The Characterization and Reductive Degradation Behavior Study of Bioreducible Crosslinked Polypropylenimine Dendrimers for Gene Delivery Systems

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ABSTRACT

Crosslinked bioreducible polymers, PPI–CBAs, are synthesized by Michael-type polyaddition of polypropylenimine (PPI) and N,N`-cystamine bisacrylamide (CBA) for gene delivery systems. PPI–CBAs could form nano-sized polyplexes, which show high stability even in reducing condition probably due to the re-crosslinking. PPI–CBAs show high transfection efficiency comparable to PEI25k in serum condition. However, cytotoxicity of PPI–CBAs is increased with the increase of concentration and crosslinking degrees of PPI–CBAs. Decrease of glutathione (GSH) levels and the following increase of reactive oxygen species (ROS) levels are also observed. PPI–CBA polyplexes also could induce the similar effects. These results suggest that the cytotoxicity of bioreducible polymers may be closely related with the polymer structures and that reduction of intracellular GSH and following increase of intracellular ROS may induce cytotoxicity by oxidative stress.

Keywords: bioireducilbe polymer, crosslink, cytotoxicity, gene delivery system, polypropylenimine

1 INTRODUCTION

Polypropylenimine (PPI) dendrimers and their derivatives have been used for gene delivery because of their properties such as cationic charge, equivalent surface functionalities, and internal cavities [1–4]. However, high generation PPIs made high cytotoxicity induced by the interaction with cell membranes; it limits their applications in vivo [5]. Generally, polymeric gene carriers with high molecular weights showed high transfection efficiency but also high cytotoxicity. Therefore, many studies also reported that polymeric gene delivery carriers composed of low molecular weight polymers with biodegradable linkages showed high transfection efficiency and low cytotoxicity due to enhanced gene condensation and intracellular degradation [6].

Especially, bioreducible crosslinkers have been used for bioreducible polymeric gene carriers. Bioreducible polymeric gene carriers, which have internal disulfide bonds, possess many advantages for gene delivery systems, including selective degradability and release control of condensed genes from polyplexes in reducing environment [7–9] such as cytoplasm containing glutathione with high concentration (0.5–10 mM) [10]. They also have been reported that they have low cytotoxicity by avoiding accumulation and undesirable interaction of toxic cationic molecules via degradation in cytoplasm.

From now on, a variety of crosslinked polymeric gene carrier possessing bioreducible linkages have been reported. However, crosslinked PPI dendrimers with bioreducible linkages have not been reported for gene delivery systems to our knowledge so far. Therefore, in this work, we synthesized high molecular weight bioreducible polymers (PPI–CBAs) by crosslinking low molecular weight PPI dendrimers with bioreducible CBA using Michael-type polyaddition in order to utilize the advantages of PPI dendrimers, achieving high transfection efficiency and maintaining low cytotoxicity. The physicochemical properties of the polymers were characterized and their potency for gene delivery systems was evaluated. Actually, interesting results about the degradation behaviors and cytotoxicity of PPI–CBAs were observed and they were studied further.

2 EXPERIMENTS

2.1 Synthesis and Characterization of Crosslinked PPIs

Crosslinked PPIs were synthesized by Michael-type addition of PPI G1 and CBA in MeOH/water solution (9:1, v/v). After 24h reaction, the products were dialyzed against distilled water and lyophilized.

The products were confirmed by ^{1}H NMR (600MHz, AVANCE 600, Brucker, Germany). The molecular weights of the polymers were measured by GPC (YL-9100, Young Lin Instrument, Korea) which have Ultrahydrogel 250 column (Waters, Milford, MA). Elemental composition of the polymers were also analyzed by elemental analyzer (Flash EA 1112, Thermo Electron Corporation, USA).

2.2 Agarose Gel Electrophoresis

To assess pDNA condensing ability of PPI-CBAs, agarose gel electrophoresis was carried out. The polyplexes were incubated for 30 min at RT. To examine degradation

PPI-CBA	Initial feed ratio [PPI:CBA]	Experimental composition ratios [PPI:CBA]	M_n	M_w	PDI	Charge density [Da/+]
P4C2	1:0.25	1:0.5	4.62	6.81	1.47	53.1
P4C3	1:0.5	1:0.75	8.67	15.5	1.78	63.9
P4C5	1:1	1:1.24	9.79	22.8	2.33	85.6
P4C9	1:2	1:2.17	18.9	66.4	3.52	129.0

Table 1: Chemical properties of synthesized PPI-CBAs.

Elements	P4C2	P4C3	P4C5	P4C9
C	39.49[a] (0.58)[b] 0.98[c]	40.21 (0.57) 0.98	40.72 (0.56) 0.98	38.97 (0.54) 0.97
H	9.19 (0.13) 1.19	8.52 (0.12) 1.12	8.38 (0.11) 1.14	8.06 (0.11) 1.21
N	14.98 (0.22) 0.96	14.43 (0.21) 0.95	13.86 (0.19) 0.96	12.48 (0.17) 0.97
S	5.00 (0.07) 0.98	7.44 (0.11) 1.07	9.94 (0.14) 1.03	12.43 (0.17) 1.01

Table 2: Elemenatal analysis result of synthesized PPI-CBAs.
a)Found value of the element (%) in each PPI-CBA; b)relative ratio of the element among total elements in each PPI-CBA; c)relative ratio between elemental analysis and NMR anlaysis of each element.

behavior of the polyplexes in reductive environment, agaros e gel electrophoresis was also carried out by using the polyplexes incubated for 30 min in the presence of 5 mM DTT.

2.3 PicoGreen Assay

To examine the degradation behavior of PPI-CBAs quantitatively, PicoGreen assay was carried out. Linear bioreducible polymer, poly(cystaminebisacrylamide-diaminobutane) (poly(CBA-DAB)) and PEI25k were used as controls. All polyplexes was formed at N/P ratio 5. Each polyplex was incubated in 5 mM DTT solution for pre-detedmined time. Fluorescence was measured with an 480 nm excitation wavelength and 520 nm emission wavelength.

2.4 Average Size and Zeta-Potential Values Measurement of PPI-CBA Polyplexes

The average sizes and Zeta-potential values of PPI-CBA polyplexes were measured by Zeta-sizer Nano ZS (Malvern Instruments, UK). 0.5 mL of polyplex solutions were prepared. After 30 min incubation, the solutions were diluted to 1 mL.

2.5 Cytotoxicity

The cytotoxicity of the polymers and the polyplexes was examined by MTT assasy. HeLa human cervical adenocarcinoma cells were used for the assay. PEI25k and PPI G1 were used as controls. The polymer solutions were teated for 4 hours. The absorbance was measured at 570 nm.

2.6 Transfection Experiments In Vitro

HeLa cells were used for the assay. The cells were exposed for 4 hours in serum-free and 10% serum condition. Luciferase assay kit and BCA protein assay reagent kit were used.

2.7 Measurement of Intracellular Glutathione (GSH) Level

Effect of PPI-CBAs or PPI-CBA polyplexes to intracellular GSH level was examined in HeLa cells. GSH assay was performed according to the slightly modified mehtod from the manufacturer's given protocol.

2.8 Measurement of Intracellular Reactive Oxygen Species (ROS) Level

Effect of PPI-CBAs or PPI-CBA polyplexes to intracellular ROS level was exmined in HeLa cells. ROS assay was performed according to the slightly modified method from the manufacturer's given protocol.

3 RESULTS AND DISCUSSION

In this proceeding, we will provide only a part of the result. It will be fully provided in the poster.

3.1 Synthesis and Characterization of PPI-CBAs

Chemical properties and elemental analysis result of synthesized PPI-CBAs were organized at Table 1 and Table 2. The experimental ratios and molecular weights of polymers were increased along with increased CBA feed ratio. The composition ratios of S were increased and those of N were decreased according to the increase of CBA portion in PPI-CBAs. These results show good correlation between ^{1}H NMR and EA result.

3.2 Agarose Gel Electrophoresis

PPI-CBA polyplexes could reatard pDNA at low weight ratios, which means the condensing ability of PPI-CBAs was improved with the increase of the charge density. In the

presence of 5 mM DTT, interesting result was obtained. We expected that the disintegration of polyplexes in reducing environment, but PPI-CBAs displayed resistance. They still could condense pDNA even at low weight ratio. From the result, we assume that some mechanism could stabilize the polymer structure.

3.3 PicoGreen Assay

PEI25k polyplex displayed very low fluorescence regardless of incubation time, which means that PEI25k polyplexes were not disintegrated in reducing condition. On the contrary, p(CBA-DAB) polyplex showed much higher fluorescence even after 30 min incubation, which means that they were easily disintegrated in reductive condition.

However, that of PPI-CBA polyplexes were relatively low even after 4 h incubation in ruductive condition. From the result, we could suggest that the disrupted PPI-CBA polyplexes might be stabilized by re-crosslinking of PPI-CBA fragments via auto-oxidation between their thiols. From the comparison with p(CBA-DAB), we could deduced that the structure of bioreducible polymers would be important factors for degradation beahvior of them.

3.4 Average Size and Zeta-Potential Values Measurement of PPI-CBA Polyplexes

Z-average size of most of PPI-CBA polyplexes was below 250 nm. Zeta-potential of PPI-CBA polyplexes was

positive at most of weight ratios for all PPI-CBA polyplexes. Zeta-potentail of PPI-CBA polyplexes were increased with the increase of weight ratio upto 50 mV at weight ratio 20. These result mean that PPI-CBA can form nano-sized, positively charged polyplexes.

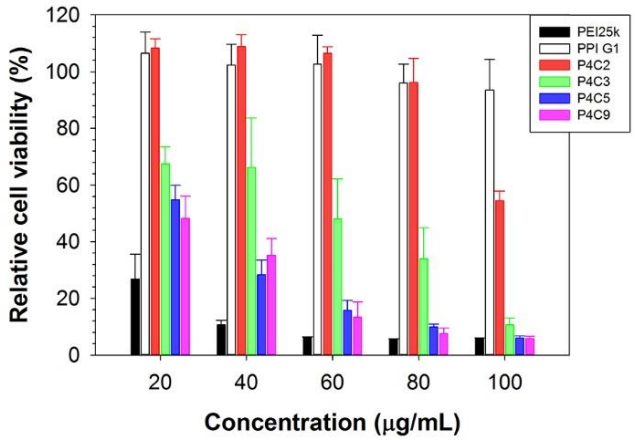

Figure 2: MTT assay result of PPI-CBAs on HeLa cells.

3.5 Cytotoxicity

The cytotoxicity of PPI-CBAs was increased with the increase in concentration and crosslinking degrees of them. It may be caused by unusual degradation behavior of the polymer in reductive condition. Therefore, additional characterizations were carried out to examine the reason of cytotoxicity.

3.6 Transfection Efficiency In Vitro

PEI25k, PPI G1, and p(CBA-DAB) polyplexes prepared at optimal ratio were used as controls. PPI-CBA polyplexes displayed lower transfection efficiency than PEI25k in the absence of serum but high transfection efficiency comparable to PEI25k in the presence of serum. The result suggest PPI-CBAs have a good potential for gene delivery systems. The results also demonstrate that high transfection efficiency could be achieved by crosslinking low molecular weight molecules.

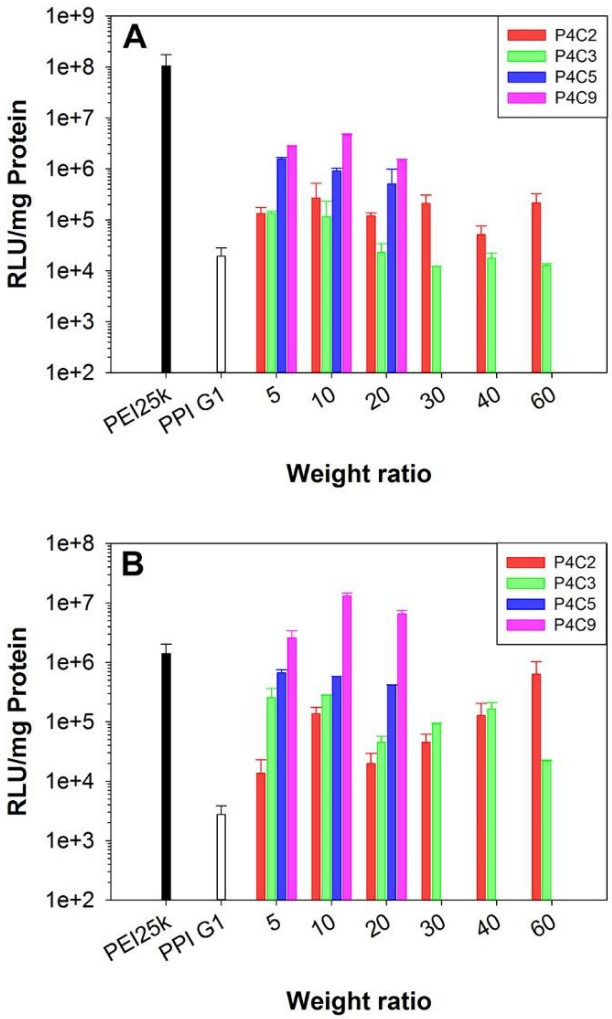

Figure 1 Transfection experiment result of PPI-CBAs on HeLa cells in serum-free condition (A) and serum condition (B).

3.7 Measurement of Intracellular GSH Level and Intracellular ROS Level

The decrease of GSH level and following increase of ROS level were also observed with the increase in concentration and crosslinking degrees of the polymers. PPI-CBA polyplexes also could induce the similar effects with less degree. These results suggest that the cytotoxicity of bioreducible polymers may be closely related with the polymer structures and that reduction of intracellular GSH quantity due to degradation of re-crosslinked bioreducible polymers and resulting increase of intracellular ROS quantity may cause cytotoxicity by oxidative stress.

4 CONCLUSION

PPI-CBAs were synthesized for gene delivery systems. They could form positively charged and nano-sized polyplexes. Interestingly, PPI-CBA polyplexes showed high stability in reductive condition, probably decause of the PPI-CBA fragment re-crosslinking. PPI-CBAs showed lower transfection efficiency than PEI25k in serum-free condition but comparable efficiency with PEI25k in serum condition, which means PPI-CBAs possessed good serum-resistance. However, cytotoxicity of PPI-CBAs was significant and increased with the increase of concentration and crosslinking degree. Decreased intracellular GSH and increased ROS levels with increase of the crosslinking degree suggest that GSH consumption due to re-crosslinking of degraded PPI-CBAs in cytosol may induce the increase of intracellular ROS. Therefore, unlike linear bioreducible polymers, crosslinked bioreducible polymer, PPI-CBAs show unique degradation and intracellular behaviors. This study would provide a deeper insight into the development of bioreducible polymeric gene carriers.

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