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GENE DELIVERY

Efficient intracellular siRNA delivery strategy through rapid and simple two steps mixing involving noncovalent post-PEGylation

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ABSTRACT

Two different and well-defined methacrylate-based (co)polymers were employed as a polymeric siRNA delivery system. siRNA, poly(2-(dimethylamino) ethyl methacrylate) homopolymers (PDMAEMA) and poly (2-(dimethylamino) ethyl methacrylate)-*b*-poly (ethyleneglycol) α-methoxy, ω-methacrylate (PDMAEMA-*b*-PMAPEG) palm-tree-like copolymer ternary complexes were prepared using a rapid and simple two-step mixing protocol involving noncovalent post-PEGylation, and physicochemical properties including hydrodynamic diameter, zeta-potential and siRNA condensation efficiency were characterized. Transfection efficiency, intracellular uptake, and cytotoxicity of ternary complexes were also evaluated. Ternary complexes provide efficient condensation and compaction of siRNA within the cationic core of complexes. Noncovalent post-PEGylation provides the ternary complexes with enzymatic and serum stability without harming complex formation and condensation of siRNA. Thereby, under an optimal N/P ratio, ternary complexes exhibited brilliant gene silencing efficiency with low cytotoxicity in media containing 10% serum. Confocal microscopy clearly showed efficient and even intracellular uptake of complexes by cells via endocytosis. This study highlights the excellent properties of noncovalent post-PEGylated ternary complexes produced by rapid and simple mixing. Accordingly, these findings suggest that the formation of ternary complexes could be utilized as a safe and effective polymeric siRNA delivery strategy.

1. Introduction

Gene therapy continues to hold promise for various gene-related and acquired disorders. In particular, RNA interference (RNAi) is a highly valuable biological process and useful tool for modulating gene expression with implications for therapeutic applications. RNAi is an evolutionarily conserved genetic surveillance mechanism that is triggered by endogenous or exogenous double-strand RNA (dsRNA). The dsRNA is cleaved by Dicer into small interfering RNA (siRNA) that can induce the highly sequence-specific posttranscriptional gene silencing of target genes [1–4]. Accordingly, the targeted silencing of disease-associated genes by using synthetic siRNA has recently emerged as powerful therapeutic strategy [2,5]. However, when it is administered intravenously, inherent instability caused by enzymatic degradation and extremely low intracellular uptake of the siRNA leads to the entrapment or destruction of a majority of the siRNA before it reaches its site of action [6–8]. Therefore, the development of safe and effective carriers and/or modification strategies is still critical to the ultimate success of siRNA-mediated gene therapy [9]. Most of all, substitution or modification by hydrophilic segments such as poly (ethylene glycol) (PEG) is a common strategy for improving their biological activity and lifetime in vivo and in vitro [10–13]. Currently, novel pH responsive (co)polymers or modifications of existing (co) polymers containing hydrophilic sequences have been generated to meet the high expectations for optimized siRNA delivery systems [13]. One of the most important polymers studied so far is undoubtedly poly(2-(dimethylamino) ethyl methacrylate) (PDMAEMA). Unfortunately the strategy used to introduce the hydrophilic polymer segment into the delivery system or siRNA markedly influences the efficiency of transfection through undesirable interactions between introduced hydrophilic segments and their counterpart molecules, leading to lower capacity to condense nucleic acids, and reduced association with cells [14,15]. In the present study, two different cationic polymers, PDMAEMA and poly 2-(dimethylamino) ethyl

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methacrylate)-*b*-poly(ethyleneglycol) α-methoxy, ω-methacrylate (PDMAEMA-*b*-PMAPEG) palm-tree-like copolymer, were employed to form the noncovalent post-PEGylated ternary complexes. We prepared the ternary complexes using a rapid two-step mixing protocol, which involves the stable initial complexation of the siRNA and PDMAEMA homopolymers, followed by substantial condensation and compaction of siRNA and noncovalent post-PEGylation through the addition of PDMAEMA-*b*-PMAPEG palm-tree-like copolymers. This strategy is used for the first time for intracellular delivery of siRNA in the presence of serum, although, in our previous report, it has been described for the transfection of polyplexes [15]. Various N/P ratios of siRNA/PDMAEMA/PDMAEMA-*b*-PMAPEG ternary complexes were prepared, and their physicochemical properties including hydrodynamic diameter, zeta-potential and condensation efficiency of siRNA as a function of N/P ratio were characterized. Furthermore, their gene silencing effect, intracellular uptake, and cytotoxicity were evaluated in the presence of 10% serum.

2. Materials and methods

2.1. Reagents

2-(Dimethylamino)ethyl methacrylate (DMAEMA), and poly (ethylene glycol) α-methoxy and ω-methacrylate (MAPEG) were purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals and reagents were of analytical grade. Fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (PBS, pH 7.4), Roswell Park Memorial Institute (RPMI) 1640 medium were obtained from Gibco-Invitrogen (Grand Island, NY, USA). RNase ONE ribonuclease was purchased from Promega (Madison, WI, USA). Human vascular endothelial growth factor VEGF (VEGF) siRNA were purchased from Qiagen (Valencia, CA, USA). The target sequence of the VEGF siRNA was GGA GTA CCC TGA TGA GAT C (human VEGF, bases 189–207); sense strand: 5′-GGA GUA CCC UGA UGA GAU CdTdT-3′ and antisense strand: 5′-GAU CUC AUC AGG GUA CUC CdTdT-3′.

2.2. Synthesis of PDMAEMA homopolymers and PDMAEMA-b-PMAPEG palm-tree-like copolymers

To synthesize two different methacrylate-based polycationic (co) polymers, we used our previously reported solvent-free atom transfer radical polymerization method [15] (see Appendix A).

2.3. Preparation of complexes

Complexes were formed with various N/P ratios (ratio between the (co)polymer nitrogen atom and the siRNA phosphate). For the preparation of ternary complexes, a proper volume of PDMAEMA stock solution was mixed gently with the desired amount of siRNA and incubating for 30 min at room temperature. After the incubation, the siRNA/PDMAEMA mixture, described above, was combined with a proper volume of PDMAEMA-*b*-PMAPEG copolymer stock solution and mixed again; the samples were then incubated for 30 min at room temperature. When siRNA/PDMAEMA binary complex was tested, PBS was added after the first 30 min incubation instead of copolymer stock solution. In addition to binary complexes, siRNA/PEI complexes were prepared, according to our previous reports as a control [10,11].

2.4. Agarose gel electrophoresis

siRNA and (co)polymer complexes, which were formulated with applicable N/P ratios, were prepared. Afterward, complexes were applied to 1% (w/v) agarose gel contacting 0.5 μg/mL ethidium bromide (EtBr). EtBr fluorescence was detected by using a gel documentation system (Gel doc 2000, Bio-Rad).

2.5. Ethidium bromide assay

siRNA (5 μg) and (co)polymer complexes were prepared at different N/P ratios. Afterward, each sample was mixed with 20 μL of 0.1 mg/mL EtBr. EtBr fluorescence was measured by using a Magellan fluorometer (Tecan, Männedorf, Switzerland) at 518 nm excitation and 605 nm emission wavelengths.

2.6. Particle size and zeta-potential measurements

Measurement of the hydrodynamic diameters and zeta-potential of the complexes was performed on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Each sample was properly diluted in order to maintain a count rate of around 300. Hydrodynamic diameter and zeta-potential were determined at least in triplicate.

2.7. Atomic force microscopy

Atomic force microscopy (AFM) was used to visualize the ternary complexes. Tapping mode AFM in air was then performed using a multimode scanning probe microscope system and scanning probe microscope controller IV (Veeco Instruments, Santa Barbara, CA, USA).

2.8. RNase protection assay

Naked siRNA (5 μg) or its complexes with (co)polymers were prepared just before the experiments. After the preparation of the complexes, each sample was incubated with 50 μg × reaction buffer (Promega, Madison, WI, USA) containing 10 units of RNase ONE ribonuclease (Promega) for 30 min or 1 h at room temperature. Nucleic acid degradation was measured by gel electrophoresis. Afterward, remaining siRNA was further confirmed by heparin-releasing experiments. RNase activity was stopped following the manufacturer's protocol and by subsequent incubation with 1 IU of heparin for 15 or 30 min at room temperature. The released siRNA was visualized by electrophoresis. The levels of remaining siRNA were quantified by densitometric analysis using Quantity One software (Bio-Rad).

2.9. Cell culture

Human prostate carcinoma (PC-3 cells) was provided from the Korea Cell Line Bank (Seoul, South Korea). PC-3 cells were cultured in RPMI 1640 medium (Gibco-Invitrogen, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS (Gibco-Invitrogen), 1% antibiotics (Gibco-Invitrogen). Cells were routinely cultivated at 37 °C in a humidified atmosphere of 5% CO_2.

2.10. Transfection and gene silencing experiments

For transfection experiments, cells were seeded in a 12-well cell culture plate at a density of 2×10^5 cells per well in growth medium (RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics) 24 h prior to the transfection. After the 24 h, incubation medium was replaced by fresh growth medium. Transfection was carried out by adding prepared complexes to the wells in a dropwise manner. After 4 h of incubation, the medium was exchanged again with fresh growth media and transfected cells were further incubated for 6 h. Afterward, the medium was aspirated in order to remove endogenously secreted VEGF and replaced with fresh growth medium containing 20 μg/mL heparin (Sigma-Aldrich). After a further 16 h of incubation, the medium was collected and the concentration of VEFG released from the cells was measured using a Quantikine human VEGF immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols. For a dose-dependent experiment, different concentrations of siRNA (0, 15, 37, 75, 100, 200, and 300 nM) were

employed for the formation of ternary complexes, which were prepared at an N/P ratio of 4.44.

2.11. Cytotoxicity test

Cytotoxicity of ternary complexes was evaluated after the transfection in triplicate using a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) assay kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocols. Measurement was performed using a Magellan ELISA microplate reading spectrophotometer (Tecan).

3. Results and discussion

3.1. Cationic polymers

Due to the excellent biocompatibility of methacrylate-based (co) polymers there has been an increased interest in using these (co) polymers as nucleic acid binding agents. In this regard, they can be used as either as pure compounds or as mixtures in other systems based on their ability to condense and compact nucleic acid within polyelectrolyte complexes and their buffering capacity in certain cellular compartments such as endosomes and lysosomes [14–21]. In this study, we used a highly controlled polymerization method and extensive purification to synthesize two different well-defined and specially designed polycationic (co)polymers. The synthesis of PDMAEMA homopolymers and palm-tree-like copolymers with PMAPEG was performed by atom transfer radical polymerization. After polymerization, extensive purification of the (co)polymers was performed by precipitation in heptane, filtration through a column of basic alumina, and dialysis against water until the monomer and the catalyst residues were completely removed. These protocols yielded (co)polymers with highly unimodal molecular weight distributions, low polydispersity indexes, and peculiar molecular characteristics (see Appendix A).

3.2. Characterization of polyelectrolyte complexes

As mentioned above, the biological fate of polymeric gene carriers are strongly influenced by their physicochemical properties. Thus, we evaluated the physicochemical properties of polyelectrolyte complexes formed with these (co)polymers, such as the nucleic acid condensation efficiency, hydrodynamic diameter, zeta-potentials, and morphology of complexes prepared at various N/P ratios. Even though siRNAs are small molecules, especially they have a greater persistence length (the length scale over which the chains behave as rigid rods) than double-stranded DNA [13]. The stiffness of siRNA prevents intact interactions and efficient condensations within polyelectrolyte complexes, when it was prepared with improper (co)polymers, resulting in the formation of undesirable large and unstable complexes. That subsequently diminishes their cellular uptake and transfection efficiency. Thus, the formation and maintenance of polyelectrolyte complexes on the nano-scale as stable colloidal dispersions using electrostatic interactions under physiological conditions is the most important prerequisite for successfully advancing the use of synthetic (co)polymers in an efficient nonviral siRNA delivery system. In this study, two different methacrylate-based polycationic (co)polymers were employed in the formulation of the polyelectrolyte complexes and these novel polyelectrolyte complexes were successfully complexed with siRNA. Briefly, polyelectrolyte ternary complexes were prepared via a rapid and simple two-step mixing protocol involving the stable initial complexation of siRNA through the formation of a polyion complex via electrostatic interactions between the anionic phosphate group of siRNA and the cationic amino groups of the PDMAEMA homopolymers. This was followed by substantial siRNA condensation, compaction, and noncovalent post-PEGylation of the complexes through the electrostatic interaction between the preformed binary complexes and PDMAEMA-*b*-PMAPEG palm-tree-like copolymers (Fig. 1). Consequently, this strategy is not only effectively avoids

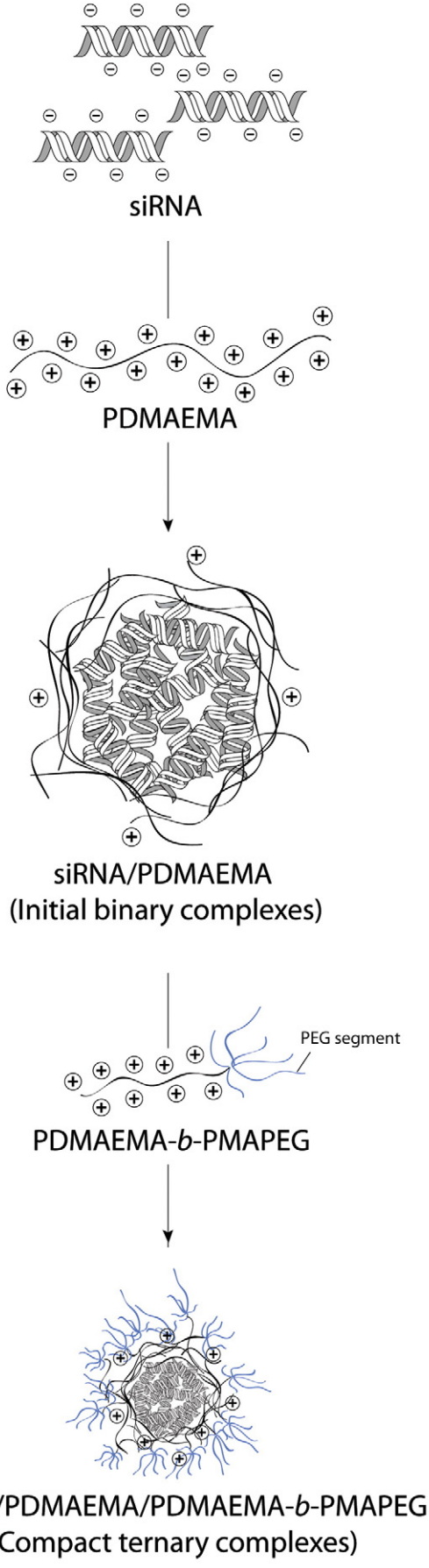

Fig. 1. Schematic diagram of the preparation of polyelectrolyte ternary complexes.

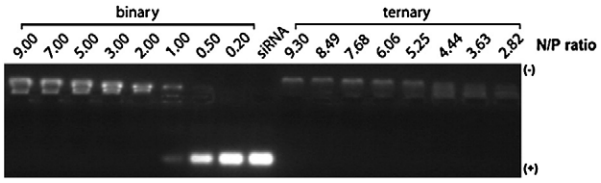

Fig. 2. The ability of (co)polymers to complex with siRNA studied using a gel retardation assay. Binary and ternary complexes were prepared at N/P ratios ranging from 0.20 to 9.00 and 2.82 to 9.30, respectively, in PBS (pH 7.4). For the formation of ternary complexes, the N/P ratio of preformed siRNA/PDMAEMA binary complexes was set at 2.01.

the negative effects that associated with the introduction of PEG segments, as mentioned above, but also effectively condenses and protects carrying siRNAs.

In the initial optimization steps, the binary complexes were prepared at N/P ratios ranging from 0.2 to 9.0 in PBS (pH 7.4) and characterized with regard to siRNA condensation as a function of the N/P ratio using an agarose gel retardation assay. As shown in Fig. 2, the PDMAEMA homopolymer completely impeded siRNA migration at an N/P ratio of around 2.0. This result demonstrates the complete combination of siRNA with PDMAEMA homopolymers via electrolytic interaction, which allows for the formation of polyelectrolyte binary complexes.

The hydrodynamic diameter and zeta-potential of the binary complexes were monitored by DLS (Fig. 3a). As the N/P ratio increased from 1.0 to 3.0, the hydrodynamic diameter and zeta-potential of the binary complexes noticeably changed from 173.25 ± 5.82 nm to 93.00 ± 1.141 nm and from −12.80 ± 1.81 mV to 4.56 ± 0.77 mV, respectively. In contrast, increasing the N/P ratio from 5.0 to 9.0 resulted in only relatively minor alterations in the hydrodynamic diameter and zeta-potential, which changed from 102.25 ± 1.11 nm to 58.220 ± 11.29 nm and from 9.09 ± 0.17 mV to 8.23 ± 1.13 mV, respectively. In this case, PDMAEMA homopolymers produced a bimodal hydrodynamic diameter distribution with a larger peak of 215.04 ± 6.07 nm and a smaller peak of 11.71 ± 0.22 nm. The intensity (%) of the smaller peak increased gradually with the N/P ratio from 12.10% ± 0.42% to 22.90% ± 0.31%. These results demonstrate that the electrostatic interactions between the polycationic PDMAEMA homopolymers and negatively charged siRNA produce stable polyelectrolyte binary complexes and that an increasing amount of homopolymer beneficially influences the condensation and/or encapsulation of siRNA into these complexes. However, at N/P ratios higher than 3.0, the PDMAEMA homopolymers seems to be toxic as reflected by a decrease in cell viability. In addition, increased N/P ratios negatively influence the formation and stability of ternary complexes. In contrast, when the binary complexes were prepared with copolymers, a higher N/P ratio (N/P ratio>4) was required to completely impede siRNA migration relative to homopolymers. In this case, an increased N/P ratio was accompanied by an increased cytotoxicity and the formation of ternary complexes were also restricted. Moreover, zeta-potential value (<5 mV) of these complexes was not suitable for efficient transfection in vitro and in vivo (data not shown). This can be explained by the lower molecular weight and undesirable effects of the introduced PEG segments of the copolymers during the formation of polyelectrolyte complexes. Thus, we did not consider the formation of preformed binary complexes using copolymers in any further experiments. For all subsequent experiments, the N/P ratio of the preformed binary complexes was fixed at 2.01 because the complexes at this N/P ratio demonstrated complete PDMAEMA/siRNA complexation and stable homogeneous binary complexes with a narrow size distribution (119 ± 4.32 nm and 38.87 nm peak width) and relatively low cytotoxicity in vitro (data not shown). In the next step, the ternary complexes were prepared by combining preformed binary complexes (N/P ratio 2.01) and PDMAEMA-*b*-MAPEG copolymers at N/P ratios ranging from 2.82 to 9.30. As expected, the ternary complexes also completely impeded siRNA migration in all cases (Fig. 2). The hydrodynamic diameter and zeta-potential were also measured in ternary complexes (Fig. 3b). Increasing the N/P ratio from 2.82 to 4.44 changed the hydrodynamic diameters and zeta-potentials of the ternary complexes dramatically from 185.00 ± 23.00 nm to 16.72 ± 1.25 nm and from 11.55 ± 0.70 mV to 16.40 ± 0.37 mV, respectively. In contrast, increasing the N/P ratio from 4.44 to 8.49 resulted in only minor alterations in hydrodynamic diameter and zeta-potential from 16.72 ± 1.25 nm to 14.57 ± 0.38 nm and from 16.40 ± 0.37 mV to 16.43 ± 0.35 mV, respectively. Increasing the N/P ratio to 9.3 decreased the zeta-potential significantly to 11.08 ± 0.19 mV. Interestingly, association between the polycationic copolymers and the preformed binary complexes decreased the hydrodynamic diameter but increased the zeta-potential markedly. This shows greater attraction of PDMAEMA-*b*-MAPEG copolymer for siRNA than for the PDMAEMA homopolymers, which should lead to the effective and substantial condensation and compaction of siRNA within the polycationic core of ternary complexes. A possible explanation of this phenomenon is the stiffer characteristic of siRNA, difference in molecular weight and flexibility between homopolymers and block copolymers in the aqueous phases. Block copolymers might be able to form efficient electrostatic interactions with preformed binary

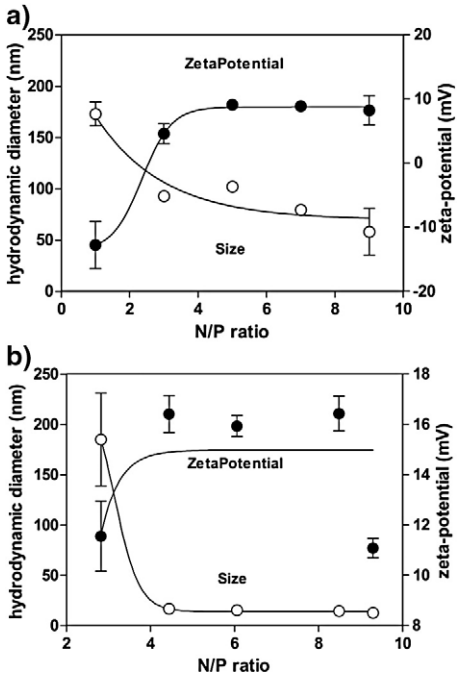

Fig. 3. Hydrodynamic diameter and zeta-potential of binary (a) and ternary (b) complexes studied by DLS. Binary and ternary complexes were prepared at N/P ratios ranging from 1.0 to 9.0 and 2.82 to 9.30 in PBS (pH 7.4). For the formation of ternary complexes, the N/P ratio of preformed binary complexes was set at 2.01.

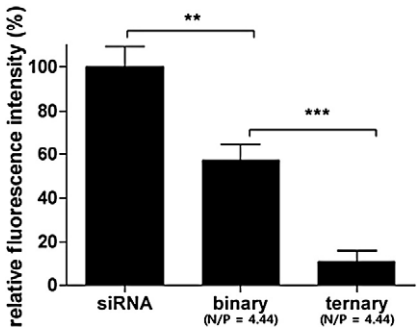

Fig. 4. Efficiency of siRNA condensation with binary and ternary complexes studied by an EtBr exclusion assay. Binary and ternary complexes were prepared at an N/P ratio of 4.44, respectively. For the formation of ternary complexes, the N/P ratio of preformed binary complexes was set at 2.01 (*, $P<0.05$, and **, $P<0.01$ vs control).

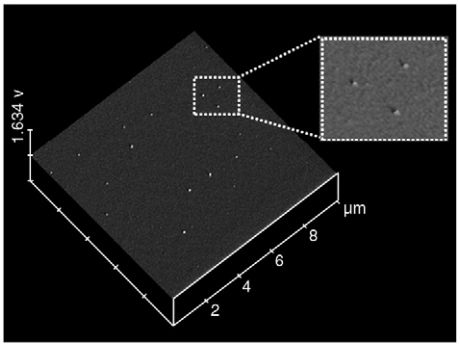

Fig. 5. AFM image of ternary complexes prepared at an N/P ratio of 4.44. Edge length: 10 µm × 10 µm.

complexes because of the relatively lower molecular weight and greater flexibility of positively charged amino side chains of copolymers. Because block copolymers are more flexible than homopolymers their interactions with negatively charged siRNA are more frequent and compact in limited volume. In contrast, the higher molecular weight and bulky amino side branches in homopolymers provide stiffness not compact, in aqueous phase but stable under strong interactions with siRNA. Consistent with this finding, significant quenching of ethidium bromide fluorescence intensity was observed in the ethidium bromide exclusion assay (Fig. 4). This can be explained by the restriction of siRNA structural flexibility through stronger condensation and compaction, which may result in a shift in the binding equilibrium of ethidium bromide, because the flexibility within the double-stranded structure of nucleic acids is an important prerequisite for the interaction between nucleic acids and ethidium bromide.

These results clearly demonstrated that the rapid two-step mixing involving noncovalent post-PEGylation results in formation of compact and stable colloidal ternary complexes under physiological conditions at an optimal N/P ratio. In addition, noncovalent post-PEGylation did not negatively influence the siRNA condensation, formation, or stability of ternary complexes in optimal conditions. For the following experiments, the N/P ratios of ternary complexes were optimized further for transfection efficiency and cytotoxicity based on a prior dose-cytotoxicity study (data not shown). The N/P ratio of 4.44 was used because at this N/P ratio, ternary complexes demonstrate stable homogeneous complexes and have the highest transfection efficiency and lowest cytotoxicity. Moreover, these complexes maintained their hydrodynamic diameter and zeta-potential in PBS over a 72 h period without any significant changes (data not shown). Earlier reports [15,22,23] show that ternary complexes at the optimal N/P ratio had both suitable zeta-potentials (ranging from 5 to 30 mV) for intracellular delivery of polyelectrolyte complexes via endocytosis and a sufficient hydrodynamic diameter to prevent rapid renal clearance in vivo (>15 nm) [24]. The simultaneous condensation and compaction of siRNA by cationic (co)polymers produced a complex of favorable size that could be taken up by cells via endocytosis and could be modified with specific targeting moieties such as antibodies and peptides. The ternary complexes were visualized using AFM (Fig. 5). AFM observations confirmed the formation of stable and uniform complexes in all cases, although, some relatively larger complexes were also observed.

3.3. RNase protection assay

Because most nucleic acids, including siRNA, are vulnerable to enzymatic digestion by nucleases, appropriate carriers that can protect the nucleic acids against nuclease attack should be used. The siRNA protection efficiency of ternary complexes was determined by incubating freshly prepared binary and ternary complexes at an N/P ratio of 4.44 for 30 min or 1 h with 10 units of ribonuclease at room temperature, respectively (Fig. 6a). For the nuclease protection assay, we employed RNase ONE ribonuclease because it cleaves the 3′ end of all bases, thus enabling a more accurate and stringent assay. As shown in the Fig. 6a, the uncomplexed naked siRNA control was degraded completely by 10 units of nuclease after 1 h incubation, whereas about 65% of siRNA complexed with PDMAEMA homopolymers at an N/P ratio of 4.44 remained after the enzymatic digestion. However, nearly 100% of the siRNA remained intact in the ternary complexes. These results were supported further by heparin-displacement experiments.

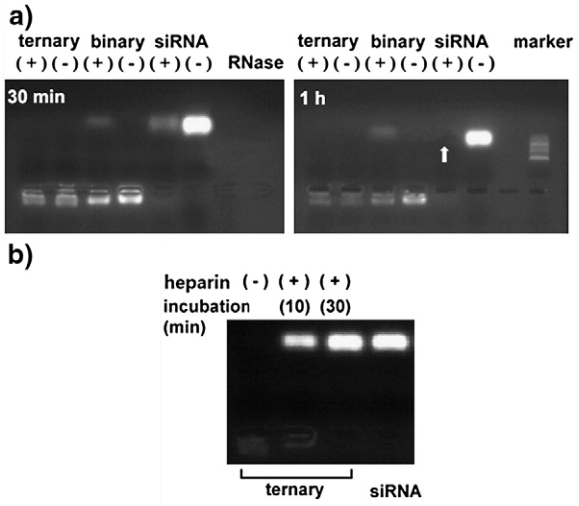

Fig. 6. RNase protection assay and heparin-releasing test. (a) RNase protection assay for binary and ternary complexes. Binary and ternary complexes were prepared at an N/P ratio of 4.44, and then incubated with 10 units of RNase ONE for 30 min or 1 h, respectively. (b) After 1 h of incubation, as described above, RNase activity was stopped and complexed siRNA was displaced by incubation with 1 IU of heparin for 10 min or 30 min.

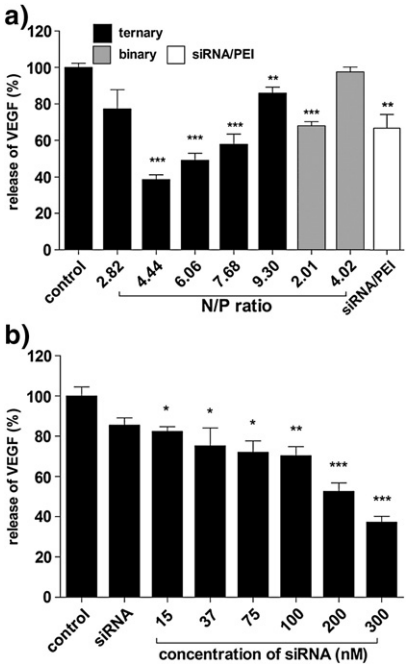

Fig. 7. Transfection efficiency of binary, ternary and PEI complexes studied by VEGF knockdown. (a) Binary and ternary complexes were prepared at N/P ratios ranging from 2.01 to 4.02 and 2.82 to 9.3 with 300 nM of siRNA, respectively, and transfected into PC 3 cells for 4 h. (b) PC 3 cells transfected with naked siRNA (300 nM) and ternary complexes prepared with different siRNA concentrations (15, 37, 75, 100, 200, and 300 nM) for 4 h. All of the transfection experiments were performed in growth medium containing 10% serum (*, $P<0.05$, and **, $P<0.01$, ***, $P<0.001$ vs control).

As expected, heparin displacement completely protected siRNA, showing clearly that the ternary complexes provide excellent protection against nuclease attack (Fig. 6b). Consistent with previous results, this phenomenon was also associated strongly with efficient siRNA condensation and noncovalently introduced PEG segments of ternary complexes. The restricted flexibility of siRNA and highly hydrated corona around the surface of the polyelectrolyte ternary complex because of noncovalent post-PEGylation strongly restrict access of nucleases to the siRNA. These results clearly demonstrate the efficiency of noncovalent post-PEGylation through electrostatic complexation between preformed binary complexes and PDMAEMA-*b*-PMAPEG palm-tree like copolymers, which provides excellent enzymatic and colloidal stability to the ternary complexes and protects the siRNA form enzymatic digestion.

3.4. Intracellular delivery of ternary complexes

To investigate the efficiency of binary and ternary complexes in intracellular siRNA delivery and gene silencing as a function of the N/P ratio, siRNA knockdown experiments were performed using human VEGF siRNA and PC3 cells in 10% serum-containing growth medium. To form the ternary complexes, the N/P ratio of preformed binary complexes and siRNA concentration were set at 2.01 and 300 nM, respectively. As in our earlier report, polyethylenimine (PEI) 25K was used as a control core-forming agent. The optimal N/P ratio of PEI/siRNA complexes and concentration of siRNA were set at 16 and 300 nM, respectively. As shown in Fig. 7a, a significant and efficient decrease in VEGF release was observed in cells transfected with ternary complexes. The most pronounced silencing efficiency was observed at an N/P ratio of 4.44, which decreased the release of VEGF by 35.58%±2.51%. However, as the N/P ratio was increased above 4.44, the silencing efficiency of ternary complexes decreased proportionally with the increasing amount of copolymers. According to a previous report [10], this phenomenon resulted from the excessively substituted PEG segments around the surface of the polyelectrolyte ternary complexes and/or excessive free copolymers, which negatively influenced the efficiency of their cellular uptake and resulted in a moderate silencing efficiency. At an N/P ratio below 4.44, ternary complexes also showed moderate silencing efficiency compared with ternary complexes at an optimal N/P ratio. In the case of binary complexes, relatively lower silencing efficiency was also observed compared with that of ternary complexes at an optimal N/P ratio. Moreover, increasing the N/P ratio from 2.01 to 4.02 decreased the silencing efficiency of binary complexes from 67.98%±2.23% to 97.44%±2.69% despite its higher zeta-potential. This phenomenon probably resulted from the increased zeta-potential of binary complexes at higher N/P ratios, which provides conditions that are more beneficial for the attraction of serum constituents and increases the interparticulate aggregation between complexes [25]. In addition to the higher zeta-potential, the excessive size resulting from the incompletely condensed and packed siRNA causes interparticulate aggregation and enzymatic degradation of carried siRNA, which act as an added barrier to intracellular uptake and efficient gene silencing. Consistent with our earlier reports, the PEI/siRNA complexes also showed moderate efficiency compared with ternary complexes at an optimal N/P ratio (Fig. 7a). As discussed previously, the moderate silencing efficiency of PEI/siRNA complexes relies mainly on the absence of PEGylation. This suggestion was supported further by our previous reports using PEG-siRNA/PEI complexes [10,11]. The dose-dependent silencing efficacy of ternary complexes prepared with different amounts of siRNA (15, 35, 75, 100, 200, or 300 nM) at an optimal N/P ratio (4.44) was also investigated (Fig. 7b). Consistent with our previous reports [10], the release of VEGF decreased gradually and significantly in a dose-dependent manner starting at 15 nM siRNA, when the VEGF expression level was 82.27%±2.34%. As expected, the most pronounced silencing was observed at 300 nM siRNA. These results demonstrate the excellent ability of ternary complexes for intracellular siRNA delivery under conditions of serum exposure (see Appendix A).

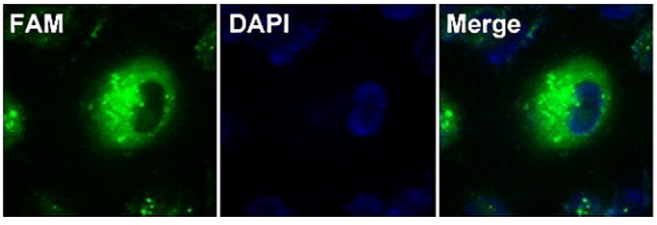

Fig. 8. Confocal laser microscopy images of PC 3 cells transfected with ternary complexes. Ternary complexes were prepared with 300 nM FAM-oligo at an N/P ratio of 4.44 and transfected into the PC 3 cells for 4 h. Blue: nucleus (DAPI); green: FAM-labeled complexes; magnification: 1200×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

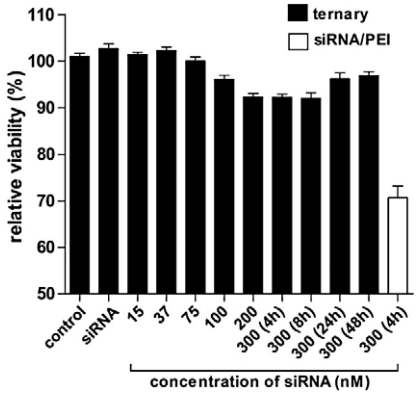

Fig. 9. Cytotoxicity of ternary complexes studied by MTT in growth medium containing 10% serum. PC 3 cells were transfected with naked siRNA and ternary complexes prepared with different siRNA concentrations at an N/P ratio of 4.44. siRNA (300 nM)/PEI complexes are also included for comparison of cytotoxicity.

3.5. Cellular uptake

FAM-labeled BLOCK-iT Fluorescent Oligo (FAM-oligo) was used to visualize the cellular uptake and subcellular localization of ternary complexes. The findings are presented in Fig. 8. Consistent with previous results, fluorescently labeled ternary complexes were internalized evenly by most of the cells without any particular aggregation. Ternary complexes were also found within intracellular vesicles and had accumulated near the nucleus in many cells. These vesicles were most likely endosomes or lysosomes, indicating uptake via endocytosis. In contrast, fluorescently labeled naked siRNA was not detected by confocal microscopy either within cells nor in the extracellular medium, suggesting that siRNA is degraded or removed by washing the cells during the incubation period (see Appendix A).

3.6. Cytotoxicity

Cytotoxicity of most synthetic polymer-based gene delivery systems limits their therapeutic application. MTT assays were performed to evaluate the metabolic activity of cells transfected with ternary complexes for 4, 8, 24, or 48 h as a function of siRNA concentration. As shown in Fig. 9, ternary complexes at an siRNA concentration of less than 100 nM caused no significant damage to the cells. However, at concentrations more than 100 nM, the cell viability decreased slightly but remained at 90% compared with the values for untreated cells. Increasing the siRNA concentration from 100 to 300 nM, cell viability decreased from 96.14%±0.80% to 92.23%±0.68%. Increasing the duration of transfection from 4 h to 48 h did not decrease cell viability significantly, but instead recovered viability

gradually. In the case of siRNA/PEI 25K complexes showed lower cell viability compared with ternary complexes at the optimal N/P ratio (relative viability <85%). The toxicity increased with an increasing N/P ratio and duration of transfection with these complexes (data not shown). In particular, although PEI 25K is a polycationic polymer used most commonly in both in vitro and in vivo polymeric gene delivery. PEI can induce early necrotic-like changes (within 30 min) and initiate the mitochondria-mediated apoptotic program (within 24 h) in a range of human cell lines [26]. In this experiment, the ternary complexes were the least cytotoxic compared with the other polyelectrolyte complexes used as controls.

4. Conclusion

In summary, we demonstrated that the formation of stable siRNA/PDMAEMAPDMAEMA-*b*-PMAPEG ternary complexes through the rapid, simplest, and noncovalent post-PEGylation of preformed binary complexes provided efficient siRNA condensation and full compaction within the cationic core of the complexes. These complexes also dramatically improved colloidal and enzymatic stability in serum-containing conditions. The ternary complexes were taken up efficiently by cells and silenced the target gene expression under conditions involving serum exposure. The ternary complexes exhibited markedly low cytotoxicity at optimal conditions in vitro and in vivo. This study highlights the development of an efficient, rapid, and simple siRNA delivery system with low cytotoxicity. The formation of ternary complexes involving noncovalent post-PEGylation might be useful as a polycationic polymer-based nonviral siRNA delivery system for therapeutic approaches because of their simple and rapid preparation, and excellent stability and efficiency.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2009.04.034.

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