Efficient siRNA delivery using osmotically active and biodegradable poly(ester amine)

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Abstract

Biodegradable and hyperbranched poly(ester amine) (PEA) was prepared by reaction of glycerol dimethacrylate (GDM) with low molecular weight polyethylenimine (LMW-PEI) by Michael addition reaction. This novel gene carrier showed excellent physicochemical properties and relatively low cytotoxicity compared with PEI 25K. It showed excellent transfection efficiency and siRNA delivery. The higher silencing efficiency of PEAs could be attributed to the synergistic effect arising from hyperosmotic glycerol and proton sponge active PEI residues in the PEA backbone.

Keywords: Poly (ester amine), hyperosmotic effect, proton sponge effect, siRNA delivery.

Introduction

Enormous research is being performed to better understand the mechanism of RNA interference (RNAi) in mammalian cells, making *in vivo* therapeutic applications of RNAi increasingly likely to emerge soon. However, systemic application of virally delivered siRNA duplexes and related RNAi products are unlikely to be viable in the near future, due to host immune responses upon repeated delivery and ineffective tumor targeting. Various groups reported non-viral systemic delivery approaches by high-volume tail vein injection or high pressure-injection of nucleic acids. However, these methods lacked the suitability and showed unacceptability in routine clinical applications in humans [1-3]. Numerous systemic administrations of siRNA to mice have been reported using naked siRNA[4, 5], plasmids expressing short hairpin RNA[6, 7] lipid- formulated siRNA [8] or polycation formulated siRNA [9-11]. There are several reports describing the *in vivo* success with the siRNA by direct delivery of naked siRNA. However, naked siRNA requires chemical stabilization for *in vivo* use [4, 12]. Like single stranded antisense agents, naked siRNAs have also non-specific biodistribution [13] which requires larger doses and repeated doses for efficacy [4]. Thus the principal challenge that remains in achieving the broadest application of RNAi therapeutics is the hurdle of delivery.

Among the several approaches for delivery of RNAi therapeutics, lipids and polymer-based nanoparticle approaches are the most widely used for the systemic delivery of siRNA *in vivo*. Although the complexity of these strategies is a key developmental challenge, they represent a promising potential approach for the development of RNAi therapeutics. With the current formulation and delivery approaches for siRNA, polymeric carriers have immense capability and therapeutic potential. Despite much recent progress, new chemistry and delivery approaches are greatly needed to systemically silence disease-causing genes in a tissue specific manner with high efficiencies and at clinically achievable [14]. To date, various cationic polymers showed their potential as a successful gene carrier owing to their versatility in chemical structure. Dynamic polyconjugates[15], cyclodextrin-based nanoparticles [16], atelocollagen (protease treated collagen) [17], chitosan [18, 19] as the various polymeric approaches have been investigated broadly as siRNA carrier because they can successfully self-assemble and condense with siRNA into the structures small enough to enter the cells through endocytosis. PEI also showed remarkable success in delivering siRNA, however, its cytotoxicity owing to its non-degradability hindered its use and gave the birth of development of biodegradable polymers [11, 20-25].

Poly(ester amine)s (PEAs) synthesized by Michael addition reaction via conjugate addition of amines to acrylate resulted in biodegradable esters [26]. The biodegradable PEAs easily undergo hydrolysis of their ester backbone leading to formation of low molecular weight bis(β-amino acids) and diol moieties which are less toxic than their parent polymers [27]. Our group has developed several biodegradable gene carriers based on LMW PEI with poly(ethylene glycol) (PEG) [28], polycaprolactone [29], poloxamer [30], glycerol dimethacrylate (GDM) [31]. All these PEAs successfully transfected pDNA and showed excellent gene expression than PEI *in vitro* and *in vivo*. We therefore, investigated siRNA delivery ability of some

of these PEAs (PEG-alt-PEI) and another new carrier with reductable polysperimine backbone which showed remarkable Akt1 silencing efficiencies [32, 33]. Both these polymers showed strong gene silencing effect in A549 cell line. Since, PEAs based on GDM and LMW PEI being superior to DNA condensation, their polyplexs to be uptaken by cells and showed better trafficking as well as subsequent dissociation of polyplexes prompted us to evaluate its plausible implications as siRNA delivery. In this study, we examined the ability of biodegradable PEAs based on GDM and LMW PEI to delivery siGFP to silence the GFP expression.

Experimental details

Materials

Branched PEI (1.2 and 25 kDa), glycerol dimethacrylate (GDM) (Mn: 228.25 Da), and anhydrous methanol were purchased from Sigma (St. Louis, MO, USA) and were used as received. siRNAs were purchased from Ambion (Austin, TX, USA) and pEGFP-N_2, which has the early promoter of CMV and enhanced green fluorescence protein (EGFP) gene, was obtained from Clontech (Palo Alto, CA, USA). The plasmid was amplified with a competent E.Coli strain JM109.

Synthesis and characterization of PEA

PEAs were successfully synthesized following modified Michael addition reaction and characterized according to the parameters reported previously [29].

Formulation and characterization of polyplexes

Plasmid DNA or siRNA was diluted, in an Eppendorf tube with either, sodium acetate buffer pH 7.4 or double distilled water in a concentration ranging from 0.5 to 3.0 µg. Polycation was added to a second Eppendorf tube in a two-fold volume of appropriate buffer/water. The nucleic acid was added to the PEA solution, mixed by vortexing, and polyplexes were formed by incubation at room temperature for 20–30 min. After polyplex preparation as described above, the morphology was studied using energy filtered transmission electron microscopy (EF TEM) (JEM 1010, JEOL, Japan) by putting one drop of PEA/siRNA complexes on copper grid and was observed after staining with 1% uranyl acetate for 1min.

GFP silencing studies

A549 and HeLa cells (ATCC; 2×10^5 cells/well) were seeded in a 12-well plate (SPL Life Sciences) and allowed to attach overnight in RPMI 1640 media (10% FBS; Hyclone, South Logan, UT). Complexes between Lipofectamine™ and plasmid pEGFP-N_2 were prepared as per the manufacture's protocol and cells were transfected for expressing stable EGFP protein. For silencing study, complexes between PEA (N/P = 30) and siGFP/scrsiRNA were prepared at different concentrations of siRNA ranging from 1 to 5 µg. Complexes were prepared in the presence of nuclease free sterile water by incubating for 30 min. Similarly, complexes were prepared between siGFP/scrsiRNA and PEI25K at 10:1 N/P ratio. After complexation, mixtures were diluted with serum free media to make a final volume of 1 ml and cells were transfected. After 48 h incubation, the efficiency of silencing was measured by FACS. The percent EGFP silencing was calculated after normalizing the results with mock and scrambled (scrsiRNA) treated cells. All experiments were performed in triplicates.

Results and discussions

Synthesis and characterization of PEA

PEA was synthesized by reacting LMW-PEI and GDM in anhydrous methanol through a Michael addition reaction **(Fig. 1).** Unlike our previous work [29], the reaction was carried out at a slightly elevated temperature (60ºC) to increase the cross-linking and hence the molecular weight of the synthesized polymer. Amine groups of PEI reacted with dimethacrylate terminals of GDM in an order of their nucleophilicity. The formation and composition of synthesized PEAs were confirmed through ^{1}H NMR spectroscopy (data not shown). The composition of PEAs was found to be variable depending on the stoichiometric feed ratio of the reactants [31]. Monomer structure, solvent choice and reaction temperature generally affect the average molecular weight of polymers which lies in the range of 2000 to 50,000 [26, 27]. However, the average molecular weights of the synthesized PEA was found not to vary widely (7,600-8,000 Da) probably indicating difficulty in controlling cross-linking by simple change of reactant feed ratios. Biodegradability of drug/gene delivery system does not imply breakdown of the carrier molecule immediately after application, but rather slow degradation in order to circumvent long term toxicity in the organism. The kinetics of ester bond degradation of PEAs was investigated by measuring the reduction in molecular weight [31]. The half-life of PEA was found to be 9 to 10 days, suggesting the controlled fashion degradation [31].

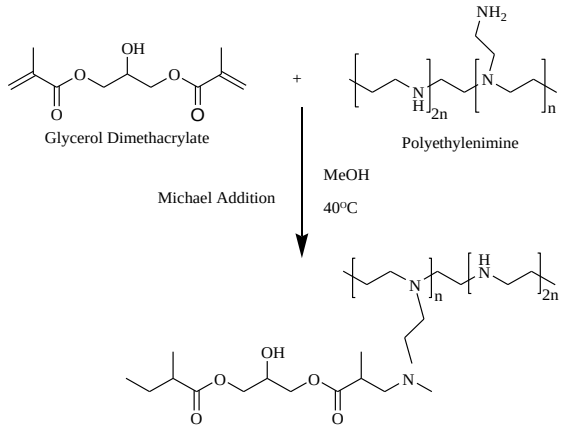

Fig. 1. Synthesis scheme for PEA.

Polyplex characteristics

The apparent ability of PEA to form more condensed siRNA complexes was demonstrated by agarose gel electrophoresis assay. As shown in **Fig. 2 (a)**, PEAs efficiently complexed siRNA at concentrations from 0.5 to 3.0 µg. In contrast, siRNA band was fully arrogated with mobility. It was observed that siRNA was retained at even 0.5 µg concentration itself. Incubation of polyplexes with 10-20% heparin resulted no exchange or leaching of siRNA. Observation of siRNA/PEA polyplexes morphologies revealed the interesting results where spherical polylexes below 100 nm were found as shown in **Fig. 2(c)**. Polyplexes revealed the uniform size distribution with particle sizes below 150 nm suitable for intracellular delivery **[Fig. 2(b)]**. PEAs showed significantly lower cytotoxicity in three different cell lines when compared with PEI 25K by MTS assay as shown in our previous reports [31].

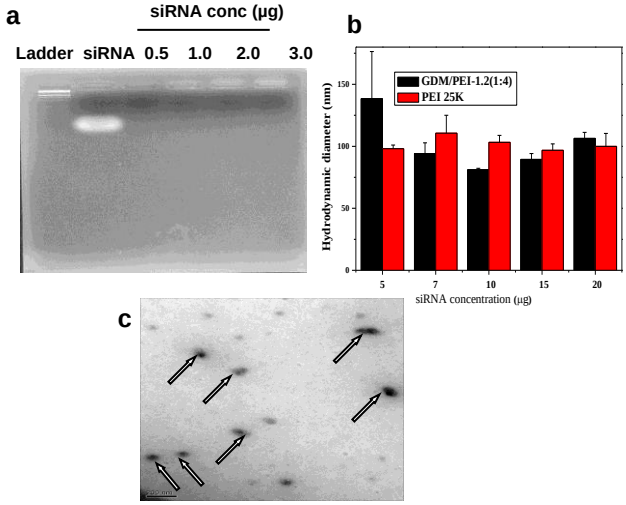

Fig. 2. (a) Agarosge gel electrophoresis study, (b) particle size measurement by DLS and (c) morphology of polyplexes.

EGFP silencing study

The ability of PEAs/siRNA complexes to induce gene silencing of GFP gene was tested in HeLa and A549 cells transfected with Lipofectamine/pGFP complexes. As a control, complexes containing non-specific siRNA (i.e. siscrGFP with no known targets in the cells) were added to the cells under identical conditions to ascertain that the reduction in the EGFP expression was due to a siGFP effect and not to non-specific effects such as INF induction. The gene silencing was observed at 38 h after the Lipofeactamine/pGFP transfection which was performed according to the manufacturer's protocol. Higher concentration of siRNA (2 µg) showed 3-4 fold higher GFP silencing than that of the 1 µg siRNA in HeLa cells. In case of A549 cells, higher amounts of siRNA revealed ~ 70% GFP silencing in a non-linear manner as per our previous reports **(Fig. 3 a & 3b)** [32, 33]. At 2 µg siGFP concentration silencing achieved with PEA was merely higher than 1 µg concentration. However, dose-dependant manner silencing was achieved when 3 µg siGFP was delivered, indicating the safety and biodegradability of ester linkage in PEA which on one hand supports the safety and non-toxicity and on the other hand facilitates the efficient gene transfer.31 In contrast PEI 25K at N/P ratio 10 using 1 µg siGFP silenced 40% GFP similar to the 1 µg siGFP concentration. PEA revealed excellent delivery of siRNA at all siGFP concentrations, indicating its potential as safe and superior gene carrier.

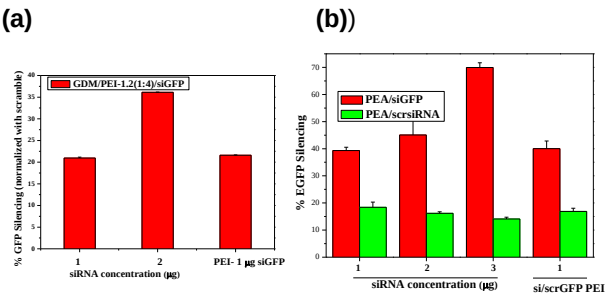

Fig. 3. *In vitro* GFP silencing efficiency of PEA/siGFP by flow cytometry assay on (a) Hela and (b) A549 cells.

Conclusions

In conclusion, we have demonstrated that usefulness of biodegradable polycations is essential for the safe and successful gene delivery. As previously described, PEAs based on GDM and LMW-PEI revealed superior transfection efficiency *in vitro* as well as *in vivo*. PEAs also exhibited excellent siRNA transfection *in vitro* for silencing studies along with excellent biophysical and physicochemical properties suitable for intracellular delivery. The higher silencing efficiency of PEAs could be attributed to the synergistic effect arising from hyperosmotic glycerol and proton sponge active PEI residues in the PEA backbone. The PEA can be used as efficient polymeric vectors which provide a versatile platform for further investigation of structure property relationship along with the controlled degradation, significant low cytotoxicity and high silencing efficiency.

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