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Reducible poly(amido ethylenimine) directed to enhance RNA interference

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Abstract

Designing synthetic macromolecular vehicles with high transfection efficiency and low cytotoxicity has been a major interest in the development of non-viral gene carriers. A reducible poly(amido ethylenimine) (SS-PAEI) synthesized by addition copolymerization of triethylenetetramine and cystamine bis-acrylamide (poly(TETA/CBA)) was used as a carrier for small interference RNA (siRNA). Poly(TETA/CBA) could efficiently condense siRNA to form stable complexes under physiological conditions and perform complete release of siRNA in a reductive environment. When formulated with VEGF-directed siRNA, poly(TETA/CBA) demonstrated significantly higher suppression of VEGF than linear-polyethylenimine (PEI) (L-PEI, 25 kDa) in human prostate cancer cells (PC-3). After 5 h of transfection, substantial dissociation and intracellular distribution of siRNA was observed in the poly(TETA/CBA) formulation, but not in the L-PEI formulation. The triggered release of siRNA by reductive degradation of poly(TETA/CBA) in the cytoplasm may affect the RNAi activity by increasing cytoplasmic availability of siRNA. These results suggest that the rational design of non-viral carriers should involve considerations for intracellular dissociation and trafficking of a nucleic acid drug to maximize its effect, in conjunction with formation of stable complexes under physiological conditions.

Keywords: Poly(amido ethylenimine); siRNA; Reducible polymer; Non-viral gene delivery

1. Introduction

RNA interference (RNAi) has attracted a lot of attention due to its ability to silence gene expression in a sequence-specific manner and shown enormous potential as a powerful therapeutic strategy for treating various gene-related diseases [1–5]. RNAi is induced by 21–23 bp short interfering RNA, which elicits RNA-mediated endonucleolytic cleavage of a target mRNA by incorporating into the RNA-induced silencing complex (RISC) [6–8]. However, due to its inherent instability and poor permeability across biological membranes, the successful application of small interference RNA (siRNA) in mammalian cells largely depends on the development of safe and efficient carriers for its delivery [9]. Several polycations have been introduced as non-viral gene carriers with a capability of forming stable complexes by electrostatic interaction with siRNA. The complex formation leads to the improved protection of the siRNA from the enzyme-mediated digestion and the enhanced intracellular delivery [10–14]. Besides the essential characteristics of the polycationic carriers, it should be noted that unpacking the complexes for the release of intact siRNA into the cytosol is required to realize the maximum gene-silencing activity of the siRNA.

Reducible cationic polymers composed of low-molecular weight polycations crosslinked with disulfide linkages have demonstrated efficient gene delivery and expression without significant cytotoxicity [15,16]. The reducible gene carriers may achieve efficient uncoupling of polymer/DNA complexes by the cleavage of disulfide linkages in a

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reductive intracellular environment, while providing protection for DNA from extracellular enzymatic digestion by forming stable complexes and facilitating endocytosis-based cellular uptake. The triggered release characteristics of the reducible polymers may meet the requirement of siRNA delivery, since siRNA should be released from the carrier in the cytosolic compartment to activate a specific RNAi by binding to the RNA-dependent endonuclease complex (RISC).

We have recently synthesized new poly(amido ethylenimine)s (SS-PAEIs) containing multiple disulfide bonds by polyaddition of cystamine bis-acrylamide and various ethylenimine monomers. The SS-PAEIs formed stable complexes with plasmid DNA and showed higher transfection efficiency with significantly lower cytotoxicity than a conventional high molecular weight PEI (25 kDa) [17]. In this study, a reducible copolymer of triethylenetetramine and cystamine bis-acrylamide (poly(TETA/CBA)) was synthesized and used as a carrier for the triggered intracellular release and activation of siRNA. The relationship between efficient intracellular unpacking of poly (TETA/CBA)/siRNA complexes and enhanced RNAi activity was investigated.

2. Materials and methods

2.1. Materials

TETA, ethidium bromide, (3-(4,5-dimethylthyazolyl-2)-2,5-diphenyl tetrazolium bromide) (MTT), and DL-buthionine sulfoxamine (BSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cystamine bisacrylamide (CBA) and linear polyethylenimne (L-PEI, M_w 25,000) was obtained from Polysciences (Warrington, PA, USA). A VEGF-targeting siRNA (target sequence: 5′-GGAGUACCCUGAUGAGAUC-3′ (bases 189–207), sense: 5′-GGAGUACCCUGAUGAGAUCdTdT-3′, antisense: 5′-GAUCUCAUCAGGGUACUCCdTdT-3′) and VEGF siRNA labeled with FITC at 5′ terminal of the sense strand was synthesized, modified and purified by Qiagen (Valencia, CA, USA). All cell culture products including fetal bovine serum (FBS) and Roswell Park Memorial Institute 1640 medium (RPMI 1640) were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Synthesis of poly(TETA/CBA)

The synthetic scheme of poly(TETA/CBA) copolymer is shown in Fig. 1. The poly(TETA/CBA) copolymer was synthesized as previously described [17]. Briefly, TETA (449 mg, 3.1 mmol) and CBA (800 mg, 3.1 mmol) dissolved in 10% aqueous methanol was placed in a light-protected flask equipped with a stirring bar. The addition reaction was performed under an argon atmosphere at 50 °C. After 16 h, excess amount of amine (TETA) was added to stop the polymerization by blocking any unreacted acrylate groups. The reaction was carried out at the same condition for an additional 2 h. The resulting product was diluted by adding 10 ml of deionized water, purified by ultrafiltration (MWCO 1000), and freeze-dried. Composition of poly(TETA/CBA) was established by using ^{1}H-NMR in D_2O: δ 2.61 (COC**H**$_2$CH$_2$NH, 4H), 2.72 (NHCH$_2$C**H**$_2$S–S, 4H), 2.90–3.21 (COCH2C**H**$_2$NHC**H**$_2$C**H**$_2$, 16H), 3.41 (NHC**H**$_2$CH$_2$S–S, 4H). The molecular weight of poly(TETA/CBA) was determined by using gel permeation chromatography (GPC). GPC analysis was performed in two serially attached columns (PL aquagel-OH 30, Polymer Labs) equipped with a thermostat at 30 °C. The mobile phase was maintained in sodium acetate buffer (0.3 M NaAc, pH 4.4) with 30% methanol (v/v) at a flow rate of 0.5 ml/min. Polyethyleneglycol (PEG) molecular weight standards were used for preparing a standard calibration curve.

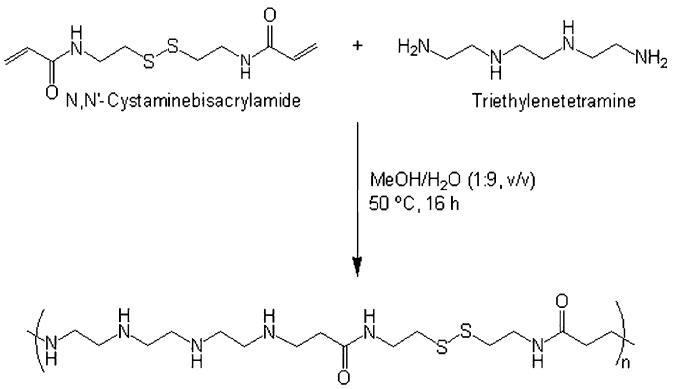

Fig. 1. Chemical structure of a reducible copolymer of triethylenetetramine (TETA) and cystamine bis-acrylamide (CBA).

2.3. Electrophoretic mobility shift assays

Varying amount of poly(TETA/CBA) and a fixed amount of siRNA (0.3 μg) were separately diluted in PBS. The diluted polymer and siRNA solutions were combined at varying weight ratios and mixed with gentle vortexing to form complexes. The complexes were allowed to be stabilized for 30 min at an ambient temperature and loaded onto 1.5% agarose gel. Electrophoresis was carried out with a current of 120 V for 20 min in TAE buffer solution (40 mM Tris/HCl, 1% (v/v) acetic acid, 1 mM EDTA). The retardation of the complexes was visualized by using an image analyzer equipped with UV transilluminator (GelDoc, BioRad, Hercules, CA, USA) after ethidium bromide staining.

2.4. Cell culture and transfection

Human prostate carcinoma cells (PC-3, American Type Culture Collection) was cultured in RPMI1640 medium supplemented with 10% FBS, streptomycin (100 μg/ml), penicillin (100 IU/ml), and 2 mM L-glutamine. The cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. The cells (1×10^5) were seeded in 12-well plates and incubated either in the presence or absence of 20 mM BSO, which decreases the intracellular level of reduced glutathione, for 24 h prior to transfection. The cell culture medium was replaced with serum-free medium prior to transfection. The complexes with a desired formulation were added to the cells. For VEGF assays, the transfection medium was removed after 3 h incubation and supplemented with fresh medium containing 10% FBS. The incubation was continued for 6 h and the medium was replaced with new RPMI1640 medium containing 10% FBS and heparin (20 μg/ml). The medium was collected after 16 h of incubation and the amount of VEGF in the medium was subjected to be analyzed using a human VEGF immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendation.

2.5. Confocal microscopy and flow cytometry

The PC-3 cells were grown in confocal imaging dishes (Glass Bottom Microwells; MatTek Corp., Ashland, MA, USA) for confocal microscopy and 35-mm dishes for flow cytometry. The transfection procedure was essentially performed as described in the transfection section except the use of siRNA labeled with FITC (100 nM).

For confocal microscopy, the transfected cells were washed five times with cold PBS, fixed by submerging the cells in 1% paraformaldehyde at 4 °C for 30 min, and washed three times with cold PBS. The subcellular

localization of the complexes were visualized by a laser scanning confocal fluorescence microscopy using Olympus Fluoview FV300 microscope (Melville, NY, USA). An argon/krypton mixed gas laser with excitation line at 495 nm was used to induce FITC fluorescence. An oil immersion objective lens was used for the epidetection configuration. Three-dimensional image rendering was done by using Velocity software (Improvision Inc., Lexington, MA, USA).

For flow cytometric analysis, the transfected cells were trypsinized, washed three times with cold PBS, and fixed by incubating the cells with 1% paraformaldehyde at 4 °C for 30 min. The fixed cells were washed two times with cold PBS and stored in 0.1% paraformaldehyde at 4 °C before carrying out flow cytometry. The extent of cellular association of the complexes was determined by using flow cytometry (FACS Caliber, Becton-Dickinson, Mountain View, CA, USA). At least 10,000 events were analyzed to generate each histogram.

3. Results and discussion

We hypothesized that the triggered release of siRNA from the complex in the cytoplasmic compartment would facilitate the access of siRNA to the RISC, and thereby enhance the corresponding RNAi in the cells. To address this hypothesis, a low molecular weight peptidomimetic PAEI, poly(TETA/CBA), containing multiple disulfide linkages, was synthesized by copolymerizing CBA with TETA via a Michael-type addition (Fig. 1). Molecular weight of poly(TETA/CBA) was 3350 (M_w) as determined by GPC. The polymer showed a relatively low polydispersity ($M_w/M_n = 1.45$) compared to the commercially available PEIs [17]. The disulfide linkages in poly(TETA/CBA) are expected to be cleaved into amidoamine monomers containing five secondary amines in the reductive cytoplasm.

The influence of poly(TETA/CBA) polymer on cell viability was monitored using an MTT assay [18]. Relative cell viability was determined against cells not receiving polymer solutions. Poly(TETA/CBA) and L-PEI showed a relative cell viability of 50% at the concentration of 500 and 50 μg/ml, respectively (Fig. 2). Low cytotoxicity of poly(TETA/CBA) is presumably due to its lower molecular weight and charge density, in conjunction with biodegradability. It was reported that the molecular weight and charge density of a polymer play a more important role in cytotoxcity than the total amount of charges [19].

The siRNA condensation property of poly(TETA/CBA) was assessed by observing the electrophoretic mobility shift of the band in an agarose gel (Fig. 3). The polymer could effectively condense siRNA at and above the weight ratio of 3:1 (polymer/siRNA). To determine the ability of poly(TETA/CBA)/siRNA complexes to be uncoupled by reductive force, the complexes were preincubated with 2.5 mM DTT prior to electrophoresis. The presence of reductive force caused the complete release of siRNA from the complex in the electrophoretic field, suggesting the

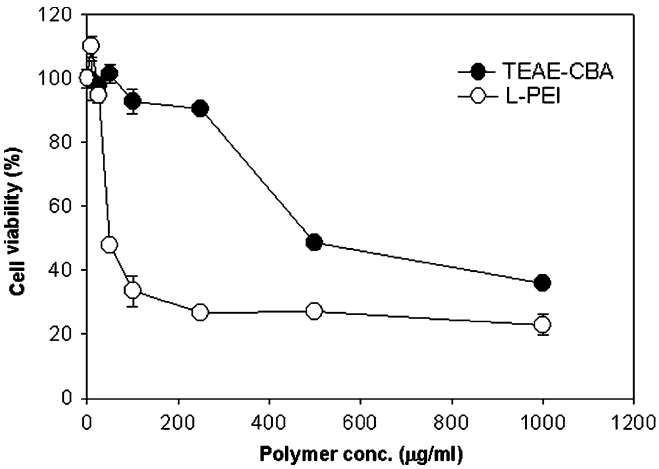

Fig. 2. Viability of PC-3 cells as a function of concentration of L-PEI 25k and TETA-CBA. Relative cell viability was determined using an MTT assay where survival percentages were calculated as compared to an untreated control (100% survival).

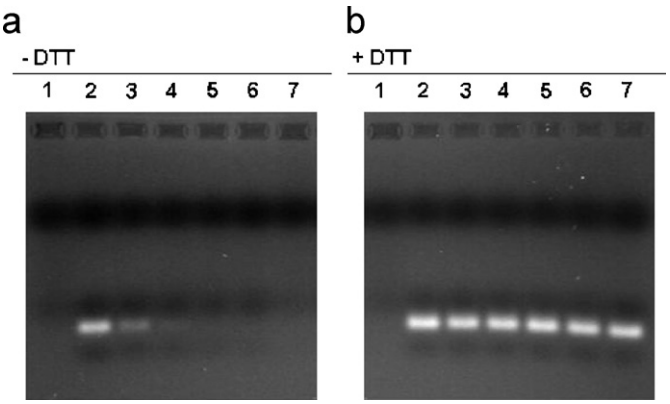

Fig. 3. Electrophoretic mobility shift assay of poly(TETA/CBA)/siRNA complexes at various polymer/siRNA weight ratios: (a) lane 1, L-PEI/siRNA at 1.2:1 (w/w); lane 2, naked siRNA; lanes 3–7, poly(TETA/CBA)/siRNA with polymer/siRNA weight ratios (w/w) of 1, 2, 3, 6, and 12; and (b) was performed in the same conditions as (a) except for the presence of 2.5 mM DTT.

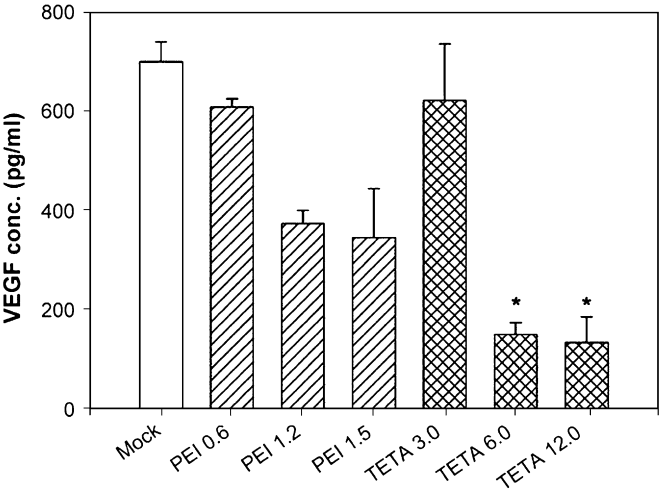

Fig. 4. RNAi-induced suppression of VEGF expression in PC-3 cells after transfection with poly(TETA/CBA) and L-PEI complex formulations. Number stands for the weight ratio (polymer/siRNA) of each formulation. Asterisk indicates statistical significance ($p < 0.01$) between the two polymers, as determined by Student's t-test.

possibility of the efficient destabilization of the complex, followed by the release of siRNA in a reductive milieu of cytosol. Previously synthesized cationic polymers containing multiple disulfide linkages also demonstrated the efficient release of a plasmid DNA in the presence of the reductive force [15,16].

The significance of poly(TETA/CBA) as a carrier for siRNA was evaluated by carrying out transfection experiments in human prostate cancer cells (PC-3). An siRNA silencing human vascular endothelial growth factor (VEGF siRNA, 30 nM) was used in the transfections and the amount of VEGF secreted from the PC-3 cells was measured by ELISA. The poly(TETA/CBA)/VEGF siRNA complexes (a weight ratio of the polymer to siRNA, w/w, of 6) exhibited much higher RNAi activity against VEGF expression than the L-PEI/VEGF siRNA complexes (w/w ratio = 1.25) (Fig. 4). The complexes prepared at higher weight ratios of the polymer to siRNA (12 and 1.5 for poly(TETA/CBA) and L-PEI, respectively) caused no significant changes in the suppression of VEGF expression. In addition, all the transfection formulations did not elicit any detectable cytotoxicity in PC-3 cells (data not shown). Based on the suggested hypothesis, the enhanced RNAi activity with the reducible poly(TETA/CBA) polymer could be due to environmentally triggered release of the siRNA in the cytoplasmic space. In similar

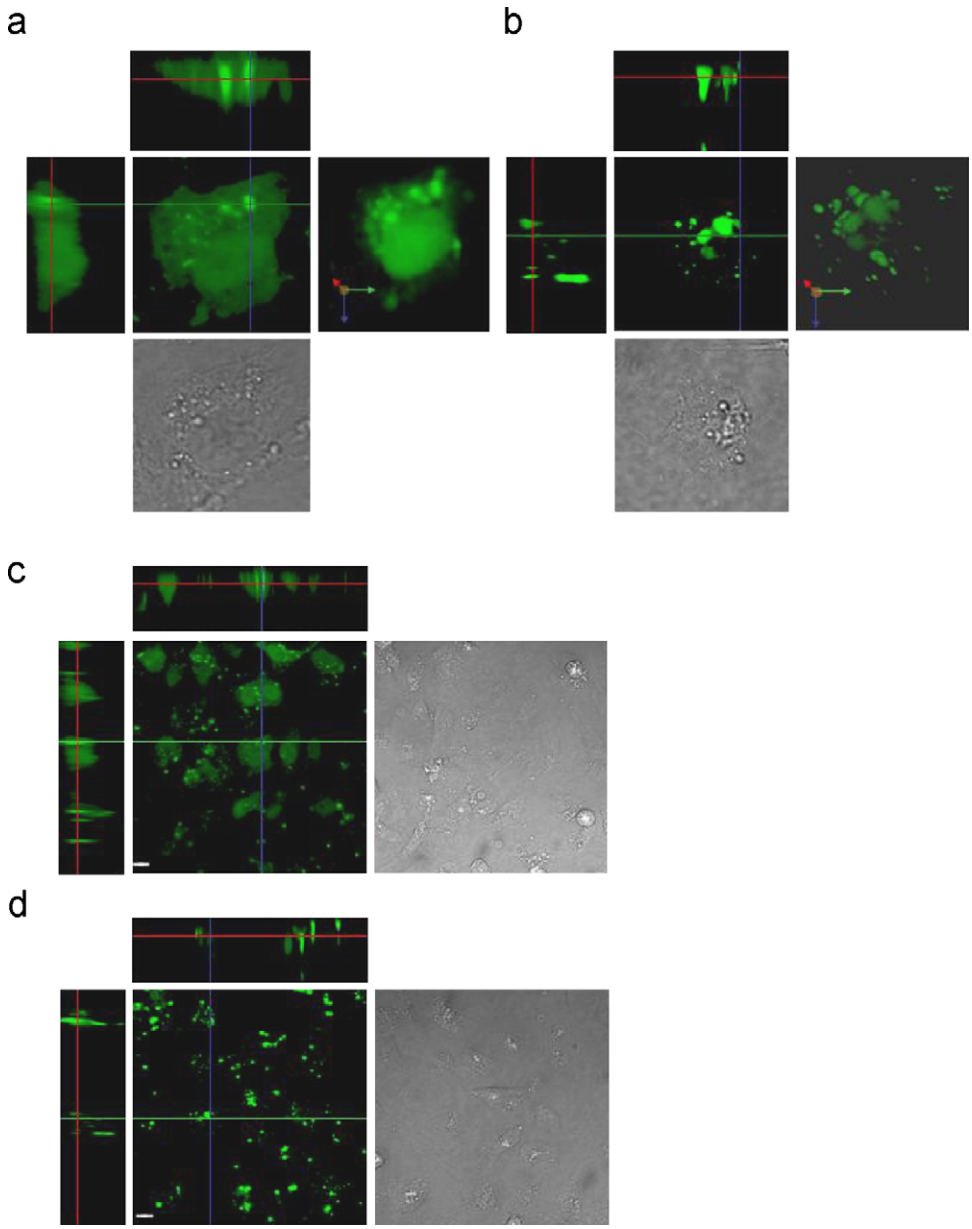

Fig. 5. Subcellular localizations of poly(TETA/CBA)/siRNA-FITC complexes (a, c) and L-PEI/ siRNA-FITC complexes (b, d) after 5 h of transfection in PC-3 cells. In panels (a) and (b), fluorescence images (600 × magnification, upper left), three-dimensionally reconstituted images of *x–y* plains (0.5 μm *z*-slices, upper right), and phase contrast images (bottom) are shown. Panels (c) and (d) present fluorescence images (left) and phase contrast images (right) at lower magnification (400 ×). Size bar = 12 μm.

circumstances, gene delivery using disulfide-crosslinked complexes achieved enhanced gene expression by complete reduction of the disulfide linkage leading to efficient dissociation of DNA from the complexes [20].

The triggered release of siRNA in the cytoplasm was visualized in PC-3 cells by confocal laser-scanning microscopy. The complexes from FITC-labeled siRNA and poly(TETA/CBA) were prepared at the weight ratio of 6 for transfection experiments. After 5 h incubation following the transfection, the siRNA was distributed over the cytoplasmic space and also localized in the nucleus, suggesting the complexes were efficiently destabilized to release siRNA by the reductive degradation of the polymer backbone (Fig. 5a and c). In contrast, the major fraction of the complexes formed from siRNA and L-PEI were preferentially localized in specific locations rather than distributed over the cytoplasm (Fig. 5b and d). The three-dimensional reconstitution of the *x–y* plains (0.5 μm *z*-slices) clearly showed most of the siRNA still exist in a form of aggregates, suggesting the PEI complexes are not prone to be decondensed to release their cargo after cellular uptake (Fig. 5b). This result agrees with previous results in which PEI/DNA complexes could obtain entry to the nucleus without a loss of the ordered structure, suggesting the limitation of PEI in the intracellular release of DNA [21,22]. The nuclear accumulation of PEI would lead to undesirable interaction with endogenous DNA or RNA, which may elicit a further cytotoxic effect.

The level of cellular association of transfection complexes could also be one of the major factors that directly affect overall transfection efficiency. Enhanced cellular association of the complexes by employing a cell-specific ligand could dramatically increase transfection efficiency [23,24]. To compare the cellular association of poly(TETA/CBA) with that of L-PEI, a fluorescently labeled siRNA was complexed with each polymer and transfected in PC-3 cells. The extent of cellular association of the respective complexes was measured using flow cytometry. The flow cytometric analysis demonstrated that there was no significant difference in the extent of the cellular association of either complex from poly(TETA/CBA) or PEI (Fig. 6). This result suggests that the enhanced RNAi activity of poly(TETA/CBA)/siRNA complexes does not come from the increased cellular uptake of the complexes.

The influence of the triggered release mechanism upon RNAi activity was further studied by altering the intracellular reducing force with BSO, which reduces intracellular level of reduced glutathione [13]. The presence of 20 mM BSO efficiently reduced the RNAi activity level of poly(TETA/CBA) formulation to that of L-PEI (Fig. 7), suggesting that the enhanced RNAi activity with the poly(TETA/CBA) polymer comes from the cytoplasmic degradation of the polymer backbone, followed by efficient dissociation of siRNA from the complexes.

Together with the results from confocal microscopy, and flow cytometry, it is reasonable to say that the enhanced RNAi activity is attributed to the improved cytosolic

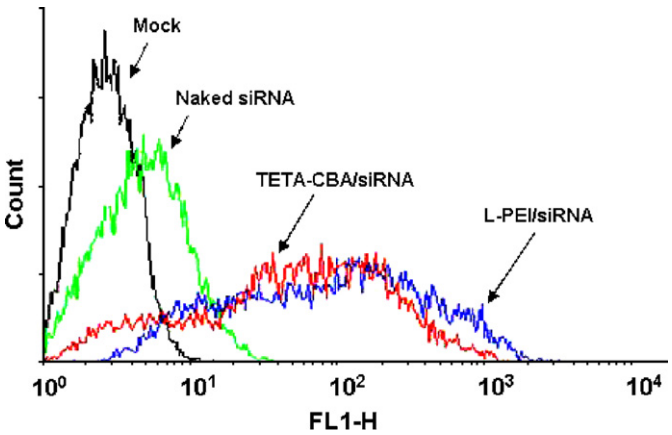

Fig. 6. Cellular association of either poly(TETA/CBA)/siRNA-FITC complexes or L-PEI/ siRNA-FITC complexes, analyzed by flow cytometry.

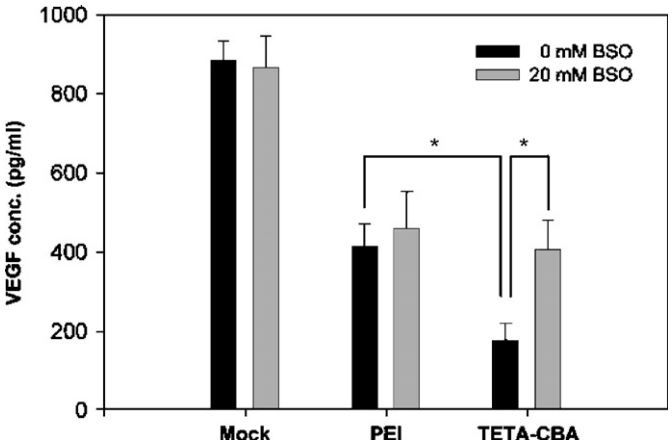

Fig. 7. Effect of DL-buthionine sulfoxamine (BSO) on the siRNA-mediate suppression of VEGF expression by poly(TETA/CBA) and L-PEI. Formulation weight ratios (polymer/siRNA) were 6.0 and 1.2 for poly(TETA/CBA) and L-PEI, respectively. Statistical difference ($p<0.01$) between the two groups was determined by Student's *t*-test (asterisk).

availability of siRNA, presumably due to the environment-responsive degradation of the disulfide-containing backbone of poly(TETA/CBA) and the release of siRNA in the reductive cytoplasmic region, which may increase the chance for the siRNA to be incorporated into RISC complex to exert the sequence-specific degradation of the target mRNA.

4. Conclusion

The incorporation of siRNA into cellular RNA-induced silencing complex (RISC) is critical event for the activation of a specific RNA-directed nuclease activity, RNAi. Since the interaction between siRNA and RISC occurs mainly in the cytoplasmic space, the availability of intact siRNA in the cytoplasm would be one of the most important factors to achieve a desired RNAi activity. This suggests that the efficient and timely unpacking of polymer/siRNA

complexes and the release of siRNA from the complexes are critical, in terms of siRNA delivery. For most of cationic polymer-based gene carriers, however, the unpacking mechanism has not been clearly understood. In this study, we demonstrated a reducible cationic polymer, poly(TETA/CBA) enhances specific RNAi by increasing the bioavailability of siRNA in the cytoplasm by a triggered activation mechanism. Poly(TETA/CBA) has an ability to condense siRNA to form stable complexes, which protect the enclosed siRNA from extracellular nuclease attacks [17] and facilitate the cellular uptake via an endocytosis. The proton-buffering capacity of poly(TETA/CBA) would help the escape of the complexes from the endosomal compartment [17]. Once located in the cytosolic space, poly(TETA/CBA) would be cleaved into amidoamine monomer units by the reduction of disulfide linkages in its backbone, owing to the relative abundance of cytosolic glutathione. This leads to destabilization and efficient unpacking of the complexes to release intact siRNA, which then interacts with RISC to initiate RNAi mechanism.

This study suggests that the approaches of using cationic polymers capable of delivering nucleic acid therapeutics to a desired subcellular location would be a promising strategy for designing efficient non-viral gene delivery systems.

Acknowledgement

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