University of Alberta

Carbohydrate and Phosphorylcholine based Polymers Prepared by Reversible Addition-Fragmentation Chain Transfer Polymerization for Gene Therapy

by

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In fulfillment of my Mother Jamil-UN-Nisa s' dream

Abstract

Reversible addition-fragmentation chain transfer polymerization (RAFT) has allowed the facile synthesis of tailor-made cationic polymers which are promising non-viral gene delivery vectors. Advanced structure-activity relationship studies between the polymers and gene expression have been possible due to the remarkable control in the design of these polymers *via* the RAFT process.

In the first study, RAFT polymerization technique allows the successful synthesis of cationic glycopolymers containing pendant sugar moieties. A library of cationic glycopolymers of pre-determined molar masses and narrow polydispersities ranging from 3-30 kDa has been synthesized. These polymers differ from each other in their architectures (*block* versus *random*), molecular weights, and monomer ratios (carbohydrate to cationic segment). It is shown that the above-mentioned parameters can largely affect the toxicity, DNA condensation ability and gene delivery efficacy of these polymers. The effect of serum proteins on statistical and diblock copolymer based polyplexes and hence gene delivery efficacy is further studied.

In the second study, 2-methacryloxyethyl phosphorylcholine (MPC) copolymers are studied for their ability to produce non-toxic and biocompatible materials. The cationic MPC copolymers of varying architectures (block *versus* random) are produced by RAFT polymerization technique. The copolymers produced are further evaluated for their, morphology, cellular uptake and gene delivery efficacy in the presence and absence of serum.

In another study, hyperbranched glycopolymers of varying molecular weights and compositions are synthesized *via* reversible addition fragmentation chain transfer (RAFT) process and are further explored for their gene expression *in vitro*. Galactose based hyperbranched polymers are compared to glucose-derived hyperbranched polymers for their cellular uptake, toxicity, lectin interactions and gene expression. Furthermore, the cellular uptake and gene expression are studied in two different cell lines in the presence of lectins.

Carbohydrate and phosphorylcholine based cationic polymers having a novel architecture, different compositions and varying molecular weights are produced and are termed as cationic '*block-statistical'* copolymers. These cationic copolymers are evaluated for their gene delivery efficacies, interactions with serum protein, cellular uptake and nuclear localization ability. In addition, MPC based '*block-statistical'* copolymers and their sugar incorporated analogues are prepared and are compared with their statistical analogues for serum interactions and gene expression.

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List of Abbreviations

RAFT; reversible addition fragmentation chain transfer
LRP; living radical polymerization
ATRP; atom transfer radical polymerization
PEI; poly(ethyleneimine)
PEG; poly(ethyleneglycol)
CTA; chain transfer agent
GAPMA; 3-glucanoamidopropyl methacrylamide
LAEMA; 2-lactobionamidoethyl methacrylamide
PGA; poly(glucoamidoamine)
AEMA; 2-aminoethylmethacrylamide
APMA; 3-aminopropyl methacrylamide
MPC; 2-methacryloxyethyl phosphorylcholine
SPDP; *N*-succinimidyl 3-(2-pyridyldithio)-propionate
PPEGA; poly(PEG-acrylate)
SNP; single nucleotide polymorphism
HPMA; 3-hydroxypropylmethacrylamide
BMA; butyl methacrylate
PEGMA; poly(ethyleneglycol) methacrylate)
pNIPAaM; poly(*N*-isopropylacrylamide),
pAA; poly(acrylic) acid
HEMA; 2-hydroxyethylmethacrylate
DMAEA; poly(2-dimethylaminoethyl acrylate)
DEAEMA; diethylaminoethyl methacrylate
DMAEMA; 2-dimethylaminoethyl methacrylate
DMAPMA; *N*-(3-(dimethylamino)propyl) methacrylamide
CS; chitosan
GSH; glutathione
PLL; poly($_{L}$-lysine)
ETC; ethyl cyanovaleric trithiocarbonate
PCL; polycarpolactone
DMSO; *N,N'*-dimethyl sulfoxide
DTT; dithiothreitol
Boc-AEMA; *N*-(tert-butoxycarbonyl)aminoethyl methacrylate
Boc-AHMA; *N*-(tert-butoxycarbonyl)aminohexyl methacrylate
GAGs; glucosaminoglycans
DIC; *N,N*′-Diisopropylcarbodiimide
ACVA; 4,4′-azobis(4-cyanovaleric acid)
OEGMA; oligo(ethyleneglycol) methacrylate
CPADN; 4-cyanopentanoic acid dithionaphthalenoate
DSDMA; disulfide-based dimethacrylate
HT; high thoroughput
LCST; lower critical solution temperature
ITC; isothermal calorimeter
AFM; atomic force microscopy
DLS; dynamic light scattering

GADPH; (glyceraldehyde-3-dehydrogenase)
pSMA; poly(styrene-*alt*-maleic anhydride)
BSPAC; 3-benzylsulfanylthiocarbonylsufanylpropionic acid chloride
MPEG; methoxyPEG
DOX; doxorubicin
GFP; green fluorescent protein
VEGF; Vascular endothelial growth factor
ONPG; *O*-Nitrophenyl *β*-D-galactopyranoside
FITC; Fluorescein isothiocyanate
CHAPS; 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
BSA; bovine serum albumin
CTP; 4-cyanopentanoic acid dithiobenzoate
DMF; *N,N'*-dimethylformamide
GPC; gel permeation chromatography
LD_{50}; lethal dose50
MWCO; molecular weight cut off

List of Publications

1. **Ahmed, M.;** Narain, R.; Rapid Synthesis of Gold Nanorods using a One Step Photochemical Strategy. 2010, *Langmuir*,*26*,18392-18399.
2. **Ahmed, M.;** Narain, R. The effect of polymer architecture, composition, and molecular weight on the properties of glycopolymer-based non-viral gene delivery systems. 2011, *Biomaterials 32*, 5279-5290.
3. Kitov, I. P.; Paszkiewicz, E.; Sadowska, M. J.; Deng, Z.; **Ahmed, M.;** Narain, R.; Griener, P. T.; Mulvey, L. G.; Armstrong, D. G.; Bundle, R. D. Impact of the Nature and Size of the Polymeric Backbone on the Ability of Heterobifunctional Ligands to Mediate Shiga Toxin and Serum Amyloid P Component Ternary Complex Formation. 2011, *Toxins. 9*, 1065-1088.
4. **Ahmed, M**.; Bhuchar, N.; Narain, R. Well-Controlled Cationic Water-Soluble Phospholipid Polymer-DNA Nanocomplexes for Gene Delivery. 2011, *Bioconjugate Chem. 22*, 1228-1238.
5. Chen, J.; **Ahmed, M**.; Liu, Q.; Narain, R. Synthesis of Cationic Magnetic Nanoparticle and Evaluation of their Gene Delivery Efficacy in Hep G2 Cells. 2012, *J. Biomed. Mat. Res. Part A. 100A*, 2342-2347.
6. **Ahmed, M**.; Narain, R. The effect of molecular weight, compositions and lectin type on the properties of hyperbranched glycopolymers as non-viral gene delivery systems. 2012, *Biomaterials 33*, 3990-4001.
7. **Ahmed, M**. Lai, F. L. B.; Kizhakkedathu, J. N.; Narain, R. Hyperbranched Glycopolymers for Blood Biocompatibility. 2012, *Bioconjugate Chem. 23*, 1050-1058.
8. **Ahmed, M.;** Jawanda, M.; Ishihara, K.; Narain, R. Carbohydrate and Phosphorylcholine Polymer based Gene Delivery Vectors with Novel Molecular Architecture. 2012, *Biomaterials*, *33*, 7858-7870.
9. **Ahmed, M.;** Narain, R. Intracellular Delivery of DNA and Enzyme in Active Form using Degradable Carbohydrate based Nanogels. 2012, *Mol. Pharmaceutics*. Accepted.
10. Kotsuchibashi, Y.; Zhang, Y.; **Ahmed, M**.; Ebara, M.; Aoyagi, T.; Narain, R. Fabrication of FITC-doped Silica Nanoparticles and their Cellular Uptake. 2012, *Soft Matter*. Submitted.

<u>Book Chapters:</u>

1. **Ahmed, M.;** Narain, R.; Cationic Glycopolymers. Engineered Carbohydrate Based Materials for Biomedical Applications: Polymers, Surfaces, Dendrimers, Nanoparticles, and Hydrogels. A John Wiley & Sons, Inc., Hoboken, New Jersey. 2011, pg. 143-166.
2. **Ahmed, M.;** Narain, R.; Glycopolymer Bioconjugates. Engineered Carbohydrate Based Materials for Biomedical Applications: Polymers, Surfaces, Dendrimers, Nanoparticles, and Hydrogels. A John Wiley & Sons, Inc., Hoboken, New Jersey. 2011, pg. 167-188.

3. **Ahmed, M.;** Narain, R.;Glycopolymer-Functionalized Carbon Nanotubes. Engineered Carbohydrate Based Materials for Biomedical Applications: Polymers, Surfaces, Dendrimers, Nanoparticles, and Hydrogels. A John Wiley & Sons, Inc., Hoboken, New Jersey. 2011, pg. 189-
212.
4. Ahmed, M.; Narain, R. Glyconanaoparticles for Gene Delivery. Nanomaterials for Biomedicine. ACS Symposium Series Book. 2012. Accepted.

Chapter 1. Progress of RAFT Based Polymers in the Field of Gene Delivery

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1.1 Introduction

Since the emergence of the concept of gene delivery in 1963, a variety of viral and non-viral gene delivery agents are produced and tested for their gene delivery efficacies.[1-13] Due to the high immunogenicity and toxicity of viral vectors in clinical trials, the development of non-viral vectors has been considered as a promising approach to produce non-toxic and highly efficient gene delivery systems [3,6,10,13] For the last two decades, a large number of synthetic vectors have been synthesized and tested for their gene delivery profiles.[2-18] However, no significant progress has been made so far in clinical trial. The synthesis of stable and effective gene delivery vectors is still a challenge, mainly due to the poor understanding of their interactions with cells at molecular level. Some of the known barriers of gene delivery vectors at the cellular level are their low cellular uptake, low endosomal escape, and translocation of DNA or polyplexes into nucleus.[3,6,10,13]

Gene therapy holds great potential for the treatment a variety of acquired and genetic disorders such as cancer, haemophilia and cystic fibrosis. The earlier focus on gene transfer was the synthesis and use of cationic polymers for gene expression.[6,13] Hence, cationic polymers such as poly(ethyleneimine) (PEI) were extensively studied for gene therapy. Due to the high proton sponge effect, various mechanisms of uptake, efficient DNA complexation and facile DNA release *in vitro* and *in vivo*, PEI has become the gold standard for gene expression experiments.[13] However, the high toxicity of cationic polymers, their non-specific interactions with cells and proteins *in vitro* and *in vivo* and

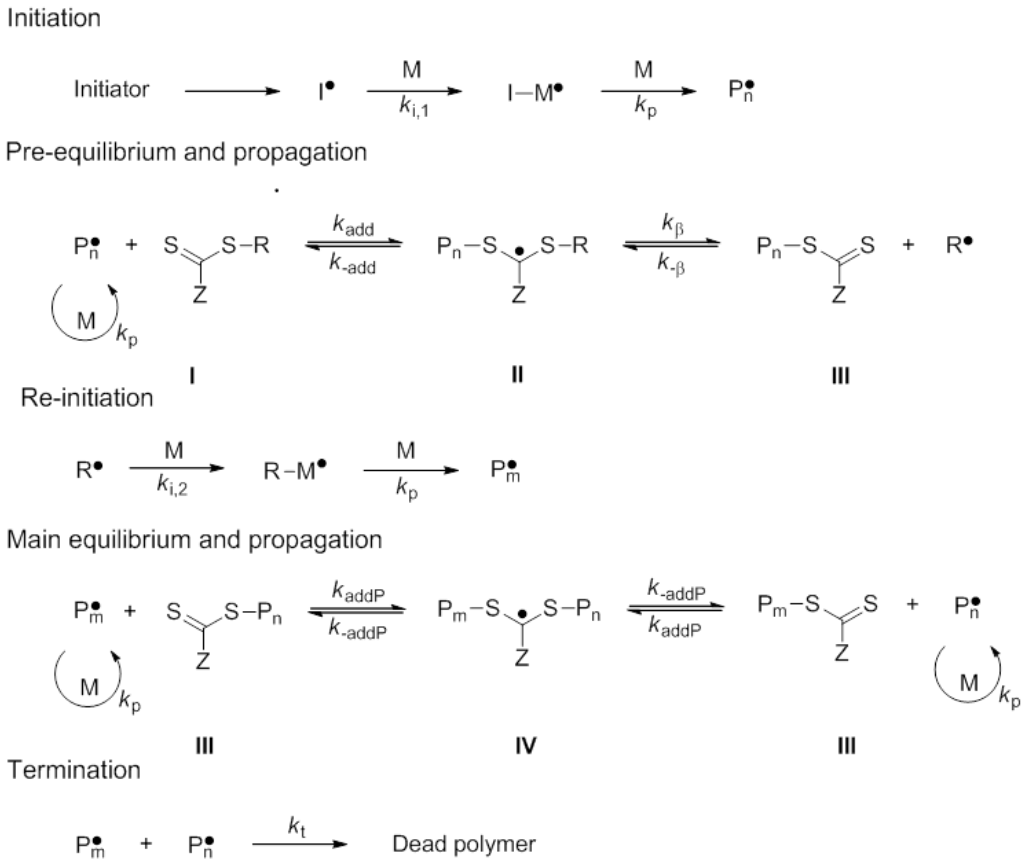
ability to elicit immune response encouraged the researchers to produce biocompatible analogues of cationic polymers.[6,13,19-22] The modification of cationic polymers with non-ionic moieties such as poly(ethyleneglycol) (PEG) has been reported. In addition, analogues of cationic polymers with natural moieties such as amino acids, lipids, and sugars have also been prepared.[6,13,19-22] These modifications are shown to increase the stability of polyplexes, and cellular uptake, along with decreasing their toxicity.

Due to the limited control over the modification sites, reproducibility, molecular weights and architecture of cationic polymers, such as PEI polymers there was an inherent need to synthesize the cationic polymers with well-tailored and well-defined properties such as molecular weights, compositions, shapes, and architectures. With the recent advances in the field of polymer chemistry, it is now possible to produce libraries of cationic vectors of varying shapes, compositions, and architectures and their gene delivery efficacies are evaluated to identify funcional structures.[12,23-25] The breakthrough in the field of gene delivery occurred with the advent of living radical polymerization (LRP) techniques in the 1990s.[12,26] LRP approaches, namely nitroxide-mediated polymerization (NMP), atom transfer radical polymerization (ATRP) and reversible addition fragmentation chain transfer polymerization (RAFT) has allowed the synthesis of libraries of polymers of controlled dimensions in a facile manner.[12,23-25] These polymers are then tested for their efficacy in transfecting cells *in vitro* and in an effort to establish the structure-activity relationships between the chemical structure of the polymers and their gene

expression profile. Hence, LRP opened new avenues in understanding gene expression, in a way which was not possible before. Various cationic polymers with gene delivery efficacies similar to PEI along with low toxicities were identified. Moreover, different analogues of cationic polymers such as reducible cationic polymer, star shaped polymers, hyperbranched polymers, and other architectures of linear cationic polymers were also prepared. In the last decade, most of the work focused on the use of cationic polymers prepared *via* ATRP technique for gene delivery. Some recent reviews described the progress in the field of gene delivery using ATRP based vectors.[12,23-25]

1.2. RAFT Polymers for Gene Delivery

In comparison to other LRP techniques, RAFT polymerization is the most versatile and convenient tool, due to its tolerance to a variety of solvents, reaction conditions and functionalities which can produce telechelic polymers in the absence of metal catalysts.[26-31] RAFT polymers are synthesized *via* a chain transfer process, which relies on the use of chain transfer agents (CTAs). CTAs are organic compounds containing thiocarbonylthiol moieties. The mechanism of RAFT polymerization is well studied and is depicted in scheme 1-1.



Scheme 1-1. Schematics of RAFT polymerization.[12] [Copyright 2011, Elsevier Ltd. reproduced with permission]

Since the emergence of RAFT polymerization approach in 1998 and its adaptation to produce water-soluble polymers in aqueous media, there has been a significant increase in the use of polymers synthesized *via* RAFT for therapeutic applications.[26-32] An overview of advancement of RAFT polymerization in the field of gene delivery is depicted in table 1-1. A number of reviews have been published on the mechanism of RAFT polymerization, choice of monomers, bioconjugation strategies and their biological applications.[29-31]

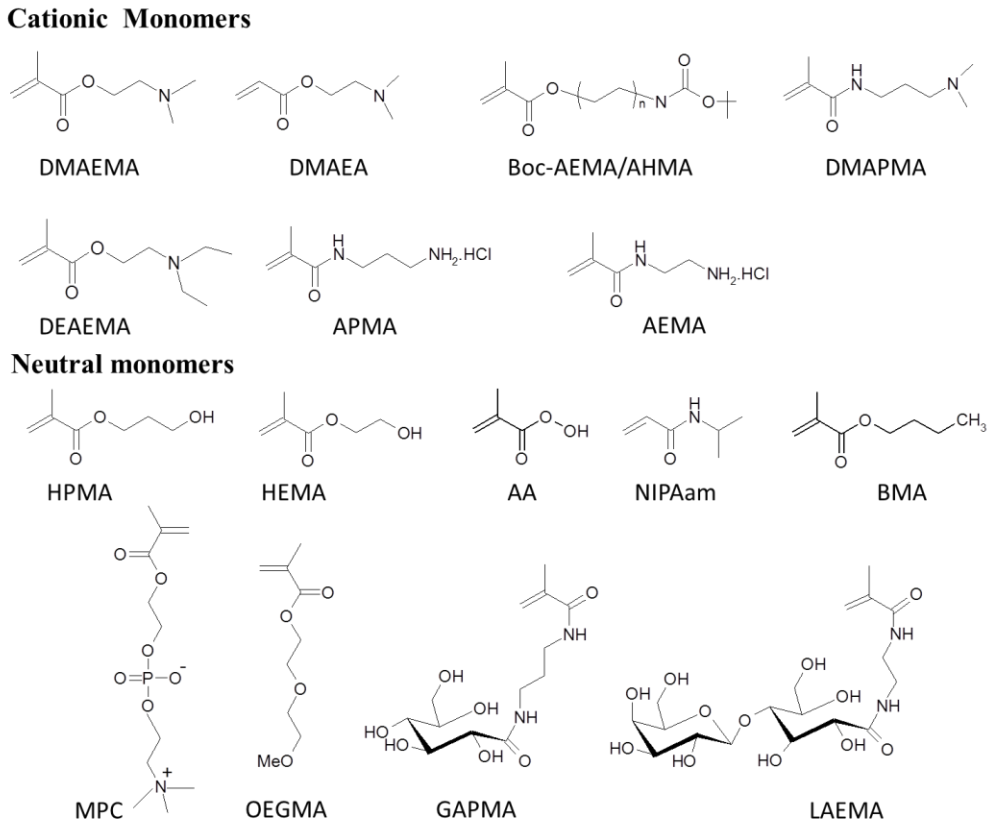
Table 1-1. A Timeline of progress made in the field of gene delivery, and the use of RAFT polymerization approach.

Time line	Progress	Reference
1963	Advent of Gene Delivery	1
1990s	Advent of LRP approach	26
1998	Aqueous RAFT Polymerization	27
2004	Synthesis of methacrylate based tertiary amine	35
2002, 2009	Synthesis of methacrylate and methacrylamide based glycopolymer in the absence of protected group chemistry	45,76
1978-1990	Synthesis of phosphorylcholine based monomer	81,82
2009	Synthesis of methacrylamide based primary amine in the absence of protected group chemistry	34
2009	Synthesis of Hyperbranched Polymers	48
2009	Use of linear polymers for DNA and siRNA delivery *in vitro*	37,96,97
2012	Use of hyperbranched polymers for DNA delivery *in vitro*	78
2011	Use of linear polymers for DNA and siRNA delivery *in vivo*	50

Recently McCormick and coworkers have provided a brief outlook on the RAFT process based copolymers for small inhibitory ribonucleic acid (siRNA) delivery.[33] The main focus of this review is to provide a comprehensive overview of the progress made by RAFT polymerization in the field of gene delivery, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) delivery.

1.2.1 Cationic Polymers *via* RAFT

Acrylates, acrylamides, styrene derivatives, and vinyl esters are some examples of monomers polymerized *via* RAFT.[26,29] The structures of monomers polymerized *via* RAFT polymerization for gene delivery applications are shown in scheme 1-2.



Scheme 1-2. Structures of monomers polymerized *via* RAFT polymerization for gene delivery application. Adapted from references [53], [41], [44], [55], [60], [63], [45], [34], [79].

The polymerization of cationic methacrylamide based monomers in aqueous solution *via* RAFT process is well-documented by our group and others.[12,34,35] The first cationic polymer prepared was a tertiary amine, namely *N*-(3-(dimethylamino)propyl) methacrylamide (DMAPMA) by McCormick and coworkers in 2004.[35] This polymer and its copolymers synthesized with 3-hydroxypropylmethacrylamide (HPMA) were then studied for their siRNA complexation efficacies and cellular uptake.[12,36] HPMA is an example of a biocompatible non-ionic moiety extensively used for gene delivery

applications to impart stealth layer to cationic vectors.[13] The copolymers of DMAPMA and HPMA were synthesized and were functionalized with folic acid. For this purpose, HPMA polymer was copolymerized with a small amount of 3-aminopropyl methacrylamide (APMA) in random fashion and the copolymer obtained was blocked with PDMAPMA macroCTA. The amine content of APMA was further functionalized with folic acid and these copolymers were complexed with siRNA. The polyplexes formulated showed effective down regulation of survivin mRNA in KB cells specific for folate receptors.[37] The synthesis of primary amine based polymers has been a significant challenge, requiring the need of protected group chemistry and various steps of de-protection and purification.[34] The synthesis of 2-aminoethylmethacrylamide (AEMA) and 3-aminopropyl methacrylamide (APMA) and their homopolymers were first reported by our group using RAFT polymerization approach in aqueous solution in the absence of protected group chemistry.[34]

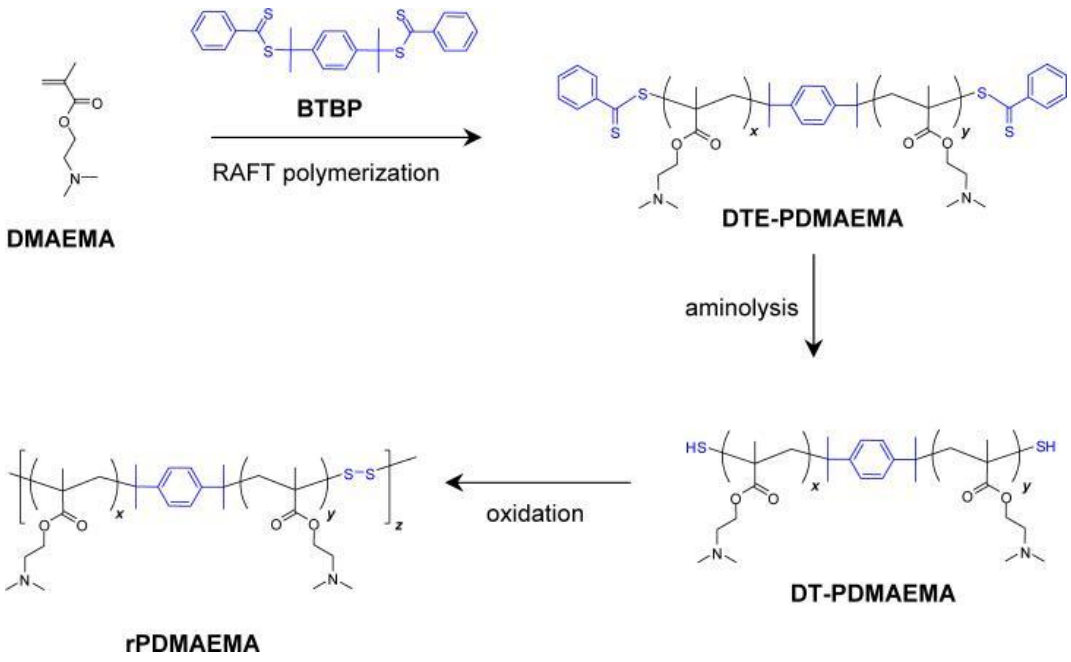
The superior gene expression of PEI is thought to be associated with the presence of a variety of amines in its polymeric structure. In order to determine the effect of the type of amine moieties on gene expression, Zhong and colleagues synthesized cationic polymers bearing pendant amine moieties (primary, tertiary).[38] 2-dimethylaminoethyl methacrylate (DMAEMA) is a well-known methacrylamide based tertiary amine, which can be polymerized by a variety of techniques to produce cationic polymer, which are studied for their gene expression profiles.[12,38] A family of PDMAEMA based polymers

containing varying amount of primary and tertiary amine groups was prepared to study the effect of type of amine type on transfection efficacies.[38] DMAEMA was copolymerized with protected primary amines, *N*-(tert-butoxycarbonyl)aminoethyl methacrylate (Boc-AEMA) or *N*-(tert-butoxycarbonyl)aminohexyl methacrylate (Boc-AHMA) in random fashion. The polymers were de-protected by acid treatment. The high gene expression of PEI is reported to be related to its high buffering capacity, due to the presence of a combination of both primary and tertiary amines in the polymer. Although, incorporation of primary amines in DMAEMA decreased the buffering capacity of DMAEMA polymer itself, the presence of primary amines significantly enhanced their DNA condensation efficacy, gene expression and cell viability, as compared to PDMAEMA alone.[38]

1.2.2 Degradable Polymers *via* RAFT Polymerization

Degradable polymers are important class of polymers because of their non-toxic and environmental friendly nature.[29] The introduction of disulfide bonds in polymeric back bone is a common approach to produce degradable polymers sensitive to reducing environment. These degradable and non-toxic forms of cationic polymers are highly desirable for gene delivery, due to the reduction of disulfide bonds in acidic environment, hence facilitating the release of DNA.[39,40] The degradable form of PDMAEMA was prepared by the RAFT process.[41] The traditional methods yield DMAEMA based polymers with broad polydispersities and poorly-defined end-groups chemistries, which greatly limit the possibility to produce reducible PDMAEMA using bis-sulfhydryl

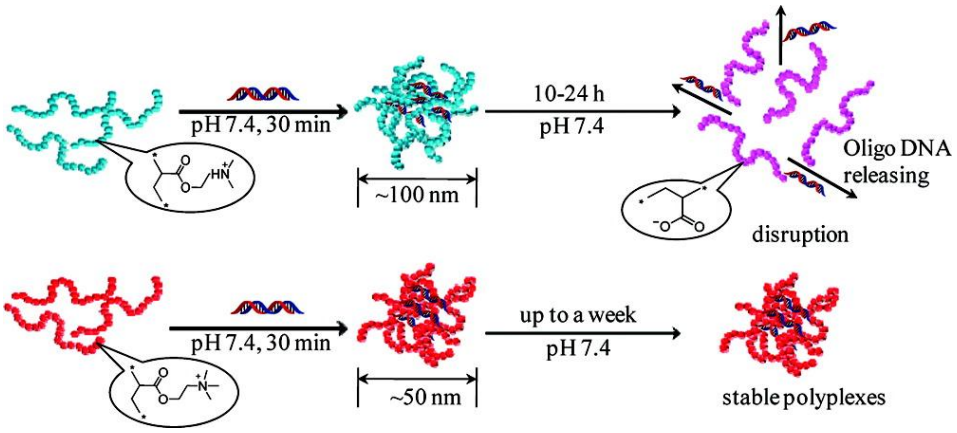
precursors. Bi-functional RAFT agent is used to obtain reducible PDMAEMA with chain transfer residues at both ends of oligomers, as shown in scheme 1-3.



Scheme 1-3. Synthesis of reducible PDMAEMA using bi-functional RAFT agent.[41] Copyright 2007, Elsevier Ltd. reproduced with permission.

Dithioester-functionalized PDMAEMA thus obtained was confirmed by ^{1}H-NMR. The conversion of dithioesters into thiol was done by aminolysis of polymer in hexane. The thiol groups of polymers were further reduced by the oxidation of terminal sulfhydryl group of polymer in *N,N'*-dimethyl sulfoxide (DMSO). The reducible PDMAEMA showed higher polydispersities than starting oligomers. The reduction of these polymers with dithiothreitol (DTT) produced low molecular weight polymers, similar to those of original oligomers used for polymerization. These copolymers showed low toxicity and superior gene expression than their non-reducible analogues.[41]

Polycations which can degrade into benign compounds over time in the absence of an intracellular trigger were also synthesized. poly(2-dimethylaminoethyl acrylate) (PDMAEA) is a tertiary amine based polymer which degrades into anionic polymer over a period of a few days, hence providing enough time for DNA release and preventing the accumulation of polymers in living tissues thereby decreasing their toxicity. The degradation of PDMAEA into poly(acrylic) acid (PAA) is independent of pH of aqueous media, as shown in scheme 1-4.



Scheme 1-4. Synthesis of PDMAEA based polyplexes, and the degradation of polymer in polyacrylic acid.[42] Copyright 2011,American Chemical Society, reproduced with permission.

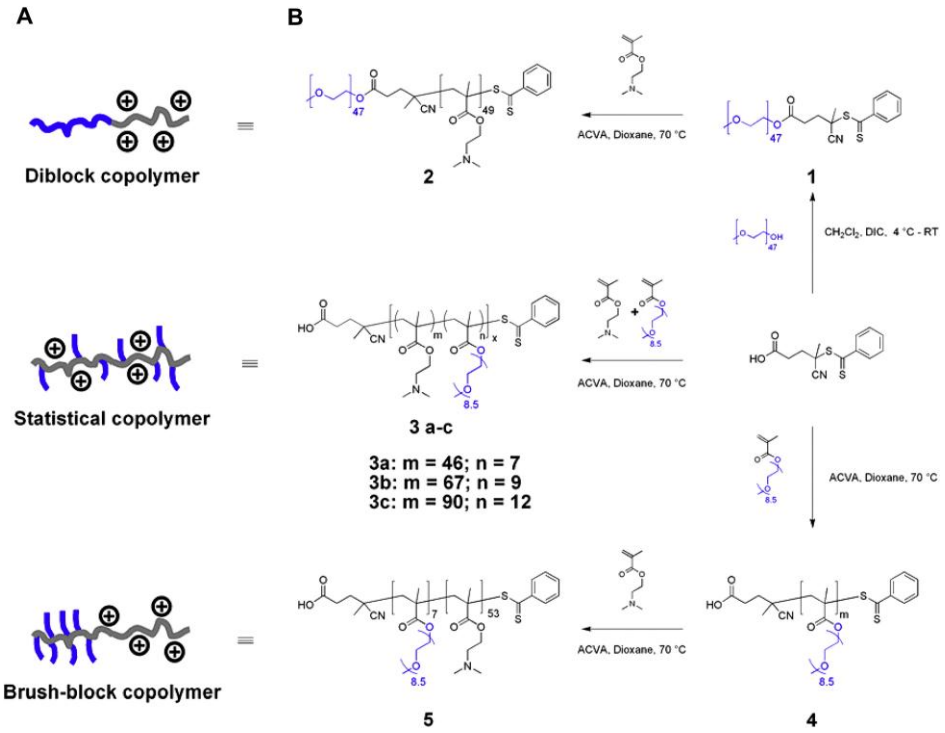
PDMAEA of 5 different molecular weights ranging from 3-11 kDa and narrow polydispersities were prepared by RAFT polymerization. For comparison purposes, quaternized analogues of these polymers were also prepared. These polymers showed excellent DNA complexation, cell viability and cellular uptake in Hela cells. The dissociation of polyplexes was occurred through self-catalysis of polymers into acrylic acid over a period of one week. The unquaternized

analogue of 11 kDa polymer showed higher cellular uptake than other analogues at low polymer/DNA ratio. However, no gene expression was studied.[42]

1.2.3 PEGylated Polymers *via* RAFT Polymerization

PEGylation of cationic polymers is a well-studied approach to mask the non-specific interactions of polymers with glucosaminoglycans (GAGs).[13] The incorporation of hydrophilic PEG layer on the surface of polyplexes decreases their interactions with anionic proteins (serum proteins, GAGs), hence increasing the stability of polyplexes in vitro and in vivo, and decreasing their non-specific interactions with cell surface.[13] The post-modification of cationic polymers with PEG based monomers has been reported. However, these post-modification strategies also compromise the gene delivery efficacies of cationic polymers.[12] RAFT polymerization technique allowed the synthesis of PEGylated cationic polymers of varying chain lengths, architectures and molecular weights to pinpoint the parameters ideal for optimum expression. Three types of polymers namely diblock, statistical, and brush-block polymers were synthesized *via* RAFT process. All of these polymers contained constant PEG weight fraction (0.2) while amine content of the polymer was varied (46-93). The polymers produced were from 10-20 kDa and of controlled polydispersities. In order to synthesize diblock copolymers, PEG chains were esterified by CTP *via N,N′*-Diisopropylcarbodiimide (DIC) coupling to yield PEG-macroCTA. The macroCTA was copolymerized with DMAEMA in the presence of 4,4′-azobis(4-cyanovaleric acid) (ACVA) to obtain diblock copolymer. The statistical copolymers were made in one pot using DMAEMA

and oligo(ethyleneglycol) methacrylate); (OEGMA). The brush-block copolymers were made by polymerizing OEGMA to obtain macro CTA, which was further copolymerized with DMAEMA yielding brush block copolymer. (Scheme 1-5)

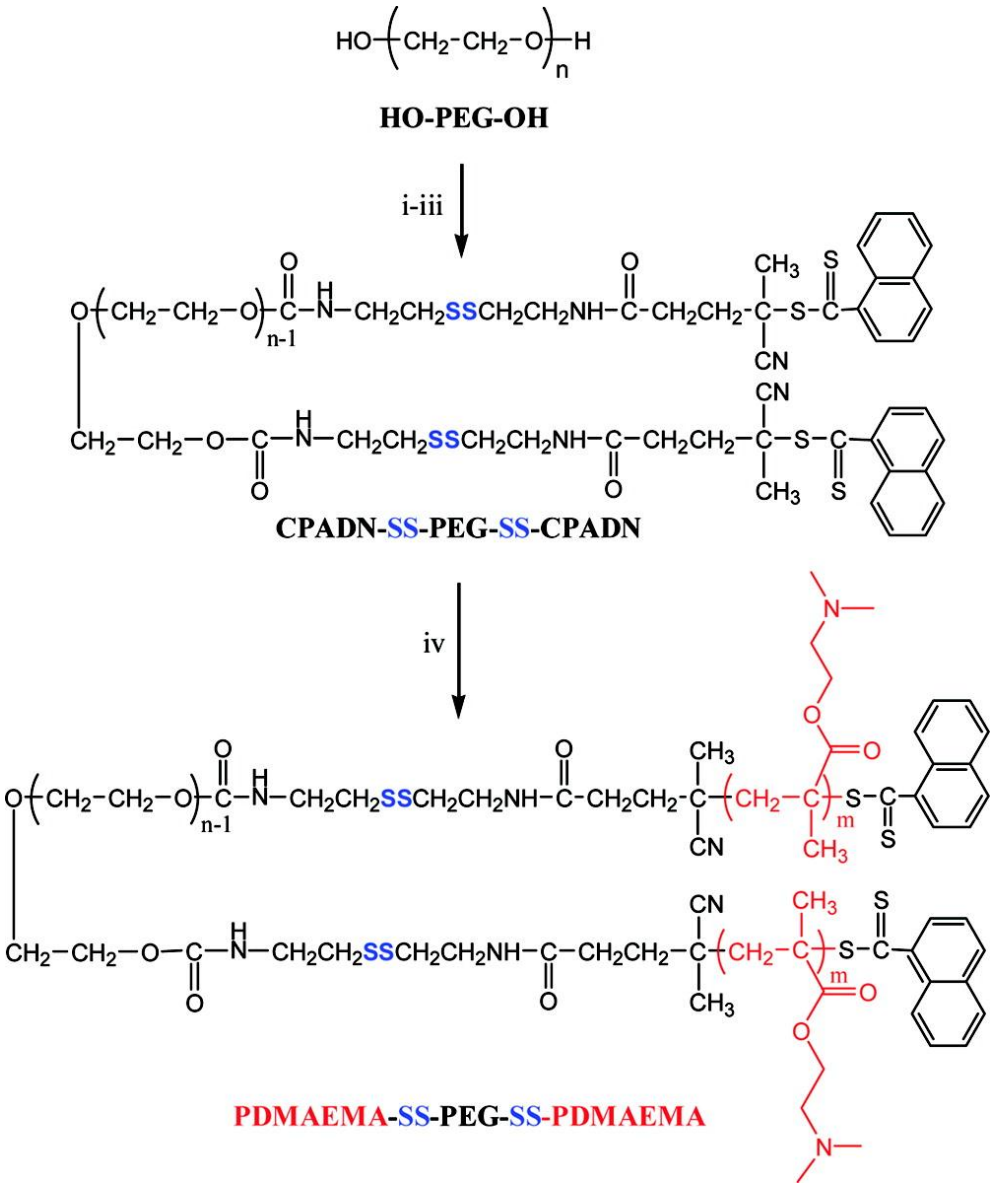


Scheme 1-5. Synthesis of diblock, statistical, and brush-block polymers *via* RAFT polymerization.[43] Copyright 2007, Elsevier Ltd. reproduced with permission.

The DNA complexation efficacies of statistical block and brush like copolymers were compared. The brush-like block copolymers were found to be more effective in DNA condensation than their statistical analogues. It is thought that steric crowding in shell due to brush-like architecture of PEG chains is responsible for fine tuning the size of polyplexes. Moreover, the gene expression was significantly higher for statistical and brush-like block copolymers in Hep

G2 and HEK 293 cells, as compared to block copolymers. However, the cell viability and cellular uptake was higher for statistical copolymers based polyplexes than their diblock or brush-like block analogues.[43]

Another major drawback of PEGylation of cationic polymers is the masking of cationic character, hence decreasing their proton sponge effect and DNA release.[13] One possible approach to solve this problem is de-shielding of PEGylated-polyplexes inside the cells, in response to endosomal pH. Zhong and coworkers employed this approach to produce reducible PDMAEMA and PEG based copolymers *via* RAFT polymerization.[44] Disulfide based reducible polymer are interesting as disulfide bonds are stable in extracellular environment but are prone to reductive intracellular environment.[39,40] The linear triblock polymers of PDMAEMA-s-s-PEG-PDMAEMA were prepared to trigger reduction in intracellular environment. Bioreducible cationic PDMAEMA were prepared using PEG macro-CTA. For this purpose, 4-cyanopentanoic acid dithionaphthalenoate (CPADN) was used as CTA to produced telechelic PEG macro-CTA, namely 'CPADN-SS-PEG-SS-CPADN'. CPADN is a versatile RAFT agent and have produced various telechelic polymers. DMAEMA monomer was then polymerized *via* RAFT process in the presence of the macroCTA to produce triblock copolymers. (Scheme 1-6) Non-reducible control using macro-CTA, CPADN-PEG-CPADN was also prepared to generate triblock PDMAEMA-PEG-PDMAEMA for the comparison purposes.



Scheme 1-6. Synthesis of reducible triblock polymers *via* RAFT polymerization. (i) p-NPC, toluene, 30 °C, 20 h; (ii) cystamine, DMSO, rt, 27 h; (iii) CPADN, NHS/DCC, DCM, rt,48 h; (iv) RAFT polymerization, THF, 60 °C, 24 h. [44] Copyright 2012,American Chemical Society, reproduced with permission.

Both reducible and non-reducible polymers showed efficient DNA complexation and produced small particles of about 120 nm in diameter, with low zeta potential values, due to the shielding of cationic polymers with PEG chains. The

de-shielding of PEGylated polyplexes is depicted in figure 1-1. The treatment of reducible polyplexes with DTT increased their size, within 1 hour of incubation along with the shift in zeta potential towards positive side, indicating the reduction of disulfide bonds in the polyplexes. In contrast, no change in size and zeta potential values was observed for their non-reducible analogues upon the treatment with DTT. As expected, reversibly shielded PEGylated polyplexes showed high cellular uptake in Cos-7 cells, as compared to non-reversible control.[44]

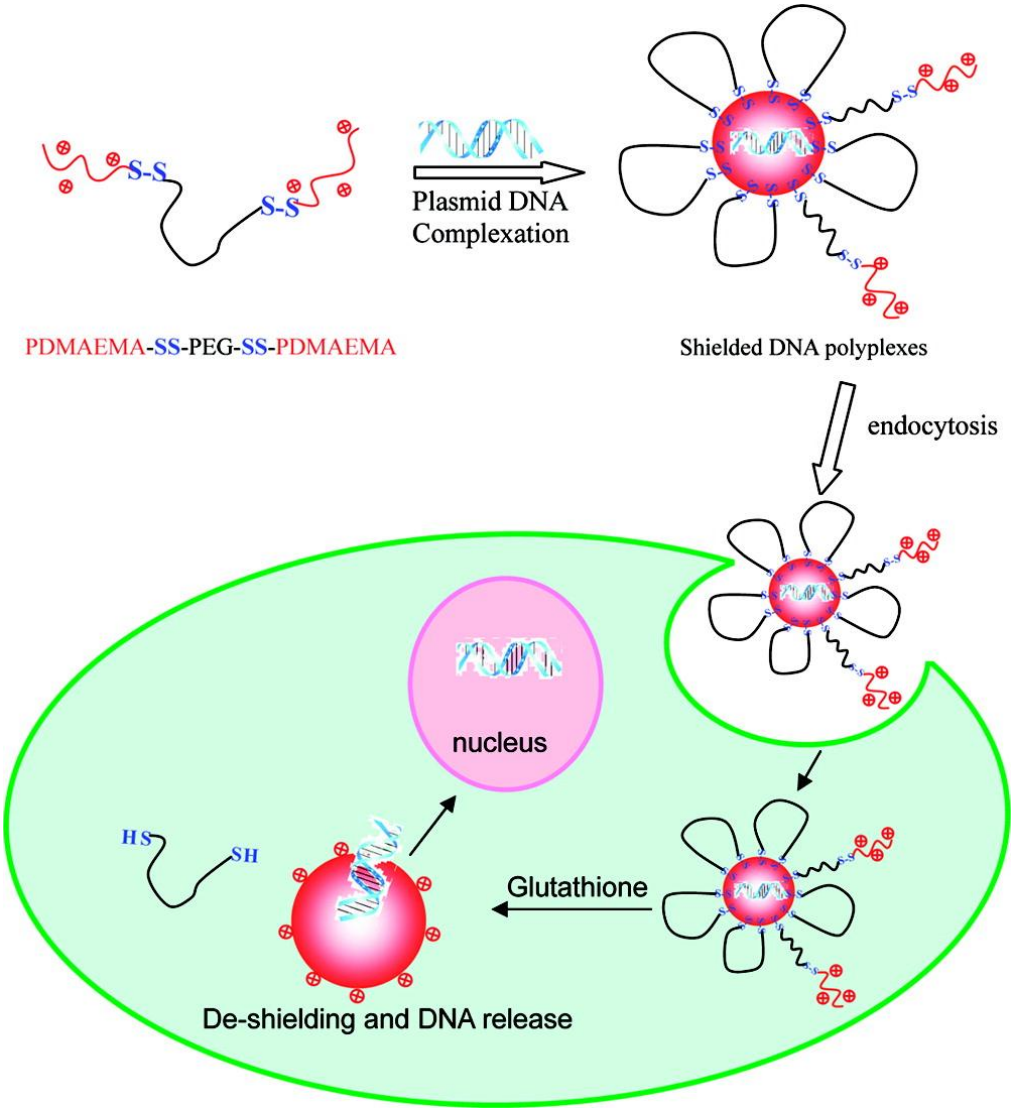


Figure 1-1. Deshielding of PEGylated polyplexes in intracellular reductive environment.(i) p-NPC, toluene, 30 °C, 20 hr.; (ii) cystamine, DMSO, rt. 27 hr.; (iii) CPADN, NHS/DCC, DCM, rt. 48 hr.; (iv) RAFT polymerization, THF, 60 °C, 24 hr.[44] Copyright 2012, American Chemical Society, reproduced with permission.

1.2.4 Copolymers of Varying Architecture for Gene Delivery

Another well-known approach to produce biocompatible non-toxic polymeric vectors is by the copolymerization of cationic monomers with a non-ionic biocompatible monomer.[43-47] The controlled polymerization approach of RAFT allows the synthesis of a variety of cationic polymers for gene delivery. The block and statistical linear copolymers are the most studied forms for DNA and siRNA delivery applications. In addition '*block-statistical'* polymers and hyperbranched copolymers of statistical and block configurations have also been synthesized and studied for their DNA complexation efficacies. Linear copolymers of 2-hydroxyethylmethacrylate (HEMA) and DMAEMA of varying molecular weights (15-290 kDa), architectures (block *versus* statistical) and cationic content were prepared by RAFT process. The DNA complexation efficacies of these polymers were studied and it was found that statistical copolymers made larger polyplexes than their block analogues. The toxicity and gene expression of these copolymers was dependent on their compositions and molecular weights. The high stability of these polyplexes is believed to reduce their gene expression, possibly due to slower dissociation of polyplexes in the

cytoplasm, as compared to PEI. The gene expression of these copolymers was significantly improved as compared to PDMAEMA alone.[46]

The synthesis of hyperbranched polymers *via* RAFT polymerization was originally reported by Perrier and coworkers.[48] Davis and coworkers reported the synthesis of biodegradable hyperbranched PDMAEMA of 290 kDa, using disulfide-based dimethacrylate (DSDMA) monomer, and 1,2-bis(2-(3-methylbuta-1,3-dien-2-yloxy)ethyl)disulfane, as crosslinking agent. The polymers produced were tested for their DNA binding efficacy however no gene expression was studied.[49]

RAFT polymerization was used as an approach to produce a library of core shell nanoparticles using high throughput (HT) polymeric nanoparticle methodology. This methodology is depicted in figure 1-2.

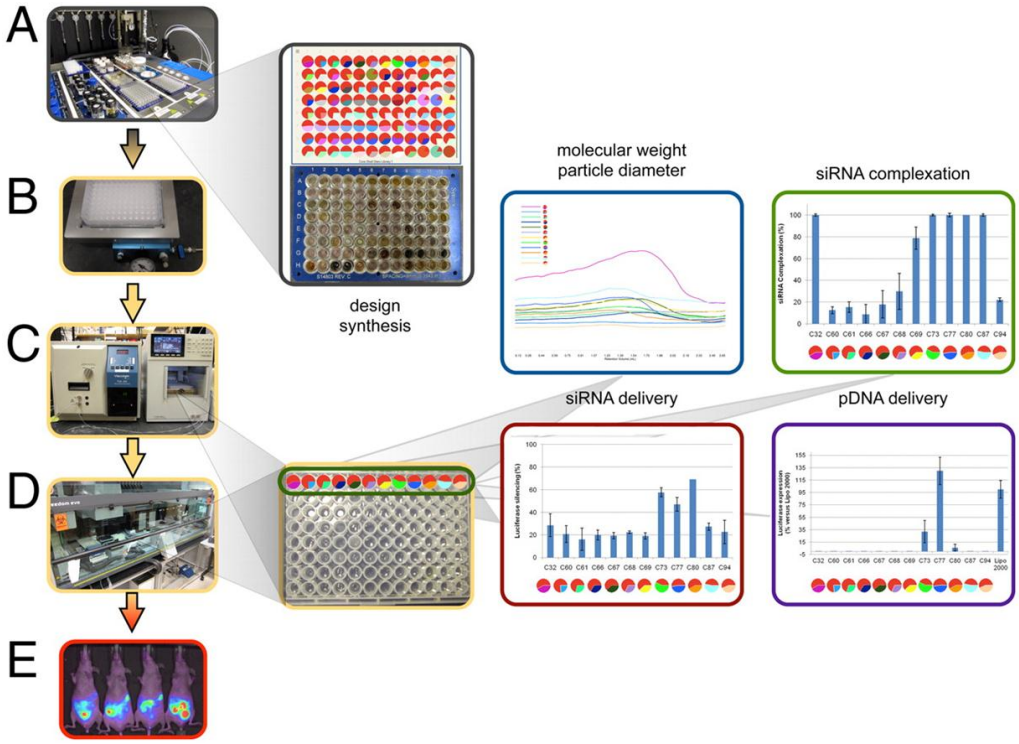


Figure 1-2. Step-by-step approach to synthesize, characterize, and screening of library of core-shell nanoparticles *in vitro* and *in vivo*.[50] Copyright 2011, National Academy of Science, reproduced with permission.

Block copolymers were designed to make a cationic core for siRNA complexation, with a biocompatible shell, which varied in chain length of polymers, number of protonizable amine and chemical properties. The block copolymers were designed to carry a non-reactive segment, and an epoxide segment, in order to crosslink the core of nanoparticles with a variety of amines (i.e. primary, secondary and tertiary amine). The non-reactive segment was varied to carry a variety of non-functional moieties such as zwitterionic polymers, PEO, hydrophobic blocks, cationic and anionic moieties. The automated HT procedure was used to develop a method for rapid synthesis, characterization and screening of these polymers using 96 well microtiter plate. Using HT system it was concluded that PEO based polymers bearing tertiary amines show high cellular uptake and gene expression *in vitro*, and the selected polymers were further evaluated for their *in vivo* gene expression.[50]

1.2.5 Stimuli Responsive Polymers *via* RAFT Polymerization

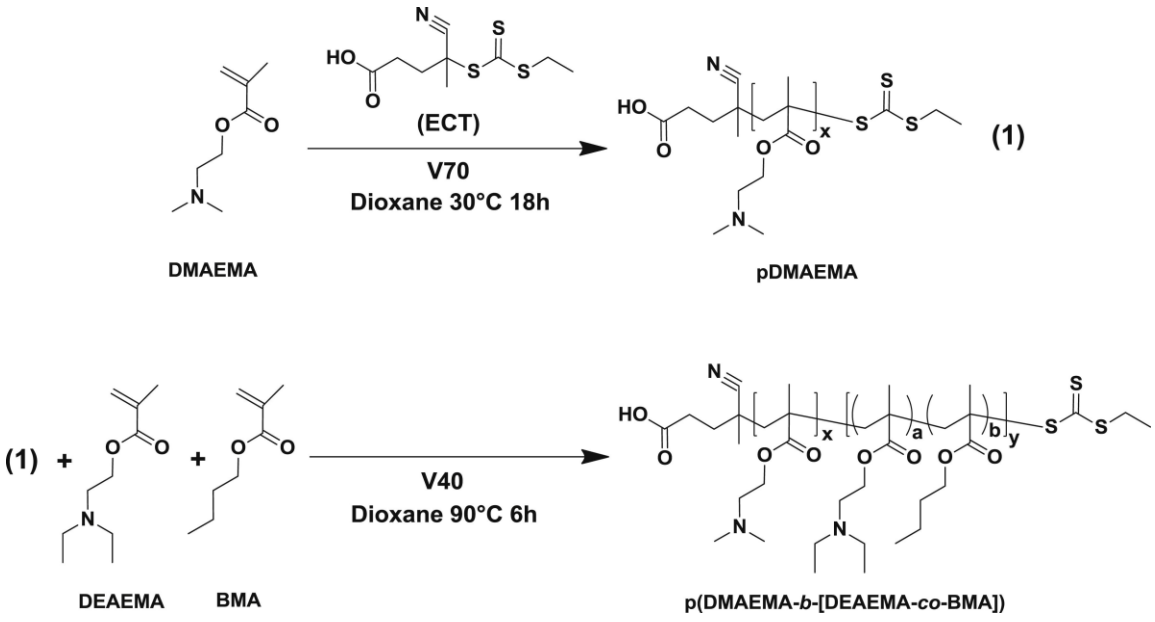
The synthesis of stimuli responsive copolymers *via* LRP is an important step towards the synthesis of successful and efficient gene delivery vectors. Stimuli responsive copolymers contain a hydrophilic segment and stimuli-responsive segment, which undergoes conformational change upon the presence of external stimuli, hence making self-assembling micelles with hydrophobic core and hydrophilic corona under aqueous conditions.[23] These modifications of

cationic polymers are found to have crucial effects on gene delivery efficacies. For example, hydrophobic components of gene delivery vectors can significantly alter their physiochemical and biological properties, including enhanced gene compaction, steric stabilization, and low toxicity.[2] A number of thermoresponsive and pH responsive copolymers have been produced by RAFT process. The thermoresponsive polymers are investigated for their DNA compaction and gene delivery efficacies. Poly(*N*-isopropylacrylamide), (PNIPAam), is a temperature responsive polymer, with lower critical solution temperature (LCST) close to physiological temperature. PNIPAam undergoes temperature induced phase transition from coil (from hydrophilic) to globular conformation (to hydrophobic) above LCST. Heterobifunctional thermoresponsive polymers of NIPAM were prepared by RAFT polymerization. For example, PEI-PNIPAam conjugates have been synthesized and studied for DNA complexation and gene expression.[51] In addition, diblock polymers of PNIPAam and single stranded DNA (ssDNA) were prepared. PNIPAam-macroCTA of 29 kDa was prepared *via* RAFT process. The obtained polymer was hydrolyzed using $NaBH_4$ to yield PNIPAam-SH. ssDNA-maleimide was synthesized and reacted with PNIPAam-SH *via* Michael addition reaction. The phase transition behavior of PNIPAam-*b*-DNA was studied and LCST was determined to be 32 °C, as compared to PNIPAam alone, for which LCST was 29 °C. The polymeric micelles of DNA were prepared above LCST, containing PNIPAam core and hydrophilic DNA corona. These micelles were highly stable

in high salt solution, and spontaneous aggregation of micelles occurred upon the addition of complementary DNA strand in solution.[52]

Poly(ethyleneglycol) methacrylate) (PEGMA) is another temperature-sensitive polymer, which has been studied for producing thermoresponsive polymers and nanogels.[53] In contrast to PNIPAam which is toxic upon its degradation into monomeric units, PEGMA is reported to be biocompatible and is more suitable for biomedical applications. Hyperbranched P(DMAEMA), P(DMAEMA-*b*-PEGMA) and folic acid functionalized P(DMAEMA-*b*-PEGMA) were prepared. The hyperbranched P(DMAEMA) was prepared using alkyne terminated RAFT agent, in the presence of cross-linker. Chain extension of P(DMAEMA) macroCTAs was performed to make P(DMAEMA-*b*-PEGMA). Folic acid was conjugated to the polymers *via* click chemistry. The polymers produced were complexed with DNA and complexation was studied using isothermal calorimetery (ITC). The amount of polymer required to complex DNA was higher for PEGMA based cationic polymers as compared to P(DMAEMA) alone, possibly due to the shielding of cationic moieties by PEG. The sizes and shape of polyplexes were studied by atomic force microscopy (AFM) and cellular uptake of complexes was studied in Hela cells. Hyperbranched P(DMAEMA) showed high cellular uptake along with high toxicity, their copolymers with PEGMA were significantly less uptaken. The functionalization of copolymers with folate residues significantly improved their cellular uptake possibly due to the specific targeting of polyplexes towards folate receptors on the surface of Hela cells.[54]

The first successful pH responsive block copolymers *via* RAFT process were reported by McCormick and coworkers. The copolymerization of DMAEMA (which has relatively low charge density (50%) at physiological pH) with diethylaminoethyl methacrylate (DEAEMA) has been found to be an effective technique to produce pH responsive micelles. Moreover, the disruption of endosome was enhanced by the incorporation of butyl methacrylate (BMA) in the copolymers. The polymers were synthesized by blocking the macroCTA of P(DMAEMA) (7 kDa) with statistical copolymer synthesized using BMA and DEAEMA as shown in Scheme 1-7.

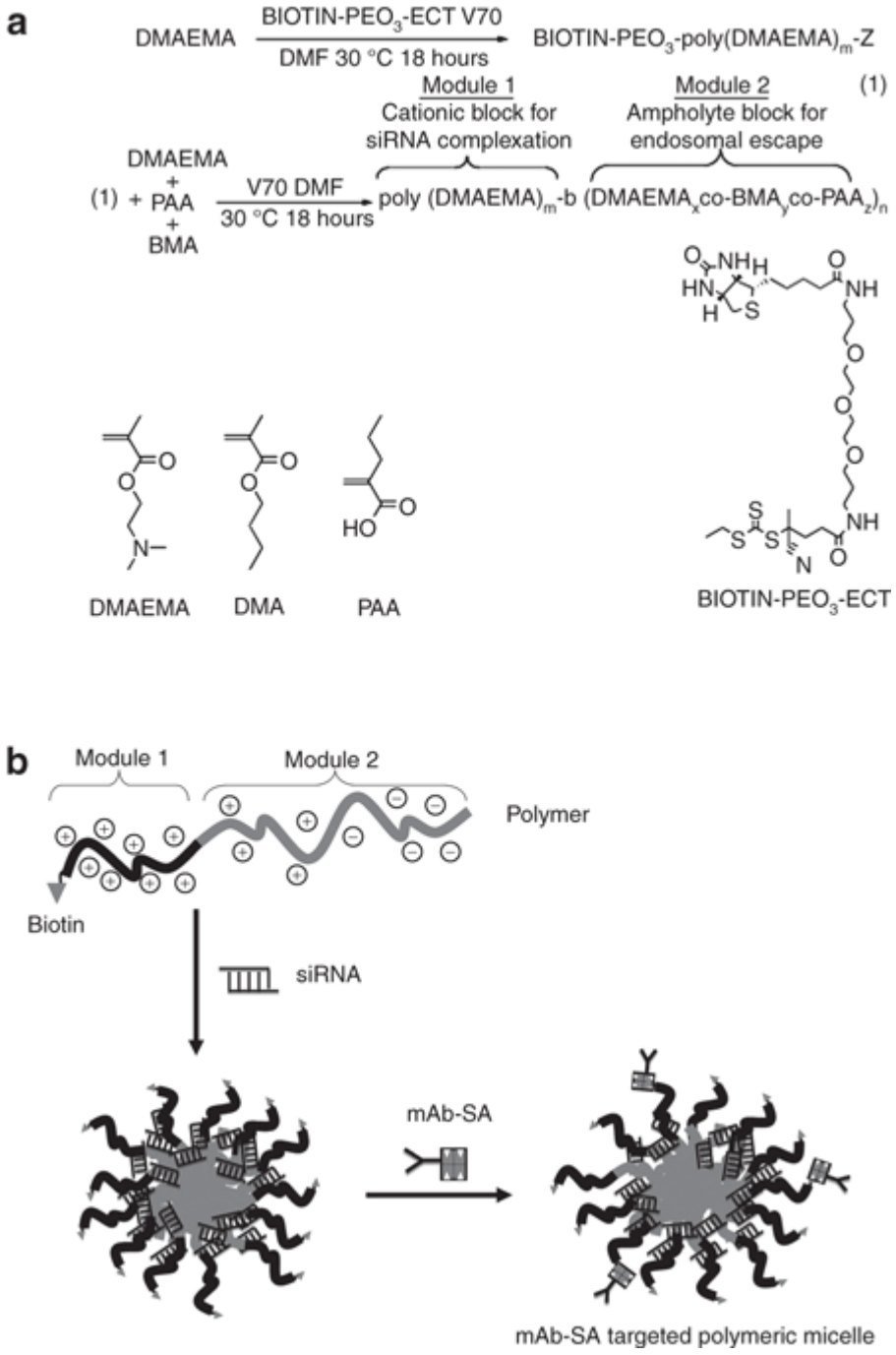


Scheme 1-7. Synthesis of stimuli responsive DMAEMA copolymers *via* RAFT polymerization.[55] Copyright 2012, Elsevier Ltd. reproduced with permission.

The copolymers of molecular weights ranging from 19-23 kDa with different feed ratios of BMA to DEAEMA were produced, which undergo pH dependent phase transition from micelles to unimers. The pH dependent phase transition of

copolymers was tuned by using different feed ratios of BMA to DEAEMA, as studied from dynamic light scattering (DLS) and hemolytic assay. The low BMA content in polymer (20%) produced micelles which showed phase transition behavior at physiological pH. In contrast high BMA content polymer showed no phase transition at studied pH values (5.8-7.4). Hence optimum BMA content (40%) produced micelles with sharp phase transition from 6.6 to 5.8. Moreover all the copolymers produced at low BMA content (20 and 40%) showed optimum gene expression in macrophages and dendritic cells. ^{1}H-NMR and D_2O titration studies were performed to confirm core-corona structure of micelles.[55]

These copolymers were further evaluated for their (glyceraldehyde-3-dehydrogenase) GADPH gene knockdown efficacies in Hela cells.[56] RAFT-CTA bearing folate functionalized group was also synthesized and was utilized to make terpolymers containing P(DMAEMA-*b*-DMAEMA-*st*-BMA-*st*-PAA). The first block of copolymers shows DNA condensation ability while second block shows endosomolytic and pH responsive behavior. These polymers were studied for GADPH knock down in Hela cells.[57] The biotinylated analogues of these polymers were prepared in another study. Biotinylated CTAs was used to make DMAEMA macro-CTA. The second block containing PAA, BMA and additional DMAEMA units was copolymerized and the micelles produced were complexed with GADPH-siRNA. The complexes were further functionalized with streptavidin modified monoclonal antibody specific for CD22 receptors, which binds to free biotin on the surface complexes as shown in scheme 1-8.

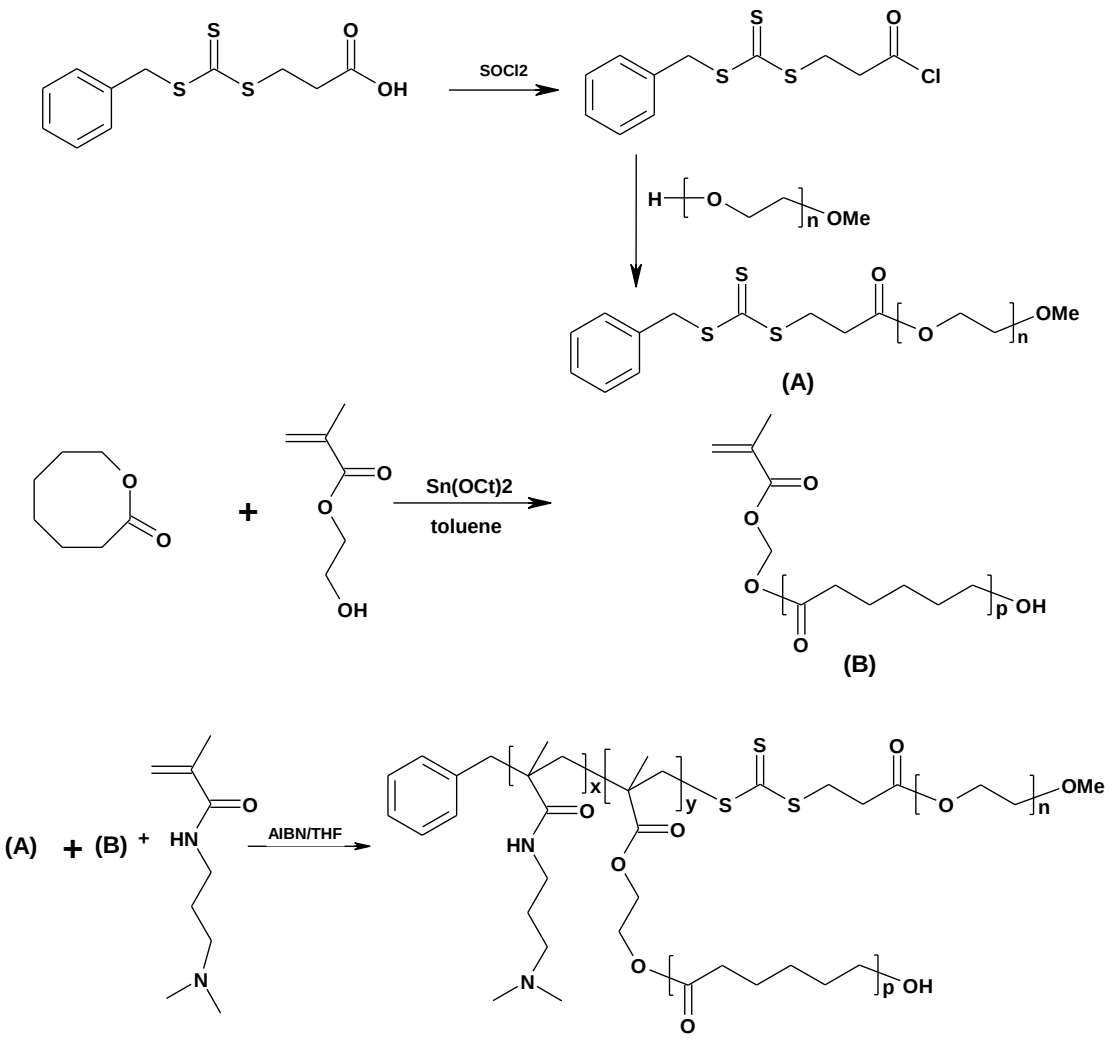


Scheme 1-8. Synthesis of biotinylated P(DMAEMA-*b*-DMAEMA-*st*-BMA-*st*-PAA) micelles (a), their complexation with siRNA and modification with $CD22^{+}$ antibody (b).[58] Copyright 2011, Nature Publishing Group, reproduced with permission.

These complexes showed specific uptake in $CD22^{+}$ expressing Hela cells.[58]

In another approach to make pH responsive micelles of block copolymers, Stayton and coworkers synthesized micelles of poly(DMAEMA-*b*-BMA). These micelles upon complexation with siRNA were stabilized with poly(styrene-*alt*-maleic anhydride) (pSMA), pSMA was used as pH sensitive component to make ternary complexes. These ternary complexes mediate effective cellular uptake and efficient knockdown of Plk1 gene in both drug sensitive and drug resistant cancer cell lines. Moreover, co-delivery of siRNA with anticancer agents was also achieved.[59]

Poly-ε-carpolactone (PCL) is a biodegradable amphiphilic polymers used to design micellar structures. Cationic micelles of PCL and poly(*N*-[3-(dimethylamino)propyl]methacrylamide) P(DMAPMA) were prepared, where PCL make a hydrophobic core to entrap drugs, while PDMAPA is used to condense DNA. The co-delivery system is PEGylated to reduce its interactions with serum proteins. (Scheme 1-9)



Scheme 1-9. Synthesis of PEGylated polycarpolactone (PCL) and DMAPMA based copolymers. Figure adapted from reference [60].

The first step in the synthesis of co-delivery system is the formation of macroRAFT-agent of PEG by reacting methoxy-PEG (MPEG) with 3-benzylsulfanylthiocarbonylsufanylpropionic acid chloride (BSPAC), as shown in scheme 1-7. HEMA-PCL was prepared by ring-opening polymerization of HEMA using initiator. NDAPM was copolymerized with HEMA-PCL using PEG-BSPAC as RAFT-agent and AIBN as initiator at 70 °C in inert atmosphere. MPEG-*b*-P(NADPM-*co*-(HEMA-PCL) of 5-7 kDa were obtained. The polymers

are prepared with decreasing hydrophobic block content and increasing cationic component. The resulting compositions of polymers were determined by ^{1}H-NMR. The micellization of MPEG-*b*-P(NADPM-*co*-(HEMA-PCL) was studied in aqueous media using pyrene as fluorescent probe. DOX-loaded micelles were prepared and release of doxorubicin (DOX) *in vitro* was studied over a function of time. The gene delivery efficacy of using green fluorescent protein (GFP) and luciferase reporter plasmids was studied in HEK 293T cells. The co-delivery of DOX and plasmid DNA in 293T cells was confirmed using confocal microscopy.[60]

Zhong and coworkers prepared triblock copolymer of PDMAEMA-PCL-PDMAEMA for co-delivery of siRNA and palcitaxel.[61] The polymers were obtained by chain extension of CPADN-PCL-CPADN with DMAEMA. CPADN-PCL-CPADN was synthesized by coupling CPADN with dihydroxy PCL. The triblock copolymers formed micelles in water. These micelles showed effective loading of palcitexal and Vascular endothelial growth factor (VEGF)-siRNA, and their delivery in PC3 cells.[61]

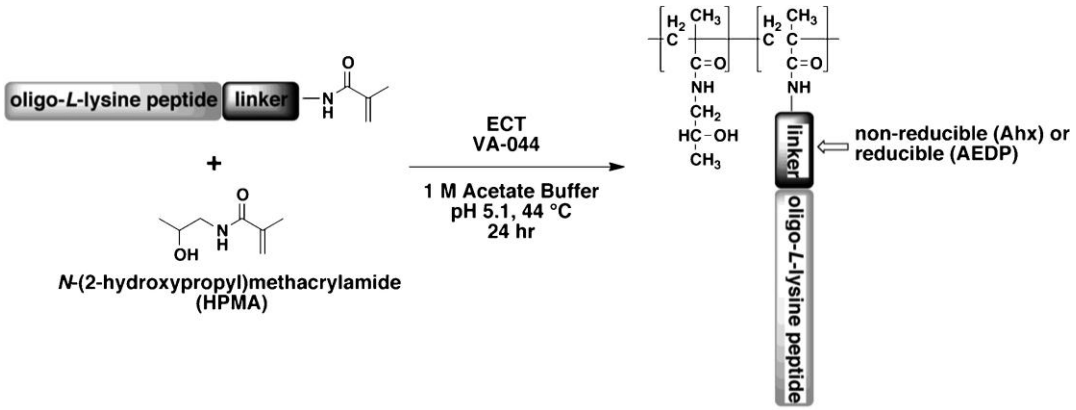
1.3. Role of Natural Polymers in Gene Delivery

1.3.1 Peptides for Gene Delivery

Another strategy to produce biocompatible gene delivery vectors is the use of natural biomolecules for gene delivery. Poly($_{L}$-lysine) (pLL) is a well-known natural degradable polypeptide for gene delivery applications. As expected, similar to other cationic polymers, high cationic content of pLL also increases its toxicity for gene delivery applications. The polymerization of lysine monomers

occur by ring opening polymerization of protected amino acid, which is then deprotected. The modifications of pLL with non-ionic moieties also compromise their amine content, hence decreasing the gene expression efficacies of the polypeptide as well.[6,13] The copolymerization of lysine monomer with other moieties *via* LRP requires the synthesis of lysine based methacrylate monomers. A library of statistical copolymers of HPMA and oligo-lysine was prepared *via* RAFT process by Pun and coworkers.[62] Oligo-lysine peptide monomer (MAhxK) was first prepared and was copolymerized with HPMA in random fashion in the presence of chain transfer agent, ethyl cyanovaleric trithiocarbonate (ETC) and initiator 2,2'-azobis[2-(2-iminazolin-2-yl)propane] dihydrochloride (VA-044). The polymers with varying oligo-lysine chain length (K5, K10 or K15), and different molecular weights were prepared. The amount of lysine was kept constant in each of these polymers (4.8-5 mmol of lysine/gram of polymer) to maintain the constant amine content in the polymer. The copolymers of high molecular weights produced stable polyplexes in physiological conditions than their low molecular weight analogues. In addition length of oligo-lysine chain was another important factor in dictating their gene expression. The polymers with shorter peptide chain produced highly stable particles and vice versa. The gene expression of these copolymers was in agreement with the results obtained for the stability of polyplexes. Hence, high molecular weight and smaller peptide chain length copolymers showed high gene expression.[62] In another study, HPMA-oligo-lysine polymers containing

either reducible or non-reducible cross-linker were prepared, as shown in scheme 1-10.[63]



Scheme 1-10. Synthesis of oligo-lysine and HPMA based copolymers in the presence and absence of reducible cross-linker.[63] Copyright 2012, Elsevier B.V. reproduced with permission.

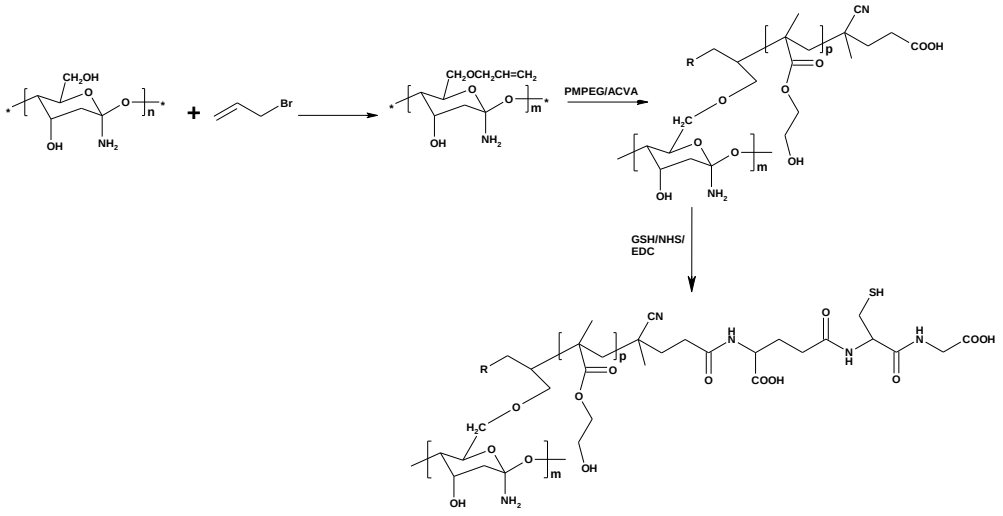
The two polymers had similar molecular weights (~ 80 kDa) and peptide compositions. The polymers were then compared for their gene expression and cell viability. Although reducible polymers showed low toxicity, they formed unstable polyplexes in physiological conditions, and hence lower gene expression.[63]

1.3.2 Polysaccharides for Gene Delivery

Carbohydrates are key structural and functional unit of living cells. In addition to providing a source of energy and metabolism, the advances at molecular biology level has provided new understanding of the role of carbohydrates in cells.[13] Carbohydrates are key component of cell membrane. All cell to cell communication and interactions of pathogens, such as viruses, and bacteria

occur at the cell surface between carbohydrates and carbohydrate specific proteins called 'lectins'.These interactions between carbohydrates and lectins are very weak with K*d* of 10^{-3} to 10^{-6} M, however, these interactions can be enhanced by multivalency also called 'cluster glycoside effect'.[64] As compared to monomers, polymers present a suitable class of materials for providing multivalent interactions.[64] Hence use of carbohydrate based copolymers is a nature inspired approach to produce biocompatible gene delivery vectors. The importance of carbohydrates based gene delivery vectors has been well-recognized in research. Dextran modified polymers were one of the first non-cationic vectors used for gene delivery by Vaheri and Panago in 1965.[65] Some other polysaccharides which are largely used for gene delivery applications are chitosan, β-cyclodextrin, schizophyllan and their cationic analogues.[13] Despite their non-toxic nature and high biocompatibility, low gene expression of these polysaccharides was a major drawback for their use as gene delivery agent. Chitosan (CS) is one of the most studied polysaccharide for gene delivery purposes. CS is composed of $_{D}$-glucosamine and *N*-acetyl-$_{D}$-glucosamine linked by β-(1,4)-glycosidic linkages.[13] The gene expression of CS is dependent on several factors such as molecular weight, degree of deacetylation, polymer/plasmid ratio, pH, serum concentration, and cell type. Moreover, modification of CS with various functional groups such as thiols, amines, PEG, lipids, and peptides has been reported to improve its transfection efficacies. The stimuli responsive chitosan was prepared by RAFT polymerization, by its modification with glutathione (GSH). Previous studies

have shown that thiolated polymers show enhanced permeation in living tissues, due to their improved muco-adhesive properties. The facile and controlled synthesis of GSH based polymers *via* RAFT process has the potential to produce a new generation of polysaccharide vectors with improved gene delivery efficacies. CS with 90% degree of deacetylation was modified with allyl bromide to yield allyl-chitosan. A brush like PMPEG was prepared by RAFT polymerization and was grafted to C6 position of allyl-chitosan *via* radical coupling reaction. (Scheme 1-11) Moreover reduced glutathione was introduced at the COOH group of hydrophilic PEG chain by amide bond. The cellular uptake of these polyplexes was studied and compared with its non-thiolated analogue. It was found that CS-PEG-GSH showed high cellular uptake than CS-PEG or CS alone in NIH3T3 cells. Moreover, in the presence of GSH (physiological conditions (10mM)) CS-PEG-GSH based polyplexes showed high DNA release, than CS-PEG based polyplexes.[66]



Scheme 1-11. Synthesis of chitosan-PEG-GSH conjugates. Figure adapted from reference [66].

1.3.3 Synthetic Sugar-based Polymers for Gene Delivery

Due to the limited control over the molecular weight, modification sites and polydispersities of polysaccharides based gene delivery vectors, it was not feasible to produce libraries of polymers with well-defined properties, such as compositions, molecular weights, shapes, and architectures. Davis and coworkers provided some insight by studying the effect of degree of modification of cyclodextrin with cationic polymers.[67,68] Later on Reineke and coworkers synthesized a variety of linear polyamides *via* polycondensation of esterified $_{D}$-glucaric acid with primary amines, in order to combine the biocompatible properties of CS with high gene expression of PEI.[16-18,69-73] Earlier studies were focused on optimizing the length of cationic segment for optimum gene expression.[69] The presence of sugar molecules in the back bone of cationic polymers provided the valuable information on the role of hydroxyl group (OH) stereochemistry on gene delivery efficacies.[16] In addition it was established through ITC that presence of OH groups in gene delivery vectors plays an important role in DNA binding-affinity of cationic polymers, possibly due to the hydrogen bonding between DNA and OH groups of polymer.[74] In order to mimic living systems, such as viruses, multivalent interactions of carbohydrate based polymers with cells are required. Prior to 1990s, synthetic techniques available to synthesize and customize carbohydrates based polymers of different design and configurations were limited.[75] Moreover, synthesis of sugar based monomers required protecting group chemistries, which were time consuming and costly. In 2000s Armes and coworkers successfully synthesized

sugar based monomers in the absence of protecting group chemistry, these monomers were further polymerized *via* ATRP to produce well-defined glycopolymers with pendant sugar moieties.[76] With the help of controlled radical polymerization techniques well-tailored glycopolymers and their copolymers of controlled architectures, molecular weights and narrow polydispersities were synthesized with the reactive functional groups, without the need of stringent reaction conditions and de-protection steps.

1.3.4 Phosphorylcholine based Copolymers

Hemocompatibility in addition to non-toxic nature of polymers is important for their use in biomedical applications. The biocompatible materials depicting the chemical structure of bio-membranes have been reported.[77,78] Nakabayashi and Ishihara *et al.* designed a methacrylate based monomer namely, 2-methacryloxyethyl phosphorylcholine (MPC) in late 1900s.[79,80] This methacrylate based monomer has been extensively polymerized using different LRP techniques and these polymers have been used for biomedical applications due to their non-toxic blood compatible nature.[79]

1.3.5 Nucleic Acid Based Polymers *via* RAFT Process

In addition to using electrostatic interactions for complexation of nucleic acid with a variety of cationic polymers, nucleic acids based polymers are also prepared *via* RAFT polymerization due to high tolerance of functional groups, facile polymerization under mild reaction conditions and facile modification of polymer end groups with biomolecules of interest. Well-defined polymer brushes on solid support have been reported. DNA-polymer bioconjugates on

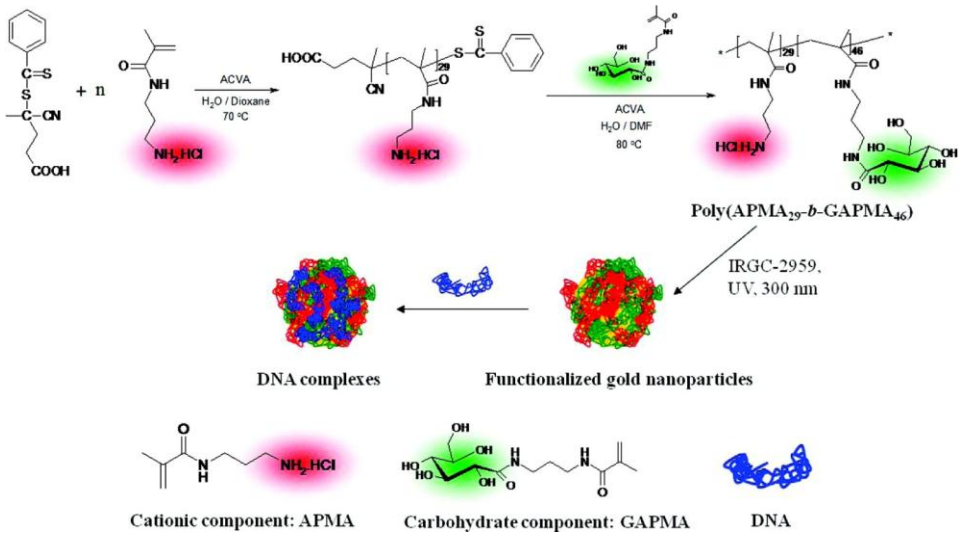
solid surface were grafted to produce a model study to show 1) the biocompatibility of RAFT polymerization technique, 2) well-controlled surface attachment chemistry and 3) the potential in DNA-based biomedical applications. ssDNA was used and modified with RAFT CTA, *via* 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) chemistry. CTA-modified-ssDNA was adsorbed in the gold surface *via* thiol bonding. The CTA modified gold surface was polymerized with OEGMA to obtain a layer of PEGMA on DNA. The surface was characterized by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), AFM.[81] The DNA functionalized copolymers were also prepared by McCormick and coworkers. The copolymers of P(HPMA-*st*-APMA) were produced *via* RAFT process in a random fashion. 89% of amine moieties of APMA were converted to active thiols using *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP). These active ends were then coupled to 5'-thiolated siRNA, *via* disulfide exchange reaction. The remaining amine content was modified with folic acid to obtain multifunctional bioconjugates.[82] Recently well-defined PEGacrylate polymers were prepared *via* RAFT technique. Pyridyldisulfide-terminated poly(PEG-acrylate) (PPEGA) were synthesized and were conjugated to thiol modified siRNA *via* disulfide linkage.[83] These conjugates showed high stability in serum containing media and optimum release of siRNA in the presence of GSH. The siRNA-PEG conjugates were further modified with KALA peptide and gene knockdown efficacy was studied in human neuroblastoma cells. KALA is a fusogenic peptide, which is well-known to

promote endosomal escape of complexes. These conjugates caused effective fluorescence inhibition in human neuroblastoma cells stably transfected with GFP.[84] DNA copolymers prepared via LRP approaches have shown a great potential to isolate specific ssDNA using affinity capillary electrophoresis method. PEG based oligodinucleotide blocks were prepared by Maeda and coworkers and were used to separate complimentary oligonucleotides specifically, using affinity capillary electrophoresis method.[85]

1.4. RAFT Polymers based Nanomaterials for Gene Delivery

Nanomaterials include a range of particles with well-defined shapes, and dimensions (2-100 nm). Metallic nanoparticles (such as gold nanoparticles, silver nanoparticles), organic nanoparticles (carbon nanotubes, fllurene, bucky balls, microgels, nanogels), inorganic materials (silica nanoparticles, iron nanoparticles, MnO particles), and semiconductors (quantum dots) are few examples of nanomaterials extensively studied for gene delivery applications.[3] A number of reviews have been published on the synthesis and unique optical, electronic, chemical and biochemical properties of these nanoparticles.[3,86-88] The use of nanomaterials for biomedical applications require their passivation with biocompatible polymers, hence telechelic polymers synthesized *via* RAFT process are used for the passivation of nanoparticles and for their use for various biomedical applications.[54,89-98] In our laboratory a variety of nanomaterials including gold nanoparticles[88,89], nanorods[102], carbon nanotubes[89], silica nanoparticles[101] and nanogels[54] are synthesized and surface functionalized with biocompatible polymers prepared *via* RAFT polymerization. These

nanomaterials are further investigated for their gene delivery efficacies in Hela cells.[89-91] The synthesis of gold nanoparticles stabilized with P(APMA-*st*-GAPMA), and their functionalization with DNA is depicted in scheme 1-12.



Scheme 1-12. Synthesis of cationic glycopolymers *via* RAFT polymerization, the synthesis of glyconanoparticles using photochemical approach and the complexation of cationic nanoparticles with ECFP plasmid.[91] Copyright 2009, American Chemical Society, reproduced with permission.

The gene expression of cationic glyconanoparticles of varying sizes is found to be dependent upon their diameter. Gold nanoparticles of 40 nm shows optimum gene expression along with high stability in physiological conditions, as compared to 10 nm nanoparticles.[92] Moreover, the mechanism of uptake of glyconanoparticles was studied and was found to be receptor mediated endocytosis.[91] In another report, McCormick and colleagues synthesized 10 nm gold nanoparticles functionalized with P(DMAPMA-*b*-HPMA) copolymer. These cationic nanoparticles were complexed with siRNA and showed effective gene silencing in Hela cells.[100] Glucose derived cationic glycopolymers were

synthesized and were used for the functionalization of carbon nanotubes. The presence of cationic glycopolymers on the surface of nanotubes significantly enhanced their water-solubility, along with their DNA condensation efficacies. These nanotubes were found to be potent gene delivery vectors in Hela cells.[90] Recently, thermosensitive nanomaterials, also called nanogels have been prepared using different polymerization approaches.[87]

1.5. Toxicity of RAFT based Polymers

Rapid advancements in the synthesis and use of polymers synthesized by RAFT process for biological applications demands a detailed study of biocompatible nature of these polymers. The hyperbranched glycopolymers synthesized *via* RAFT process are shown to be hemocompatible, as well as non-cytotoxic at certain concentrations. Maynard and colleagues have reported that toxicity of RAFT based polymers is dependent on the CTA used for its synthesis.[101] Dithioester based macroCTAs of poly(ethylene glycol acrylate) (PEGA) showed higher toxicity than trithiocarbonate based PEGAs. This change in toxicity of PEG based polymers was found to be dependent on the hydrolysis of macro CTAs, as free thioester groups in living tissues can interact with a variety of protein, hence causing acute toxicity. End group modification of RAFT based polymers is described as an invaluable tool before their use for systemic applications.[101] Later on Bulmus and coworkers provided a comprehensive study on the toxicity of RAFT based polymers in different cell lines, using different polymeric groups and CTAs. It was concluded that toxicity of RAFT based polymers was dependent not only on the type of CTA used for the

synthesis, but is also dependent on type of cell line used for the study, and type of functional units present in the polymer.[99] Fortunately, facile functionalization of polymeric end-groups provides an appropriate solution to the toxicity issues related with these polymers, along with incorporating the functional properties to these polymers.[27,102-104]

1.6. Concluding Remarks and Future Directions

The concept of gene delivery has been introduced in 1963 by Joshua Ledberg, and within 26 years the first human gene therapy trial, using viral vectors, has been performed by Rosenberg *et al.* However, the field of gene therapy entered into a sluggish phase in 2002, upon the development of adverse side effects of viral based gene therapies.[1] The focus has shifted on the synthesis and applications of non-viral vectors. The inherent toxicities, poor control over the molecular weights, polydispersities, architectures and shapes of cationic polymers synthesized along with their low gene expression posed major challenges in the advancement of field of gene delivery. LRP offers a feasible solution to produce a variety of cationic vectors of controlled dimensions. RAFT RAFT allows the synthesis of well-defined polymers and copolymers of varying architectures, molecular weights, shapes and compositions, and their resultant polyplexes are well-defined. These polymer libraries are independently synthesized and studied by different groups using different types of non-ionic monomers and biocompatible residues. All these results obtained within the last eight years show the great potential of RAFT based polymers for gene therapy. Some promising gene delivery vectors have been identified by the *in vitro*

studies. Carbohydrate based polymers are interesting candidates for gene delivery applications. However, due to the complex synthetic procedures involved in the preparation of glycopolymers, natural polysaccharides are mostly used for gene delivery application. The facile synthesis of glycopolymers by LRP allows the preparation of cationic glycopolymers of varying architectures, molecular weight, and compositions. RAFT polymerization approach is an appealing technique to produce cationic glycopolymers for biomedical applications. The linear statistical, and block cationic glycopolymers can be prepared by RAFT polymerization and are studied for their gene delivery applications. In addition, hyperbranched cationic glycopolymers and '*block-statistical'* analogues can also be synthesized *via* RAFT polymerization and their gene delivery efficacies can be studied. The *in vitro* experiments performed by different research groups conclude that in addition to the molecular weight and composition of polyplexes, their architecture is an important parameter to obtain optimum gene expression.

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1.7. References

[1] M.L. Edelstein, M.R. Abedi, J. Wixon, M.R. Edelstein, Gene therapy clinical trials worldwide 1989–2004- an overview. J. Gene Med. 6 (2004) 597-602.

[2] Z. Liu, Z. Zhang, C. Zhou, Y. Jio, Hydrophobic modifications of cationic polymers for gene delivery. Prog. Polym. Sci. 35 (2010) 1144-1162.

[3] X. Guo, L. Huang, Recent advances in nonviral vectors for gene delivery. Acc. Chem. Res. (2012) ASAP.

[4] D. Putnam, Polymers for gene delivery across length Scales. (5) 2006, 439-451.

[5] A.C. Roche, I. Fajac, S. Grosse, N. Frison, C. Rondanino, R. Mayer, N. Monsigny, Glycofection: facilitated gene transfer by cationic Glycopolymers. Cell. Mol. Life Sci. 60 (2003) 288-297.

[6] S.Y. Wong, J.M. Pelet, D. Putnam, Polymer systems for gene delivery-past, present, and future. Prog. Polym. Sci. 32 (2007) 799-837.

[7] T.M. Reineke, Poly(glycoamidoamine)s: cationic glycopolymers for DNA delivery. J. Poly Sci. Part A: Polym. Chem. 44 (2006) 6895-6908.

[8] A. Sizovs, P.M. McLendon, S. Srinivasachari, T.M. Reineke, Carbohydrate polymers for nonviral nucleic acid delivery. Top. Curr. Chem. 296 (2010) 131-190.

[9] Y. Nakayama, Hyperbranched polymeric “star vectors” for effective DNA or siRNA delivery. Acc. Chem. Res. (2012) ASAP.

[10] E. Wagner, J. Koleckner, Gene delivery using polymer therapeutics. Adv. Polym. Sci. 192 (2006) 135-173.

[11] D. Schaffert, E. Wagner, Gene therapy progress and prospects: synthetic polymer-based systems. Gene Ther. 15 (2008) 1131-1138.

[12] F.J. Xu, W.T. Yang, Polymer vectors via controlled/living radical polymerization for gene delivery. Prog. Polym. Sci. 36 (2011) 1099-1131.

[13] M.A. Mintzer, E.E. Simanek, Nonviral vectors for gene delivery. Chem. Rev. 109 (2009) 259-302.

[14] P.M. McLendon, D.J. Buckwalter, E.M. Davis, T.M. Reineke, Interaction of poly(glycoamidoamine)-DNA delivery vehicles with cell-surface glycosaminoglycans leads to polyplex internalization in a manner not solely dependent on charge. Mol. Pharmaceutics 7 (2010)1757-1768.

[15] G. Grandinetti, N.P. Ingle, T.M. Reineke, Interaction of poly(ethylenimine)-DNA polyplexes with mitochondria: implications for a mechanism of cytotoxicity. Mol. Pharmaceutics 8 (2011) 1709-1719.

[16] Y. Liu, T.M. Reineke, Hydroxyl stereochemistry and amine number within poly(glycoamidoamine)s affect intracellular DNA delivery. J. Am. Chem. Soc. 127 (2005) 3004-3015.

[17]S. Srinivasachari, Y. Liu, G. Zhang, L. Prevette, T.M. Reineke, Trehalose click polymers inhibit nanoparticle aggregation and promote pDNA delivery in serum. J. Am. Chem. Soc. 128 (2006) 8176-8184.

[18] S. Srinivasachari, K.M. Fichter, T.M. Reineke, Polycationic β-cyclodextrin "click clusters": monodisperse and versatile scaffolds for nucleic acid delivery. J. Am. Chem. Soc. 130 (2008) 4618-4627.

[19] S. Grosse, Y. Aron, I. Honore, G. Thevenot, C. Danel, A-C. Roche, M. Monsigny, I. Fajac, Lactosylated polyethylenimine for gene transfer into airway epithelial cells: role of the sugar moiety in cell delivery and intracellular trafficking of the complexes. J. Gene Med. 6 (2004) 345-356.

[20] Y.H. Choi, F. Liu, J.S. Park, S.W. Kim, Lactose-poly(ethylene glycol)-grafted poly-$_L$-lysine as hepatoma cell-targeted gene carrier. Bioconjugate Chem. 9 (1998) 708-718.

[21] K. Wada, H. Arima, T. Tsutsumi. Y. Chihara, K. Hattori, F. Hirayama, K. Uekama, Improvement of gene delivery mediated by mannosylated dendrimer/α-cyclodextrin conjugates. J. Control. Release 104 (2005) 397-413.

[22] T. Anno, T. Higashi, K. Motoyama, F. Hirayama, K. Uekama, H. Arima, Possible enhancing mechanisms for gene transfer activity of glucuronylglucosyl-β-cyclodextrin/dendrimer. Intl. J. Pharma. 426 (2012) 239-247.

[23] A.W. York, S.E. Kirkland, C.L. McCormick, Advances in the synthesis of amphiphilic block copolymers via RAFT polymerization: stimuli-responsive drug and gene delivery. Adv. Drug Del. Rev. 60 (2008)1018-1036.

[24] D.S.H. Chu, J.G. Schellinger, J. Shi, A.J. Convertine, P.S. Stayton, S.H. Pun, Application of living free radical polymerization for nucleic acid delivery. Acc. Chem. Res. (2012) ASAP.

[25] G. Moad, E. Rizzardo, S.H. Thang, Toward living radical polymerization. Acc. Chem. Res. 41 (2008) 1133-1142.

[26] G. Moad, The emergence of RAFT polymerization. Aust. J. Chem. 59 (2006) 661-662.

[27] G. Moad, E. Rizzardo, S.H. Thang, Living radical polymerization by the RAFT process. Aust. J. Chem. 58 (2005) 379-410.

[28] C. Boyer, J. Liu, V. Bulmus, T.P. Davis, RAFT polymer end-group modification and chain coupling/conjugation via disulfide bonds. Aust. J. Chem. 62 (2009) 830-847.

[29] M. Semsarilar, S. Perrier, 'Green' reversible addition-fragmentation chain-transfer (raFt) polymerization. 2 (2010) 811-820.

[30] V. Bulmus, RAFT polymerization mediated bioconjugation strategies. Polym. Chem. 2 (2011)1463-1472.

[31] C. Boyer, V. Bulmus, T.P. Davis, V. Ladmiral, J. Liu, S. Perrier, Bioapplications of RAFT polymerization. Chem. Rev. 109 (2009) 5402-5436.

[32] S. Pearson, N. Allen, M.H. Stenzel, Core-shell particles with glycopolymer shell and polynucleoside core via RAFT: from micelles to rod. J. Poly. Sci. Part A: Polym. Chem. 47 (2009) 1706-1723.

[33] D. Smith, A.C. Holley, C.L. McCormick, RAFT-synthesized copolymers and conjugates designed for therapeutic delivery of siRNA. Polym. Chem. 2 (2011)1428-1441.

[34] Z. Deng, H. Bouchekif, K. Babooram, A. Housni, N. Choytun, R. Narain, Facile synthesis of controlled-structure primary amine-based methacrylamide polymers via the reversible addition-fragmentation chain transfer process. J. Poly. Sci. Part A: Polym. Chem. 46 (2008) 4984-4996.

[35] Y.A. Vasilieva, D.B. Thomas, C.W. Scales, C.L. McCormick, Direct controlled polymerization of a cationic methacrylamido monomer in aqueous media via the RAFT process. Macromolecules 37 (2004) 2728-2737.

[36] C.W. Scales, F. Huang, N. Li, Y.A. Vasilieva, J. Ray, A.J. Convertine, C.L. McCormick, Corona-stabilized interpolyelectrolyte complexes of siRNA with nonimmunogenic, hydrophilic/cationic block copolymers prepared by aqueous RAFT polymerization. Macromolecules 39 (2006) 6871-6881.

[37] A.W. York, Y. Zhang, A.C. Holley, Y. Guo, F. Huang, C.L. McCormick, Facile synthesis of multivalent folate-block copolymer conjugates via aqueous RAFT polymerization: targeted delivery of siRNA and subsequent gene suppression. Biomacromolecules 10 (2009) 936-943.

[38] C. Zhu, S. Jung, G. Si, R. Cheng, F. Meng, X. Zhu, T.G. Park, Z. Zhong, Cationic methacrylate copolymers containing primary and tertiary amino side groups: controlled synthesis via RAFT polymerization, DNA condensation, and in vitro gene transfection. J. Poly. Sci. Part A: Polym. Chem. 48 (2010) 2869-2877.

[39] C. Lin, J.F.J. Engbersen, The role of the disulfide group in disulfide-based polymeric gene carriers. Expert Opin. Drug Deliv. 6 (2009) 421-439.

[40] S. Son, R. Namgung, J. Kim, K. Singha, W.J. Kim, Bioreducible polymers for gene silencing and delivery. Acc. Chem. Res. (2012) ASAP, DOI: 10.1021/ar200248u.

[41] Y-Z. You, D.S. Manickam, Q-H. Zhou, D. Oupicky, Reducible poly(2-dimethylaminoethyl methacrylate): synthesis, cytotoxicity, and gene delivery activity. J. Control. Release 122 (2007) 217-225.

[42] N.P. Truong, Z. Jia, M. Burgess, L. Payne, N.A.J. McMillan, M.J. Monteiro, Self-catalyzed degradable cationic polymer for release of DNA. Biomacromolecules 12 (2011) 3540-3548.

[43] S. Venkataraman, W.L. Ong, Z.Y. Ong, S.C. Loo, P.L.R. Ee, Y.Y. Yang, The role of PEG architecture and molecular weight in the gene transfection performance of PEGylated poly(dimethylaminoethyl methacrylate) based cationic polymers. Biomaterials 32 (2011) 2369-2378.

[44] C. Zhu, M. Zheng, F. Meng, F.M. Mickler, N. Ruthardt, X. Zhu, Z. Zhong, Reversibly shielded DNA polyplexes based on bioreducible PDMAEMA-SS-PEG-SS-PDMAEMA triblock copolymers mediate markedly enhanced nonviral gene transfection. Biomacromolecules 13 (2012) 769-778.

[45] Z. Deng, M. Ahmed, R. Narain, Novel well-defined glycopolymers synthesized via the reversible addition fragmentation chain transfer process in aqueous media. J. Polym. Sci. Part A: Polym. Chem. 47 (2009) 614-627.

[46] O. Samsonova, C. Pfeiffer, M. Hellmund, O.M. Merkel, T. Kissel, Low molecular weight pDMAEMA-block-pHEMA block-copolymers synthesized via RAFT-polymerization: potential non-viral gene delivery agents? Polymers 3 (2011) 693-718.

[47] A.H. Alidedeoglu, A.W. York, C.L. McCormick, S.E. Morgan, Aqueous RAFT polymerization of 2-aminoethyl methacrylate to produce well-defined, primary amine functional homo- and copolymers. J. Polym. Sci. Part A: Polym. Chem. 47 (2009) 5405-5415.

[48] D. Konkolewicz, A. Gray-Weale, S. Perrier, Hyperbranched polymers by thiol-yne chemistry: from small molecules to functional polymers. J. Am. Chem. Soc. 131 (2009) 18075-18077.

[49] L. Tao, J. Liu, B.H. Tan, T.P. Davis, RAFT synthesis and DNA binding of biodegradable, hyperbranched poly(2-(dimethylamino)ethyl methacrylate. Macromolecules 42 (2009) 4960-4962.

[50] D.J. Siegwart, K.A. Whitehead, L. Nuhn, G. Sahay, H. Cheng, S. Jiang, M. Ma, A. Lytton-Jean, A. Vegas, P. Fenton, C.G. Levins, K.T. Love, H. Lee, C. Cortez, S.P. Collins, Y.F. Li, J. Jang, W. Querbes, C. Zurenko, T. Novobrantseva, R. Langer D.G. Andersona, Combinatorial synthesis of chemically diverse core-shell nanoparticles for intracellular delivery. PNAS 108 (2011) 12996-13001.

[51] H.S. Bisht, D.S. Manickam, Y. You, D. Oupicky, Temperature-controlled properties of DNA complexes with poly(ethylenimine)-graft-poly(N-isopropylacrylamide). Biomacromolecules 7 (2006) 1169-1178.

[52] K. Isoda, N. Kanayama, D. Miyamoto, T. Takarada, M. Maeda, RAFT-generated poly(N-isopropylacrylamide)–DNA block copolymers for temperature-responsive formation of polymer micelles. Reactive & Functional Polymers 71 (2011) 367-371.

[53] N. Bhuchar, Z. Deng, K. Ishihara, R. Narain, Detailed study of the reversible addition–fragmentation chain transfer polymerization and co-polymerization of 2-methacryloyloxyethyl phosphorylcholine. Polym. Chem. 2 (2011) 632-639.

[54] J.H. Tan, N.A.J. McMillan, E. Payne, C. Alexander, F. Heath, A.K. Whittaker, K.J. Thurecht, Hyperbranched polymers as delivery vectors for oligonucleotides. J. Polym. Sci. Part A: Polym. Chem. 50 (2012) 2585-2595.

[55] M.J. Manganiello, C. Cheng, A.J. Convertine, J.D. Bryers, P.S. Stayton, Diblock copolymers with tunable pH transitions for gene delivery. Biomaterials 33 (2012) 2301-2309.

[56] A.J. Convertine, D.S.W. Benoit, C.L. Duvall, A.S. Hoffman, P.S. Stayton, Development of a novel endosomolytic diblock copolymer for siRNA delivery. J. Control. Release, 133 (2009) 221-229.

[57] D.S.W. Benoit, N. Srinivasan, A.D. Shubin, P.S. Stayton, Synthesis of folate-functionalized RAFT polymers for targeted siRNA delivery. Biomacromolecules 12 (2011) 2708-2714.

[58] M.C. Palanca-Wessels, A.J. Convertine, R. Cutler-Strom, G.C. Booth, F. Lee, G.Y. Berguig, P.S. Stayton, O.W. Press, Anti-CD22 antibody targeting of pH-responsive micelles enhances small interfering RNA delivery and gene silencing in lymphoma cells. Molecular Ther. 19 (2011) 1529-1537.

[59] D.S.W. Benoit, S.M. Henry, A.D. Shubin, A.S. Hoffman, P.S. Stayton, pH-Responsive polymeric siRNA carriers sensitize multidrug resistant ovarian cancer cells to doxorubicin via knockdown of polo-like kinase 1. Molecular Pharm. 7 (2010)442-455.

[60] J-L. Zhu, H, Cheng, Y. Jin, S-X. Cheng, X-Z. Zhang, R-X. Zhou, Novel polycationic micelles for drug delivery and gene transfer. J. Mater. Chem. 18 (2008) 4433-4441.

[61] C. Zhu, S. Jung, S. Luo, F. Meng, X. Zhu, T.G. Park, Co-delivery of siRNA and paclitaxel into cancer cells by biodegradable cationic micelles based on PDMAEMA–PCL–PDMAEMA triblock copolymers. Biomaterials 31 (2010)2408-2416.

[62] R.N. Johnson, D.S.H. Chu, J. Shi, J.G. Schellinger, P.M. Carlson, S.H. Pun, HPMA-oligolysine copolymers for gene delivery: Optimization of peptide length and polymer molecular weight. J. Control. Release 155 (2011) 303-311.

[63] J. Shi, R.N. Johnson, J.G. Schellinger, P.M. Carlson, S.H. Pun, Reducible HPMA-co-oligolysine copolymers for nucleic acid delivery. Intl. J. Pharma. 427 (2012) 113-122.

[64] S.G. Spain, N.R. Cameron, A spoonful of sugar: the application of glycopolymers in therapeutics. Polym. Chem. 2 (2011) 60-68.

[65] A. Vaheri, J.S. Pagano, Infectious poliovirus RNA: a sensitive method of assay. Virology 17 (1965) 456-464.

[66] C. Li, T. Guo, D. Zhou, Y. Hu, H. Zhou, S. Wang, J. Chen, Z. Zhang, A novel glutathione modified chitosan conjugate for efficient gene delivery. J. Control. Release 154 (2011) 177-188.

[67] T.M. Reineke, M.E. Davis, Structural effects of carbohydrate-containing polycations on gene delivery. 2. charge center type. Bioconjugate Chem. 14 (2003)255-261.

[68] T.M. Reineke, M.E. Davis, Structural effects of carbohydrate-containing polycations on gene delivery. 1. carbohydrate size and its distance from charge centers. Bioconjugate Chem. 14 (2003) 247-254.

[69] A.E. Smith, A. Sizovs, G. Grandinetti, L. Xue, T.M. Reineke, Diblock glycopolymers promote colloidal stability of polyplexes and effective pDNA and siRNA delivery under physiological salt and serum conditions. Biomacromolecules 12 (2011) 3015-3022.

[70] Y. Liu, L. Wenning, M. Lynch, T.M. Reineke, New poly(D-glucaramidoamine)s induce DNA nanoparticle formation and efficient gene delivery into mammalian cells. J. Am. Chem. Soc. 126 (2004) 7422-7423.

[71] C-C. Lee, Y. Liu, T.M. Reineke, General structure-activity relationship for poly(glycoamidoamine)s: the effect of amine density on cytotoxicity and DNA delivery efficiency. Bioconjugate Chem. 19 (2008) 428-440.

[72] P.M. McLendon, D.J. Buckwalter, E.M. Davis, T.M. Reineke, Interaction of poly(glycoamidoamine) DNA delivery vehicles with cell-surface glycosaminoglycans leads to polyplex internalization in a manner not solely dependent on charge. Mol.Pharmaceutics 7 (2010) 1757-1768.

[73] G. Grandinetti, N.P. Ingle, T.M. Reineke, Interaction of poly(ethylenimine)DNA polyplexes with mitochondria: implications for a mechanism of cytotoxicity. Mol. Pharmaceutics 8 (2011) 1709-1719.

[74] L.E. Prevette, T.E. Kodger, T.M. Reineke, M. L. Lynch, Deciphering the role of hydrogen bonding in enhancing pDNA-polycation interactions. Langmuir 23 (2007) 9773-9884.

[75] Q. Wang, J.S. Dordick, R.J. Linhardt, Synthesis and application of carbohydrate-containing polymers. Chem. Mater. 14 (2002) 3232-3244.

[76] R. Narain, S.P. Armes, Synthesis of low polydispersity, controlled-structure sugar methacrylate polymers under mild conditions without protecting group chemistry. Chem. Comm. (2002) 2776-2777.

[77] M. Ahmed, F.L.B. Lai, J.N. Kizhakkedathu, R. Narain, Hyperbranched glycopolymers for blood biocompatibility. Bioconjugate Chem. 23 (2012) 1050-1058.

[78] Y. Iwasaki, K. Ishihara, Phosphorylcholine-containing polymers for biomedical applications. Anal. Bioanal. Chem. 381 (2005) 534-546.

[79] Y. Kadoma, N. Nakabayashi, E. Masuhara, J. Yamauchi. "Synthesis and hemolysis test of the polymer containing phosphorylcholine groups. Koubunshi Ronbunshu (Jpn. J. Polym. Sci. Technol.) 35 (1978) 423-427.

[80] K. Ishihara, T. Ueda, N. Nakabayashi, Synthesis of surface-anchored DNA-polymer bioconjugates using reversible addition-fragmentation chain transfer polymerization Polym. J. 22 (1990) 355-360.

[81] P. He, L. He, Synthesis of surface-anchored DNA-polymer bioconjugates using reversible addition-fragmentation chain transfer polymerization. Biomacromolecules 10 (2009)1804-1809.

[82] A.W. York, F. Huang, C.L. McCormick, Rational design of targeted cancer therapeutics through the multiconjugation of folate and cleavable siRNA to RAFT-synthesized (HPMA-s-APMA) copolymers. Biomacromolecules 11 (2010) 505-514.

[83] K.L. Heredia, T.H. Nguyen, C-W. Chang, V. Bulmus, T.P. Davis, H.D. Maynard, Reversible siRNA–polymer conjugates by RAFT polymerization. Chem. Commun. (2008) 3245-3247.

[84] K. Gunasekaran, T.H. Nguyen, H.D. Maynard, T.P. Davis, V. Bulmus, Conjugation of siRNA with comb-type PEG enhances serum stability and gene silencing efficiency. Macromol. Rapid Commun. 32 (2011) 654-659.

[85] N. Kanayama, H. Shibata, A. Kimura, D. Miyamoto, T. Takarada, M. Maeda, RAFT-generated polyacrylamide-DNA block copolymers for single-nucleotide polymorphism genotyping by affinity capillary electrophoresis. Biomacromolecules 10 (2009) 805-813.

[86] K.S. Ghosh, T. Pal, Interparticle coupling effect on the surface plasmon resonance of gold nanoparticles: from theory to applications. Chem. Rev. 107 (2007) 4797-4862.

[87] M.H. Smith, L.A. Lyon, Multifunctional nanogels for siRNA delivery. Acc. Chem. Res. (2012) ASAP, DOI: 10.1021/ar200216f.

[88] S.S. Kelkar, T.M. Reineke, Theranostics: combining imaging and therapy. Bioconjugate Chem. 122 (2011) 1879-1903.

[89] K. Babooram, R. Narain, Fabrication of SWNT/silica composites by the sol-gel process. ACS Applied Mater. Interface. 1 (2009) 181-186.

[90] M. Ahmed, X. Jiang, Z. Deng, R. Narain, Cationic glyco-functionalized single walled carbon nanotubes as efficient gene delivery vehicles. Bioconjugate Chem. 20 (2009) 2017-2022.

[91] M. Ahmed, Z. Deng, S. Liu, R. Lafrenie, R. Narain, Cationic glyconanoparticles: their complexation with DNA, cellular uptake, and transfection efficiencies. Bioconjugate Chem. 20 (2009) 2169-2176.

[92] M. Ahmed, Z. Deng, R. Narain, Study of size dependent transfection efficiencies of gold nanoparticles in human cell line. ACS Applied Mater. Interface 1 (2009) 1980-1987.

[93] X. Jiang, M. Ahmed, Z. Deng, R. Narain, Biotinylated glyco-functionalized quantum dots: synthesis, characterization, and cyto-toxicity studies. 20 (2009) 994-1001.

[94] F.B. Lollmahommed, R. Narain, Photochemical approach toward deposition of gold nanoparticles on functionalized carbon nanotubes. Langmuir 27 (2011) 12642-12649.

[95] X. Jiang, A. Housni, G. Gody, P. Boullanger, M-T. Charreyre, T. Delair, R. Narain, Synthesis of biotinylated alpha-d-mannoside or N-acetyl beta-d-glucosaminoside decorated gold nanoparticles: study of their biomolecular recognition with Con A and WGA lectins. 21 (2010) 521-530.

[96] A. Housni, M. Ahmed, S. Liu, R. Narain, Monodisperse protein stabilized gold nanoparticles via a simple photochemical process. J. Phys. Chem. C 112 (2008) 12282-12290.

[97] R. Narain, A. Housni, G. Gody, P. Boullanger, M-Y. Charreyre, T. Delair, Preparation of biotinylated glyconanoparticles via a photochemical process and study of their bioconjugation to streptavidin. Langmuir 23 (2008) 12835-12841.

[98] Y. Kotsuchibashi, Y. Zhang, M. Ahmed, M. Ebara, T. Aoyagi, R. Narain, Fabrication of FITC-doped Ssilica nanoparticles and their cellular uptake. Soft Matter. (2012) Submitted.

[99] M. Ahmed, R. Narain, Rapid Synthesis of gold nanorods using a one step photochemical strategy. Langmuir 26 (2010) 18392-18399.

[100] S. Kirkland-York, Y. Zhang, A.E. Smith, A.W. York, F. Huang, C.L. McCormick, Tailored design of Au nanoparticle-siRNA carriers utilizing reversible addition-fragmentation chain transfer polymers. Biomacromolecules 11 (2010) 1052-1059.

[101] C.W. Chang, E. Bays, L. Tao, S.N.S. Alconel, H.D. Maynard, Differences in cytotoxicity of poly(PEGA)s synthesized by reversible addition–fragmentation chain transfer polymerization. Chem. Commun. (2009) 3580-3582.

[102] D. Pissuwan, C. Boyer, K. Gunasekaran, T.P. Davis, V. Bulmus, In vitro cytotoxicity of RAFT polymers. Biomacromolecules 11 (2010) 412-420.

[103] J. Hentschel, K. Bleek, O. Ernst, J-F. Lutz, G.H. Borner, Easy access to bioactive peptide-polymer conjugates via RAFT. Macromolecules 41 (2008) 1073-1075.

[104] H. Willock, R.K. O'Reilly, End group removal and modification of RAFT polymers. Polym. Chem. 1 (2010) 149-157.

Chapter 2. Objectives

Early studies on viral particles for gene expression have been established as remarkable vectors for clinical uses. However, the high toxicities and immune responses associated with viral vectors have hampered their further uses. Much attention in recent years has therefore been devoted to the development of non-viral vectors for gene delivery. A variety of non-viral vectors is synthesized and is evaluated for their gene expression, in an effort to produce efficient systems with high gene expression and low toxicities. Cationic vectors are the ideal choice, mainly due to the facile condensation of DNA, formulation of stable polyplexes due to electrostatic repulsion and rapid uptake by mammalian cells. The toxicity is one of the major and the most studied drawback of cationic vectors. The modification of cationic vectors with stealth layers such as PEG has been reported to reduce their toxicities, however, this approach also compromises their gene delivery efficacies. In this study, carbohydrates or phosphorylcholine based polymers of varying molecular weights, compositions and architectures are synthesized *via* RAFT to produce gene delivery vectors with low toxicities and high gene expression. The use of carbohydrates in gene delivery applications has been limited to targeting applications, possibly due to the stringent reaction conditions required in the past to produce well-defined carbohydrate based vectors. With the advances in the field of chemistry, it is now possible to synthesize carbohydrate based monomers in the absence of protected group chemistry. The variety of monomers polymerized *via* RAFT, high tolerance to functional end groups, and absence of metal catalysts, allowed

the synthesis of carbohydrates and phosphorylcholine based gene delivery vectors with well-defined architectures, molecular weights and compositions. The cationic glyco- and phosphorylcholine-based polymers of varying molecular weights, morphologies, architectures, and compositions were prepared and were studied in detail to determine the ideal characteristics required for gene delivery.

The first part of the thesis focuses on the synthesis of cationic glycopolymers of varying architectures (block *versus* statistical configurations) and molecular weights. The linear cationic glycopolymers are synthesized using 2-aminoethyl methacrylamide (AEMA) and 3-aminopropyl methacrylamide (APMA) and 3-glucanoamidopropyl methacrylamide (GAPMA). Copolymers of similar molecular weights, and compositions but of different architectures (*block versus statistical*) are prepared and are complexed with β-galactosidase plasmid. These complexes are studied for their gene delivery efficacies, as a function of amine component and molecular weights in hepatocytes and Hela cells. Moreover, the interaction of serum proteins with gene delivery vectors is explored in detail. It is concluded that high molecular weight statistical copolymers show high gene expression and low toxicities, as compared to their block analogues. However, their aggregation in serum containing media can be a significant drawback for their use for *in vivo* applications.

In an attempt to produce cationic glycopolymer with improved gene expression, cationic hyperbranched glycopolymers are synthesized in another study. The polymers of branched architecture are reported to show high gene expression than their linear analogues. In this Chapter, glucose-derived or galactose-based

hyperbranched cationic polymers are prepared *via* RAFT polymerization for gene delivery. This is the first approach, where hyperbranched polymers synthesized *via* RAFT process are studied for their gene expression in Hep G2 and HEK293T cells. These polymers indeed show low toxicities and enhanced gene expression, along with low interactions with serum proteins, possibly due to high carbohydrate residues on the polyplex surface. The presence of carbohydrates on the surface of polyplexes is further confirmed by studying the interaction of these hyperbranched cationic glycopolymer-DNA complexes with galactose specific plant lectins, namely *Riccinus Communis* (RCA_{120}) and *Jacalin*.

The use of MPC based copolymers for gene delivery purposes have been explored by others. In an attempt to develop non-toxic polymer, a library of MPC-based copolymers of statistical and block configurations is synthesized. The copolymers produced are studied in detail for their toxicities, gene expression and cellular uptake in Hep G2 cells. These polymers based polyplexes (regardless of their architecture) showed high stability in serum containing media. In contrast to cationic glycopolymers of statistical configuration, MPC based statistical copolymers showed no gene expression.

To further investigate the significant differences in gene expression of MPC and carbohydrate based copolymers, MPC and carbohydrates based analogues are prepared in Chapter 6. The cationic glycopolymers or MPC based cationic polymers of a novel architecture termed as '*block statistical'* polymers are prepared *via* RAFT polymerization. The polymers are then tested for their

cellular uptake, nuclear localization ability and gene delivery. It is found that '*block statistical*' polymers show low interactions with serum proteins, along with low toxicities and low gene expression, as compared to their statistical analogues. MPC-Sugar based analogues were also prepared and it was possible to enhance the gene expression of MPC based polymer by incorporating sugar residues in the copolymers, at low polymer/plasmid ratios.

Chapter 3.

The Effect of Polymer Architecture, Composition, and Molecular Weight on the Properties of Glycopolymer-based Non-Viral Gene Delivery Systems

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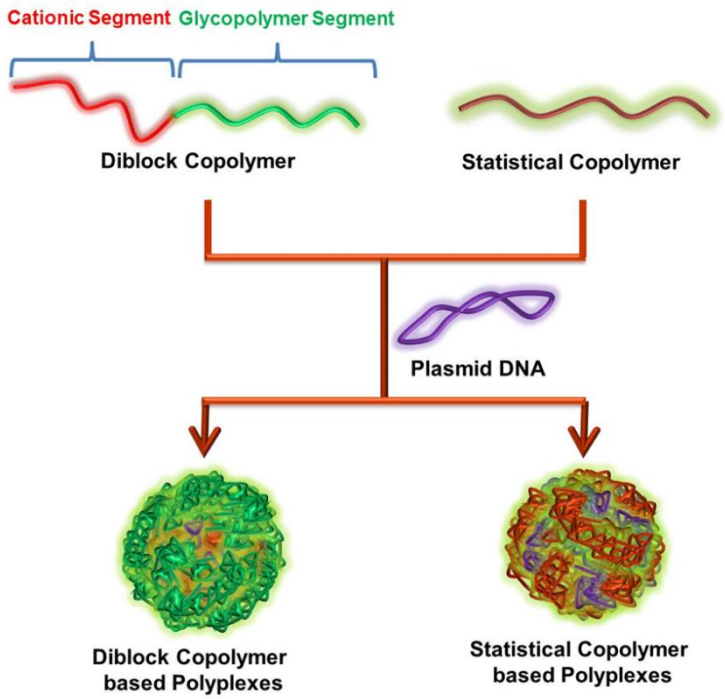
3.1. Introduction

Gene therapy has the potential to treat both acquired and inherited genetic disorders like cancer, and cystic fibrosis. [1,2] The successful delivery of exogenous DNA into malignant cells can also help to replace chemotherapeutic treatment for these lethal diseases. A variety of non-viral vectors have been synthesized, however, they all lack the inherent properties of viral vectors, including their ability to bypass cellular barriers and immune defence mechanisms, which are key to their success. [1,3] The control on the design of the non-viral vectors is crucial for enhanced biological functions, in the development of cationic polymeric vehicles the following parameters such as molecular weights and spacing of charged groups need to be set. Therefore, the synthesis of well-defined polymers with pre-determined compositions and structures are essential in improving the transfection efficiencies. [1,3] The cationic polymer vehicles synthesized and largely utilized for gene delivery today has been chosen from the library of polymers. Poly(ethyleneimine) (PEI), poly(*β*-amino esters), poly(*N,N'*-dimethylaminoethyl methacrylate) poly(DMAEMA) are few examples of polymers that have been selected by the screening method and now some of them are used as standard for gene delivery purpose. [4-6]

The use of natural cationic polysaccharides for gene delivery are the focus of research from decades, as carbohydrates are thought to be involved in condensing DNA *via* hydrogen bonding, thus reducing the need of excess cationic charge and hence decreasing the toxicity of the system. [7-13] The

monomeric carbohydrate units are also found to interact with specific cell receptors and may serve as targeting agent without the need for further modification of gene delivery vehicle with receptors. [14,15] A variety of natural cationic polysaccharides has been modified and studied for gene delivery. Chitosan, dextran and β-cyclodextrin are a few examples of natural carriers that are extensively studied. [7,9,16-19] These macromolecules have been modified by a variety of cationic or hydrophobic molecules and the structure-activity relationship of these modified biomolecules is studied in detail. [20-22] However, due to the limited control on the structure of these naturally occurring carbohydrate-based carriers, it is desirable to produce synthetic cationic glycopolymers of controlled molecular weight and architecture to provide a clear and concise overview of structure related gene delivery efficacies. Reineke and coworkers synthesized poly(glucoamidoamine) PGAs by polymerizing oligoethyleneamine monomers with monosaccharides *via* amide linkage in random fashion, yielding cationic glycopolymers with high polydispersities. [10-12] Some systematic investigations of these PGAs were performed based upon the hydroxyl group stereochemistry of the polymers to determine how the molecular structure are related to the biological activity. [12] To better understand the role of carbohydrates in cationic gene delivery carriers, it is desirable to produce well-defined glycopolymers of different architecture. In the past, synthesis of carbohydrate based polymers was largely limited due to the excessive steps of protection and deprotection of carbohydrate groups, which does not allow the synthesis of library of cationic glycopolymer of varying

properties by any facile method. [23] With the advent of living radical polymerization, synthesis of carbohydrate based polymers is facilitated as the reactions can now occur in the absence of protected group chemistry, in aqueous media and the polymers produced are of controlled dimensions. [24,25] In this work, well-defined cationic glycopolymers of varying molecular weight, cationic chain length, carbohydrates to cationic contents and different architecture (block versus random polymers) are synthesized by RAFT polymerization. The cationic glycopolymers ranging from 3-30 kDa synthesized are then complexed with *β*-galactosidase plasmid and the resulting polyplexes are analyzed using dynamic light scattering (DLS) and zeta potential for their size and charge, respectively, as shown in scheme 3-1.



Scheme 3-1. Synthesis of statistical and diblock copolymers based polyplexes using β-galactosidase plasmid.

The polyplexes obtained and polymers alone are subjected to cell viability assay. It is found that the cell viability and the size and charge of polyplexes formed are dependent on the copolymer architectures. Moreover, the transfection efficiencies of these cationic glycopolymers are determined by using *β*-galactosidase assay, the gene delivery efficacies of these copolymers are evaluated as a function of polymer architecture. The effects of serum proteins on polyplexes were studied in detail.

3.2. Material and Methods.

3.2.1. Materials

Branched PEI (M_w= 25 kDa,), *O*-Nitrophenyl *β*-D-galactopyranoside, (ONPG) (enzymatic), 37 wt. % formalin, Fluorescein isothiocyanate (FITC), *β* -Mercaptoethanol, MTT assay kit to determine cell viability, and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were purchased from Sigma Aldrich. Linear PEI (M_w= 25 kDa) was purchased from Polysciences Inc. Cell Culture media Dulbecco's Modified Eagle Medium (DMEM; high and low glucose with L-glutamine and sodium pyruvate), penicillin (10000 U/mL), and streptomycin (10 mg/mL), 0.25% trypsin, Dulbecco's modified Phosphate Buffer Saline (DPBS) and Fetal Bovine Serum (FBS) were from Invitrogen. Micro BCA assay kit was obtained from Fisher Scientific. Gwiz *β*-galactosidase plasmid was purchased from Aldevron. Bovine serum albumin (BSA) was from Promega Corporation.

3.2.2. Synthesis of Monomers and Polymers.

The polymers of varying degree of polymerization and architecture were synthesized using RAFT polymerization according to previously reported protocols. [26,27] The statistical copolymerization of 3-aminopropyl methacrylamide (APMA) or 2-amino ethyl methacrylamide (AEMA) and 3-gluconamidopropyl methacrylamide (GAPMA) was performed at 70 °C in the presence of 4-cyanopentanoic acid dithiobenzoate (CTP) and 4,4'-azobis(4-cyanovaleric acid) (ACVA) as chain transfer agent and initiator, respectively. In a typical protocol APMA (0.1 g, 0.56 mmol) and GAPMA (0.3 g, 0.93 mmol) was dissolved in doubly distilled water (1.5 mL) followed by the addition of CTP (2.4 mg, 7.2 μmol) and ACVA (1.2 mg, 3.6 μmol) in 0.3 mL of DMF (targeted DP_n = 105, Targeted molecular weight = 25 kDa). The mixture was subjected to three freeze-thaw cycles in 25 mL flask and flask was placed in oil bath for polymerization under nitrogen for 12 hours. The polymerization was quenched using liquid nitrogen and polymer was precipitated in acetone. The product was extensively washed with methanol and *N,N'*-dimethylformamide (DMF) to remove the excess of monomers and was dried. The polymer was then analyzed using Varian 500 NMR. The molecular weight and molecular weight distributions of homo and copolymers were determined using aqueous GPC; Viscotek GPC system.0.5 M sodium acetate/0.5M acetic acid buffer was used as eluents at room temperature and at a flow rate of 1.0 mL/min and pullulan standards (M_w = 500-404,000 g/mol) were used for calibration.

In a typical diblock copolymerization experiment, APMA (3 g, 17 mmol) was dissolved in double distilled water (6 mL), followed by the addition of CTP (

0.078 g, 0.3 mmol) and ACVA (0.026 g, 0.1mmol) in 1,4 dioxane (1.2 mL) (Targeted DP_n = 60 , Targeted M_w = 10.7 kDa). The mixture was subjected to three freeze thaw cycles in 10 mL flask and was placed in oil bath for polymerization at 70 °C, under nitrogen for 6 hours. The polymerization was quenched by submerging the flask in liquid nitrogen and polymer was precipitated in acetone. The excess monomer was removed by washing the polymer with methanol and polymer was dried and analyzed by GPC as described above. The macro CTA obtained (0.1 g, 9.3 μmol) and GAPMA (0.2 g, 0.6 mmol) were dissolved in double distilled water, followed by the addition of ACVA (3 mg, 9.3 μmol) in DMF (0.2 mL) (Targeted DP_n = 60 , Targeted M_w = 20 kDa). The polymerization was carried out at 70 °C overnight after three freeze-thaw cycles. The polymer was precipitated in acetone and excess monomer and macro CTA were removed by extensive washing with DMF and methanol respectively. The final product was dried and analyzed by GPC as described above.

3.2.3. Formation of Polyplexes.

Nanocomplexes were made by mixing varying concentrations (at +/- ratio 40-60 depending on the type of polymers used) of polymers with 0.4 mg/mL of *β*-galactosidase plasmid in OMEM. The complexes were vortexed and incubated for 30 minutes before their analysis. The particle sizes and surface charges were measured using Brookhaven Zeta Plus (zeta potential and particle size analyzer) instrument.

3.2.4. Cell Culture.

Hela and Hep G2 cells were maintained in DMEM supplemented with 5 and 10% FBS, respectively and 1% penicillin/streptomycin solution in a humidified atmosphere in the presence of 5% CO_2 at 37 °C. Upon 80% confluency the cells were trypsinized with 0.25% trypsin and were seeded in tissue culture plates.

3.2.5. Determination of Lethal Dose50 (LD_{50}) Values.

Hela cells were seeded in 96 well tissue culture plates at the density of 9,000 cells per well. The cells were incubated overnight and were treated with varying concentrations of polymers in the presence of serum containing media. The cells were further incubated for 12 hours and 25 µL of MTT dye was added per well. After 2 hours 100 µL of lysis buffer was added and cells were allowed to lyse overnight. The absorbance was read at 570 nm using TECAN Genios pro microplate reader. The untreated cells and media alone were used as positive and negative controls, respectively. The percent cell viability was calculated (% cell viability = (treated cells – negative control)/ (untreated cells – negative control). LD_{50} values for different polymers were determined using Origin Pro software and data was analyzed using Boltzman function to provide a sigmoidal fit.

3.2.6. Transfection.

Hep G2 and Hela cells were seeded at the density of 100,000 and 50,000 cells per well respectively, in 24 well tissue culture plates and were incubated overnight. The media was removed and 150 µL of serum free OMEM or serum containing media was added followed by the addition of varying concentrations of polyplexes prepared at varying +/- ratio. The cells were incubated for four hours and polyplexes containing media were replaced with fresh serum

containing media. The cells were allowed to grow for 48 hours before their lysis (CHAPS in Sodium Phosphate lysis buffer) (pH=7.5) followed by a freeze thaw cycle. The activity of *β*-galactosidase was detected using *β*-galactosidase assay. Briefly, (150 µL of 4 mg/mL) ONPG solution was added to varying amounts of lysate volume in the presence of 4.5 µL 100X Mg solution (5.12 µL of *β*-Mercaptoethanol in 400 µL of 0.1M $MgCl_2$) in 96 well plate and plate was incubated at 37 °C. The yellow color developed was detected using TECAN Genios Pro microplate reader at 420 nm after four hours interval. The total content of protein in cell lysate was detected using micro BCA assay (Pierce). For PEI transfection, polyplexes were incubated for 24 hours with cells in the presence or absence of serum, the media was removed next day and replaced with fresh serum containing media and cells were allowed to grow for another 12 hours before the detection of *β*-galactosidase activity.

3.2.7. Toxicity after Transfection.

Hep G2 Cells were seeded in 24 well tissue culture plates as described above and were transfected with polyplexes in the manner identical to transfection conditions. 48 hours post-transfection125 µL MTT dye was added per well followed by the addition of 500 µL of lysis buffer after two hours of incubation. The absorbance was read at 570 nm using TECAN plate reader. The untreated cells and media alone were used as positive and negative controls respectively and percent cell viability was determined as mentioned in LD_{50} section.

3.2.8. Quantification of Serum proteins on the Surface of Polyplexes.

P($AEMA_{40}$-*st*-$GAPMA_{36}$) and P($AEMA_{52}$-*b*-$GAPMA_{49}$) based polyplexes were synthesized as mentioned above in the presence of 150 mM NaCl (rather than in OMEM) and were incubated with 10% serum in OMEM for 30 minutes. The mixture was centrifuged at 14000 rpm for two hours and supernatant was removed, followed by the addition of OMEM without serum. The step was repeated five more times to ensure the complete removal of free serum proteins from the polyplexes surfaces. The amount of serum proteins in the supernatant and on the surface of polyplexes was determined quantitatively using BCA assay.

3.2.9. Fluorescence labelling of BSA.

Fluorescein isothiocyanate (FITC) was dissolved in DMSO at 1 mg/mL concentration. BSA (2 mg/mL) was dissolved in 4% Na_2CO_3 (pH adjusted to 8.5). Small aliquots of FITC (100 µL of FITC per mL of BSA solution) were slowly added to BSA solution in dark while vortexing. The solution was incubated in the dark overnight. The solution was dialyzed in dark (MWCO = 6,000-8,000 Da) against de-ionized water and purified solution was stored in the dark at room temperature.

3.2.10. Uptake of Polyplexes using Confocal Microscope.

Hela and Hep G2 cells were seeded in 6 well tissue culture plates containing glass coverslips. Upon 80% confluency media was removed and cells were treated with FITC-labelled BSA alone, or with P($AEMA_{40}$-*st*-$GAPMA_{36}$) and

P($AEMA_{52}$-*b*-$GAPMA_{49}$) based polyplexes in the presence of FITC-labelled BSA. The untreated cells were used as negative control. The cells were incubated for 4 hours, media was removed and the cells were washed 2X with DPBS before their fixation using 3.7% formalin in DPBS. The cells were rehydrated with DPBS and fixed on microscope slides. The samples were analyzed using Fluoview FV10i Olympus confocal microscope and samples were excited at 485 nm and emission spectra were detected at 535 nm, using 60X objective.

3.2.11. Flow Cytometer.

Hep G2 cells were grown on 24 well tissue culture plate at the density of 100,000 cells per well and were allowed to adhere overnight. The media was then replaced with 150 μL of OMEM followed by the treatment with polyplexes in the presence of 10% FITC-BSA solution. The cells were further incubated for 3-4 hours. The cells were rinsed with DPBS two times to remove the adherent polyplexes from the surface of cells, and were trypsinized and suspended in DPBS containing 3.7% formalin. The uptake of polyplexes in the presence of FITC-BSA was quantified using Beckman Coulter Quanta SC flow cytometer using FL1 channel. The untreated cells and FITC-BSA- treated cells were used as negative control. The percentage of cells exhibiting the fluorescence and mean fluorescence of cells in population was determined.

3.2.12. Agarose gel Electrophoresis.

The polyplexes were loaded in 1% agarose gel containing 1μg/mL ethidium bromide in 1X tris acetate/EDTA buffer and the gel was run at 140 V for 30

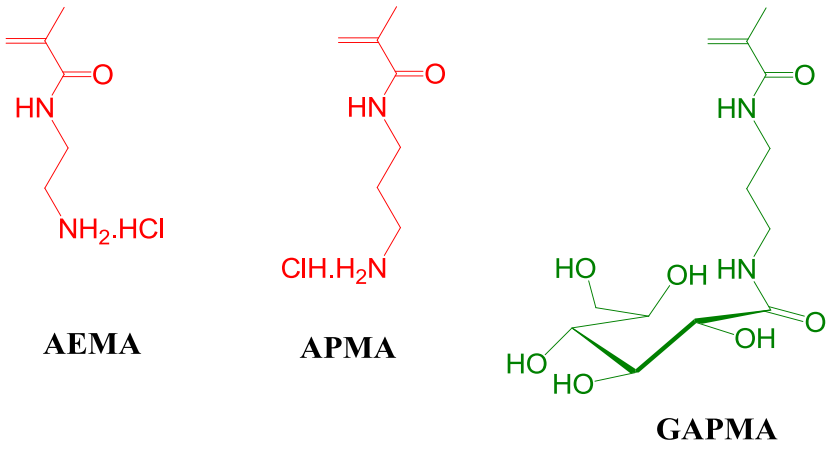
minutes and the DNA bands were visualized using UV transilluminator (Alpha Innotech; San Leandro, CA).

3.3. Results and Discussion.

The role of natural cationic polysaccharides and their derivatives for gene delivery purposes is well explored field of research, however there is no facile method that determines structure based gene delivery efficacy of these compounds. The polydisperse nature of biomolecules is a major hindrance to this approach. A facile approach to produce cationic glycopolymers of varying molecular weights, cationic to glycopolymer ratios and architecture (block versus random polymers) with narrow polydispersities is required. With the advent of living radical polymerization, synthesis of carbohydrate based polymers is largely facilitated as the reactions can now occur in the absence of protected group chemistry in aqueous media and the polymers produced are of controlled molecular weight. [24,25] Reversible addition-fragmentation chain transfer (RAFT) polymerization is a controlled radical polymerization technique that allows the synthesis of a variety of polymeric structures of desired molecular weight and narrow polydispersity due to the tolerance to various solvents, monomers and mild reaction conditions. This polymerization approach is also highly tolerant to a variety of functional groups and allows the synthesis of well-designed telechelic block or random copolymers. [24,25] The successful and facile synthesis of library of cationic glycopolymers by RAFT process further allows the complexation of these polymers with DNA and their use as a gene delivery vehicle. The benefits of using synthetic glycopolymer as pendant

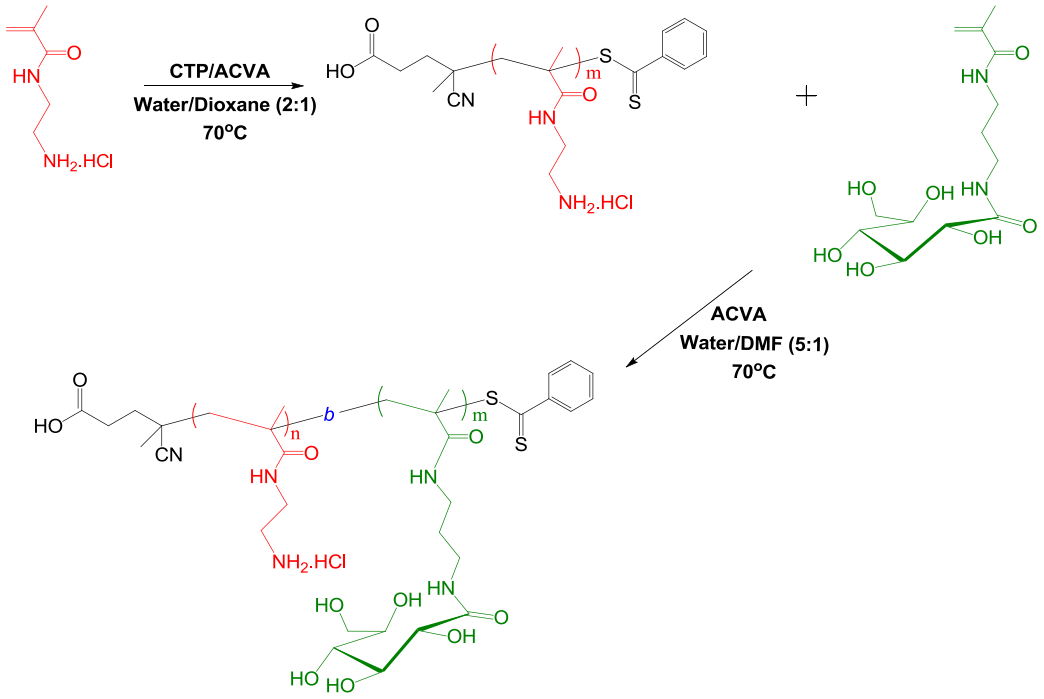
groups as gene delivery agent is that carbohydrate residues are accessible on polyplexes and are available for cell targeting even when complexes with DNA.

The first step in evaluating the synthetic cationic glycopolymers for gene delivery purposes of varying architectures is the synthesis cationic monomers (AEMA & APMA) and glycomonomer (GAPMA). The structures of these monomers are shown in scheme 3-2.



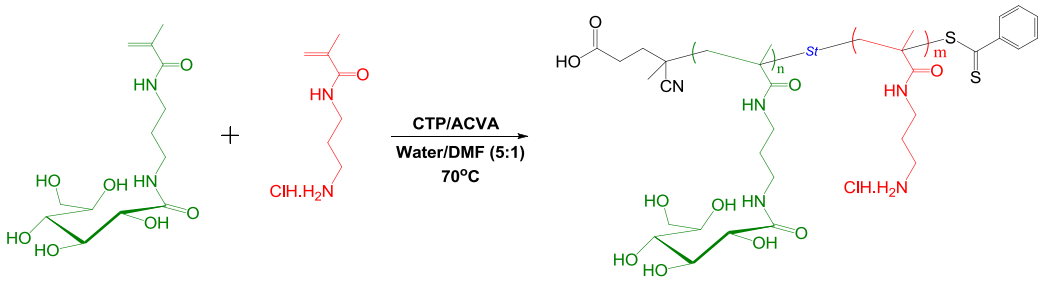
Scheme 3-2. Structures of monomers: 2-aminoethyl methacrylamide (AEMA), 3-aminopropyl methacrylamide (APMA) and 3-gluconamidopropyl methacrylamide (GAPMA).

Cationic homopolymers and copolymers of block and random architecture with varying degree of polymerization are synthesized. (Scheme 3-3 & 3-4) For this purpose, AEMA and APMA are polymerized by RAFT process to yield homopolymers of low and high degrees of polymerization. GAPMA is polymerized in the presence of PAEMA or PAPMA macroCTAs to yield their corresponding diblock copolymers.



Scheme 3-3. RAFT synthesis of block cationic glycopolymer using 4-cyanopentanoic acid dithiobenzoate (CTP) as chain transfer agent and 4,4'-azobis(4-cyanovaleric acid) (ACVA) as initiator.

The statistical copolymers of AEMA or APMA and GAPMA are synthesized by RAFT polymerization having similar DP_n as the diblock copolymers for comparison purposes.



Scheme 3-4. RAFT Synthesis of statistical cationic glycopolymers using 4-cyanopentanoic acid dithiobenzoate (CTP) as chain transfer agent and 4,4'-azobis(4-cyanovaleric acid) (ACVA) as initiator.

The carbohydrate segments in copolymers are varied to determine to role of carbohydrate segment in masking the toxicity of cationic segment. Furthermore, the role of carbohydrate segment in gene delivery efficacy is evaluated. The polymers synthesized are tabulated in table 3-1.

Table 3-1. Molar masses and molecular weight distribution for RAFT synthesized homopolymers and copolymers.

Three Carbon Chain Length		
Polymer Composition	GPC M_n kDa	PDI
Homopolymers[1]		
$P(APMA)_{18}$	3.2	1.40
$P(APMA)_{47}$	10.2	1.20
$P(AEMA)_{54}$	8.9	1.4
Statistical Copolymers[1]		
$P(APMA_{13}$-*st*-$GAPMA_{20})$	8.9	1.30
$P(APMA_{34}$-*st*-$GAPMA_{25})$	14.1	1.22
$P(APMA_{37}$-*st*-$GAPMA_{54})$	24.0	1.19
$P(APMA_{34}$-*st*-$GAPMA_{73})$	29.5	1.29
$P(AEMA_{40}$-*st*-$GAPMA_{36})$	18.2	1.31
$P(APMA_{18}$-*b*-$GAPMA_{22})$	10.4	1.18
$P(APMA_{18}$-*b*-$GAPMA_{37})$	15.1	1.16
$P(APMA_{47}$-*b*-$GAPMA_{67})$	29.6	1.36
$P(APMA_{47}$-*b*-$GAPMA_{55})$	26.1	1.3
$P(APMA_{57}$-*b*-$GAPMA_{54})$	27.6	1.14
$P(AEMA_{52}$-*b*-$GAPMA_{49})$	24.5	1.13

1The composition of homopolymers and block copolymers is determined using gel permeation chromatography (GPC) and of statistical copolymers is determined using 1H-NMR

The determination of lethal dose 50 (LD_{50}) values for newly synthesized biomaterials is essential before their use for gene delivery. Toxicity studies are performed using an MTT assay on Hela cells in order to determine LD_{50} values of those synthesized polymers. The toxicity of polymers is found to be dependent on the length of cationic, copolymers architecture and carbohydrate segments and molecular weights. As shown in Table 3-2, the cell viabilities increase with decreasing molecular weights of the homo or copolymers.

Table 3-2. Lethal dose 50 (LD_{50}) values of the series of polymers obtained using MTT assay of cell viability using Hela cells.

Polymer Composition	LD_{50} (μM)
$p(APMA)_{18}$	40 ± 1.4
$p(APMA_{18}$-*b*-$GAPMA_{22})$	92.2 ± 1.11
$p(APMA_{18}$-*b*-$GAPMA_{37})$	>373
$P(APMA_{13}$-*st*-$GAPMA_{20})$	>373
$P(APMA)_{47}$	<12.4
$(APMA_{47}$-*b*-$GAPMA_{55})$	46.6 ± 1.02
$P(APMA_{47}$-*b*-$GAPMA_{67})$	52.9 ± 1.19
$P(APMA_{57}$-*b*-$GAPMA_{54})$	<12.4
$P(APMA_{34}$-*st*-$GAPMA_{25})$	105 ± 1.09
$P(APMA_{37}$-*st*-$GAPMA_{54})$	138.6 ± 1.2
$P(APMA_{34}$-*st*-$GAPMA_{73})$	125.3 ± 1.13
$P(AEMA)_{54}$	12.4 ± 2.2
$P(AEMA_{52}$-*b*-$GAPMA_{49})$	134.9 ± 1.97
$P(AEMA_{40}$-*st*-$GAPMA_{36})$	123.03 ± 2.13

As expected, lower molar masses of cationic homopolymers namely $P(APMA)_{18}$ show higher cell viability than those of higher molar masses such as $P(APMA)_{47}$ and $P(AEMA)_{54}$. It is also found that AEMA based polymers (homo, statistical and block) have higher LD_{50} values than those APMA based copolymers. For

example LD_{50} values of $P(APMA)_{47}$ is determined to be less than 12.4, as compared to $P(AEMA)_{54}$, which has LD_{50} value of 12.4±2.2 (despite of higher cationic character of the later). Similarly, LD_{50} values of $P(APMA_{57}$-*b*-$GAPMA_{54})$ is less than that of $(PAEMA_{52}$-*b*-$GAPMA_{49})$ (less than 12.4 versus 134.9±1.97 respectively). Furthermore, the cell viability studies clearly indicate that the presence of carbohydrates indeed reduce the toxicity of these cationic copolymers. The incorporation of carbohydrate segment in $P(APMA)_{18}$ to form $P(APMA_{18}$-b-$GAPMA_{22})$ increases LD_{50} value from 40±1.4 to 92.2±1.11 µM. Further increase in carbohydrate content produces biocompatible copolymers with LD_{50} value higher than 373 µM. The effect of carbohydrate content on cell viability is less pronounced for higher molecular weight copolymers. For example, incorporation of carbohydrate segment in $P(APMA)_{47}$ to produce $P(APMA_{47}$-*b*-$GAPMA_{55})$ increases LD_{50} values considerably (from less than 12.4 to 46.6 ± 1.02). Further increase in carbohydrate segment such as in copolymer, $P(APMA_{47}$-*b*-$GAPMA_{67})$, does not have a pronounced effect on cell viability. Previous data based on structure-activity relationship of natural polysaccharides based cationic polymers showed similar trend, and increase in polysaccharide to cationic polymers ratio, showed increased cell viability. [9,25] However, in our case with pendant carbohydrate residues, statistical copolymers showed greater cell viability than their corresponding diblock copolymers. To the best of our knowledge this is the first study, which has evaluated the role of glycopolymers architecture on gene expression. Therefore, the cell viability of these polymers is not only dependent on their molecular weights, type of cationic

monomers used, and composition of carbohydrate segment, but also on the architecture of the copolymers. This study may provide valuable information about the design of carbohydrate based cationic polymers for optimal gene expression and low toxicity.

The increase in cell death by increasing the cationic chain length (DP_n), or by decreasing the GAPMA content is expected due to increase in net cationic character of these polymers. Hwang *et al*. has synthesized *β*-cyclodextrin conjugated poly(amides) of varying cationic chain lengths, and it has been reported that the increase in charge density of these polymers, decrease their IC_{50} values. [19] The increased cell viability for AEMA based polymers compared to APMA based polymers is thought to be due to the greater distance between amine group and back bone of polymers in the case of APMA compared to AEMA, hence contributing towards higher toxicity. The architecture of polymers is also found to contribute towards polymer toxicity. The block polymers are more toxic than the statistical polymers, indicating that block configuration is less able to mask the cationic component of copolymers effectively than those of statistical configuration. The only exception to this trend is AEMA based copolymers where P($AEMA_{40}$-*st*-$GAPMA_{36}$) showed slightly lower cell viability than P($AEMA_{52}$-*b*-$GAPMA_{49}$). These homopolymers and copolymers are then incubated with *β*-galactosidase plasmid at varying polymer to DNA ratios in Opti MEM for 30 minutes, and stable polyplexes obtained are characterized using DLS instrument. It should be noted that although the complete complexation of (1.2 μg) plasmid with polymers was obtained at very low

concentrations of polymers (5 µg). DLS showed the presence of micron particles under these conditions. (data not shown) Hence, the addition of slight excess of polymers to the complexes was necessary to obtain nanometer sized polyplexes as determined by DLS. The +/- ratios required to make nanometer sized polyplexes is indicated and is used elsewhere to determine net charge, transfection data and toxicity after transfection. (supporting information table S1) It is found that under these conditions the size of polyplexes formed is dependent on the architecture of polymer, length of cationic chain and molecular weight of the polymers. (Figure 3-1)

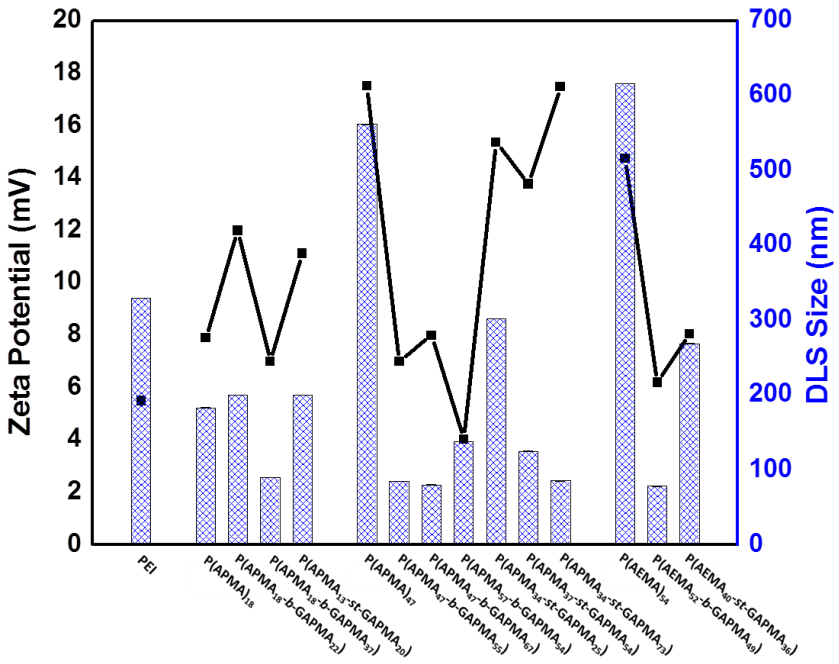


Figure 3-1. Dynamic light scattering (DLS) and zeta potential data for the glycopolymer-DNA polyplexes. All samples were prepared in Opti-MEM media at fixed plasmid concentration, while polymer concentrations were varied to obtain stable particles.

Regardless of the type of cationic monomer used, low molecular weight homopolymer $(PAPMA)_{15}$ form smaller particles (~ 200 nm) with plasmid DNA than higher molecular weights counterparts $P(APMA)_{47}$ and $P(AEMA)_{54}$ (~ 600 nm). These results are in agreement with previous literature, where effect of polymer molecular weight on polyplex formation is studied. [1] The polyplexes formed with block and statistical copolymers are significantly smaller in size than the corresponding high molecular weight homopolymers. Moreover, it is found that copolymers of higher molecular weight form smaller particles. For example, polyplexes formed using $P(APMA_{18}$-*b*-$GAPMA_{22})$ (~ 200 nm) are smaller in size as compared to $P(APMA_{18}$-*b*-$GAPMA_{37})$ (~80 nm). The increase in molecular weight of copolymers does not affect the size of polyplexes and polyplexes formed using $P(APMA_{47}$-*b*-$GAPMA_{55})$ and $P(APMA_{47}$-*b*-$GAPMA_{67})$ are about 80 nm in diameter. The sizes of polyplexes formed are also found to be dependent on the architecture of polymers. The statistical copolymers produce particles of larger sizes than their corresponding diblock copolymers, indicating the difference in DNA condensation ability of these copolymers. It is hypothesized that the diblock copolymers condense DNA efficiently in their core with a shell of the carbohydrate segment and hence yielding smaller particles as compared to statistical copolymers.

The net charge of polyplexes is an important parameter that dictates their transfection efficiencies. The zeta potential of polyplexes varies as a function of polymer architecture and their molecular weights. As expected, homopolymers of higher DP_n produce polyplexes with higher net positive zeta potential values.

In general, statistical copolymers (regardless of molecular weights) produce polyplexes with higher positive zeta potential values than their corresponding block copolymers. This further indicates that the complexation of plasmid DNA to diblock copolymers is different than the statistical copolymers. Therefore, in the case of diblock copolymers, the plasmid is more likely complexed with the cationic segment of the copolymer in the core of the nanoparticles leaving a shell of carbohydrate segment. In contrast, in the case of statistical copolymers, the complexation to DNA is random and hence there is an equal probability that carbohydrates and cationic segments will be displayed on the surface of polyplexes, indicating the higher zeta potential values. The support to this core-shell hypothesis of diblock copolymer based polyplexes comes from a recent work, a molecular dynamics simulation of complexation of anionic macromolecules with diblock copolymer has been performed. The results indicate the complexation of diblock copolymers with anionic macromolecules like DNA, where one block of the polymer is cationic while the other block is hydrophilic, this system is found to produce small particles with a well-defined core-corona structure. [28]

The modification of cationic polymers with various biocompatible moieties is intensely studied approach in order to reduce the toxicity of former, without disrupting their gene delivery efficacy. [29-36] One of the most common modification used for this purpose is PEGylation (polyethyleneglycol modification). Bon *et al*. has produced diblock and statistical copolymers of PEG and cationic telomers. The conjugation of amines with PEG, regardless of

copolymer architecture significantly decreased their transfection efficiencies, compared to the cationic homopolymer. The shielding effect provided by PEG was found to decrease the interaction of polyplexes with cell surface proteins. [30] In an effort to produce carbohydrate based gene delivery vehicles with less toxicity and enhanced gene expression, Azzam *et al.* has modified the backbone of various polysaccharides with different cationic polymers. [24] The backbones of arabinogalactan, dextran, and pullulan are modified with PEI, spermine or with other cationic polymers and it has been shown that charge ratio and carbohydrates type play important role in gene delivery efficacy of the gene delivery agent. Dextran based conjugates are found to show better gene delivery efficacy than other polysaccharide conjugates. Study of structure activity relationship for cationic polysaccharides was explored by Davis *et al.* work. [9] Cyclodextrin molecules are functionalized with polycation of DP 10 or lower and it has been shown that length of cationic component and size of carbohydrate molecule plays important role in determining the toxicity and gene transfer ability of these polymeric vehicles. These are some examples of natural polysaccharides based cationic systems, which are studied in literature for gene delivery purposes, however none of these reports discuss the effect of polysaccharides based copolymer architecture on gene expression. The polyplexes, formed at +/- ratio of 40-60 depending upon polymer types, showed size range of 80 nm to 600 nm, with net positive zeta potential values and are further subjected to transfection. (Figure 3-1) The gene expression is detected using β-galactosidase assay in Hep G2 and Hela cells. The type of cationic

monomer (AEMA vs. APMA), DP_n, content of GAPMA residues in copolymers, DNA dose, presence and absence of serum are evaluated during the gene expression experiments and will be discussed in detail here.

Gene expression is found to be dependent on the molecular weight of the copolymers studied, which is in agreement with previous literature. [30] As a result, high molecular weight copolymers are found to be better transfecting agent as compared to their lower molecular weights counterparts (Supporting information, Figures S4- S6). It is interesting to note that copolymers of high DP_n show transfection efficiencies comparable to linear PEI which is used as positive control. The gene expression for the linear homopolymers and copolymers are found to be higher than linear PEI of similar molecular weights at the studied DNA doses and are comparable to branched PEI (M_w = 25 kDa) at DNA dose of 0.6 µg. (Figure 3-2) This is quite remarkable results, considering the lower toxicity of these cationic glycopolymers as compared to PEI, therefore these high molecular weight glycopolymers are considered promising gene delivery agents.

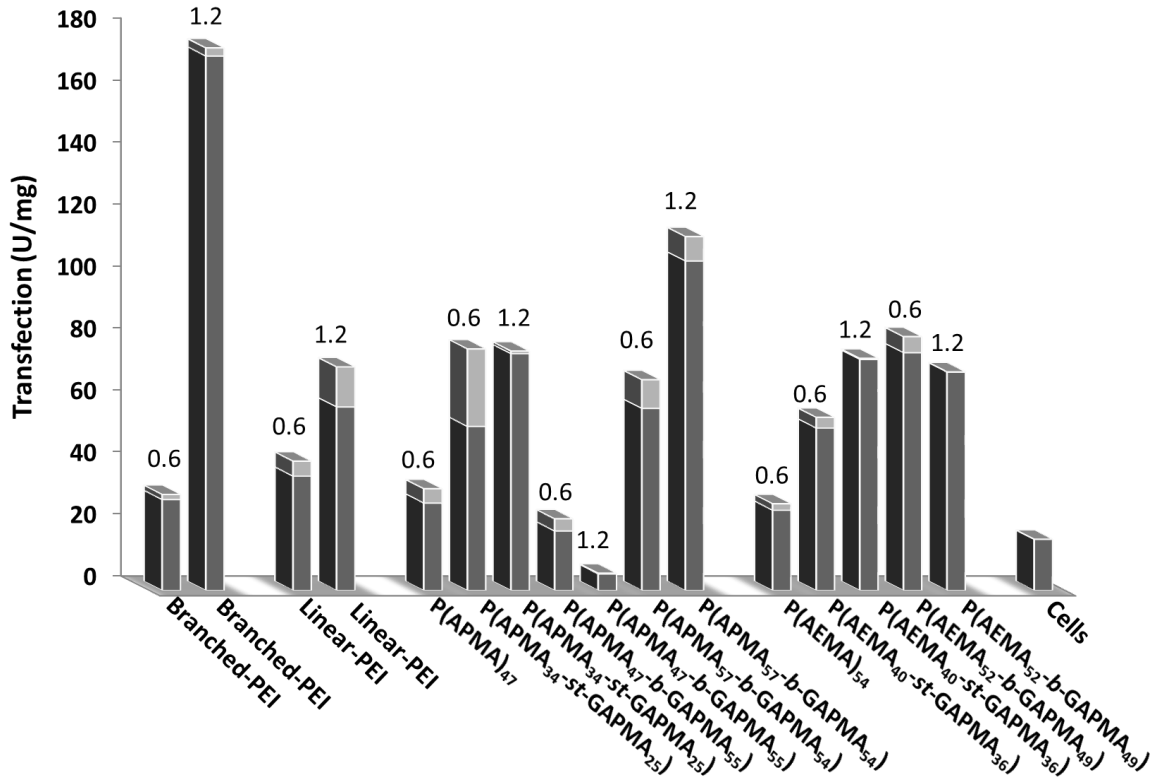


Figure 3-2. Gene expression of high molecular weights homopolymers and copolymers, in the absence of serum using Hep G2 cells. Gene expression is evaluated using β-galactosidase assay at DNA dose 0.6 & 1.2 µg as shown.

The gene expression of homopolymers with the corresponding copolymers is compared. As shown in figure 3-2, homopolymers of AEMA and APMA show low transfection efficiencies (20-25 U/mg) in the absence of serum, compared to their copolymers. In the case of homopolymers, higher DNA doses (1.2 µg) lead to very high toxicities, which further compromised gene expression. (data not shown) The general trend is that, regardless of cationic monomer type, the statistical copolymers show higher transfection than their corresponding diblock copolymers. In contrast, diblock and statistical copolymers of PEG and cationic monomer did not show any difference in gene expression as a function of

polymer architecture. The gene expression of copolymers was lower than their corresponding homopolymers. [30] This indicates that, regardless of architecture of polymer, PEG masks the net cationic character of the polyplexes, hence decreasing their cellular uptake. In contrast, the presence of carbohydrate residues on the surface of polyplexes in statistical architecture not only helps to mask the cationic charge, but also enhance the gene expression of these copolymers. For example, the transfection efficiency of P($APMA_{34}$-*st*-$GAPMA_{25}$) (52.9 U/mg) is higher than P($APMA_{47}$-*b*-$GAPMA_{55}$) (19.21 U/mg). However, further increase in DP_n of polymers improved the transfection ability of block copolymers specifically in the absence of serum. As shown in figure 3-3, P($APMA_{54}$-*b*-$GAPMA_{55}$) copolymers show transfection efficiency of up to 106 U/mg, while gene expression for p($AEMA_{52}$-*b*-$GAPMA_{49}$) are 77 U/mg. The enhanced gene delivery of high molecular weight diblock copolymers in the absence of serum, further indicates that surface carbohydrate of polyplexes interacts with cell surface proteins, and aids in enhanced gene expression, however a longer cationic segment in copolymers is required. Similar to APMA based copolymers, P($AEMA_{52}$-*b*-$GAPMA_{49}$) show higher transfection (77 U/mg) than P($AEMA_{40}$-*st*-$GAPMA_{36}$) (52.4 U/mg). The increase in DNA dose (1.2 μg) significantly decreased cell viabilities and transfection efficiencies in the case of diblock copolymers, but statistical copolymers show slight increase in transfection efficiencies.

The role of GAPMA content of cationic glycopolymers on transfection efficacies is also studied and results obtained are consistent with previous literature.

[29,37] The increase in carbohydrate content decreased the transfection efficiencies of these copolymers. (Figure 3-3) The decrease in gene expression upon increasing the carbohydrate residues is thought to be due to increased hydrophilicity of the polyplexes. [37] As shown in figure 3, for constant cationic component, increase in carbohydrate content leads to a proportional decrease in transfection efficiencies. It is estimated that ideal ratio for carbohydrate to cationic content of copolymers regardless of polymer architecture is 0.9-1.0. However, an increase in carbohydrate residues of copolymer increases cell viability but significantly decreases transfection efficiencies of these copolymers.

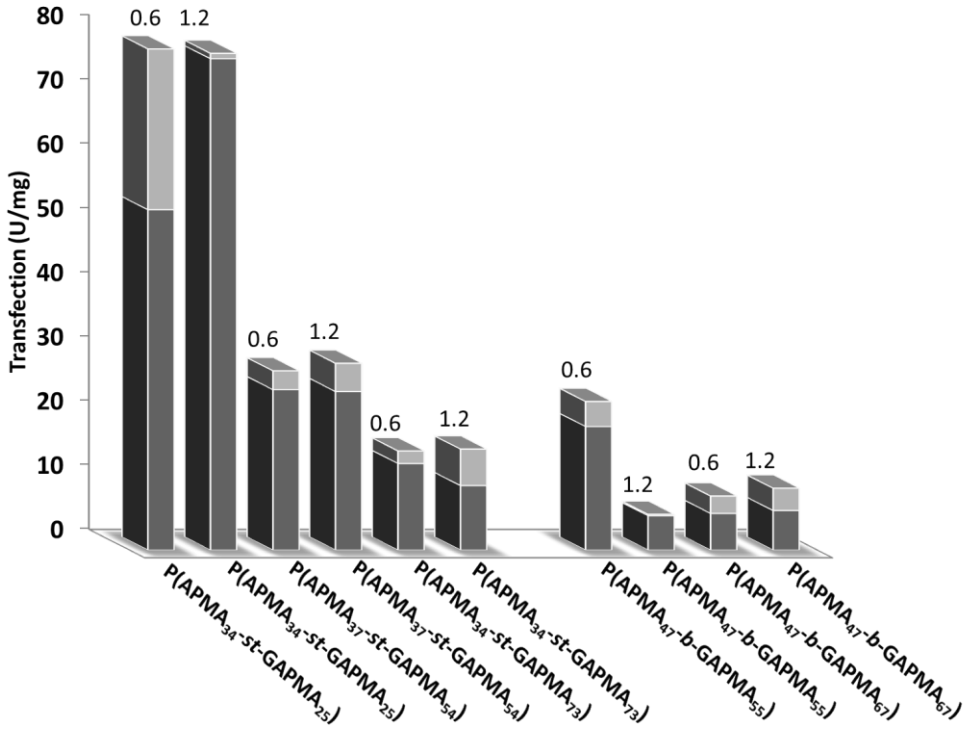


Figure 3-3. Effect of carbohydrate content in the absence of serum on the transfection efficiencies of high DPn polymers using Hep G2 cells. Gene

expression is determined using β-galactosidase assay at DNA dose 0.6 and 1.2 μg as shown on graph.

These results are in agreement with previous work, where effect of carbohydrates content of cationic polysaccharides on gene delivery efficacies was studied and it was found that carbohydrate to cationic component of 0.5:1-0.75:1 is required for optimum gene expression. [29,37] The copolymers used for gene delivery evaluation have a cationic to carbohydrate content of 0.9-1.0 or higher as those compositions are found to be more cell viable as compared to cationic homopolymers. Toxicity studies were also performed after gene expression and the results will be discussed later.

The stability of polyplexes in the presence of serum is an important criterion for gene delivery vehicles. It has been found that PGAs and some carbohydrates based gene delivery agents show low or no gene delivery in the presence of serum. [11] The gene delivery efficacy of chitosan in the presence of serum is enhanced by incorporating oligosaccharides to the chitosan structure. [7] The high molecular weight homopolymers and copolymers synthesized by RAFT polymerization are tested for their gene transfer efficacy in the presence of serum. (Figure 3-4, Figure S6) Interestingly, the gene expression for copolymers in the presence of serum is dependent on polymer architecture.

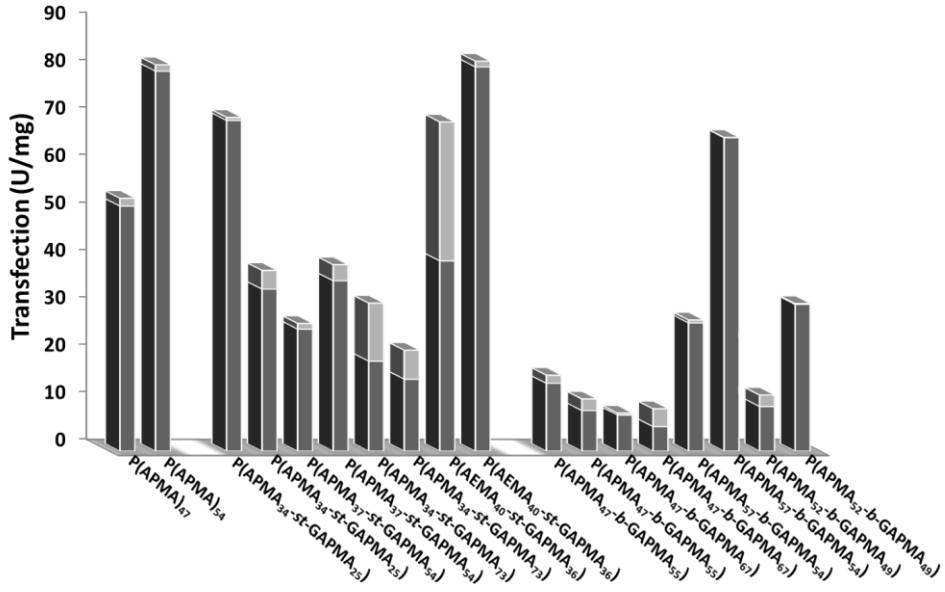


Figure 3-4. Gene expression as a function of high molecular weight copolymer architectures in the presence of serum proteins using Hep G2 cells. The transfection efficiencies are determined using β-galactosidase assay at DNA dose 0.6 and 1.2 µg as shown on graph.

The homopolymers showed improved transfection efficacies in the presence of serum. In general, block copolymers of any DP_n show very low transfection efficiencies in the presence of serum, while transfection is enhanced or remain unaffected by the presence of serum for statistical copolymers. For example, gene expression of P($APMA_{47}$-*b*-$GAPMA_{55}$)-polyplexes decreased from 8.5 U/mg to 5.3 U/mg and 19 U/mg to 14 U/mg, when transfection is done in the presence of serum containing media at DNA dose of 1.2 and 0.6 µg, respectively. However, no significant difference in gene expression was observed in the presence and absence of serum for P($APMA_{47}$-*b*-$GAPMA_{67}$), as the transfection was minimal and was close to the negative control under these conditions. The effect of serum on transfection was more pronounced for

P(APMA$_{57}$-*b*-GAPMA$_{54}$) copolymer, the transfection efficiency decreased by half at low DNA dose (27 U/mg versus 58U/mg). The higher DNA dose also showed significant decrease in transfection (66U/mg versus 106U/mg) Similar trend was found for P(AEMA$_{52}$-*b*-GAPMA$_{49}$) copolymer based polyplexes. The presence of serum decreased the transfection efficacy of this copolymer from 77U/mg to 30 U/mg at DNA dose of 1.2 µg. In the case of statistical copolymers, presence of serum did not affect the transfection efficiency significantly. The exception to this trend is P(APMA$_{40}$-*st*-GAPMA$_{36}$)-polyplexes at higher DNA dose (1.2 µg), where a slight decrease in gene expression in the presence of serum has been observed. We attribute this decrease due to the slight aggregation of these polyplexes (due to strong net cationic character) in serum containing media, as shown by DLS data below. These results are further supported by the transfection data that lower DNA dose of this copolymer based polyplexes (0.6 µg) slightly enhance the gene expression in the presence of serum. This indicates that serum proteins interact differently with statistical and block copolymer based polyplexes. It should be noted that effect of serum on copolymer architecture has not been studied in detail, in previous literature based on gene expression of copolymers as a function of architecture. [30] Although, some natural polysaccharides based polyplexes are found to work in the presence of serum, the significance of synthetic glycopolymer based polyplexes is their monodispersity and presence of carbohydrate segments on the surface of polyplexes, which can additionally serve for targeting purposes in specific cell types. We further explored the role of serum proteins in gene expression as a

function of polymer architecture. Several experiments were performed to evaluate the interaction between serum proteins with statistical and block copolymer based polyplexes. The idea is to determine whether the serum proteins are interacting with the surface of the polyplexes and if so, whether they are responsible for the differences in gene expression for those two copolymer systems.

The interaction of the serum proteins with the polyplexes is first evaluated using confocal microscopy. For this experiment, Hela and Hep G2 grown on glass coverslips are treated with FITC-BSA alone or with FITC-BSA incubated polyplexes formed using P($AEMA_{52}$-*b*-$GAPMA_{49}$) and P($AEMA_{40}$-*st*-$GAPMA_{36}$) and *β*-galactosidase plasmid respectively. (Figure 3-5) The images show that statistical copolymer based polyplexes are well uptaken by both cells lines (Figure 3-5-1, images B,E) in the presence of BSA, as compared to block copolymers based polyplexes (Figure 3-5-1, images C,F). To obtain quantitative data, the polyplexes in the presence of FITC-BSA are analyzed using flow cytometer. (Figure 3-5-2) The results show that lower fluorescene of block polymer based polyplexes may be due to the lower uptake of these polyplexes in the presence of BSA than statistical copolymer based polyplexes, which further explain the lower gene expression of block copolymers based polyplexes in the presence of serum. It should also be noted that these polyplexes (block and statistical) aggregate over time in the presence of serum proteins as revealed by DLS data, for both copolymer types. (Supporting information, Table S2) However, this aggregation of polyplexes in the presence of serum proteins is not

an issue for the gene expression, possibly because the uptake of polyplexes occurs before their aggregation. The size of polyplexes is not thought to be the major factor of difference in the uptake of diblock and statistical based polyplexes, as diblock copolymer based polyplexes are only ~200 nm in diameter after their aggregation, while size of statistical copolymers based polyplexes increases upto ~900 nm. We speculate that lower uptake of diblock copolymer based polyplexes is due to their lower zeta potential values which is further reduced in the presence of negatively charged serum proteins, as compared to their corresponding statistical copolymer based polyplexes.

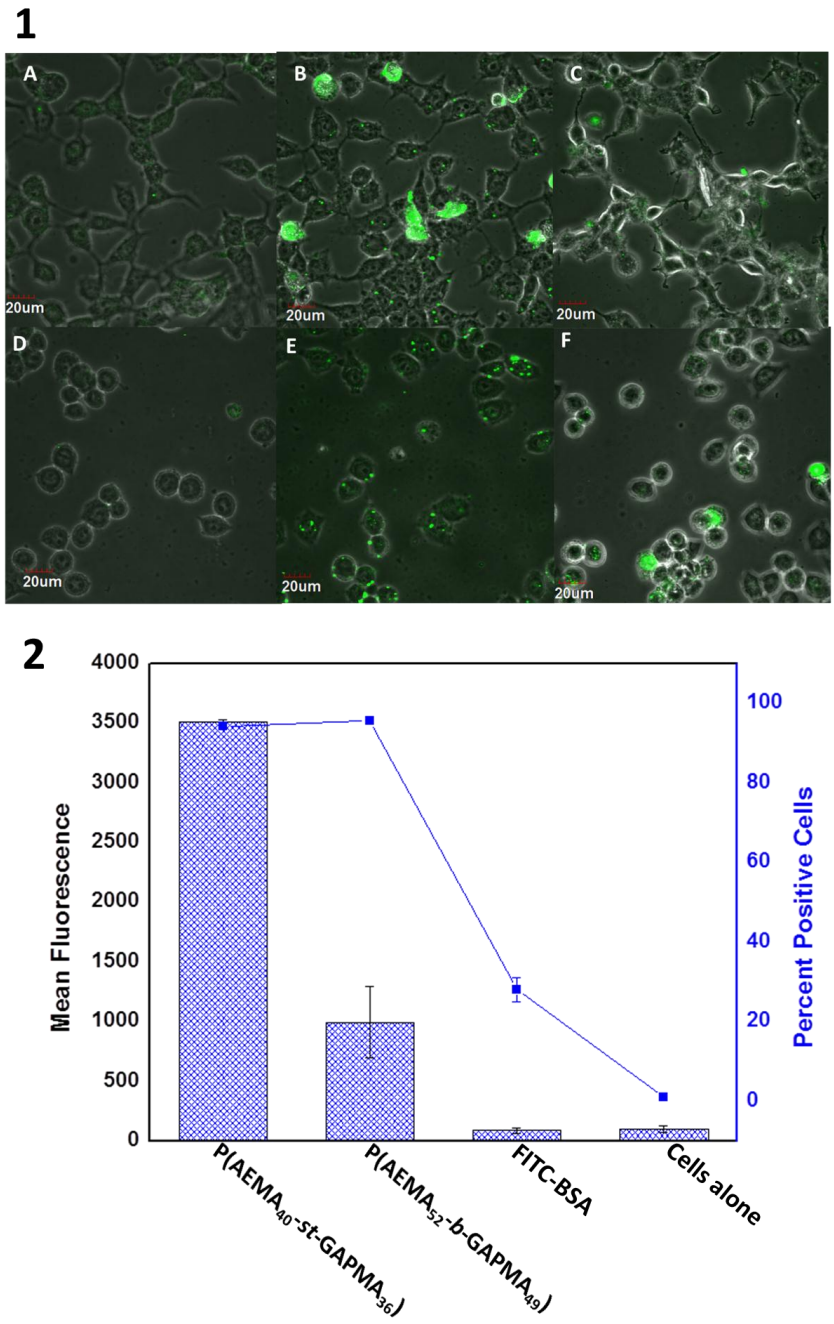


Figure 3-5. The study of uptake of polyplexes synthesized using copolymers of varying architectures. The uptake of polyplexes-BSA complexes using confocal microscope (1) and flow cytometer (2). Confocal microscope images (5-1) A-C show the treated Hep G2 cells, while D-F show treated Hela cells. Images A & D represent cells treated with FITC-BSA (in the absence of polyplexes). Images B

& E represents P($AEMA_{40}$-*st*-$GAPMA_{36}$)-plasmid complexes uptaken by cells in the presence of BSA-FITC. Images C & F represents P($AEMA_{52}$-*b*-$GAPMA_{49}$)-plasmid complexes uptaken in the presence of FITC-BSA.

The interactions of polyplexes with serum proteins are studied. The polyplexes are incubated with 10% FBS for 30 minutes. The polyplexes are subsequently washed and centrifuged to remove excess serum proteins. The purified polyplexes and supernatant are analyzed by agarose gel electrophoresis to confirm that the polyplexes are actually well separated from free serum proteins after centrifugation. (Figure S8) In order to determine the protein content on the polyplexes and supernatant, a bicinchoninic acid (BCA) assay is performed. The results indicate that, in the case of statistical copolymers, content of serum proteins is higher on the polyplexes than in supernatant. In contrast, block copolymers based polyplexes show 4X less serum proteins on their surface. The interactions of serum proteins with two types of polyplexes can be explained in terms of their zeta potential values. The statistical copolymers based polyplexes possessing higher net positive zeta values strongly interact with negatively charged serum proteins, as compared to block copolymers based polyplexes, which have lower positive zeta values. (Supporting information, Figure S9-S10)

The selected polymer samples which showed enhanced gene delivery in Hep G2 cells were used to transfect Hela cells. (Figure 3-6) It is found that statistical copolymers show enhanced gene delivery than the corresponding block copolymers. Gene expression in Hela cells follow a similar trend as in Hep G2 cells with transfection efficiencies for statistical glycopolymers better the blocks

The difference in gene expression values in the two cell lines has been reported previously and is referred as a cell line dependent effect on gene expression. [38]

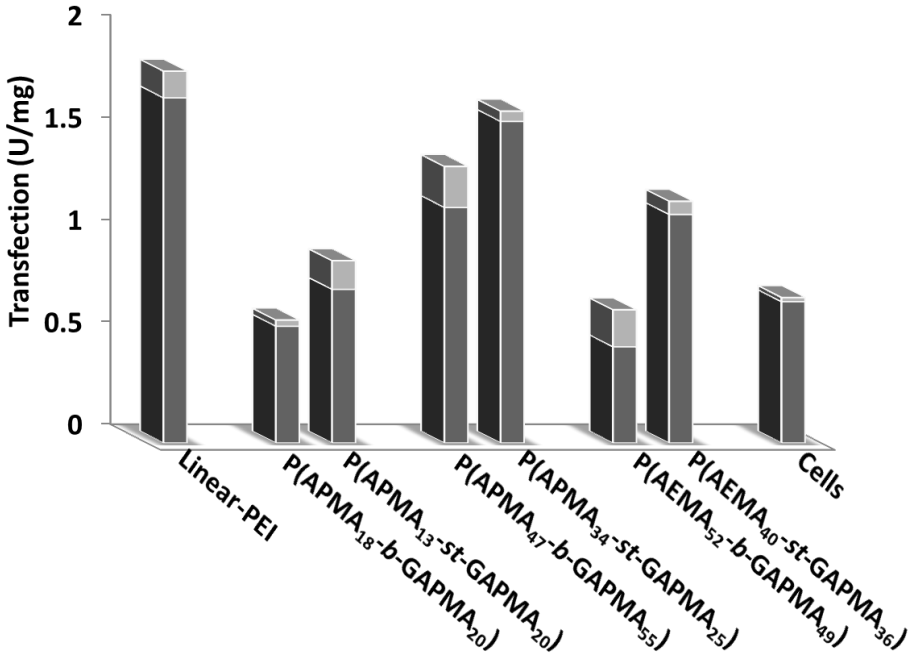


Figure 3-6. Gene expression of statistical and diblock copolymers of varying molecular weight, using Hela cells in the absence of serum.

Cytotoxicity studies are performed after transfection using Hep G2 cells for the selected samples which showed enhanced gene delivery. It is found that statistical copolymers based polyplexes show better cell viability post transfection than block copolymers based polyplexes, as determined by MTT assay. (Supporting information Figure S11)

3.4. Conclusion

In short, a library of cationic glycopolymers is synthesized using RAFT polymerization technique and the synthesized polymers are evaluated for their

cytotoxicities and for their ability to serve as non-viral gene delivery carriers. It is interesting to note that the physiological properties of these polymers are dependent on the polymer architecture as well as on their molecular weight and content of carbohydrate residues. We have identified that the statistical copolymers show similar gene expression as PEI, in the presence of serum with significantly lower toxicity. It should be noted that the high molecular weight statistical copolymers such as, P($AEMA_{40}$-*st*-$GAPMA_{36}$) and P($APMA_{34}$-*st*-$GAPMA_{25}$) show greater cell viabilities and gene expression, as compared to the diblock copolymers of similar compositions. Furthermore, in the case of the diblock copolymers, the gene expression is significantly reduced in the presence of serum. The interaction of serum proteins with statistical and diblock copolymers based polyplexes is studied in detail, and it has been found that serum proteins do not affect the uptake of statistical based polyplexes by cells. The block copolymers based polyplexes show less interactions with BSA, and their gene expression is significantly low. The study provides a platform for the synthesis of cationic glycopolymers with specific carbohydrate residues for cell targeting purposes. Overall, the statistical configuration of the cationic glycopolymers is found to be the ideal candidate for gene delivery, where pendant carbohydrate residues are present on the surface of polyplexes for enhanced cell-surface interactions and therefore can interact to specific cell receptors as well as masking the toxicity. In the near future, *in vivo* studies will be conducted to determine the efficacy and stability of statistical copolymers. It

is thought that statistical copolymer based polyplexes will selectively target the specific cells and will successfully deliver the gene of interest *in vivo*.

3.5. References

[1] Mintzer AM, Simanek EE. Nonviral vectors for gene delivery. Chem Rev 2009; 109(2): 259-02.

[2] Zhang S, Zhao Y, Zhao B, Wang B. Hybrids of nonviral vectors for gene delivery. Bioconjugate Chem 2010; 21(6): 1003-9.

[3] Tao L, Liu J, Tan HB, Davis PT. RAFT synthesis and DNA binding of biodegradable, hyperbranched poly(2-(dimethylamino)ethyl methacrylate. Macromolecules 2009; 42(14): 4960-62.

[4] Green JJ, Langer R, Anderson GD. A combinatorial polymer library approach yields insight into nonviral gene delivery. Acc Chem Res 2008; 41(6): 749-59.

[5] Barua S, Joshi A, Banerjee A, Matthews D, Sharfstein TS, Cramer MS, Kane SR, Rege K. Parallel synthesis and screening of polymers for nonviral gene delivery. Mol Pharmaceutics 2009; 6(1): 86-97.

[6] Lynn MD, Anderson GD, Putnam D, Langer R. Accelerated discovery of synthetic

transfection vectors: parallel synthesis and screening of a degradable polymer library.

J Am Chem Soc 2001; 123(33): 8155-56.

[7] Strand PS, Issa MM, Christensen EB, Varum MK, Artursson P. Tailoring of chitosans for gene delivery: novel self-branched glycosylated chitosan oligomers with improved functional properties. Biomacromolecules 2008; 9(11): 3268-76.

[8] Toita S, Morimoto N, Akiyoshi K. Functional Cycloamylose as a polysaccharide-based biomaterial: application in a gene delivery system. Biomacromolecules 2010; 11(2) : 397-01.

[9] Popielarski RS, Mishra S, Davis EM. Structural effects of carbohydrate-containing polycations on gene delivery. 3. cyclodextrin type and functionalization. Bioconjugate Chem 2003; 14(3): 672-8.

[10] Liu Y, Reineke MT. Degradation of poly(glycoamidoamine) DNA delivery vehicles: polyamide hydrolysis at physiological conditions promotes DNA release. Biomacromolecules 2010; 11(2): 316-25.

[11] Liu Y, Wenning L, Lynch M, Reineke MT. New poly(d-glucaramidoamine)s induce DNA nanoparticle formation and efficient gene delivery into mammalian cells. J Am Chem Soc 2004; 126(24): 7422-23.

[12] Liu Y, Reineke MT. Poly(glycoamidoamine)s for gene delivery. structural effects on cellular internalization, buffering capacity, and gene expression. Bioconjugate Chem 2007; 18(1): 19-30.

[13] Liu Y, Reineke MT. Poly(glycoamidoamine)s for gene delivery: stability of polyplexes and efficacy with cardiomyoblast cells. Bioconjugate Chem 2006; 17(1): 101-08.

[14] Zanta MA, Bossif O, Abdennaji A, Behr JP. In vitro gene delivery to hepatocytes with galactosylated polyethylenimine. Bioconjugate Chem 1997; 8(6): 839-44.

[15] Anderson K, Fernandez C, Rice GK. N-glycan targeted gene delivery to the dendritic cell SIGN receptor. Bioconjugate Chem 2010; 21(8): 1479-85.

[16] Azzam T, Raskin A, Makovitzki A, Brem H, Vierling P, Lineal M, Domb JA. Cationic polysaccharides for gene delivery. Macromolecules 2002; 35(27): 9947-53.

[17]Wong K, Sun G, Zhang X, Dai H, Liu Y, He C, Leong WK. PEI-g-chitosan, a novel gene delivery system with transfection efficiency comparable to polyethylenimine in vitro and after liver administration in vivo. Bioconjugate Chem 2006; 17(1): 152-8.

[18] Pun HS, Bellocq CN, Liu A, Jensen G, Machemer T, Quijano E, Schluep T, Wen S, Engler H, Heidel J, David EM. Cyclodextrin-modified polyethylenimine polymers for gene delivery.

Bioconjugate Chem 2004; 15(4): 831-40.

[19] Hwang JS, Bellocq CN, Davis EM. Effects of structure of β-cyclodextrin-containing polymers on gene delivery. Bioconjugate Chem 2001; 12(2) 280-90.

[20] Tseng WC, Jong MC. Improved stability of polycationic vector by dextran-grafted branched polyethylenimine. Biomacromolecules 2003; 4(5): 1277-84.

[21] Wang ZH, Li BW, Ma J, Tang PG, Yang TW, Xu JF. Functionalized nonionic dextran backbones by atom transfer radical polymerization for efficient gene delivery. Macromolecules 2011; 44(2) : 230-39.

[22] Youdovin-Farber I, Domb JA. Cationic polysaccharides for gene delivery. Materials Science and Engineering C 2007; 27(3): 595-8.

[23] Wang Q, Dordick SJ, Linhardt JR. Synthesis and application of carbohydrate-containing polymers. Chem Mater 2002; 14(8) 3232-44.

[24] Lowe BA, Wang R. Synthesis of controlled-structure AB diblock copolymers of 3-*O*-methacryloyl-1,2:3,4-di-*O*-isopropylidene-d-galactopyranose and 2-(dimethylamino)ethyl methacrylate. Polymer 2007; 48(8) 2221-30.

[25] Albertin L, Stenzel M, Barner-Kowollik C, John L, Foster R, Davis TP. Well-defined glycopolymers from RAFT polymerization: poly(methyl 6-*O*-methacryloyl-α-d-glucoside) and its block copolymer with 2-hydroxyethyl methacrylate. Macromolecules 2004; 37(20): 7530-7.

[26] Deng Z, Ahmed M, Narain R. Novel well-defined glycopolymers synthesized via the reversible addition fragmentation chain transfer process in aqueous media. J Poly Sci Part A Polym Chem 2008; 46(15): 4984-4996.

[27] Deng Z, Bouchekif H, Babooram K, Housni A, Choytun N, Narain R. Facile synthesis of controlled-structure primary amine-based methacrylamide polymers via the reversible addition-fragmentation chain transfer process. J Poly Sci Part A Polym Chem 2009; 47(2): 614-27.

[28] Ziebarth J, Wang Y. Coarse-grained molecular dynamics simulations of DNA condensation by block copolymer and formation of core−corona structures. J. Phys Chem B 2010; 114(19): 6225-32.

[29] Boussif O, Delair T, Brua C, Veron L, Pavirani A, Kolbe JVH. Synthesis of polyallylamine derivatives and their use as gene transfer vectors in vitro. Bioconjugate Chem 1999; 10(5): 877-83.

[30] Le Bon B, Van Craynest N, Boussif O, Vierling P. Polycationic diblock and random polyethylene glycol- or tris(hydroxymethyl)methyl-grafted (co)telomers

for gene transfer: synthesis and evaluation of their in vitro transfection efficiency. Bioconjugate Chem 2002; 13(6): 1292-1301.

[31] Zugates TG, Peng W, Zumbuehl A, Jhunjhunwala S, Huang YH Langer R, Sawicki AJ, Anderson GD. Rapid optimization of gene delivery by parallel end-modification of poly(*β*-amino ester)s. Molec Ther 2007; 15(7): 1306-12.

[32] Wolfert AM, Dash RP, Nazarova O, Oupicky D, Seymour WL, Smart S, Strohalm J, Ulbrich K. Polyelectrolyte vectors for gene delivery: influence of cationic polymer on biophysical properties of complexes formed with DNA. Bioconjugate Chem 1999; 10(6): 993-1004.

[33] Sharma R, Lee SJ, Bettencourt CR, Xiao C, Konieczny FS, Won YY. Effects of the incorporation of a hydrophobic middle block into a PEG-polycation diblock copolymer on the physicochemical and cell interaction properties of the polymer−DNA complexes. Biomacromolecules 2008; 9(11): 3294-3307.

[34] Nakayama Y, Kakei C, Ishikawa A, Zhou MY, Nemoto Y, Uchida K. Synthesis and in vitro evaluation of novel star-shaped block copolymers (blocked star vectors) for efficient gene delivery. Bioconjugate Chem 2007; 18(6): 2037-44.

[35] Shuai X, Merdan T, Unger F, Wittmar M, Kissel T. Novel biodegradable ternary copolymers *hy*-PEI-*g*-PCL-*b*-PEG: synthesis, characterization, and potential as efficient nonviral gene delivery vectors. Macromolecules 2003; 36(15): 5751-9.

[36] Hsiue HG, Chinag ZH, Wang HC, Juang MT. Nonviral gene carriers based on diblock copolymers of poly(2-ethyl-2-oxazoline) and linear polyethylenimine. Bioconjugate Chem 2006; 17(3): 781-6.

[37] Azzam T, Eliyahu H, Shapira L, Linial M, Barenholz Y, Domb JA. Polysaccharide-oligoamine based conjugates for gene delivery. J Med Chem 2002; 45(9): 1817-24.

[38] De Smedt CS, Demeester J, Hennink EW. Cationic polymer based gene delivery systems. Pharm Res 2000; 17(2): 113-26.

Chapter 4. Well-controlled Cationic Water-soluble Phospholipid Polymer-DNA Nano-complexes for Gene Delivery

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4.1. Introduction

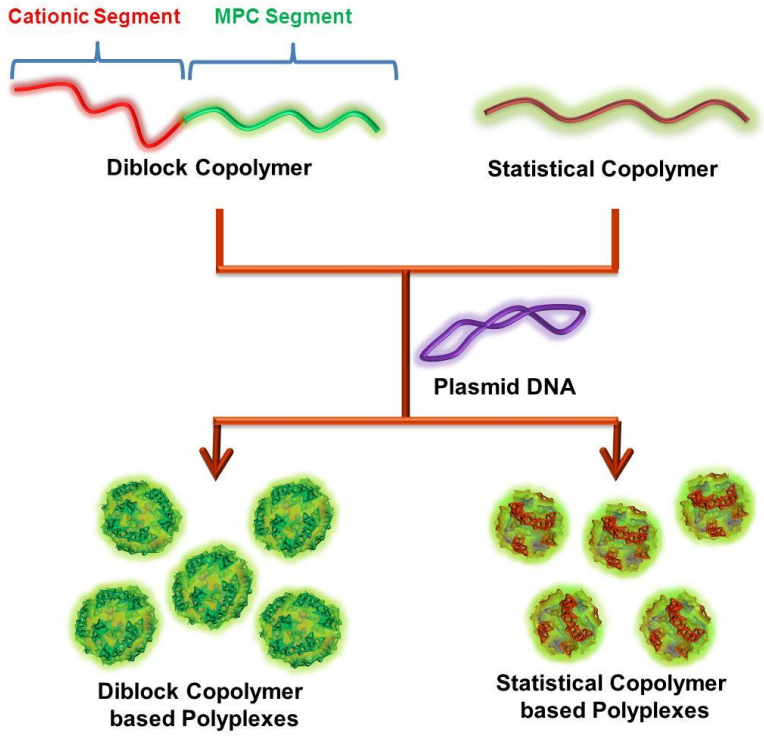
The successful treatment of genetic disorders by gene therapy is dependent on the facile synthesis of non-immunogenic and non-toxic gene delivery vectors in reproducible manner.[1-2] A variety of non-viral gene delivery vectors have been produced and their transfection efficiencies are being evaluated, in an effort to determine the ideal gene delivery vector with enhanced gene expression and low toxicity.[2-11] Poly(ethylene glycol) (PEG) is one of the water-soluble and low-toxic polymers that has been extensively explored in cationic gene delivery vectors to decrease the immunogenicity and toxicity of the later.[3,5,8,10,12] The drawbacks associated with PEGylated gene delivery systems are the low uptake of polyplexes and hence a lower gene expression. Therefore, there is a need to develop alternative biocompatible polymers, which can be used as cationic gene delivery vehicles.[5]

The role of phospholipid polymers for biological functions is well studied and it has been found that these polymers have the potential to produce bio-inert surfaces for specific biological applications.[13,14] 2-Methacryloxyethyl phosphorylcholine (MPC) has phosphorylcholine group in the molecule and shows very high water-solubliity[15]. The MPC can be polymerized by a variety of radical polymerization techniques to yield the phospholipid polymers.[16] Hence, the production of the cationic MPC copolymer based vectors for gene delivery purposes is thought to be a useful technique to produce gene delivery agents of desired properties.[17-21] Lam *et al*. and others have produced block-type MPC copolymers by incorporation of tertiary amines with MPC polymer

segments *via* Atom transfer Radical Polymerization Technique (ATRP) and their role for gene and drug deliveries are investigated.[18] The incorporation of MPC units to tertiary amines is found to impact the DNA condensation ability and gene transfer efficiencies of cationic polymers.[17, 21] Chim *et al.* has further explored the role DNA condensation abilities of cationic MPC block copolymers using atomic force microscopy (AFM) and transmission electron microscopy (TEM).[19] The increase in MPC polymer segment in diblock copolymers is found to decrease the DNA condensation efficacies and hence the gene expression of the corresponding block copolymers.[17, 20] It is shown that approximately three-fold increase in tertiary amine component of copolymers is required to produce enhanced gene expression *in vitro*.[20] However, an increase in cationic character also induces severe toxicity, and hence produce a major barrier towards the synthesis of biocompatible non-viral gene delivery vector.

We propose the synthesis of water-soluble and cationic polymers composed of the MPC and methacrylate with primary amine as block and random architectures by reversible addition fragmentation chain transfer (RAFT) method. The RAFT process is extensively studied for the synthesis of a range of polymeric materials for biomedical applications[22] and is chosen here due to the ease of synthesis of those hydrophilic polymers under aqueous conditions. The role of primary amines in gene delivery is well studied and is found to be superior than tertiary amines.[2] The synthesis of cationic polymers composed of the MPC and methacrylate with primary amine for gene delivery purposes are thought to improve the gene expression of the MPC copolymers, by promoting

enhanced DNA condensation abilities. The predetermined ratio of the MPC units to primary amine component is targeted to be one. The determination of this ratio of monomers for copolymer synthesis is based on the previous work, which shows that polymers of this composition can deliver the gene of interest with higher efficacies and low toxicity and their gene expression is dependent on the architecture of the polymers.[23] The cationic polymers and MPC based copolymers of varying degree of polymerization were synthesized and their toxicity profiles were determined. These polymers were then tested for their DNA condensation ability and gene expression. To the best of our knowledge, this is the first approach where cationic MPC copolymers of similar compositions and of different architectures (block versus random) are synthesized by RAFT method and their gene transfer abilities are explored in detail.



Scheme 4-1. Synthesis of statistical and diblock copolymers based polyplexes using *β*-galactosidase plasmid.

The polyplexes were synthesized at varying polymer to DNA ratios, as shown in scheme 4-1, and their sizes and net charges were determined using transmission electron microscopy (TEM), dynamic light scattering (DLS) instrument and zeta potential, respectively. The polyplexes synthesized were subjected to agarose gel electrophoresis to determine their stability in electrophoretic environment. The gene expression of polyplexes synthesized using copolymers of block and random architectures in the presence and absence of serum were then compared and their toxicities after transfection were evaluated. Moreover, the uptake of polyplexes in Hep G2 cells was studied in detail using confocal microscopy and flow cytometer.

4.2. Material and Methods

4.2.1. Materials.

O-Nitrophenyl *β*-D-galactopyranoside, (ONPG) (enzymatic), 37 wt% formalin, Fluorescein isothiocyanate (FITC), *β*-Mercaptoethanol, MTT assay kit to determine cell viability, Phosphotungstic acid (PTA), and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were purchased from Sigma Aldrich. Linear polyethyleneimine (PEI) (M_w= 25 kDa) was purchased from Polysciences Inc. Cell Culture media Dulbecco's Modified Eagle Medium (DMEM; low glucose with glutamine and sodium pyruvate), penicillin (10000 U/mL), and streptomycin (10 mg/mL), 0.25% trypsin, Dulbecco's modified Phosphate Buffer Saline (DPBS) and Fetal Bovine Serum

(FBS) were obtained from Invitrogen. Micro BCA assay kit was obtained from Fisher Scientific. Gwiz β-galactosidase plasmid was purchased from Aldevron. MPC was obtained from NOF Co, Tokyo, Japan, which was produced by the previous reported method.[15, 16] Cy5' plasmid labelling kit was purchased from Bio Mirus. 4-cyanopentanoic acid dithiobenzoate (CTP) was synthesized in lab and 4,4'-azobis(4-cyanovaleric acid) (ACVA) was from Sigma Aldrich.

4.2.2. Synthesis of monomers and polymers

The cationic monomers, and polymers of varying degree of polymerization and architecture were synthesized according to previously reported protocols.[21,23]

4.2.3. Synthesis of P(AEMA) macroCTA by RAFT polymerization:

MPC macroRAFT agent was synthesized using our previous protocol.[21] 2-Aminoethyl methacrylamide hydrochloride (AEMA) (1.0 g, 6.0 x 10^{-3} moles), 4-cyanopentanoic acid dithiobenzoate (CTP) (0.042 g, 1.5 x 10^{-4} moles) and 4,4'-azobis(4-cyanovaleric acid) (ACVA) (0.014 g, 5.0 x 10^{-5} moles) were dissolved in 2.0 mL water in a 10 mL reactor. The solution was purged with nitrogen for 30 min. After degassing, the solution was placed in an oil bath at a temperature of 60 °C. The reaction was stopped after 6 hours by quenching in liquid nitrogen. Polymer was obtained by precipitating the mixture in acetone and washing 3-4 times to obtain a white powder. Any remaining traces of monomer were removed by washing the solution in water: acetone (1:7). The final powder was freeze-dried. The P(MPC_{18}) macroCTA molecular weight and PDI were found to be 2.5 x 10^3 g/mol and 1.2, respectively, by Gel Permeation Chromatography.

4.2.4. Synthesis of P(AEMA-*b*-MPC) by RAFT polymerization:

Chain extension was carried out using MPC as the second block and AEMA/APMA macroRAFT agent. In a typical reaction procedure, MPC (0.40 g, 2.4 x 10^{-3} moles), and AEMA macroCTA (0.20 g, 8.0 x 10^{-5} moles) were dissolved in 2 mL water. Stock solution of ACVA (0.011 g, 4.0 x 10^{-5} moles) in 2-propanol was prepared and mixed with the reaction solution. The reactor was placed in an oil bath at 70 °C for 20 h. The di-block co-polymer P($AEMA_{15}$-*b*-MPC_{17}) was obtained by precipitating the solution in acetone and washing the solution with methanol: acetone (1:7). The final powder was freeze-dried. The molecular weight of the resulting copolymer was found to be 7.6 x 10^3 g/mol and PDI 1.3.

4.2.5. Synthesis of P(AEMA-*st*-MPC) by RAFT polymerization:

The random copolymers of MPC and AEMA were synthesized by adding the monomers in the required molar ratio. A typical polymerization procedure for the copolymerization of MPC and AEMA in ratio 4:3 is given as follows: MPC (0.48 g, 1.6 x 10^{-3} mol), and AEMA (0.20 g, 1.2 x 10^{-3} mol) were dissolved in 3 mL water. CTP (0.022 g, 8.1 x 10^{-5} mol) and ACVA (7.0 mg, 2.7 x 10^{-5} mol) were dissolved in 1.0 mL of 2-propanol and mixed with the aqueous solution of monomers. The reaction mixture was degased and is kept at 70 °C in an oil bath for 20 h. The reaction was stopped by quenching in liquid nitrogen. The polymer was precipitated in acetone and washed repeatedly to obtain a powder. Any un-reacted monomer was removed by washing the powder with water:acetone (1:7). The powder was freeze-dried to remove any traces of acetone. The molecular

weight of the resultant copolymer P($AEMA_{15}$-*st*-MPC_{10}) was found to be 6.0 x 10^3 g/mol and the PDI was found to be 1.2 by aqueous GPC.

The molecular weight and molecular weight distributions of homo and copolymers were determined using aqueous GPC; Viscotek GPC system. The sodium acetate/acetic acid (0.50 mol/L / 0.50 mol/L) buffer was used as eluents at room temperature and at a flow rate of 1.0 mL/min and pullulan standards (M_w = 5.0 x 10^2 – 4.0 x10^6 g/mol) were used for calibration. The compositions of statistical copolymers were analyzed using ^{1}H-NMR.

4.2.6. Formation of polyplexes

Polyplexes were made by mixing varying concentrations of polymers with 0.4 mg/mL of *gwiz-β*-galactosidase plasmid in OMEM. The complexes were vortexed and incubated for 30 min before their analysis. The particle sizes and surface charges were measured using Brookhaven Zeta Plus (zeta potential and particle size analyzer) instrument.

4.2.7. Cell culture

Hela and Hep G2 cells were maintained in DMEM supplemented with 5.0 and 10 % FBS, respectively and 1.0 % penicillin/streptomycin solution in a humidified atmosphere in the presence of 5% CO_2 at 37 °C. Upon 80% confluency the cells were trypsinized with 0.25 % trypsin and were seeded in tissue culture plates.

4.2.8. Determination of lethal dose50 (LD_{50}) values

Hela cells were seeded in 96 well tissue culture plates at the density of 9.0 x 10^3 cells per well. The cells were incubated overnight and were treated with varying

concentrations of polymers in the presence of serum containing media. The cells were further incubated for 12 h and 25 µL of MTT dye was added per well. After 2 h 100 µL of lysis buffer was added and cells were allowed to lyse overnight. The absorbance was read at 570 nm using TECAN Genios pro microplate reader. The untreated cells and media alone were used as positive and negative controls, respectively. The percent cell viability was calculated (% cell viability = (treated cells – negative control)/ (untreated cells – negative control). LD_{50} values for different polymers were determined using Origin Pro software and data was analyzed using Boltzman function to provide a sigmoidal fit.

4.2.9. Transfection

Hep G2 cells were seeded at the density of $1.0X10^5$ cells per well in 24 well tissue culture plates and were incubated overnight. The media was removed and 150 µL of serum free OMEM or serum containing media was added followed by the addition of varying concentrations of polyplexes. The cells were incubated for four hours and polyplexes containing media were replaced with fresh serum containing media. The cells were allowed to grow for 48 h before their lysis (CHAPS in sodium phosphate lysis buffer) (pH = 7.5) followed by a freeze-thaw cycle. The activity of *β*-galactosidase was detected using *β*-galactosidase assay, as described before.[23]

4.2.10. Toxicity after transfection

Hep G2 Cells were seeded in 24 well tissue culture plates as described above and were transfected with polyplexes in the manner identical to transfection conditions. 48 h post-transfection, 125 µL MTT dye was added per well

followed by the addition of 500 µL of lysis buffer after two hours of incubation. The absorbance was read at 570 nm using TECAN plate reader. The untreated cells and media alone were used as positive and negative controls respectively and percent cell viability was determined as mentioned in LD_{50} section.

4.2.11. Fluorescence labelling of copolymers

Fluorescein isothiocyanate (FITC) was dissolved in DMSO at 1.0 mg/mL concentration. P($AEMA_{26}$-*b*-MPC_{27}), and P($AEMA_{19}$-*st*-MPC_{21}) (57 mg/mL) were dissolved in 4.0 % Na_2CO_3 (pH was adjusted to 8.5). Small aliquots of FITC (2.4 mg of FITC per 100 g of polymer) were slowly added to the polymer solutions in dark while vortexing. The solution was incubated in the dark overnight. The solution was dialyzed in the dark (MWCO = 6,000-8,000 Da) against de-ionized water and purified solution was stored in the dark at room temperature.

4.2.12. Fluorescent labelling of plasmid

Gwiz-*β*-Galactosidae plasmid was labelled according to the manufacturer protocol. In brief, plasmid DNA (1.0 µg) was incubated with DNA labelling buffer and cy5' fluorescent dye at 37 °C for 2 h. The cy5'labelled plasmid was purified and labelling of plasmid was confirmed using confocal microscope.

4.2.13. Uptake of Polymers and Polyplexes using Confocal Microscope

Hep G2 cells were seeded in 6 well tissue culture plates containing glass coverslips. Upon 80 % confluency media was removed and cells were treated with FITC-labelled polymers alone, FITC-labelled polyplexes, or with cy5'-

labelled polyplexes in the presence or absence of serum. The untreated cells were used as negative control. The cells were incubated for 4 h, media was removed and the cells were washed twice with DPBS before their fixation using 3.7 % formalin in DPBS. The cells were rehydrated with DPBS and fixed on microscope slides. The samples were analyzed using Fluoview FV10i Olympus confocal microscope and samples were excited at 485 & 650 nm and were detected at 535 and 670 nm, for FITC and Cy5'-labelled samples respectively, using 60X objective.

4.2.14. Flow Cytometer

Hep G2 cells were grown on 24 well tissue culture plate at the density of 1.0 x 10^5 cells per well and were allowed to adhere overnight. The media was then replaced with 150 μL of OMEM followed by the treatment with FITC-labelled polymers or polyplexes and cy5' labelled polyplexes in the presence or absence of serum. The cells were further incubated for 3-4 h. The cells were rinsed with DPBS two times to remove the adherent polyplexes from the surface of cells, and were trypsinized and suspended in DPBS containing 3.7 % formalin. The uptake of polymers and polyplexes was quantified using Beckman Coulter Quanta SC flow cytometer & FACS Quanta using FL1 channel for FITC and FL-4 channel for cy5'-labelled samples, respectively. The untreated cells were used as negative control.

4.2.15. Agarose gel Electrophoresis

The polyplexes were synthesized using conditions identical to transfection experiment and were loaded in 1.0 % agarose gel containing 1.0 μg/mL ethidium

bromide in 1X tris acetate/EDTA buffer and the gel was run at 140 V for 30 min and the DNA bands were visualized using UV transilluminator (Alpha Innotech; San Leandro, CA).

4.2.16. Stability of Polyplexes in the Presence of Serum Proteins

P($AEMA_{26}$-*b*-MPC_{27}) & P($AEMA_{19}$-*st*-MPC_{21}) polyplexes were prepared in OMEM as described above and their size was determined using DLS. The polyplexes were then incubated with 10 % fetal bovine serum and change in size and polydisperisty of polyplexes as a function of time was monitored for four hours period.

4.2.17. Determination of Protein Content on the Surface of Polyplexes

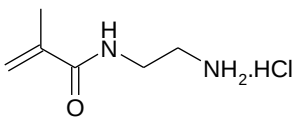
P($AEMA_{26}$-*b*-MPC_{27}) and P($AEMA_{19}$-*st*-MPC_{21}) polyplexes were prepared in deionized water under conditions used for transfection purposes. The polyplexes were incubated with 10 % fetal bovine serum for 30 min. The polyplexes were centrifuged at 14,000 g for 60 min and supernatant was collected. The polyplexes were redispersed in deionized water and were washed two more times to ensure the removal of free proteins in solution. The supernatant and polyplexes were subjected to BCA assay to determine the amount of protein in supernatant and on the surface of polyplexes. The percent protein in solutions was determined by dividing the protein in solution by total amount of protein added to the sample.

4.2.18. Transmission Electron Microscopy

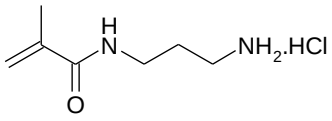
Transmission electron microscope (TEM) images of polyplexes samples stained with Phosphotungstic acid (PTA) (synthesized using conditions identical to transfection experiments) were obtained using Philips Transmission electron microscope equipped with a CCD camera.

4.3. Results and Discussion

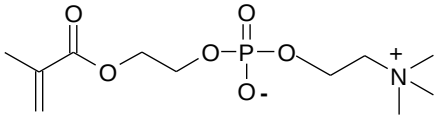
Phospholipid polymers, in particular, the phosphorylcholine groups bearing polymers have high potential in the biomedical field due to their excellent biocompatibility of the resulting biomaterials[24-27] Among them, the water-soluble MPC polymers are attractive for the development of gene delivery vectors that show biocompatible properties, including low toxicity and low protein activation ability. The copolymers composed of the MPC unit andAEMA & APMA units were prepared by reversible addition fragmentation chain transfer (RAFT) polymerization method. The series of cationic copolymers of controlled dimensions and architecture in the absence of metal catalysts were prepared.[21, 23] The chemical structures of MPC, APMA and AEMA were shown. (Scheme 4-2)



AEMA



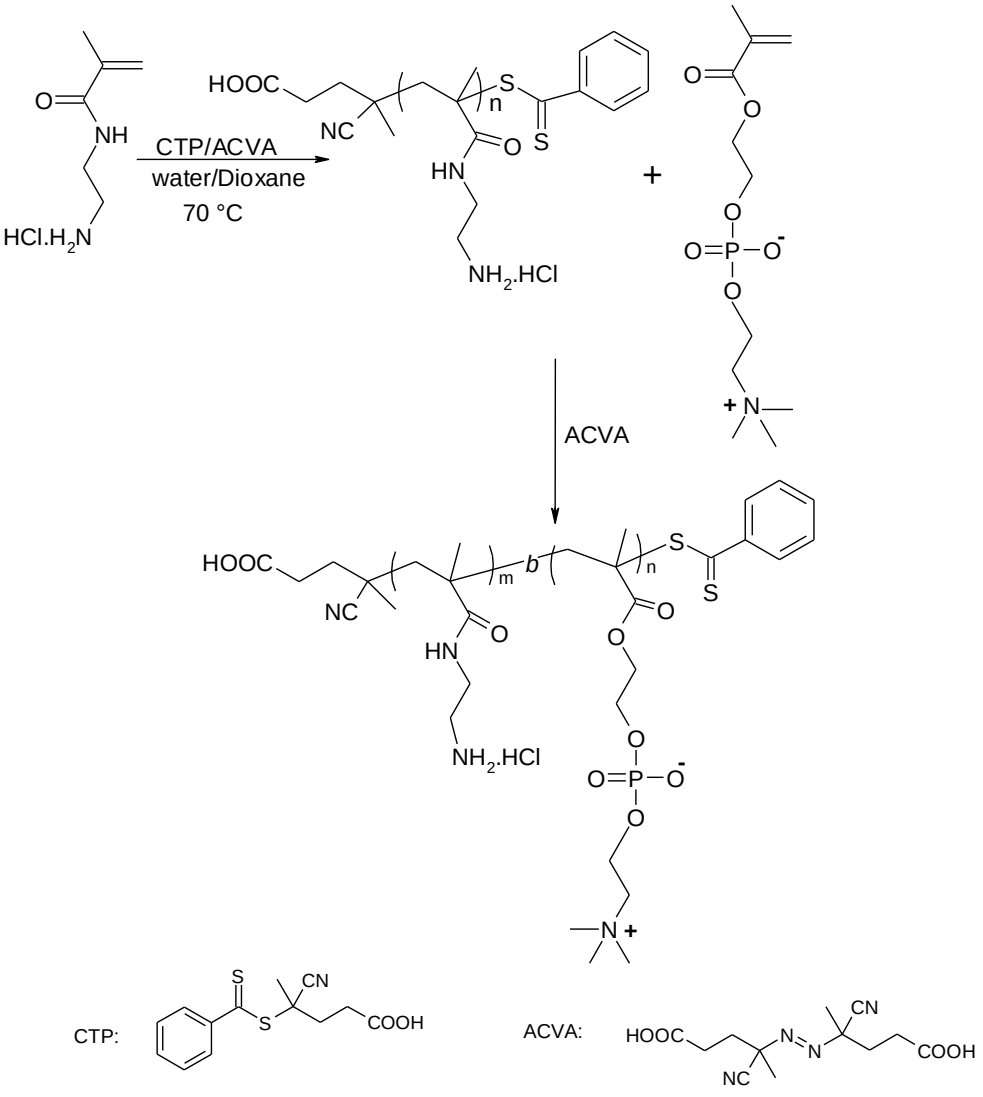
APMA



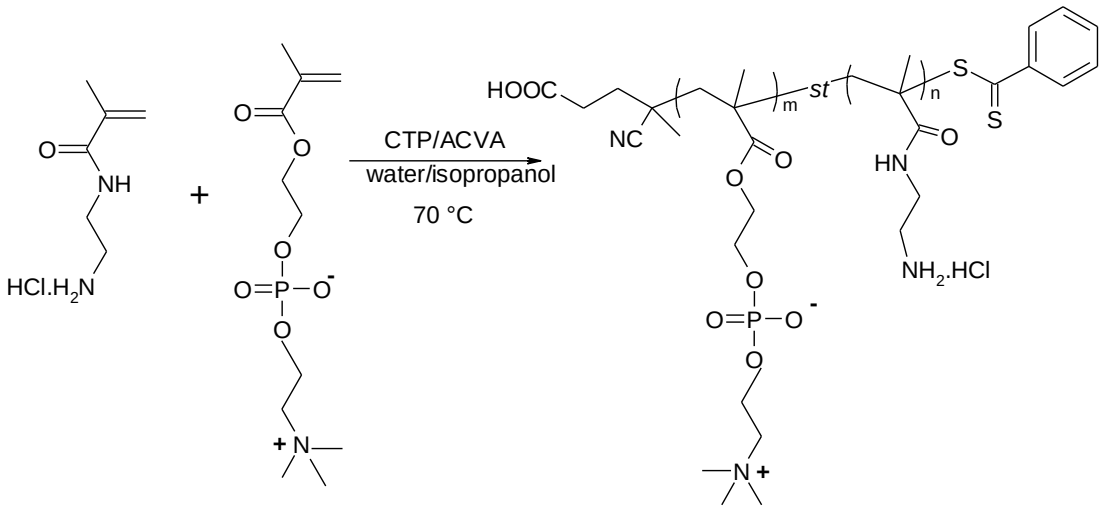
MPC

Scheme 4-2. Structures of monomers, 2-aminoethylmethacrylamide (AEMA), 3-aminopropylmethacrylamide (APMA) and 2-methacryloxyethyl phosphorylcholine (MPC).

The synthesis of statistical and diblock copolymers composed of the MPC polymer segment and AEMA polymer or APMA polymer segment was depicted in schemes 4-3 & 4-4.



Scheme 4-3. RAFT synthesis of diblock cationic MPC polymers using CTP as chain transfer agent and ACVA as initiator at 70 °C.



Scheme 4-4. RAFT synthesis of statistical cationic MPC copolymers using CTP as chain transfer agent and ACVA as initiator at 70 °C.

The cationic homopolymers and their corresponding MPC copolymers of varying degrees of polymerization and molecular weights were synthesized and their GPC data were shown in Table 4-1. (Supporting Information Figure S1 & S2) Subsequently, the copolymers were evaluated for their transfection efficiencies as a function of their molecular weight and architectures.

Table 4-1. GPC data showing the molecular weights and PDI of cationic homopolymers and their corresponding copolymer synthesized by RAFT method.

Polymer Samples	Targeted M_n (kDa)	Achieved M_n (kDa)	PDI
$P(AEMA)_{15}$	2.5	2.5	1.2
$P(PAEMA)_{36}$	6.6	6.0	1.2
$P(AEMA_{15}$-*b*-$MPC_{17})$	8.3	7.6	1.3
$P(AEMA_{26}$-*b*-$MPC_{27})$	1.9	12	1.3
$P(AEMA_{36}$-*b*-$MPC_{40})$	18.4	18	1.3
$P(AEMA_{10}$-*st*-$MPC_{15})$	8.3	6.0	1.2
$P(AEMA_{19}$-*st*-$MPC_{21})$	12.9	9.0	1.2
$P(AEMA_{40}$-*st*-$MPC_{36})$	18.3	17	1.2
$P(APMA)_{18}$	2.7	3.3	1.2
$P(APMA)_{37}$	7.1	6.7	1.1
$P(APMA_{18}$-*b*-$MPC_{16})$	8.6	8.2	1.1
$P(APMA_{22}$-*b*-$MPC_{37})$	13.3	15	1.3
$P(APMA_{37}$-*b*-$MPC_{40})$	20.4	18	1.3
$P(APMA_{7}$-*st*-$MPC_{14})$	8.6	5.4	1.2
$P(APMA_{14}$-*st*-$MPC_{22})$	13.3	9.0	1.2
$P(APMA_{32}$-*st*-$MPC_{32})$	20.4	15	1.2
$P(MPC)_{54}$	15.9	16	1.1

The copolymers (statistical and blocks) were designed in such a way that they have a similar content of cationic and phosphorylcholine groups. Such a composition was targeted to generate cationic vectors of low toxicity and improved DNA condensation ability and hence gene expression. Evaluation of a series of polymers for gene delivery is one way to select and understand the gene delivery efficacy of such materials.[29-32] Lam *et al*. synthesized a variety of MPC

block copolymers with tertiary amine groups by ATRP and their detailed study allowed the selection of copolymers with superior gene expression. However, the study revealed that a very high content of tertiary amine to the MPC unit is required to produce stable polyplexes for moderate gene expression.[18] In this work, we evaluated the transfection efficiencies of primary amine based cationic homopolymers and their corresponding the MPC copolymers of varying architecture (block *vs*. statistical) and chain length.

One of the important criteria that determine the biocompatibility of these copolymers was their cytotoxicity. The homopolymer and copolymers synthesized were subjected to cell viability assay and their LD_{50} values were determined in Hela cells (Table 4-2). As expected, the cationic homopolymers P(AEMA) and P(APMA) showed high transfection efficacy and high toxicity as compared to the P(MPC) which were non-toxic but showed no gene expression. [2, 17, 18] The combination of these two systems were therefore expected to produce non-toxic and efficient gene delivery vectors, once the copolymers of specific architecture and compositions were identified. As expected, the presence of MPC polymer segment in cationic copolymers strongly enhanced the cell viability, regardless of the copolymers architecture (Table 4-2). The previous studies showed the synthesis of the MPC copolymers as gene delivery vectors, however, no toxicity data was provided.[20] In general, homopolymers of low molecular weights $P(AEMA)_{15}$ and $P(APMA)_{18}$ showed lower toxicity than their corresponding higher molecular weight counterparts. In contrast, P(MPC) showed no significant toxicity at the concentration of 750 μM. The statistical

copolymers were found to be more cell viable than their corresponding block copolymers. For example, P($AEMA_{19}$-*st*-MPC_{21}) showed higher cell viability, indicating LD_{50} of 165 µM as compared to P($AEMA_{26}$-*b*-MPC_{27}), for which LD_{50} was 92.6 µM Moreover, P(APMA) and their corresponding copolymers were found to be more toxic than P(AEMA). The LD_{50} values for $P(AEMA)_{15}$ was found to be 141 µM, as compared to $P(APMA)_{18}$ for which LD_{50} was 12.4 µM.

Table 4-2. Determination of lethal dose 50 (LD50) values of cationic homopolymers and their corresponding MPC copolymers using MTT assay.

Polymer Samples	**LD_{50} (µM)**
$P(AEMA)_{15}$	141 ± 1.16
$P(AEMA)_{26}$	< 12.4
P($AEMA_{15}$-*st*-MPC_{20})	188 ± 1.33
P($AEMA_{15}$-*b*-MPC_{17})	546 ±1.42
P($AEMA_{19}$-*st*-MPC_{21})	165 ± 1.97
P($AEMA_{26}$-*b*-MPC_{27})	92.6 ± 1.1
P($AEMA_{40}$-*st*-MPC_{36})	316 ± 1.23
P($AEMA_{40}$-*b*-MPC_{36})	266 ± 1.06
$P(APMA)_{18}$	< 12.4
$P(APMA)_{37}$	< 12.4
P($APMA_{18}$-*st*-MPC_{16})	520 ± 1.5
P($APMA_{18}$-*b*-MPC_{16})	112 ± 1.43
P($APMA_{14}$-*st*-MPC_{22})	>745
P($APMA_{26}$-*b*-MPC_{27})	131 ± 1.04
P($APMA_{32}$-*st*-MPC_{32})	271 ± 1.2
P($APMA_{37}$-*b*-MPC_{40})	< 12.4
$P(MPC)_{54}$	> 745

The increased cell viability for AEMA based polymers compared to APMA based polymers was thought to be due to the greater distance between amine group and back bone of polymers in the case of APMA compared to AEMA, hence contributing towards higher toxicity.[29] These results were consistent with previous study, where the effect of architecture of cationic glycopolymers on cell viability was studied.[29] The MPC copolymers synthesized were then studied for their DNA complexation ability at various w/w ratios. The complexation of plasmid DNA with MPC copolymers was achieved by titrating the copolymers with fixed amount (1.2 µg) of DNA and polyplexes formed were studied by DLS for their sizes. At lower plasmid/polymers w/w ratios, aggregates ranging from 2-5 micron was observed. The increase in w/w ratio lead to formation of stable polyplexes and the sizes of these polyplexes were measured using DLS and their charges were studied with zeta potential analyzer. The DLS data showed the presence of stable nano-plexes of in the range of 50-200 nm in diameter in deionized water with strong net positive zeta values. (Figure 4-1) These results were consistent with previous study, where polyplexes formation using MPC block copolymers was studied.[19] In general, polyplexes synthesized using statistical copolymers were larger in diameter, as compared to the corresponding diblock copolymers. In contrast, P(MPC) did not allow the formation of stable polyplexes at any concentration of polymers to DNA ratios, as studied by DLS

(data not shown).

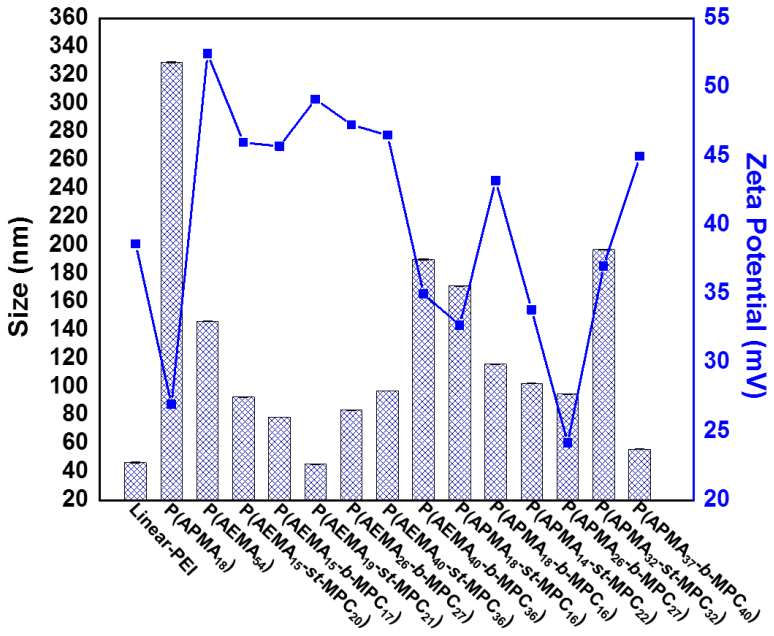


Figure 4-1. Dynamic light scattering (DLS) and zeta potential data for the MPC copolymers-DNA polyplexes. All samples were prepared in deionized water at fixed plasmid concentration, while polymer concentrations were varied to obtain stable particles.

The instability of MPC diblock copolymers based polyplexes in physiological conditions was found to be a major hurdle for their usage for gene delivery purposes.[16] Thus only a few reports were available that showed the use of MPC diblock copolymers for gene or drug delivery purposes.[17, 18-20] The conditions were optimized for the formation of polyplexes with a net positive zeta values in OMEM media. The polyplexes were also subjected to agarose gel electrophoresis to determine their stability in the media. (Supporting

information, Figure S3) The TEM images of polyplexes were then obtained to determine the role of polymer architectures on polyplexes morphology. (Figure 4-2)

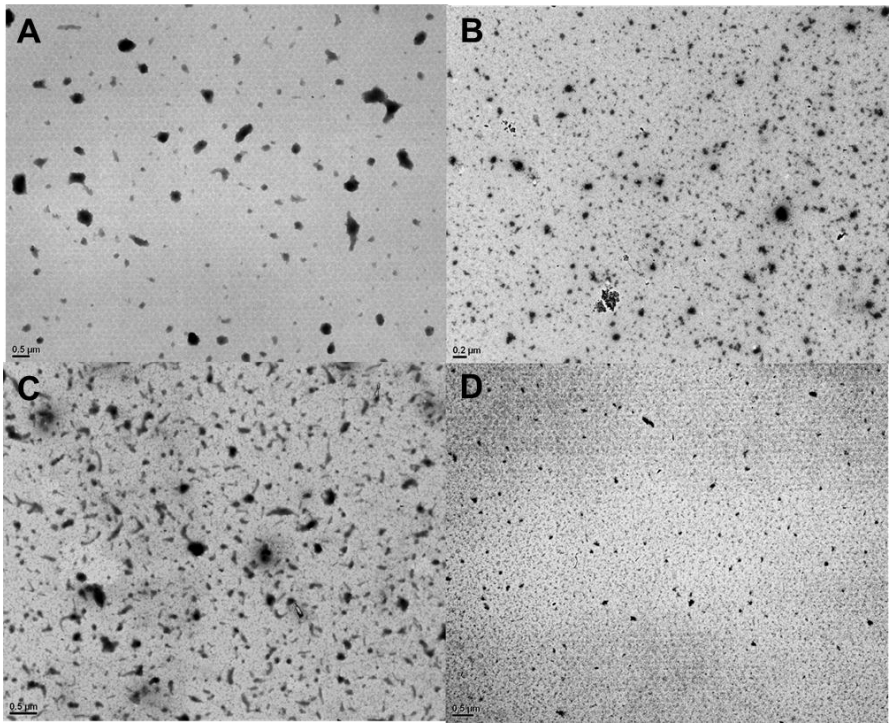


Figure 4-2. TEM images of polyplexes were stained with PTA; A) P($AEMA_{19}$-*st*-MPC_{21})-polyplexes, B) P($AEMA_{26}$-*b*-MPC_{27})-polyplexes, C) P($APMA_{14}$-*st*-MPC_{22})-polyplexes, D) P($APMA_{26}$-*b*-MPC_{27})-polyplexes. The scale bar is 0.5 µm for images A, C and D and 0.2 µm for B.

Diblock copolymers based polyplexes such as P($AEMA_{26}$-*b*-MPC_{27}) and P($APMA_{26}$-*b*-MPC_{27}) showed the presence of spherical particles as compared to their statistical copolymers based polyplexes, such as P($AEMA_{19}$-*st*-MPC_{21}) and P($APMA_{14}$-*st*-MPC_{22}) based polyplexes, which showed irregular shapes. Hence, in addition to the composition of copolymers, their architecture was found to play a dominant role in determining the shape of polyplexes. These results were

also in agreement with DLS data, where net diameter of statistical copolymers based polyplexes was found to be greater than their corresponding diblock copolymers. The DNA condensation ability of MPC block copolymers was discussed by others with the help of TEM images.[18, 19] It was shown that for tertiary amine and MPC block copolymers, content of the MPC unit to amine ratios higher than 2 were required to produce spherical well-condensed particles, MPC unit to amine ratios lower than 2 produced irregular structures ranging from toroids, to rods.[18, 19] It should be noted that in our case the diblock copolymers with an equal amount of primary amine and the MPC polymer segment produce spherical well condensed particles, as revealed by TEM images.

The stable polyplexes synthesized were tested for their gene delivery efficacy in Hep G2 cells using *β*-galactosidase assay. The hydrolysis of substrate due to the production of *β*-galactosidase enzymes was detected by measuring the absorbance at λ=420 nm. Figure 4-3 showed the gene transfection ability of MPC copolymers of varying degrees of polymerizations. The gene expression of these copolymers was found to be dependent on the copolymer architectures and molecular weights of the copolymers. The copolymers of low molecular weights (6-7 kDa) showed lower gene expression, as compared to the copolymers of high molecular weights (10-12 kDa), for DNA dose of 0.6 μg and the gene expression was comparable to that of linear PEI, which was used as a positive control. Further increase in molecular weights of copolymers (17-18 kDa) led to a

decrease in gene expression most likely due the high toxicity of these copolymers.

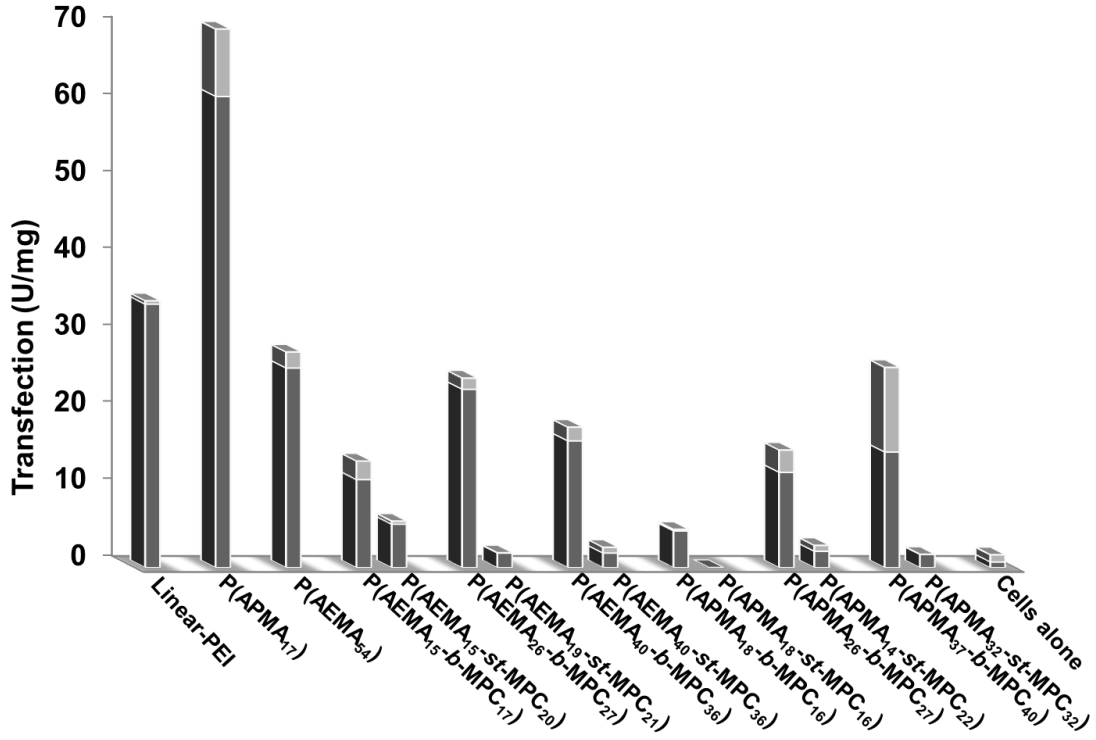


Figure 4-3. Gene expression as a function of copolymer architectures in the absence of serum proteins using Hep G2 cells. The transfection efficiencies are determined using β-galactosidase assay at DNA dose 0.6 μg. The standard deviations are shown by light color on the columns.

Hence the copolymers P($AEMA_{26}$-*b*-MPC_{27}) and P($APMA_{26}$-*b*-MPC_{27}) showed superior gene transfection as compared to the other copolymers of varying molecular weights. The gene expression of low molecular weight cationic homopolymer was higher than other homopolymers and copolymers, however the toxicity of the homopolymers was a major issue for their use for gene delivery agents. Moreover, the statistical copolymers showed lower gene expression as compared to their corresponding block copolymers. For example, gene expression was found to be 23 ± 1 U/mg for P($AEMA_{26}$-*b*-MPC_{27}) and was 1.9 ± 0.1 U/mg for P($AEMA_{19}$-*st*-MPC_{21}). This significant difference in gene expression was attributed to the architecture of copolymers, which was also shown to affect the polyplexes morphology. Sakaki *et al*. produced the MPC copolymers having primary amino group of statistical architecture, which were shown to possess some gene delivery efficacy.[17] The block copolymers composed of the MPC polymer segment and polymer segments with tertiary amines have also been prepared and are shown to possess gene expression, and the optimum gene expression is obtained at tertiary amine to cationic ratio of 2 or higher.[18] However, to the best of our knowledge, there is no study where the effect of MPC copolymers architecture on gene expression has been studied. Here in, it is clearly shown that, in addition to the copolymer compositions, the architecture of copolymers plays a significant role in determining the gene expression. The effect of DNA dose on gene expression was further studied, in an effort to produce enhanced gene delivery using these copolymers.

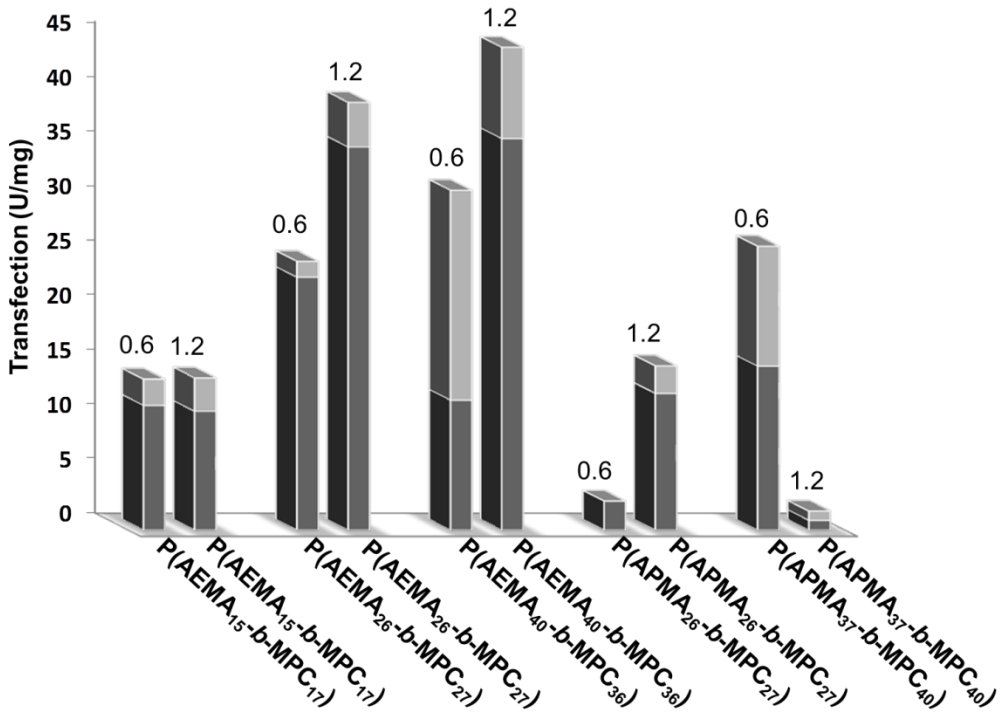


Figure 4-4. Gene expression of diblock copolymers in the absence of serum proteins using Hep G2 cells. The transfection efficiencies are determined using β-galactosidase assay at DNA dose 0.6 and 1.2 μg, as indicated on the graph. The standard deviations are indicated by light colors on the columns.

As shown in Figure 4-4, the AEMA copolymers showed improved gene transfection ability at higher DNA does (1.2 μg) as compared to APMA based copolymers. The gene expression was found to be highest (35 ± 4 U/mg) for P($AEMA_{26}$-*b*-MPC_{27}) based copolymers, although P($AEMA_{40}$-*b*-MPC_{36}) based polyplexes also showed enhanced gene expression (38 ± 8 U/mg), the toxicity of these copolymers was a major drawback at the higher DNA dose.

An important factor that determined the gene expression of polymeric vectors was their cellular uptake, which was dictated by a number of factors including

the net charge of polyplexes, their morphology and content of serum proteins on the surface of polyplexes.[1, 2] Although all MPC copolymers based polyplexes showed strongly positive zeta potential values, as determined, the morphology of these polyplexes was found to vary as a function of copolymer architecture, as shown by TEM images. To determine if lower gene expression of statistical copolymers based polyplexes was related to their cellular uptake, β-galactosidase plasmid and copolymers individually were labelled to fluorescent dyes and their uptake by HepG2 cells was explored using confocal microscopy and flow cytometry. The FITC-labelled copolymers of P($AEMA_{26}$-*b*-MPC_{27}) and P($APMA_{26}$-*b*-MPC_{27}) and cy-5' labelled plasmid were used for this purpose. The polyplexes were formed by the incubation of cy-5'-label free plasmid with FITC-labelled polymers. These polyplexes and polymers alone were incubated with Hep G2 in the absence of serum and uptake of polymers and polyplexes was studied using confocal microscope and flow cytometer. (Figure 4-5, Supporting Information Figure S5)

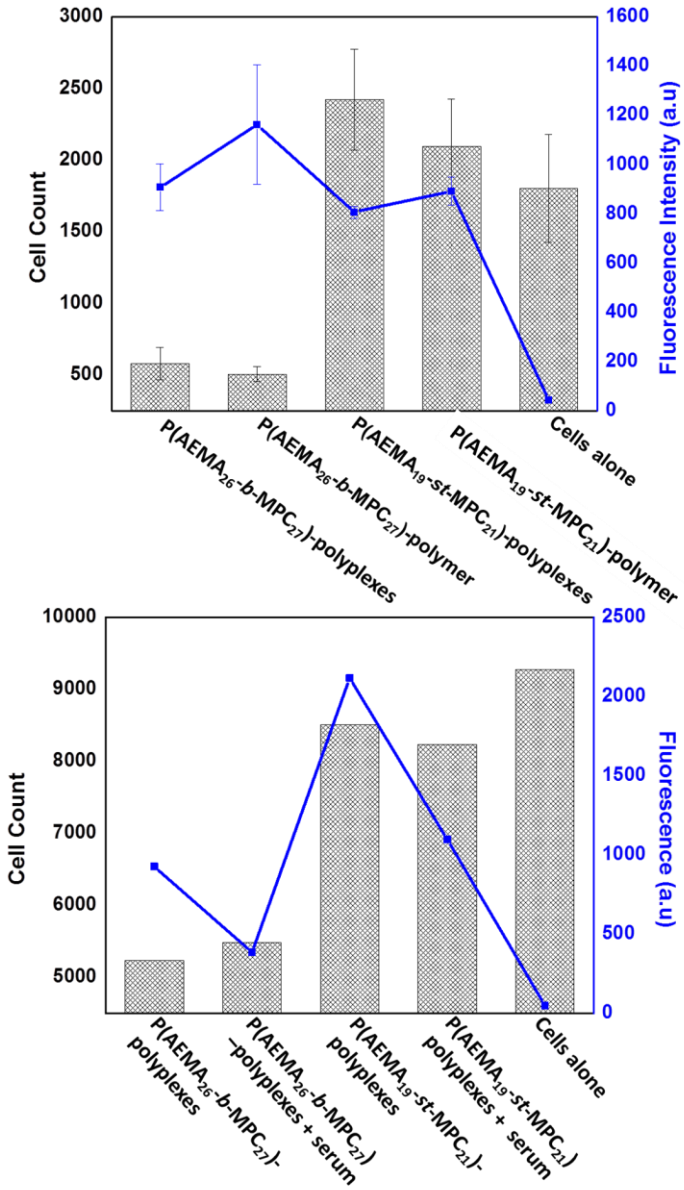


Figure 4-5. Polyplexes uptake by Hep G2 cells after four hours of incubation by flowcytometer analysis. A) FITC labelled polymers and polyplexes in the absence of serum proteins, B) Cy5'-labelled polyplexes in the presence and absence of serum proteins, the data indicates the amount of plasmid delivered to the cells (in line) and number of cells counted (in bars).

These images showed that FITC-labelled polymers and polyplexes were well uptaken by HepG2 cells, regardless of polymer architecture. The uptake of FITC-labelled copolymers and their corresponding polyplexes was further confirmed and quantified by flow cytometer analysis. It was shown that P($AEMA_{26}$-*b*-MPC_{27}) and P($AEMA_{19}$-*st*-MPC_{21}) and their polyplexes were uptaken by Hep G2 cells, and lower transfection of statistical copolymer was not related to their lower uptake.

To further confirm that these results reflected the uptake of plasmid DNA and not the polymers alone (although these polyplexes were found to be stable as determined by agarose gel electrophoresis, the association of polyplexes with cell membrane proteins can lead to dissociation of plasmid from polyplexes, hence attributing towards lower uptake of polyplexes). β-galactosidase plasmid was labelled with cy-5' fluorescent dye and cy-5'-labelled polyplexes were then prepared by incubating label free copolymers with labelled plasmid. The uptake of cy5'-labelled polyplexes was then studied using confocal microscope and flow cytometer as before. (Figure 4-5 & 4-6)

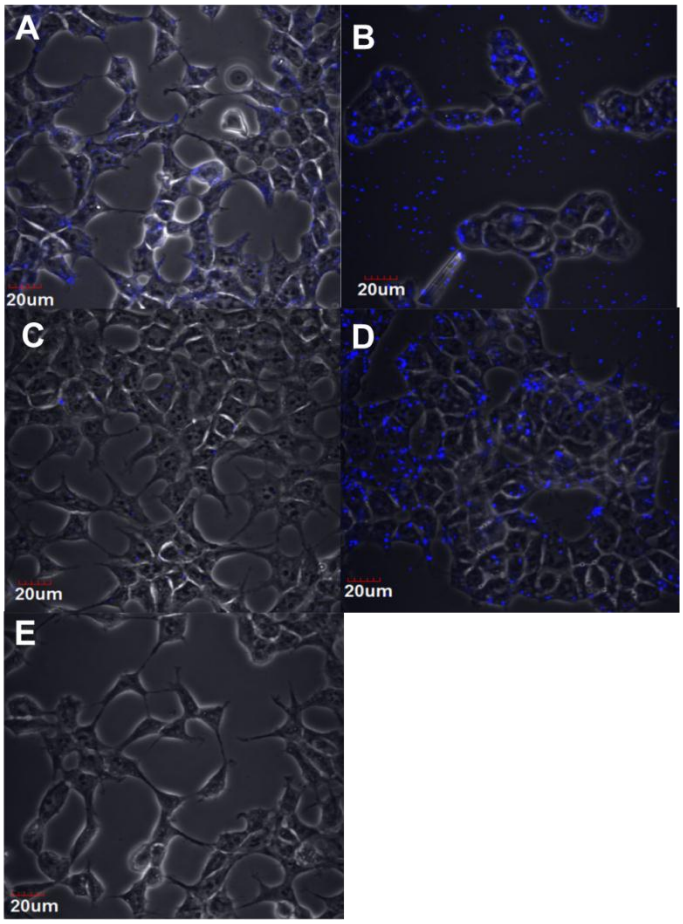


Figure 4-6. Confocal Microscope images of polyplexes uptake by Hep G2 cells after four hours of incubation. A) P($AEMA_{26}$-*b*-MPC_{27})-cy5' labelled polyplexes in the absence of serum proteins, B) P($AEMA_{26}$-*b*-MPC_{27})-cy5' labelled polyplexes in the presence of serum proteins, C) P($AEMA_{19}$-*st*-MPC_{21})-cy-5' labelled polyplexes in the absence of serum proteins, D) P($AEMA_{19}$-*st*-MPC_{21})-cy-5' labelled polyplexes in the presence of serum proteins, E) Untreated cells.

The results obtained further supported the previous results that uptake of polyplexes by Hep G2 cells was independent on copolymers architecture and polyplexes produced as both block and statistical copolymer were well uptaken.

Hence, lower gene expression of statistical copolymers was not related to their lower uptake. The modification of living cells by exogenous DNA is a complex process, and successful uptake of foreign gene does not always results in its expression. Layman, *et al.* and others have also studied the relation between the cellular uptake of polyplexes and their gene expression, and it has been shown that successful uptake of polyplexes is not always related to the transfection.[33, 34] It was thought that the mechanisms of uptake of block and statistical copolymer based polyplexes were different. The diblock copolymer based polyplexes were probably uptaken by endocytosis owing to the smaller size of the particles and hence the facile release of DNA from the polyplexes, which contribute towards their higher gene expression, as compared to the statistical copolymer based polyplexes.

The main purpose to produce MPC copolymers for gene delivery purposes was to produce gene delivery vectors that showed minimum toxicity with enhanced gene expression, as well as minimum or no interactions with serum proteins.[2] The previous studies indicated that MPC polymers served as an excellent stealth layer and did not allow the adsorption of proteins on cationic or anionic surfaces modified with MPC polymer segments.[13] Although, a handful of studies show the significance of synthesis of cationic MPC copolymers, no data has been shown so far that indicates the successful use of these copolymers based polyplexes in the presence of serum.[14-17] Chim *et al*. produced tertiary amine and MPC copolymers and it was shown that incorporation of MPC polymer segment in cationic segment greatly compromise the DNA protection ability of these

copolymers from serum proteins and nucleases.[19] This data indicated that MPC copolymers were not ideal polymeric vectors to deliver the exogenous DNA in physiological conditions. The synthesis of MPC copolymers with primary amines was expected to change their physiological properties. The primary amines based polymers show better DNA condensation ability, DNA protection and thus show better gene expression.[2] It was thought that synthesis of primary amine based copolymers would offer greater DNA protection from serum proteins and nucleases. However, a major concern in this approach was to determine if MPC polymer segment was able to mask the cationic component of these copolymers. For this purpose, selected MPC copolymers based polyplexes, P($AEMA_{26}$-*b*-MPC_{27}) and P($AEMA_{19}$-*st*-MPC_{21}) based polyplexes were prepared under conditions identical to those used for transfection purposes and these polyplexes were incubated with 10% serum for four hours. The polyplexes were centrifuged to remove the excess of serum proteins and were washed three times with deionized water. The amount of serum proteins in the supernatant and on the surface of polyplexes was determined quantitatively using BCA assay. (Supporting information, Table S1) It was found that regardless of polymer architecture, almost 89-90% of protein was present in supernatant 1, and minimal amount of serum protein was detected in supernatant 2, & 3 and on polyplexes surfaces. These results further confirmed the antifouling properties of MPC polymer based vectors, indicating that these copolymers had the potential to be used for gene delivery purposes site specifically. Moreover the stability of these polyplexes in serum media as a function of time was determined using

DLS. The polyplexes synthesized using MPC copolymers were found to be relatively stable in serum containing media, the slight aggregation of these polyplexes was reflected by higher polydispersity of the polyplexes upon the addition of serum proteins. (Supporting Information, Table S2) This slight aggregation of polyplexes was also apparent by confocal microscope images (Figure 4-6 B& D), the increase in polyplexes size in these images, as compared to no serum treatments (Figure 4-6 A & C) further supported the DLS data obtained. It should be noted that after the addition of serum, polyplexes were strongly associated to the cell surface, however their aggregation did not hinder the uptake of polyplexes by cells completely, which was further confirmed using flow cytometer analysis. (Figure 4-6 B, D, & F) The data indicated that fluorescence intensity reduced by almost half upon the addition of serum to the labelled polyplexes, possible due to the aggregation of polyplexes. The results obtained showed that this trend of polyplexes uptake and aggregation was independent of copolymer architecture. P($AEMA_{26}$-*b*-MPC_{27}) and P($AEMA_{19}$-*st*-MPC_{21}) based polyplexes were then subjected to transfection and as expected a slight decrease in gene expression was observed possible due to the lower uptake of these polyplexes in the presence of serum. However, it should be noted that the gene expression significantly was still comparable to serum free treatments.

The toxicity after transfection of selected copolymer samples was then determined in Hep G2 cells. (Supporting information, Figure S6) These results further concluded that P($AEMA_{26}$-*b*-MPC_{27}) based copolymers were excellent

gene delivery agent and showed enhanced gene expression with minimum toxicity (~60% cell viability).

4.4. Conclusions

In short, the synthesis of a variety of MPC copolymers by RAFT process allowed the careful selection of copolymer of specific architecture and molecular weight, which was found to show enhanced cell viability and gene expression. It was found that type of cationic monomer, molecular weight of copolymers and their architecture plays significant role in determining the transfection efficacies of these copolymers. The low molecular weight copolymers of MPC (~ 7 KDa) were found to be less efficient in gene transfer, while in the case of high molecular weight copolymers (~18 KDa) the gene expression was compromised due to their high toxicities. In addition to molecular weight architecture of copolymers was also found to be an important criteria in gene delivery. The statistical copolymers (regardless of their molecular weights) did not show any gene expression. P($AEMA_{26}$-*b*-MPC_{27}) based polyplexes were found to be ideal MPC copolymer for gene delivery purposes, which retained antifouling properties of MPC units, in combination with enhanced gene expression of amine component. These polymers were found to work well in the presence and absence of serum. These diblock copolymers and their corresponding polyplexes also showed high cellular uptake in the presence and absence of serum, which further supported their role as an ideal candidate for higher gene expression. Further, studies will be performed to produce MPC copolymer for targeting

purposes and their role as specific gene delivery agent *in vitro* and *in vivo* will be evaluated.

4.5. References

1. Pathak, A., Patnaik, S., and Gupta, C. K. (2009) Recent trends in non-viral vector-mediated gene delivery. *Biotechnol. J. 4*, 1559-1572.
2. Mintzer, A. M., and Simanek, E. E. (2009) Nonviral vectors for gene delivery. *Chem. Rev. 109*, 259-302.
3. Blessing, T., Kursa, M., Holzhauser, R., Kircheis, R., and Wagner, E. (2001) Different strategies for formation of PEGylated EGF-conjugated PEI/DNA complexes for targeted gene delivery. *Bioconjugate Chem*. *12*, 529-537.
4. Boussif, O., Delair, T., Brua, C., Veron, L., Pavirani, A., and Kolbe, J. V. H. (1999) Synthesis of polyallylamine derivatives and their use as gene transfer vectors in vitro. *Bioconjugate Chem*. *10*, 877-883.
5. Le Bon, B., Van Craynest, N., Boussif, O., and Vierling, P. (2002) Polycationic diblock and random polyethylene glycol- or tris(hydroxymethyl)methyl-grafted (co)telomers for gene transfer: synthesis and evaluation of their in vitro transfection efficiency. *Bioconjugate Chem. 13*, 1292-1301.
6. Zugates, T. G., Peng, W., Zumbuehl, A., Jhunjhunwala, S., Huang, Y-H., Langer, R., Sawicki, A. J., and Anderson, G. D. (2007) Rapid optimization of gene delivery by parallel end-modification of poly(β-amino ester)s. *Mol. Ther. 15*, 1306-1312.

7. Wolfert. A. M., Dash, R. P., Nazarova, O., Oupicky, D., Seymour, W. L., Smart, S., Strohalm, J., and Ulbrich, K. (1999) Polyelectrolyte vectors for gene delivery: influence of cationic polymer on biophysical properties of complexes formed with DNA. *Bioconjugate Chem*. *10*, 993-1004.
8. Sharma R., Lee, S-J., Bettencourt, C. R., Xiao, C., Konieczny, F. S., and Won, Y-Y. (2008) Effects of the incorporation of a hydrophobic middle block into a PEG−polycation diblock copolymer on the physicochemical and cell interaction properties of the polymer−DNA complexes. *Biomacromolecules 9*, 3294-3307.
9. Nakayama, Y., Kakei, C., Ishikawa, A., Zhou, M-Y., Nemoto, Y., and Uchida, K. (2007) Synthesis and *in Vitro* evaluation of novel star-shaped block copolymers (blocked star vectors) for efficient gene delivery. *Bioconjugate Chem*. *18*, 2037-2044.
10. Shuai, X., Merdan, T., Unger, F., Wittmar, M., and Kissel, T. (2003) Novel biodegradable ternary copolymers *hy*-PEI-*g*-PCL-*b*-PEG: synthesis, characterization, and potential as efficient nonviral gene delivery vectors. *Macromolecules 36*, 5751-5759.
11. Hsiue, H-G., Chinag, Z-H., Wang, H-C., and Juang, M-T. (2006) Nonviral gene carriers based on diblock copolymers of poly(2-ethyl-2-oxazoline) and linear polyethylenimine. *Bioconjugate Chem. 17*, 781-786.

12. Lin, S., Du, F., Wang, Y., Ji, S., Liang, D., Yu, L., and Li, Z. (2008) An acid-labile block copolymer of PDMAEMA and PEG as potential carrier for intelligent gene delivery systems. *Biomacromolecules 9*, 109-115.
13. Xu, Y., Takai, M., and Ishihara, K. (2009) Protein adsorption and cell adhesion on cationic, neutral, and anionic 2-methacryloyloxyethyl phosphorylcholine copolymer surfaces. *Biomaterials 30*, 4930-4938.
14. Goda, T., Goto, Y., and Ishihara, K. (2010) Cell-penetrating macromolecules: Direct penetration of amphipathic phospholipid polymers across plasma membrane of living cells. *Biomaterials 31*, 2380-2387.
15. Ishihara, K., Ueda, T., Nakabayashi, N. Ishihara K, Ueda T, and Nakabayashi N. (1990) Preparation of phospholipid polymers and their properties as polymer hydrogel membrane. *Polym. J. 22*, 355-360.
16. Ueda T, Oshida H, Kurita K, Ishihara K, and Nakabayashi N. (1992) Preparation of 2-methacryloyloxyethyl phosphorylcholine copolymers with alkyl methacrylates and their blood compatibility. *Polym. J. 24*, 1259-69.
17. Sakaki, S., Tsuchida, M., Iwasaki, Y., and Ishiara, K. (2004) A water-soluble phospholipid polymer as a new biocompatible synthetic DNA carrier. *Bull. Chem. Soc. Jpn. 77*, 2283-2288.
18. Licciardi, M., Giammona, G., Du, J., Armes, P. S., Tang, Y., and Lewis, L. A. (2006) New folate-functionalized biocompatible block copolymer

micelles as potential anti-cancer drug delivery systems. *Polymer 47*, 2946-2955.

19. Chim, A. T. Y., Lam, W. K. J., Ma, Y., Armes, S. P. Lewis, L. A., Roberts, J. C., Stolnik, S., Tendler, B. J. S., and Davies, C. M. (2005) Structural study of DNA condensation induced by novel phosphorylcholine-based copolymers for gene delivery and relevance to DNA protection. *Langmuir 21*, 3591-3598.
20. Lam, W. K. J., Ma, Y., Armes, S. P., Lewis, L. A., Baldwin, T., and Stolnik, S. (2004) Phosphorylcholine–polycation diblock copolymers as synthetic vectors for gene delivery *J. Control. Release 100*, 293-312.
21. Bhuchar, N., Deng, Z., Ishihara, K., and Narain, R. (2011) Detailed study of the reversible addition–fragmentation chain transfer polymerization and co-polymerization of 2-methacryloyloxyethyl phosphorylcholine. *Polym. Chem. 2,* 632-639.
22. Boyer, C., Bulmus, V., Davis, P. T., Ladmiral, V., Liu, J., and Perrier, S. (2009) Bioapplications of RAFT Polymerization. *Chem. Rev. 109*, 5402-5436.
23. Ahmed, M., and Narain, R. (2011) Synthetic glycopolymer-based non-viral gene delivery systems: evaluation of polymer architectures, compositions and molecular weights on gene expression. *Biomaterials In Press*.

24. Ishihara, K., and Inoue Y. (2010) Essential factors to make excellent biocompatibility of phospholipid polymer materials, *Adv. Sci. Technol.* 76, 1-9.

25. Ishihara K., Goto Y., Matsuno R., Inoue Y., and Konno T. (2011) Novel polymer biomaterials and interfaces inspired from cell membrane functions, *Biochim. Biophys. Acta-General, 1810*, 268-275.

26. Ishihara K. (2000) Bioinspired phospholipid polymer biomaterials for making high performance artificial organs. *Sci. Technol. Adv. Mater.1*,131-138.

27. Lewis AL. (2000) Phosphorylcholine-based polymers and their use in the prevention of biofouling. *Colloids Surf. B: Biointerf. 18*, 261-275.

28. Deng, Z.; Bouchekif, H., Babooram, K., Housni, A., Choytun, N., and Narain, R. (2008) Facile synthesis of controlled-structure primary amine-based methacrylamide polymers via the reversible addition-fragmentation chain transfer process. *J. Poly. Sci. Part A. Polym. Chem. 46*, 4984-4996.

29. Reineke, M. T., and Davis, E. M. (2003) Structural effects of carbohydrate-containing polycations on gene delivery. 2. charge center type. *Bioconjugate Chem*. *14*, 255-261.

30. Green, J. J., Langer, R., and Anderson, G. D. (2008) A combinatorial polymer library approach yields insight into nonviral gene delivery. *Acc. Chem. Res., 41*, 749-759.

31. Barua, S., Joshi, A., Banerjee, A., Matthews, D., Sharfstein, T. S., Cramer, M. S., Kane, S. R., and Rege, K. (2009) Parallel synthesis and screening of polymers for nonviral gene delivery. *Mol. Pharm. 6*, 86-97.
32. Lynn, M. D., Anderson, G. D., Putnam, D., and Langer, R. (2001) Accelerated discovery of synthetic transfection vectors: parallel synthesis and screening of a degradable polymer library. *J. Am. Chem. Soc. 123*, 8155-8156.
33. Layman, M. J., Ramirez, M. S., Green, D. M., and Long, E. T. (2009) Influence of polycation molecular weight on poly(2-dimethylaminoethylmethacrylate)-mediated DNA deliver in vitro. *Biomacromolecules 10,* 1244-1252.
34. Srinivasachari, S., Liu, Y., Prevette, L. E., and Reineke, T. M. (2007) Effects of trehalose click polymer length on pDNA complex stability and delivery efficacy. *Biomaterials 28*, 2885-2898.

Chapter 5. The Effect of Molecular Weight, Compositions and Lectin Type on the Properties of Hyperbranched Glycopolymers as Non-Viral Gene Delivery Systems

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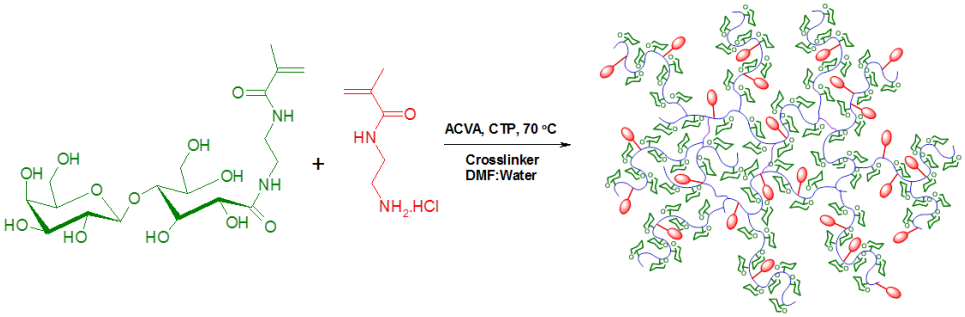
5.1. Introduction

The synthesis of gene delivery vectors and the study of their gene delivery efficacies is essential to treat genetic disorders like cancer and cystic fibrosis.[1-3] The progress in the field of chemistry and biology has allowed the preparation of well-tailored gene delivery vectors and their structure activity relationships *in vitro* are established.[3-16] Reversible Addition Fragmentation Chain Transfer (RAFT) polymerization is a living radical polymerization technique which has allowed the synthesis of cationic carbohydrate-based polymers of controlled molecular weight, compositions and architectures in the absence of protected group chemistry.[17-19] These carbohydrate based polymers and the polymers functionalized nanoparticles are then well studied for their gene delivery efficacies and are found to be an excellent materials with decreased toxicity as well as enhanced gene expression.[5,13-16] When compared to poly(ethyleneglycol) (PEG), carbohydrate stealth layer incorporated into cationic vectors enhances the cellular uptake and DNA condensation ability of these gene delivery agents, hence resulting in high gene expression.[1] In addition, the architecture of cationic glycopolymers is also found to play an important role in determining their gene delivery efficacies.[3-8] It is shown that linear statistical copolymers show higher cell viability and gene expression as compared to their corresponding linear block copolymers.[5] The gene expression of these linear statistical copolymer is found to be higher than linear poly(ethyleneimine) (PEI), which is used as a positive control for gene delivery experiments.[5] The compositions, architectures and designs of gene delivery vectors are known to

alter their biological properties.[3,7,8] For instance, cationic branched polymers have excellent DNA binding ability and show higher gene expression than their linear analogues. Dendrimers, branched PEI and branched poly(amidoamines) are a handful of examples, which are studied for gene expression in comparison to their linear analogues.[6,17-24] Recently, hyperbranched polymers of poly(dimethyl aminoethyl methacrylate) (PDMAEMA) has been synthesized by atom transfer radical polymerization (ATRP) and it is found that although hyperbranched homopolymer of PDMAEMA fails to show enhanced gene expression, as compared to its linear analogue, copolymerization of PDMAEMA with ethyleneglycol dimethacrylate (EGDMA) enhances the gene expression of its branched structure in the absence of serum.[9] It is concluded that incorporation of second monomer increases the degree of branching and hence the gene expression in this specific example.[9] Branched PEI is the most commonly used gene delivery vector and it is a very effective gene carrier. However, the major drawback for the use of branched PEI for gene delivery is its high toxicity and immunogenicity. To overcome this issue, branched PEIs are often modified by various moieties, including carbohydrates to improve the cell viability.[6,23,25] However, these modifications require complex chemical approaches, which decrease the charge density of the polymers and often compromise their gene expression, depending upon the extent of substitution.[6] Hence, it is desirable to produce hyperbranched cationic glycopolymers in one step, in the absence of protected group chemistry. Therefore, hyperbranched cationic glycopolymers of desired compositions and molecular weights are

synthesized *via* RAFT polymerization and are studied for their gene expression. The synthesis of hyperbranched glycopolymers and MPC based hyperbranched polymers has been reported. [26-28] However, there are no reports of the synthesis of hyperbranched cationic glycopolymers and of their use for biological applications. These hyperbranched glycopolymers are thought to be excellent gene delivery vectors studied for gene delivery applications, as branched architecture of polymers exhibits multivalent functional groups (carbohydrates) on their surface, as well as provide higher DNA condensation ability.[27]



Scheme 5-1. Synthesis of hyperbranched cationic glycopolymers by RAFT process.

The hyperbranched glycopolymers are synthesized by RAFT polymerization using 2-aminoethyl methacrylamide (AEMA), 3-glucoamidopropyl methacrylamide (GAPMA) and 2-lactobionamidoethyl methacrylamide (LAEMA) as shown in scheme 5-1. The hyperbranched polymers of varying molecular weights and compositions are synthesized and their gene expressions are evaluated in two different cell lines. Furthermore, the roles of two different lectins (Jacalin and RCA_{120}) on the interactions with the polyplexes and hence their effect on cellular uptake and gene expression are studied in detail.

5.2. Materials and Methods

5.2.1. Materials

Branched PEI (M_w = 25 kDa,), *O*-Nitrophenyl *β*-D-galactopyranoside, (ONPG) (enzymatic), 37 wt. % formalin, *β* -Mercaptoethanol, MTT assay kit to determine cell viability, agglutinin (lectin from *Ricinus communis*) RCA_{120}, β-lactose, Phosphotungstic acid (PTA), Dulbecco's phosphate buffer saline (DPBS) and 3-[(3-Cholamidopropyl) dimethylammonio-1-propanesulfonate (CHAPS) were purchased from Sigma Aldrich. Jacalin was from vectors lab. Cell Culture media Dulbecco's Modified Eagle Medium (DMEM) low glucose with L-glutamine and sodium pyruvate), Opti-MEM (OMEM), penicillin (10000 U/mL), and streptomycin (10 mg/mL), 0.25% trypsin, Dulbecco's modified Phosphate Buffer Saline (DPBS) and Fetal Bovine Serum (FBS) were from Invitrogen. Micro BCA assay kit was obtained from Fisher Scientific. *Gwiz β*-galactosidase plasmid was purchased from Aldevron *N*,*N'*-Methylenebis(acrylamide) was purchased from BioRad Laboratories Inc.

5.2.2. Synthesis of Hyperbranched Cationic Glycopolymers

The monomers 3-gluconamidopropyl methacrylamide (GAPMA), 2-aminoethyl methacrylamide (AEMA) and 2-lactobionamidoethyl methacrylamide (LAEMA) are synthesized according to previously reported protocols.[17,18,29] The hyperbranched cationic glycopolymers of 2-aminoethyl methacrylamide (AEMA) and 3-gluconamidopropyl methacrylamide (GAPMA) or 2-lactobionamidoethyl methacrylamide (LAEMA) are synthesized at 70 °C in the presence of 4-cyanopentanoic acid dithiobenzoate (CTP) and 4,4'-azobis(4-

cyanovaleric acid) (ACVA) as chain transfer agent and initiator, respectively. *N*,*N*′-Methylenebis(acrylamide) is used as a cross-linking agent. In a typical protocol, AEMA (0.25 g, 1.5 mmol) and GAPMA (0.25 g, 0.75 mmol) are dissolved in doubly distilled water (1.25 mL) followed by the addition of *N*,*N*′-methylenebis(acrylamide) (27.5 mg, 0.18mmol), CTP (25 mg, 89 µmol) and ACVA (5 mg, 17.8 µmol) in 1 mL of DMF (targeted DP_n = 25, Targeted molecular weight = 7.5 kDa). The mixture is subjected to three freeze-thaw cycles in 25 mL flask and is placed in oil bath for polymerization under nitrogen atmosphere for 24 hours. The polymerization is quenched using liquid nitrogen and polymer is precipitated in acetone. The product is extensively washed with methanol to remove the excess of monomers and dried under vacuum. The polymer is then analyzed using Varian 500 ^{1}H-NMR. The molecular weight and molecular weight distributions of hyperbranched glycopolymers are determined using aqueous GPC; Viscotek GPC system.0.5 M sodium acetate/0.5 M acetic acid buffer is used as eluents at room temperature and at a flow rate of 1.0 mL/min and pullulan standards (M_w = 500-404,000 g/mol) are used for calibration.

Similarly, hyperbranched statistical copolymers of LAEMA and AEMA monomers are synthesized as follows. AEMA (0.16 g, 0.96 mmol) and LAEMA (0.45g, 0.96mmol) are dissolved in double distilled water (3.5 mL), followed by the addition of CTP (21 mg, 74 µmol) and ACVA (4.2 mg, 14.8 µmol) and *N*,*N*′-methylenebis(acrylamide) (10.5 mg, 68 µmol) in DMF (3 mL) (Targeted DP_n = 25 , Targeted M_w = 8 kDa). The mixture is subjected to three freeze thaw

cycles in 10 mL flask and is placed in oil bath for polymerization at 70 °C, under nitrogen for 24 hours. The polymerization is quenched by submerging the flask in liquid nitrogen and polymer is precipitated in acetone. The excess monomer is removed by washing the polymer with methanol and polymer is dried and analyzed by GPC and ^{1}H-NMR as described above.

5.2.3. Polyplexes Formation

Polyplexes are made by mixing varying concentrations of polymers with 1.2 µg of *β*-galactosidase plasmid) in deionized water, PBS or OMEM. The complexes are vortexed and incubated for 30 minutes before their analysis. The particle sizes and surface charges are measured using Brookhaven Zeta Plus (zeta potential and particle size analyzer) instrument in deionized water. The polyplexes are also formulated in OMEM and their hydrodynamic diameter is measured before and after the addition of 10% fetal bovine serum (FBS).

5.2.4. Transmission Electron Microscopy (TEM) of Polyplexes

Hyperbranched glycopolymers are complexed with β-galactosidase plasmid in PBS as described above. TEM images of polyplexes stained with 1% Phosphotungstic acid (PTA) are obtained using Philips Transmission electron microscope equipped with a CCD camera.

5.2.5. Agarose Gel Electrophoresis

Hyperbranched glycopolymer-plasmid complexes are synthesized in OMEM at varying polymer/plasmid molar ratio. The complexes are allowed to formulate for 30 minutes, followed by the addition of DNA loading buffer. DNA alone (400 ng/well) is used as a control. The polyplexes are loaded in 1.0 % agarose

gel containing 1.0 µg/mL ethidium bromide in 1X tris-acetate/EDTA buffer and the gel is run at 140 V for 45 min. The DNA bands are visualized using UV transilluminator (Alpha Innotech; San Leandro, CA).

5.2.6. Study of Polyplexes-lectin Interactions using DLS and BCA Assay

Hyperbranched glycopolymer or PEI based polyplexes (DNA concentration 20 µg/mL) are synthesized at predetermined polymer/plasmid molar ratios in Ca^{2+} and Mg^{2+} containing DPBS and are allowed to stabilize for 30 minutes. The size of complexes is measured using DLS, followed by the addition of lectins, Jacalin and RCA_{120} at final concentrations 160 and 180 µg/mL, respectively. The polyplexes are allowed to stabilize for 20 minutes, and their sizes are measured using DLS.

To determine the content of lectins on the surface, the polyplexes synthesized above are centrifuged to remove the excess of polymers and the complexes are re-dispersed in Ca^{2+} and Mg^{2+} ions containing DPBS to maintain the concentration of DNA (20 µg/mL). The complexes are incubated with Jacalin (final concentration 160 µg/mL) or RCA_{120} (180 µg/mL) for 20 minutes in buffer at room temperature. The complexes are centrifuged and amount of lectins in the supernatant and on the surface of polyplexes is determined using BCA assay.

5.2.7. Transfection

Hep G2 and HEK293 are maintained in low glucose DMEM supplemented with 10% FBS, and 1% penicillin/streptomycin solution in a humidified atmosphere

in the presence of 5% CO_2 at 37 °C. Upon 80% confluency the cells are trypsinized with 0.25% trypsin and are seeded in tissue culture plates.

Hep G2 and HEK293 cells are seeded at the density of 100,000 and 150,000 cells per well, respectively, in 24 well tissue culture plates and are incubated overnight. The media is removed and 150 µL of serum free OMEM or serum containing media is added followed by the addition of polyplexes prepared at varying polymer/plasmid molar ratios, in the presence and absence of lectins. The cells are incubated for 2, 4 or 24 hours and polyplexes containing media is replaced with fresh serum containing media. The cells are allowed to grow for 48 hours before their lysis (CHAPS in Sodium Phosphate lysis buffer) (pH=7.5) followed by two freeze-thaw cycles. The activity of *β*-galactosidase is detected using *β*-galactosidase assay. Briefly, ONPG solution (150 µL of 4 mg/mL) is added to 50 µL of lysate volume in the presence of 4.5 µL 100X Mg solution (1.3 µL of *β*-mercaptoethanol in 400 µL of 0.1M $MgCl_2$) in 96 well plate and plate is incubated at 37 °C. The yellow color developed is detected using TECAN Genios Pro microplate reader at 420 nm after 4 hours. The total protein content in cell lysate is detected using microBCA assay (Pierce).

5.2.8. Lectin Induced Aggregation of Hyperbranched copolymer-polyplexes

RCA_{120} induced aggregation of polyplexes is studied using UV-spectrophotometer as a function time. Briefly, polyplexes are made at predetermined polymer/plasmid molar ratios at final DNA concentration of 20 µg/mL in 250 µL of DPBS. The polyplexes are allowed to stabilize for 35

minutes, RCA_{120} at final concentration of 180 µg/mL is added, and change in UV absorbance at 450 nm is studied as a function of time for 35 minutes. β-D-Lactose (200 µL of 10 mg/mL) is then added and change in UV spectra as a function of time is observed for 5 minutes.

5.2.9. Uptake of Polyplexes by Flow Cytometer

G-wiz β-galactosidase plasmid is labeled with Cy-3' labeling dye, according to manufacturer protocol. The labeled polyplexes are formulated using Cy-3' labeled plasmid and hyperbranched glycopolymers, as described above. Hep G2 and HEK293 cells are seeded in 24 well tissue culture plates. At about 80% confluency, the cells are treated with polyplexes alone (DNA dose 1.2 µg) or RCA_{120} (45µg/mL), or Jacalin, (160 µg/mL) or lactose containing polyplexes and are incubated for two hours. The cells are then washed twice with serum containing DPBS, and are rinsed with DPBS. The cells are trypsinized, followed by the addition of 3.7% formalin in PBS. The uptake of polyplexes is studied using FL2 channel of flow cytometer.

5.2.10. Confocal Microscopic Imaging of Cellular Uptake of Polyplexes

Hep G2 cells are seeded in 6 well tissue culture plates containing glass slides. The cells are allowed to adhere overnight. The media is changed and fresh serum containing media containing Cy-3' labeled polyplexes (DNA dose 1.2 µg) or Cy-3' labeled polyplexes-lectin conjugates are added. After two hours, cells are washed twice with DPBS, and are fixed with 3.7 wt. % formalin in DPBS. The cells are rehydrated and are fixed on glass slides. The slides are imaged using

Fluoview FV10i Olympus confocal microscope and samples are excited at 512 nm and are detected at 570 nm, for Cy3'-labeled samples using 60X objective.

5.2.11. Toxicity of hyperbranched glycopolymers, Polyplexes, Lectins, and polyplexes-lectin Conjugates Post-Transfection

Hep G2 Cells are seeded in 96 well tissue culture plates in complete serum containing media. Upon 80% confluency the cells are treated with varying concentration of Jacalin (0.01µg/mL – 570 µg/mL), RCA_{120}, polyplexes or RCA_{120}-polyplexes conjugates for 2 hours or with hyperbranched glycopolymers for 24 hours. The media is then replaced with fresh serum containing media and cells are allowed to grow for 48 hours. 25 µL MTT dye is then added per well followed by the addition of 100 µL of lysis buffer after two hours of incubation. The absorbance is read at 570 nm using TECAN plate reader. The untreated cells and media alone are used as positive and negative controls respectively. The percent cell viability is calculated (% cell viability = (treated cells – negative control)/ (untreated cells – negative control). Lethal dose 50 (LD_{50}) of polymers is then calculated using sigmoidal fit curve.

For the study of toxicity post transfection, polyplexes are formulated at predetermined polymer/plasmid ratios. Hep G2 cells are transfected using method described above. After 48 hours of incubation time, toxicity of polyplexes post transfection is detected using MTT assay.

5.3. Results and Discussion

The use of hyperbranched polymers for gene delivery is well explored. A variety of cationic hyperbranched polymers have been synthesized and studied for gene

delivery.[20,21] Poly(ethylenimine) (PEI) is one of the most commonly used branched polymers that has shown great potential towards gene delivery. However, the toxicity and immunogenicity associated with PEI requires its modifications with PEG, which can compromise the gene expression.[6,23,30-32] In our recent study, we have shown that linear statistical cationic glycopolymers of controlled molecular weight and compositions by RAFT polymerization are excellent alternative to produce gene delivery vectors which also show low toxicity and enhanced gene expression.[5] As branched polymers are usually better than their linear counterparts, it is hypothesized that hyperbranched cationic glycopolymers can be excellent gene delivery vectors. Therefore, hyperbranched glycopolymers of statistical architectures are synthesized and evaluated for their efficacy in gene expression.

5.3.1. Synthesis of Hyperbranched Cationic Glycopolymers

The cationic hyperbranched polymer of varying molecular weights and compositions are synthesized by RAFT polymerization using AEMA and LAEMA monomers and are analyzed by GPC for their molecular weights and polydispersities (PDI). The hyperbranched polymers are also characterized by ^{1}H-NMR for their compositions which is critical in this study. The statistical hyperbranched glycopolymers are synthesized in a one pot approach *via* the RAFT process and in the absence of protecting group chemistry. These hyperbranched polymers, once purified and characterized, are then explored for their potential to serve as gene delivery vectors. The gene expression of these glycopolymers is evaluated in the presence of two different lectins, namely

Jacalin and RCA_{120}. Two types of hyperbranched cationic glycopolymers are synthesized having either galactose or glucose-derived open sugar as pendent groups. The PDI of these copolymers are ≥1.5 which indicate their branching nature, as shown in table 5-1.

Table 5-1. Molar masses and molecular weight distribution for RAFT synthesized hyperbranched cationic glycopolymers.

Polymer Composition[a]	Cross-linker (mol-%)	GPC M_n (kDa)	M_w/M_n
P(AEMA$_{175}$-*st*-LAEMA$_{51}$)	3.5	53	3.60
P(AEMA$_{84}$-*st*-LAEMA$_{28}$)	3.5	27	1.82
P(AEMA$_{54}$-*st*-LAEMA$_{5}$)	3.5	11	1.55
P(AEMA$_{18}$-*st*-LAEMA$_{6}$)	3.5	6	1.26
P(AEMA$_{111}$-*st*-LAEMA$_{70}$)	7.0	51	2.60
P(AEMA$_{70}$-*st*-LAEMA$_{41}$)	7.0	31	2.10
P(AEMA$_{31}$-*st*-LAEMA$_{17}$)	7.0	13	1.70
P(AEMA$_{144}$-*st*-GAPMA$_{85}$)	7.0	60	11.2
P(AEMA$_{42}$-*st*-GAPMA$_{25}$)	3.5	27	2.50
P(AEMA$_{11}$-*st*-GAPMA$_{17}$)	7.0	4.5	2.80

[a]The compositions of copolymers were determined by ^{1}H-NMR.

It should be noted that, in the absence of the cross-linker *N,N'*-methylene bisacrylamide, linear statistical copolymers (M_w/M_n < 1.3) are obtained by RAFT process under same experimental conditions.[5] The molecular weight of the copolymers are further controlled by varying the degree of polymerization, monomer compositions and concentration of cross-linker. The synthesis of hyperbranched polymers by living radical polymerization (LRP) is relatively new and carries great potential to produce biologically active

macromolecules.[9,23,26] The use of RAFT process to synthesize hyperbranched polymers offers better control on the branching process, as primary chains are relatively monodisperse.[27] Hyperbranched cationic homopolymers have previously been prepared by RAFT polymerization and are shown to possess DNA binding capability, however no gene expression for these polymers has been evaluated.[23]

Cationic galactose-based hyperbranched polymers of two compositions (amine to galactose ratio 1.7:1 and 3:1) and varying molecular weights (M_n = 6-53 kDa) are obtained by varying the concentration of cross-linker from 3.5 -7 mol% (Table 5-1, Supporting Information Figure S1-S4). Further increase in cross-linker concentration leads to the formation of macrogels, especially for higher targeted degree of polymerization. The galactose-based copolymer P($AEMA_{18}$-*st*-$LAEMA_6$) show low PDI (similar to linear copolymers) under these conditions. For the comparison of gene expression of galactose-based hyperbranched copolymers with glucose-derived copolymers, a series of glucose-derived hyperbranched copolymers (M_n=5-60 kDa) are also produced under same conditions. In this study, two groups of galactose based hyperbranched copolymers of varying molecular weight (5-60 kDa) are produced which differ from each other by their compositions. The role of galactose residues for hepatocytes targeting has been studied previously.[31-36] Galactose based linear cationic polymers has been synthesized by RAFT polymerization and are found to show optimum gene expression in Hela and HepG2 cells in the absence of serum.[33] The stability and transfection

efficiencies of galactose modified cationic polyplexes is found to be dependent upon the extent of galactosylation. The increase in galactose residue on polymers, is associated with lower stability of polyplexes formed and hence low gene expression, possibly due to decrease in cationic content of copolymers.[35] The galactose based hyperbranched polymers are interesting, as no further modification of amine groups is required to introduce targeting moieties, and branched architecture of copolymers is

expected to increases the availability of galactose residues on the surface of polyplexes, after the complexation of polymers with DNA.

5.3.2. Determination of LD_{50} values

The modification of cationic polymers with carbohydrate moieties decrease their toxicity.[5] However, this involves complex chemical reactions and compromise the amine content as well as gene delivery efficacy of the polymers. The linear statistical cationic glycopolymers synthesized *via* RAFT process show high cell viability than their block analogues. [5] LD_{50} values of hyperbranched statistical cationic glycopolymers has been studied using MTT assay and are found to dependent on the molecular weight of the polymers, as well as type of carbohydrates used for the synthesis. (Table 5-2)

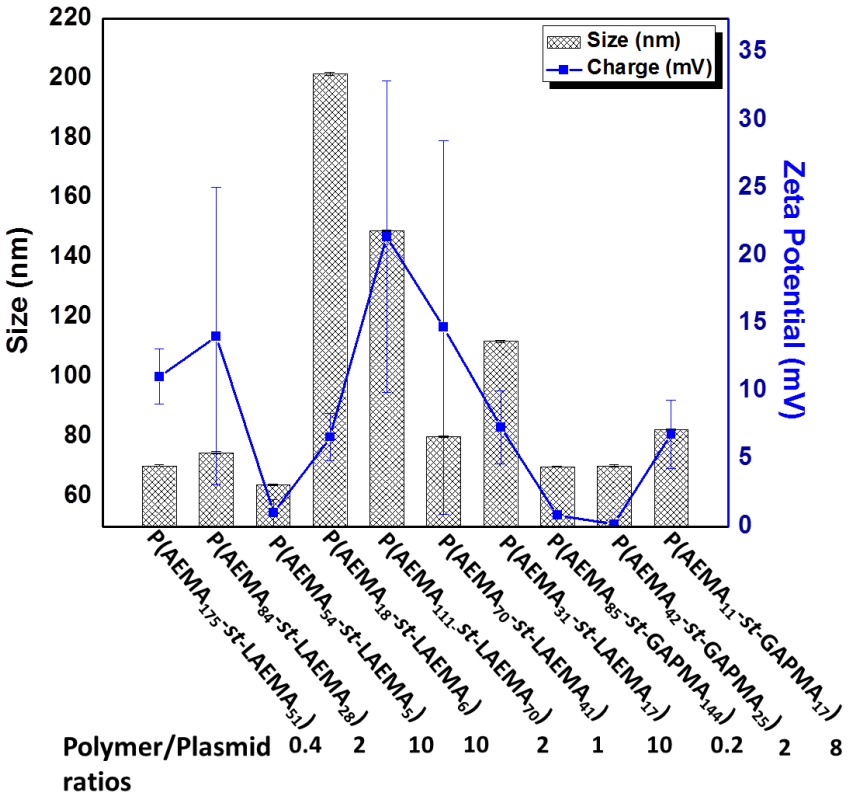
Table 5-2. Lethal Dose 50 (LD50) values of hyperbranched cationic glycopolymers, as determined by MTT assay.

Polymer Composition[a]	GPC M_n (kDa)	M_w/M_n	Lethal Dose50 (LD_{50}) µM
P($AEMA_{175}$-*st*-$LAEMA_{51}$)	53	3.60	527
P($AEMA_{84}$-*st*-$LAEMA_{28}$)	27	1.82	407
P($AEMA_{54}$-*st*-$LAEMA_{5}$)	11	1.55	>870
P($AEMA_{18}$-*st*-$LAEMA_{6}$)	6	1.26	>870
P($AEMA_{111}$-*st*-$LAEMA_{70}$)	51	2.60	<15
P($AEMA_{70}$-*st*-$LAEMA_{41}$)	31	2.10	631
P($AEMA_{31}$-*st*-$LAEMA_{17}$)	13	1.70	>870
P($AEMA_{85}$-*st*-$GAPMA_{144}$)	60	11.2	<15
P($AEMA_{42}$-*st*-$GAPMA_{25}$)	27	2.50	<15
P($AEMA_{11}$-*st*-$GAPMA_{17}$)	4.5	2.80	>870
PEI	10	2.5	8

In general, high molecular weight polymers (50-60 kDa) are more toxic than low molecular weight polymers. The galactose derived hyperbranched polymers show high LD_{50} values than glucose derived analogues. This is due to the longer carbohydrate chain of galactose derived polymers which mask the toxicity of cationic component better than glucose derived glycopolymers. Hence, P($AEMA_{42}$-*st*-$GAPMA_{25}$) show high toxicity than corresponding galactose based polymers. Low molecular weight hyperbranched polymers are very cell viable even at high concentrations.

5.3.3. Polyplexes Formulation using Hyperbranched Cationic Glycopolymers

The hyperbranched copolymers produced are complexed with β-galactosidase plasmid at varying polymer/plasmid molar ratios in OMEM. The DNA binding efficacies of these hyperbranched polymers are studied using agarose gel electrophoresis. (Supporting Information Figure S5-S13, Table S1) As expected, the higher the molecular weight of the copolymers, the lower the amount of polymer required to complex the plasmid. These results are in agreement with the previous literature, where the effect of molecular weight of copolymers on DNA complexation and gene delivery is studied.[1,33] The net surface charge and size of polyplexes at polymer/plasmid molar ratios (showing optimum gene expression) is also measured in water. It is found that regardless of molecular weight of hyperbranched copolymers, the polyplexes produced in water are about 70-200 nm in diameter (see Figure 5-1).



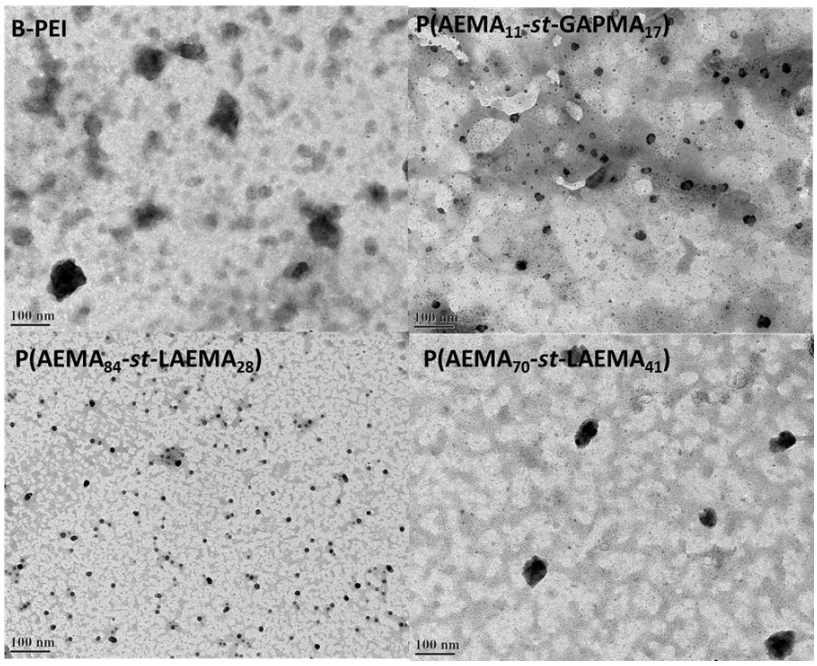


Figure 5-1. Dynamic light scattering (DLS) and zeta potential data for the hyperbranched glycopolymer-DNA polyplexes. All samples are prepared in

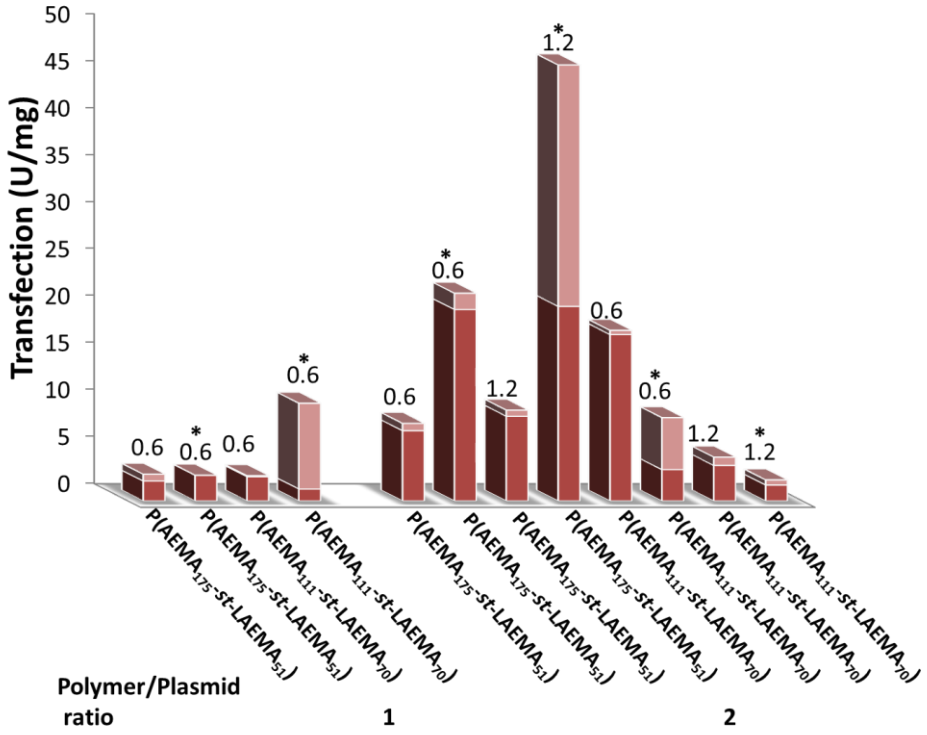
deionized water at varying polymer/DNA molar ratios. Transmission Electron Microscope (TEM) images of polyplexes formulated in PBS using branched PEI and hyperbranched glycopolymers, and stained with 1% PTA solution (bottom).

The polyplexes show low net surface charge and in some cases the charge is close to neutral. The higher polydispersitises of zeta potential values may be associated with polydisperse nature of hyperbranched copolymers produced. This data is in contrast with the polyplexes formulated using linear statistical copolymers, where monodisperse particles with strongly positive zeta potential values are obtained.[5] The block copolymers based polyplexes show lower net cationic charge, as compared to statistical copolymers based polyplexes, due to the presence of larger number of carbohydrate residues on the surface of polyplexes.[5] TEM images are obtained for the selected samples. These images confirm the formation spherical polyplexes in PBS (Figure 5-1). It should be noted that TEM images show polyplexes of smaller sizes, as compared to DLS. DLS measures the hydrodynamic radius of polyplexes in solution, while polyplexes are in dried state for TEM, this may account for the change in diameter of these polyplexes.

5.3.4. Transfection Efficacies of Hyperbranched Cationic Glycopolymers

The polyplexes produced using hyperbranched copolymers and β-galactosidase plasmid are then studied in detail for their gene expression using β-galactosidase assay. The activity of β-galactosidase enzyme in mammalian cells is determined in Units/milligram (U/mg) of protein. The galactose based hyperbranched

copolymers are first compared as a function of their molecular weights and compositions. It is found that molecular weights of these hyperbranched copolymers play an important role in determining their gene expression. All statistical hyperbranched copolymers of high molecular weights (50-60 kDa) show very low gene expression, regardless of composition and polymer/plasmid ratio used (Supporting information Figure S14-S15). The glycopolymers, P(AEMA$_{175}$-*st*-LAEMA$_{51}$), and P(AEMA$_{111}$-*st*-LAEMA$_{70}$) are studied at varying polymer/plasmid ratios. Although, the toxicity of these polyplexes is dependent upon their polymer/plasmid ratios (Supporting information Figure S14), they show low gene expression under all conditions, as shown in Figure 5-2.



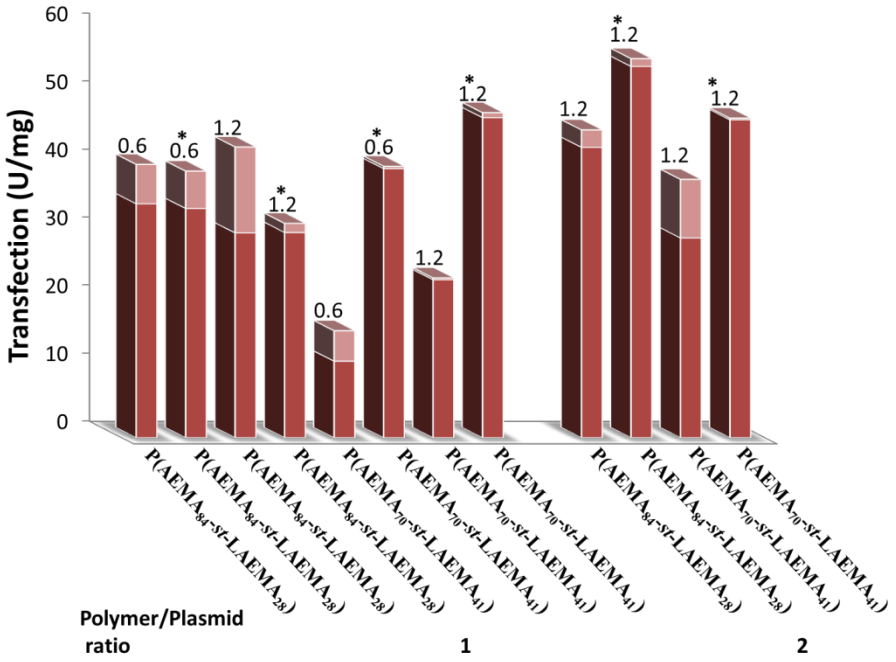


Figure 5-2. Gene expression of galactose based polymers of varying molecular weights, in the presence (*) and absence of serum using Hep G2 cells. Gene expression is evaluated using *β*-galactosidase assay at DNA dose 0.6 and 1.2 µg and varying polymer/plasmid molar ratios as shown.

The galactose-based hyperbranched copolymers of molecular weights 5, 15 and 30 kDa show high gene expression, at different polymer/plasmid molar ratios studied. P($AEMA_{84}$-*st*-$LAEMA_{28}$) and P($AEMA_{70}$-*st*-$LAEMA_{41}$) based polyplexes show optimum gene expression at low polymer/plasmid ratios (molar ratios of 1 and 2 respectively), along with high cell viability. (Figure 5-2, Supporting information figure S14) Further increase in polymer/plasmid molar ratios (up to 12) of this copolymer does not improve the transfection efficiencies. (Supporting Information Figure S15, S16, and S17) This difference in gene expression of copolymers of similar molecular weights at varying polymer/plasmid molar ratios is attributed to the high specificity of P($AEMA_{70}$-

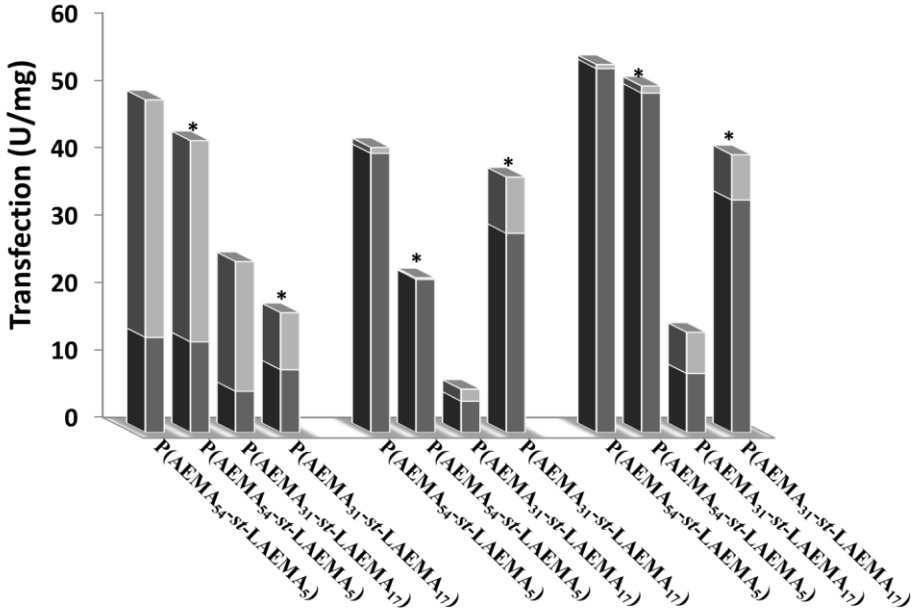
st-LAEMA$_{41}$) towards Hep G2 cells, as compared to P(AEMA$_{84}$-*st*-LAEMA$_{28}$) based polyplexes.

The galactose based hyperbranched copolymers of 15 kDa or lower are found to show low gene expression at low polymer/plasmid molar ratios. This is in agreement with agarose gel electrophoresis data, which show that high polymer/plasmid ratios are required to complex DNA for lower molecular weight copolymers. The statistical copolymers of 11-15 kDa show low gene expression at polymer/plasmid molar ratio of 2 (Supporting Information Figures S14 & S15). The polymer/plasmid molar ratios of 6 or higher are required to show efficient gene expression using low molecular weight polymers, in the presence of serum, however, the increase in polymer/plasmid ratio of these low molecular weight polymers also compromise their cell viability (Figure 5-3, supporting information Figure S14). From these results, it can be concluded that galactose based hyperbranched polymers of molecular weights ~30 kDa are ideal to obtain high gene expression at low polymer/plasmid molar ratios. Morimoto *et al*. has compared the gene expression of galactosylated PEI of varying molecular weights (M_n=1.8, 10 and 70 kDa) and has concluded that galactosylated PEI of molecular weight 10 kDa shows optimum gene expression in hepatocytes, due to the balance between cytotoxicity and availability of ligands on the surface of polyplexes.[30] The galactose based hyperbranched copolymers of molecular weights 5-60 kDa are then compared to glucose derived copolymers having similar molecular weight. It is found that in addition

to the extent of glycosylation of cationic polymers, their molecular weight and N/P ratios are also important in determining their gene expression.

As discussed above, the high molecular weight copolymers (60 kDa) show low gene expression, regardless of the type of monomers (GAPMA versus LAEMA) and composition of copolymers used for the study. (Supporting Information, Figures S15, S16, S17, and S18). The galactose based copolymers P($AEMA_{84}$-*st*-$LAEMA_{28}$) and P($AEMA_{70}$-*st*-$LAEMA_{41}$) show high gene expression than copolymer P($AEMA_{42}$-*st*-$GAPMA_{25}$). The glycopolymer, P($AEMA_{18}$-*st*-$LAEMA_{6}$), is then compared with P($AEMA_{11}$-*st*-$GAPMA_{17}$) for their gene expression. P($AEMA_{11}$-*st*-$GAPMA_{17}$) shows high gene expression at high polymer/plasmid molar ratios (8-12) than other GAPMA based hyperbranched copolymers of higher molecular weights. In contrast, P($AEMA_{18}$-*st*-$LAEMA_{6}$) based polyplexes do not show significant increase in gene expression at polymer/plasmid molar ratio of 10. This difference in gene expression of LAEMA and GAPMA based copolymers of similar molecular weights can be explained in terms of their polyplexes stability and size. Although both of these polyplexes show similar surface charge in water (+6), the polyplexes produced by P($AEMA_{18}$-*st*-$LAEMA_{6}$) are three times larger in size as compared to P($AEMA_{11}$-*st*-$GAPMA_{17}$) based polyplexes. (Figure 5-1) This difference in transfection efficacies of glucose derived and galactose based copolymers is probably due to lower ability of P($AEMA_{18}$-*st*-$LAEMA_{6}$) to produce stable particles as compared to P($AEMA_{11}$-*st*-$GAPMA_{17}$). The transfection efficiencies of these hyperbranched glycopolymers are also studied in the presence and

absence of serum. It has been found that linear cationic glycopolymers of statistical architecture show higher gene expression or their gene expression is not affected in the presence of serum as compared to the corresponding block copolymers.[5] In this study, hyperbranched glycopolymers of varying molecular weights synthesized are expected to serve as excellent gene delivery agent in the presence of serum. (Figure 5-3) It is found that the presence of serum indeed improves the gene expression of these copolymers regardless of molecular weights and compositions.



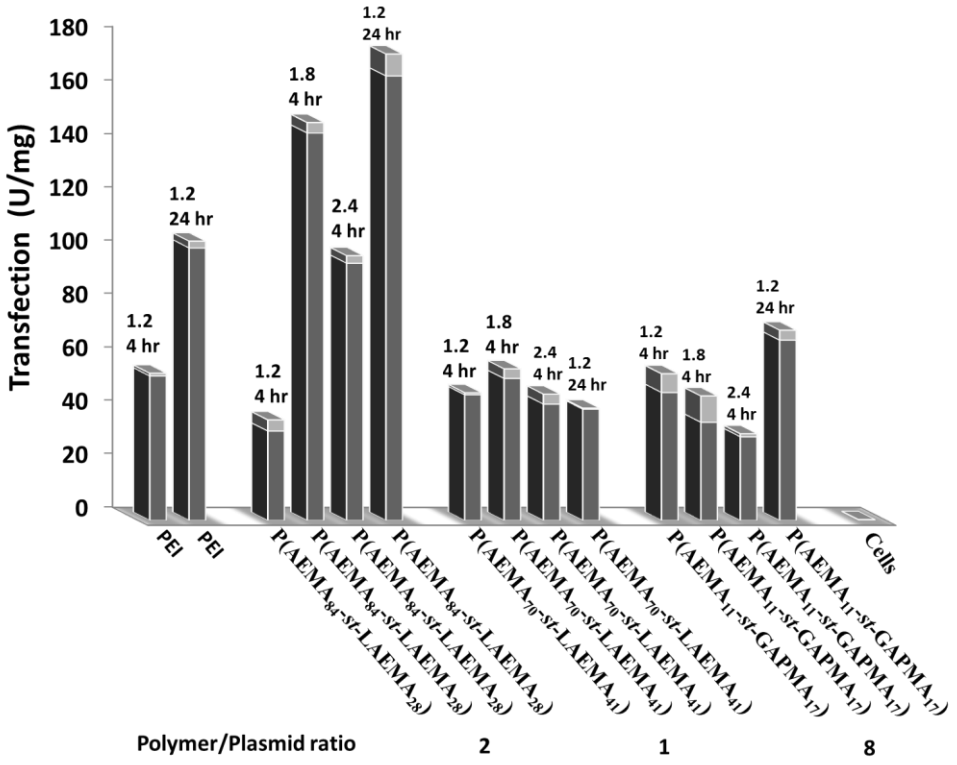


Figure 5-3. Comparison of gene expression of 11-13 kDa galactose based polymers, in the presence (*) and absence of serum using Hep G2 cells, at DNA dose 1.2 μg and varying polymer/plasmid ratios. Gene expression of glucose-derived and galactose based polymers of varying molecular weights and compositions, in the presence of serum using Hep G2 cells, at DNA dose 1.2, 1.8 and 2.4 μg, and at incubation times of 4 and 24 hours.

The selected galactose based and glucose derived copolymers, P($AEMA_{84}$-*st*-$LAEMA_{28}$), P($AEMA_{70}$-*st*-$LAEMA_{41}$), and P($AEMA_{11}$-*st*-$GAPMA_{17}$), which show high gene expression and low toxicity in Hep G2 cells are then compared to branched PEI, as a function of DNA dose and incubation time. (Figure 5-3) Figure 5-3 shows that gene expression of all these selected polymers is indeed comparable to branched PEI at DNA dose of 1.2 μg and for an incubation time

of 4 hours. An increase in incubation time with the polyplexes to 24 hours for branched PEI and P($AEMA_{84}$-*st*-$LAEMA_{28}$), significantly increases the transfection efficacies these polyplexes. The increase in DNA dose for these polyplexes is also studied and it is found that P($AEMA_{84}$-*st*-$LAEMA_{28}$) show higher gene expression than branched PEI at high DNA dose (1.8 and 2.4 µg).

The gene expression of the selected copolymers is also studied in HEK293 cells which do not have asialoglycoprotein receptors (ASGP-R). It is found that transfection efficiencies of these copolymers in HEK293 cells are dependent on the extent of carbohydrate content on the polyplexes surface (Supporting Information Figure S19). The copolymers with high carbohydrate content show lower gene expression. P($AEMA_{70}$-*st*-$LAEMA_{41}$) shows lower gene expression in HEK293 cells as compared to P($AEMA_{84}$-*st*-$LAEMA_{28}$). It should be noted that P($AEMA_{70}$-*st*-$LAEMA_{41}$) polymer shows optimum gene expression at polymer/plasmid molar ratio of 1. P($AEMA_{84}$-*st*-$LAEMA_{28}$) shows comparable gene expression to branched PEI, indicating that increasing the amine content in the polymers is associated with the high gene expression, and hence there is no specificity of these polyplexes towards HEK293 cell line. These results are in agreement with others, where the role of galactose and other sugars on gene delivery is investigated. [31-33] Bettinger *et al*. has compared the transfection efficacies of galactose and glucose substituted hyperbranched PEI in different cell lines and it is shown that cationic polymers with high galactose content can effectively express gene in BNL.CL2 murine hepatocytes at any polymer/plasmid ratios studied. In contrast glucose modified PEI show lower

gene expression in hepatocytes. However, the gene expression is not dependent upon the type of glycosylation in other non-specific cell lines.[31] These studies discuss the cationic homopolymers of same molecular weight substituted with different carbohydrates, and role of molecular weight of different glycosylated copolymers in DNA condensation ability, serum stability and gene expression is not explored in detail.

5.3.5. Toxicity Post-transfection

MTT assay is performed to determine toxicity post transfection of hyperbranched glycopolymer-polyplexes at polymer/plasmid ratios which show high gene expression. (supporting information Figure S14) It is found that toxicity of polyplexes is dependent upon their molecular weights as well as polymer/plasmid ratios used for the study. High molecular weight hyperbranched glycopolymers (50-60 kDa) show high toxicity (at polymer/plasmid ratio 2) than low molecular weight polymers. The relatively low cell viability of low molecular weight hyperbranched glycopolymers (13, 6 kDa) is due to the high polymer/plasmid ratios required for optimum transfection. It should be noted that P($AEMA_{70}$-*st*-$LAEMA_{41}$) and P($AEMA_{84}$-*st*-$LAEMA_{28}$), and P($AEMA_{11}$-*st*-$GAPMA_{17}$) maintain high cell viability (60-80%), along with high gene expression, as compared to branched PEI (cell viability is 35%).

5.3.6. Interactions of Lectins with Polyplexes

The clustered arrangement of carbohydrates around proteins produces multivalent interactions, which further increases their affinities and

specificity.[36] The measurement of binding affinities of synthetic glyco-conjugates can be done using agglutination assays.[36] RCA_{120} (lectin from *Ricinus communis)* is a plant lectin which specifically interacts with galactose residues on the surface of glyco-conjugates, these carbohydrate-protein interactions can be studied using agglutination assays.[31-33,37] Jacalin is another galactose specific tetrameric protein that has four galactose binding sites per molecule.[38] Others have utilized this method to detect the presence of galactose residues on the surface of galactose-copolymers based polyplexes.[31-33,35] To determine the role of galactose residue for targeting purposes, P($AEMA_{84}$-*st*-$LAEMA_{28}$) and P($AEMA_{70}$-*st*-$LAEMA_{41}$) based polyplexes are subjected to agglutination assay and P($AEMA_{11}$-*st*-$GAPMA_{17}$) is used as a control (see Figure 5-4).

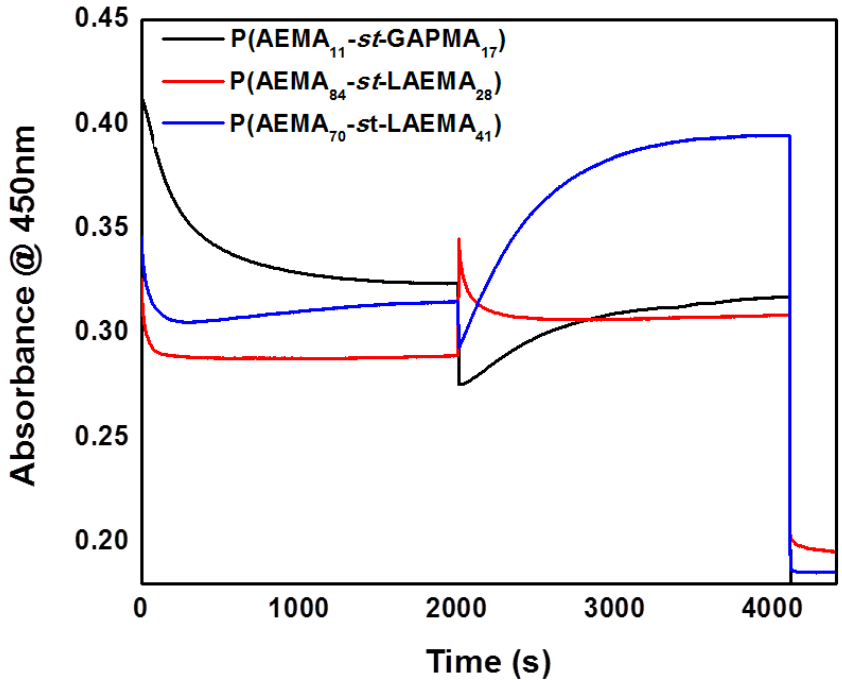


Figure 5-4. Lectin induced aggregation of polyplexes. Polyplexes are allowed to stabilize for 2000 seconds and RCA_{120} at 180 μg/mL was added, followed by the addition of lactose at 4000 sec.

It is found that P($AEMA_{84}$-*st*-$LAEMA_{28}$) based polyplexes do not aggregate in the presence of RCA_{120}, and the result is similar for P($AEMA_{11}$-*st*-$GAPMA_{17}$) based polyplexes which lack galactose specific residues on the surface. However, significant aggregation is observed for P($AEMA_{70}$-*st*-$LAEMA_{41}$) based polyplexes in the presence of RCA_{120}.

This data is in agreement with previous literature, showing that optimum concentration of galactose residues are required on the surface of polyplexes to induce the cluster glycoside effect.[31-33, 6] The interaction of polyplexes with RCA_{120} and Jacalin are further studied by DLS and BCA assay. It is found that, agglutination assay do not show the aggregation of PEI, P($AEMA_{84}$-*st*-$LAEMA_{28}$), P($AEMA_{11}$-*st*-$GAPMA_{17}$) polyplexes in the presence of RCA_{120}, all these polyplexes interact with RCA_{120} and Jacalin as revealed by the DLS and BCA analysis (Supporting Information Table S2, S3). Therefore, the polyplexes do interact with lectins either specifically or non-specifically in solution, however these interactions are insufficient to cause agglutination, except when there is a high density of galactose residues on the surface of the polyplexes.

5.3.7. Lectin Mediated Gene Expression

To determine the significance of the lectins on the gene expression, transfection experiments are carried out with Hep G2 and HEK293 cell lines. It is found that gene expression is dependent on the type of polyplexes-lectin conjugates (Jacalin

vs. RCA_{120}). Conjugates of Jacalin with P($AEMA_{70}$-*st*-$LAEMA_{41}$) based polyplexes show a significant increase in gene expression in HEK293 cells and remarkably, the gene expression is comparable to that of branched PEI. It should be noted that, however, in the absence of Jacalin this polyplex shows low transfection in HEK293 cells (see Supporting Information Figure S18, S21). Hence, this three-fold increase in gene expression may be associated with high uptake of Jacalin mediated interactions of polyplexes with HEK293 cells. PEI and P($AEMA_{11}$-*st*-$GAPMA_{17}$) based polyplexes show slight decrease in gene expression in the presence of Jacalin. Gene expression is unaffected for P($AEMA_{84}$-*st*-$LAEMA_{28}$) based polyplexes in the presence of Jacalin (see Supporting Information, Figure S21). In contrast, these Jacalin-polyplexes conjugates show no significant change in gene expression in Hep G2 cells (Supporting Information, Figure S21). These differences in transfection are attributed to cell line dependent effect. Further mechanistic studies are required to investigate the role of Jacalin on transfection. RCA_{120}-polyplexes conjugates are then studied for their role on gene expression. It is found that the gene expression is completely knocked down (even for that of PEI) in the presence of RCA_{120} in Hep G2 cells at a concentration of 45 μg/mL (data not shown).

RCA_{120} is well-known to inhibit protein synthesis in cells [37] and, therefore inhibition of transfection in Hep G2 cells is most likely due to RCA_{120} at this concentration. The effect of RCA_{120} on transfection and toxicity is found to be concentration dependent (Figure 5-5).

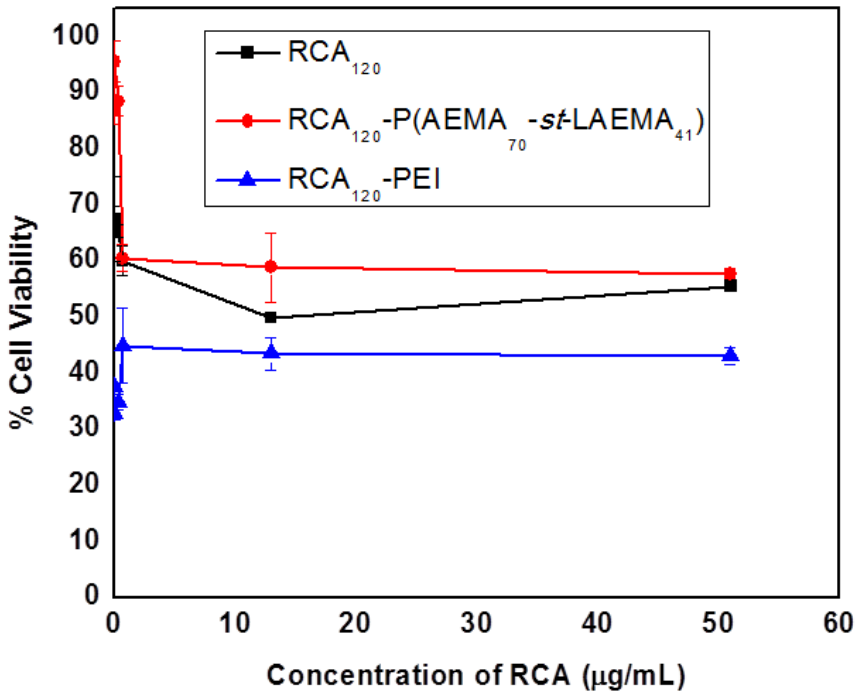


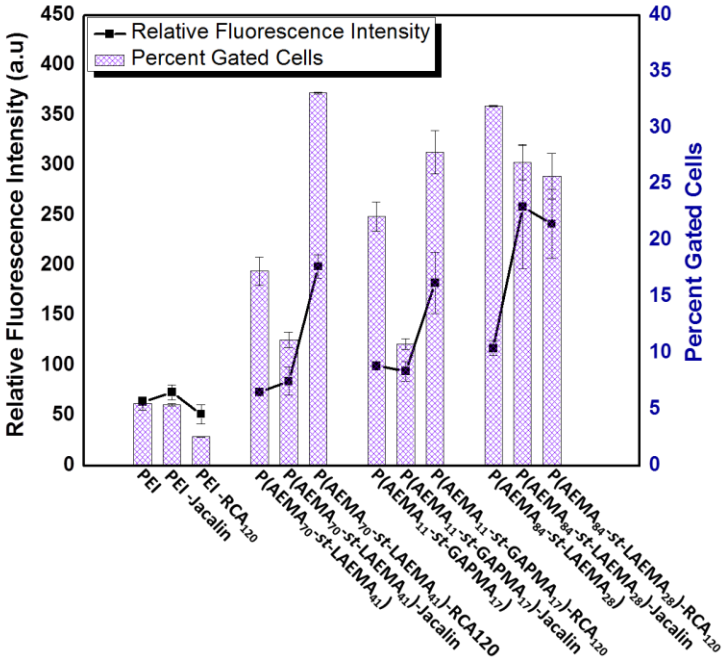
Figure 5-5. MTT assay for toxicity of RCA_{120}, RCA_{120}-P($AEMA_{70}$-*st*-$LAEMA_{41}$) and RCA_{120}-PEI based polyplexes conjugates.

The gene expression and toxicity of RCA_{120}-polyplexes conjugates is studied at varying concentration of RCA_{120} and it is found that decreasing the concentration to 0.5 µg/mL does not compromise the cell viability. However, the gene expression is significantly reduced in Hep G2 cells for hyperbranched copolymer based polyplexes. The gene expression is not significantly reduced for PEI based polyplexes at the concentration of 0.5 µg/mL of RCA_{120} (Supporting Information, Figure S20). In contrast, HEK293 cells show lower sensitivity in the presence of RCA_{120} for all polyplexes at a concentration of 45

μg/mL as the gene expression is not completely inhibited (Supporting Information, Figure S21).

5.3.8. Cellular Uptake of Polyplexes in the Presence of Lactose and Lectins

To investigate, if impaired gene expression of polyplexes-lectins conjugates is associated with lower uptake of polyplexes, flow cytometer studies are conducted. The uptake of PEI, P(AEMA$_{84}$-*st*-LAEMA$_{28}$), P(AEMA$_{11}$-*st*-GAPMA$_{17}$), and P(AEMA$_{70}$-*st*-LAEMA$_{41}$) based polyplexes is studied in both Hep G2 and HEK293 cells (Figure 5-6).



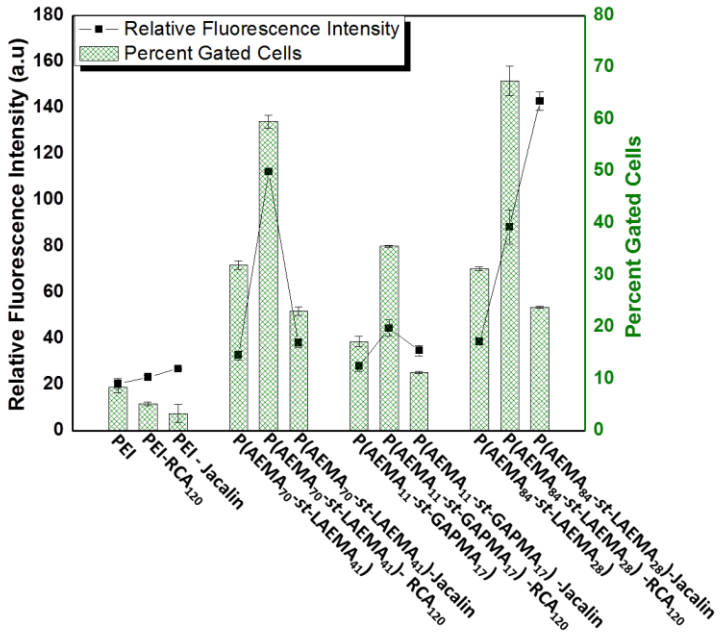


Figure 5-6. Flow cytometer analysis showing the uptake of Cy-3' labelled uptake of polyplexes, in Hep G2 (top) and HEK 293 (bottom) cells in the presence of lectins (45 µg/mL) after incubation time of 120 minutes.

It is found that the polyplexes uptake in both cell lines is higher in the presence of lectins, irrespective of the chemical compositions of the polymers. PEI polyplexes do not show significant increase in uptake in the presence of lectins. The uptake of polyplexes in the presence of Jacalin is most significant in the case of P($AEMA_{84}$-*st*-$LAEMA_{28}$) based polyplexes, as compared to P($AEMA_{70}$-*st*-$LAEMA_{41}$) based polyplexes. This high uptake of polyplexes with low galactose residues is probably due to their aggregation in the presence of Jacalin. This is in agreement with gene expression data, for P($AEMA_{84}$-*st*-$LAEMA_{28}$) based polyplexes, which show no increase in gene expression in the presence of Jacalin. In contrast to gene expression results, flow cytometer analysis show that the cellular uptake of polyplexes is significantly increased for hyperbranched

polyplexes-RCA_{120} conjugates. To further confirm flow cytometer analysis, confocal microscopy is performed to get a better picture of cellular uptake of polyplexes.

Moreover, inhibition of cellular uptake of galactose based polyplexes in the presence of free lactose was studied in Hep G2 cells, to investigate the role of ASGPR in the uptake of polyplexes. It is shown that in the presence of free lactose, uptake of galactose based polyplexes significant decreased in Hep G2 cells, as compared to PEI based polyplexes, indicating the specific uptake of galactose-based polyplexes by ASGPR receptors of Hep G2. (Supporting information Figure S22)

5.3.9. Confocal Microscopic Imaging of RCA_{120}-Polyplexes Uptake

Confocal microscopic images are obtained for PEI and P($AEMA_{70}$-*st*-$LAEMA_{41}$) based polyplexes in the absence and at two different concentrations of RCA_{120} (0.5 and 45 µg/mL). The addition of RCA_{120} at 0.5 µg/mL, shows slight aggregation of polyplexes on the cell surface as revealed by the confocal images. However, no significant effect on the uptake PEI polyplexes is observed at the studied concentrations of RCA_{120} (Figure 5-7).

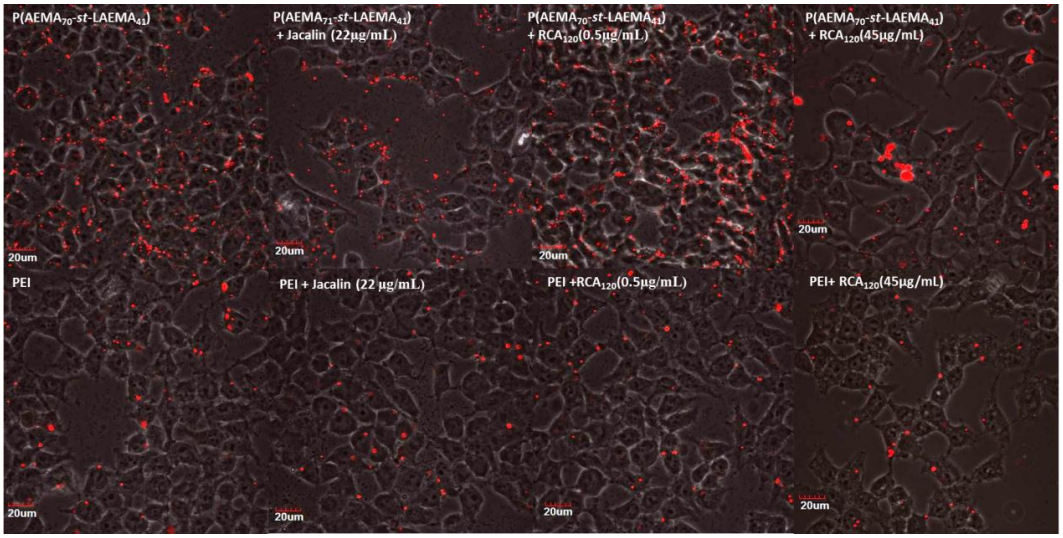


Figure 5-7. Confocal Images of Cy-3' labeled polyplexes in the presence and absence of lectins.

The increase in concentration of RCA_{120} to 45 µg/mL significantly increases the aggregation of polyplexes for P($AEMA_{70}$-*st*-$LAEMA_{41}$) and the resulting large aggregates are found to adhere to the surface of cells, while the number of particles internalized in cells are significantly small (Figure 5-7). From these results, it is concluded that reduced gene expression for the galactose based polyplexes in the presence of RCA_{120} is due to the *aggregation* of polyplexes and their *low internalization* in cells.

5.4. Conclusion

This study reports the efficacy of cationic hyperbranched glycopolymers for gene delivery. The role of glucose-derived and galactose based hyperbranched polymers in DNA complexation, polyplexes stability in serum and gene expression is evaluated in detail. It is found that, in addition to the architecture, the compositions and molecular weight can significantly influence the stability

of polyplexes and hence gene expression. The hyperbranched polymer with high galactose content, P($AEMA_{70}$-*st*-$LAEMA_{41}$) can efficiently transfect hepatocytes at low polymer/plasmid ratios in the presence and absence of serum. In contrast, hyperbranched polymers with high amine content are found to show enhanced gene expression in both cell lines at higher polymer/plasmid ratios. Moreover, P($AEMA_{70}$-*st*-$LAEMA_{41}$) polyplexes also show significant interactions towards galactose specific lectins. These lectin conjugated polyplexes are further explored for gene delivery purposes. Jacalin is found to improve the uptake of galactose based polyplexes and subsequently an increase in gene expression is noted for those polyplexes. However, due to the protein inhibitory role of RCA_{120} and aggregation, the gene expression is strongly compromised in the presence of RCA_{120}. Further studies will be done to explore the role of lectins on gene expression in different cell lines.

Supporting Information Available.

GPC curves and ^{1}H NMR data for polymer synthesis, molecular weight dependent gene expression, DLS data, BCA assay, MTT assay for jacalin, gene expression of polyplexes in the presence and absence of lectins in Hep G2 and HEK293 and agarose gel electrophoresis. This information is available at http://elsevier.com

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5.5. References

[1] Mintzer AM, Simanek EE. Nonviral vectors for gene delivery. Chem Rev 2009; 109: 259-02.

[2] Troiber C, Wagner E. Nucleic acid carriers based on precise polymer conjugates. Bioconjugate Chem 2011;22:1737-1752.

[3] Grigsby LG, Leong WK. Balancing protection and release of DNA: Tools to address a bottleneck of non-viral gene delivery. J R Soc Interface 2010;6:S67-S82.

[4] Gao Y, Yin Q, Chen L, Zhang Z, Li Y. Linear cationic click polymers/DNA nanoparticles: in vitro structure activity relationship and in vivo evaluation for gene delivery. Bioconjugate Chem 2011;22:1153-1161.

[5] Ahmed M, Narain R. The effect of polymer architecture, composition, and molecular weight on the properties of glycopolymer-based non-viral gene delivery systems. Biomaterials 2011;32:5279-5290.

[6] Hobel S, Loos A, Appelhans D, Schwarz S, Seidel J, Voit B, et al. Maltose- and maltotriose-modified, hyperbranched poly(ethylene imine)s (OM-PEIs): physicochemical and biological properties of DNA and siRNA complexes. J Control Release 2011;149:146-158.

[7] Popielarski RS, Mishra S, Davis EM. Structural effects of carbohydrate-containing polycations on gene delivery. 3. cyclodextrin type and functionalization. Bioconjugate Chem 2003;14:672-678.

[8] Liu Y, Reineke MT. Poly(glycoamidoamine)s for gene delivery. structural effects on cellular internalization, buffering capacity, and gene expression. Bioconjugate Chem 2007;18:19-30.

[9] Newland B, Tai H, Zheng Y, Velasco D, Luca DA, Howdle MS, et al. A highly effective gene delivery vector-hyperbranched poly(2-(dimethylamino)ethyl methacrylate from in situ deactivation enhanced ATRP. Chem Commun 2010;46:4698-4700.

[10] Uchida H, Miyata K, Oba M, Ishii T, Suma T, Itaka K, et al. Odd-even effect of repeating aminoethylene units in the side chain of N-substituted polyaspartamides on gene transfection profiles. J. Am. Chem. Soc 2011;133:15524-15532.

[11] Chen Q-R, Zhang L, Luther WP, Mixson JA. Optimal transfection with the HK polymer depends on its degree of branching and the pH of endocytic vesilces. Nucleic Acids Res 2002;30:s1338-1345.

[12] Synatschke VC, Schallon A, Jérôme V, Freitag R, Müller AHE. Influence of polymer architecture and molecular weight of poly(2-(dimethylamino)ethyl methacrylate) polycations on transfection efficiency and cell viability in gene delivery. *Biomacromolecules* 2011;ASAP.

[13] Ahmed M, Bhuchar N, Narain R. Well-controlled cationic water-soluble phospholipid polymer-DNA nanocomplexes for gene delivery. Bioconjugate Chem 2011;22:1228-1238.

[14] Ahmed M, Jiang X, Deng Z, Narain R. Cationic glyco-functionalized single walled carbon nanotubes as efficient gene delivery vehicles. Bioconjugate Chem 2009;20:2017-2022.

[15] Ahmed M, Deng Z, Liu S, Narain R. Cationic glyconanoparticles: their complexation with DNA, cellular uptake, and transfection efficiencies. Bioconjugate Chem 2009;20:2169-2176.

[16] Ahmed M, Deng Z, Narain R. Study of size dependent transfection efficiencies of gold nanoparticles in human cell line. ACS Appl. Mater. Interface 2009;1:1980-1987.

[17] Deng Z, Ahmed M, Narain R. Novel well-defined glycopolymers synthesized via the reversible addition fragmentation chain transfer process in aqueous media. J Poly Sci Part A Polym Chem 2009;47:614-627.

[18] Deng Z, Li S, Jiang X, Narain R. Well-define galactose-containing multifunctional copolymers and glyconanoparticles for biomolecular recognition processes. Macromolecules 2009;42:6393-6305.

[19] Boyer C, Bulmus V, Davis PT, Ladmiral V, Liu J, Perrier S. Bioapplications of RAFT polymerization. Chem Rev 2009;109:5402-5436.

[20] Arote BR, Lee E-S, Jiang H-L, Kim Y-K, Choi Y-J, Cho M-H, et al. Efficient gene delivery with osmotically active and hyperbranched poly(ester amine)s. Bioconjugate Chem 2009;20:2231-2241.

[21] Chen J, Wu C, Oupicky D. Bioreducible hyperbranched poly(amido amine)s for gene delivery. Biomacromolecules 2009;10:2921-2927.

[22] Klaykruayat B, Siralertmukul K, Srikulkit K. Chemical modification of chitosan with cationic hyperbranched dendritic polyamidoamine and its antimicrobial activity on cotton fabric. Carbohydrate Polymers 2010;80:197-207.

[23] Zhang L, Hu H-C, Cheng X-S, Zhou X-R. PEI grafted hyperbranched polymers with polyglycerol as a core for gene delivery. Colloids Surf B: Biointerf 2010;76:427-433.

[24] Chen Y, Zhou L, Pang Y, Huang W, Qiu F, Jiang X, et al. Photoluminescent hyperbranched poly(amido amine) containing β-cyclodextrin as a nonviral gene delivery vector. Bioconjugate Chem 2011;22:1162-1170.

[25] Tian YH, Deng C, Lin H, Sun J, Deng M, Chen X, et al. Biodegradable cationic PEG–PEI–PBLG hyperbranched block copolymer: synthesis and micelle characterization. Biomaterials 2005;26:4209-4217.

[26] Muthukrishnan S, Mori H, Muller EH. Synthesis and characterization of methacrylate-type hyperbranched glycopolymers via self-condensing atom transfer radical copolymerization of sugar carrying acrylate. Macromolecules 2005;38:3108-3119.

[27] Luzon M, Boyer C, Peinado C, Corrales T, Whittaker M, Tao L, et al. Water-soluble, thermoresponsive, hyperbranched copolymers based on PEG-methacrylates: synthesis, characterization, and LCST behavior. J Poly Sci Part A: Polym Chem 2010;48:2783-2792.

[28] Muthukrishnan S, Dominik P, Erhard HM, Müller AHE. Synthesis and characterization of surface-grafted hyperbranched glycomethacrylates. Macromolecules 2006;39:2743-2750.

[29] Deng Z, Bouchekif H, Babooram K, Housni A, Choytun N, Narain R. Facile synthesis of controlled-structure primary amine-based methacrylamide polymers via the reversible addition-fragmentation chain transfer process. J Poly Sci Part A Polym Chem 2008;46:4984-4996.

[30] Bettinger P, Remy J-S, Erbacher P. Size reduction of galactosylated PEI/DNA complexes improves lectin-mediated gene transfer into hepatocytes. Bioconjugate Chem 1999;10:558-561.

[31] Tao L, Liu J, Tan HB, Davis PT. RAFT synthesis and DNA binding of biodegradable, hyperbranched poly(2-(dimethylamino)ethyl methacrylate. Macromolecules 2009;42:4960-4962.

[32] Zhang Q-X, Wang L-X, Zhang C-P, Liu L-Z, Zhou X-R, Mao Q-H, et al. Galactosylated ternary/DNA polyphosphoramidate nanoparticles mediate high gene transfection efficiency in hepatocytes. J Control Release 2005;102:749-763.

[33] Morimoto K, Nishikawa M, Kawakami S, Nakano T, Hattori Y, Fumoto S, et al. Molecular weight-dependent gene transfection activity of unmodified and galactosylated polyethyleneimine on hepatoma cells and mouse liver. Mol Ther 2003;7:254-261.

[34] Kuiper J, Bakkeren FH, Biessen LAE, Van Berkel CJT. Characterization of the interaction of galactose exposing particles with rat kupffer cells. J Biochem 1994;299:285-290.

[35] Qin Z, Liu W, Li L, Guo L, Yao C, Li X. Galactosylated N-2-hydroxypropyl methacrylamid-b-N-3-guanidinopropyl methacrylamide block

copolymers as hepatocytes-targeting gene carriers. Bioconjugate Chem 2011;22:1503-1512.

[36] Zanta MA, Bossif O, Abdennaji A, Behr JP. In vitro gene delivery to hepatocytes with galactosylated polyethylenimine. Bioconjugate Chem 1997;8:839-844.

[37] Stripe F. Ribosome-inactivating proteins. Toxicon 2004;44:371-383.

[38] Komath SS, Bhanu K, Maiya GB, Swamy JM. Binding of porphyrins by the tumor-specific lectin, jacalin [jack fruit (artocarpus integrifolia) agglutinin] Biosci Rep 2000;20:265-276.

Chapter 6. Impact of the Nature, Size and Chain Topologies of Carbohydrate-Phosphorylcholine Polymeric Gene Delivery Systems

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6.1. Introduction

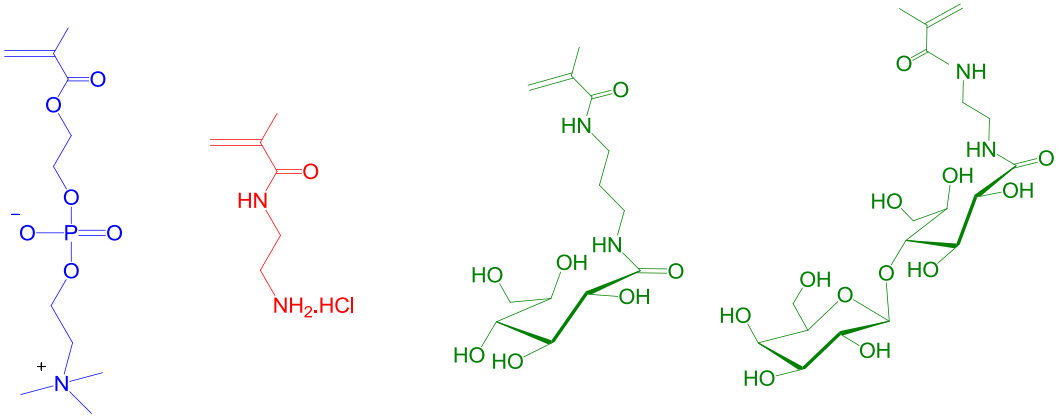
The synthesis of non-toxic cationic polymers of controlled molecular weights and desired architectures is important to produce efficient gene delivery vectors.[1-2] The modification of materials with phospholipids and carbohydrates is a nature-inspired approach to produce materials with antifouling and biocompatible properties.[3-5] For instance, the modification of cationic vectors with mono-saccharides or polysaccharides is a well-studied approach to produce gene delivery vectors with low toxicity.[6-9] With the recent advances in the field of polymer chemistry, synthetic carbohydrate and phospholipid based polymers of pre-determined molecular weights and narrow polydispersities can be synthesized by facile approaches.[10-15] These carbohydrate and phosphorylcholine based polymers are extensively studied to produce effective gene delivery carriers with low toxicities.[16-24] Recently, carbohydrates and phosphorylcholine based synthetic gene delivery vectors of controlled molecular weights and architectures are evaluated for their structure activity relationships.[17-19] It has been reported that linear statistical cationic glycopolymers are superior gene transfecting agents as compared to their block analogues. The statistical cationic glycopolymers show enhanced gene expression along with low toxicities, as compared to the block copolymer systems.[17]

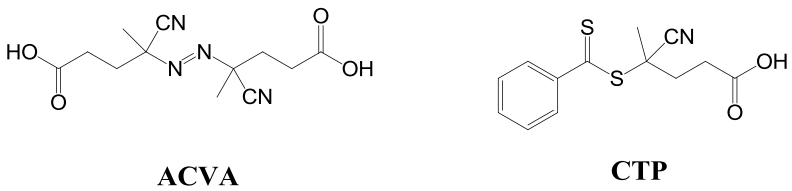
However, as compared to diblock copolymers which show higher stability in serum containing media, statistical cationic glycopolymers show high interaction with serum proteins and hence show an aggregation over time, which could

significantly compromise their uses for *in vivo* applications. [17] Hence, it is important to produce a copolymer with a novel architecture, which can show high gene expression, along with the low interactions with serum proteins. To solve this issue, we have designed linear cationic glycopolymers with novel architectures, which are expected to maintain high gene delivery efficacies, along with low toxicities and high stability in physiological conditions.

Phosphorylcholine-based polymers are well-known for imparting antifouling properties to materials.[3-4] Their low interactions with serum proteins and non-toxic nature make them ideal candidates for gene delivery vectors. A number of studies have shown that phosphorylcholine based cationic polymers can show some gene expression and their efficacy is found to be dependent upon the composition and architecture of the copolymers.[19-21] In a detailed study of structure-activity relationship of phosphorylcholine-based polymers, it is reported that the linear diblock copolymers show some gene expression, along with high toxicities under *in vitro* conditions. In contrast, their statistical analogues show high cell viability but poor gene expression.[19] In this study, we have developed a new type of copolymers and the gene expression is studied in hepatocytes and human dermal fibroblasts. First, carbohydrate and phosphorylcholine based homopolymers are synthesized via the reversible addition fragmentation chain transfer (RAFT) polymerization of 3-gluconamidopropyl methacrylamide (GAPMA), 2-lactobionamidoethyl methacrylamide (LAEMA) or 2- methacryloxyethyl phosphorylcholine (MPC)

and are subsequently used as macro chain transfer agents (macroCTAs). The structure of the monomers is shown in scheme 6-1.





Scheme 6-1. Chemical structure of monomers, 2-methacryloxyethyl phosphorylcholine (MPC), 3-glucanoamidopropyl methacrylamide (GAPMA), 2-lactobionamidoethyl methacrylamide (LAEMA), and 2-amino ethyl methacrylamide (AEMA), chain transfer agent (CTP) and initiator (ACVA).

These macroCTAs are then used in the RAFT co-polymerization of 2-aminoethyl methacrylamide (AEMA) with GAPMA or LAEMA or MPC to produce *block-statistical* copolymers of varying molecular weights. These novel cationic *block-statistical* copolymers are then investigated for their DNA condensation efficacies. The size and net charges of the resulting polyplexes are determined using dynamic light scattering (DLS) and zeta potential analyzer, respectively. The gene expression of these copolymer-based polyplexes is

evaluated at varying polymer/plasmid ratios using a β-galactosidase assay in hepatocytes and human dermal fibroblasts. Moreover, the interaction of these polyplexes with serum proteins, their cellular uptake and nuclear localization efficacies of these novel polymers based polyplexes is studied. The toxicities post-transfection of these polyplexes at varying polymer/plasmid ratios is determined using MTT assay.

6.2. Materials and Methods

6.2.1 Materials

2-aminoethyl methacrylamide (AEMA), 3-gluconamidopropyl methacrylamide (GAPMA), 4-cyanopentanoic acid dithiobenzoate (CTP)and 2-lactobionamidoethyl methacrylamide (LAEMA) were synthesized according to previously reported procedure.[11-13] 2-Methacryloxyethyl phosphorylcholine (MPC) was obtained from NOF Co, Tokyo, Japan, which was produced by the previous reported method. [14] Branched PEI (M_w = 25 kDa,), *O*-Nitrophenyl β-D-galactopyranoside, (ONPG) (enzymatic), ethidium bromide, 4'-azobis(4-cyanovaleric acid) (ACVA), 37 wt. % formalin, β -Mercaptoethanol, Ethylenediaminetetraacetic acid (EDTA), MTT assay kit to determine cell viability, Dulbecco's phosphate buffer saline (DPBS), formalin (37 wt. %), and 3-[(3-Cholamidopropyl) dimethylammonio-1-propanesulfonate (CHAPS) were purchased from Sigma Aldrich. Cell Culture media Dulbecco's Modified Eagle Medium (DMEM) low glucose with L-glutamine and sodium pyruvate), Opti-MEM (OMEM), penicillin (10000 U/mL), and streptomycin (10 mg/mL), 0.25% trypsin, Dulbecco's modified Phosphate Buffer Saline (DPBS), 0.25% trypsin

with EDTA and Fetal Bovine Serum (FBS) were from Invitrogen. Phenylmethanesulfonyl fluoride (PMSF), HEPES, and Micro BCA assay kit were obtained from Fisher Scientific. *Gwiz* β-galactosidase plasmid was purchased from Aldevron. Cy-3' and Cy-5' DNA labeling kit were from Mirus Bio.

6.2.2 Methods

6.2.3 Synthesis of Macro Chain Transfer Agents (macroCTA)

The macroCTAs of GAPMA, LAEMA or MPC were synthesized according to previously reported protocols. [12,13,15] In a typical protocol, GAPMA (1 g, 3.1 mmol) was dissolved in 5 mL of distilled water. CTP (58 mg, 0.2 mmol) and ACVA (19 mg, 0.07 mmol) were dissolved in 1 mL of DMF. The solutions were mixed in Schlenk tube and mixture was purged with nitrogen for 45 minutes. The tube was sealed and polymerization was carried out in inert atmosphere in an oil bath at 70 °C for 12 hours. The polymerization was quenched using liquid nitrogen and the macroCTA was precipitated in acetone. The macroCTA was washed with excess amount of methanol to remove residual monomers. The polymer was dried and was analyzed for its molecular weight using aqueous GPC: The chromatograms were obtained using conventional Viscotek GPC system, Sodium acetate (0.5 M) / acetic acid (0.5 M) buffer as eluent, two Waters WAT011545 columns at room temperature and a flow rate of 1.0 mL/min. Pullulan standards (M_w = 500-404,000 g/mol) were used for calibration. In a typical MPC macroCTA synthesis, MPC (1 g, 3.4 mmol) was dissolved in 3 mL of distilled water. CTP (63 mg, 0.23 mmol) and ACVA (21 mg, 0.08 mmol)

were dissolved in 1 mL of 2-propanol. The solutions were mixed in a Schlenk tube and the mixture was purged with nitrogen gas for 45 minutes. The tube was put in oil bath in inert atmosphere at 70 °C for 12 hours. The polymerization was quenched using liquid nitrogen, and MPC macroCTA was precipitated in acetone. The macro CTA was dried and was analyzed for its molecular weight using aqueous GPC, as described above.

6.2.4 Synthesis of *Block-statistical* Cationic Glycopolymers

In a typical synthesis, the macroCTAs synthesized above were used as chain transfer agent and were blocked with statistical copolymer of AEMA, and GAPMA. In a typical protocol, GAPMA or MPC (0.5 g, 1.5 mmol) and AEMA (0.5 g, 3 mmol) were dissolved in 2.5 mL distilled water. GAPMA macro CTA (0.25 g, 50 µmol) and ACVA (7mg, 25 µmoles) were dissolved in 0.5 mL DMF. The solutions were mixed in Schlenk tube and mixture was purged with nitrogen gas for 45 minutes. The tube was sealed and polymerization was carried out in Schlenk tube for 24 hours in inert atmosphere at 70 °C. The polymerization was quenched using liquid nitrogen and polymer was precipitated in acetone, followed by excessive washing with methanol. The polymer was dried and was analyzed for its molecular weight using aqueous GPC as described above. The composition of polymer was determined using Varian 500 ^{1}H NMR.

6.2.5 Synthesis of *Block-Statistical* Phosphorylcholine Based Cationic Polymers

In a typical synthesis, the macro CTAs of MPC synthesized above were used as chain transfer agent and were blocked with statistical copolymer of AEMA, and

GAPMA, or MPC. In a typical protocol, MPC (0.25 g, 0.75 mmol) and AEMA (0.125 g, 0.75 mmol) were dissolved in 2.5 mL distilled water. GAPMA macroCTA (80mg, 17 μmoles) and ACVA (3mg, 8 μmol) were dissolved in 700 μL of 2-propanol. The solutions were mixed in Schlenk tube and the mixture was purged with nitrogen gas for 45 minutes. The tube was sealed and polymerization was carried out in Schlenk tube for 24 hours in inert atmosphere at 70 °C. The polymerization was quenched using liquid nitrogen and polymer was precipitated in acetone. The polymer was dried and was analyzed for its molecular weight using aqueous GPC as described above. The composition of polymer was determined using Varian 500 ^{1}H NMR.

6.2.6 Polyplexes Formation

Polyplexes of cationic *block-statistical* copolymers with β-galactosidase plasmid were synthesized in opti-MEM or deionized water. Varying concentrations of polymers were mixed with 1.2 μg of plasmid to obtain different polymer/plasmid molar ratios. The mixture was slightly vortexed and was allowed to complex for 30 minutes. The polyplexes formed were analyzed for their sizes (in OMEM, as a function of time) and surface charge (in DI water) using Brookhaven Zeta Plus (zeta potential and particle size analyzer) instrument.

6.2.7 Agarose Gel Electrophoresis

The polyplexes of formed with β-galactosidase plasmid were formulated in OMEM at varying polymer/plasmid ratios, as described above. The polyplexes were loaded in 1% agarose gel containing 1μg/mL ethidium bromide in 1X tris acetate/EDTA buffer. The gel was run for 45 minutes at 130 V. The gel was

illuminated with UV light and the DNA bands were visualized using UV transilluminator (Alpha Innotech; San Leandro, CA).

6.2.8 Stability of Polyplexes in Serum

The polyplexes were synthesized at different polymer/plasmid ratios, as described above in OMEM. The complexes were allowed to stabilize for 30 minutes, followed by the addition of 10% FBS. The change in size of polyplexes in the presence of serum proteins, as a function of time was studied using DLS instrument.

6.2.9 Gene Expression in Hepatocytes

Hep G2 cells were grown in low glucose DMEM, supplemented with 10% FBS and 1% antibiotic in humidified atmosphere, containing 5 % CO_2 at 37 °C. Upon 80% confluency, cells were trypsinized using trypsin with EDTA and were seeded in 24 well tissue culture plates at the density of 60,000 cells/well. The cells were allowed to adhere overnight. The media was then removed and was supplemented with OMEM or OMEM supplemented with 10% FBS. The polyplexes formulated as described above were added to the wells and the cells were incubated for 4 hours in the presence of the polyplexes at 37 °C. The media was then removed and was supplemented with fresh serum containing DMEM. The cells were allowed to grow for another 48 hours. The cells were then lysed and were analyzed for β-galactosidase activity, using O-nitrophenyl-β-D-galactopyranosidase, as a substrate, according to previously established protocol. [17]

6.2.10 Gene Epression in Human Dermal Fibroblasts

Human dermal fibroblasts (CRL-2522) were maintained in low glucose DMEM, supplemented with 10% FBS and 1% antibiotic, 5% CO_2, in humidified atmosphere. The cells in their passage 24 were trypsinized and 0.2×10^5 cells/mL were diluted and were seeded in 24 well tissue culture plates, cells are at 70% confluency in the well. The cells were allowed to adhere overnight. *Block-statistical* copolymers based polyplexes were prepared at polymer/plasmid molar ratio of 0.4, as described above. For PEI-based polyplexes, the complexes were prepared at polymer/plasmid w/w ratio of 2.5. The media was removed and serum free OMEM media was added in well, followed by the addition of preformed complexes. The cells were incubated for 8 hours. The treatment was then removed and fresh serum containing media was added per well. The cells were allowed to grow for another 48 hours followed by their lysis. The amount of β-galactosidase was determined in terms of mU/mg, as described above.

6.2.11 Cellular Uptake of Polyplexes

Hep G2 cells were seeded in 24 well tissue culture plate at cell density of 60,000 cells/well. The cells were allowed to adhere overnight at 37 °C, in humidified atmosphere, and 5% CO_2. The polyplexes were formulated using Cy-3' labelled plasmid at varying polymer/plasmid ratios. The media was replaced with polyplexes containing media, and cells were incubated with polyplexes for 4 hours in the presence of serum proteins. The media was then replaced with fresh serum containing media and cells were further incubated for 2 hours. The media was then removed, and cells were washed with PBS, followed by their

suspension in deionized water. The cells were lysed by 3-4 freeze thaw cycles and centrifuged at 14,000 rpm for 10 minutes. The supernatant was detected for the presence of Cy-3' labeled plasmid using TECAN microplate reader. The untreated cells were used at negative control. The amount of plasmid in microgram was determined by the graph constructed using the fluorescence intensity as a function of plasmid concentration. The fluorescence intensity of polyplexes treated supernatant was normalized with fluorescence intensity of untreated cells and final value was read from the graph, and was expressed in terms of microgram of plasmid present in the sample.

6.2.12 Confocal Microscopy

Hep G2 cells were seeded in 6 well tissue culture plates containing glass cover slips. The cells were allowed to adhere overnight at 37 °C, 5% CO_2 in humidified atmosphere. The cells were treated with fresh serum containing media containing Cy-5' labeled polyplexes as described above. The cells were incubated for two hours. The media was removed and cells were allowed to incubate for two hours in fresh serum containing media. The cells were washed 2X with PBS, and were fixed on glass cover slips using 3.7 wt. % formalin. The cells were rehydrated and were mounted on glass slides using 90% glycerol. The samples were analyzed using Fluoview FV10i Olympus confocal microscope and samples were excited at 485 & 650 nm and were detected at 535 and 670 nm, for FITC and Cy5'-labelled samples respectively, using 60X objective.

6.2.13 Quantification of Cy-3' labeled DNA in Nucleus and Cytoplasm

Hep G2 cells were seeded in 24 well tissue culture plates, as described above. The cells were treated with Cy-3' labeled polyplexes for four hours in the presence of serum proteins. The media was then replaced with fresh serum containing media and cells were further incubated for two hours. The cells were then wash with PBS and were suspended in ice cold hypotonic buffer (10mM Hepes, pH 7.5; 2 mM $MgCl_2$; 25mM KCl) containing 1mM DTT and 1mM PMSF. The cells were centrifuged at 2000 rpm for 10 minutes at 4 °C. The supernatant was removed and pellet was re-suspended in hypotonic buffer, and cells were allowed to swell on ice for 10 minutes. The cells were lysed on ice by gentle strokes and were centrifuged at 1000 rpm for 10 minutes at 4 °C. The pellet containing nuclei was collected, suspended in hypotonic buffer and was probed for the presence of Cy-3' labeled plasmid. Similarly, cytoplasmic extracts of samples (supernatant) were collected and were probed for the presence of Cy-3' labeled plasmid, as described above. The untreated cells were used as controls. The percent fluorescence intensity in cytoplasm and nuclei of samples were collected from total normalized fluorescence intensity values of samples.

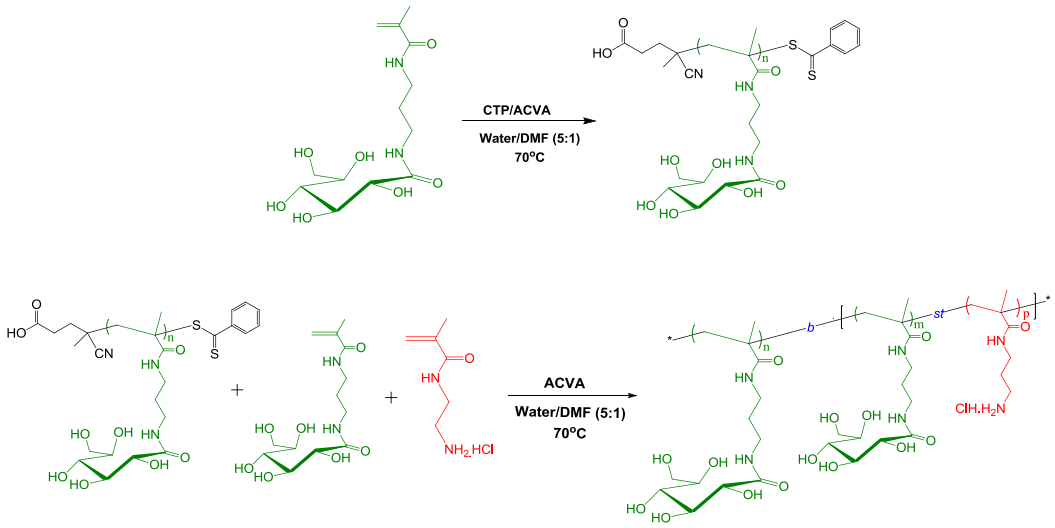
6.2.14. Toxicity Post-Transfection

Hep G2 cells and human dermal fibroblasts were seeded in 24 well tissue culture plate, and were transfected with polyplexes under conditions used for gene expression experiments. After 4 and 8 hours for Hep G2 and fibroblasts respectively, the media was replaced with fresh serum containing media and

cells were allowed to grow for another 48 hours. 125 µL of MTT dye/well was added, and cells were incubated for 2 hours, followed by the addition of lysis buffer. The plate was read at 570 nm using TECAN micro plate reader.

6.3 Results and Discussion

The ability to synthesize well-defined and advanced gene delivery vectors with novel architectures and compositions has allowed a better understanding of the structure-activity relationship of those non-viral gene delivery systems.[8-9, 16-21, 25-27] Major advances in the field of gene delivery have therefore been made by careful engineering of the materials which have overcome some of the major drawbacks of previously studied vectors.[17,19] Recently, we have reported synthetic carbohydrate and phosphorylcholine based non-viral vectors of different architectures, molecular weights and compositions.[17-19]. We have shown that changes in those parameters can significantly affect their performance in gene expression *in vitro*. For instance, gene expression of phosphorylcholine-based vectors is significantly lower than carbohydrate-based carriers. Moreover, carbohydrate-based *statistical* cationic polymers show significant aggregation in the presence of serum proteins, as compared to their *diblock* analogues. However, these block copolymers showed high toxicity post transfection under *in vitro* conditions. Herein, we report a novel architecture of these polymers prepared by reversible addition-fragmentation chain transfer polymerization (RAFT) process as depicted in scheme 6-2.



Scheme 6-2. Synthesis of linear block-statistical copolymers by RAFT process.

To design these cationic polymers of novel *block-statistical* configurations, low molecular weight homopolymers of GAPMA, LAEMA or MPC were first synthesized *via* RAFT polymerization and subsequently used as macroCTA for the copolymerization with AEMA and GAPMA, LAEMA or MPC. The resulting *block-statistical* copolymers were characterized by gel permeation chromatography (GPC) for their molar masses which revealed to be between 14-40 kDa. (Supporting information Figure S1-2) The copolymers were also characterized by ^{1}H NMR to determine their compositions. These results are summarized in Table 6-1. (Supporting Information Figure S3-S6). The compositions of the statistical segment are carefully designed for better performance in DNA condensation ability and hence for high gene expression. As shown in our previous reports, a higher amine to carbohydrate or MPC content is necessary for optimal gene expression. [17,19]

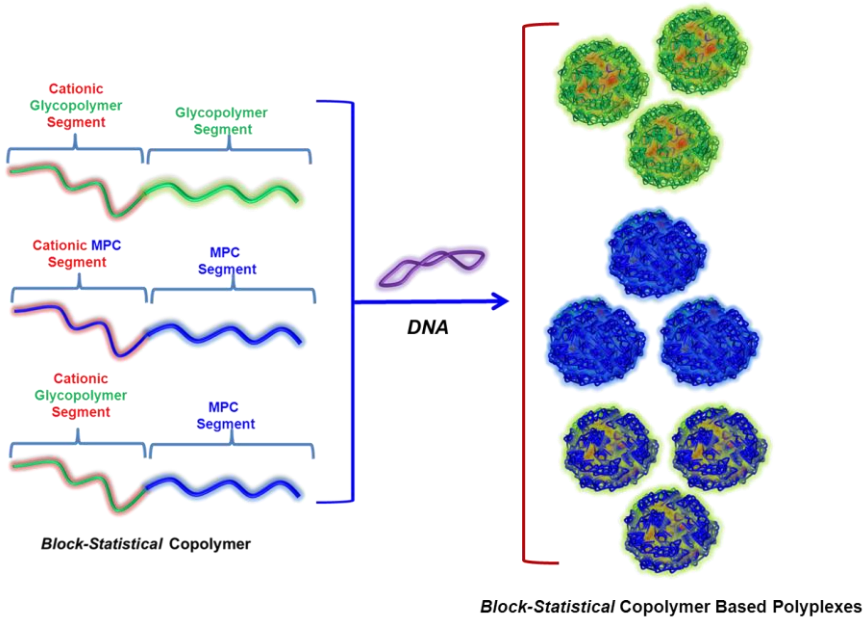
The polymers produced were studied for their DNA complexation by agarose gel electrophoresis. The results obtained were consistent with previous studies, and

showed that low polymer/plasmid ratios were required to complex plasmid DNA for high molecular weight copolymers as compared to low molecular weight copolymers.[18]

Table 6-1. Molecular weights, molecular weight distributions, and compositions of *block-statistical* copolymers as determined from gel permeation chromatography (GPC) and ^{1}H NMR.

Polymer Compositions	M_n **(kDa)**	M_w/M_n
P(AEMA$_{87}$-*st*-GAPMA$_{48}$)	30	1.3
P(AEMA$_{30}$-*st*-MPC$_{30}$)	14	1.3
P[(AEMA$_{65}$-*st*-GAPMA$_{39}$)-*b*-GAPMA$_{15}$]	28	1.4
P[(AEMA$_{147}$-*st*-GAPMA$_{34}$)-*b*-GAPMA$_{15}$]	40	1.4
P[(AEMA$_{19}$-*st*-LAEMA$_{10}$)-*b*-LAEMA$_{15}$]	20	1.3
P[(AEMA$_{27}$-*st*-LAEMA$_{26}$)-*b*-LAEMA$_{15}$]	42	1.3
P[(MPC$_{16}$-*st*-AEMA$_{34}$)-*b*-MPC$_{12}$]	14	1.4
P[(MPC$_{41}$-*st*-AEMA$_{64}$)-*b*-MPC$_{12}$]	26	1.5
P[(AEMA$_{64}$-*st*-GAPMA$_{64}$)-*b*-MPC$_{15}$]	34	1.5
P[(AEMA$_{66}$-*st*-MPC$_{70}$)-*b*-GAPMA$_{15}$]	39	1.5

These molecular weight dependent DNA complexations were not affected by the type of copolymer used, as GAPMA, LAEMA and MPC based copolymers showed similar DNA condensation efficacies. (Supporting information Figure S9-S14) The formation of polyplexes using *block-statistical* copolymers is depicted in scheme 6-3.



Scheme 6-3. Polyplexes formation using cationic block-statistical copolymers.

As discussed earlier, '*the block-statistical'* polymers produced are of varying molecular weights. The gene expression of the polymers is dependent on their polymer/plasmid ratio, which in turn is dependent on their molecular weights. In general, the polymers of high molecular weights and amine content show optimum gene expression and vice versa. A range of polymer/plasmid ratios for each polymer sample were studied to obtain optimum gene expression. The polymer/plasmid ratios which showed high gene expression were then tested for their net size and charges and stability in the presence of serum containing proteins. These results are depicted in figure 6-1. All of these polyplexes showed strong positive zeta potential values, in deionized water. The sizes of the polyplexes in media were dependent on the molecular weight of copolymers; low molecular weight copolymers produced large polyplexes as compared to high molecular weight copolymers. (Figure 6-1a)

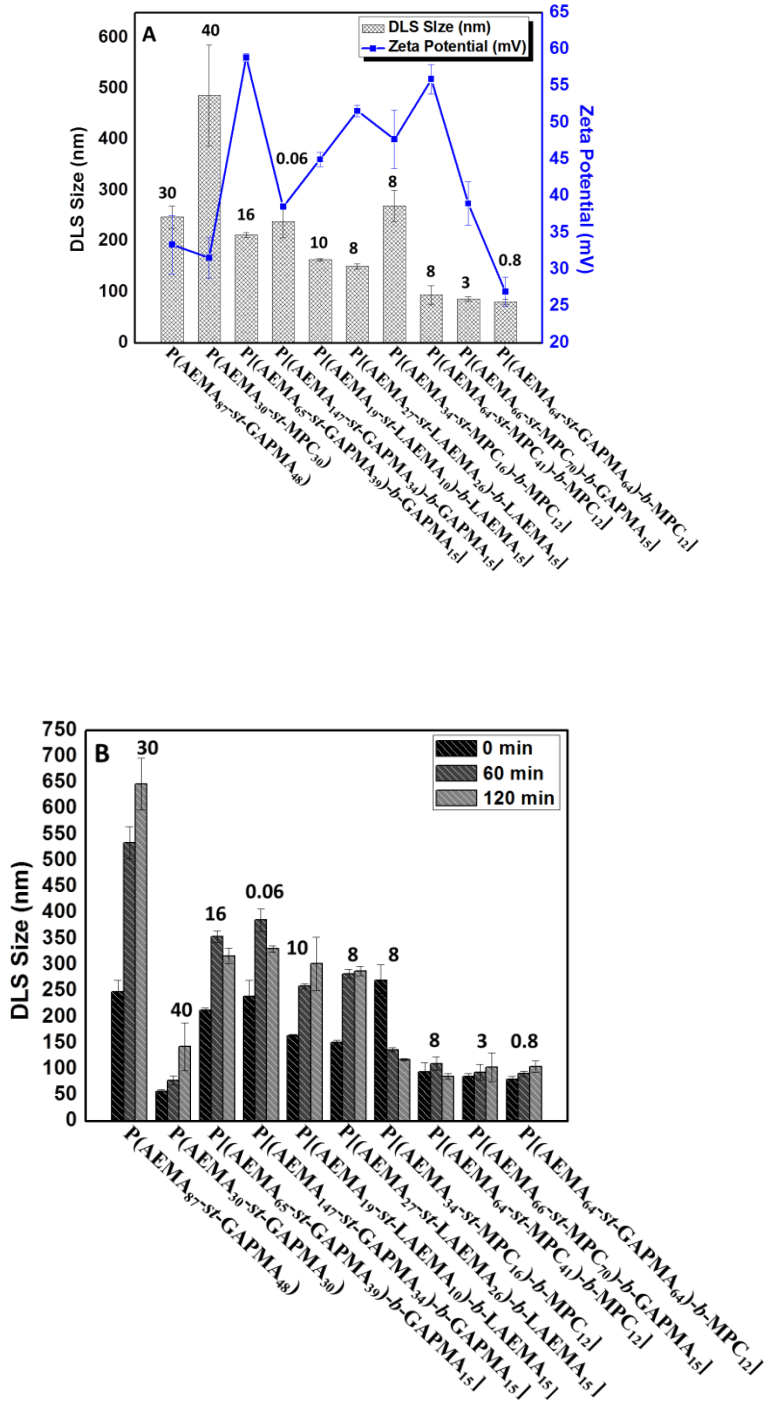


Figure 6-1. Dynamic light scattering (DLS) (in media) and zeta potential (in deionized water) values of polyplexes formulated at varying polymer/plasmid ratios (shown at the top of bars) (A). DLS analysis of aggregation of polyplexes

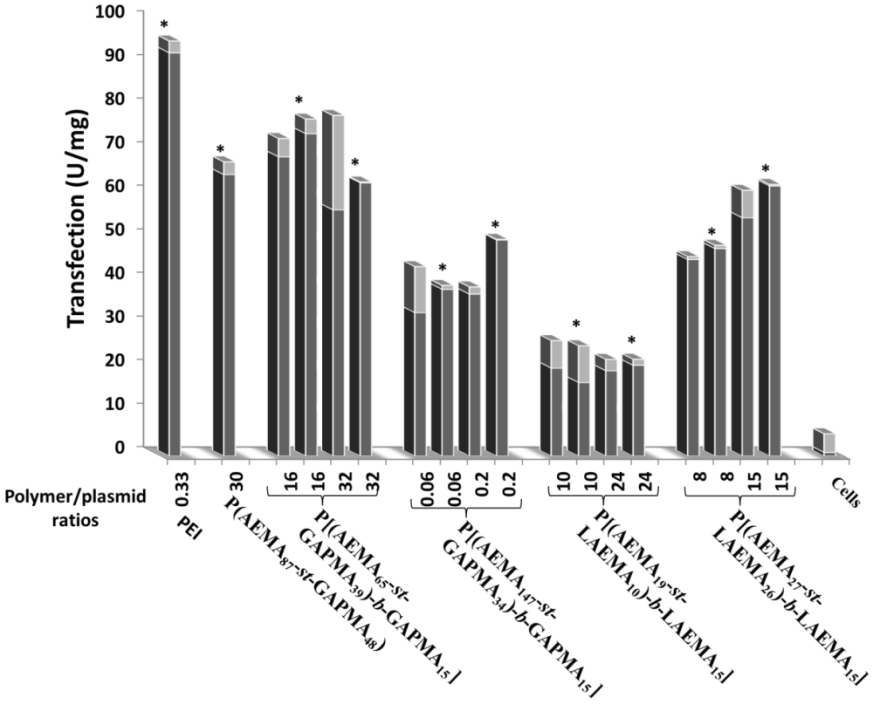
formulated at varying polymer/plasmid ratios (shown at the top of bars) in serum containing media as a function of time (B).

The *statistical* copolymers, although they can condense DNA efficiently, are susceptible to aggregation over time in the presence of serum proteins.[17] Polyplexes produced using *block-statistical* polymers is expected to show high stability in serum containing media due to the presence of carbohydrate/MPC shell on the surface of the polyplexes hence preventing the aggregation of polyplexes. The aggregation of these polyplexes is studied in the presence of serum containing media over a 2-hour time period. A *statistical* cationic glycopolymer, P($AEMA_{87}$-*st*-$GAPMA_{48}$) and a phosphorylcholine based cationic polymer P($AEMA_{30}$-*st*-MPC_{30}) are used as a control to evaluate the sizes of *block-statistical* polymers based polyplexes in comparison to *statistical* copolymers based polyplexes in serum containing media. The size of P($AEMA_{87}$-*st*-$GAPMA_{48}$) based polyplexes increase over time, due to the aggregation of polyplexes in serum containing media and continue to increase over a period of 120 minutes.[17] In contrast, all *block-statistical* cationic glycopolymers show no significant aggregation in serum containing media. The polyplexes formulated with MPC based copolymers show better stability in serum containing media than those formulated with carbohydrate-based copolymers. (Figure 6-1b) These results are consistent with previous literature where interactions of MPC-based materials with serum proteins are studied.[19] It should be noted that *block-statistical* copolymers significantly improved the

stability of both phosphorylcholine and carbohydrate-based polyplexes in high serum conditions.

The gene expression of these *block-statistical* copolymers in the presence and absence of serum proteins is then evaluated at varying polymer/plasmid ratios, and is compared with statistical glycopolymer P($AEMA_{87}$-*st*-$GAPMA_{48}$), statistical MPC-based copolymer P($AEMA_{30}$-*st*-MPC_{30}) and branched PEI, as controls. Diblock copolymers show low gene expression along with higher toxicity, as compared to their statistical analogues, possibly due to the high density of amine in the core.[17] Therefore, *block-statistical* copolymers are designed with a statistical cationic segment which can condense DNA and is significantly less toxic. The gene delivery efficacies of these *block-statistical* copolymers are studied in Hep G2 cells using β-galactosidase assay. It is found that carbohydrate-based copolymers show high gene expression than the MPC-based analogues. The gene expression of GAPMA and LAEMA based *block-statistical* polymers was dependent upon their molecular weights. P[($AEMA_{65}$-*st*-$GAPMA_{39}$)-*b*-$GAPMA_{15}$] (M_n = 28 kDa) based polyplexes showed higher gene expression than P[($AEMA_{147}$-*st*-$GAPMA_{34}$)-*b*-$GAPMA_{15}$] based polyplexes. In contrast, P[($AEMA_{27}$-*st*-$LAEMA_{26}$)-*b*-$LAEMA_{15}$] based polyplexes showed higher gene expression than P[($AEMA_{19}$-*st*-$LAEMA_{10}$)-*b*-$LAEMA_{15}$] based polyplexes. (Figure 6-2) The low gene expression of the high molecular weight GAPMA based copolymer P[($AEMA_{147}$-*st*-$GAPMA_{34}$)-*b*-$GAPMA_{15}$] is probably due to its high toxicity at high polymer/plasmid ratio.

Further increase in polymer/plasmid ratios for this system, did not improve the gene expression.



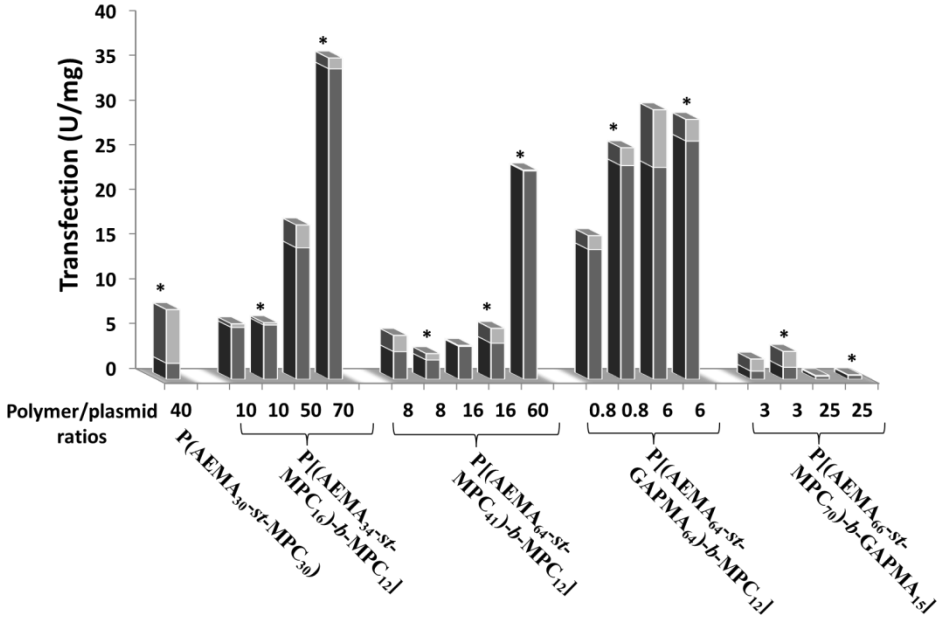
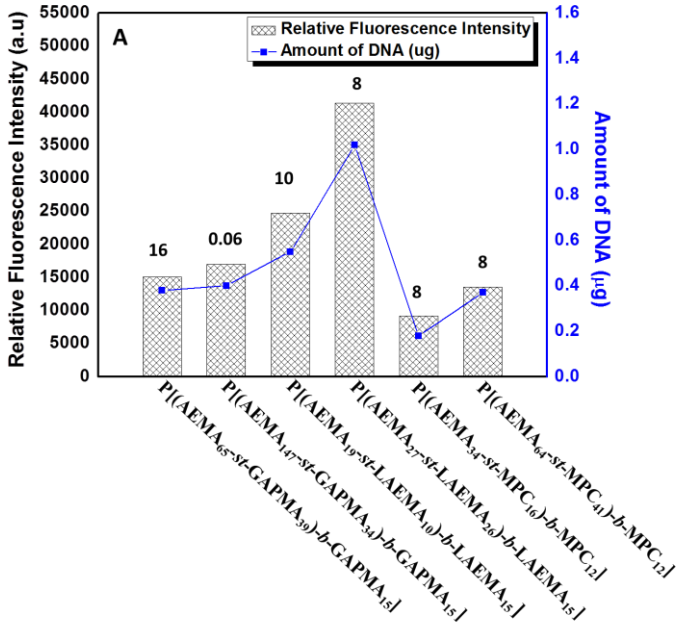


Figure 6-2. Transfection efficacies of *block-statistical* copolymers in Hep G2 cells at varying polymer/plasmid ratios, as determined by β-galactosidase assay, in the presence (*) and absence of serum.

MPC based *block-statistical* polymers efficiently condensed DNA and produced strongly positive polyplexes of 80-270 nm in diameter, they showed poor gene expression, at polymer/ plasmid ratios comparable to the carbohydrate-based analogues. However, the increase in polymer/plasmid ratio (polymer/plasmid ratio of 50-70) of these MPC based copolymers significantly improved their gene expression, as compared to P($AEMA_{30}$-*st*-MPC_{30}) based polyplexes (which showed low gene expression, at all studied polymer/plasmid ratios and showed high toxicity at high polymer/plasmid ratio). The several fold increase in transfection efficacy of both carbohydrate and MPC based *block-statistical* polymers as compared to their statistical analogues, and their superior stability towards serum containing media, show the effect of architecture and design of copolymers on gene expression.

Although, MPC-based block-statistical copolymers based polyplexes showed superior gene expression than their statistical analogues, the gene expression is still low as compared to carbohydrates based polyplexes. The low adhesion of proteins and living cells on MPC modified cationic surfaces is well-studied.[4] The low gene expression of MPC based copolymers may be associated with the poor uptake of these complexes in hepatocytes. Two factors that may be responsible for poor gene expression of MPC based copolymers are low interaction of MPC based vectors with proteins and living cells, as well as poor

DNA condensation ability of MPC based *statistical* polymers. [4,19] The cellular uptake of these polyplexes has been studied in detailed and will be discussed below.



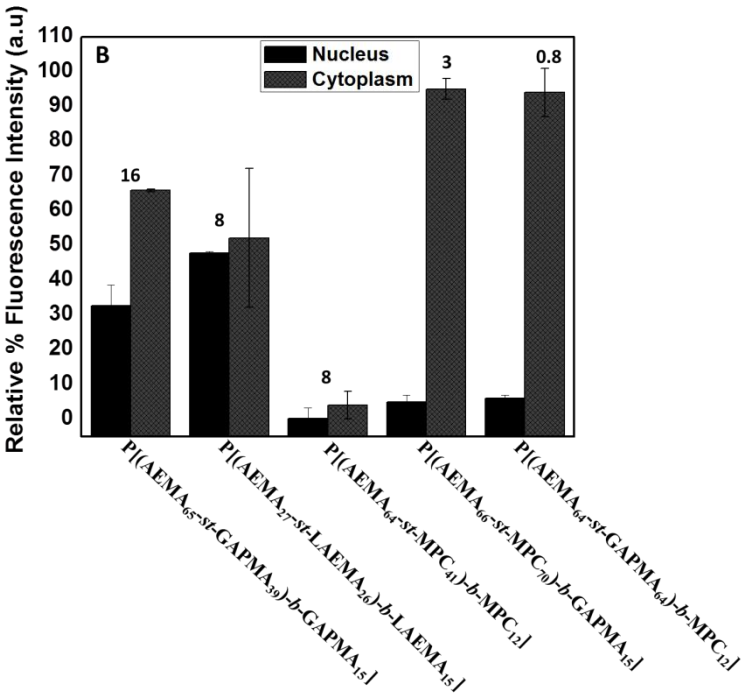


Figure 6-3. Cellular uptake of polyplexes by Hep G2 cells at varying polymer/plasmid ratios as indicated on the top of bars (A). The nuclear and cytoplasmic localization of polyplexes in Hep G2 cells at varying polymer/plasmid ratios, as indicated on the top of bars (B).

To improve the low cellular uptake of MPC based copolymer, new MPC and GAPMA based copolymers were designed, as shown in table 6-1. (Supporting information Figure S7-8) Both P[($AEMA_{64}$-*st*-$GAPMA_{64}$)-*b*-MPC_{12}], and P[($AEMA_{66}$-*st*-MPC_{70})-*b*-$GAPMA_{15}$] formed polyplexes with positive zeta potential values and showed high stability in serum containing media. (Figure 6-1) It was found that, P($AEMA_{66}$-*st*-MPC_{70})-*b*-$GAPMA_{15}$ showed no gene expression at all studied polymer/plasmid ratios, P[($AEMA_{64}$-*st*-$GAPMA_{64}$)-*b*-MPC_{12}] based polyplexes showed improved gene expression at low polymer/plasmid ratios (6), and gene expression was comparable to MPC based copolymers at high polymer/plasmid ratio (70). (Figure 6-2B) Hence, incorporation of GAPMA in MPC based cationic polymers significantly improved their gene expression at low polymer/plasmid ratios. To investigate the effect of MPC and carbohydrate based *block-statistical* polymers on gene expression, we studied their cellular uptake, and nuclear localization ability.

It is found that uptake of polyplexes and their nuclear localization efficacy is directly related to their gene expression. As expected, the uptake of LAEMA based polyplexes is higher in Hep G2 cells as compared to other polyplexes. (Figure 6-3a) This enhanced uptake of LAEMA-based polyplexes is possibly due to the presence of the high density of asialoglycoprotein receptors (ASGP-R)

on the surface of Hep G2 cells, which show galactose specific targeting properties.[18] In contrast, MPC based polyplexes (at all studied polymer/plasmid ratios) showed poor cellular uptake than carbohydrate based polyplexes. (see figure 6-3 and supporting information Figure S16) The incorporation of GAPMA in MPC and AEMA copolymers in block or statistical configuration, significantly improved the cellular uptake of polyplexes. However, this high cellular uptake of P[($AEMA_{66}$-*st*-MPC_{70})-*b*-$GAPMA_{15}$] based polyplexes did not translate into their high gene expression. To further investigate this relationship between the uptake of polyplexes and gene expression, nuclear localization ability of these polyplexes was studied after 4 hours of incubation of polyplexes with hepatocytes. (Supporting information figure S15) It was found that, although GAPMA based polyplexes show less cellular uptake than LAEMA based polyplexes, GAPMA based polyplexes show high nuclear localization efficacies than LAEMA based analogues, which is also translated into their high gene expression in hepatocytes. (Figure 6-3b) This high nuclear localization efficacy of GAPMA based complexes is attributed to the high cationic content of this vector as compared to LAEMA based vector. As discussed above, P[($AEMA_{64}$-*st*-MPC_{41})-*b*-MPC_{12}], P([$AEMA_{64}$-*st*-$GAPMA_{64}$)-*b*-MPC_{12}] and P[($AEMA_{70}$-*st*-MPC_{66})-*b*-$GAPMA_{15}$] showed poor cellular uptake, and significantly low nuclear localization ability.

The cellular uptake of polyplexes in Hep G2 cells was visualized by confocal microscopy to explore the uptake of these polyplexes in detail. (Figure 6-4)

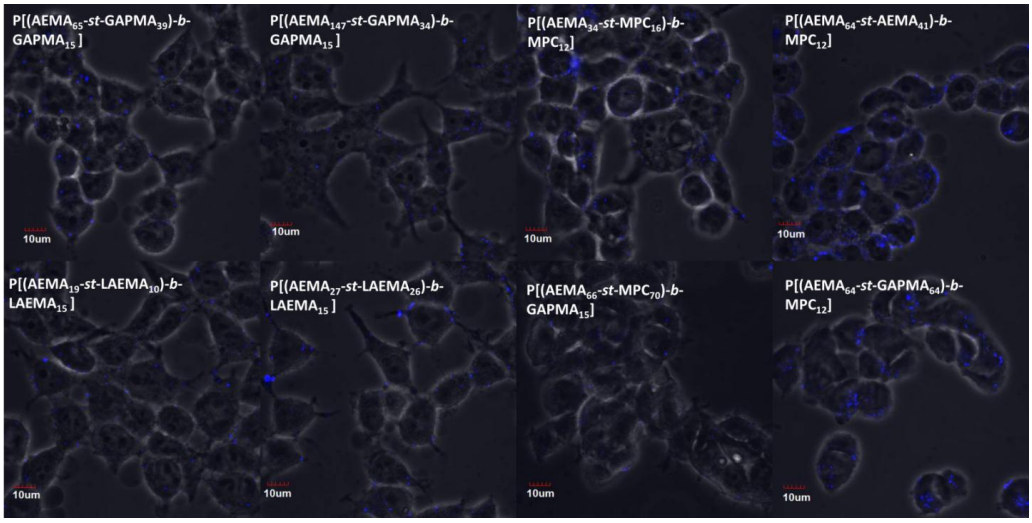


Figure 6-4. Confocal images of uptake of *block-statistical* based polyplexes, in Hep G2 cells.

The confocal images revealed that GAPMA and LAEMA derived *block-statistical* polymers based polyplexes are well internalized in the cells, and most of these polyplexes are visible in the nucleus of the cells, after only 4 hours of incubation. In contrast, MPC based *block-statistical* polymers show diffused fluorescent particles at low polymer/plasmid ratios, these particles are located on the cell surface, and are not well internalized. The low transfection efficiency of MPC based complexes is consistent with their low DNA uptake in Hep G2 cells. In contrast, at high polymer/plasmid ratios, MPC based *block-statistical* polymers were well internalized in the cells, and consequently a high gene expression is recorded. (Supporting information Figure S17) P[(AEMA$_{64}$-*st*-GAPMA$_{64}$)-*b*-MPC$_{12}$] based polyplexes showed relatively higher uptake in cells and hence improved gene expression at low polymer/plasmid ratio is noted. In contrast, P[(AEMA$_{66}$-*st*-MPC$_{70}$)-*b*-GAPMA$_{15}$] based polyplexes showed low

fluorescent particles in cells, and no polyplexes were observed associated with the cells .

The toxicity post-transfection of these polyplexes was studied at various polymer/plasmid ratios and was compared to branched PEI and *statistical* cationic copolymer as controls. (See Figure 6-5)

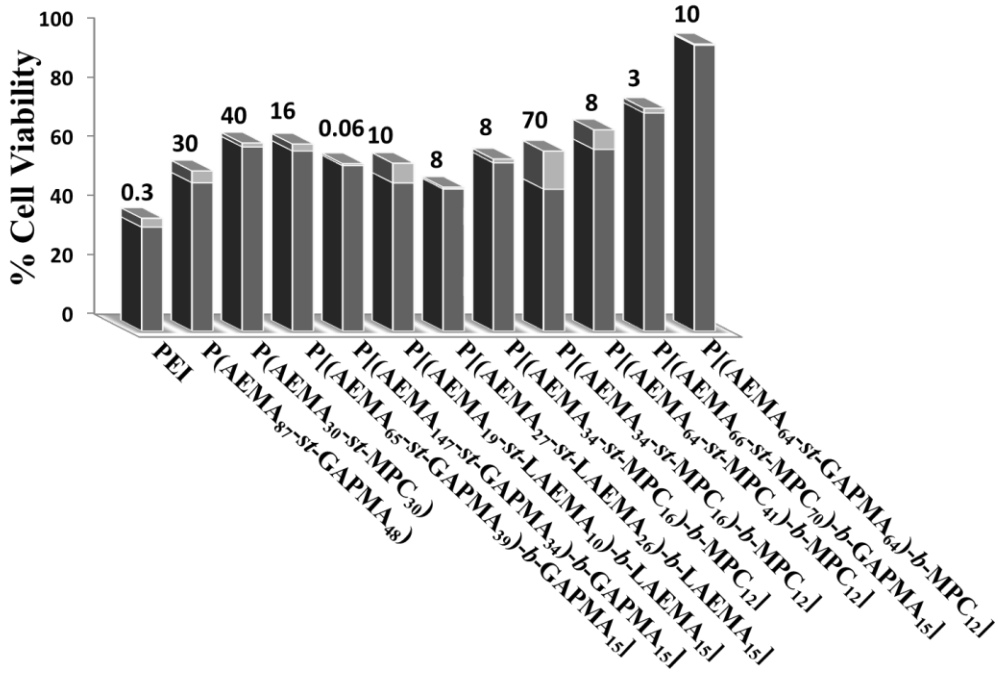


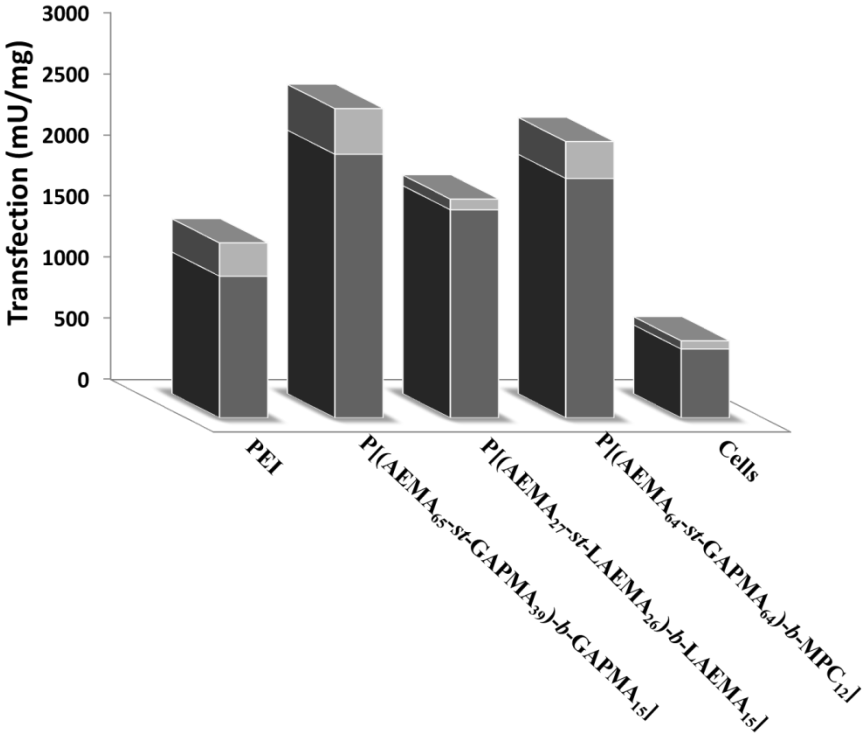
Figure 6-5. Toxicity post-transfection of *block-statistical* copolymers based polyplexes at varying polymer/plasmid ratios (shown at the top of bars), as determined using MTT assay.

The results show that *block-statistical* polymers show high cell viability (60-90%) along with high gene expression as compared to their statistical copolymer analogues. The toxicity of these copolymers was dependent upon their polymer/plasmid ratios. P[(AEMA$_{66}$-*st*-MPC$_{70}$)-*b*-GAPMA$_{15}$] based polyplexes showed high cell viability than other polyplexes, possibly due to their low

nuclear localization ability and gene delivery efficacy. As confocal images reveal that these polyplexes are less uptaken and show DNA release in cytoplasm, which may translate into their low toxicity. These results are in agreement with recent literature, where mechanism of toxicity of gene delivery vectors is studied in detail and it is found that nuclear membrane disruption ability of polymers is related to their high gene expression as well as toxicity. [29] Although, P[($AEMA_{64}$-*st*-MPC_{41})-*b*-MPC_{12}] based polyplexes showed improved gene expression at high polymer/plasmid ratio, this high gene expression was also associated with high toxicity (cell viability is 47±10%). P[($AEMA_{64}$-*st*-$GAPMA_{64}$)-*b*-MPC_{12}] (MPC based sugar analogue) showed improved gene expression at low polymer/plasmid ratio along with high cell viability (73±1.5%).

The high gene expression of these *block-statistical* polymers and their high nuclear localization efficiency make them suitable for gene expression in progenitor and pluripotent cells, which show slow mitotic activities. Human dermal fibroblasts are important for cell therapies, as they can be induced as pluripotent stem cells, to prepare grafts for skin burns. [30] However, genetic modification of these cells is a major issue, which can further enhance their therapeutic efficacies. The low gene expression of human dermal fibroblasts has been reported previously. The incorporation of lipids into cationic vectors only slightly improved their gene expression.[31] The gene expression of the selected polymers, which show high nuclear localization efficacies was studied in human dermal fibroblasts. In contrast, for Hep G2 cells, where incubation of polyplexes

for 4 hours produced significant gene expression, possibly due to the presence of high number of carbohydrate specific receptors on the surface of Hep G2 cells, longer incubation times (up to 8 hours) were required to obtain high gene expression in human dermal fibroblasts. It was found that *block-statistical* copolymers studied show high gene expression, as compared to PEI as positive control. (Figure 6)



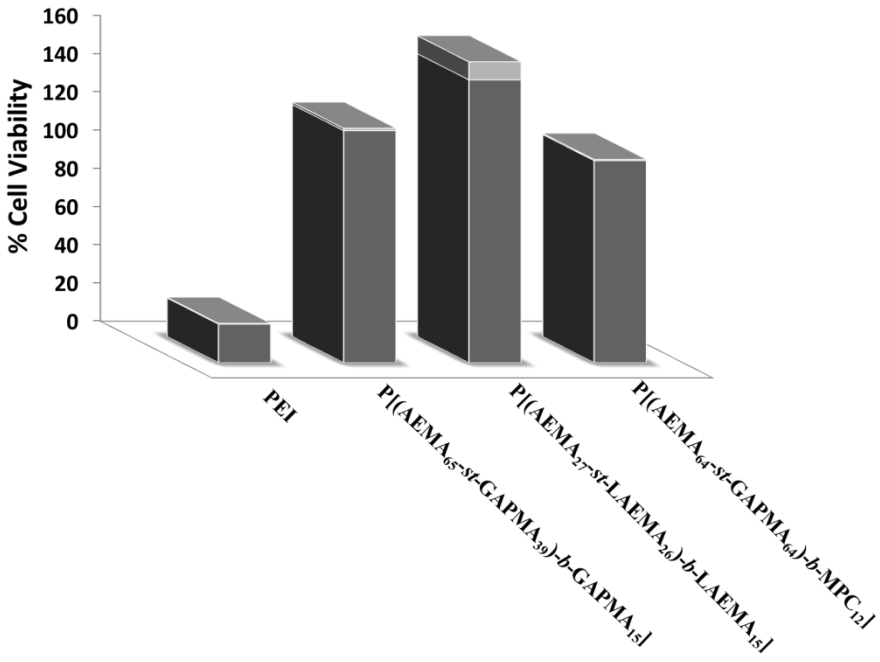


Figure 6-6. Transfection efficacies of *block-statistical* copolymers in fibroblasts, studied at polymer/plasmid ratio of 0.4, and determined by β-galactosidase assay (top). Toxicity post-transfection of *block-statistical* copolymers based polyplexes as determined by MTT assay (bottom).

The gene expression is significantly improved for P[(AEMA$_{65}$-*st*-GAPMA$_{39}$)-*b*-GAPMA$_{15}$] based polyplexes (1.5 fold better than PEI). Galactose copolymer and sugar based MPC analogue showed gene expression higher or similar to PEI. The overall low transfection values of fibroblasts for controls and *block-statistical* polyplexes, as compared to Hep G2 can be associated with low protein content of the cells itself, as well as their low metabolic activities. These results are supported by another study where, trehalose based polymers are shown to have superior gene expression ability in fibroblasts, as compared to PEI.[30] MPC based *block-statistical* copolymers were not chosen for the study, due to

high polymer/plasmid ratio required for their transfection, as well as due to their low cellular uptake.

The toxicity post-transfection of these polyplexes is further studied in human dermal fibroblasts. (See figure 6) It is found that all *block-statistical* copolymer based polyplexes maintain high cell viabilities (90-100%) along with high gene expression, as discussed above. The low gene expression of PEI based complexes is probably associated with their high toxicity (cell viability is ~20%).

6.4. Conclusions

The study provides a comprehensive account on the synthesis and gene delivery application of *block-statistical* copolymers of carbohydrate and phosphorylcholine based moieties in hepatocytes and human dermal fibroblasts. These cationic *block-statistical* copolymers provide an excellent system for the formation of polyplexes which are stable and show low interactions with serum proteins. In contrast to cationic diblock copolymers, these *block-statistical* copolymers are significantly less toxic and revealed to be excellent DNA carriers to cells. The detailed study of cellular uptake, nuclear localization ability, and gene expression of these vectors revealed that high gene expression of carbohydrate based vectors is directly related to their cellular uptake, and high nuclear localization ability in hepatocytes. MPC based vectors show poor cellular uptake and gene transfection efficacies at low polymer/plasmid ratios. The increase in polymer concentration of these polyplexes improve their gene expression and confocal images show the fluorescent particles which are well

internalized. However, the toxicity of polyplexes also increased at high concentration of polymers. The incorporation of carbohydrate in the core of MPC based *block-statistical* copolymers significantly enhanced their gene expression at low polymer/plasmid ratios, possibly due to their higher uptake and high cell viability as compared to their MPC based analogues. The high nuclear localization ability of the carbohydrate-based *block-statistical* copolymers suggests their inherent ability to transfect progenitor stem cells. The glucose-derived *block-statistical* copolymers are found to show high gene expression in fibroblasts, as compared to PEI. The low gene expression of PEI based polyplexes in fibroblasts is associated with their high toxicity.

Supporting Information Available. GPC curves and ^{1}H NMR data for polymer synthesis, confocal images of nuclear localization, and agarose gel electrophoresis. This information is available at www.elsevier.com.

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6.5. References

[1] Ingle NP, Malone B, Reineke TM. Poly(glycoamidoamine)s: a broad class of carbohydrate-containing polycations for nucleic acid delivery. Trends Biotechnol 2011;29:443-453.

[2] Grigsby LG, Leong WK. Balancing protection and release of DNA: Tools to address a bottleneck of non-viral gene delivery. J R Soc Interface 2010;6:S67-S82.

[3] Lewis AL. Phosphorylcholine-based polymers and their use in the prevention of biofouling. Colloids Surf B Biointerf 2000;18:261-275.

[4] Xu Y, Takai M, Ishihara K. Protein adsorption and cell adhesion on cationic, neutral, and anionic 2-methacryloyloxyethyl phosphorylcholine copolymer surfaces. Biomaterials 2009;30:4930-4938.

[5] Marques AP, Reis RL, Hunt JA. The biocompatibility of novel starch-based polymers and composites: in vitro studies. Biomaterials 2002;23:1471-1478.

[6] Hobel S, Loos A, Appelhans D, Schwarz S, Seidel J, Voit B, et al. Maltose- and maltotriose-modified, hyperbranched poly(ethylene imine)s (OM-PEIs): physicochemical and biological properties of DNA and siRNA complexes. J Control Release 2011;149:146-158.

[7] Popielarski RS, Mishra S, Davis EM. Structural effects of carbohydrate-containing polycations on gene delivery. 3. cyclodextrin type and functionalization. Bioconjugate Chem 2003;14:672-678.

[8] Srinivasachari S, Liu Y, Prevette LE, Reineke TM. Effects of trehalose click polymer length on pDNA complex stability and delivery efficacy. Biomaterials 2007;28:2885-2898.

[9] Reineke MT, Davis EM. Structural effects of carbohydrate-containing polycations on gene delivery. 2. charge center type. Bioconjugate Chem 2003;14:255-261.

[10] Boyer C, Bulmus V, Davis PT, Ladmiral V, Liu J, Perrier S. Bioapplications of RAFT polymerization. Chem Rev 2009;109:5402-5436.

[11] Deng Z, Bouchekif H, Babooram K, Housni A, Choytun N, Narain R. Facile synthesis of controlled-structure primary amine-based methacrylamide polymers via the reversible addition-fragmentation chain transfer process. J Poly Sci Part A Polym Chem 2008;46:4984-4996.

[12] Deng Z, Li S, Jiang X, Narain R. Well-defined galactose-containing multifunctional copolymers and glyconanoparticles for biomolecular recognition processes. Macromolecules 2009;42:6393-6405.

[13] Deng Z, Ahmed M, Narain R. Novel well-defined glycopolymers synthesized via the reversible addition fragmentation chain transfer process in aqueous media. J Poly Sci Part A Polym Chem 2009;47:614-627.

[14] Ishihara K, Goto Y, Matsuno R, Inoue Y, Konno T. Novel polymer biomaterials and interfaces inspired from cell membrane functions. Biochim Biophys Acta-General 2011;1810:268-275.

[15] Bhuchar N, Deng Z, Ishihara K, Narain R. Detailed study of the reversible addition–fragmentation chain transfer polymerization and co-polymerization of 2-methacryloyloxyethyl phosphorylcholine. Polym Chem 2011;2:632-639.

[16] Liu Y, Reineke MT. Poly(glycoamidoamine)s for gene delivery. structural effects on cellular internalization, buffering capacity, and gene expression. Bioconjugate Chem 2007;18:19-30.

[17] Ahmed M, Narain R. The effect of polymer architecture, composition, and molecular weight on the properties of glycopolymer-based non-viral gene delivery systems. Biomaterials 2011;32:5279-5290.

[18] Ahmed M, Narain R. The effect of molecular weight, compositions and lectin type on the properties of hyperbranched glycopolymers as non-viral gene delivery systems. Biomaterials 2012;In press.

[19] Ahmed M, Bhuchar N, Narain R. Well-controlled cationic water-soluble phospholipid polymer-DNA nanocomplexes for gene delivery. Bioconjugate Chem 2011:22:1228-1238.

[20] Lam WKJ, Ma Y, Armes SP, Lewis LA, Baldwin T, Stolnik S. Phosphorylcholine–polycation diblock copolymers as synthetic vectors for gene delivery. J Control Release 2004;100:293-312.

[21] Chim ATY, Lam WKJ, Ma Y, Armes SP, Lewis LA, Roberts JC, et al. Structural study of DNA condensation induced by novel phosphorylcholine-based copolymers for gene delivery and relevance to DNA protection. Langmuir 2005;21:3591-3598.

[22] Ahmed M, Deng Z, Narain R. Study of size dependent transfection efficiencies of gold nanoparticles in human cell line. ACS Appl. Mater. Interfaces 2009;1:1980-1987.

[23] Ahmed M, Deng Z, Liu S, Lafrenie R, Kumar A, Narain R. Cationic glyconanoparticles: their complexation with DNA, cellular uptake, and transfection efficiencies. Bioconjugate Chem 2009;20:2169-2176.

[24] Ahmed M, Jiang X, Deng Z, Narain R. Cationic glyco-functionalized single walled carbon nanotubes as efficient gene delivery vehicles. Bioconjugate Chem 2009;20:2017-2022.

[25] Uchida H, Miyata K, Oba M, Ishii T, Suma T, Itaka K, et al. Odd-even effect of repeating aminoethylene units in the side chain of N-substituted polyaspartamides on gene transfection profiles. J Am Chem Soc 2011;133:15524-15532.

[26] Chen Q-R, Zhang L, Luther WP, Mixson JA. Optimal transfection with the HK polymer depends on its degree of branching and the pH of endocytic vesilces. Nucleic Acids Res 2002;30:1338-1345.

[27] Synatschke VC, Schallon A, Jérôme V, Freitag R, Müller AHE. Influence of polymer architecture and molecular weight of poly(2-(dimethylamino)ethyl methacrylate) polycations on transfection efficiency and cell viability in gene delivery. *Biomacromolecules* 2011;12:4247-4255.

[28] Gao Y, Yin Q, Chen L, Zhang Z, Li Y. Linear cationic click polymers/DNA nanoparticles: in vitro structure activity relationship and in vivo evaluation for gene delivery. Bioconjugate Chem 2011;22:1153-1161.

[29] Grandinetti G, Smith AE, Reineke TM. Membrane and nuclear permeabilization by polymeric pDNA vehicles: Efficient method for gene delivery or mechanism of cytotoxicity? Mol. Pharmaceutics 2012;9;523-538.

[30] Kizjakina K, Bryson, JM, Grandinetti G, Reineke TM. Cationic glycopolymers for the delivery of pDNA to human dermal fibroblasts and rat mesenchymal stem cells. Biomaterials 2012;33;1851-1862.

[31] Abbasi M, Uludag H, Incani V, Olson C, Lin X, Clements BA, et al. Palmitic acid modified poly-L-lysine for non-viral delivery of plasmid DNA to skin fibroblasts. Biomacromolecules 2007;8;1059-1063.

Chapter 7. Conclusion and Future Directions

RAFT polymerization is inarguably the most versatile approach of LRP techniques, due to its tolerance to a variety of functional groups, mild reaction conditions, polymerization in the presence of aqueous solution and the absence of metal catalysts. The development of cationic monomers in the absence of protected group chemistry and their facile polymerization in aqueous solution has opened new horizons in the field of gene delivery. It has been feasible to produce libraries of cationic polymers, bearing different amine content, chain length, and polymer architectures and shapes in the presence of biocompatible moieties.

The carbohydrates based linear cationic polymers are synthesized *via* RAFT polymerization approach. The copolymers of varying molecular weights, compositions and narrow polydispersities are synthesized and are analyzed by GPC and ^{1}H-NMR. The architecture of copolymers (block *versus* statistical) is compared for their toxicity, gene expression, and interactions with serum proteins. It is found that high amine to sugar content (1:1 or higher) is required for the high gene expression of these copolymers. The cationic glycopolymers of block architectures show low LD_{50} values as compared to their statistical analogues. The statistical copolymers show superior gene expression than the corresponding block copolymers, in the presence and absence of serum proteins. In contrast, block copolymers show reduced interactions with serum proteins, as well as low gene expression in the presence of serum.

Similarly, MPC based linear cationic polymers of varying molecular weights, are prepared *via* RAFT polymerization at MPC to amine content 1:1. The MPC based cationic polymers are compared for their gene expression and toxicity profile as a function of polymer architecture. The statistical MPC based copolymers behaved similar to statistical cationic glycopolymers and showed high LD_{50} values than their block analogues. In general the gene expression of MPC based cationic polymers is significantly lower than cationic glycopolymers. The statisical MPC based cationic polymers showed poor gene expression, as compared to their block copolymers. This poor gene expression of MPC based statistical cationic polymers is associated with their poor DNA condensation ability.

In an effort to enhance the gene expression of linear cationic glycopolymers, hyperbranched statistical cationic glycopolymers are prepared *via* RAFT polymerization at high amine to carbohydrate content. The hyperbranched statistical cationic glycopolymers bearing pendant glucose residues or galactose residues are synthesized and are studied for their gene expression and lectin interactions. The glucose-derived cationic polymers and galactose based cationic polymers showed high gene expression in both Hep G2 cells and HEK 293T cells. However, the addition of RCA_{120} caused the aggregation galactose based based polyplexes only, indicating the specific interactions of galactose residues with ASGPR receptors of Hep G2 cells.

The cationic '*block-statistical'* copolymers using carbohydrates and MPC monomers are also synthesized *via* RAFT polymerization. The copolymers are

designed to enhance the gene expression of linear statistical copolymers and to decrease their interactions with serum proteins. The glucose-derived, galactose based, and MPC based '*block-statistical'* analogues are produced. These copolymers of selected molecular weights showed high gene expression along with reduced interactions with serum proteins. MPC based cationic block-statistical copolymers showed improved gene expression than their statistical analogues, however high polymer/plasmid ratios were required, which also compromised their cell viability.To overcome this problem, sugar analogues of MPC based cationic polymers are prepared. The incorporation of sugar in statistical architecture of MPC based cationic polymers significantly improved their gene expression at low polymer/plasmid ratios.

The study discusses a range of architectures, compositions and molecular weights of carbohydrate and MPC based cationic polymers for gene delivery applications. The carbohydrate based hyperbranched cationic polymers showed superior gene expression than other copolymers. The glucose-derived hyperbranched cationic polymers of low molecular weight (4.5 kDa) showed improved gene expression in a variety of cell lines. In contrast, galactose-derived hyperbranched cationic polymers of high molecular weight (30 kDa) showed improved gene expression, specifically in Hep G2 cells. The increase in cationic content in galactose based hyperbranced cationic polymers improves their gene expression in other cell lines.

The drawbacks associated with linear cationic copolymers (MPC or carbohydrate based) are successfully addressed by synthesizing '*block-*

statistical' analogues' of these polymers. These copolymers showed superior gene expression than their linear statistical analogues.

Future studies should focus on the understanding the mechanism of interaction of these copolymers with cell surface. It will be interesting to note the effect of carbohydrate shell ('*block statistical'* copolymers *versus* statistical copolymers) on the mechanism of cellular uptake. Moreover, synthesis of cationic glycopolymers bearing nuclear localization signal, or fusogenic peptide can enhance their gene expression efficacies. The results show that cationic glycopolymers (branched architecture) show high cellular uptake than the positive control (PEI). It will be interesting to note if the incorporation of these peptides aid in their endosomal release or nuclear localization, hence improving their gene expression.

The cellular uptake studies of '*block-statistical'* copolymers in Hep G2 cells have shown that galactose based copolymers show high cellular uptake, possibly due to the cell specific interactions with ASGPR, than their glucose-derived analogues. In contrast, the gene expression was higher for glucose-derived copolymers. The nuclear localization studies further revealed high localization of glucose-derived copolymers in cell nuclie than galactose based analogues. The high cellular uptake but poor nuclear localization ability of galactose based copolymers can make them suitable candidate for siRNA delivery in Hep G2 cells. The study of type of carbohydrate residues, as well as architecture of copolymers may help in identifying the copolymers for enhanced siRNA delivery *in vitro*. In addition, there is only one report on the study of RAFT

based polymers for *in vivo* applications. Further *in vivo* studies are required to determine the role of these gene delivery vectors of different architecture for systemic applications

7.1. General Bibliography

[1] M.L. Edelstein, M.R. Abedi, J. Wixon, M.R. Edelstein, Gene therapy clinical trials worldwide 1989–2004- an overview. J. Gene Med. 6 (2004) 597-602.

[2] Z. Liu, Z. Zhang, C. Zhou, Y. Jio, Hydrophobic modifications of cationic polymers for gene delivery. Prog. Polym. Sci. 35 (2010) 1144-1162.

[3] X. Guo, L. Huang, Recent advances in nonviral vectors for gene delivery. Acc. Chem. Res. (2012) ASAP.

[4] D. Putnam, Polymers for gene delivery across length Scales. Nature Materials (5) 2006, 439-451.

[5] A.C. Roche, I. Fajac, S. Grosse, N. Frison, C. Rondanino, R. Mayer, N. Monsigny, Glycofection: facilitated gene transfer by cationic Glycopolymers. Cell. Mol. Life Sci. 60 (2003) 288-297.

[6] S.Y. Wong, J.M. Pelet, D. Putnam, Polymer systems for gene delivery-past, present, and future. Prog. Polym. Sci. 32 (2007) 799-837.

[7] T.M. Reineke, Poly(glycoamidoamine)s: cationic glycopolymers for DNA delivery. J. Poly Sci. Part A: Polym. Chem. 44 (2006) 6895-6908.

[8] A. Sizovs, P.M. McLendon, S. Srinivasachari, T.M. Reineke, Carbohydrate polymers for nonviral nucleic acid delivery. Top. Curr. Chem. 296 (2010) 131-190.

[9] Y. Nakayama, Hyperbranched polymeric “star vectors” for effective DNA or siRNA delivery. Acc. Chem. Res. (2012) ASAP.

[10] E. Wagner, J. Koleckner, Gene delivery using polymer therapeutics. Adv. Polym. Sci. 192 (2006) 135-173.

[11] D. Schaffert, E. Wagner, Gene therapy progress and prospects: synthetic polymer-based systems. Gene Ther. 15 (2008) 1131-1138.

[12] F.J. Xu, W.T. Yang, Polymer vectors via controlled/living radical polymerization for gene delivery. Prog. Polym. Sci. 36 (2011) 1099-1131.

[13] M.A. Mintzer, E.E. Simanek, Nonviral vectors for gene delivery. Chem. Rev. 109 (2009) 259-302.

[14] P.M. McLendon, D.J. Buckwalter, E.M. Davis, T.M. Reineke, Interaction of poly(glycoamidoamine)-DNA delivery vehicles with cell-surface glycosaminoglycans leads to polyplex internalization in a manner not solely dependent on charge. Mol. Pharmaceutics 7 (2010)1757-1768.

[15] G. Grandinetti, N.P. Ingle, T.M. Reineke, Interaction of poly(ethylenimine)-DNA polyplexes with mitochondria: implications for a mechanism of cytotoxicity. Mol. Pharmaceutics 8 (2011) 1709-1719.

[16] Y. Liu, T.M. Reineke, Hydroxyl stereochemistry and amine number within poly(glycoamidoamine)s affect intracellular DNA delivery. J. Am. Chem. Soc. 127 (2005) 3004-3015.

[17]S. Srinivasachari, Y. Liu, G. Zhang, L. Prevette, T.M. Reineke, Trehalose click polymers inhibit nanoparticle aggregation and promote pDNA delivery in serum. J. Am. Chem. Soc. 128 (2006) 8176-8184.

[18] S. Srinivasachari, K.M. Fichter, T.M. Reineke, Polycationic β-cyclodextrin "click clusters": monodisperse and versatile scaffolds for nucleic acid delivery. J. Am. Chem. Soc. 130 (2008) 4618-4627.

[19] S. Grosse, Y. Aron, I. Honore, G. Thevenot, C. Danel, A-C. Roche, M. Monsigny, I. Fajac, Lactosylated polyethylenimine for gene transfer into airway epithelial cells: role of the sugar moiety in cell delivery and intracellular trafficking of the complexes. J. Gene Med. 6 (2004) 345-356.

[20] Y.H. Choi, F. Liu, J.S. Park, S.W. Kim, Lactose-poly(ethylene glycol)-grafted poly-$_L$-lysine as hepatoma cell-targeted gene carrier. Bioconjugate Chem. 9 (1998) 708-718.

[21] K. Wada, H. Arima, T. Tsutsumi. Y. Chihara, K. Hattori, F. Hirayama, K. Uekama, Improvement of gene delivery mediated by mannosylated dendrimer/α-cyclodextrin conjugates. J. Control. Release 104 (2005) 397-413.

[22] T. Anno, T. Higashi, K. Motoyama, F. Hirayama, K. Uekama, H. Arima, Possible enhancing mechanisms for gene transfer activity of glucuronylglucosyl-β-cyclodextrin/dendrimer. Intl. J. Pharma. 426 (2012) 239-247.

[23] A.W. York, S.E. Kirkland, C.L. McCormick, Advances in the synthesis of amphiphilic block copolymers via RAFT polymerization: stimuli-responsive drug and gene delivery. Adv. Drug Del. Rev. 60 (2008)1018-1036.

[24] D.S.H. Chu, J.G. Schellinger, J. Shi, A.J. Convertine, P.S. Stayton, S.H. Pun, Application of living free radical polymerization for nucleic acid delivery. Acc. Chem. Res. (2012) ASAP.

[25] G. Moad, E. Rizzardo, S.H. Thang, Toward living radical polymerization. Acc. Chem. Res. 41 (2008) 1133-1142.

[26] G. Moad, The emergence of RAFT polymerization. Aust. J. Chem. 59 (2006) 661-662.

[27] G. Moad, E. Rizzardo, S.H. Thang, Living radical polymerization by the RAFT process. Aust. J. Chem. 58 (2005) 379-410.

[28] C. Boyer, J. Liu, V. Bulmus, T.P. Davis, RAFT polymer end-group modification and chain coupling/conjugation via disulfide bonds. Aust. J. Chem. 62 (2009) 830-847.

[29] M. Semsarilar, S. Perrier, 'Green' reversible addition-fragmentation chain-transfer (raFt) polymerization. 2 (2010) 811-820.

[30] V. Bulmus, RAFT polymerization mediated bioconjugation strategies. Polym. Chem. 2 (2011)1463-1472.

[31] C. Boyer, V. Bulmus, T.P. Davis, V. Ladmiral, J. Liu, S. Perrier, Bioapplications of RAFT polymerization. Chem. Rev. 109 (2009) 5402-5436.

[32] S. Pearson, N. Allen, M.H. Stenzel, Core-shell particles with glycopolymer shell and polynucleoside core via RAFT: from micelles to rod. J. Poly. Sci. Part A: Polym. Chem. 47 (2009) 1706-1723.

[33] D. Smith, A.C. Holley, C.L. McCormick, RAFT-synthesized copolymers and conjugates designed for therapeutic delivery of siRNA. Polym. Chem. 2 (2011)1428-1441.

[34] Z. Deng, H. Bouchekif, K. Babooram, A. Housni, N. Choytun, R. Narain, Facile synthesis of controlled-structure primary amine-based methacrylamide polymers via the reversible addition-fragmentation chain transfer process. J. Poly. Sci. Part A: Polym. Chem. 46 (2008) 4984-4996.

[35] Y.A. Vasilieva, D.B. Thomas, C.W. Scales, C.L. McCormick, Direct controlled polymerization of a cationic methacrylamido monomer in aqueous media via the RAFT process. Macromolecules 37 (2004) 2728-2737.

[36] C.W. Scales, F. Huang, N. Li, Y.A. Vasilieva, J. Ray, A.J. Convertine, C.L. McCormick, Corona-stabilized interpolyelectrolyte complexes of siRNA with nonimmunogenic, hydrophilic/cationic block copolymers prepared by aqueous RAFT polymerization. Macromolecules 39 (2006) 6871-6881.

[37] A.W. York, Y. Zhang, A.C. Holley, Y. Guo, F. Huang, C.L. McCormick, Facile synthesis of multivalent folate-block copolymer conjugates via aqueous RAFT polymerization: targeted delivery of siRNA and subsequent gene suppression. Biomacromolecules 10 (2009) 936-943.

[38] C. Zhu, S. Jung, G. Si, R. Cheng, F. Meng, X. Zhu, T.G. Park, Z. Zhong, Cationic methacrylate copolymers containing primary and tertiary amino side groups: controlled synthesis via RAFT polymerization, DNA condensation, and in vitro gene transfection. J. Poly. Sci. Part A: Polym. Chem. 48 (2010) 2869-2877.

[39] C. Lin, J.F.J. Engbersen, The role of the disulfide group in disulfide-based polymeric gene carriers. Expert Opin. Drug Deliv. 6 (2009) 421-439.

[40] S. Son, R. Namgung, J. Kim, K. Singha, W.J. Kim, Bioreducible polymers for gene silencing and delivery. Acc. Chem. Res. (2012) ASAP, **DOI:** 10.1021/ar200248u.

[41] Y-Z. You, D.S. Manickam, Q-H. Zhou, D. Oupicky, Reducible poly(2-dimethylaminoethyl methacrylate): synthesis, cytotoxicity, and gene delivery activity. J. Control. Release 122 (2007) 217-225.

[42] N.P. Truong, Z. Jia, M. Burgess, L. Payne, N.A.J. McMillan, M.J. Monteiro, Self-catalyzed degradable cationic polymer for release of DNA. Biomacromolecules 12 (2011) 3540-3548.

[43] S. Venkataraman, W.L. Ong, Z.Y. Ong, S.C. Loo, P.L.R. Ee, Y.Y. Yang, The role of PEG architecture and molecular weight in the gene transfection performance of PEGylated poly(dimethylaminoethyl methacrylate) based cationic polymers. Biomaterials 32 (2011) 2369-2378.

[44] C. Zhu, M. Zheng, F. Meng, F.M. Mickler, N. Ruthardt, X. Zhu, Z. Zhong, Reversibly shielded DNA polyplexes based on bioreducible PDMAEMA-SS-PEG-SS-PDMAEMA triblock copolymers mediate markedly enhanced nonviral gene transfection. Biomacromolecules 13 (2012) 769-778.

[45] Z. Deng, M. Ahmed, R. Narain, Novel well-defined glycopolymers synthesized via the reversible addition fragmentation chain transfer process in aqueous media. J. Polym. Sci. Part A: Polym. Chem. 47 (2009) 614-627.

[46] O. Samsonova, C. Pfeiffer, M. Hellmund, O.M. Merkel, T. Kissel, Low molecular weight pDMAEMA-block-pHEMA block-copolymers synthesized via RAFT-polymerization: potential non-viral gene delivery agents? Polymers 3 (2011) 693-718.

[47] A.H. Alidedeoglu, A.W. York, C.L. McCormick, S.E. Morgan, Aqueous RAFT polymerization of 2-aminoethyl methacrylate to produce well-defined,

primary amine functional homo- and copolymers. J. Polym. Sci. Part A: Polym. Chem. 47 (2009) 5405-5415.

[48] D. Konkolewicz, A. Gray-Weale, S. Perrier, Hyperbranched polymers by thiol-yne chemistry: from small molecules to functional polymers. J. Am. Chem. Soc. 131 (2009) 18075-18077.

[49] L. Tao, J. Liu, B.H. Tan, T.P. Davis, RAFT synthesis and DNA binding of biodegradable, hyperbranched poly(2-(dimethylamino)ethyl methacrylate. Macromolecules 42 (2009) 4960-4962.

[50] D.J. Siegwart, K.A. Whitehead, L. Nuhn, G. Sahay, H. Cheng, S. Jiang, M. Ma, A. Lytton-Jean, A. Vegas, P. Fenton, C.G. Levins, K.T. Love, H. Lee, C. Cortez, S.P. Collins, Y.F. Li, J. Jang, W. Querbes, C. Zurenko, T. Novobrantseva, R. Langer D.G. Andersona, Combinatorial synthesis of chemically diverse core-shell nanoparticles for intracellular delivery. PNAS 108 (2011) 12996-13001.

[51] H.S. Bisht, D.S. Manickam, Y. You, D. Oupicky, Temperature-controlled properties of DNA complexes with poly(ethylenimine)-graft-poly(N-isopropylacrylamide). Biomacromolecules 7 (2006) 1169-1178.

[52] K. Isoda, N. Kanayama, D. Miyamoto, T. Takarada, M. Maeda, RAFT-generated poly(N-isopropylacrylamide)–DNA block copolymers for temperature-responsive formation of polymer micelles. Reactive & Functional Polymers 71 (2011) 367-371.

[53] N. Bhuchar, Z. Deng, K. Ishihara, R. Narain, Detailed study of the reversible addition–fragmentation chain transfer polymerization and co-

polymerization of 2-methacryloyloxyethyl phosphorylcholine. Polym. Chem. 2 (2011) 632-639.

[54] J.H. Tan, N.A.J. McMillan, E. Payne, C. Alexander, F. Heath, A.K. Whittaker, K.J. Thurecht, Hyperbranched polymers as delivery vectors for oligonucleotides. J. Polym. Sci. Part A: Polym. Chem. 50 (2012) 2585-2595.

[55] M.J. Manganiello, C. Cheng, A.J. Convertine, J.D. Bryers, P.S. Stayton, Diblock copolymers with tunable pH transitions for gene delivery. Biomaterials 33 (2012) 2301-2309.

[56] A.J. Convertine, D.S.W. Benoit, C.L. Duvall, A.S. Hoffman, P.S. Stayton, Development of a novel endosomolytic diblock copolymer for siRNA delivery. J. Control. Release, 133 (2009) 221-229.

[57] D.S.W. Benoit, N. Srinivasan, A.D. Shubin, P.S. Stayton, Synthesis of folate-functionalized RAFT polymers for targeted siRNA delivery. Biomacromolecules 12 (2011) 2708-2714.

[58] M.C. Palanca-Wessels, A.J. Convertine, R. Cutler-Strom, G.C. Booth, F. Lee, G.Y. Berguig, P.S. Stayton, O.W. Press, Anti-CD22 antibody targeting of pH-responsive micelles enhances small interfering RNA delivery and gene silencing in lymphoma cells. Molecular Ther. 19 (2011) 1529-1537.

[59] D.S.W. Benoit, S.M. Henry, A.D. Shubin, A.S. Hoffman, P.S. Stayton, pH-Responsive polymeric siRNA carriers sensitize multidrug resistant ovarian cancer cells to doxorubicin via knockdown of polo-like kinase 1. Molecular Pharm. 7 (2010)442-455.

[60] J-L. Zhu, H, Cheng, Y. Jin, S-X. Cheng, X-Z. Zhang, R-X. Zhou, Novel polycationic micelles for drug delivery and gene transfer. J. Mater. Chem. 18 (2008) 4433-4441.

[61] C. Zhu, S. Jung, S. Luo, F. Meng, X. Zhu, T.G. Park, Co-delivery of siRNA and paclitaxel into cancer cells by biodegradable cationic micelles based on PDMAEMA–PCL–PDMAEMA triblock copolymers. Biomaterials 31 (2010)2408-2416.

[62] R.N. Johnson, D.S.H. Chu, J. Shi, J.G. Schellinger, P.M. Carlson, S.H. Pun, HPMA-oligolysine copolymers for gene delivery: Optimization of peptide length and polymer molecular weight. J. Control. Release 155 (2011) 303-311.

[63] J. Shi, R.N. Johnson, J.G. Schellinger, P.M. Carlson, S.H. Pun, Reducible HPMA-co-oligolysine copolymers for nucleic acid delivery. Intl. J. Pharma. 427 (2012) 113-122.

[64] S.G. Spain, N.R. Cameron, A spoonful of sugar: the application of glycopolymers in therapeutics. Polym. Chem. 2 (2011) 60-68.

[65] A. Vaheri, J.S. Pagano, Infectious poliovirus RNA: a sensitive method of assay. Virology 17 (1965) 456-464.

[66] C. Li, T. Guo, D. Zhou, Y. Hu, H. Zhou, S. Wang, J. Chen, Z. Zhang, A novel glutathione modified chitosan conjugate for efficient gene delivery. J. Control. Release 154 (2011) 177-188.

[67] T.M. Reineke, M.E. Davis, Structural effects of carbohydrate-containing polycations on gene delivery. 2. charge center type. Bioconjugate Chem. 14 (2003)255-261.

[68] T.M. Reineke, M.E. Davis, Structural effects of carbohydrate-containing polycations on gene delivery. 1. carbohydrate size and its distance from charge centers. Bioconjugate Chem. 14 (2003) 247-254.

[69] A.E. Smith, A. Sizovs, G. Grandinetti, L. Xue, T.M. Reineke, Diblock glycopolymers promote colloidal stability of polyplexes and effective pDNA and siRNA delivery under physiological salt and serum conditions. Biomacromolecules 12 (2011) 3015-3022.

[70] Y. Liu, L. Wenning, M. Lynch, T.M. Reineke, New poly(D-glucaramidoamine)s induce DNA nanoparticle formation and efficient gene delivery into mammalian cells. J. Am. Chem. Soc. 126 (2004) 7422-7423.

[71] C-C. Lee, Y. Liu, T.M. Reineke, General structure-activity relationship for poly(glycoamidoamine)s: the effect of amine density on cytotoxicity and DNA delivery efficiency. Bioconjugate Chem. 19 (2008) 428-440.

[72] P.M. McLendon, D.J. Buckwalter, E.M. Davis, T.M. Reineke, Interaction of poly(glycoamidoamine) DNA delivery vehicles with cell-surface glycosaminoglycans leads to polyplex internalization in a manner not solely dependent on charge. Mol.Pharmaceutics 7 (2010) 1757-1768.

[73] G. Grandinetti, N.P. Ingle, T.M. Reineke, Interaction of poly(ethylenimine)DNA polyplexes with mitochondria: implications for a mechanism of cytotoxicity. Mol. Pharmaceutics 8 (2011) 1709-1719.

[74] L.E. Prevette, T.E. Kodger, T.M. Reineke, M. L. Lynch, Deciphering the role of hydrogen bonding in enhancing pDNA-polycation interactions. Langmuir 23 (2007) 9773-9884.

[75] Q. Wang, J.S. Dordick, R.J. Linhardt, Synthesis and application of carbohydrate-containing polymers. Chem. Mater. 14 (2002) 3232-3244.

[76] R. Narain, S.P. Armes, Synthesis of low polydispersity, controlled-structure sugar methacrylate polymers under mild conditions without protecting group chemistry. Chem. Comm. (2002) 2776-2777.

[77] M. Ahmed, R. Narain, The effect of polymer architecture, composition, and molecular weight on the properties of glycopolymer-based non-viral gene delivery systems. Biomaterials 32 (2011) 5279-5290.

[78] M. Ahmed, R. Narain, The effect of molecular weight, compositions and lectin type on the properties of hyperbranched glycopolymers as non-viral gene delivery systems. Biomaterials 33 (2012) 3990-4001.

[79] M. Ahmed, F.L.B. Lai, J.N. Kizhakkedathu, R. Narain, Hyperbranched glycopolymers for blood biocompatibility. Bioconjugate Chem. 23 (2012) 1050-1058.

[80] Y. Iwasaki, K. Ishihara, Phosphorylcholine-containing polymers for biomedical applications. Anal. Bioanal. Chem. 381 (2005) 534-546.

[81] Y. Kadoma, N. Nakabayashi, E. Masuhara, J. Yamauchi. "Synthesis and hemolysis test of the polymer containing phosphorylcholine groups. Koubunshi Ronbunshu (Jpn. J. Polym. Sci. Technol.) 35 (1978) 423-427.

[82] K. Ishihara, T. Ueda, N. Nakabayashi, Synthesis of surface-anchored DNA-polymer bioconjugates using reversible addition-fragmentation chain transfer polymerization Polym. J. 22 (1990) 355-360.

[83] M. Ahmed, N. Bhuchar, R. Narain, Well-controlled cationic water-soluble phospholipid polymer-DNA nanocomplexes for gene delivery. Bioconjugate Chem. 22 (2011) 1228-1238.

[84] M. Ahmed, M. Jawanda, K. Ishihara, R. Narain, Carbohydrate and phosphorylcholine polymer based gene delivery vectors with novel molecular architecture. Biomaterial (2012) Accepted.

[85] P. He, L. He, Synthesis of surface-anchored DNA-polymer bioconjugates using reversible addition-fragmentation chain transfer polymerization. Biomacromolecules 10 (2009)1804-1809.

[86] A.W. York, F. Huang, C.L. McCormick, Rational design of targeted cancer therapeutics through the multiconjugation of folate and cleavable siRNA to RAFT-synthesized (HPMA-s-APMA) copolymers. Biomacromolecules 11 (2010) 505-514.

[87] K.L. Heredia, T.H. Nguyen, C-W. Chang, V. Bulmus, T.P. Davis, H.D. Maynard, Reversible siRNA–polymer conjugates by RAFT polymerization. Chem. Commun. (2008) 3245-3247.

[88] K. Gunasekaran, T.H. Nguyen, H.D. Maynard, T.P. Davis, V. Bulmus, Conjugation of siRNA with comb-type PEG enhances serum stability and gene silencing efficiency. Macromol. Rapid Commun. 32 (2011) 654-659.

[89] N. Kanayama, H. Shibata, A. Kimura, D. Miyamoto, T. Takarada, M. Maeda, RAFT-generated polyacrylamide-DNA block copolymers for single-nucleotide polymorphism genotyping by affinity capillary electrophoresis. Biomacromolecules 10 (2009) 805-813.

[90] K.S. Ghosh, T. Pal, Interparticle coupling effect on the surface plasmon resonance of gold nanoparticles: from theory to applications. Chem. Rev. 107 (2007) 4797-4862.

[91] M.H. Smith, L.A. Lyon, Multifunctional nanogels for siRNA delivery. Acc. Chem. Res. (2012) ASAP, **DOI:** 10.1021/ar200216f.

[92] S.S. Kelkar, T.M. Reineke, Theranostics: combining imaging and therapy. Bioconjugate Chem. 122 (2011) 1879-1903.

[93] K. Babooram, R. Narain, Fabrication of SWNT/silica composites by the sol-gel process. ACS Applied Mater. Interface. 1 (2009) 181-186.

[94] M. Ahmed, X. Jiang, Z. Deng, R. Narain, Cationic glyco-functionalized single walled carbon nanotubes as efficient gene delivery vehicles. Bioconjugate Chem. 20 (2009) 2017-2022.

[95] M. Ahmed, Z. Deng, S. Liu, R. Lafrenie, R. Narain, Cationic glyconanoparticles: their complexation with DNA, cellular uptake, and transfection efficiencies. Bioconjugate Chem. 20 (2009) 2169-2176.

[96] M. Ahmed, Z. Deng, R. Narain, Study of size dependent transfection efficiencies of gold nanoparticles in human cell line. ACS Applied Mater. Interface 1 (2009) 1980-1987.

[97] J. Chen, M. Ahmed, Q. Liu, R. Narain, Synthesis of cationic magnetic nanoparticle and evaluation of their gene delivery efficacy in Hep G2 cells. J. Biomed. Mat. Res. Part A. (2012) ASAP article, doi: 10.1002/jbm.a.34176.

[98] M. Ahmed, R. Narain, Intracellular delivery of DNA and enzyme in active form using degradable carbohydrate based nanogels Mol. Pharmaceutics (2012) Submitted.

[99] R. Sunasee, P. Wattanaarsakit, M. Ahmed, F. Lollmohamed, R. Narain, Biodegradable, non-toxic cationic nanogels as non-viral gene delivery systems. Bioconjugate Chem. (2012) Submitted.

[100] X. Jiang, M. Ahmed, Z. Deng, R. Narain, Biotinylated glyco-functionalized quantum dots: synthesis, characterization, and cyto-toxicity studies. 20 (2009) 994-1001.

[101] F.B. Lollmahommed, R. Narain, Photochemical approach toward deposition of gold nanoparticles on functionalized carbon nanotubes. Langmuir 27 (2011) 12642-12649.

[102] X. Jiang, A. Housni, G. Gody, P. Boullanger, M-T. Charreyre, T. Delair, R. Narain, Synthesis of biotinylated alpha-d-mannoside or N-acetyl beta-d-glucosaminoside decorated gold nanoparticles: study of their biomolecular recognition with Con A and WGA lectins. 21 (2010) 521-530.

[103] A. Housni, M. Ahmed, S. Liu, R. Narain, Monodisperse protein stabilized gold nanoparticles via a simple photochemical process. J. Phys. Chem. C 112 (2008) 12282-12290.

[104] R. Narain, A. Housni, G. Gody, P. Boullanger, M-Y. Charreyre, T. Delair, Preparation of biotinylated glyconanoparticles via a photochemical process and study of their bioconjugation to streptavidin. Langmuir 23 (2008) 12835-12841.

[105] Y. Kotsuchibashi, Y. Zhang, M. Ahmed, M. Ebara, T. Aoyagi, R. Narain, Fabrication of FITC-doped Silica nanoparticles and their cellular uptake. Soft Matter. (2012) Submitted.

[106] M. Ahmed, R. Narain, Rapid Synthesis of gold nanorods using a one step photochemical strategy. Langmuir 26 (2010) 18392-18399.

[107] S. Kirkland-York, Y. Zhang, A.E. Smith, A.W. York, F. Huang, C.L. McCormick, Tailored design of Au nanoparticle-siRNA carriers utilizing reversible addition-fragmentation chain transfer polymers. Biomacromolecules 11 (2010) 1052-1059.

[108] C.W. Chang, E. Bays, L. Tao, S.N.S. Alconel, H.D. Maynard, Differences in cytotoxicity of poly(PEGA)s synthesized by reversible addition–fragmentation chain transfer polymerization. Chem. Commun. (2009) 3580-3582.

[109] D. Pissuwan, C. Boyer, K. Gunasekaran, T.P. Davis, V. Bulmus, In vitro cytotoxicity of RAFT polymers. Biomacromolecules 11 (2010) 412-420.

[110] J. Hentschel, K. Bleek, O. Ernst, J-F. Lutz, G.H. Borner, Easy access to bioactive peptide-polymer conjugates via RAFT. Macromolecules 41 (2008) 1073-1075.

[111] H. Willock, R.K. O'Reilly, End group removal and modification of RAFT polymers. Polym. Chem. 1 (2010) 149-157.

[112] S. Zhang, Y. Zhao, B. Zhao, B. Wang, Hybrids of nonviral vectors for gene delivery. Bioconjugate Chem. 21 (2010) 1003-1009.

[113] J. J. Green, R. Langer, G. D. Anderson, A combinatorial polymer library approach yields insight into nonviral gene delivery. Acc. Chem. Res. 41 (2008) 749-759.

[114] S. Barua, A. Joshi, A. Banerjee, D. Matthews , T. S. Sharfstein, M. S. Cramer, S. R. Kane, K. Rege, Parallel synthesis and screening of polymers for nonviral gene delivery. Mol. Pharmaceutics 6 (2009) 86-97.

[115] M. D. Lynn, G. D. Anderson, D. Putnam, R. Langer, Accelerated discovery of synthetic transfection vectors: parallel synthesis and screening of a degradable polymer library. J. Am. Chem. Soc. 123 (2001) 8155-8156.

[116] P. S. Strand, M. M. Issa, E. B. Christensen, M. K. Varum, P. Artursson, Tailoring of chitosans for gene delivery: novel self-branched glycosylated chitosan oligomers with improved functional properties. Biomacromolecules 9 (2008) 3268-3276.

[117] S. Toita, N. Morimoto, K. Akiyoshi, Functional cycloamylose as a polysaccharide-based biomaterial: application in a gene delivery system. Biomacromolecules 11 (2010) 397-401.

[118] R. S. Popielarski, S. Mishra, E. M. Davis, Structural effects of carbohydrate-containing polycations on gene delivery. 3. cyclodextrin type and functionalization. Bioconjugate Chem. 14 (2003) 672-678.

[119] Y. Liu, M. T. Reineke, Degradation of poly(glycoamidoamine) DNA delivery vehicles: polyamide hydrolysis at physiological conditions promotes DNA release. Biomacromolecules 11 (2010) 316-325.

[120] Y. Liu, M. T. Reineke, Poly(glycoamidoamine)s for gene delivery: stability of polyplexes and efficacy with cardiomyoblast cells. Bioconjugate Chem. 17 (2006) 101-108.

[121] M. A. Zanta, O. Bossif, A. Abdennaji, J. P. Behr, In vitro gene delivery to hepatocytes with galactosylated polyethylenimine. Bioconjugate Chem. 8 (1997) 839-844.

[122] K. Anderson, C. Fernandez, G. K. Rice, N-glycan targeted gene delivery to the dendritic cell SIGN receptor. Bioconjugate Chem. 21 (2010) 1479-1485.

[123] T. Azzam, A. Raskin, A. Makovitzki, H. Brem, P. Vierling, M. Lineal, J. A. Domb Cationic polysaccharides for gene delivery. Macromolecules 35 (2002) 9947-9953.

[124] K. Wong, G. Sun, X. Zhang, H. Dai, Y. Liu, C. He, W. K. Leong, PEI-g-chitosan, a novel gene delivery system with transfection efficiency comparable to polyethylenimine in vitro and after liver administration in vivo. Bioconjugate Chem. 17 (2006) 152-158.

[125] H. S. Pun, C. N. Bellocq, A. Liu, G. Jensen, T. Machemer, E. Quijano, T. Schluep, S. Wen, H. Engler, J. Heidel, E. M. David, Cyclodextrin-modified polyethylenimine polymers for gene delivery. Bioconjugate Chem. 15 (2004) 831-840.

[126] J. S. Hwang, C. N. Bellocq, E. M. Davis, Effects of structure of β-cyclodextrin-containing polymers on gene delivery. Bioconjugate Chem. 12 (2001) 280-290.

[127] W. C. Tseng, M. C. Jong, Improved stability of polycationic vector by dextran-grafted branched polyethylenimine. Biomacromolecules 4 (2003) 1277-1284.

[128] Z. H. Wang, B. W. Li, J. Ma, P. G. Tang, T. W. Yang, J. F. Xu, Functionalized nonionic dextran backbones by atom transfer radical polymerization for efficient gene delivery. Macromolecules 44 (2011) 230-239.

[129] I. Youdovin-Farber, J. A. Domb, Cationic polysaccharides for gene delivery. Materials Science and Engineering C 27 (2007) 595-598.

[130] B. A. Lowe, R. Wang, Synthesis of controlled-structure AB diblock copolymers of 3-*O*-methacryloyl-1,2:3,4-di-*O*-isopropylidene-d-galactopyranose and 2-(dimethylamino)ethyl methacrylate. Polymer 48 (2007) 2221-2230.

[131] L. Albertin, M. Stenzel, C. Barner-Kowollik, L. John, R. Foster, T. P. Davis, Well-defined glycopolymers from RAFT polymerization: poly(methyl 6-*O*-methacryloyl-α-d-glucoside) and its block copolymer with 2-hydroxyethyl methacrylate. Macromolecules 37 (2004) 7530-7537.

[132] J. Ziebarth, Y. Wang, Coarse-grained molecular dynamics simulations of DNA condensation by block copolymer and formation of core−corona structures. J. Phys. Chem. B 114 (2010) 6225-6232.

[133] O. Boussif, T. Delair, C. Brua, L. Veron, A. Pavirani, J. V. H. Kolbe, Synthesis of polyallylamine derivatives and their use as gene transfer vectors in vitro. Bioconjugate Chem. 10 (1999) 877-883.

[134] B. Le Bon, N. Van Craynest, O. Boussif, P. Vierling, Polycationic diblock and random polyethylene glycol- or tris(hydroxymethyl)methyl-grafted

(co)telomers for gene transfer: synthesis and evaluation of their in vitro transfection efficiency. Bioconjugate Chem. 13 (2002) 1292-1301.

[135] T. G. Zugates, W. Peng, A. Zumbuehl, S. Jhunjhunwala, Y. H. Huang, R. Langer, A. J. Sawicki, G. D. Anderson, Rapid optimization of gene delivery by parallel end-modification of poly(*β*-amino ester)s. Molec. Ther. 15 (2007) 1306-1312.

[136] A. M. Wolfert, R. P. Dash, O. Nazarova, D. Oupicky, W. L. Seymour, S. Smart, J. Strohalm, K. Ulbrich, Polyelectrolyte vectors for gene delivery: influence of cationic polymer on biophysical properties of complexes formed with DNA. Bioconjugate Chem. 10 (1999) 993-1004.

[137] R. Sharma, S. J. Lee, C. R. Bettencourt, C. Xiao, F. S. Konieczny, Y. Y. Won, Effects of the incorporation of a hydrophobic middle block into a PEG-polycation diblock copolymer on the physicochemical and cell interaction properties of the polymer-DNA complexes. Biomacromolecules 9 (2008) 3294-3307.

[138] Y. Nakayama, C. Kakei, A. Ishikawa, M. Y. Zhou, Y. Nemoto, K. Uchida, Synthesis and in vitro evaluation of novel star-shaped block copolymers (blocked star vectors) for efficient gene delivery. Bioconjugate Chem. 18 (2007) 2037-2044.

[139] X. Shuai, T. Merdan, F. Unger, M. Wittmar, T. Kissel, Novel biodegradable ternary copolymers *hy*-PEI-*g*-PCL-*b*-PEG: synthesis, characterization, and potential as efficient nonviral gene delivery vectors. Macromolecules 36 (2003) 5751-5759.

[140] H. G. Hsiue, Z. H. Chinag, H. C. Wang, M. T. Juang, Nonviral gene carriers based on diblock copolymers of poly(2-ethyl-2-oxazoline) and linear polyethylenimine. Bioconjugate Chem. 17 (2006) 781-786.

[141] T. Azzam, H. Eliyahu, L. Shapira, M. Linial, Y. Barenholz, J. A. Domb, Polysaccharide-oligoamine based conjugates for gene delivery. J. Med. Chem. 45 (2002) 1817-1824.

[142] C. S. De Smedt, J. Demeester, E. W. Hennink, Cationic polymer based gene delivery systems. Pharm. Res. 17 (2000) 113-126.

[143] A. Pathak, S. Patnaik, C. K. Gupta, Recent trends in non-viral vector-mediated gene delivery. Biotechnol. J. 4 (2009) 1559-1572.

[144] T. Blessing, M. Kursa, R. Holzhauser, R. Kircheis, E. Wagner, Different strategies for formation of PEGylated EGF-conjugated PEI/DNA complexes for targeted gene delivery. Bioconjugate Chem. 12 (2001) 529-537.

[145] S. Lin, F. Du, Y. Wang, S. Ji, D. Liang, L. Yu, Z. Li, An acid-labile block copolymer of PDMAEMA and PEG as potential carrier for intelligent gene delivery systems. Biomacromolecules 9 (2008) 109-115.

[146] Y. Xu, M. Takai, K. Ishihara, Protein adsorption and cell adhesion on cationic, neutral, and anionic 2-methacryloyloxyethyl phosphorylcholine copolymer surfaces. Biomaterials 30 (2009) 4930-4938.

[147] T. Goda, Y. Goto, K. Ishihara, Cell-penetrating macromolecules: Direct penetration of amphipathic phospholipid polymers across plasma membrane of living cells. Biomaterials 31 (2010) 2380-2387.

[148] T. Ueda, H. Oshida, K. Kurita, K. Ishihara, and N. Nakabayashi, Preparation of 2-methacryloyloxyethyl phosphorylcholine copolymers with alkyl methacrylates and their blood compatibility. Polym. J. 24 (1992) 1259-1269.

[149] S. Sakaki, M. Tsuchida, Y. Iwasaki, K. Ishiara, A water-soluble phospholipid polymer as a new biocompatible synthetic DNA carrier. Bull. Chem. Soc. Jpn. 77 (2004) 2283-2288.

[150] M. Licciardi, G. Giammona, J. Du, P. S. Armes, Y. Tang, L. A. Lewis, New folate-functionalized biocompatible block copolymer micelles as potential anti-cancer drug delivery systems. Polymer 47 (2006) 2946-2955.

[151] A. T. Y. Chim, W. K. J. Lam, Y. Ma, S. P. Armes, L. A. Lewis, J. C. Roberts, S. Stolnik, B. J. S. Tendler, C. M. Davies, Structural study of DNA condensation induced by novel phosphorylcholine-based copolymers for gene delivery and relevance to DNA protection. Langmuir 21 (2005) 3591-3598.

[151] W. K. J. Lam, Y. Ma, S. P. Armes, L. A. Lewis, T. Baldwin, S. Stolnik, (2004) Phosphorylcholine–polycation diblock copolymers as synthetic vectors for gene delivery J. Control. Release 100 (2005) 293-312.

[152] K. Ishihara, Y. Inoue Essential factors to make excellent biocompatibility of phospholipid polymer materials, Adv. Sci. Technol. 76 (2010) 1-9.

[153] K. Ishihara, Y. Goto, R. Matsuno, Y. Inoue, T. Konno, Novel polymer biomaterials and interfaces inspired from cell membrane functions. Biochim. Biophys. Acta-General, 1810 (2011) 268-275.

[154] Ishihara K. Bioinspired phospholipid polymer biomaterials for making high performance artificial organs. Sci. Technol. Adv. Mater.1 (2000) 131-138.

[155] A. L. Lewis, Phosphorylcholine-based polymers and their use in the prevention of biofouling. Colloids Surf. B: Biointerf. 18 (2000) 261-275.

[156] S. Barua, A. Joshi, A. Banerjee, D. Matthews, T. S. Sharfstein, M. S. Cramer, S. R. Kane, and K. Rege, Parallel synthesis and screening of polymers for nonviral gene delivery. Mol. Pharm. 6 (2009) 86-97.

[157] M. J. Layman, M. S. Ramirez, D. M. Green, E. T. Long, Influence of polycation molecular weight on poly(2-dimethylaminoethylmethacrylate)-mediated DNA deliver in vitro. Biomacromolecules 10 (2009) 1244-1252.

[158] S. Srinivasachari, Y. Liu, L. E. Prevette, T. M. Reineke, Effects of trehalose click polymer length on pDNA complex stability and delivery efficacy. Biomaterials 28 (2007) 2885-2898.

[159] C. Troiber, E. Wagner, Nucleic acid carriers based on precise polymer conjugates. Bioconjugate Chem. 22 (2011) 1737-1752.

[160] L. G. Grigsby, W. K. Leong, Balancing protection and release of DNA: Tools to address a bottleneck of non-viral gene delivery. J. R. Soc. Interface 6 (2010) S67-S82.

[161] Y. Gao, Q. Yin, L. Chen, Z. Zhang, Y. Li, Linear cationic click polymers/DNA nanoparticles: in vitro structure activity relationship and in vivo evaluation for gene delivery. Bioconjugate Chem. 2011 (22) 1153-1161.

[162] S. Hobel, A. Loos, D. Appelhans, S. Schwarz, J. Seidel, B. Voit, et al. Maltose- and maltotriose-modified, hyperbranched poly(ethylene imine)s (OM-PEIs): physicochemical and biological properties of DNA and siRNA complexes. J. Control. Release 149 (2011)146-158.

[163] Y. Liu, T. M. Reineke, Poly(glycoamidoamine)s for gene delivery. structural effects on cellular internalization, buffering capacity, and gene expression. Bioconjugate Chem. 18 (2007) 19-30.

[164] B. Newland, H. Tai, Y. Zheng, D. Velasco, D. A. Luca, M. S. Howdle, et al. A highly effective gene delivery vector-hyperbranched poly(2-(dimethylamino)ethyl methacrylate from in situ deactivation enhanced ATRP. Chem. Commun. 46 (2010) 4698-4700.

[165] H. Uchida, K. Miyata, M. Oba, T. Ishii, T. Suma, K. Itaka et al. Odd-even effect of repeating aminoethylene units in the side chain of N-substituted polyaspartamides on gene transfection profiles. J. Am. Chem. Soc. 133 (2011) 15524-15532.

[166] Q-R. Chen, L. Zhang, W. P. Luther, J. A. Mixson, Optimal transfection with the HK polymer depends on its degree of branching and the pH of endocytic vesilces. Nucleic Acids Res. 30 (2002) s1338-1345.

[162] V. C. Synatschke, A. Schallon, V. Jérôme, R. Freitag, A. H. E. Müller, Influence of polymer architecture and molecular weight of poly(2-(dimethylamino)ethyl methacrylate) polycations on transfection efficiency and cell viability in gene delivery. Biomacromolecules 12 (2011) 4247-4255.

[163] B. R. Arote, E-S. Lee, H-L. Jiang, Y-K. Kim, Y-J. Choi, M-H. Cho, et al. Efficient gene delivery with osmotically active and hyperbranched poly(ester amine)s. Bioconjugate Chem. 20 (2009) 2231-2241.

[164] J. Chen, C. Wu, D. Oupicky, Bioreducible hyperbranched poly(amido amine)s for gene delivery. Biomacromolecules 10 (2009) 2921-2927.

[165] B. Klaykruayat, K. Siralertmukul, K. Srikulkit, Chemical modification of chitosan with cationic hyperbranched dendritic polyamidoamine and its antimicrobial activity on cotton fabric. Carbohydrate Polymers 80 (2010) 197-207

[166] L. Zhang, H-C. Hu, X-S. Cheng, X-R. Zhou, PEI grafted hyperbranched polymers with polyglycerol as a core for gene delivery. Colloids Surf B: Biointerf. 76 (2010) 427-433.

[167] Y. Chen, L. Zhou, Y. Pang, W. Huang, F. Qiu, X. Jiang, et al. Photoluminescent hyperbranched poly(amido amine) containing β-cyclodextrin as a nonviral gene delivery vector. Bioconjugate Chem. 22 (2011) 1162-1170.

[168] Y. H. Tian, C. Deng, H. Lin, J. Sun, M. Deng, X. Chen, et al. Biodegradable cationic PEG–PEI–PBLG hyperbranched block copolymer: synthesis and micelle characterization. Biomaterials 26 (2005) 4209-4217.

[169] S. Muthukrishnan, H. Mori, E. H. Muller, Synthesis and characterization of methacrylate-type hyperbranched glycopolymers via self-condensing atom transfer radical copolymerization of sugar carrying acrylate. Macromolecules 38 (2005) 3108-3119.

[170] M. Luzon, C. Boyer, C. Peinado, T. Corrales, M. Whittaker, L. Tao, et al. Water-soluble, thermoresponsive, hyperbranched copolymers based on PEG-methacrylates: synthesis, characterization, and LCST behavior. J. Poly. Sci. Part A: Polym. Chem. 48 (2010) 2783-2792.

[171] S. Muthukrishnan, P. Dominik, H.M. Erhard, A. H. E. Müller Synthesis and characterization of surface-grafted hyperbranched glycomethacrylates. Macromolecules 39 (2006) 2743-2750.

[172] P. Bettinger, J-S. Remy, P. Erbacher, Size reduction of galactosylated PEI/DNA complexes improves lectin-mediated gene transfer into hepatocytes. Bioconjugate Chem. 10 (1999) 558-561.

[173] Q-X. Zhang, L-X. Wang, C-P. Zhang, L-Z. Liu, X-R. Zhou, Q-H. Mao, et al. Galactosylated ternary/DNA polyphosphoramidate nanoparticles mediate high gene transfection efficiency in hepatocytes. J. Control. Release 102 (2005) 749-763.

[174] K. Morimoto, M. Nishikawa, S. Kawakami, T. Nakano, Y. Hattori, S. Fumoto, et al. Molecular weight-dependent gene transfection activity of unmodified and galactosylated polyethyleneimine on hepatoma cells and mouse liver. Mol. Ther. 7 (2003) 254-261.

[175] J. Kuiper, F. H. Bakkeren, L. A. E. Biessen, C. J. T. Van Berkel, Characterization of the interaction of galactose exposing particles with rat kupffer cells. J. Biochem. 299 (1994) 285-290.

[176] Z. Qin, W. Liu, L. Li, L. Guo, C. Yao, X. Li, Galactosylated N-2-hydroxypropyl methacrylamid-b-N-3-guanidinopropyl methacrylamide block copolymers as hepatocytes-targeting gene carriers. Bioconjugate Chem. 22 (2011) 1503-1512.

[177] F. Stripe, Ribosome-inactivating proteins. Toxicon 44 (2004) 371-383.

[178] S. S. Komath, K. Bhanu, G. B. Maiya, J. M. Swamy, Binding of porphyrins by the tumor-specific lectin, jacalin [jack fruit (artocarpus integrifolia) agglutinin] Biosci. Rep. 20 (2000) 265-276.

[179] N. P. Ingle, B. Malone, T. M. Reineke, Poly(glycoamidoamine)s: a broad class of carbohydrate-containing polycations for nucleic acid delivery. Trends Biotechnol. 29 (2011) 443-453.

[180] A. P. Marques, R. L. Reis, J. A. Hunt, The biocompatibility of novel starch-based polymers and composites: in vitro studies. Biomaterials 23 (2002) 1471-1478.

[181] W. K. J. Lam, Y. Ma, S. P. Armes, L. A. Lewis, T. Baldwin, S. Stolnik, Phosphorylcholine–polycation diblock copolymers as synthetic vectors for gene delivery. J. Control. Release 100 (2004) 293-312.

[182] G. Grandinetti, A. E. Smith, T. M. Reineke, Membrane and nuclear permeabilization by polymeric pDNA vehicles: Efficient method for gene delivery or mechanism of cytotoxicity? Mol. Pharmaceutics 9 (2012) 523-538.

[183] K. Kizjakina, J. M. Bryson, G. Grandinetti, T. M. Reineke, Cationic glycopolymers for the delivery of pDNA to human dermal fibroblasts and rat mesenchymal stem cells. Biomaterials 33 (2012) 1851-1862.

[184] M. Abbasi, H. Uludag, V. Incani, C. Olson, X. Lin, B. A. Clements, et al. Palmitic acid modified poly-L-lysine for non-viral delivery of plasmid DNA to skin fibroblasts. Biomacromolecules 8 (2007) 1059-1063.

7.2 Appendix
Supporting Information

Supplementary Materials

"The Effect of Polymer Architecture, Composition, and Molecular Weight on the Properties of Glycopolymer-based Non-Viral Gene Delivery Systems"

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Table S1. N/P ratio of polyplexes used for transfection purposes.

Polymer Samples	+/- ratio used form Transfection Purposes
$P(APMA)_{18}$	40
$P(APMA)_{47}$	63
$P(AEMA)_{54}$	60.3
$P(APMA_{13}$-*st*-$GAPMA_{22})$	40
$P(APMA_{34}$-*st*-$GAPMA_{25})$	45
$P(APMA_{37}$-*st*-$GAPMA_{54})$	49
$P(APMA_{34}$-*st*-$GAPMA_{73})$	45
$P(AEMA_{40}$-*st*-$GAPMA_{36})$	45
$P(APMA_{18}$-*b*-$GAPMA_{22})$	40
$P(APMA_{18}$-*b*-$GAPMA_{37})$	40
$P(APMA_{47}$-*b*-$GAPMA_{67})$	50
$P(APMA_{47}$-*b*-$GAPMA_{55})$	63
$P(APMA_{57}$-*b*-$GAPMA_{54})$	39
$P(AEMA_{52}$-*b*-$GAPMA_{49})$	58

Table S2. DLS data indicating the effect of serum on polyplexes size as a function of time.

Time	Block- polymer P($AEMA_{52}$-*b*-$GAPMA_{49}$) DLS (nm)	Statistical polymer P($AEMA_{40}$-*st*-$GAPMA_{36}$) DLS (nm)
0 min	78.3±0.305	269.9±0.09
30 min	218±0.38	279±0.4
90 min	164±0.373	230±0.358
210 min	300±0.257	950±0.247

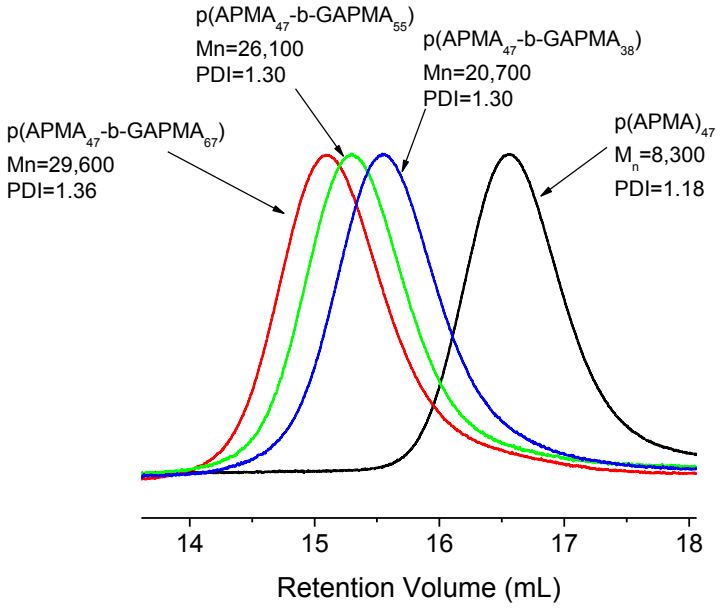

Figure S1. GPC traces showing the homopolymer poly(APMA)$_{47}$ and the resulting diblock copolymers P(APMA$_{47}$-*b*-GAPMA$_{38}$), P(APMA$_{47}$-*b*-GAPMA$_{55}$) & P(APMA$_{47}$-*b*-GAPMA$_{67}$) by RAFT at 70 °C.

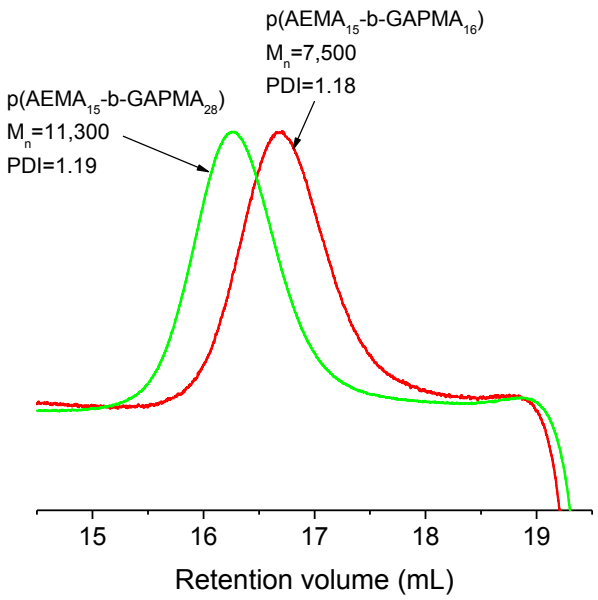

Figure S2. GPC traces showing diblock copolymers P($AEMA_{15}$-*b*-$GAPMA_{16}$), P($AEMA_{15}$-*b*-$GAPMA_{28}$) by RAFT at 70 °C.

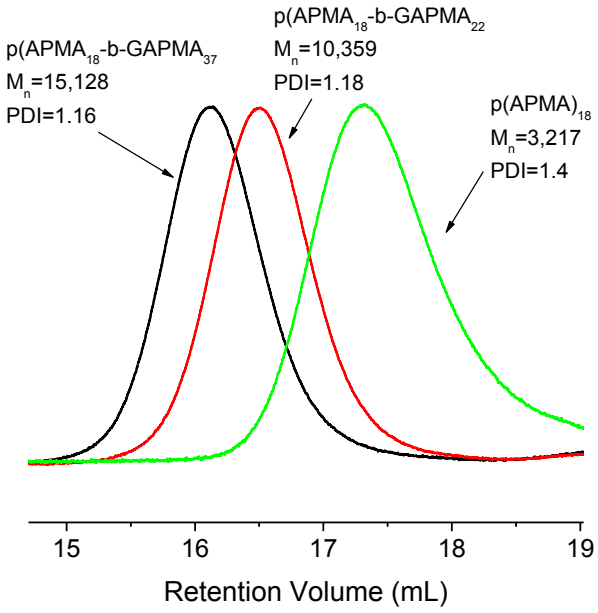

Figure S3. GPC traces showing the homopolymer P(APMA)$_{18}$ and the resulting diblock copolymers P(APMA$_{18}$-*b*-GAPMA$_{22}$), P(APMA$_{18}$-*b*-GAPMA$_{37}$).

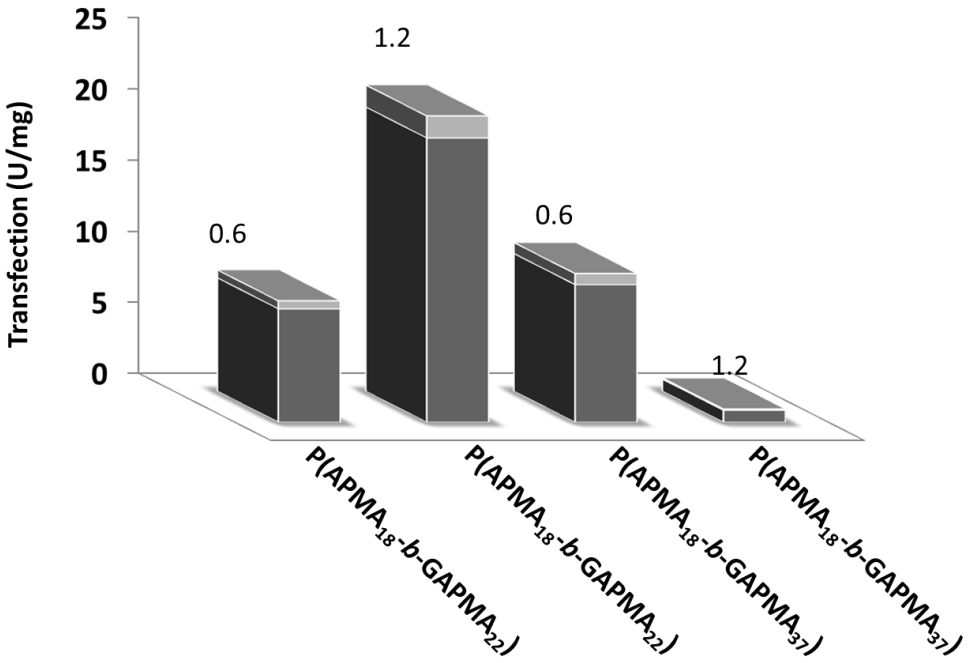

Figure S4. Transfection efficiencies of low DP_n cationic glycopolymers in the absence of serum in Hep G2 cells. Gene expression is evaluated at DNA dose 0.6 and 1.2 µg as shown.

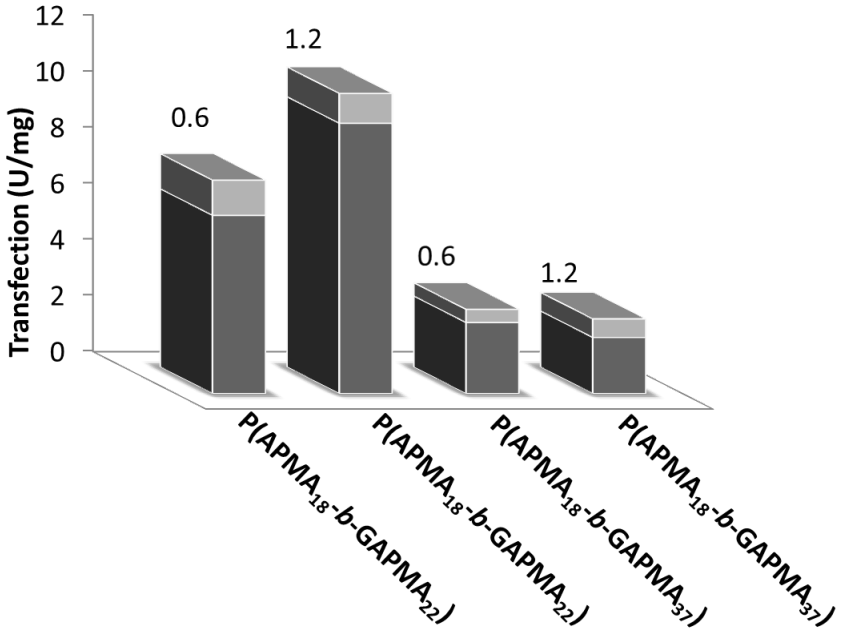

Figure S5. Transfection efficiencies of low DP_n cationic glycopolymers in the presence of serum in Hep G2 cells. The gene expression is evaluated using 0.6 and 1.2 µg of DNA as shown.

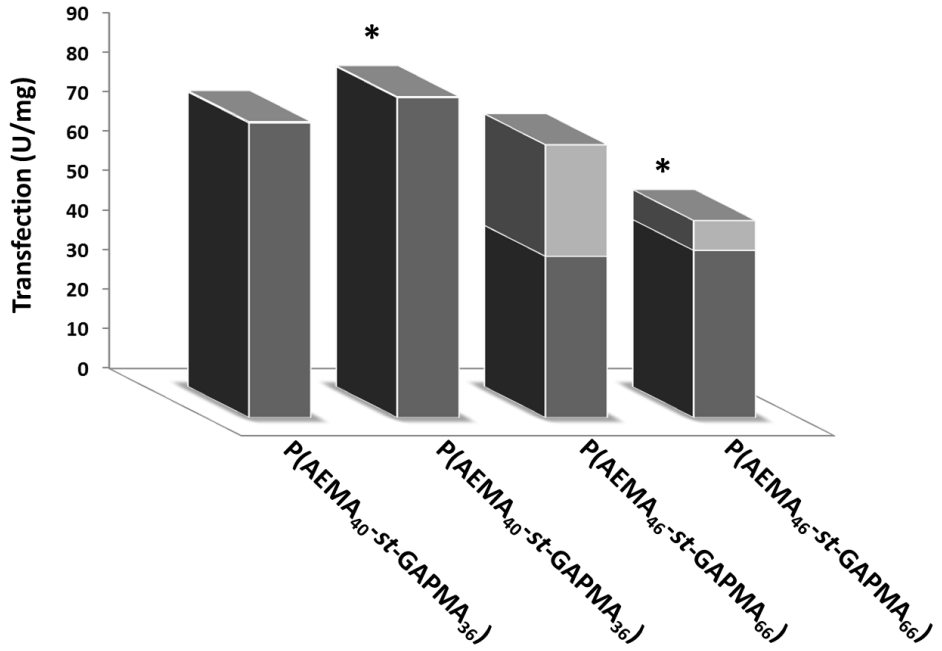

Figure S6. Transfection efficiencies of AEMA based copolymer in the presence (*) or absence of serum at DNA dose 1.2 µg, showing the effect of carbohydrates on transfection ability in Hep G2 cells.

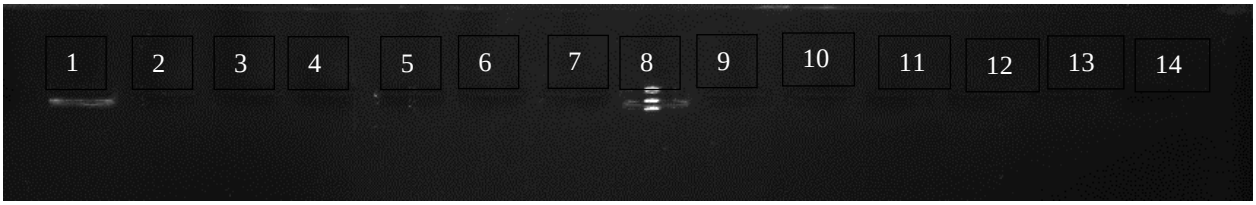

Figure S8. Agarose gel electrophoresis to confirm the presence of polyplexes in pellet. Wells 1-7 indicate polyplexes of P($AEMA_{40}$-*st*-$GAPMA_{36}$) and supernatants after each wash, respectively. Wells 8-14 indicate polplexes of P($AEMA_{52}$-*b*-$GAPMA_{49}$) and supernatants after each wash ,respectively.

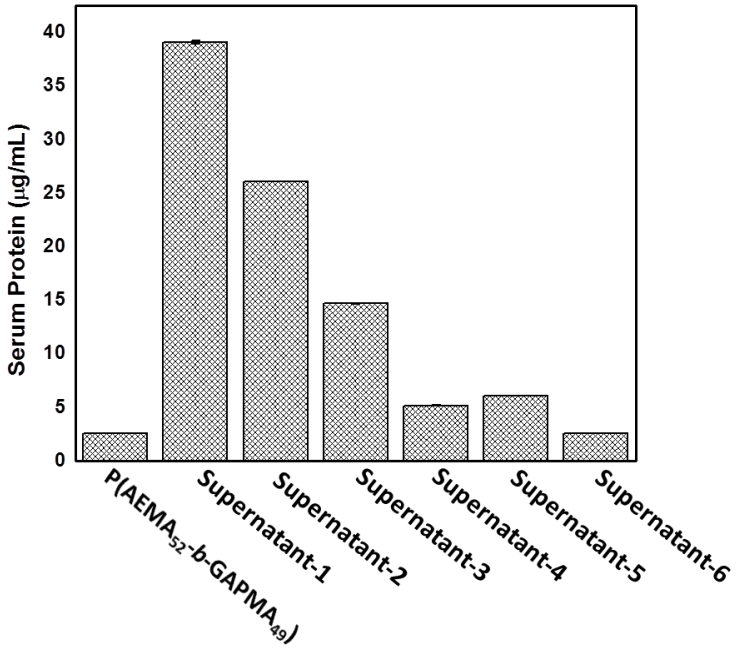

Figure S9. Determination of serum protein (μg/mL) present on the surface of polyplexes formed using P($AEMA_{52}$-*b*-$GAPMA_{49}$) and on each wash after centrifuge. The polyplexes were incubated with serum proteins, the excess serum proteins were removed by centrifuge and the amount of serum proteins on polyplexes and in supernatant was determined by BCA assay.

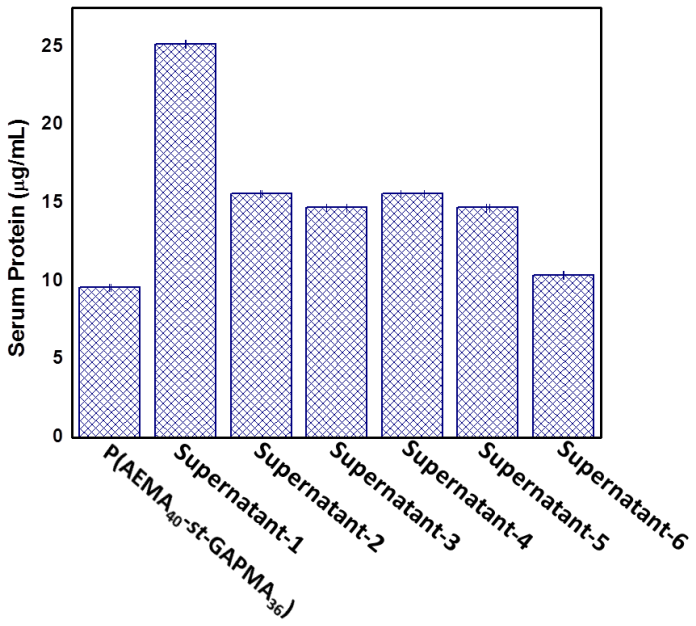

Figure S10. Determination of serum protein (μg/mL) present on the surface of polyplexes formed using P($AEMA_{40}$-*st*-$GAPMA_{36}$) and in each supernatnat after centrifuge. The polyplexes were incubated with serum proteins, the excess serum proteins were removed by centrifuge and the amount of serum proteins on polyplexes and in supernatant was determined by BCA assay.

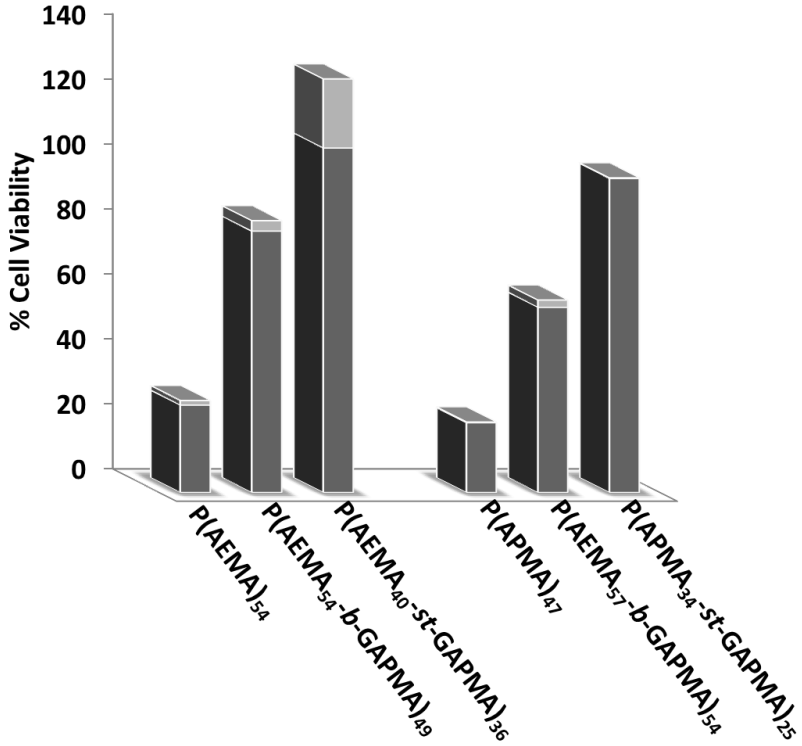

Figure S11. Determination of toxicity after transfection of homo and copolymers using Hep G2 cells**.**

Supporting Information

Well-Controlled Cationic Water-soluble Phospholipid Polymer-DNA Nano-complexes for Gene Delivery

Marya Ahmed[a], Neha Bhuchar[a], Kazuhiko Ishihara[b] and Ravin Narain[a*]
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Table S1. Quantification of serum proteins present in the supernatant and on the surface of polyplexes.

Samples	Relative amount of serum in solution (%)	samples	Relative amount of serum in solution (%)
Supernatant-1	89	Supernatant-1	90
Supernatant-2	3.6	Supernatant-2	4.5
Supernatant-3	3.6	Supernatant-3	1.8
p($AEMA_{26}$-*b*-MPC_{27})polyplexes	3.6	p($AEMA_{19}$-*st*- MPC_{21})-polyplexes	4.5

Table S2. Change in size of polyplexes in the presence of serum proteins as a function of time.

Time (min)	**P($AEMA_{26}$-*b*-MPC_{27}) polyplexes (nm)**	**P($AEMA_{19}$-*st*-MPC_{21}) polyplexes (nm)**
0	78.4 ± 0.3	388 ± 0.25
5	35.6 ± 0.3	148 ± 0.3
30	56.4 ± 0.4	181 ± 0.35
60	75.6 ± 0.4	185 ± 0.3
150	86.1 ± 0.4	184 ± 0.4
240	104 ± 0	185 ± 0.4

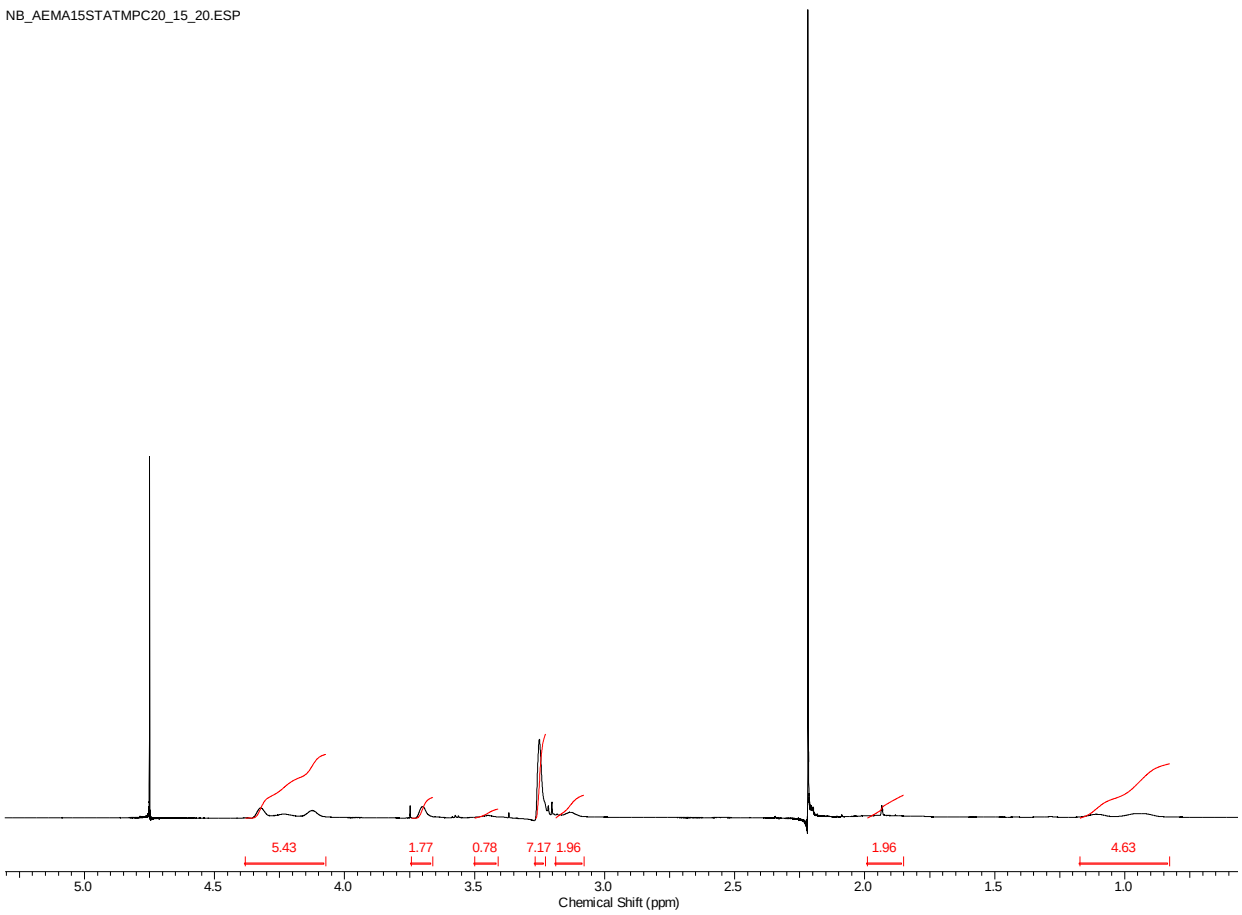

Figure S1: ^{1}H-NMR spectrum of random copolymer P($AEMA_{10}$-*st*-MPC_{10})

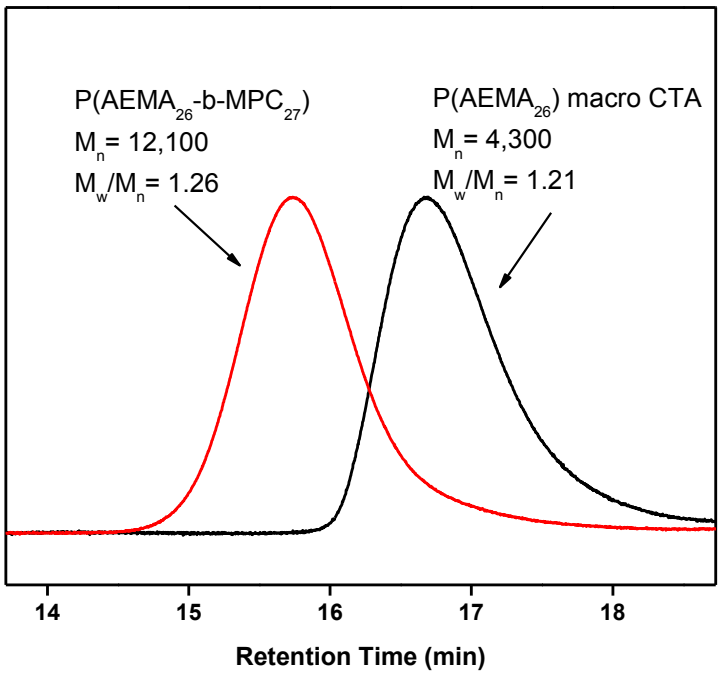

Figure S2. GPC traces showing the homopolymer $P(AEMA)_{26}$ and the resulting diblock polymer $P(AEMA_{26}$-*b*-$MPC_{27})$ by RAFT at 70 °C.

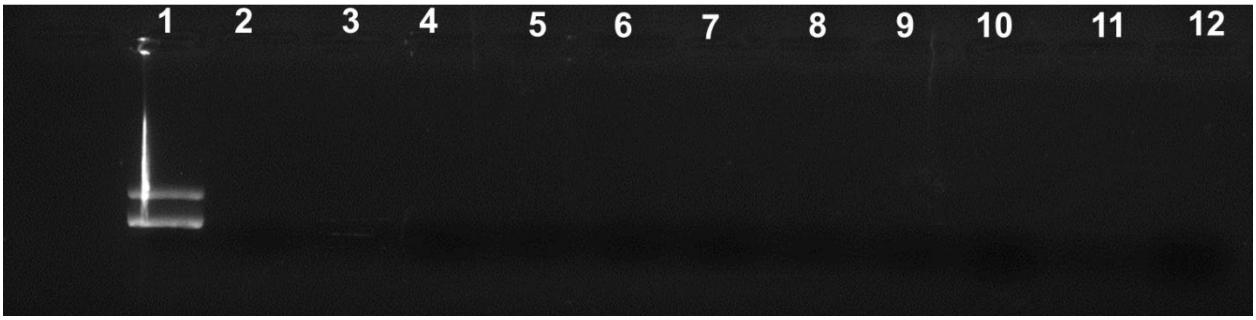

Figure S3. 1) DNA alone 2) $P(AEMA_{25}$-*st*-$MPC_{30})$ polyplexes 3) $P(AEMA_{25}$-*b*-$MPC_{30})$ polyplexes 4) $P(APMA_{15}$-*b*-$MPC_{20})$ polyplexes 5) $P(AEMA_{15}$-*b*-$MPC_{20})$ polyplexes 6) $P(APMA_{25}$-*b*-$MPC_{30})$ polyplexes 7) $P(APMA_{25}$-*st*-$MPC_{30})$ polyplexes 8) $P(APMA_{40}$-*st*-$MPC_{40})$ polyplexes 9)

P($AEMA_{40}$-*st*-MPC_{40}) polyplexes 10) P($APMA_{15}$-*st*-MPC_{20}) polyplexes 11) P(AEMA15-*st*-MPC_{20}) polyplexes 12) P($APMA_{40}$-*b*-MPC_{40}) polyplexes.

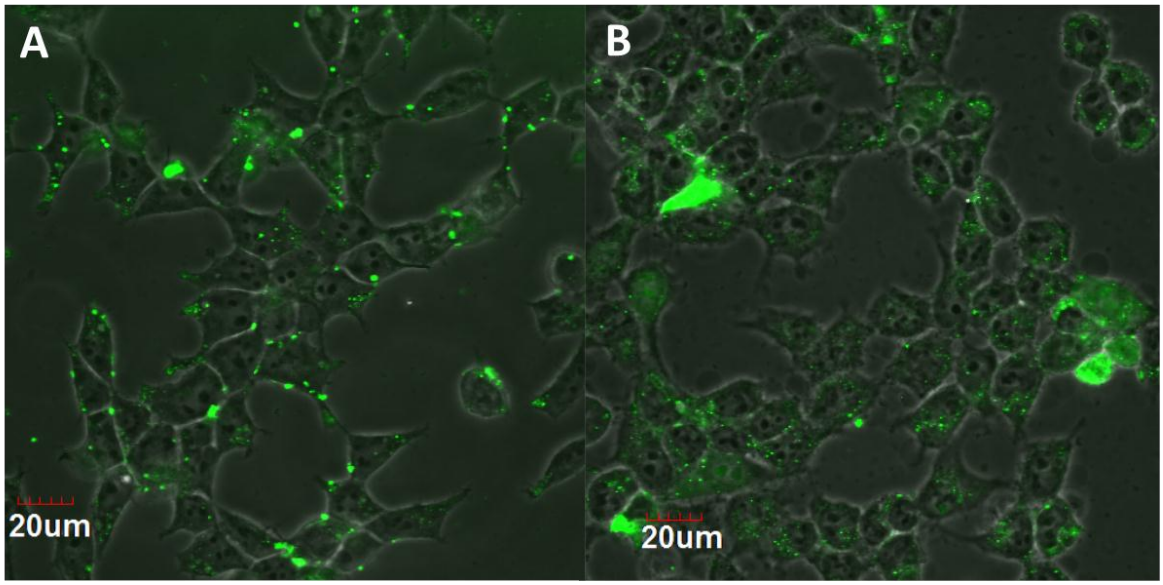

Figure S5. Confocal images showing the uptake of FITC-labelled polyplexes by Hep G2, A) FITC-labelled P($AEMA_{26}$-*b*-$APMA_{27}$)-polyplexes, B) FITC-labelled P($AEMA_{19}$-*st*-MPC_{21}) based polyplexes.

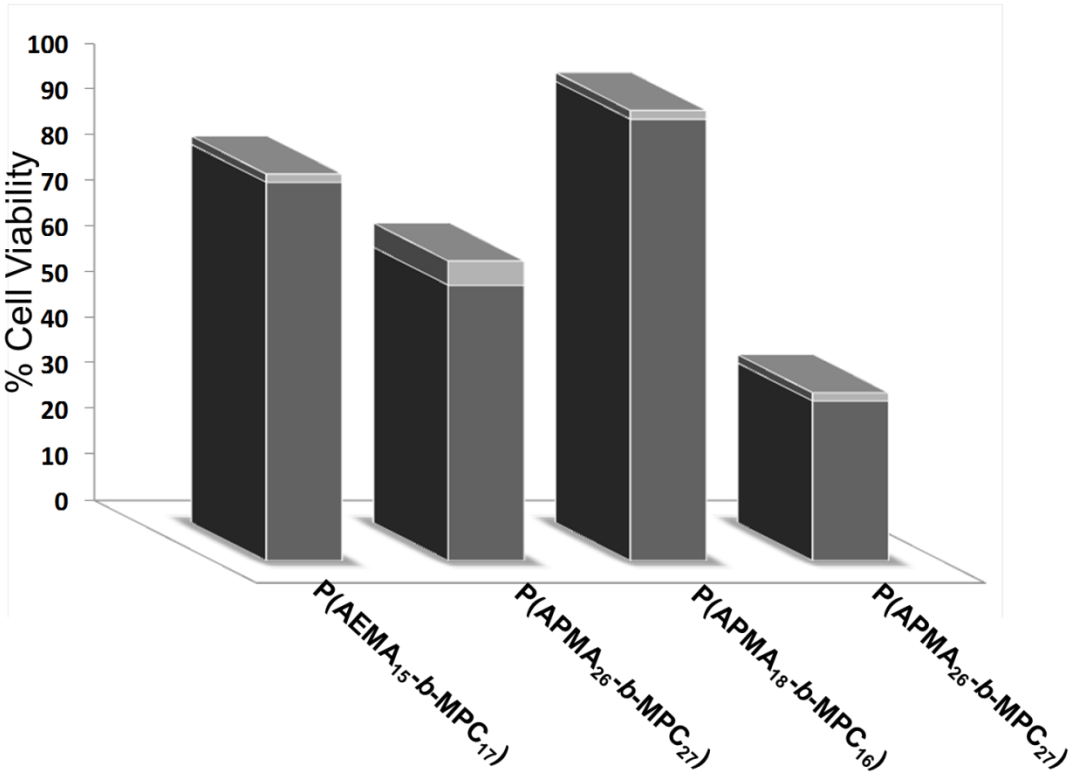

Figure S6. Cell Viability of Hep G2 cells studied post transfection.

Supporting Information

"The Effect of Molecular Weight, Compositions and Lectin Type on the Properties of Hyperbranched Glycopolymers as Non-Viral Gene Delivery Systems"

Marya Ahmed and Ravin Narain*

Department of Chemical and Materials Engineering and Alberta Ingenuity Centre for Carbohydrate Science, University of Alberta, 116 St and 85 Ave, Edmonton, AB, T6G 2G6, Canada

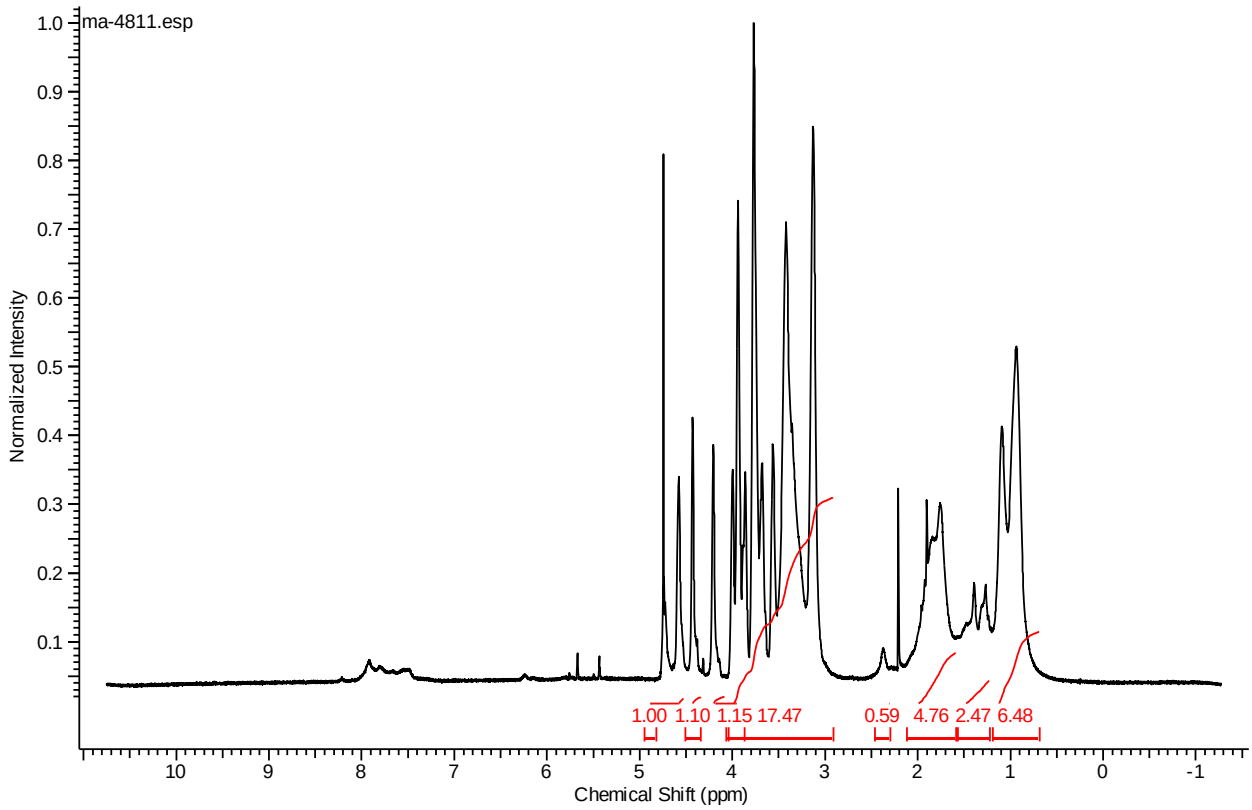

Figure S1. ^{1}H NMR spectrum of hyperbranched glycopolymer P(AEMA$_{84}$-*st*-LAEMA$_{28}$).

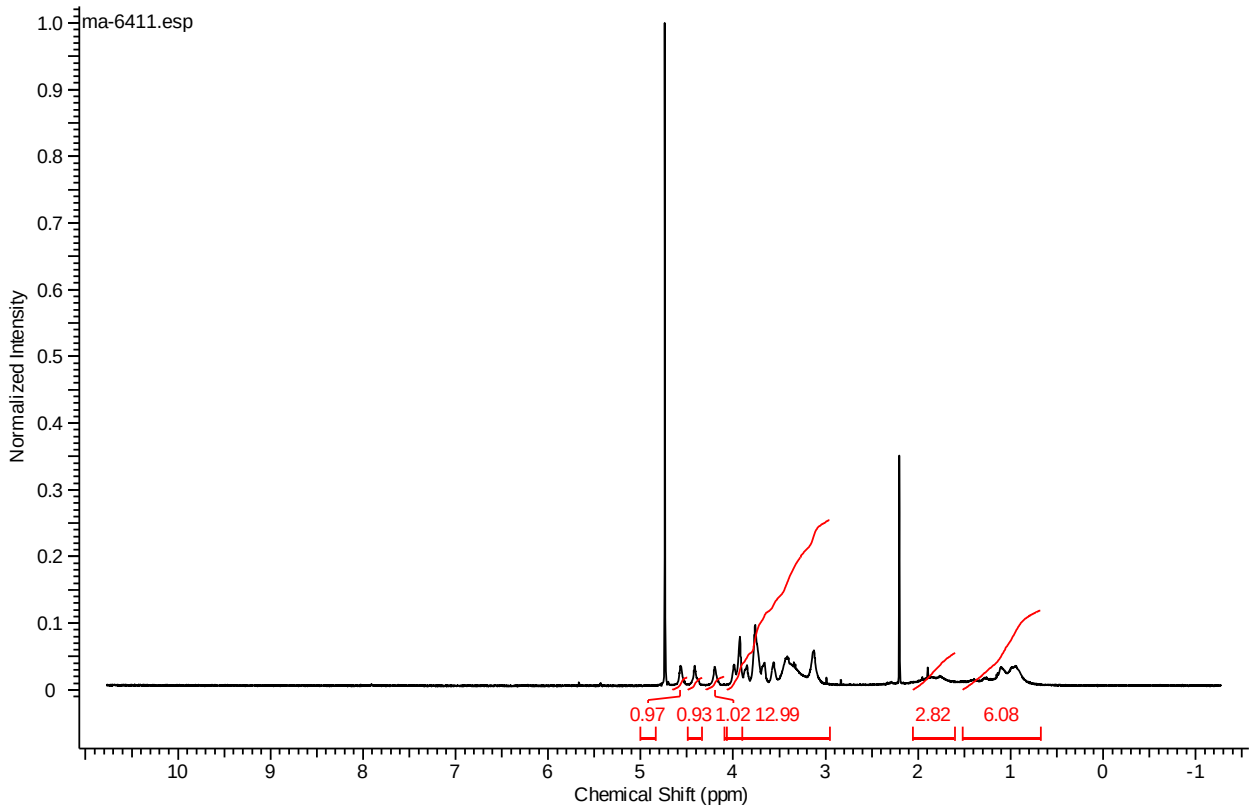

Figure S2. ^{1}H NMR spectrum of hyperbranched glycopolymer P(AEMA$_{111}$-*st*-LAEMA$_{70}$).

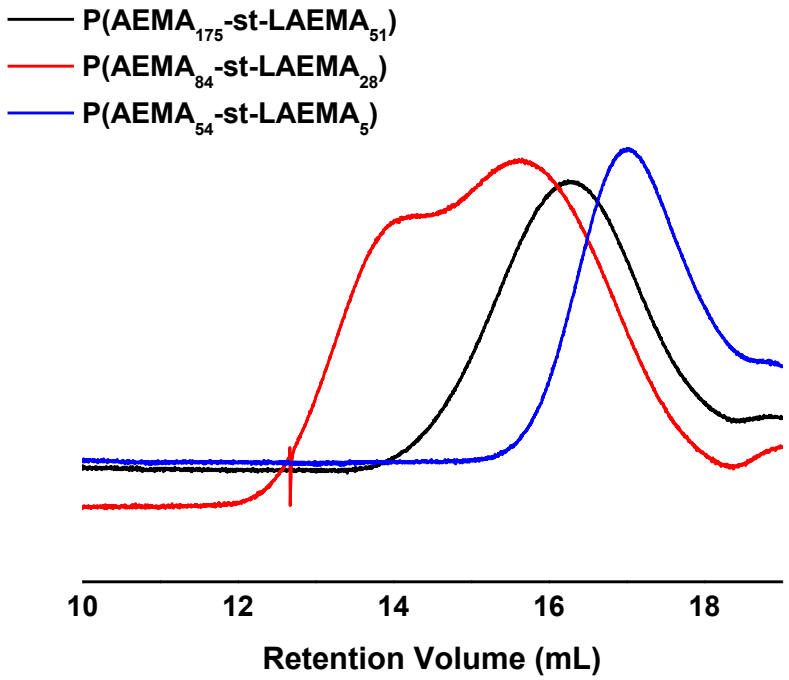

Figure S3. GPC traces showing galactose based hyperbranched polymers of varying molecular weights produced by RAFT at 70 °C.

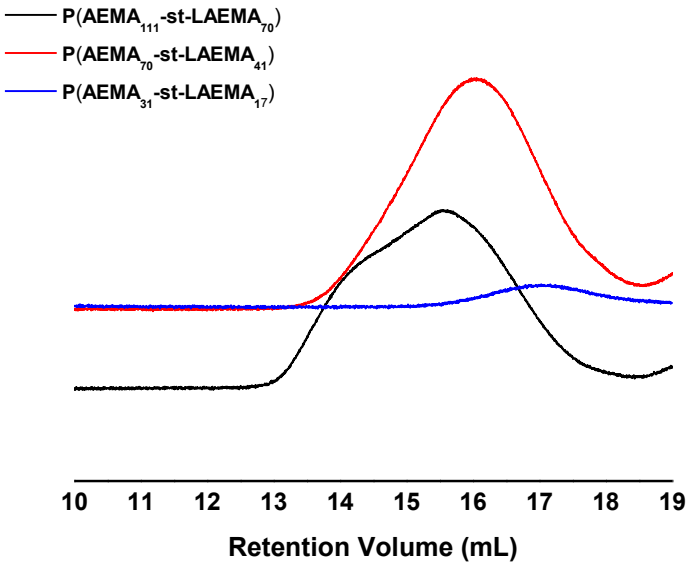

Figure S4. GPC traces showing galactose based hyperbranched polymers of varying molecular weights produced by RAFT at 70 °C.

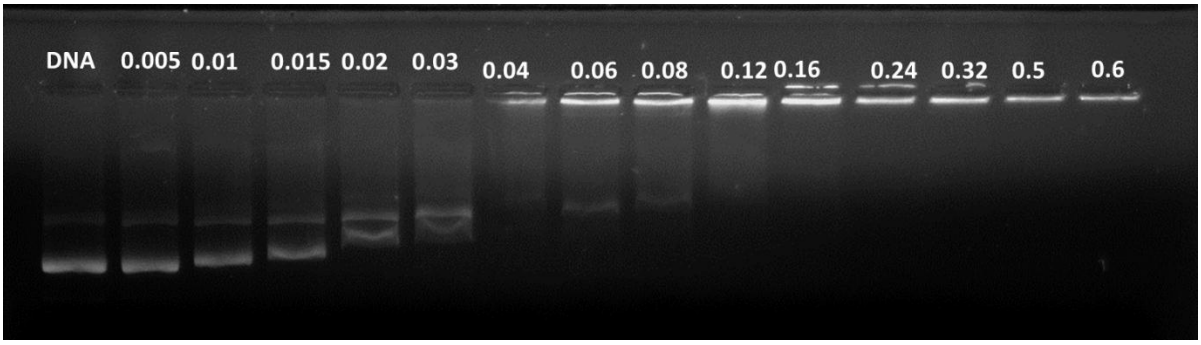

Figure S5. DNA binding affinities of P($AEMA_{11}$-*st*-$GAPMA_{17}$) at varying polymer/plasmid ratios (as shown above), as determined by agarose gel electrophoresis.

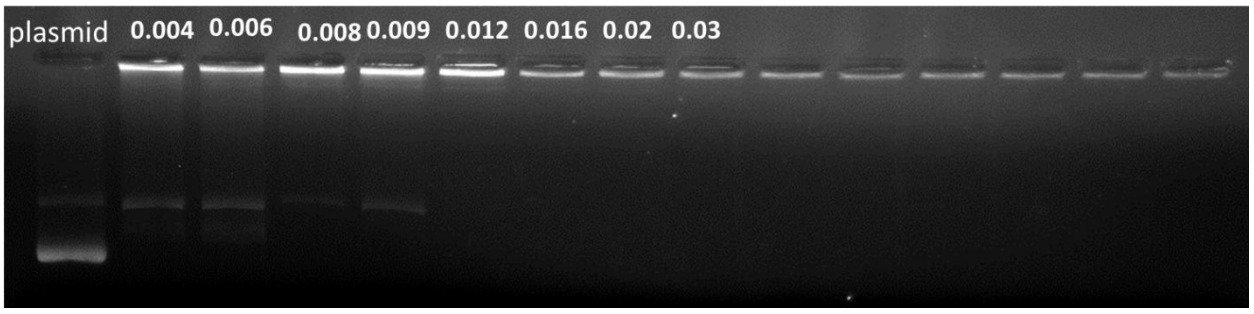

Figure S6. DNA binding affinities of P($AEMA_{84}$-*st*-$LAEMA_{28}$), as determined by agarose gel electrophoresis.

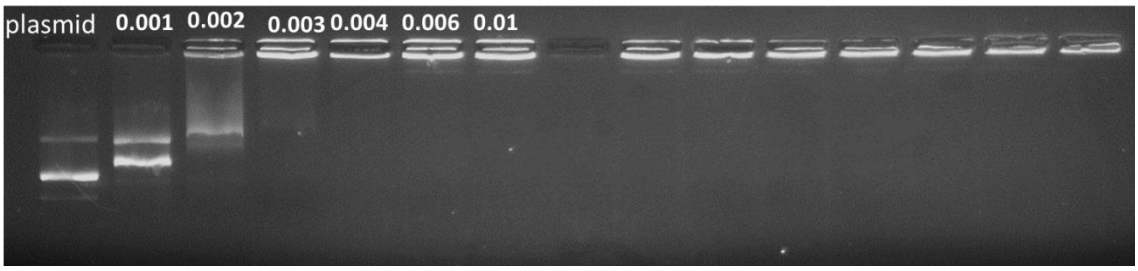

Figure S7. DNA binding affinities of P($AEMA_{70}$-*st*-$LAEMA_{41}$), as determined by agarose gel electrophoresis.

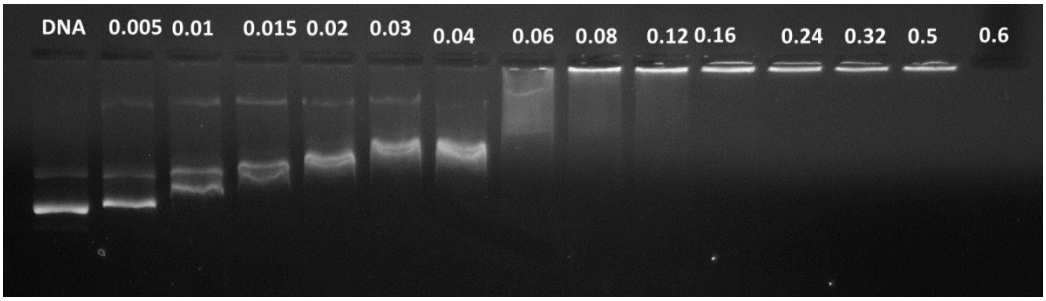

Figure S8. DNA binding affinities of P($AEMA_{18}$-*st*-$LAEMA_{6}$), as determined by agarose gel electrophoresis.

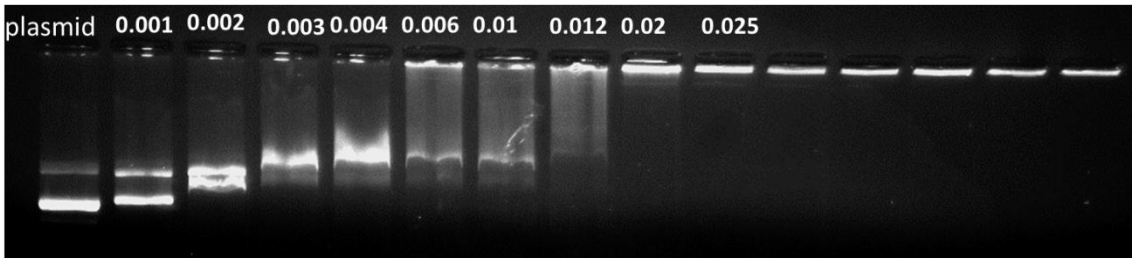

Figure S9. DNA binding affinities of P($AEMA_{54}$-*st*-$LAEMA_{5}$), as determined by agarose gel electrophoresis.

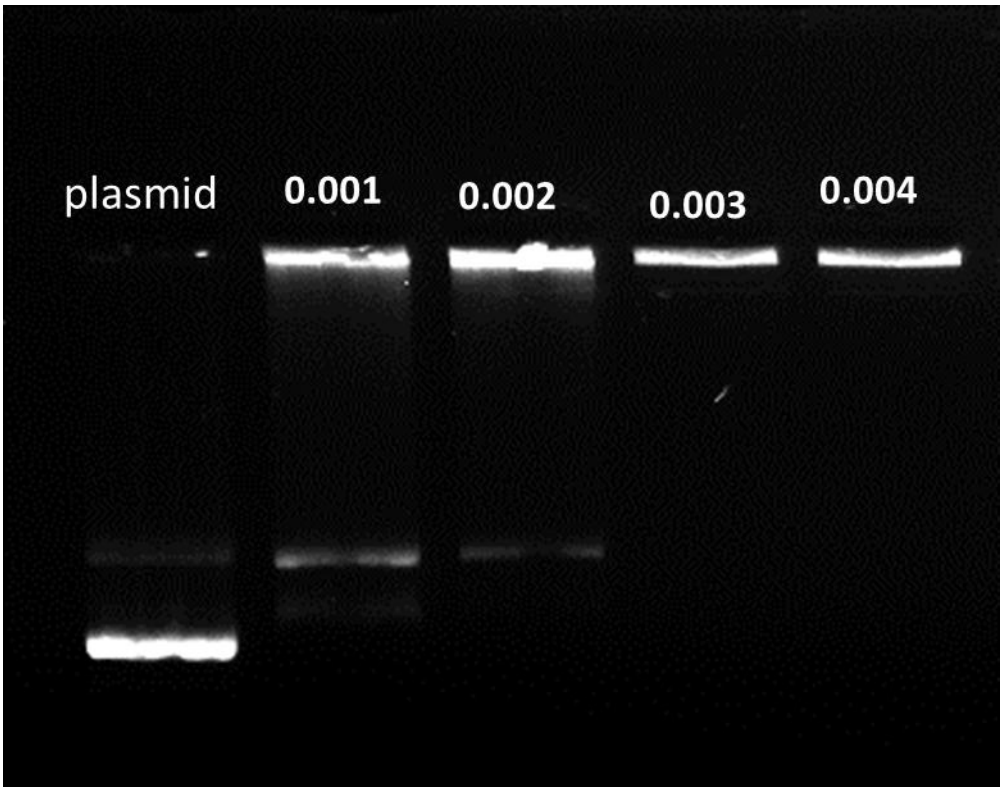

Figure S10. DNA binding affinities of P($AEMA_{42}$-*st*-$GAPMA_{25}$), as determined by agarose gel electrophoresis.

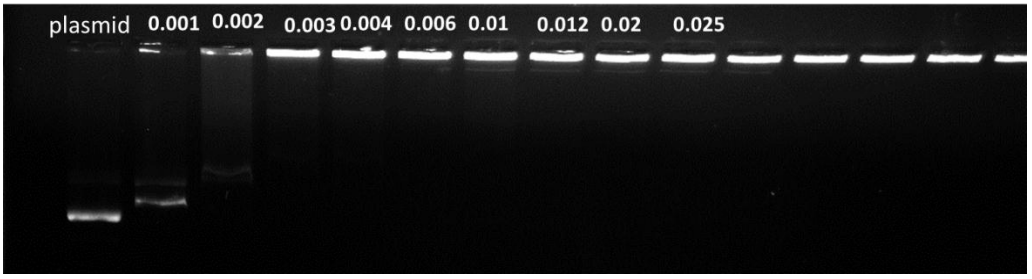

Figure S11. DNA binding affinities of P($AEMA_{31}$-*st*-$LAEMA_{17}$), as determined by agarose gel electrophoresis.

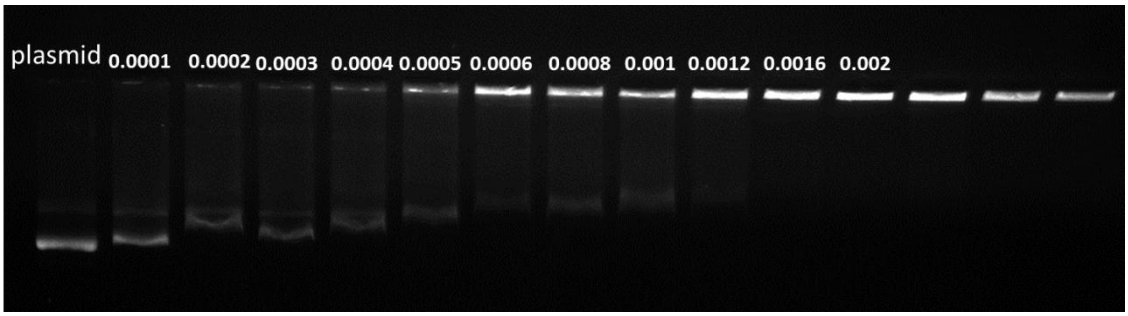

Figure S12. DNA binding affinities of P(AEMA$_{111}$-*st*-LAEMA$_{70}$), as determined by agarose gel electrophoresis.

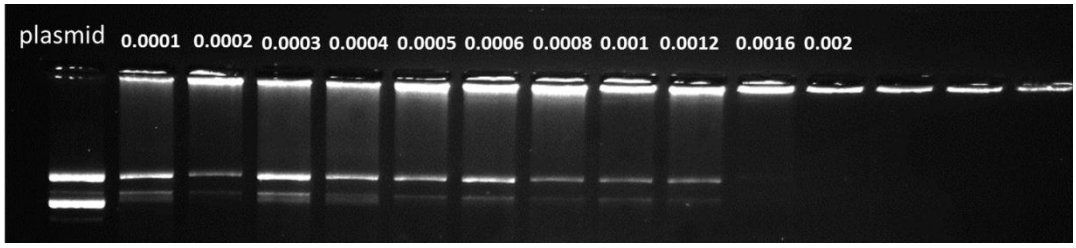

Figure S13. DNA binding affinities of P(AEMA$_{175}$-*st*-LAEMA$_{51}$), as determined by agarose gel electrophoresis

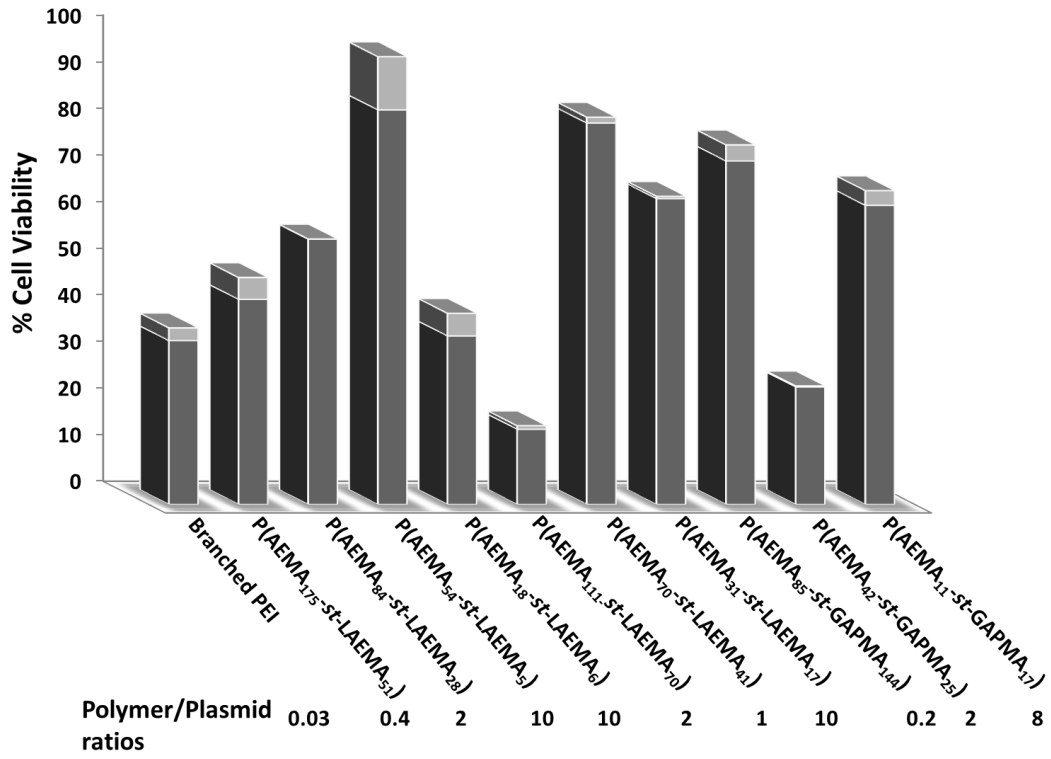

Figure S14. Toxicity post transfection of hyperbranched polyplexes at varying polymer/plasmid ratios as determined by MTT assay.

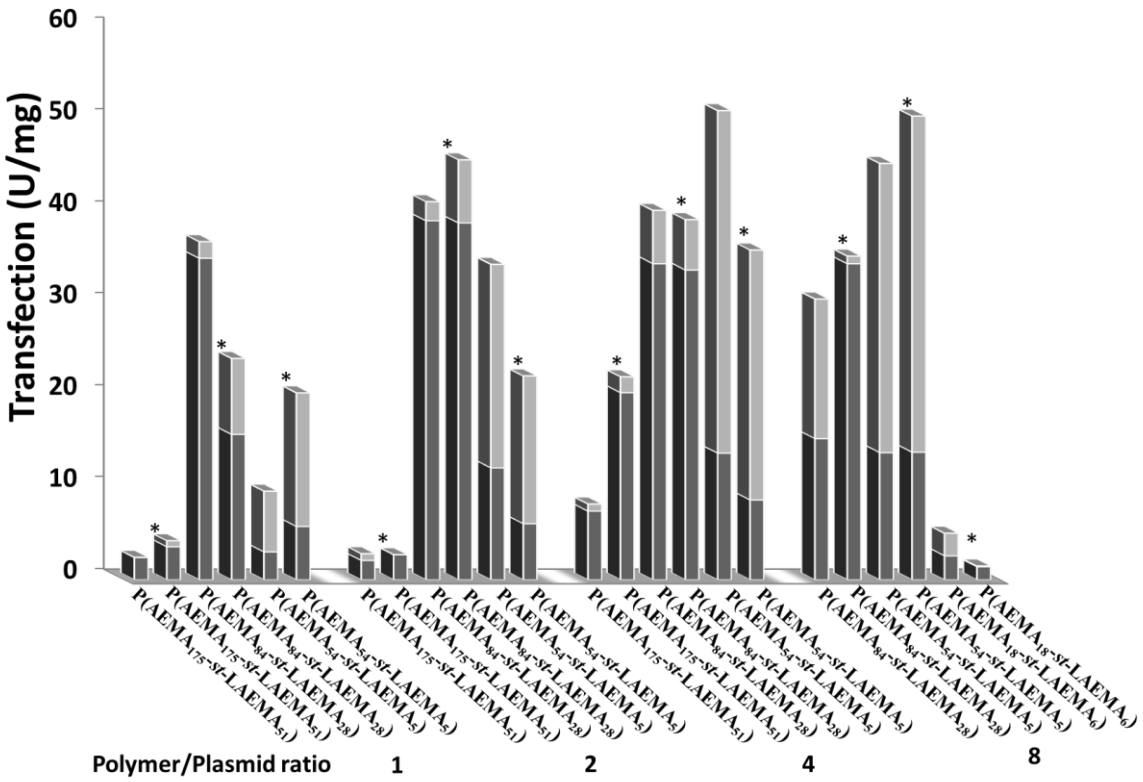

Figure S15. Gene expression of galactose based hyperbranched polymers of varying molecular weight, in the presence (*) and absence of serum using Hep G2 cells. Gene expression is evaluated using *β*-galactosidase assay at DNA dose 0.6 µg and at varying polymer/plasmid ratios as shown. High error bars are obtained for gene expression of low molecular weight copolymers at low polymer/plasmid ratios.

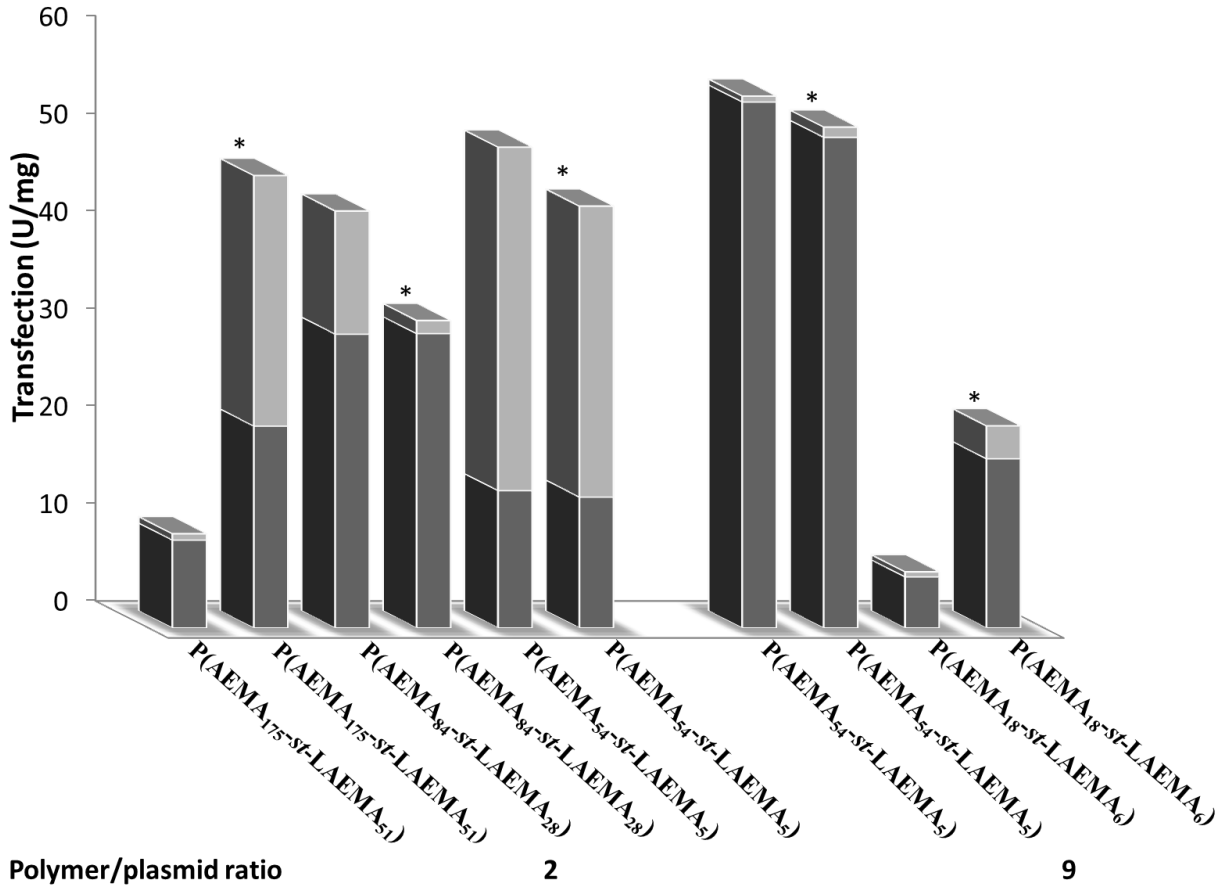

Figure S16. Gene expression of galactose based hyperbranched polymers, in the presence (*) and absence of serum using Hep G2 cells. Gene expression is evaluated using β-galactosidase assay at DNA dose 1.2 µg at varying polymer/plasmid ratios. High error bars are obtained for gene expression of low molecular weight copolymers at low polymer/plasmid ratios.

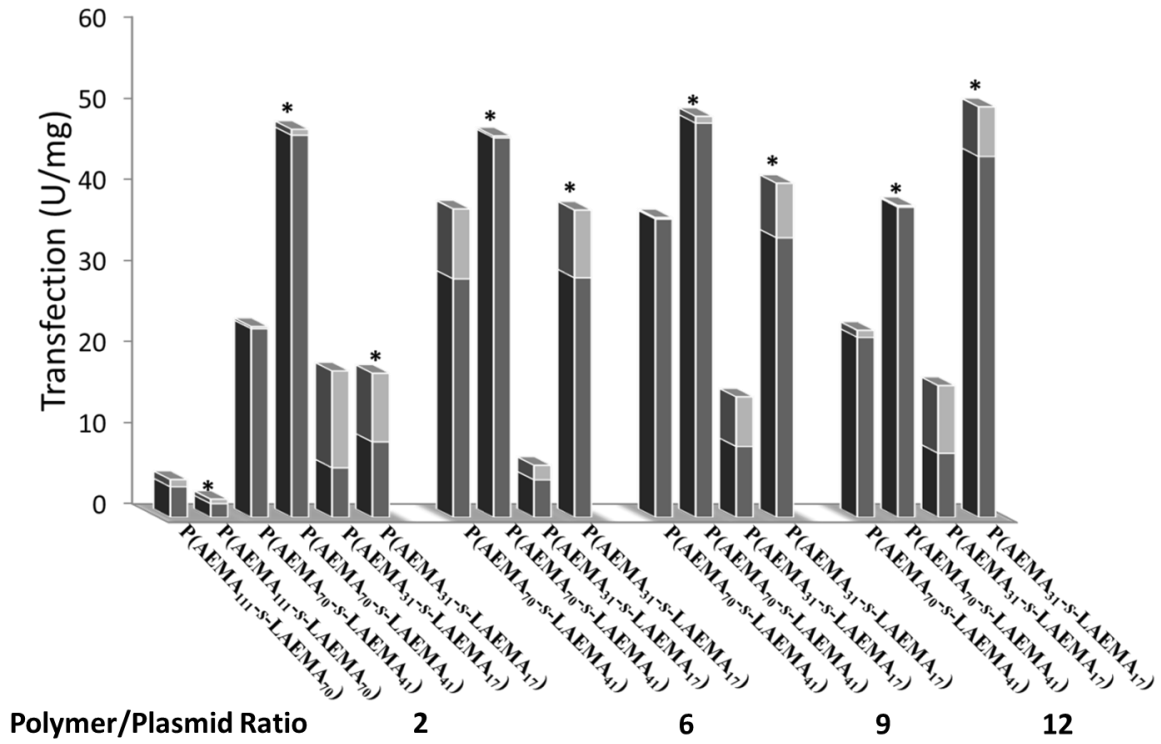

Figure S17. Gene expression of galactose based hyperbranched polymers, in the presence (*) and absence of serum using Hep G2 cells. Gene expression is evaluated using *β*-galactosidase assay at DNA dose 1.2 µg at varying polymer/plasmid ratio.

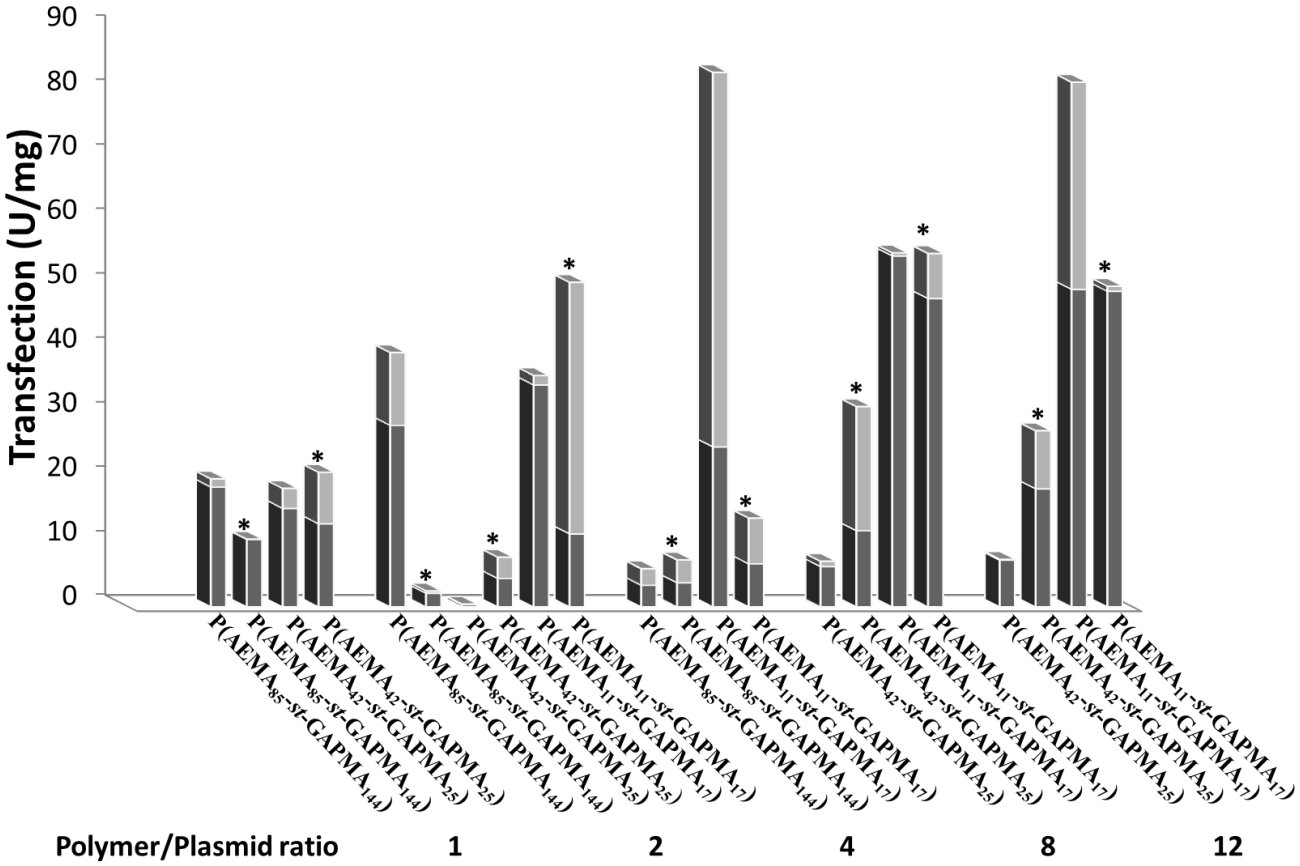

Figure S18. Gene expression of glucose-derived hyperbranched polymers of varying molecular weights, in the presence (*) and absence of serum using Hep G2 cells. Gene expression is evaluated using β-galactosidase assay at DNA dose 1.2 µg. High error bars are obtained for gene expression of low molecular weight copolymers at low polymer/plasmid ratios.

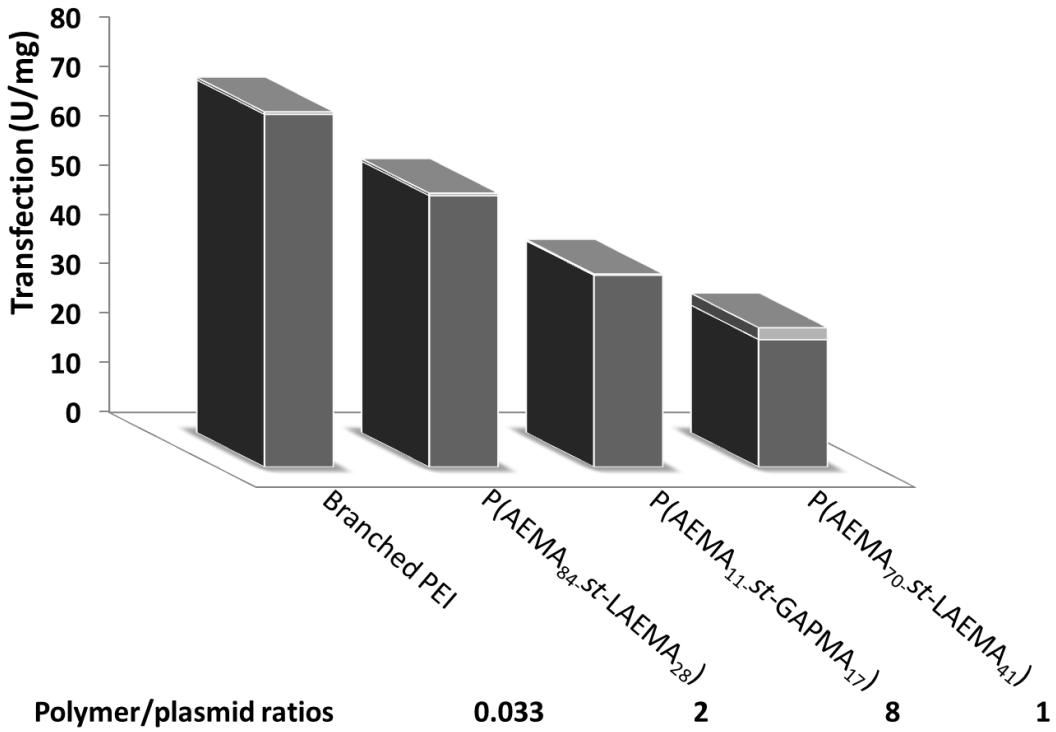

Figure S19. Gene expression selected hyperbranched glycopolymers of varying molecular weights, in the presence of serum in HEK293 cells at different polymer/plasmid ratios. Gene expression is evaluated using β-galactosidase assay at DNA dose of 1.2 µg.

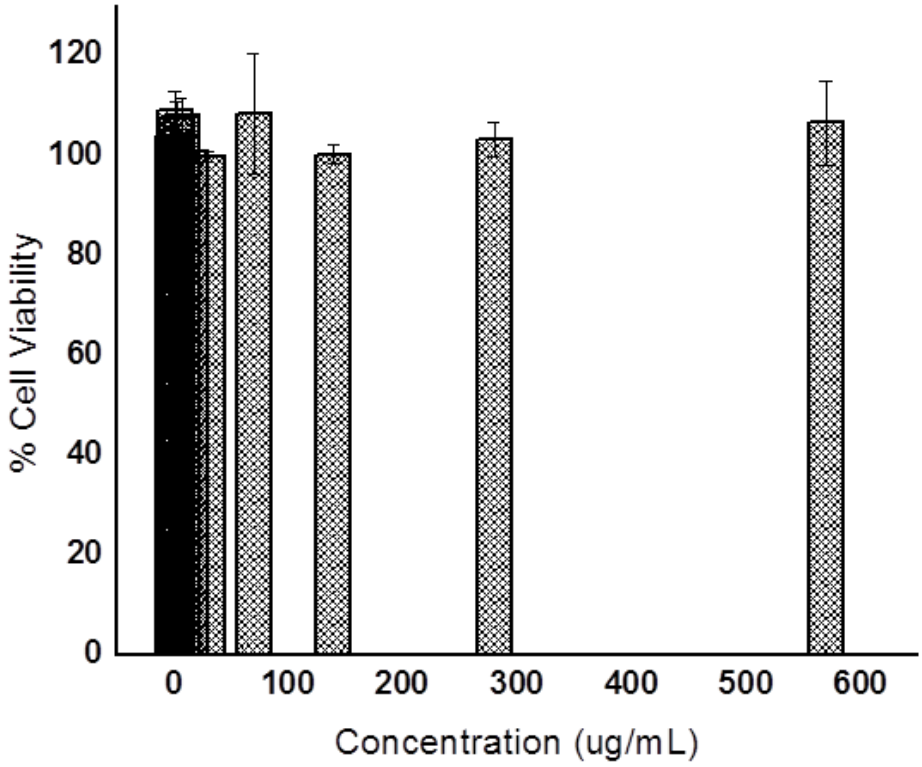

Figure S20. MTT assay on Jacalin using Hep G2 cells.

Table S2. DLS data for the interaction of RCA_{120} with polyplexes at given polymer/plasmid ratios.

Samples	DLS before adding RCA	DLS after adding RCA
PEI	358 ± 0.06	693 ± 0.005
P($AEMA_{11}$-*st*-$GAPMA_{17}$)	268 ± 0.005	878 ± 0.04
P($AEMA_{84}$-*st*-$LAEMA_{28}$)	163 ± 0.36	132 ± 0.26
P($AEMA_{70}$-*st*-$LAEMA_{41}$)	107 ± 0.35	520 ± 0.24

Table S3. BCA results for the interaction of polyplexes with RCA_{120} at given polymer/plasmid ratios

Samples	**Polymer/plasmid Molar ratio**	**Amount of RCA on the surface of polyplexes**
PEI	0.033	72%
P($AEMA_{11}$-*st*-$GAPMA_{17}$)	8	94%
P($AEMA_{84}$-*st*-$LAEMA_{28}$)	2	77%
P($AEMA_{70}$-*st*-$LAEMA_{41}$)	1	100%

Table S4. BCA results for the interaction of Polyplexes with Jacalin at given polymer/plasmid ratios.

Samples	M_n **(kDa)**	**Polymer/Plasmid Molar Ratio**	**Amount of Jacalin on the Surface of Polyplexes**
PEI	10	0.033	91%
P($AEMA_{11}$-*st*-$GAPMA_{17}$)	4.5	8	88%
P($AEMA_{84}$-*st*-$LAEMA_{28}$)	27	2	86%
P($AEMA_{70}$-*st*-$LAEMA_{41}$)	31	1	90%

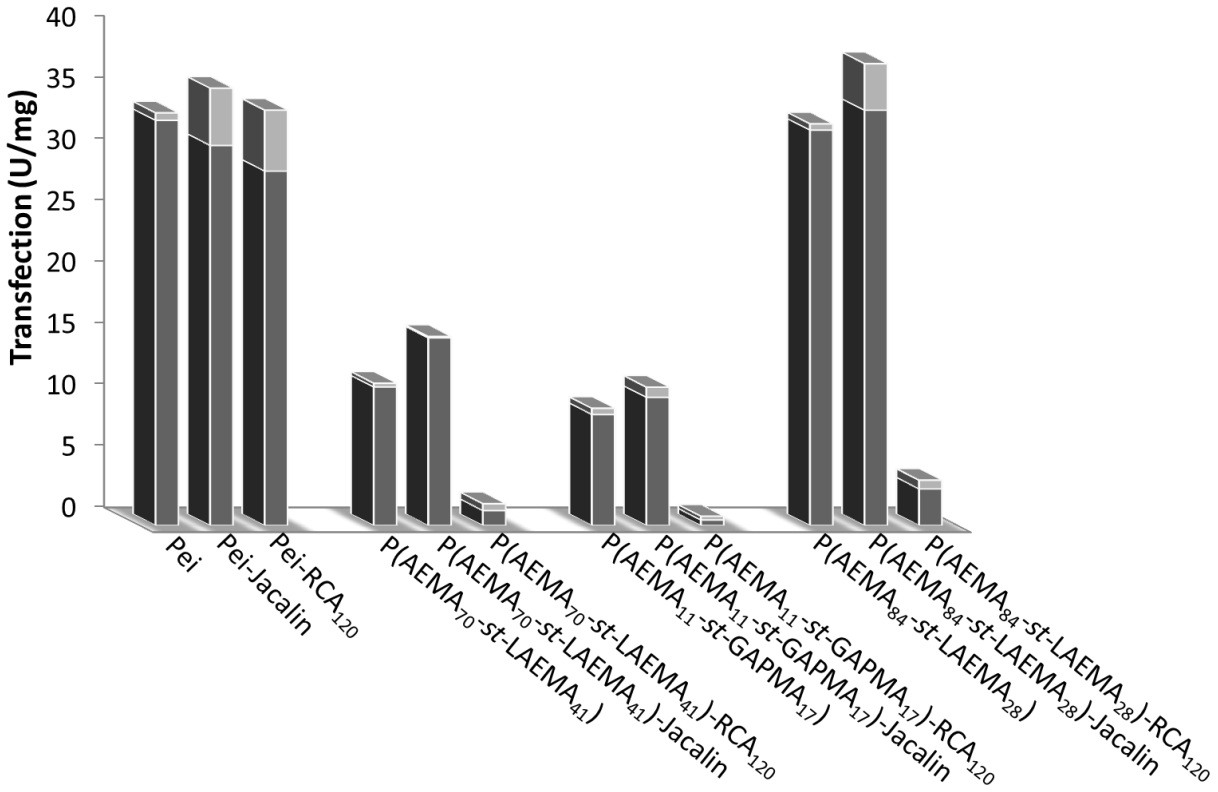

Figure S20. Gene expression selected polyplexes-lectin conjugates in the presence of serum in Hep G2 cells at different polymer/plasmid ratios. Gene expression is evaluated using β-galactosidase assay at DNA dose 1.2 μg. The concentration of RCA_{120} is 0.5 μg/mL.

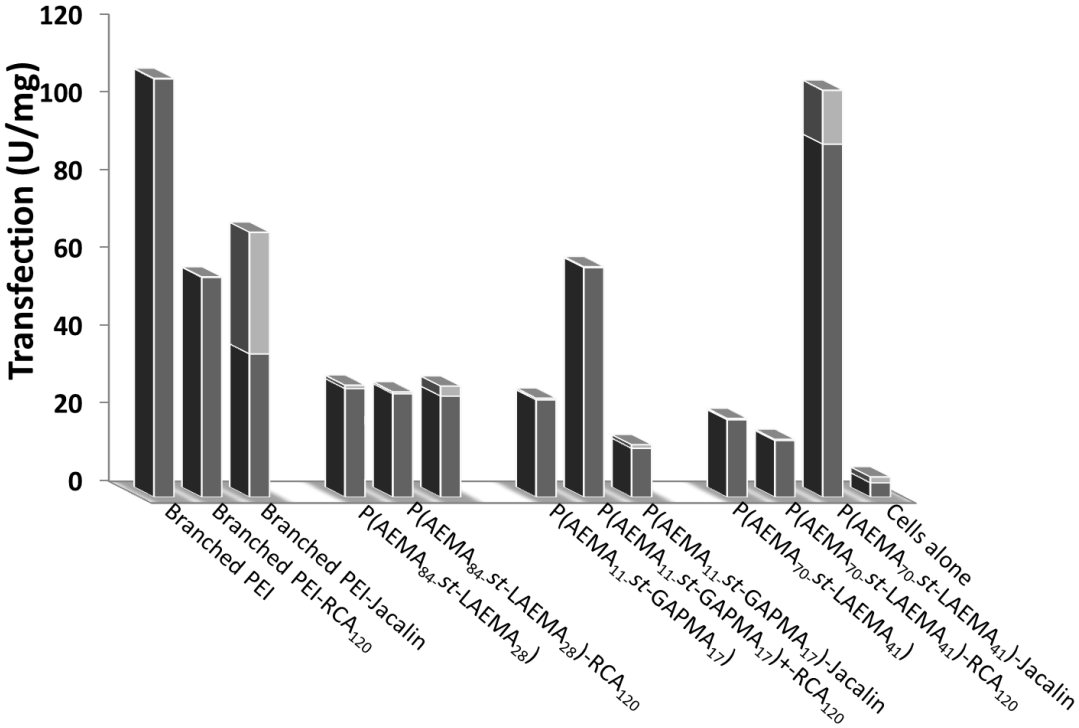

Figure S21. Gene expression selected polyplexes-lectin conjugates in the presence of serum in HEK293 cells at different polymer/plasmid ratios. Gene expression is evaluated using β-galactosidase assay at DNA dose 1.2 µg. The concentration of RCA_{120} is 45 µg/mL.

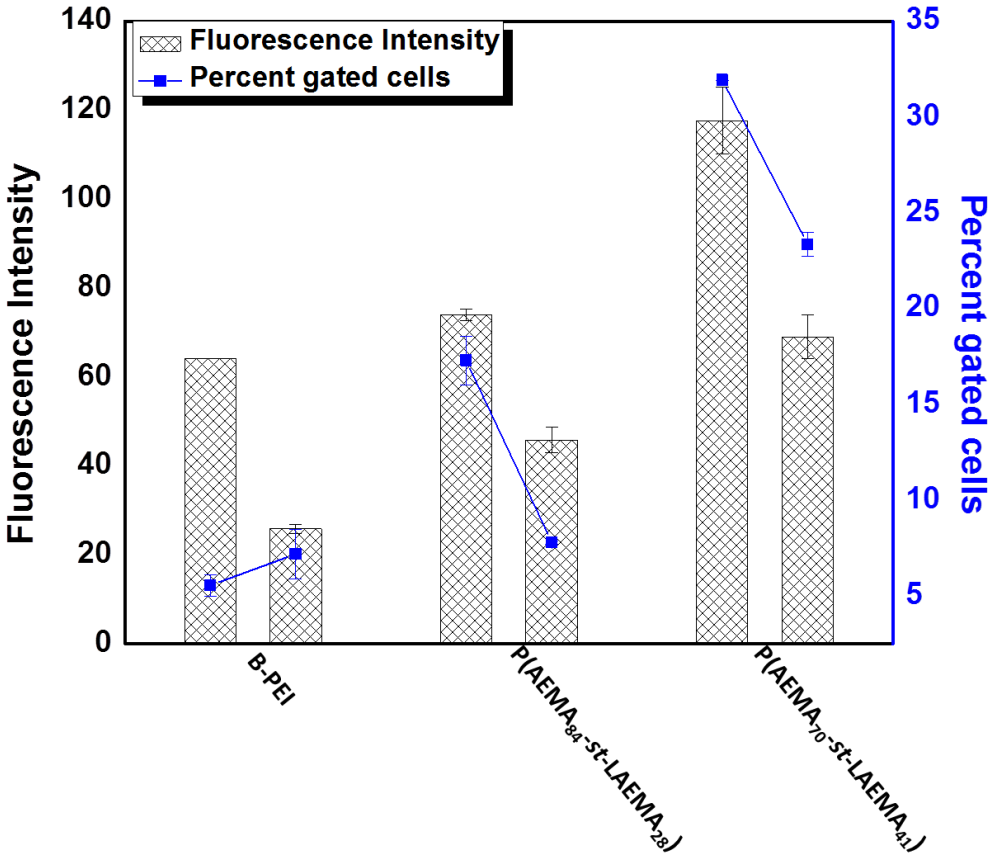

Figure S22. Inhibition of cellular uptake of polyplexes in the presence of free galactose.

Supporting Information

"Impact of the Nature, Size and Chain Topologies of Carbohydrate-Phosphorylcholine Polymeric Gene Delivery Systems"

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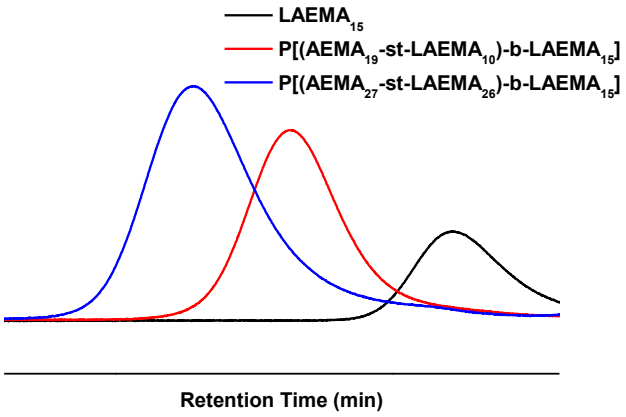

Figure S1: GPC traces, showing blocking of statistical copolymers with LAEMA macroCTA.

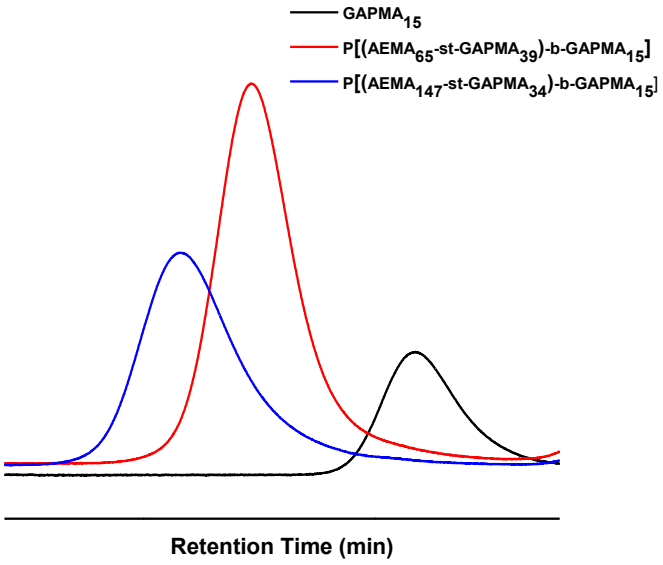

Figure S2: GPC traces, showing blocking of statistical copolymers with GAPMA macroCTA.

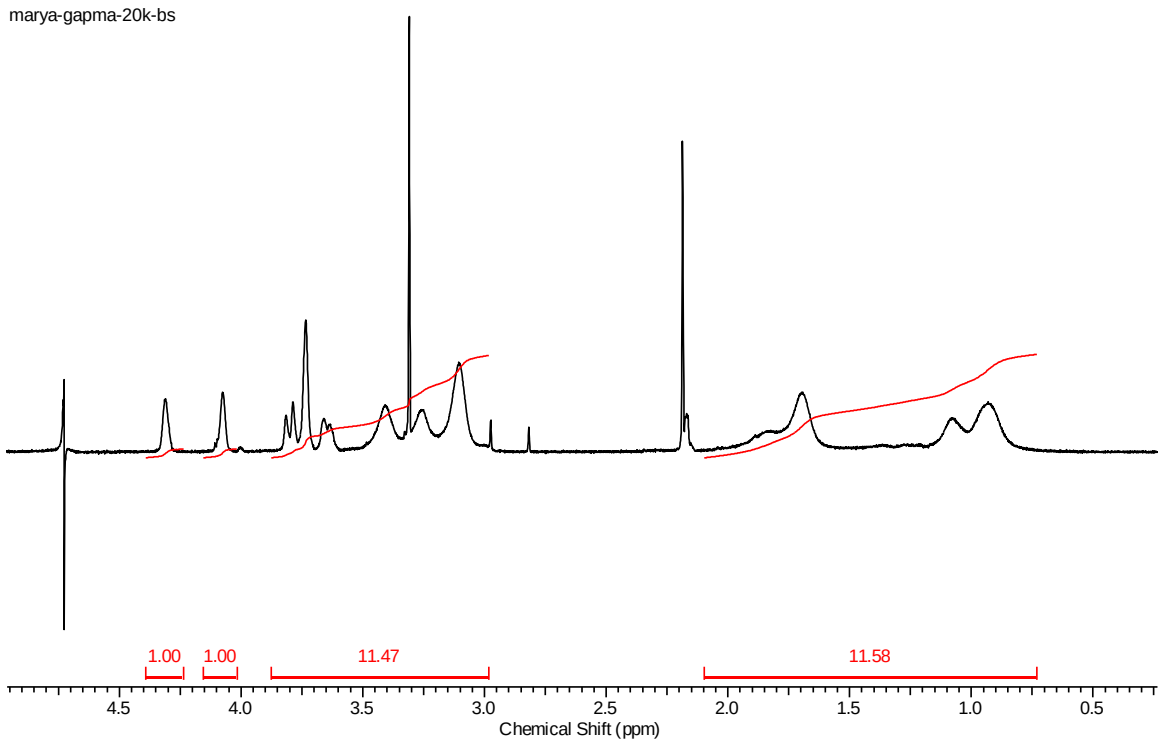

Figure S3: ^{1}H-NMR spectrum of P[(AEMA$_{65}$-*st*-GAPMA$_{39}$)-*b*-GAPMA$_{15}$].

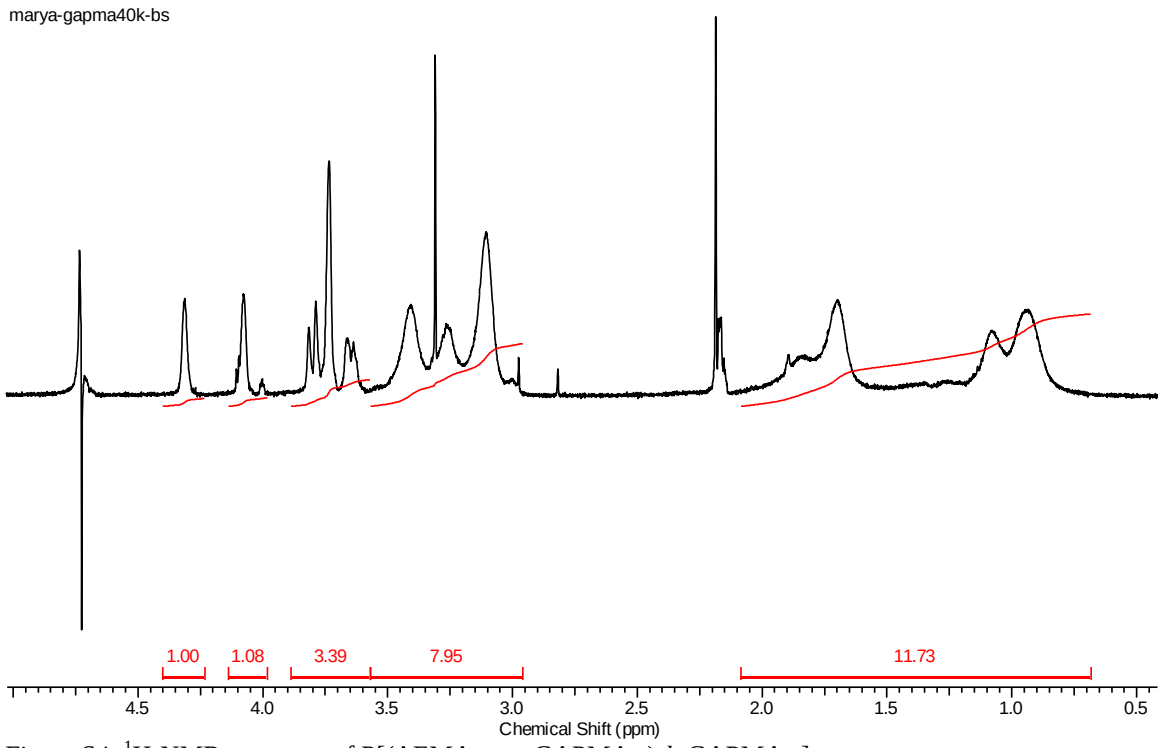

Figure S4: ^{1}H-NMR spectrum of P[(AEMA$_{147}$-*st*-GAPMA$_{34}$)-*b*-GAPMA$_{15}$].

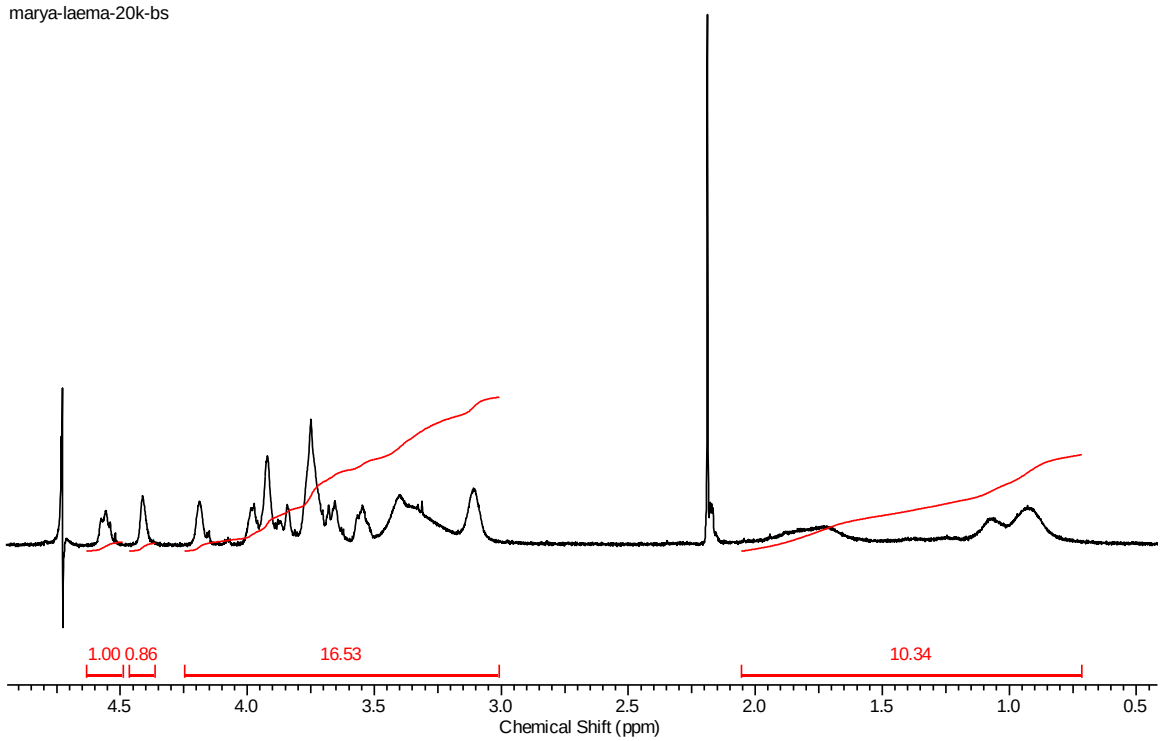

Figure S5: ^{1}H-NMR spectrum of P[(AEMA$_{19}$-*st*-LAEMA$_{10}$)-*b*-LAEMA$_{15}$].

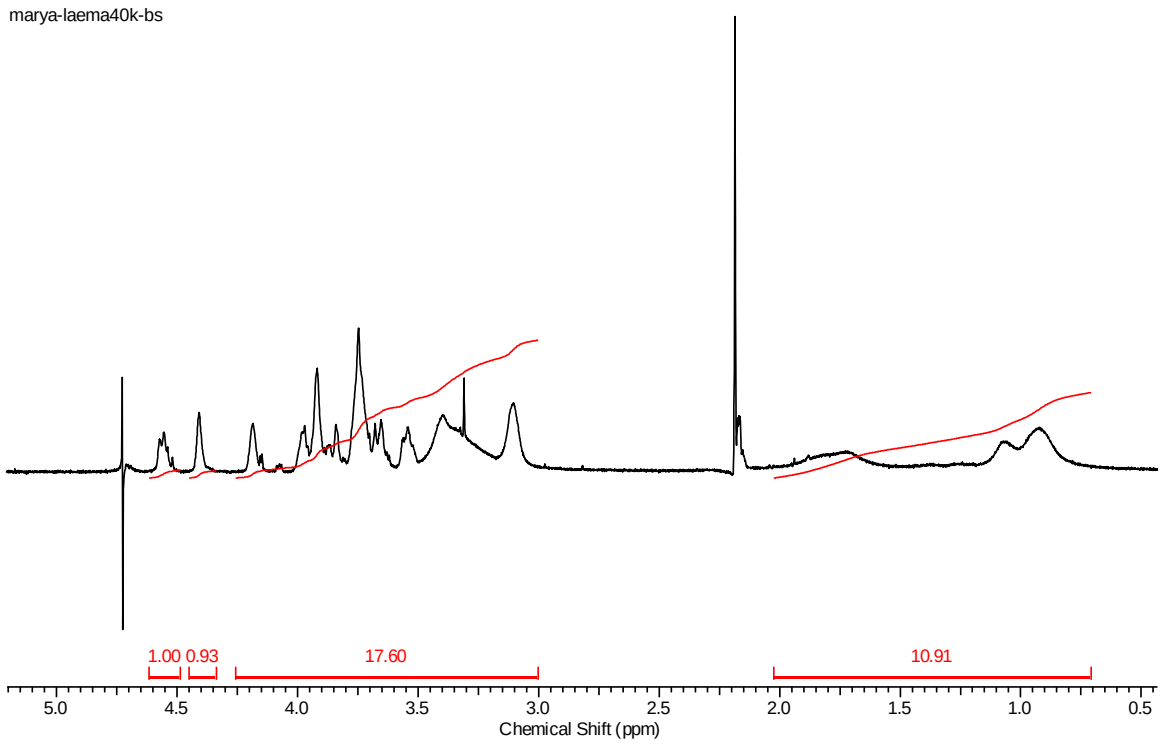

Figure S6: ^{1}H-NMR spectrum of P[(AEMA$_{27}$-st-LAEMA$_{26}$)-b-LAEMA$_{15}$].

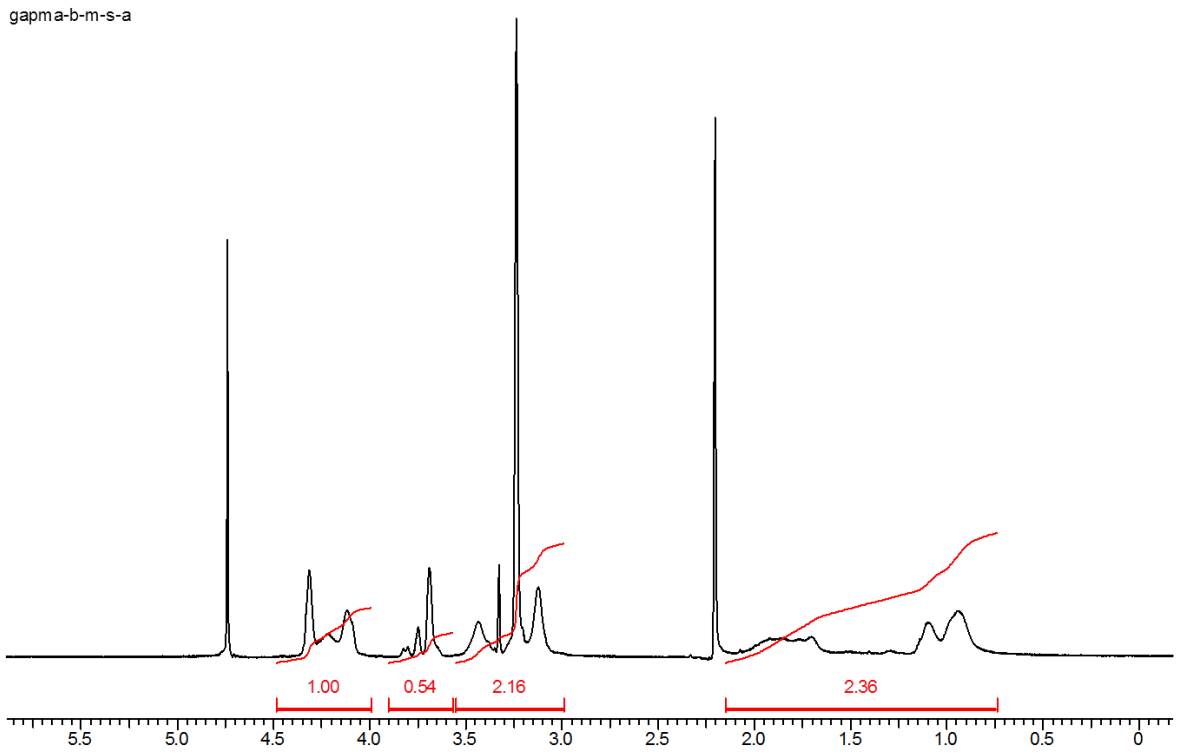

Figure S7: ^{1}H-NMR spectrum of P[(AEMA$_{66}$-*st*-MPC$_{70}$)-*b*-GAPMA$_{15}$].

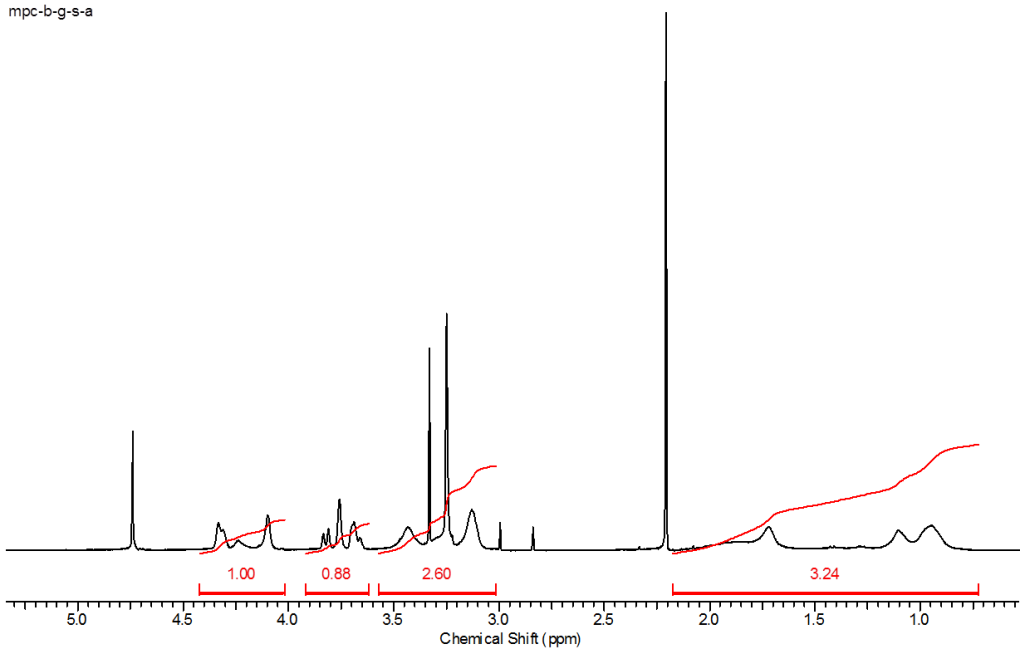

Figure S8: ^{1}H-NMR spectrum of P[(AEMA$_{64}$-*st*-GAPMA$_{64}$)-*b*-MPC$_{15}$].

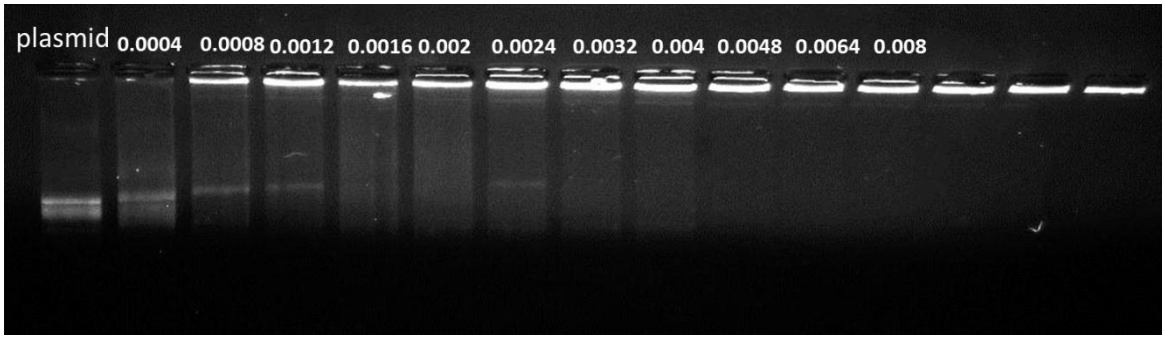

Figure S9: DNA binding efficiency of P[(AEMA$_{19}$-*st*-LAEMA$_{10}$)-*b*-LAEMA$_{15}$], as determined by agarose gel electrophoresis.

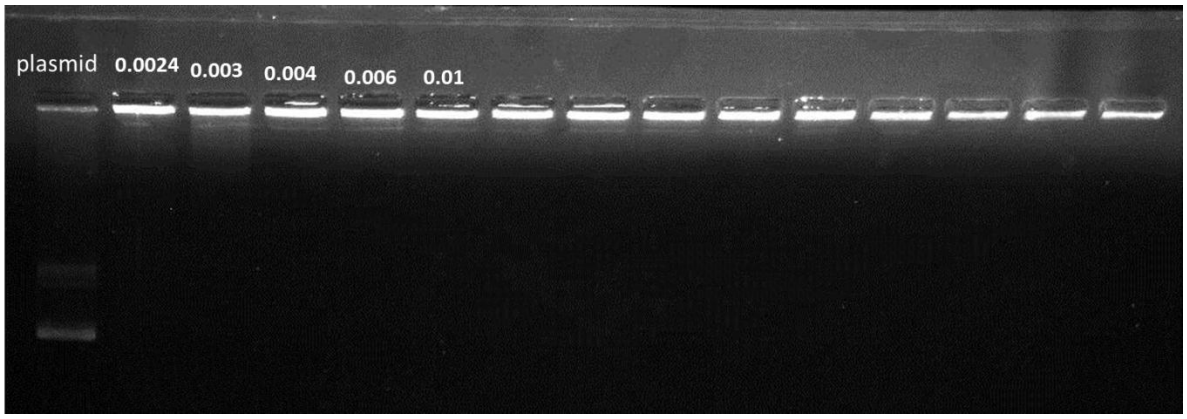

Figure S10: DNA binding efficiency of P[(AEMA$_{65}$-*st*-GAPMA$_{39}$)-*b*-GAPMA$_{15}$], as determined by agarose gel electrophoresis.

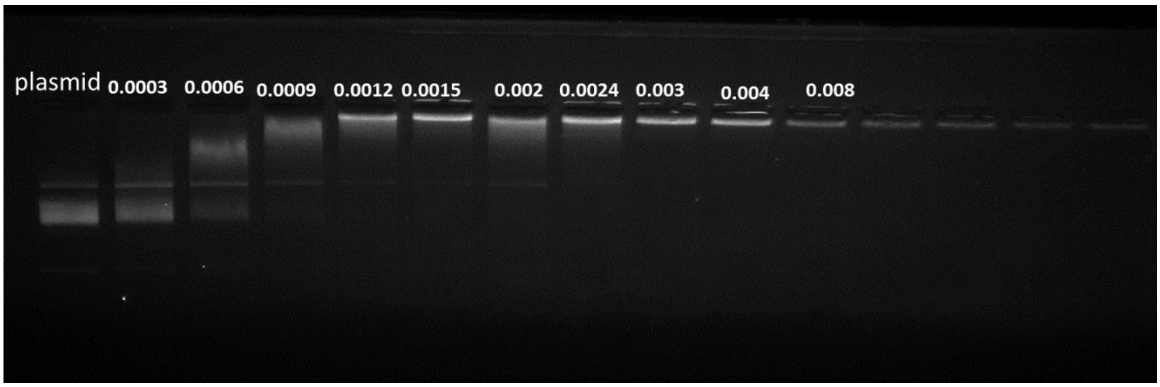

Figure S11: DNA binding efficiency of P[(AEMA$_{120}$-*st*-GAPMA$_{60}$)-*b*-GAPMA$_{15}$], as determined by agarose gel electrophoresis.

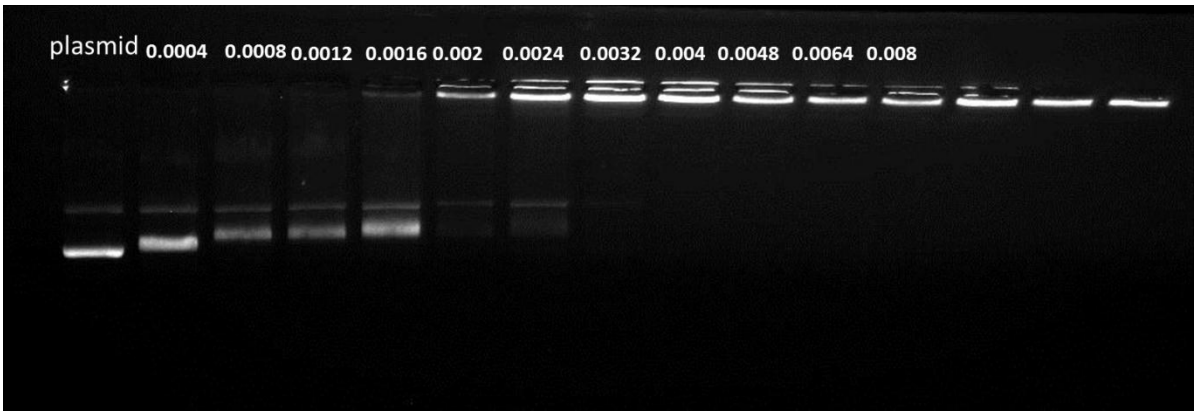

Figure S12: DNA binding efficiency of P[(AEMA$_{27}$-*st*-LAEMA$_{26}$)-*b*-LAEMA$_{15}$], as determined by agarose gel electrophoresis.

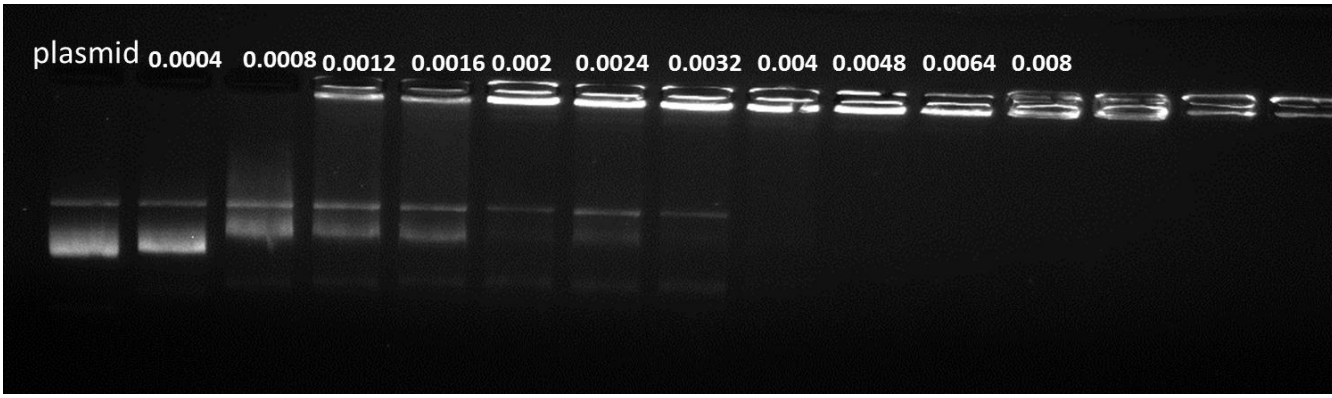

Figure S13: DNA binding efficiency of P[(AEMA$_{64}$-*st*-MPC$_{41}$)-*b*-MPC$_{12}$], as determined by agarose gel electrophoresis.

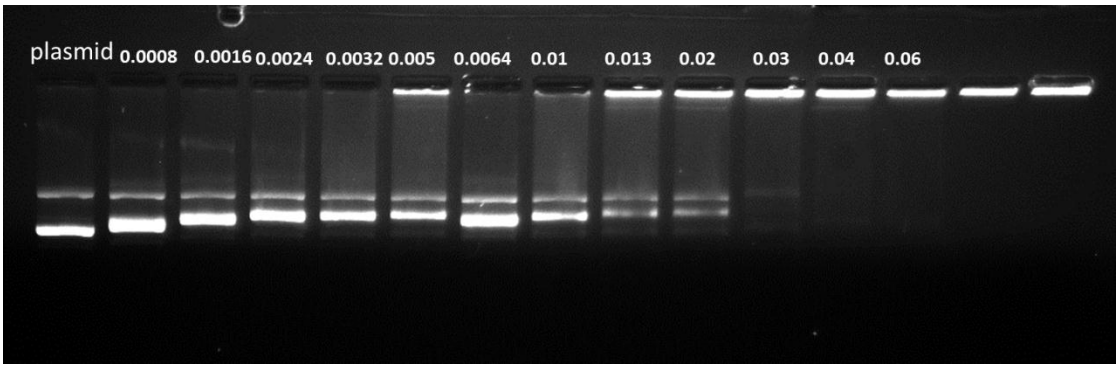

Figure S14: DNA binding efficiency of P[(AEMA$_{34}$-*st*-MPC$_{16}$)-*b*-MPC$_{12}$], as determined by agarose gel electrophoresis.

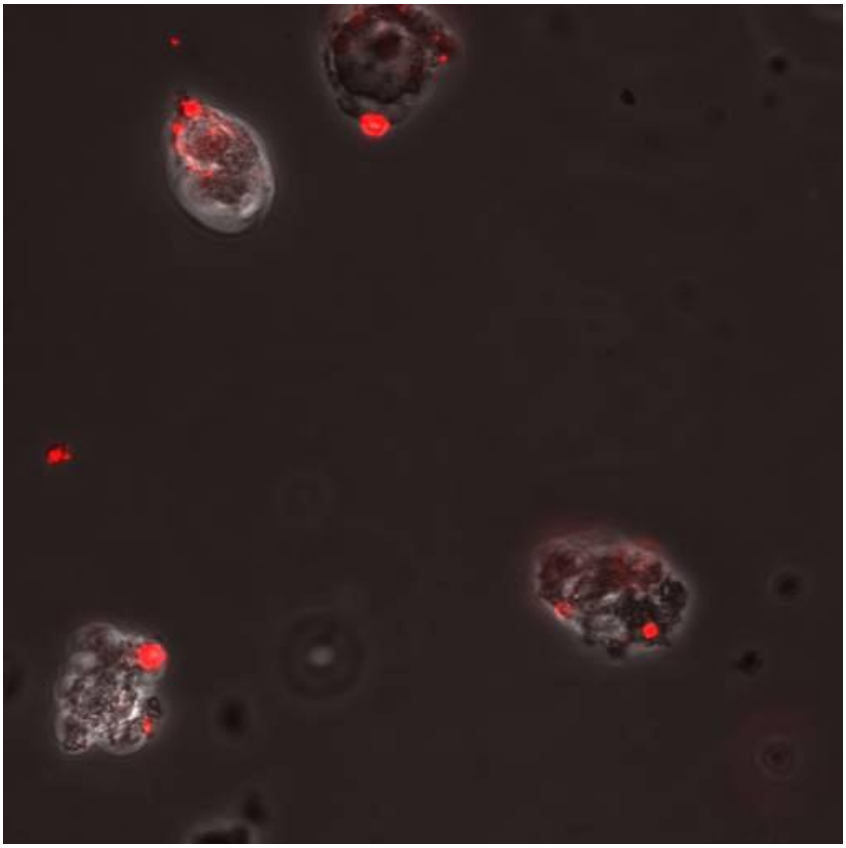

Figure S15: Nuclear isolation and the uptake of polyplexes, as studied by confocal images.

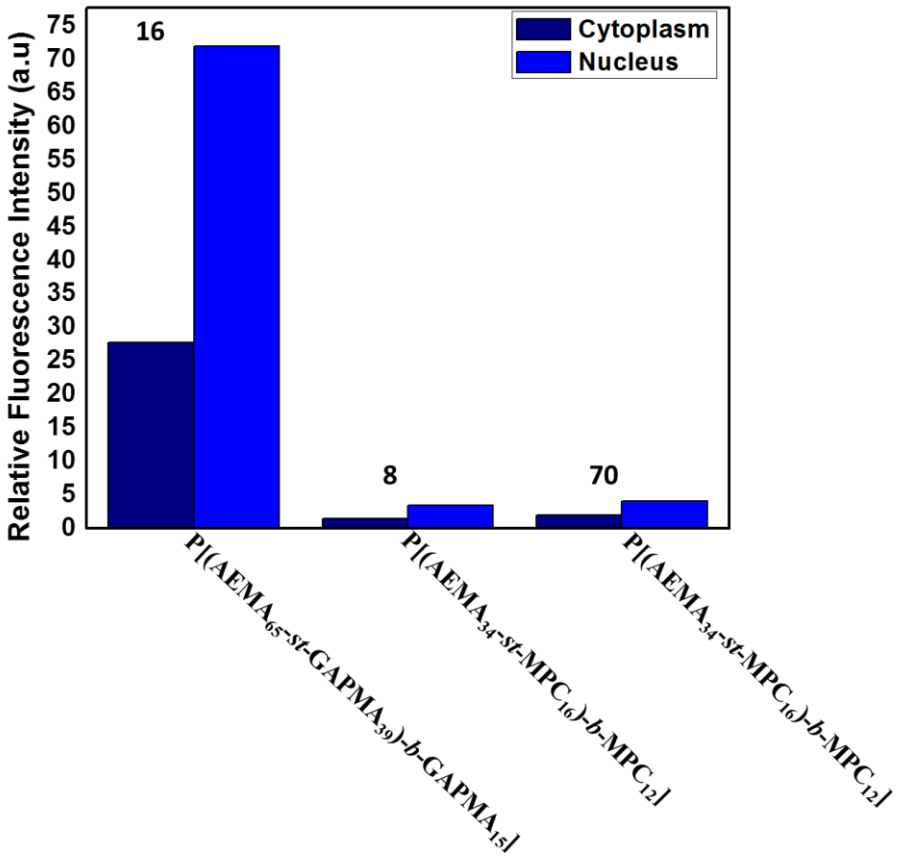

Figure S16: Cellular uptake of Cy-3'-labelled polymers at varying polymer/plasmid ratio (polymer/plasmid ratio = 70)

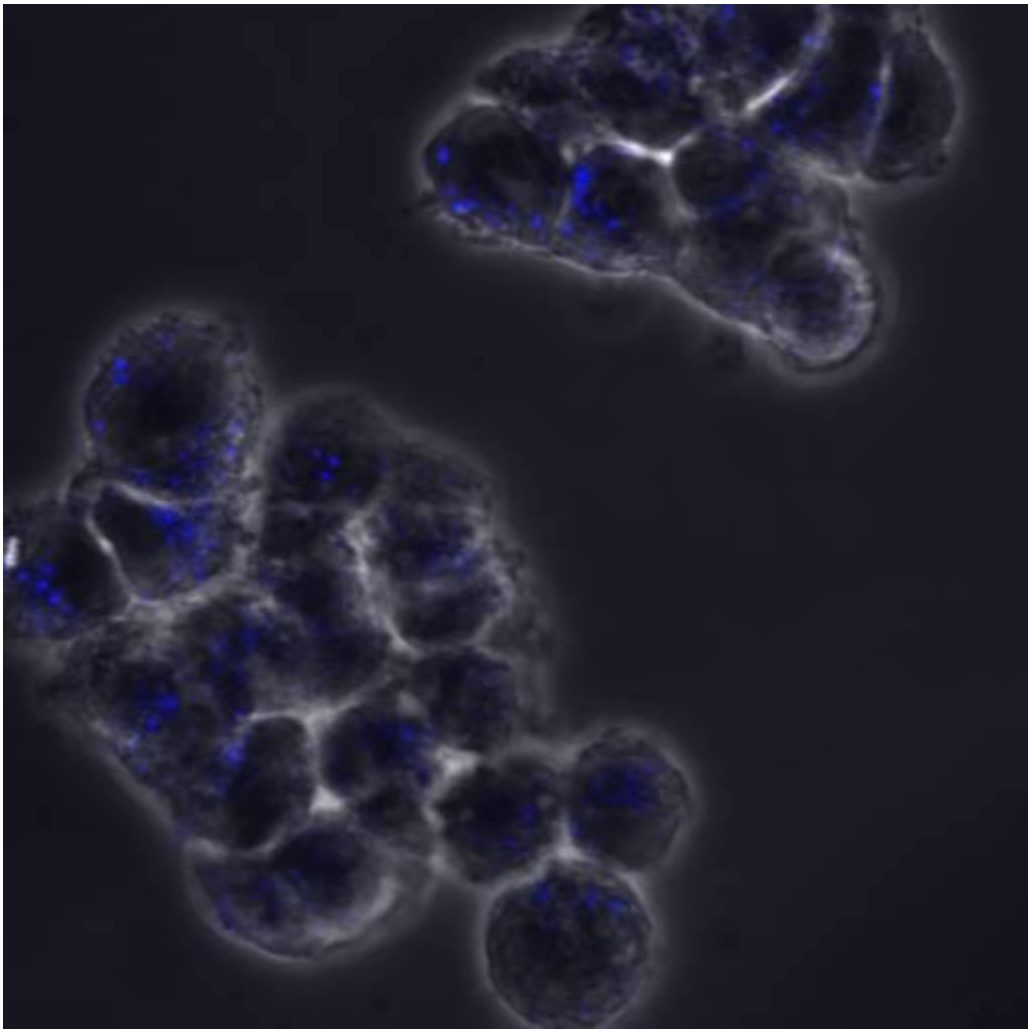

Figure S17: Cellular uptake of Cy-5'-labelled P[(AEMA$_{34}$-*st*-MPC$_{16}$)-*b*-MPC$_{12}$] based polyplexes at high polymer/plasmid ratio (polymer/plasmid ratio.