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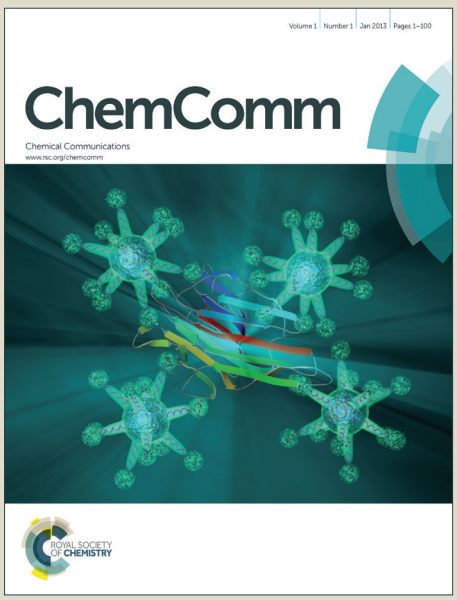

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Calcium Mediated Formation of Phosphorylcholine-based Polyplexes for Efficient Knockdown of Epidermal Growth Factor Receptors (EGFR) in HeLa Cells

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2-Methacryloxyethyl phosphorylcholine (MPC) materials are well studied due to their excellent biocompatibility and are currently being used in many clinical applications. In this study, MPC based homopolymers and copolymers are prepared and are subsequently evaluated for their charge inversion properties in the presence of cations and subsequent DNA binding efficacies. These polymers are then studied for their epidermal growth factor receptor (EGFR) specific siRNA delivery in Hela cells. The homopolymers of MPC and their copolymer show efficient EGFR knockdown efficacies in Hela cells both in the presence and absence of serum proteins.

The development of siRNA based therapeutics for specific, non-toxic, non-immunogenic silencing of genes offers great potential for the treatment of a variety of genetic and malignant disorders.[1,2] Cationic lipids and polymers are the most commonly used vectors for siRNA delivery.[2] The requirement of high N/P ratio of lipo- and polyplexes for effective siRNA delivery and low charge contribution of siRNA cause adverse side-effects to the treated cells/tissue, due to the severe toxicity of the complexes.[1] Hence, there is an inherent need to develop a non-toxic system to obtain effective siRNA delivery with minimum or ideally no side effects. The incorporation of non-toxic residues in cationic polymers are well-known to reduce the gene delivery efficacies of the polymeric system.[3] For instance, 2-methacryloxyethyl phosphorylcholine (MPC) based polymers are safe and biocompatible material[4,5], but are found to reduce the gene expression of cationic copolymers.[3] The zwitterionic MPC with structural similarities to phosphatidylcholine lipids of cell membrane was introduced by Nakabayashi and Ishihara in the 1990s.[4,6] Due to the high biocompatibility and anti-fouling properties, the use of MPC in biomedical applications has been significantly increased.[5,7]

MPC coated implants, and devices are largely used for tissue engineering and drug delivery applications. [7-8] However, the potential of MPC polymers in gene therapy is limited due to their poor DNA condensation ability and low cellular uptake.[9,10] The poor cellular adhesion properties of MPC modified charged surfaces is also well-documented.[10] The incorporation of carbohydrate moieties in MPC copolymers of '*block-statistical*' architecture is found to improve their gene expression profiles by improving their cellular uptake.[3c] The charge reversal properties of phosphatidylcholine (PC) based lipids are reported by Prettu *et al*.[11] A family of PC based zwitterionic lipids are prepared and are characterized for their pH and calcium ions dependent charge reversal properties. The inversion of PC head group occurs at acidic pH or in the presence of divalent cations, hence altering the overall surface charge of liposomes and release profile of encapsulated biomolecules.[11] The concentration dependent interaction of divalent cations (such as Ca^{2+} ions) with zwitterionic head groups of PC are shown to produce cationic liposomes.[11] We herein exploit this strategy to change the zwitterionic nature of the phosphorylcholine groups into a cationic system for DNA condensation and gene expression.

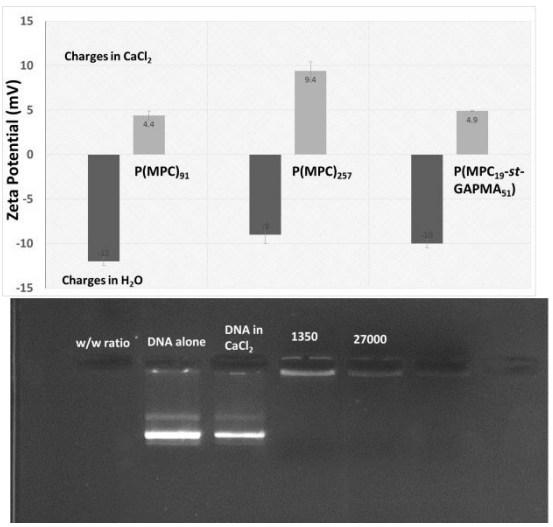
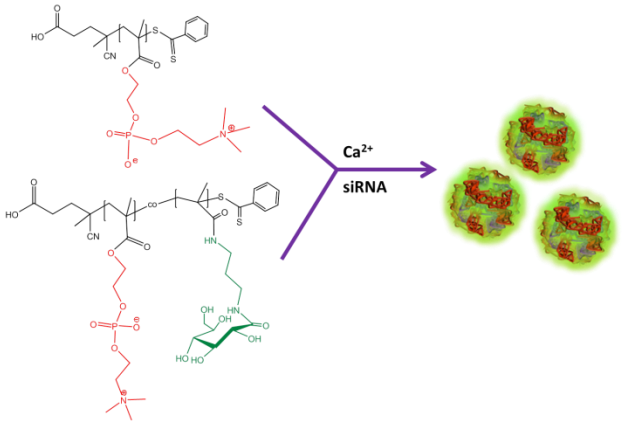


Fig. 1 Charges of MPC homopolymers and copolymer in water and 10 mM $CaCl_2.2H_2O$ solution, at 20 mg/mL concentration. (top) Agarose gel electrophoresis, showing the condensation of 10mM $CaCl_2$ and $P(MPC_{19}$-*st*-$GAPMA_{51}$ with 400 ng of β-galactosidase plasmid. (bottom)

Homopolymers of MPC of varying molecular weights and their copolymers with gluconamidopropyl methacrylate (GAPMA) are prepared by RAFT polymerization. (Supporting information scheme

S1 & S2) We have selected the RAFT process to prepare a variety of MPC polymers of controlled dimensions under mild polymerization conditions.[12-14] Well-defined polymers of molecular weights ranging from 20-76 kDa are obtained, as determined by gel permeation chromatography (GPC). (Supporting information Table S1)



Scheme 1. Formation of polyplexes with RAFT synthesized MPC based polymers and EGFR specific siRNA in the presence of calcium ions.

Solubilization of MPC polymers in 10 mM $CaCl_2$ solution produced cationic species as revealed by the zeta potential data and the efficient DNA condensation was determined by agarose gel electrophoresis (Figure 1). It is hypothesized that the divalent calcium ions interact with the negative phosphate groups on the MPC polymers strongly, allowing the ammonium residues to bind to the negatively charges DNA or siRNA. The formulation of polyplexes (with siRNA (Figure 1) and DNA (Figure S1) was studied by DLS and discrete particles ranging from 400-900 nm with close neutral charge were obtained. (Figure 1,2, Scheme 1, Supporting information Figure S1)

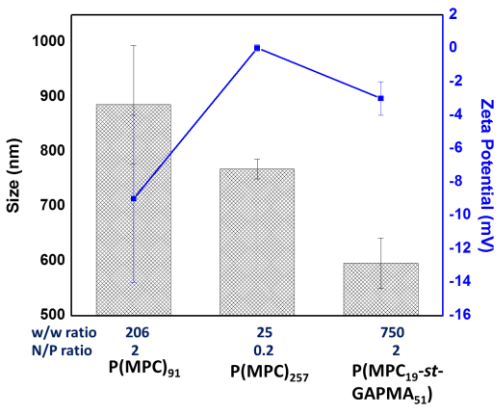
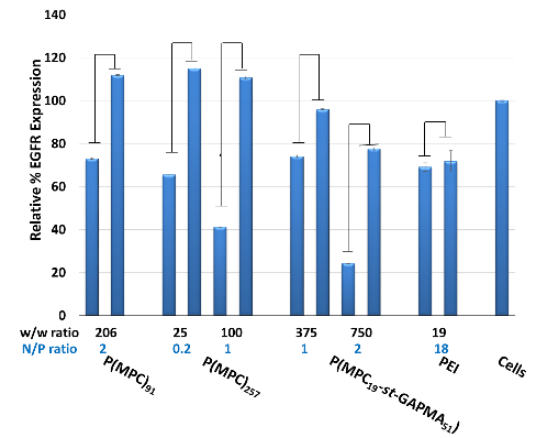


Fig. 2 Sizes and charges of polymer-siRNA complexes in deionized water, as determined by DLS and zeta potential instrument.

siRNA complexes with low zeta potential values (-10 to 10mV) are considered ideal candidates for gene delivery. PEGylation of cationic polymers has been employed as a technique to acquire these stealth properties.[1d] The low zeta potential values of MPC based siRNA complexes indicate their ability to serve as ideal siRNA delivery agents in physiological conditions. The sizes of siRNA complexes produced by PMPC condensation were analyzed by DLS. As expected, the high molecular weight polymers $P(MPC)_{257}$ produced smaller particles, as compared to low molecular weight copolymers. The formulation of well-defined polyplexes was further confirmed by transmission electron microscopy (TEM). (Supporting information Figure S2).

It should be noted that calcium ions alone at varying w/w ratios of Ca^{2+} to siRNA (16-800) could not condense, as revealed by the agarose gel electrophoresis. (Supporting information Figure S3) Ca2+/siRNA complexes migrated towards cathode similar to siRNA alone, however presence of Ca^{2+} ions (regardless of concentration of $CaCl_2$) enhanced the stability of siRNA in 1% agarose gel. siRNA alone formed a smear in gel, in contrast, siRNA-Ca^{2+} complexes maintained their discrete bands. However as shown by the gel, no complexation of siRNA by Ca^{2+} ions at all studied w/w ratios was observed. MPC based polyplexes formulated with DNA and siRNA were subjected for their gene expression analysis and gene knockdown efficacies, respectively in a variety of cell lines. siRNA is small anionic molecule (20-25 bp), which can effectively and specifically knock down a gene of interest in targeted cells.[1,2] The high efficacies of siRNA therapeutics are assessed by the statistics, showing that in less than decade of discovery of siRNA, a number of siRNA based therapeutics are now in clinical trials.[1d] Epidermal growth factor receptors (ErBb1/EGFR) is a 170 kDa protein which is largely overexpressed in a variety of cancer types, such as ovarian, lung, and breast carcinomas. EGFR knockdown efficacies are associated with tumor regression, either by blocking EGFR mediated cell proliferation pathways or by inhibiting the cross-talks between ErBb family members, responsible for cell growth and survival.[15]



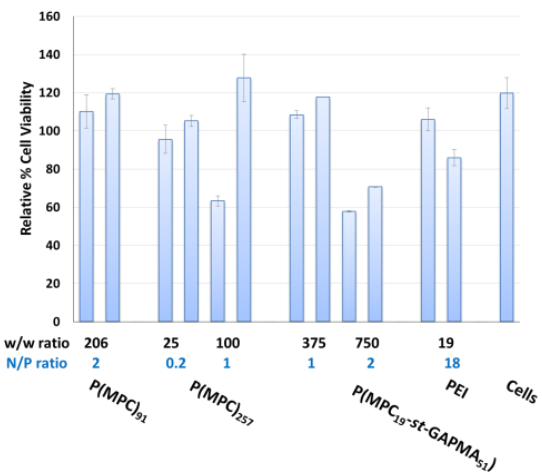


Fig. 3 Relative % EGFR expression on the surface of Hela cells 48 hours post-treatment with EGFR-specific siRNA (left bar) or control-siRNA (right bar) at dose of 0.1 nmoles of siRNA. Relative % cell viability of Hela cells 48 hours post-treatment with siRNA or control siRNA polyplexes, as determined by Janus green assay.

In this study, EGFR specific siRNA is chosen to evaluate the gene knock down efficacies in Hela cells, by enzyme linked immunosorbent assay (ELISA). In contrast to DNA-based polyplexes, which showed low gene expression in a variety of cell lines (data not shown), enhanced gene knock-down efficacies were obtained for MPC-siRNA complexes in Hela cells. (Figure 3,4)

The gene knockdown efficacies of MPC based EGFR-specific and EGFR non-specific siRNA complexes are studied at varying weight/weight or N/P ratios and in the presence and absence of serum proteins, at the dose of 0.1 nmoles of siRNA per treatment. The high molecular weight PMPC, $P(MPC)_{257}$ showed efficient gene knockdown efficacies (~ 40%) at low N/P ratios, as compared to $(PMPC)_{91}$, which showed ~30% gene knockdown efficacy at high N/P ratio. These results are consistent with previous reports, which show that low N/P ratio are required for optimum gene expression of high molecular weight polymers.[12,13a,c] Interestingly, A high $P(MPC_{19}$-*st*-$GAPMA_{51})$ copolymer showed optimum (~60%) gene knockdown in Hela Cells at similar N/P ratio (2).

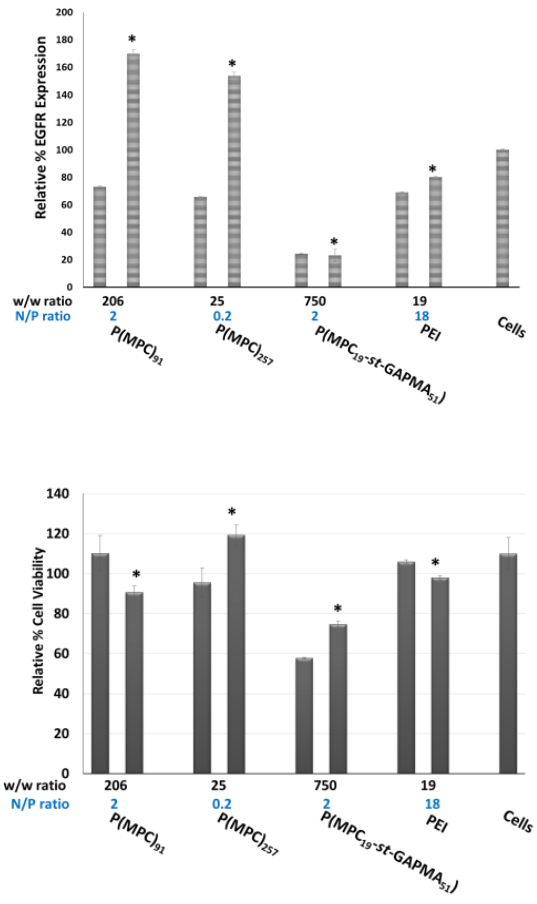


Fig. 4 Relative % EGFR expression of polyplexes in the absence and presence of (*) serum proteins on the surface of Hela cells as determined by In-cell ELISA assay. Cell viability 48 hours post-transfection with MPC-polyplexes, as determined by Janus green assay. The dose of siRNA was 0.1 nmoles per treatment.

The toxicities of transfected cells were further studied as shown in figure 3 (Supporting information figure S3). As expected, the toxicities of MPC based polyplexes were dependent on their molecular weight. The low molecular weight MPC based polymers showed high cell viability at high N/P ratios, as compared to high molecular weight based MPC polymers (Supporting information figure S3). Moreover, the toxicity of $P(MPC_{19}$-*st*-$GAPMA_{51})$ was not associated with EGFR-specific siRNA, as both EGFR specific siRNA and negative control showed similar toxicity profiles. (Figure 3) MPC homopolymers of the studied molecular weights showed low gene knockdown efficacies in the presence of serum proteins. However, $P(MPC_{19}$-*st*-$GAPMA_{51})$ copolymer showed high gene knock down efficacies in the presence of serum proteins, along with low toxicities. (Figure 4) The improved gene expression of glycopolymers based cationic vectors in the presence of serum proteins has been studied.[13a] It is hypothesized that the high gene knockdown efficacies of sugar based copolymers of MPC (as compared to homopolymers of MPC) is associated with their high stability under physiological conditions.

We describe here a unique strategy for the preparation of non-toxic siRNA delivery vectors. MPC based polymers was found to condense and form discrete particles with EGFR specific siRNA in the presence of divalent cations. The polyplexes produced show efficient gene knockdown efficacies in Hela cells, in the presence and absence of serum proteins. The incorporation of carbohydrate residues in the MPC polymers was found to enhance the knock-down of the EGFR receptors on the surface of Hela cells, possibly by enhancing the stability of complexes under physiological conditions. Further studies will be focused on the synthesis of MPC based copolymers of varying molecular weights and architectures and their role in siRNA delivery will be studied.

Notes and references

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