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Tuning Surface Charge and PEGylation of Biocompatible Polymers for Efficient Delivery of Nucleic Acid or Adenoviral Vector

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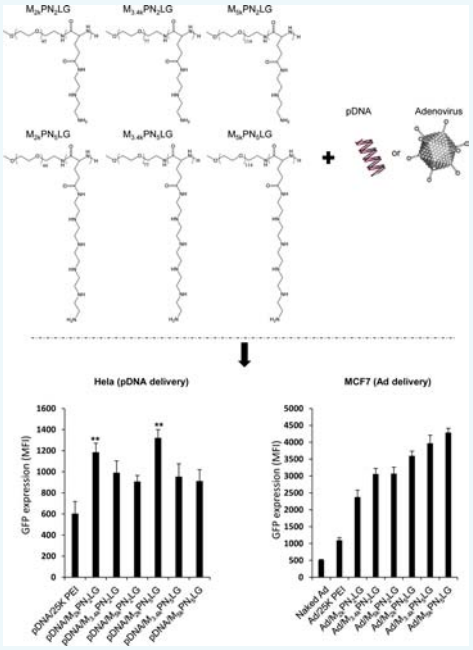
Supporting Information

ABSTRACT: As an effective and safe strategy to overcome the limits of therapeutic nucleic acid or adenovirus (Ad) vectors for in vivo application, various technologies to modify the surface of vectors with nonimmunogenic/biocompatible polymers have been emerging in the field of gene therapy. However, the transfection efficacy of the polymer to transfer genetic materials is still relatively weak. To develop more advanced and effective polymers to deliver not only Ad vectors, but also nucleic acids, 6 biocompatible polymers were newly designed and synthesized to different sizes (2k, 3.4k, or 5k) of poly(ethylene) glycol (PEG) and different numbers of amine groups (2 or 5) based on methoxy poly(ethylene glycol)-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]-L-glutamate (PNLG). We characterized size distribution and surface charge of 6 PNLGs after complexation with either nucleic acid or Ad. Among all 6 PNLGs, the 5 amine group PNLG showed the strongest efficacy in delivering nucleic acid as well as Ad vectors. Interestingly, cellular uptake results showed higher uptake ability in Ad complexed with 2 amine group PNLG than Ad/5 amine group PNLG, suggesting that the size of Ad/PNLGs is more essential than the surface charge for cellular uptake in polymers with charges greater than 30 mV. Moreover, the endosome escape ability of Ad/PNLGs increased depending on the number of amine groups, but decreased by PEG size. Cancer cell killing efficacy and immune response studies of oncolytic Ad/PNLGs showed 5 amine group PNLG to be a more effective and safe carrier for delivering Ad. Overall, these studies provide new insights into the functional mechanism of polymer-based approaches to either nucleic acid or Ad/nanocomplex. Furthermore, the identified ideal biocompatible PNLG polymer formulation (5 amine/2k PEG for nucleic acid, 5 amine/5k PEG for Ad) demonstrated high transduction efficiency as well as therapeutic value (efficacy and safety) and thus has strong potential for in vivo therapeutic use in the future.



INTRODUCTION

Gene therapy is a promising strategy to treat human genetic and acquired diseases. The key to success in gene therapy is to develop an efficient and safe vector system.[1,2] For nonviral delivery, cationic polymers, such as poly(L-lysine),[3] chitosan and its derivatives,[4,5] and poly(ethylene imine) (PEI),[6] have been widely explored as plasmid DNA or siRNA delivery vectors. Among them, 25K PEI has been regarded as the gold standard because of its high transfection efficiency, which is due to the proton sponge effect.[7] Transfection efficiency of the PEI-nucleic acid complex increases depending on molecular weight, but cytotoxicity of PEI also increases.[8] To overcome this issue, biodegradable cationic polymers have been designed with reducible degradable disulfide linkages.[9] Recently, unique polypeptides derived from natural source conjugated biomaterials have been developed for numerous biomedical applications such as controlled drug release, gene delivery, tissue engineering, and regenerative medicine.[10] Otherwise, adenovirus (Ad) is highlighted as a potential therapeutic agent for viral vector system among the viral vectors. Recently,

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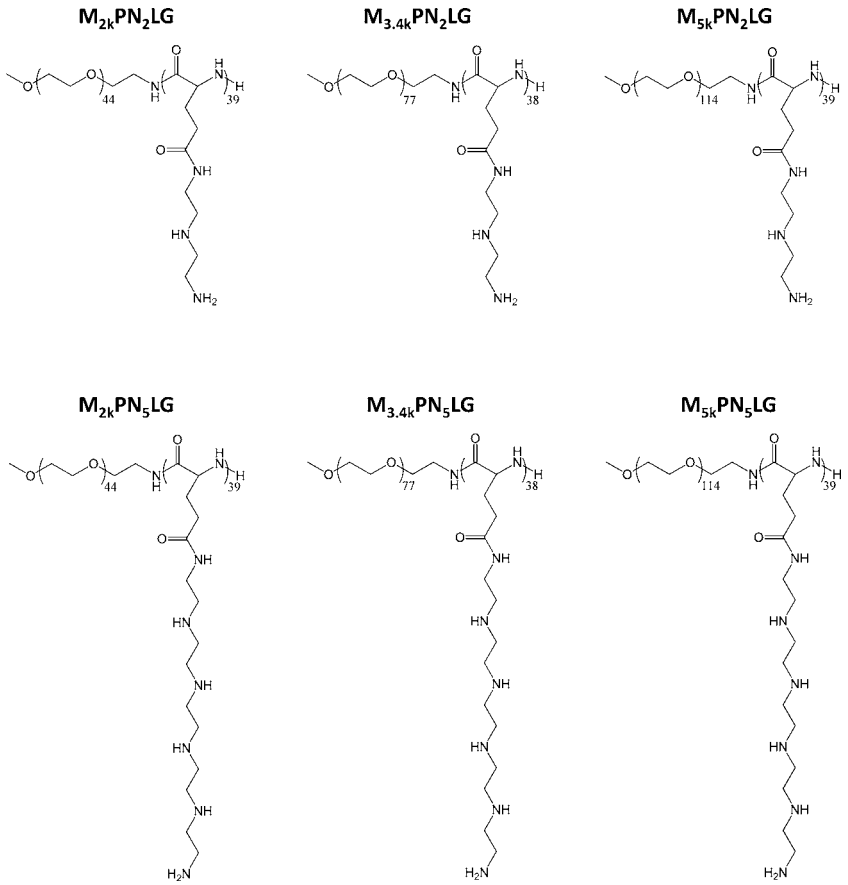

Figure 1. Structures of 6 polymers. All 6 polymers include amine groups, PEG chains, and peptide bonds in their structures. The polymers $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, and $M_{5k}PN_2LG$ have 2 amine groups, and the polymers $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, and $M_{5k}PN_5LG$ have 5 amine groups. All the polymers have different sizes (2k, 3.4k, or 5k) of PEG (2k PEG for $M_{2k}PN_2LG$ and $M_{2k}PN_5LG$; 3.4k PEG for $M_{3.4k}PN_2LG$ and $M_{3.4k}PN_5LG$; 5k PEG for $M_{5k}PN_2LG$ and $M_{5k}PN_5LG$).

oncolytic Ad has been extensively investigated for cancer gene therapy.

The most attractive characteristic of oncolytic adenovirus (Ad) vector system would be the selective replication and the killing ability in cancer cells among many other advantages such as broad host range, easy genetic modification, and rare host genome integration.[11,12] This cancer selective killing Ad, the oncolytic Ad, has also been utilized to transfer suicide genes, tumor suppressor genes, small interfering RNA (siRNA), or immune-stimulator genes to eliminate tumor cells.[13−17] The conditional replication and progeny viral production of the oncolytic Ad after infection in the cancer cells, while sparing normal cells, can be enabled because of the dysfunctional and/or malfunctional tumor suppressor systems of the cancer cells.[18] Moreover, multiplied Ad genomes after the replication can induce amplified therapeutic transgene expression. Also, the reproduced progeny virions are released and spread to neighboring cancer cells.[18] Although the broad utilization of the oncolytic Ad vector systems has been highlighted on in vivo application for cancer gene therapy, a route of administration is limited only to exposed tumors such as head and neck cancer.[19] To become more effective and safe therapeutic vectors, both primary and metastatic tumors in disseminated tissues throughout the whole body must be considered via systemic administration of the vectors. The restricted in vivo application of the oncolytic Ad for the cancer treatment has been issued because of induction of liver toxicity by the inherent Ad tropism, and fast clearance by host immune responses.[20−24]

To overcome the obstacles of the therapeutic Ad vectors for systemic administration, a multitude of techniques for shielding and masking immunogenic surfaces of the Ad with non-immunogenic and biocompatible materials have been introduced.[25−31] The most popular and effective way to deliver the Ad vectors is using synthetic polymers such as poly(ethylene glycol) (PEG) or bioreducible cationic polymers. These technologies used to modify the Ad vectors with the polymers are fully required to protect the surface of the Ad for evading immune responses as well as localization within liver tissues.

We previously introduced a biodegradable cationic polymer, methoxy poly(ethylene glycol)-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]-L-glutamate} (PNLG or $M_{5k}PN_2LG$) for delivering Ad vector systems.[29] The therapeutic Ad particles shielded with the PNLG polymer exhibited enhanced transduction efficiency, resulting in improved cancer killing effect and amplified viral reproduction. Not only that, the remarkable suppression of the tumor growth by the oncolytic Ad delivered by PNLG (Ad/PNLG) was observed in murine xenograft models. Evasion of host immune responses and liver-detargeting by the Ad/PNLG polymer was also successfully accomplished. However, this PNLG polymer was not sufficient to deliver genetic materials such as plasmid DNA (pDNA) or siRNA. For the purpose of developing a more effective polymer system to deliver genetic materials as well as Ad vectors, we designed and synthesized 5 additional polymers related to PNLG, conjugated with different sizes of the PEG chains (2k, 3.4k, or 5k) and/or different numbers (2 amines or 5 amines) of amine groups. In this study,

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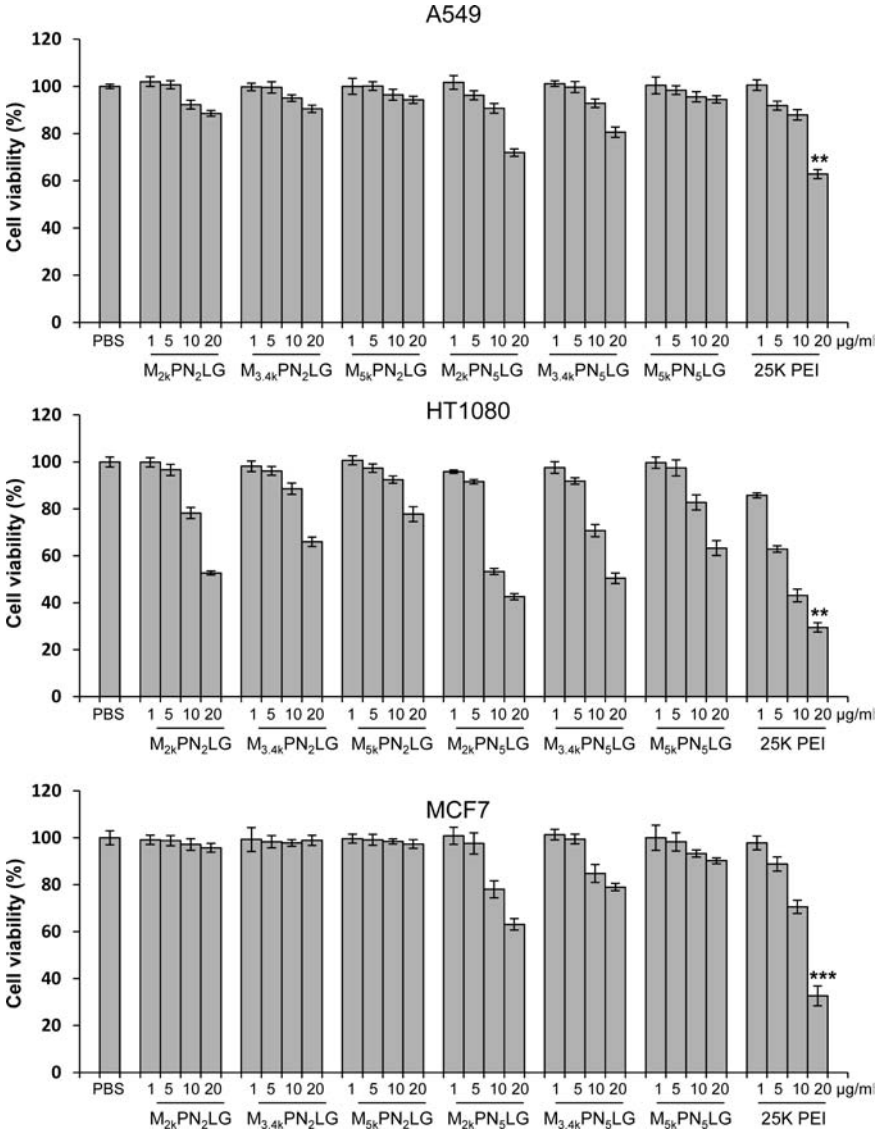

Figure 2. Cytotoxicity of 6 polymers. Cytotoxicity of polymers on A549, HT1080, and MCF7 cell viability. Cells were treated with PBS, 25K PEI, $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$, followed by an MTT cell viability assay 2 days post-treatment. Cytotoxicity results were normalized against the negative control. The data represent the means ± SD of triplicate experiments. Symbols: $^{**}P < 0.01$, $^{***}P < 0.001$.

we compared the efficacy of the 6 PNLGs not only for delivering pDNA, but also for delivering the Ad particles. Additionally, the 6 PNLG chemical structure and pDNA/PNLG or Ad/PNLG nanocomplex size and zeta potential were characterized. We also explored and compared the transduction efficiency and selective cancer cell killing effect of Ad/6PNLGs nanocomplex. Furthermore, we demonstrated cellular uptake and endosome escape ability of 6 PNLGs. These studies discovered new insights on the structure−function interplay of different molecular weight PEGylation and surface charge to promising tuned biodegradable polymer for efficient application in both viral vector and nonviral vector.

RESULTS AND DISCUSSION

Synthesis of Biocompatible Polymers. The polymer precursors, MPEG-PBLG ($MPEG_{2k}PBLG$, $MPEG_{3.4k}PBLG$, $MPEG_{5k}PBLG$), were synthesized by ring-opening polymerization of BLG-NCA monomer using different MPEG-NH_2 ($MPEG_{2k}$, $MPEG_{3.4k}$ and $MPEG_{5k}$) as macroinitiators. The result structures and molecular weight were analyzed via ^{1}H NMR data, which were shown in Supporting Information Figure S1. The degree of polymerization of $MPEG_{2k}PBLG$, $MPEG_{3.4k}PBLG$, and $MPEG_{5k}PBLG$ was calculated to be 39, 38, and 39, respectively. Subsequently, those diblock prepolymers were aminolyzed using different diamine compounds to achieve the final polypeptide. DETA or PEHA was employed to prepare 6 cationic polymers ($M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$) with 2-hydroxypyridine as a bifunctional catalyst (Figure 1). The aminolysis conversion or the replacement of benzyl groups was calculated by ^{1}H NMR (Supporting Information Figure S2). By using an excessive amount of diamine compound, the benzyl groups were completely replaced after 72 h. In the Supporting Information data, the identifying peak of benzyl group (7.3 ppm) totally disappeared in ^{1}H NMR data of the final polymer, while characteristic peaks of amine pendant groups appeared (2.52−3.55 ppm). The

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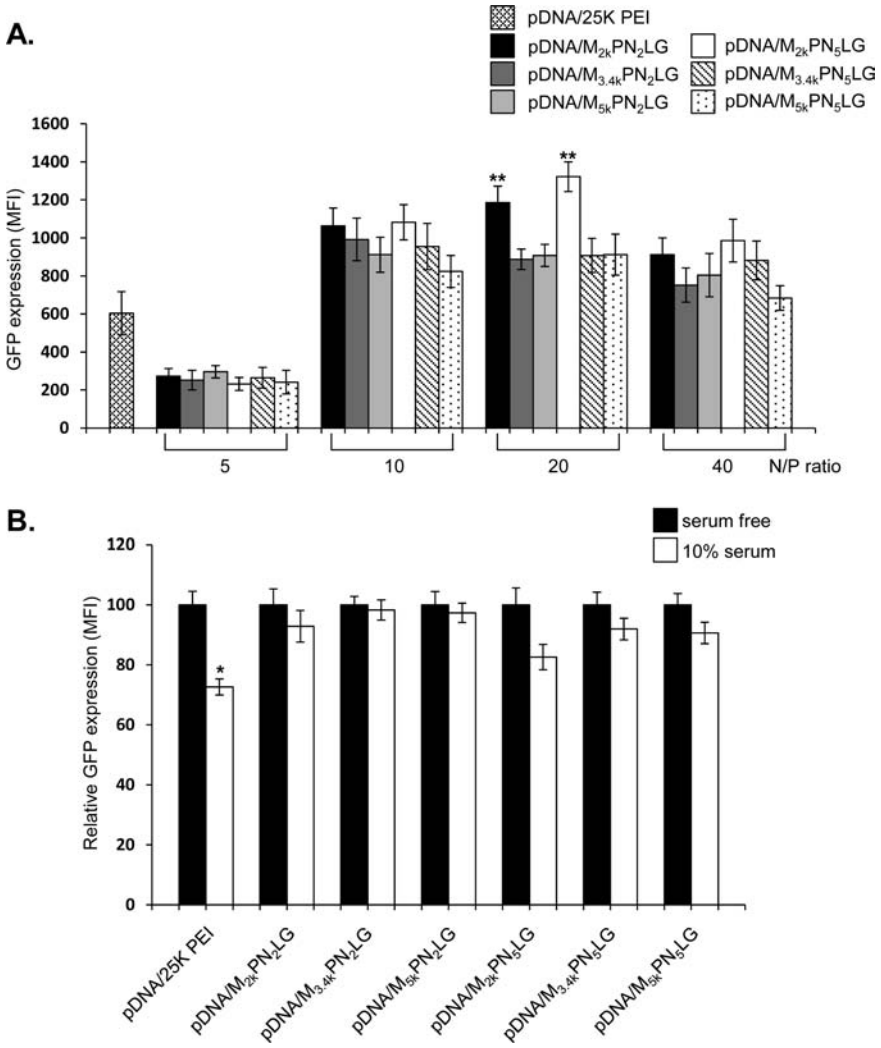

Figure 3. Enhanced transfection efficiency of pDNA encoding GFP gene with the polymers. A. GFP expressing pDNA polyplexed with the polymers (25K PEI, $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$, N/P ratio = 5 for 25K PEI, 5, 10, 20, and 40 for PNLGs) were transfected to HeLa cancer cells. Each GFP expression after the transfection with pDNA/polymer was quantified by measuring the fluorescence intensity. B. Comparison of transfection in serum free and serum supplemented media using in HeLa with pDNA/polymers complexes (N/P ratio = 20). The data represent the means ± SD of triplicate experiments. Symbols: *P < 0.05 versus pDNA/25 K PEI transfected 10% serum contained media.

molecular weights of PNLG series were calculated in Supporting Information Figure S3.

Cytotoxicity of Biocompatible Polymers. It is reported that the cytotoxicity of cationic polymer is caused by their positive charge and adhesion to cellular membrane that causes significant necrosis. It was reported that the size of PEG conjugation and surface charge of cationic gene carriers are related to cytotoxicity. In order to evaluate the cytotoxicity of 6 PNLGs, the viabilities of A549, HT1080, and MCF7 cells were measured by MTT assay, using 25K PEI as control. From this result, 25K PEI treated cells exhibited significance in all cancer cell lines (Figure 2). A much lower cytotoxicity was observed for 6 PNLGs at concentrations below 20 μg/mL (**P < 0.01 for A549 and HT1080, ***P < 0.001 for MCF7). Meanwhile, PN_5LG treated cells exhibited more toxicity than PN_2LG, due to the surface charge on the particle surface. Interestingly, gradually increasing the high molecular weight of PEG conjugated PNLG decreased its cytotoxicity to larger molecular weight PEG molecules in order to achieve efficient charge shielding of both PN_2LG and PN_5LG. These results suggested that lower amine and high molecular weight PEG might contribute to a decrease in cytotoxicity. Further studies will focus on improving Ad-mediated gene delivery by exploring different conjugation options by changing the surface charges and the molecular weight of PEG of PNLG.

Characterization of pDNA/Polymer Complex. To be effective as gene delivery vectors, cationic polymers should have the capacity to bind and condense to plasmid DNA delivering it into the cells. To determine the PNLGs condensed with plasmid DNA, average sizes, and the surface charge values of the plasmid DNA encoding GFP gene (pDNA) polyplexed with the polymers (25K PEI, $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$), the DLS assay was conducted. Because positively charged cationic polymers easily interact with negatively charged pDNA to form tight complex via electrostatic interaction, all PNLG polyplexes were stably formed below 121 nm (Supporting Information Figure S4, Table 1). Also, the surface charges of the polyplex were measured to be about +26.2 mV to +31.4 mV, indicating that negatively charged pDNA was completely condensed with

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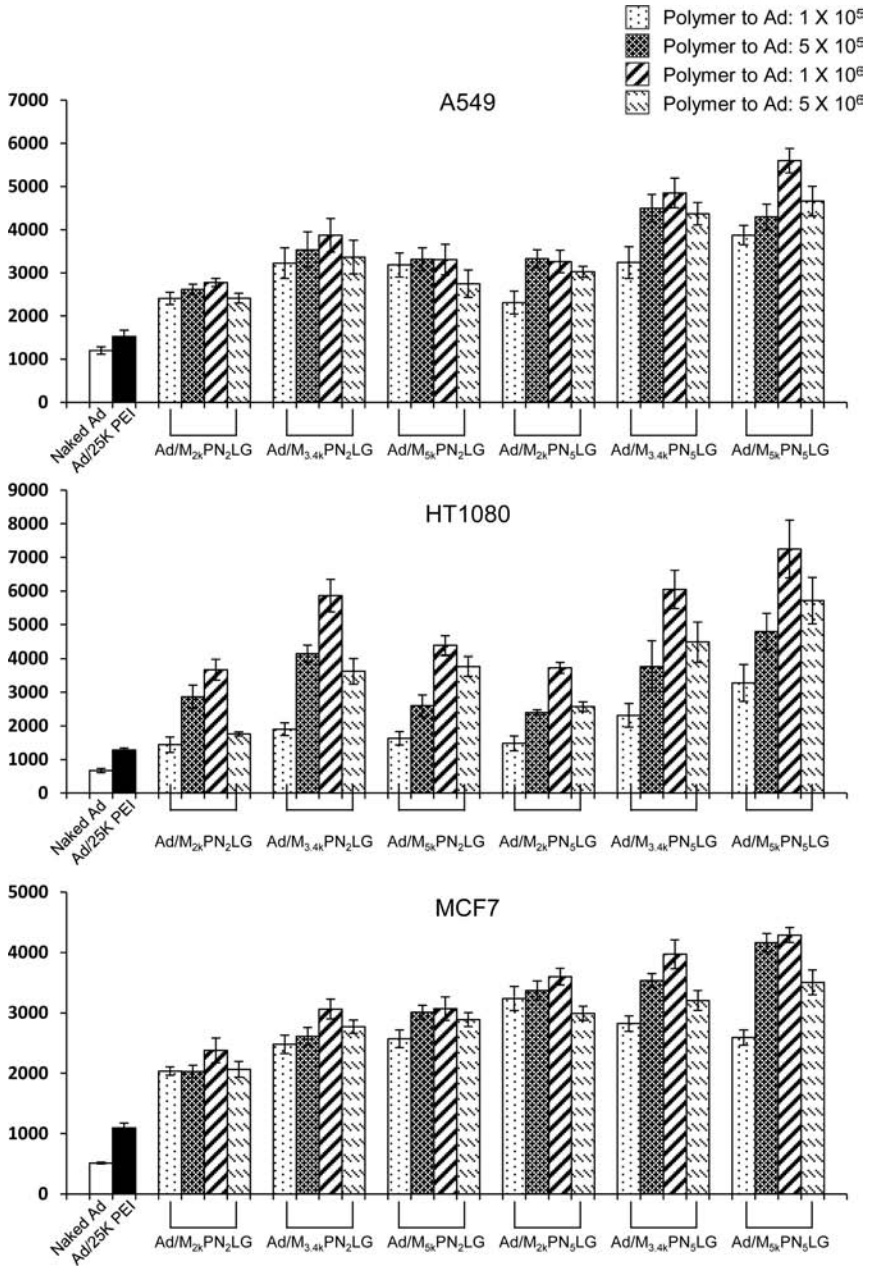

Figure 4. Improved transduction efficacy of Ad expressing GFP coated with the polymers in cancer cells. The Ad expressing GFP was complexed with the polymers (25K PEI, $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$), indicating polymer to Ad ratio at room temperature for 30 min and treated to the cancer cells (50 MOI for A549; 100 MOI for HT1080; 250 MOI for MCF7) for 4 h. After incubation for 2 days, the GFP expression levels from the treated cells were quantified. The data represent the means ± SD of triplicate experiments.

all the cationic polymers (Supporting Information Figure S4, Table 2). Interestingly, the polyplex size increased for conjugation concomitant to the increase in PEG molecular weight. However, these diameters are in a useful range for EPR-based tumor delivery. Similarly, an increase in the PEG molecular weight for PNLGs decreased the surface charge. Moreover, pDNA complexed with PN5LG showed less size and more positive surface charge than pDNA/PN_2LG; 5 amines may cause strong condensation between pDNA and polymer. These data suggest that both relative molecular weight of the PEG and number of amines can contribute to the size and surface charge of the polymer.

Transfection Efficiency of the pDNA/Polymer Polyplex. Previously, we reported that PNLG polymers can efficiently deliver the therapeutic Ad in vitro and in vivo, showing enhanced antitumor efficacy in the xenograft models.[23] To compare the transfection efficiency of the polymers delivering pDNA to the cancer cells, the GFP expression levels from the cells treated with the various N/P ratios of pDNA/polymer polyplexes were measured. As shown in Figure 3A, HeLa human cancer cells transfected with pDNA/$M_{2k}PN_5LG$ displayed much higher GFP expression than that of other polymers (pDNA/25K PEI, pDNA/$M_{2k}PN_2LG$, pDNA/$M_{3.4k}PN_2LG$, pDNA/$M_{5k}PN_2LG$, pDNA/$M_{3.4k}PN_5LG$, or pDNA/$M_{5k}PN_5LG$). However, GFP expression levels of the cells treated with the polymers (pDNA/$M_{2k}PN_2LG$, pDNA/$M_{3.4k}PN_2LG$, or pDNA/$M_{5k}PN_2LG$) including 2 amine groups were slightly increased over that of the cells treated with

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pDNA/25K PEI. However, high-molecular-weight PEGylation can decrease the transfection efficiency, due to shielding ability of PEG. There was a remarkable tendency between the polymers related with the lengths (PEG 2K, PEG 3.4K, or PEG 5K) of the PEG chain between the 2 amine-polymer groups (pDNA/$M_{2k}PN_2LG$, pDNA/$M_{3.4k}PN_2LG$, or pDNA/$M_{5k}PN_2LG$) or 5 amine-polymer groups (pDNA/$M_{2k}PN_5LG$, pDNA/$M_{3.4k}PN_5LG$, or pDNA/$M_{5k}PN_5LG$). Furthermore, many cationic polymers are known to be sensitive to serum, which is one of the major hurdles for in vivo gene delivery.[32] As shown in Figure 3B, it can be found that 10% serum has little effect on the GFP expression for pDNA/PNLGs; meanwhile, GFP expression induced pDNA/25K PEI decreased by serum. Interestingly, both pDNA/PN_2LG and pDNA/PN_5LG complexes can tolerate the presence of negatively charged molecules such as serum proteins following increased molecular weight of PEG. It is worth mentioning that PEGylation of cationic polymer can contribute to increasing the safety as well as serum resistance. These results suggest that fine-tuning both the amine number and the PEG molecular weight of synthesized polymer can be utilized to optimize the efficiency of gene delivery.

Characterization of Ad/Polymer Complex. For efficient cellular uptake and blood circulation, a proper size (<200 nm) of the gene delivery vectors or therapeutic materials is required for systemic administration. To characterize the Ad/polymer complex, the average sizes and the surface charges of the Ad particles complexed with the polymers were determined by DLS assay. As expected, the average size of the naked Ad was determined at about 102 nm. The average sizes of all Ad/polymer groups were less than 207 nm (Supporting Information Figure S5, Table 3). The negatively charged Ad surfaces were changed to the positively charged surfaces after coating with all cationic polymers (Supporting Information Figure S5, Table 4). Interestingly, the 5 amine-polymer groups ($M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$) coated with the Ad showed both larger size and higher positive charges than 2 amine-polymer groups ($M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, or $M_{5k}PN_2LG$). These results demonstrate that the sizes of all polymer groups delivering the Ad vectors would be appropriate for systemic delivery and the negatively charged Ad particles were completely condensed with the cationic polymers.

Transduction Efficiency of Ad/Polymers. The entry pathway of Ad into cell is majorly dependent on the level of coxsackie virus and adenovirus receptor (CAR) expression on the surface of the target cell membrane.[11,12] However, masking Ad surface with the cationic polymers is widely used to overcome the limit of Ad vector's application and can bypass the CAR-mediated transduction, because the CAR expression on the malignant cancer cells is lacking or down-regulated, resulting in poor Ad infectivity into the tumor.[11,12,26] Thus, we selected 3 types of human cancer cells (high CAR expressing cancer cells: A549; low/lack CAR expressing cancer cells: HT1080 and MCF7) to compare the transduction efficiency depending on their CAR expression levels (Supporting Information Figure S6). As we demonstrated in the previous publication,[29] the enhanced transduction efficacy of the Ad coated with the $M_{5k}PN_2LG$ polymer was already confirmed using the 3 cancer cell lines (A549, HT1080, and MCF7). As described in the Introduction, we synthesized 5 more additional polymers related to the $M_{5k}PN_2LG$ polymers (Figure 1) to develop more effective polymers for delivering therapeutic genetic materials or viral vectors. To compare the transduction efficiency of the Ad delivered by all 6 polymers, the GFP expression levels from the various ratio of polymers to Ad treated cancer cells were determined (Figure 4). When compared to the GFP expression levels from the cells treated with the naked Ad, the cells treated with Ad/polymer groups showed higher transgene expression in all the cells, whereas there was no significant difference between the naked Ad and the Ad/25K PEI. In the cells (A549) expressing high CAR, the GFP expression levels of Ad with the polymers were increased to 2.3 (for Ad/$M_{2k}PN_2LG$), 3.2 (for Ad/$M_{3.4k}PN_2LG$), 2.8 (for Ad/$M_{5k}PN_2LG$), 2.8 (for Ad/$M_{2k}PN_5LG$), 4 (for Ad/$M_{3.4k}PN_5LG$), or 4.7 (for Ad/$M_{5k}PN_5LG$) -fold higher than that of the naked Ad, respectively. Moreover, in the cells (HT1080 or MCF7) expressing weak or lacking CAR, the GFP expression levels of Ad with the polymers were increased to 5.4 or 4.6 (for Ad/$M_{2k}PN_2LG$), 8.7 or 6 (for Ad/$M_{3.4k}PN_2LG$), 6.5 or 6 (for Ad/$M_{5k}PN_2LG$), 5.6 or 7 (for Ad/$M_{2k}PN_5LG$), 9 or 7.7 (for Ad/$M_{3.4k}PN_5LG$), or 11 or 8.4 (for Ad/$M_{5k}PN_5LG$) -fold higher than that of the naked Ad, respectively. These data demonstrated that the Ad vector coated with all the polymers exhibited dramatically enhanced transduction efficiency. Especially, the efficacy of the transgene expression by the Ad/polymers was much more effective in the cancer cells expressing low or lacking CAR than in the high CAR expressing cells. Also, there was a tendency that the polymers including 5 amine groups ($M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$) delivering Ad vectors showed stronger GFP expression in each cell line. Moreover, the strongest transgene expression was detected from all the cells treated with the 2 polymers ($M_{3.4k}PN_5LG$ or $M_{5k}PN_5LG$) coated with the Ad. Although it would be natural that the 5 amine polymers show better transfection/transduction efficacy than the 2 amine-polymers; there was enhanced transduction tendency depending on the molecular weight of PEG of the PNLG, whereas the efficacy of the 5 amine polymers ($M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$) was slightly increased, elongating the PEG sizes. Generally, the longer PEG chain of a polymer could show less delivery efficacy than shorter PEG chain polymers, even though the polymer conjugated with longer PEG chain can show better pharmacokinetics in in vivo systems.[33] Thus, newly synthesized PN_5LG polymer can be an attractive vector for Ad delivery in vitro as well as in vivo.

Cellular Uptake and Endosome Escape Efficiency of Ad/Polymers. Cellular uptake of FIC-labeled Ad complexed with 6 PNLG was evaluated by fluorescence intensity in A549 cancer cells (Figure 5A). Cellular uptake results inversely correlated with the molecular weight of PEG and amine number of PNLG. Increasing the molecular weight of the PEG decreased surface charge, which was likely a contributor to effects on the extent of cellular uptake. This result is consistent with a previous report that electrostatic interactions between cationic polymer vector systems and anionic cellular membrane promote internalization.[34] Interestingly, 5 amine group of PNLGs ($M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$) showed more positive charge than 2 amine group of PNLGs ($M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, or $M_{5k}PN_2LG$) on their surface (Supporting Information Table 4); in contrast, cellular uptake result showed $^*P < 0.05$. It has been demonstrated that nanoparticle size and inorganic nanostructure play a role in cellular uptake.[35−37] So, this result demonstrated that the smaller size of PN_2LG, in particular regarding cellular uptake, was a more critical factor than surface charge for Ad/cationic polymer delivery system.

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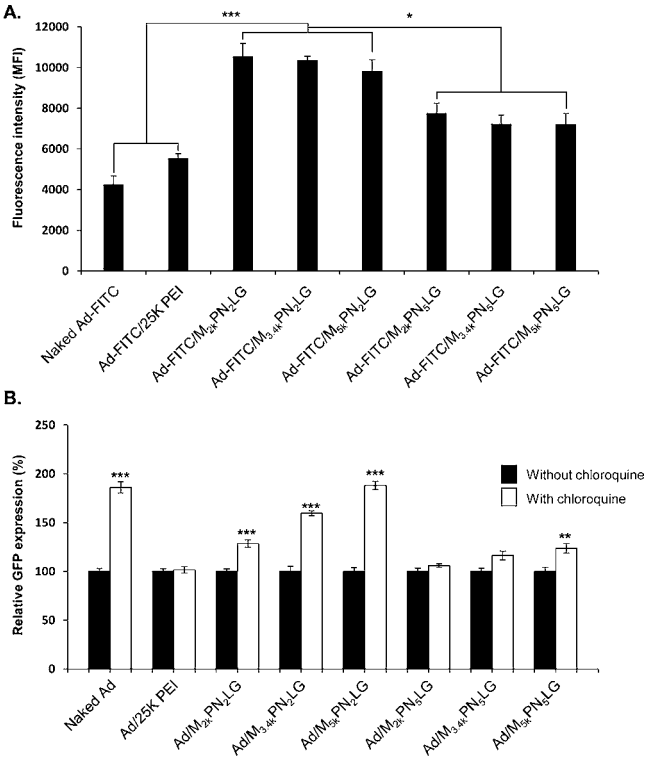

Figure 5. Cellular uptake and endosome escape of Ad/PNLGs. A. A549 cells were treated with Ad-FITC or Ad-FITC/polymers (25K PEI, $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$) for 2 h, then fluorescence intensity was measured. The data represent the means ± SD for three replicates. Symbols: $***P < 0.001$ versus naked Ad or Ad/25K PEI. $*P < 0.05$ versus Ad-FITC/PN_5LGs. B. Transduction efficiency of naked Ad or Ad/polymers (25K PEI, $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$) in the presence or absence of chloroquine in A549 cells. After incubation for 2 days, the GFP expression levels from the treated cells were quantified at the Tecan Infinite M200 plate reader. The data represent the means ± SD for three replicates. Symbols: $**P < 0.01$, $***P < 0.001$ versus treatment with chloroquine.

Next, to investigate the relationship between transduction efficiency and cellular uptake, we focused on endosomal escape ability of PNLGs. According to the proton sponge hypothesis, A549 cells were transduced in the presence of chloroquine. As shown in Figure 5B, Ad/25K PEI did not increase transduction efficiency in the presence of chloroquine, suggesting little involvement of Ad/25K PEI in late endosome and lysosome pathway. However, naked Ad and Ad/PN_2LGS showed markedly enhancing transduction efficiency, suggesting that Ad and Ad/PN_2LGs showed poor endosomal escape activity compared to Ad/PN_5LGs. Meanwhile, Ad/PN_5LG was less affected by chloroquine. One interesting finding is that increasing molecular weight of PEG decreased the endosomal escape activity. In sum, this implies that the Ad/PNLG transduction mechanism did not depend strongly on the surface charge or the molecular weight of PEG. The higher PEG molecular weight in Ad/PN_5LG showed gradually enhanced transduction (Figure 4), although cellular uptake was lower than Ad/PN_2LGs (Figure 5A). Thus, for efficient delivery of cationic polymer into the cells, endosomal escape activity is needed as well as cellular uptake efficiency. Moreover, the lack of correlation between the transduction and uptake profiles of different Ad/PNLGs suggests that different weight of PEGylation may also have an effect on adenoviral delivery. The transduction efficiency using cationic polymer is highly related to their surface charge. However, higher positive charge can induce cytotoxicity. Moreover, several previous studies reported that PEGylated nanoparticles increased tumor accumulation by EPR effect due to prolonged blood circulation time, resulting in enhanced therapeutic efficacy in the tumor model in vivo; however, cellular transduction ability decreased compared to that of un-PEGylated nanoparticles in vitro.[38] These results suggest that PEGylation can be advantageous in terms of enhancing tumor accumulation of nanoparticles, while it has a disadvantage in terms of decreasing intracellular trafficking of cellular uptake and endosomal escape. The crucial issue caused by the use of PEG is referred to as the PEG dilemma. As shown in Figures 4 and 5, transduction efficiency and endosomal escape ability decreased following increased molecular weight of PEG. One of the major problems associated with PEGylation is the low uptake of the nanoparticles into the target cells. To overcome this issue, targeting ligand conjugated on the PEGylated nanoparticle is needed to display ligands for their specific receptors on the surface of the targeted cells. This technique would improve the selectivity and uptake of the carriers to targeted cells. Thus, targeting moiety conjugated PNLGs will need to be evaluated separately for targeting and therapeutic efficacy both in vitro and in vivo.

Cancer Killing Effect of Oncolytic Ad with the Polymers. Although the oncolytic Ad vectors have been highlighted in the field of cancer therapy due to reasons such as the selective cancer killing effect in the cancer cells harboring tumor suppressor-defective pathway and ability to spread to neighboring cancer cells; in vivo application has been strictly restricted because of highly immunogenic surfaces of the Ad vectors. To overcome the limits of the vectors, many different technologies have been introduced for shielding the Ad surface with biocompatible materials such as PEG,[33,39] lipid,[40,41] chitosan,[42] and bioreducible polymers.[30,43] In order to investigate the cancer killing effect of the oncolytic Ad (oAd) when delivered by the polymers, the oncolytic Ad vector was physically coated with the polymers used and treated to the cancer cells (A549, HT1080, or MCF7) (Figure 6). The cancer cells infected with only the naked oAd were killed with the percentage of 42.3% (A549), 15.8% (HT1080), or 7.7% (MCF7), relatively. However, the killing effects of the cells treated with the oAd/polymers (oAd/$M_{2k}PN_2LG$, oAd/$M_{3.4k}PN_2LG$, oAd/$M_{5k}PN_2LG$, oAd/$M_{2k}PN_5LG$, oAd/$M_{3.4k}PN_5LG$, or oAd/$M_{5k}PN_5LG$) were dramatically enhanced in all cancer cells, whereas oAd/25K PEI showed quite similar effects (46.9% in A549; 18.2% in HT1080; 9.6% in MCF7) with the naked oAd. In contrast to the killing effects (57.8%, 55.7%, 53.5% in A549; 65.7%, 63.3%, 57.6% in HT1080; 56.8%, 53.3%, 50.2% in MCF7) by the oAd with the 2 amine-polymer groups (oAd/$M_{2k}PN_2LG$, oAd/$M_{3.4k}PN_2LG$ or oAd/$M_{5k}PN_2LG$), the oAd with the 5 amine-polymer groups (oAd/$M_{2k}PN_5LG$, oAd/$M_{3.4k}PN_5LG$, or oAd/$M_{5k}PN_5LG$) showed much higher killing effects (59.9%, 72.5%, 76.5% in A549; 63.5%, 85.8%, 88.7% in HT1080; 55.7%, 84.4%, 87.2% in MCF7) in all the cancer cells, respectively. Moreover, 2 groups of the oAd with 5 amine-polymers (oAd/$M_{3.4k}PN_5LG$, or oAd/$M_{5k}PN_5LG$) conjugated with 3.4k PEG or 5k PEG showed the strongest killing effects in all cancer cells (72.5% or 76.5% in A549; 85.8% or 88.7% in HT1080; 84.4% or 87.2% in MCF7) ($*P < 0.05$). Therefore, we could conclude that the transduction and cancer-killing efficacy of the Ad vectors

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Bioconjugate Chem. 2015, 26, 1818−1829

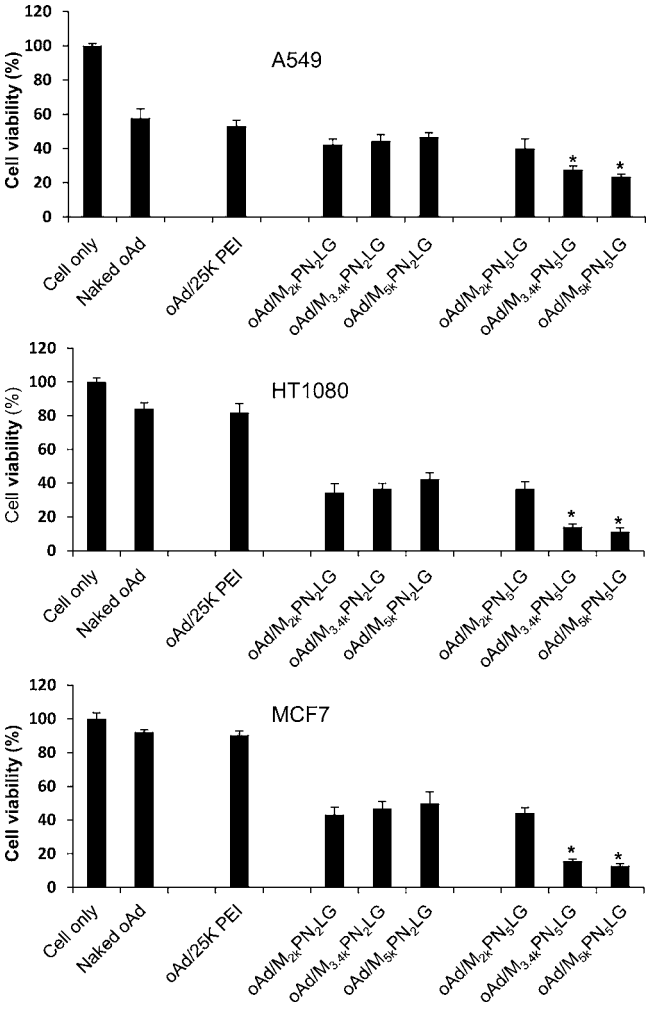

Figure 6. Comparison of cancer killing effect of Ad/polymer complexes in cancer cells. The oncolytic Ad (oAd) was complexed with the polymers (25K PEI, $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$) at room temperature for 30 min and treated to the cancer cells (50 MOI for A549; 100 MOI for HT1080; 250 MOI for MCF7) for 4 h. The treated solution was exchanged with the fresh media containing 5% FBS. After incubation for 2 days (A549 or HT1080) or 3 days (MCF7), the cells were monitored under optical microscopy and the cell-viability was quantified by MTT assay. The data represent the means ± SD of triplicate experiments. Symbols: $*P < 0.05$ versus other treated groups.

could be markedly improved by the coating with all of the polymers. Especially, longer PEG (3.4k or 5k) conjugated 5 amine-polymer groups ($M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$) delivering the Ad vectors showed the highest transduction efficiency and cancer killing effects in all cancer cells, whether high, low, or even lacking CAR expression.

In the past decade, nonviral vectors delivering therapeutic vectors via surface modification would not be sufficient to expect a successful therapeutic index due to the loss of targeting moiety. Although prolonged blood circulation time escaping from host immune responses by coating with the polymers can be achieved, an endowment of the targeting ligands for those delivering vectors can maximize the selective capture by target cell-specific receptors, followed by enhanced therapeutic efficacy and reduced side effects.[26] Therefore, the combinatorial approaches of these polymers and tumor-homing ligands are strongly considered for cancer gene therapy as promising agents to deliver genetic materials or viral vectors for further study.[26,28,43]

Immune Response of Oncolytic Ad with the Polymers. To assess whether the polymeric layer on Ad nanocomplexes reduces the induction of the adaptive immune response, we determined the level of Ad-specific neutralizing antibodies induced by each of the Ad preparations. For clinical applications of cured metastatic cancer, Ad must be injected intravenously; however, intravenous Ad injection can activate the innate immune response in the host, which limits the therapeutic efficiency of Ad. To assess whether the Ad/PNLG nanocomplexes reduce the induction of innate immune response, we determined the level of proinflammatory cytokine IL-6 secretion from macrophage after treating Ad or Ad/PNLGs. Naked oAd induced a significant increase in IL-6 level, 4.3-fold compared to PBS treated group ($**P < 0.01$) (Figure 7). In marked contrast, oAd/PNLGs treatment showed a

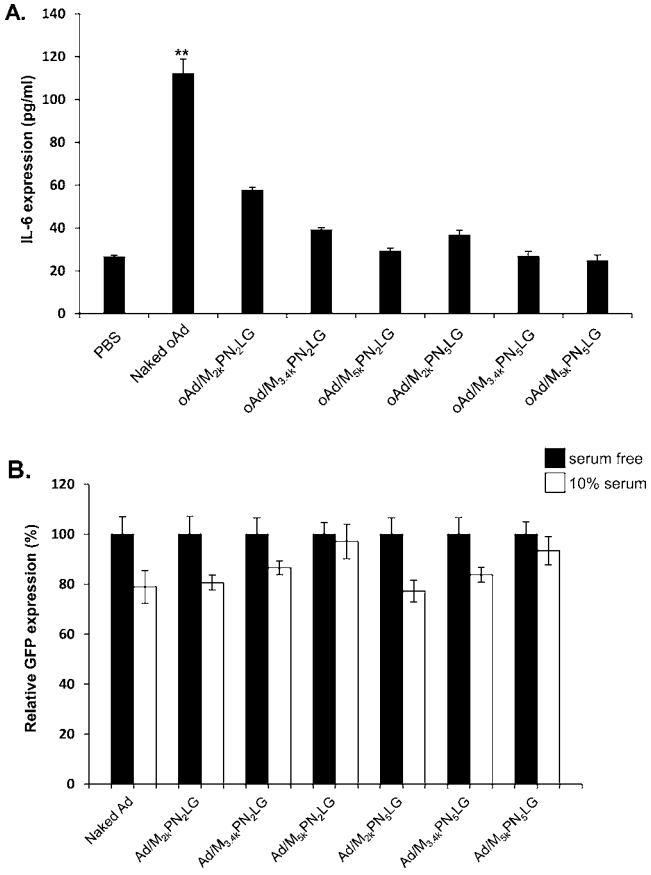

Figure 7. Innate immune response against Ad and serum tolerance of Ad/polymers. A. Amount of IL-6 secretion from Raw264.7 macrophage after transduction with naked oAd or oAd/polymers ($M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$). The data represent the means ± SD for three replicates. Symbols: $**P < 0.01$ versus PBS treated group. B. GFP gene expression infected by Ad/polymers in the presence or absence of serum. The data represent the means ± SD for three replicates.

significant decrease in IL-6 levels depending on the molecular weight of PEG; moreover, oAd/PN_2LG induced macrophage IL-6 secretion more than Ad/PN_5LGs, which is correlated with our cellular uptake results (Figure 5A). Thus, secretion of IL-6 is highly affected by viral uptake tendency, not transduction efficiency.

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Nonviral vectors deliver therapeutic genes to targeting cells often through the blood circulation system.[44] One of the major obstacles is the nonspecific interaction between polymer complexes and serum or other negatively charged components. This interaction causes complex aggregation, resulting in decreased transduction efficiency. Thus, enhancing the serum resistant capacity is an essential premise of the cationic polymer for in vivo study. To evaluate the transduction efficiency of PNLGs in the presence of serum, PNLGs were complexed with Ad treated with or without 10% FBS containing media, and transgene expression efficiency was estimated. As shown in Figure 7B, the transduction efficiency of naked Ad decreased in the presence of 10% serum, whereas Ad/PNLGs were less negatively influenced by serum. Moreover, PN_2LG is less affected by serum than PN_5LG, indicating that the surface charge is related with serum resistance. Following increased molecular weight of PEG, serum resistant ability of PNLG would be increased as well, implying that the hindrance effect of PEG can increase evasion of polymer against serum. These results demonstrate that coating the surface of Ad with PNLGs polymer can protect Ad from serum, supporting the potential use of PNLGs-coated Ad for in vivo study. Altogether, these data indicate that Ad surface coating with PNLG can attenuate the innate immune response against Ad and increased serum stability.

CONCLUSIONS

The present study evaluated a cationic biodegradable polymer tuned surface charge and PEGylation for efficient viral and nonviral delivery. In the cytotoxicity profile, PN_5LG showed more toxicity than PN_2LG, but it can recover by higher molecular weight PEGylation. For nonviral delivery, we evaluated pDNA/M_2PN_5LG at <100 diameter, highest zeta potential, and strongest transduction among the other PNLGs, suggesting that surface charge contributes the most to nonviral delivery. The higher molecular weight of PEG in the PNLG complexed with Ad resulted in decreased zeta potential and decreased cellular uptake for both 2 amine and 5 amine of PNLG, in vitro. In spite of the lower cellular uptake, Ad/PNLGs with higher molecular weight PEG showed strongest transduction efficiency as well as cancer cell killing effect. Based on the immune response result, the Ad/PNLGs is ideally suited to overcome the adverse effect of Ad. Overall, these studies provide newly synthesized structure−function insights for tuning to evaluate for both nonviral vector and viral vectors.

EXPERIMENTAL PROCEDURES

Human Cancer Cells and Ad Vectors. Human cancer cell lines (lung adenocarcinoma, A549; breast adenocarcinoma, MCF7; fibrosarcoma, HT1080; cervical cancer, HeLa) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in high glucose Dulbecco's modified Eagle's Media (DMEM) containing 10% fetal bovine serum (FBS) in 37 °C incubator with 5% CO_2. The construction and production of a replication-incompetent Ad expressing GFP (dE1/GFP, referred here as Ad) is described in our previous report.[25] The oncolytic Ad (Ad-ΔB7-U6shIL8, referred to here as oAd) expressing short hairpin RNA (shRNA) against interleukin-8 (IL-8) RNA was used as mentioned in ref 45.

Cationic Polymers. Methoxy poly(ethylene glycol) amine with different molecular weights (MPEG-NH_2, Mw 2000, 3400, or 5000) was purchased from SunBio Corporation. L-Glutamic acid γ-benzyl ester (BLG) and 2-hydroxypyridine (2-HP) were obtained from Sigma-Aldrich. Triphosgene, diethylenetriamine (DETA), and pentaethylenehexamine (PEHA) were obtained from TCI. Both 6 cationic polymers were synthesized according to the previously reported literature with slight modification.[29] First, cyclic monomer BLG-NCA was prepared by the reaction between 10 g BLG and 6.255 g triphosgene in 100 mL anhydrous tetrahydrofuran for 2 h at 50 °C under nitrogen environment. The crude product was collected by precipitation three times in hexane, and purified by recrystallization twice in 2:1 hexane/ethyl acetate. Second, diblock copolymers methoxy poly(polyethylene glycol)-*block*-poly(γ-benzyl-L-glutamate) (MPEG-PBLG) with different MPEG block lengths ($MPEG_{2k}$, $MPEG_{3.4k}$, or $MPEG_{5k}$) were prepared by the ring-opening polymerization of BLG-NCA initiated by MPEG-NH_2 with correlative molecular weights. Briefly, MPEG-NH_2 (1 equiv) and BLG-NCA (40 equiv) were dissolved in anhydrous chloroform. The reaction was kept at room temperature for 72 h under nitrogen protection, followed by concentration and precipitation in excess cold ether. Third, 6 cationic polymers were obtained by aminolysis of 3 diblock copolymers ($MPEG_{2K}$PBLG, $MPEG_{3.4K}$PBLG, or $MPEG_{5K}$PBLG) with DETA or PEHA. MPEG-PBLG and 2-HP (5× mol ratio to the ester groups of MPEG-PBLG) were dissolved in anhydrous *N*,*N*′-dimethylformamide and warmed up in an oil bath at 50 °C. DETA or PEHA was subsequently injected and the reaction was allowed to proceed for 72 h before precipitating in excess cold ether. Precipitated product was further purified by dialysis again in 0.05 M HCL (1 day) and deionized water (2 days) using dialysis bag at 3.5 kDa molecular weight cut off. The final products (methoxy poly(ethylene glycol)-*b*-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]-L-glutamate}and methoxy poly-(ethylene glycol)-*b*-poly[*N*-(*N*-{*N*-(*N*-[*N*-(2-aminoethyl)-2-aminoethyl]-2-aminoethyl)-2-aminoethyl}2-aminoethyl)-L-glutamate} $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$ (Figure 1) were collected by lyophilization. ^1H NMR spectra was recorded on Varian Unity Inova 500NB (500 MHz) in deuterium water. bPEI (Mw 25 000) was purchased from Sigma-Aldrich (St. Louis, MO).

Cytotoxicity of Polymers. A549, HT1080, and MCF7 cells were seeded at 50% confluence in 24 well plates and then treated with 25K PEI or 6 PNLGs at concentrations ranging 1−20 μg/mL. Two days following polymer treatment, 200 μL of 2 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to each well and incubated for 4 h at 37 °C. The precipitate was dissolved in 500 μL dimethyl sulfoxide (DMSO, Sigma-Aldrich). Plates were read on a microplate reader (Tecan Infinite M200; Tecan Deutschland GmbH, Crailsheim, Germany) at 540 nm.

Dynamic Light Scattering (DLS) Assay. Average particle sizes and surface charges of the pDNA encoding GFP gene (1 μg) or the naked Ad encoding GFP gene (pEGFP-N1, Clontech Lifescience Inc., Mountain View, CA) complexed with the polymers (25K PEI, $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$; N/P ratio = 5 for 25K PEI, N/P ratio = 20 for PNLGs) were determined by the Zetasizer 3000HS (Malvern Instrument Inc., Worcestershire, UK) with a He−Ne Laser beam (633 nm, fixed scattering angle of 90°) at room temperature. The obtained sizes were presented as the average values of 3 runs. For coating the Ad particles with the polymers, millions of molecules of the polymers versus a viral particle (VP) (25K PEI: 1×10^5

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Bioconjugate Chem. 2015, 26, 1818−1829

molecules/VP; $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$: 1×10^5, 5×10^5, 1×10^6, or 5×10^6 molecules/VP) were complexed with the Ad particles (1×10^9 VP (initial titer)), diluted with phosphate-buffered saline (PBS) for 30 min at room temperature before the DLS assay.

DNA Transfection Assay. The HeLa cells were seeded on 24-well plates at 80% of confluence, 1 day before the transfection. The pDNA encoding GFP gene (1 μg) was polyplexed with the polymers (25K PEI: N/P ratio = 5, $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$: N/P ratio = 5−40 for 30 min at room temperature before the transfection. The HeLa cells were transfected with each polyplex solution diluted with the serum free DMEM media at 37 °C incubator. For evaluation of serum tolerance, pDNA/polymer polyplex (25K PEI: N/P ratio = 5, $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$: N/P ratio = 20) transfected into the HeLa cell in either serum free media or 10% FBS contained media. The fresh media containing 10% FBS were exchanged after incubation for 4 h, and the treated cells were further incubated for 2 days. To determine the transgene expression levels from the cells, each GFP expression was quantified by measuring the absorbance with a plate reader (Tecan Infinite M200).

Ad Transduction Efficiency in Human Cancer Cells. The human cancer cells (A549, HT1080, or MCF7) were seeded on 24-well plates at 80% confluence, 1 day before the transduction assay. The cells were treated with the naked Ad (Ad-ΔE1/GFP) at multiplicity of infection (MOI) of 50 (A549), 100 (HT1080), and 250 (MCF7) or the Ad/polymers (25K PEI: 1×10^5 molecules/VP; $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$: 1×10^5, 5×10^5, 1×10^6, or 5×10^6 molecules/VP) in serum free condition at 37 °C. For evaluation of serum tolerance, Ad/polymer complex (25K PEI: 1×10^5 molecules/VP; $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$: 1×10^6 molecules/VP) infected the A549 cell in either serum free media or 10% FBS containing media. Fresh media containing 5% FBS were exchanged after the incubation for 4 h. The treated cells were further incubated for 2 days. For quantifying the transgene expression, each GFP expression was photographed under fluorescence microscopy and quantified as described above.

Cellular Uptake of Ad/Polymers. The naked Ad was conjugated with fluorescein isothiocyanate (FITC, Sigma-Aldrich) for 4 h, then dialysis (10K cut off, Slide-A-Lyzer Dialysis Cassettes, Life Technologies, Grand Island, NY) to remove unbound FITC. A549 cells were treated with Ad-FITC or Ad-FITC/polymers (25K PEI: 1×10^5 molecules/VP; $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$: 1×10^6 molecules/VP) MOI of 200 for 2 h, then washed 3 times with PBS to remove Ad/polymers settled onto the surfaces of the cells. Cellular uptake activity was quantified by measuring the fluorescence intensity with a plate reader (Tecan Infinite M200).

Endosome Escape of Ad/Polymers. The A549 cells were seeded on 24-well plates at 80% of confluence. One day later, cells were pretreated with chloroquine at 50 μM for 30 min. Naked Ad or Ad complexed with polymers (25K PEI: 1×10^5 molecules/VP; $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$: 1×10^6 molecules/VP) MOI of 100 were then added in the absence or presence of chloroquine for an additional 4 h. Cells were washed with PBS and incubated with fresh DMEM for 2 days. GFP expression was quantified as described above.

Cancer Cell Killing Effect. The human cancer cells (A549, HT1080, or MCF7) were seeded on 24-well plates at 80% confluence, 1 day before the treatment. The cells were treated with the naked oncolytic Ad (oAd) at a multiplicity of infection (MOI) of 50 (A549), 100 (HT1080), and 250 (MCF7) or the Ad/polymers (25K PEI: 1×10^5 molecules/VP; $M_{2k}PN_2LG$, $M_{3.4k}PN_2LG$, $M_{5k}PN_2LG$, $M_{2k}PN_5LG$, $M_{3.4k}PN_5LG$, or $M_{5k}PN_5LG$: 1×10^6 molecules/VP) at 37 °C. Fresh media containing 5% FBS were exchanged after incubation for 4 h. After incubation for 2 days (A549, and HT1080) or 3 days (MCF7) allowing viral replication and cell lysis, the medium was replaced with fresh medium containing MTT in PBS. Subsequently, the cells were incubated at 37 °C for 4 h in the dark. The MTT solution was removed and replaced with 500 μL of DMSO to solubilize the produced formazan crystals. The absorbency was determined using a microplate reader (Tecan Infinite M200) at 540 nm. The cell viability was expressed as the percentage of untreated cells as a negative control.

Evaluation of Innate Immune Response. RAW264.7 macrophage cells were seeded on a six-well plate at a density of 1×10^6 cells/well. One day later, cells were treated with 1×10^{10} viral particles of naked oAd, or oAd/polymers (1×10^6 molecules/VP) for 12 h at 37 °C. The level of IL-6 secretion by macrophages was determined from culture media supernatant using an IL-6 enzyme-linked immunosorbent assay (ELISA) kit (R&D Quantikine, Minneapolis, MN).

Statistical Analysis. The data was expressed as the mean ± standard deviation (SD) where indicated. Comparisons between two samples were analyzed for homogeneity of variance using Levene's test and analyzed by Student's *t*-test. Groups with *p* values less than 0.05 were considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR of MPEG-PBLG (A), MPEG-PN_2LG (B), and MPEG-PN_5LG, degree of polymerization and molecular weight of MPEG-PBLG diblock copolymers, average molecular weight of cationic polymers, status of CAR expression on the cancer cells, average size distribution (nm) and surface charge of pDNA/polymer polyplex, and average size distribution (nm) and surface charges (mV) of naked Ad or Ad/complex. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00357.

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Notes

The authors declare no competing financial interest.

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Bioconjugate Chem. 2015, 26, 1818−1829

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REFERENCES

(1) Mintzer, M. A., and Simanek, E. E. (2009) Nonviral vectors for gene delivery. *Chem. Rev. 109*, 259−302.
(2) Hunt, K. K., and Vorburger, S. A. (2002) Tech.Sight. Gene therapy. Hurdles and hopes for cancer treatment. *Science 297*, 415−6.
(3) Trentin, D., Hubbell, J., and Hall, H. (2005) Non-viral gene delivery for local and controlled DNA release. *J. Controlled Release 102*, 263−75.
(4) Borchard, G. (2001) Chitosans for gene delivery. *Adv. Drug Delivery Rev. 52*, 145−50.
(5) Zhang, X., Ercelen, S., Duportail, G., Schaub, E., Tikhonov, V., Slita, A., Zarubaev, V., Babak, V., and Mely, Y. (2008) Hydrophobically modified low molecular weight chitosans as efficient and nontoxic gene delivery vectors. *J. Gene Med. 10*, 527−39.
(6) Neu, M., Fischer, D., and Kissel, T. (2005) Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. *J. Gene Med. 7*, 992−1009.
(7) Akinc, A., Thomas, M., Klibanov, A. M., and Langer, R. (2005) Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J. Gene Med. 7*, 657−63.
(8) Godbey, W. T., Wu, K. K., and Mikos, A. G. (1999) Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. *J. Biomed. Mater. Res. 45*, 268−75.
(9) Ou, M., Wang, X. L., Xu, R., Chang, C. W., Bull, D. A., and Kim, S. W. (2008) Novel biodegradable poly(disulfide amine)s for gene delivery with high efficiency and low cytotoxicity. *Bioconjugate Chem. 19*, 626−33.
(10) Deng, C., Wu, J., Cheng, R., Meng, F., Klok, H.-A., and Zhong, Z. (2014) Functional polypeptide and hybrid materials: Precisionsynthesis via -amino acid N-carboxyanhydridepolymerization and emerging biomedical applications. *Prog. Polym. Sci. 39*, 330−364.
(11) Volpers, C., and Kochanek, S. (2004) Adenoviral vectors for gene transfer and therapy. *J. Gene Med. 6* (Suppl 1), S164−71.
(12) Wu, Q., Moyana, T., and Xiang, J. (2001) Cancer gene therapy by adenovirus-mediated gene transfer. *Curr. Gene Ther. 1*, 101−22.
(13) Choi, K. J., Kim, J. H., Lee, Y. S., Kim, J., Suh, B. S., Kim, H., Cho, S., Sohn, J. H., Kim, G. E., and Yun, C. O. (2006) Concurrent delivery of GM-CSF and B7−1 using an oncolytic adenovirus elicits potent antitumor effect. *Gene Ther. 13*, 1010−20.
(14) Lee, Y. S., Kim, J. H., Choi, K. J., Choi, I. K., Kim, H., Cho, S., Cho, B. C., and Yun, C. O. (2006) Enhanced antitumor effect of oncolytic adenovirus expressing interleukin-12 and B7−1 in an immunocompetent murine model. *Clin. Cancer Res. 12*, 5859−68.
(15) Shewach, D. S., Zerbe, L. K., Hughes, T. L., Roessler, B. J., Breakefield, X. O., and Davidson, B. L. (1994) Enhanced cytotoxicity of antiviral drugs mediated by adenovirus directed transfer of the herpes simplex virus thymidine kinase gene in rat glioma cells. *Cancer gene therapy 1*, 107−12.
(16) Yoo, J. Y., Kim, J. H., Kwon, Y. G., Kim, E. C., Kim, N. K., Choi, H. J., and Yun, C. O. (2007) VEGF-specific short hairpin RNA-expressing oncolytic adenovirus elicits potent inhibition of angiogenesis and tumor growth. *Mol. Ther. 15*, 295−302.
(17) Zhang, W. W., Alemany, R., Wang, J., Koch, P. E., Ordonez, N. G., and Roth, J. A. (1995) Safety evaluation of Ad5CMV-p53 in vitro and in vivo. *Hum. Gene Ther. 6*, 155−64.
(18) Kim, J., Kim, J. H., Choi, K. J., Kim, P. H., and Yun, C. O. (2007) E1A- and E1B-Double mutant replicating adenovirus elicits enhanced oncolytic and antitumor effects. *Hum. Gene Ther. 18*, 773−86.
(19) Nemunaitis, J., and O'Brien, J. (2002) Head and neck cancer: gene therapy approaches. Part II: genes delivered. *Expert Opin. Biol. Ther. 2*, 311−24.
(20) Alemany, R., Suzuki, K., and Curiel, D. T. (2000) Blood clearance rates of adenovirus type 5 in mice. *Journal of general virology 81*, 2605−9.
(21) Kiang, A., Hartman, Z. C., Everett, R. S., Serra, D., Jiang, H., Frank, M. M., and Amalfitano, A. (2006) Multiple innate inflammatory responses induced after systemic adenovirus vector delivery depend on a functional complement system. *Mol. Ther. 14*, 588−98.
(22) Molnar-Kimber, K. L., Sterman, D. H., Chang, M., Kang, E. H., ElBash, M., Lanuti, M., Elshami, A., Gelfand, K., Wilson, J. M., Kaiser, L. R., et al. (1998) Impact of preexisting and induced humoral and cellular immune responses in an adenovirus-based gene therapy phase I clinical trial for localized mesothelioma. *Hum. Gene Ther. 9*, 2121−33.
(23) Muruve, D. A., Barnes, M. J., Stillman, I. E., and Libermann, T. A. (1999) Adenoviral gene therapy leads to rapid induction of multiple chemokines and acute neutrophil-dependent hepatic injury in vivo. *Hum. Gene Ther. 10*, 965−76.
(24) Sumida, S. M., Truitt, D. M., Lemckert, A. A., Vogels, R., Custers, J. H., Addo, M. M., Lockman, S., Peter, T., Peyerl, F. W., Kishko, M. G., et al. (2005) Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. *J. Immunol. 174*, 7179−85.
(25) Choi, J. W., Jung, S. J., Kasala, D., Hwang, J. K., Hu, J., Bae, Y. H., and Yun, C. O. (2015) pH-sensitive oncolytic adenovirus hybrid targeting acidic tumor microenvironment and angiogenesis. *J. Controlled Release 205*, 134−143.
(26) Choi, J. W., Lee, J. S., Kim, S. W., and Yun, C. O. (2012) Evolution of oncolytic adenovirus for cancer treatment. *Adv. Drug Delivery Rev. 64*, 720−9.
(27) Jung, S. J., Kasala, D., Choi, J. W., Lee, S. H., Hwang, J. K., Kim, S. W., and Yun, C. O. (2015) Safety profiles and antitumor efficacy of oncolytic adenovirus coated with bioreducible polymer in the treatment of a CAR negative tumor model. *Biomacromolecules 16*, 87−96.
(28) Kim, J., Kim, P. H., Kim, S. W., and Yun, C. O. (2012) Enhancing the therapeutic efficacy of adenovirus in combination with biomaterials. *Biomaterials 33*, 1838−50.
(29) Kim, J., Li, Y., Kim, S. W., Lee, D. S., and Yun, C. O. (2013) Therapeutic efficacy of a systemically delivered oncolytic adenovirus - biodegradable polymer complex. *Biomaterials 34*, 4622−31.
(30) Kim, P. H., Kim, T. I., Yockman, J. W., Kim, S. W., and Yun, C. O. (2010) The effect of surface modification of adenovirus with an arginine-grafted bioreducible polymer on transduction efficiency and immunogenicity in cancer gene therapy. *Biomaterials 31*, 1865−74.
(31) Moon, C. Y., Choi, J. W., Kasala, D., Jung, S. J., Kim, S. W., and Yun, C. O. (2015) Dual tumor targeting with pH-sensitive and bioreducible polymer-complexed oncolytic adenovirus. *Biomaterials 41*, 53−68.
(32) Zhang, Y., Satterlee, A., and Huang, L. (2012) In vivo gene delivery by nonviral vectors: overcoming hurdles? *Mol. Ther. 20*, 1298−304.
(33) O'Riordan, C. R., Lachapelle, A., Delgado, C., Parkes, V., Wadsworth, S. C., Smith, A. E., and Francis, G. E. (1999) PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. *Hum. Gene Ther. 10*, 1349−58.
(34) Mislick, K. A., and Baldeschwieler, J. D. (1996) Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc. Natl. Acad. Sci. U. S. A. 93*, 12349−54.
(35) Chithrani, B. D., Ghazani, A. A., and Chan, W. C. (2006) Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. *Nano Lett. 6*, 662−8.

DOI: 10.1021/acs.bioconjchem.5b00357
Bioconjugate Chem. 2015, 26, 1818−1829

(36) Osaki, F., Kanamori, T., Sando, S., Sera, T., and Aoyama, Y. (2004) A quantum dot conjugated sugar ball and its cellular uptake. On the size effects of endocytosis in the subviral region. *J. Am. Chem. Soc. 126*, 6520−1.
(37) Xu, X. H., Brownlow, W. J., Kyriacou, S. V., Wan, Q., and Viola, J. J. (2004) Real-time probing of membrane transport in living microbial cells using single nanoparticle optics and living cell imaging. *Biochemistry 43*, 10400−13.
(38) Hatakeyama, H., Akita, H., and Harashima, H. (2011) A multifunctional envelope type nano device (MEND) for gene delivery to tumours based on the EPR effect: a strategy for overcoming the PEG dilemma. *Adv. Drug Delivery Rev. 63*, 152−60.
(39) Croyle, M. A., Chirmule, N., Zhang, Y., and Wilson, J. M. (2002) PEGylation of E1-deleted adenovirus vectors allows significant gene expression on readministration to liver. *Hum. Gene Ther. 13*, 1887−900.
(40) Chillon, M., Lee, J. H., Fasbender, A., and Welsh, M. J. (1998) Adenovirus complexed with polyethylene glycol and cationic lipid is shielded from neutralizing antibodies in vitro. *Gene Ther. 5*, 995−1002.
(41) Singh, R., Tian, B., and Kostarelos, K. (2008) Artificial envelopment of nonenveloped viruses: enhancing adenovirus tumor targeting in vivo. *FASEB J. 22*, 3389−402.
(42) Kwon, O. J., Kang, E., Choi, J. W., Kim, S. W., and Yun, C. O. (2013) Therapeutic targeting of chitosan-PEG-folate-complexed oncolytic adenovirus for active and systemic cancer gene therapy. *J. Controlled Release 169*, 257−65.
(43) Kim, J., Nam, H. Y., Choi, J. W., Yun, C. O., and Kim, S. W. (2014) Efficient lung orthotopic tumor-growth suppression of oncolytic adenovirus complexed with RGD-targeted bioreducible polymer. *Gene Ther. 21*, 476−83.
(44) Yin, H., Kanasty, R. L., Eltoukhy, A. A., Vegas, A. J., Dorkin, J. R., and Anderson, D. G. (2014) Non-viral vectors for gene-based therapy. *Nat. Rev. Genet. 15*, 541−55.
(45) Yoo, J. Y., Kim, J. H., Kim, J., Huang, J. H., Zhang, S. N., Kang, Y. A., Kim, H., and Yun, C. O. (2008) Short hairpin RNA-expressing oncolytic adenovirus-mediated inhibition of IL-8: effects on antiangiogenesis and tumor growth inhibition. *Gene Ther. 15*, 635−51.

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Bioconjugate Chem. 2015, 26, 1818−1829