,586**
HRRP 4 (Orange)	**55.1**	**22, 294**

Figure 5S5. Flow cytometry results of cellular internalization of FITC-labeled polyplexes (HRRP 2[Red] and HRRP 4[Orange]) in comparison to untreated Cells (Green).