Increased Transfection Efficiency of Lipid-Coated Methacrylate-Based Gene Delivery Systems

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Abstract

Purpose: To develop a safe, lipid-based non-viral gene delivery system that achieves high transfection efficiency in the presence of serum proteins.

Methods: Polyplexes with the pAcGFP1-C1 plasmid were formed in phosphate buffered saline, pH 7.4 (PBS) using the novel poly[N-(2-hydroxypropyl)methacrylamide]-poly(N,N-dimethylaminoethylmethacrylate) diblock copolymer (pHPMA-b-pDMAEMA) at N/P=4. Cationic-Liposomes were prepared from a dried lipid film comprised of equimolar 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). Lipopolyplexes were fabricated at lipid/DNA weight ratios up to 40. Particle size distribution and zeta potential of lipopolyplexes were determined by dynamic light scattering. HeLa cells viability in the presence and absence of lipopolyplexes was quantified using the CellTiter-Glo® luminescent assay. HeLa cell transfection efficiency in the presence and absence of FBS was visually assessed by confocal microscopy and quantitatively compared to the TurboFect™ control.

Results: pHPMA-b-pDMAEMA exhibited a high condensation capacity of 1 µg of pDNA per 0.513 µg of polymer (N/P=1). Lipid-coating of polyplexes at lipid/DNA weight ratios up to 40 resulted in particle sizes <200 nm and zeta potential >+25 mV. Exposure to FBS significantly increased mean particle size to >300 nm, reduced zeta potential to -10 mV, and augmented polydispersity. Lipid coating of polyplexes only decreased HeLa cell viability at lipid/DNA ratios >20. HeLa transfection with lipopolyplexes was most effective at lipid/DNA = 20 and was significantly greater in the presence of FBS than measured for lipid-free polyplexes.

Conclusion: Lipid coating of pHPMA-b-pDMAEMA/DNA polyplexes with an equimolar DOTAP/DOPE mixture at a lipid/DNA ratio = 20 effectively enhances in vitro transfection efficiency of HeLa cells in the presence of serum proteins.

Keywords: lipopolyplex, DOTAP, DOPE, pHPMA-b-pDMAEMA copolymer

Introduction

Development of gene carriers that deliver genetic material effectively into cells is one of the main objectives of gene therapy. Although substantial progress has been achieved in this field over the past decade, clinical gene therapy applications are still limited largely due to inadequate safety of viral carriers and unsatisfactory efficacy results with existing nonviral gene delivery systems [1][2]. The premise of nonviral vectors is based on successfully condensation of the bulky DNA structure to the nanoscales for effective cellular internalization. Simultaneously, the gene carrier must protect nucleic acids from enzymatic degradation mediated by both extra- and intracellular nucleases [3]. To date, transfection efficiency of nonviral vectors is still substantially lower than is achieved with viral vectors, especially in the presence of serum [1]. Cationic polymers are among the most widely used components of nonviral vectors due to their high propensity to form electrostatically-driven association complexes (polyplexes) when combined with DNA [4]. In polyplexes; cationic polymers facilitate cellular entry of negatively charged DNA [5]. However, surface adsorption of ubiquitous serum proteins has the ability to destabilize polyplexes resulting in dissociation and/or aggregation [6, 7]. As a consequence, serum is recognized as a major obstacle to efficient gene transfection in a clinical setting.

A new generation of nonviral vectors attempts to overcome this serum sensitivity by surface shielding of polyplexes using various chemical moieties. Lipopolyplexes represent a ternary complex comprised of at least one cationic lipid, cationic polymer, and the negatively-charged genetic material. Although lipid-based gene carriers may also suffer from low transfection efficiency [8], selection of a suitable lipid composition has been demonstrated to protect polyplexes from undesired serum effects [9]. Specifically, gene carriers containing the cationic phospholipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) appear to be more effective in reducing serum-catalyzed polyplex dissociation and/or aggregation [9, 10]. Inclusion of 1,2-dioleoyl-sn-glycero-

3-phosphoethanolamine (DOPE) as "helper lipid" into these complexes is predicted to enhance electrostatic association between the cationic polymer and the negatively charged DNA via hydrophobic interactions. As a consequence, serum protein adsorption to the surface of these lipopolyplexes is thermodynamically less favorable, thus, enhancing intracellular uptake of these nonviral gene delivery systems in the presence of plasma [11]. Despite the fact that lipid-based carriers are effective in augmenting intracellular uptake of genetic material, results from previous liposome trials revealed ineffective nuclear transport of DNA due to the presence of high levels of cationic lipid [12, 13]. It is, therefore, predicted that lipid coating of pre-condensed DNA within polyplexes will overcome the limitations of pure polymer- and lipid-based gene delivery carriers in the presence of serum [1, 14].

The objective of this research study is to investigate whether coating of the pre-condensed pHPMA-b-pDMAEMA/DNA polyplexes with an equimolar DOTAP/DOPE lipid mixtures improves transfection efficiency of this nonviral gene delivery system in the presence of serum proteins.

Materials and Methods

Materials

The poly[N-(2-hydroxypropyl)methacrylamide]-poly(N,N-dimethylaminoethylmethacrylate) diblock copolymer (pHPMA-b-pDMAEMA) was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization using a procedure adapted from Duvall and colleagues [15]. The average molecular weight of pHPMA-b-pDMAEMA determined by gel permeation chromatography was = 21.2 kDa. The cationic 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, chloride salt) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). The pAcGFP1-C1 expression plasmid was obtained from Clontech Laboratories (Mountain View, CA) and amplified according to the manufacturer`s protocols. TurboFect™ Transfection Reagent, DRAQ5™, 1kb DNA ladder, Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline pH 7.4 (PBS), agarose, agarose gel loading dye, ethidium bromide (EtBr), Tris/EDTA (TE) buffer, and cell supplements such as trypsin/EDTA, penicillin-streptomycin, L-glutamine, and non-essential amino acids were purchased from ThermoFisher Scientific (Pittsburgh, PA). Tris-acetate-EDTA (TAE) running buffer and CellTiter-Glo® cytotoxicity assay kit were purchased from Promega (Madison, WI). Heat-inactivated fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA). All other chemicals were of analytical grade and used as received.

Cell Culture

HeLa cells, which were derived from cervical cancer cells, were obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37°C in a humidified 5% (v/v) CO_2 atmosphere using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 1% (w/v) L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 1 % (v/v) non-essential amino acids.

Ethidium Bromide Intercalation

The ability of cationic polymers to condense pDNA was monitored by fluorescence quenching of the pDNA-EtBr interaction as described previously [16]. 1 µg of pDNA suspended in 50 µL of either PBS or serum-free DMEM (SFM) was combined with 50 µL of an aqueous EtBr solution (5 µg/mL) and incubated at RT for 15 minutes. pHPMA-b-pDMAEMA polymer aliquots were sequentially added and incubated for 30 minutes at RT before fluorescence emission was quantified at λ = 590 nm (EX = 544 nm) using the POLARstar microplate reader (BMG Labtech, Cary, NC).

Agarose Gel Retardation

Electrophoretic mobility of pHPMA-b-pDMAEMA/pDNA complexes was determined by agarose gel electrophoresis [17]. Catiomer/pDNA complexes containing 0.4 µg of pAcGFP1-C1 were combined with 2 µL of the gel loading dye, and suspension was loaded onto a 0.5% (w/v) agarose gel. pAcGFP1-C1 plasmid without polymer was used as a control. Separation was carried out for 100 minutes at 90 V in TAE running buffer using the Power Pac® 200 (Bio-Rad, Hercules, CA). Following electrophoresis, gels were stained for 40 minutes using a 0.005% (v/v) aqueous EtBr solution. pDNA bands were visualized after 30 mintes destaining in water at λ = 254 nm using the UVP Bioimaging System (UVP, Upland, CA).

Lipopolyplex Fabrication

To prepare lipopolyplexes, pHPMA-b-pDMAEMA/DNA polyplexes and cationic DOTAP/DOPE liposomes were prepared first separately. Polymer/DNA association complexes were formed by combining the pAcGFP1-C1 plasmid with an appropriate amount of the pHPMA-b-pDMAEMA polymer in PBS, pH 7.4 resulting in polyplexes with N/P=4 as defined by the results from the EtBr intercalation assay. Electrostatic association was allowed for 1 hour at RT with occasional vortexing (20 seconds every 15 minutes). Cationic liposomes were prepared by hydrating a thin dry lipid film of equimolar DOTAP and DOPE with PBS, pH 7.4. Small bilayer vesicles were obtained after subsequent sonication for 10 minutes at 40 kHz using a sonication

bath. Lipopolyplexes were fabricated by combining cationic liposomes with freshly prepared polyplexes at a lipid/DNA weight ratio of 10, 20, and 40, respectively.

Physicochemical Properties

Particle size distribution and zeta potential of fabricated lipopolyplexes were estimated before and after addition of 10% FBS-containing DMEM by dynamic laser light scattering using the Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.) according to the manufacturer's instructions. All particle size values reported in this study refer to the equivalent hydrodynamic diameter.

Cell Viability

Viability of HeLa cells after exposure to pHPMA-b-pDMAEMA polyplexes and lipopolyplexes was quantified using the CellTiter-Glo® luminescent assay that measures total cellular ATP. HeLa cells were seeded in white 96-well plate at a density of 1×10^4 cells/well. Following overnight attachment, cells were incubated for 4 hours at 37°C in the presence of various concentrations of lipopolyplexes and lipid-free polyplexes at N/P=4 in 10% (v/v) FBS-containing DMEM. Subsequently, cells were washed using FBS-containing DMEM and incubated for additional 44 hours in maintenance media. Following addition of the CellTiter-Glo® reagent, luminescence was quantified using the POLARstar microplate reader (BMG Labtech, Cary, NC). Cells incubated with a 1% (v/v) Triton X-100 solution prepared in PBS were used as negative control. Cell viability was normalized to vehicle-treated controls.

In vitro Transfection

HeLa cells were seeded in 16-well chamber slides at a density of 1×10^4 cells/well. Following overnight attachment, cells were incubated for 4 hours at 37°C with different concentrations of lipopolyplexes and corresponding lipid-free polyplexes suspended in 10% (v/v) FBS-containing DMEM (0.2 µg DNA/well). Naked DNA and the commercial transfection reagent (TurboFect™) were used as negative and positive control, respectively. After this incubation period, cells were washed with FBS-containing DMEM and incubated for additional 44 hours in maintenance media to allow transgene expression. Cells were washed with PBS and counterstained with DRAQ5 (red nuclear stain). The percentage of positive, green fluorescent cells was determined by counting green cells versus total count for red-stained nuclei in three randomly selected sections using the Zeiss LSM510 Confocal Microscope (Zeiss, Germany). The LSM 510 consists of a Zeiss Axiovert 200M microscope Meta detector head (range 410-750 nm)For green and red fluorescence, we used green: Argon Laser (λex = 488 nm and λem 505-530 nm) + BP 505-530 nm filter; red: HeNe543 Laser (λex = 543 nm and λem 560-615nm) + Meta 550-615 nm detection.

Statistical Analysis

Experiments were performed at least in triplicate, and results are reported as mean ± standard deviation (SD). Statistical difference among various treatment groups was assessed using one-way ANOVA or two-sided Student's t-test for pairwise comparison. A probability of $p<0.05$ was considered statistically significant (GraphPad Prism 6.0, GraphPad, San Diego, CA).

Results and Discussion

pHPMA-b-pDMAEMA/pDNA Condensation

Polyplex formation is thermodynamically driven by electrostatic interactions between negatively charged phosphate groups of nucleic acids and positively charged amino groups present on the polymers [18]. To quantitatively determine the complexation capacity of the novel pHPMA-b-pDMAEMA polymer, the EtBr intercalation and gel retardation assays were performed. The stoichiometric relationship of this interaction was determined by the fluorescence quenching of EtBr/pDNA complexes in the presence of various amounts of pHPMA-b-pDMAEMA, which was assumed to have significantly greater affinity for DNA than EtBr. As shown in Fig. 1A, fluorescence intensity decreased with increasing amounts of pHPMA-b-pDMAEMA present in the mixture prepare in PBS and SFM, respectively. In PBS, maximum condensation of 1 µg of pDNA was effectively achieved in the presence of 0.513 µg of the polymer (N/P=1). These results were consistent with agarose gel retardation data where the fluorescence intensity of EtBr/pDNA bands associated with relaxed and coiled nucleic acid strands dramatically decreased following addition of polymer amounts >0.128 µg (Fig. 1B). In the presence of 0.513 µg of pHPMA-b-pDMAEMA (Fig. 1B, Lane 4), electrophoretic mobility of EtBr/pDNA was visually absent suggesting effective pDNA condensation with the cationic polymer [16, 19]. In contrast, the stoichiometry of pDNA/pHPMA-b-pDMAEMA interactions in SFM was significantly different. Based on EtBr intercalation results (Fig. 1A), it appears that the presence of media components interfered with condensation of pDNA with this cationic polymer. Addition of pHPMA-b-pDMAEMA >0.5 µg was only moderately successful in increasing EtBr displacement greater than 40%. Even in the presence of 1.5 µg of this polymer, electrostatic neutrality (i.e., N/P=1) was never reached. Agarose gel retardation assay confirmed the inability of pHPMA-b-pDMAEMA to fully condensate pDNA in SFM by the presence of bright, fluorescent EtBr-stained pDNA bands (Fig. 1C). It is hypothesized that zwitterionic amino acids in SFM afforded

electrostatic shielding of positively charged amine groups in the DMAEMA moieties, thereby reducing the pDNA condensation ability of this polymer [20, 21].

Physicochemical Properties

Cellular internalization of lipopolyplexes is a necessary prerequisite for successful gene delivery. Previous studies identified the critical relationship between particle size and cellular uptake rates [22]. Sensitivity of gene carriers to serum is an important parameter for the in vivo transfection efficiency. To directly assess the effect of serum on the structure of these complexes, the particle size and zeta potential were determined in the absence and presence of 10% (v/v) FBS. In PBS, a relatively homogenous population of complexes (PDI ≤0.2) was measured with a mean hydrodynamic diameter of 101±6 nm (Fig. 2A) and a zeta potential of 0.16±0.1 mV (Fig. 2B). After inclusion of FBS, the polydispersity index (PDI) of the polyplex suspension dramatically increased to ~1 suggesting significant serum-induced dissociation and/or aggregation [7, 23].

The mean particle size of lipopolyplexes determined in the absence of serum was 185-200 nm, which represents a 2-fold increase in hydrodynamic diameter as compared to the lipid-free polyplexes (Fig. 2A). The zeta potential of these lipopolyplexes increased from +25 to +40 mV at increasing lipid/DNA weight ratio (Fig. 2B). The increased particle size and significantly positive zeta potential suggest that these cationic lipids successfully formed an outer coat around the polyplexes, which is consistent with the "core-shell" structure of lipopolyplexes proposed by other investigators [10].

Exposure to FBS moderately increased in mean size of these lipopolyplexes but did not alter the homogeneous size distribution as demonstrated by a PDI of approx. 0.2 under those conditions. However, the surface charge dramatically decreased to about -10 mV (Fig. 2) suggesting adsorption of negatively-charged serum proteins and ions to the lipopolyplex surface. Most importantly, the absence of a corresponding increase in polydispersity implies substantially fewer dissociation and/or aggregation of lipopolyplexes in the presence of serum when compared to lipid-free polyplexes [24]. This may be attributed to the presence of a phospholipid "corona" surrounding the polyplex core, which is predicted to stabilize the electrostatic association between pHPMA-b-pDMAEMA and DNA by hydrophobic interaction [9, 11]. Consequently, this phopholipid "corona" renders the delicate polyplexes structure more resistant to serum-induced dissociation and/or aggregation.

Cell Viability

Cellular safety is an essential prerequisite for any successful drug delivery system. The cellular safety of lipopolyplexes and corresponding polyplexes was assessed by quantifying total cellular ATP levels using the CellTiter-Glo® assay. Non-lipidated pHPMA-b-pDMAEMA/pDNA polyplexes (N/P=4) did not significantly compromise cellular safety (Fig. 3). Lipopolyplexes at lipid/DNA ratios ≤20 exhibited cell viability comparable to polyplexes (i.e., cell viability >80%). These results imply that addition of DOTAP/DOPE lipids up to 20-fold weight ratio did not significantly compromise cellular safety of HeLa cells. However, lipopolyplexes prepared at a lipid/DNA ratio of 40 significantly decreased cell viability of HeLa cells by 33% (Fig. 3). DOTAP and DOPE are preferred lipid-based gene carriers due to apparent low cytotoxicity [25, 26]. However, DOTAP was demonstrated to cause cellular changes when used in high concentrations [26]. Cationic lipids may alter the net charge of the cell membranes which, in turn, can negatively affect the functional activity of ion channels, membrane receptors, and enzymes. Cationic lipids are also reported to interfere with cell attachment to the extracellular matrix resulting in reduce anchorage of cells on solid support during in vitro cell culture studies [27].

In-vitro Transfection Efficiency

Transfection efficiency of gene delivery systems in vivo is generally reduced due surface adsorption of plasma proteins that alters surface charge and particle size distribution of polyplexes [7]. To assess transfection efficiency of lipopolyplexes prepared at different lipid/DNA weight ratios, HeLa cells were incubated with these nonviral gene delivery systems for the pAcGFP1-C1 reporter gene in the presence of 10% (v/v) FBS. The commercially available TurboFect™ transfection reagent was used as a positive control. Transfection efficiencies of DNA-containing lipopolyplexes were compared with those of corresponding lipid-free polyplexes. Quantitative assessment of the percentage of GFP-expressing cells is summarized in Figure 4.

The novel pHPMA-b-pDMAEMA polymer effectively condensated pDNA into electrostatically stabilized association complexes with hydrodynamic diameters ranging from 101 to 197 at N/P=4. The transfection efficiency of HeLa cells with these polyplexes was only 40%, which was significantly lower than the transfection efficiency of TurboFect™/pAcGFP1-C1 control polyplexes (i.e., 55%, Fig. 4). Consistent with earlier reports, it is predicted that this reduced transfection efficiency may be the result of surface-adsorbed serum proteins [7, 23]. This hypothesis is supported by experimental evidence included in this manuscript that the particle size distribution of polyplexes in the presence of FBS dramatically increased (PDI of ~ 1) suggesting interaction of positively charged nanoassemblies with oppositely charged ions or serum proteins that can lead to polyplex dissociation and/or aggregation [7, 23].

The lipopolyplexes prepared at a lipid/DNA weight ratio = 20 demonstrated greater transfection efficiency than unlipidated pHPMA-b-pDMAEMA polyplexes. These biological results were comparable to those obtained with TurboFect™ control polyplexes. Naked DNA failed to induce effective GFP expression, which is generally attributed to the polyanionic macromolecular nature of DNA and susceptibility to enzymatic degradation by nucleases [29, 30]. Earlier, Simberg and colleagues assessed the dynamic properties of lipid–serum interactions [9] and demonstrated a reversible mode of serum protein binding to cationic lipids. Following intravenous administration, serum proteins are predicted to instantaneously bind to lipid-based nanocarriers. During cellular uptake, however, surface-adsorbed serum proteins appear to dissociate from the lipid "corona" as a consequence of molecular interactions with extracellular proteoglycans. Interestingly, the lipid-enhancing effect was critically dependent on the specific lipid/DNA ratio selected. In the presence of reduced lipids (i.e., lipid/DNA = 10, w/w), transfection efficiency was not significantly different from that of pHPMA-b-pDMAEMA polyplexes. This may be attributed to formation of an incomplete lipid "corona" surrounding the polyplex core and/or insufficient thickness of the lipid layer that could effectively prevent surface adsorption of serum proteins and oppositely charged ions [28]. In contrast, transfection efficiency at a lipid/DNA weight ratio of 40 was significantly reduced. This may be attributed to competition of excessive cationic lipids with pHPMA-b-pDMAEMA polymer for DNA molecules, which induces destabilization of the polyplex core [24]. As a consequence, oppositely charged ions or serum proteins are capable of interfering with the delicate charge balance present in these electrostatically stabilized association complexes inducing dissociation followed by subsequent aggregation [8], which is consistent with the experimentally determined increase in particle size distribution measured in the presence of FBS.

Conclusion

In this study, coating of pHPMA-b-pDMAEMA polyplexes with an equimolar mixture of DOTAP/DOPE at lipid/DNA weight ratios up to 40 resulted in lipopolyplexes with a mean hydrodynamic diameter between 100-200 nm. The lipid/DNA weight ratio = 20 induced maximum polyplex stability in the presence of serum while limiting undesired cytotoxicity on HeLa cells. Most importantly, pHPMA-b-pDMAEMA-based lipopolyplexes fabricated at a lipid/DNA weight ratio of 20 induced significantly greater transgene expression in HeLa cells in the presence of serum when compared to unlipidated control polyplexes prepared with the commercially available TurboFect™ transfection reagent. The demonstrated resistance towards serum-induced dissociation and/or aggregation of these electrostatically stabilized association complexes, combined with the high transfection efficiency in vitro, render the fabricated lipopolyplexes promising candidates for in vivo gene delivery applications.

Acknowledgements

The authors would like to thank Dr. Helen Jones and Charles Klanke (Cincinnati Children`s Hospital Medical Center, Cincinnati, OH) for providing the GFP-expression plasmid. This research was supported in part by a predoctoral fellowship from the Egyptian Ministry of Higher Education awarded to Noura H. Abd Ellah.

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Figure 1

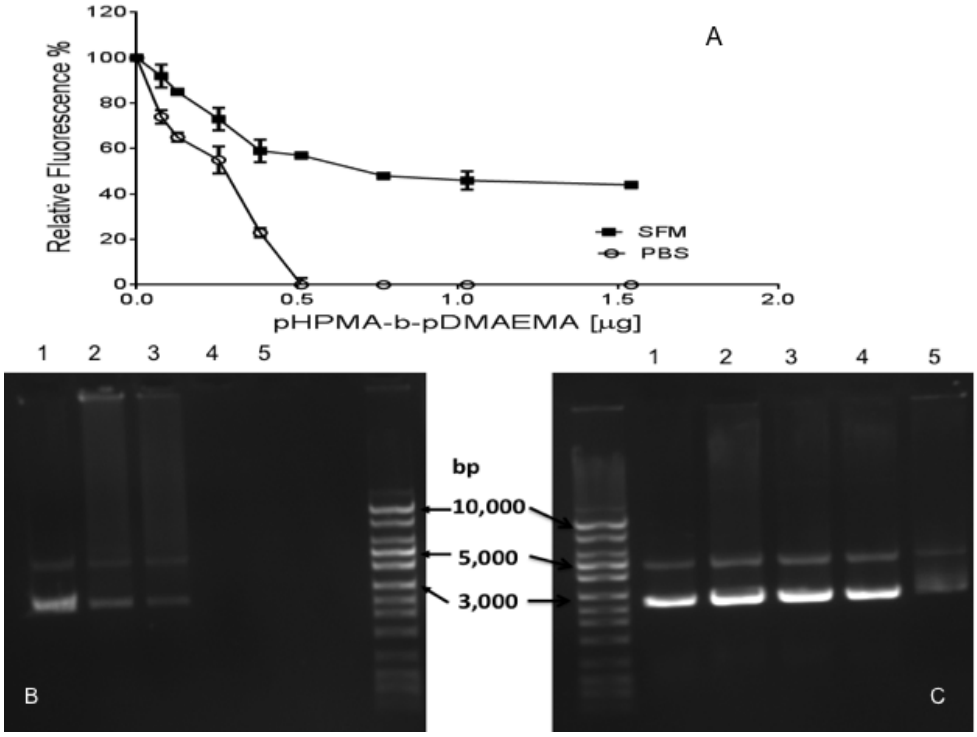

Fig. 1. Vehicle effect on polyplex formation with pHPMA-b-pDMAEMA. EtBr displacement from pDNA in PBS (○) and SFM (■) was quantified in the presence of 0-1.5 µg of pHPMA-b-pDMAEMA using fluorescence spectrophotometry (Panel A). Results are shown as mean ± SD (n=3). Representative agarose gel pictures after electrophoretic separation of pHPMA-b-pDMAEMA/pDNA complexes prepared at different mass ratios with 1 µg of pAcGFP1-C1 in PBS and SFM are shown in Panel B and C, respectively. Lane 1: DNA control, Lane 2: 0.128 µg polymer, Lane 3: 0.256 µg polymer, Lane 4: 0.513 µg polymer, Lane 5: 0.2052 µg polymer.

Figure 2

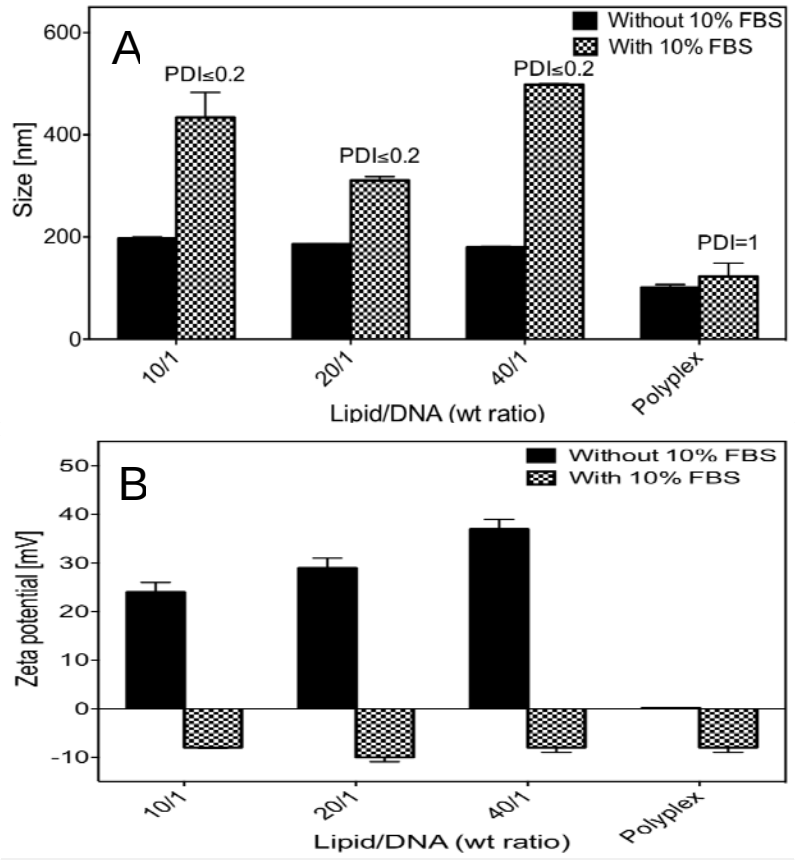

Fig. 2. Effect of lipid/DNA weight ratio on physicochemical properties of lipopolyplexes. Average particle size (Panel A) and zeta potential (Panel B) of lipopolyplexes before and after incubation with 10% (v/v) FBS.

Figure 3

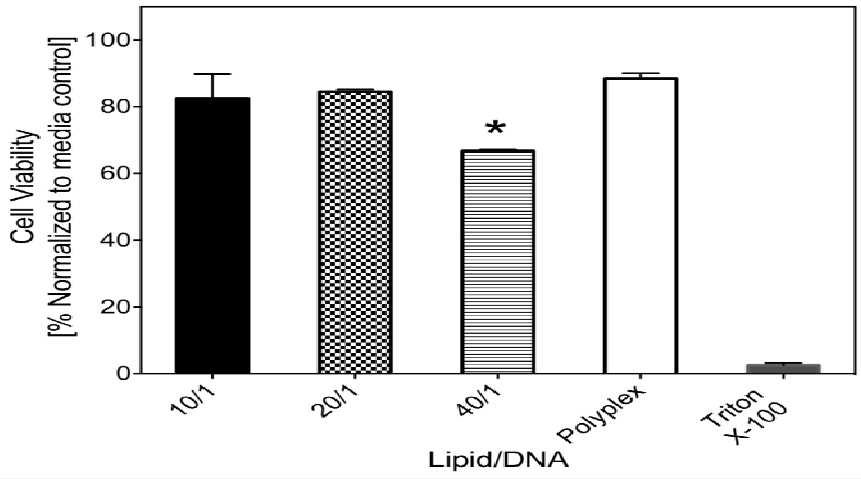

Fig. 3. Cellular safety of lipopolyplexes fabricated at lipid/DNA ratios ranging from 0-40 (w/w). HeLa cells were incubated for 4 hrs in 10% (v/v) FBS with the lipopolyplexes. Cell viability was quantified 48 hrs post-treatment using the CellTiter-Glo® assay. Results are normalized to media control and shown as mean ± SD (n=3). *Significantly different ($p<0.05$).

Figure 4

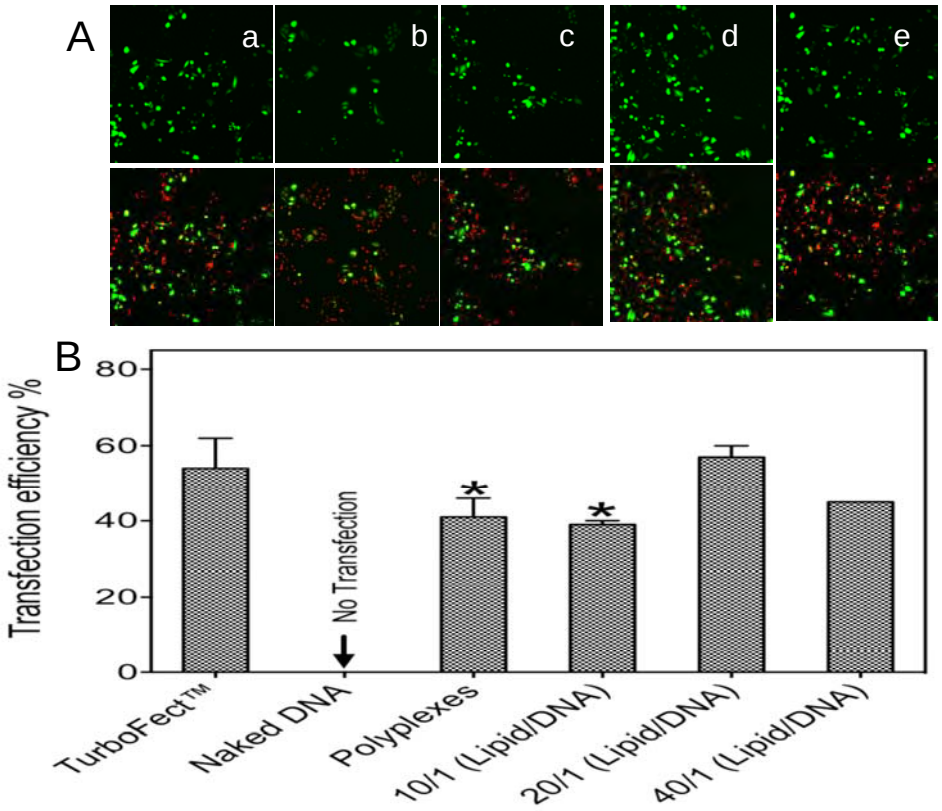

Fig. 4. Transfection efficiency of pHPMA-b-pDMAEMA-based polyplexes and lipopolyplexes in HeLa cells in the presence of serum. HeLa cells were incubated for 4 hrs in the presence of 10% (v/v) FBS with polyplexes fabricated with pHPMA-b-pDMAEMA at N/P=4 or lipopolyplexes at lipid/DNA ratios up to 40 (w/w). Nanoassemblies were prepared with 1 µg of pAcGFP1-C1. TurboFect™/pDNA polyplexes were used as positive control. GFP-expressing cells were identified 48 hrs post-treatment by confocal microscopy. Panel A shows representative confocal images (top row: GFP channel, bottom row: merged GFP and DRAQ5 (nuclear stain) picture; a = TurboFect™ polyplexes (control), b = pHPMA-b-pDMAEMA polyplexes, N/P=4, c, d, and e = Lipopolyplexes at lipid/DNA weight ratio of 10, 20 and 40, respectively). Transfection efficiency was quantified by determining the percentage of GFP-positive cells in three random sections (Panel B). Data are shown as mean ± SD (n = 3).