pH-Sensitive PEG-modification onto cell signal-responsive polyplex for improved colloidal stability and cell specificity in gene delivery

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ABSTRACT SUMMARY
We have reported disease-cell specific gene delivery systems that are responsive to target intracellular signals (protein kinase or protease) abnormally activated in target disease cells. In this system, cationic peptide-polymer conjugates are used as signal-responsive gene regulating carrier. In this research, PEG was modified onto the carrier in pH-sensitive manner to improve colloidal stability of carrier/gene complex. Resulting carrier showed improved disease cell-specificity of the transgene expression as well as improved colloidal stability in serum.

INTRODUCTION
Although current gene therapy possesses a great potential for the treatment of cancer, it still has serious problem with its target cell specificity. To access this issue, passive and active targeting strategies have been widely reported. However, cell-specificity has not fully satisfied to avoid side effects which disturb clinical application of gene therapy. While passive targeting, which uses enhanced permeability of neovasculature in tumor tissue, actually enhances the accumulation of gene/carrier complex into tumor, the accumulation is only several % of the total dosage which means more than 90% of gene might distribute to other normal organs. On the other hand, active targeting suffers from heterogeneity of tumor tissue and still large amount of gene complex would deliver into non-target cells and tissues. These facts suggest that it will be nearly impossible to send gene complex only into target cancer cells so that suppression of the transgene activity in non-target cells and site-selective activation will be crucial to achieve real cell specificity. In this context, we have developed intracellular signal-responsive gene carriers which can release transgene only after reacting with target signal enzymes hyperactivated in target disease cells. When this strategy was applied to cancers which contain active protein kinase Cα, highly cancer-specific gene expression was observed in vitro and in vivo.¹,² However, colloidal stability of the polyplex was not stable enough for i.v. administration. Therefore we tried here to introduce PEG chains onto the carrier. However, the PEG modification attenuated the signal responsiveness of the gene regulation and transfection ability into living cells, although the colloidal stability was improved.³ Thus, we designed pH-labile PEG modification onto the carrier. Resulting carrier showed high stability in serum condition and also improved its cell specificity.

EXPERIMENTAL METHODS
pH-nonresponsive or pH-labile PEG modification was performed onto peptide-grafted polymer, where the grafted peptide was PKCα-specific substrate. For the pH-
nonresponsive PEG modification, peptide and amino-PEG (M.W. 5000) was reacted with poly(N-isopropylacrylamide)-co-(secinimidloxy-acrylate) random copolymer. On the other hand, blanched-polyethyleneimine (PEI) was reacted with protected-PKCα substrate peptide which has carboxylethyl-amide group at the N-terminus and PEG(M.W. 5000)-carbonylbenzaldehyde after which the peptide side chains were deprotected to obtain pH-labile PEG modified polymer (bPEI-PEG).

Gene regulation ability was investigated by cell-free expression experiment and *in vitro* expression using HepG2 cell. Luciferase-encoding plasmid was used as reporter gene. In the cell-free experiment, luciferase expression was measured in the presence and absence of PKCα. For bPEI-PEG, luciferase expression was compared at pH 7.4 and 6.5.

**RESULTS AND DISCUSSION**

For the synthesis of NPAK-PEG (pH-nonresponsive), some polymers with different content of PEG (0, 0.6, 3.3 and 6.8 mol%) were prepared. The peptide content was 3.5 mol%. On the other hand, peptide and PEG content in bPEI-PEG (pH-labile) were 4.5 and 7.8 mol%, respectively.

Effect of PEG on the colloidal stability of the complex was evaluated by using NPAK-PEG/plasmid. Colloidal stability of the complex was improved dependent on the PEG content (Fig. 1 left panel). However, gene suppression of the polymer was attenuated with PEG introduction (Fig. 1, right panel) probably due to the decrease of flexibility of the polymer backbone. Lack of flexibility in the polymer backbone will prohibit the formation of compact polyplex which is essential for the gene suppression. This result suggests that PEG chains should be removed before the transfection of the complex in living cell, while it will be required when the complex is circulating in blood stream. Thus we designed a new PKC-responsive carrier with PEG chain in pH-labile manner. In this carrier, PEG was introduced into polymer through benzoic imine linker. This moiety is known to be hydrolyzed at pH below 6.5, which is often observed near tumor tissue. Fig. 2 shows luciferase expression level in bPEI-PEG system in HepG2 cell at pH 7.4 and 6.5. bPEI-PEG activated the luciferase expression in 250 folds in response to PKCα signaling when the expression level was compared with that in negative control bPEI-PEG(A), which isn’t phosphorylated with PKC. On the other hand, when the cell was treated with the complex at neutral pH, the expression level was very low due to the lack of PEG cleavage. Another experiment evaluating of cell internalization of the complex using fluorescently labeled DNA indicated PEG chain prohibited the transfection efficiency. These results indicated that this carrier will achieve 1500 times activation of transgene in cancer cell comparing with normal organs.

**Reference**