Enhancement of colloidal stability of cell signal-responsive gene carriers for disease cell-specific delivery

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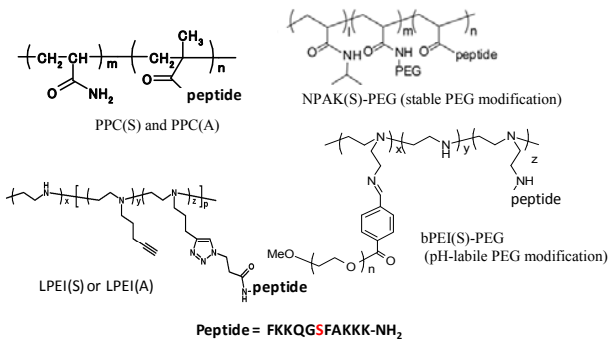
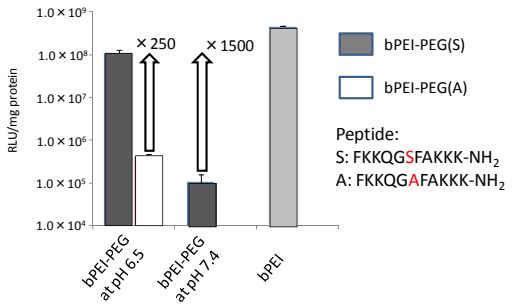
Statement of Purpose: Although gene therapy has a great potential, it is still suffering from its side effects due to the undesired delivery into non-target normal tissue or cells. To access this issue, we recently have developed intracellular signal-responsive gene carriers. These systems can release DNA in response to target protein kinase or protease, which is hyper-activated in target disease cell specifically, so that gene medicine can be activated only in target cell. The carriers are polymers grafted with cationic peptide that is designed as specific substrate of target enzyme. Therefore, the polymer forms nano-polyplex with anionic genetic medicine and suppresses its activity, while phosphorylation or cleavage of grafted peptide with target enzyme decreases the electrostatic interaction between the polymer and DNA. However, the polyplex can't be applied to intravenous (*i.v.*)injection, because the complex tends to aggregate with serum components. Here we tried the stabilization of such polyplex by changing the polymer backbone and introducing PEG chain.

Methods: Following four kinds of polymers were synthesized. These polymers possess protein kinase Cα specific substrate peptide as side chains. PPC and NPAK-PEG were prepared by radical polymerization of corresponding monomers. On the other hand, for the synthesis of LPEI and bPEI-PEG, peptide was introduced with click chemistry. Molecular weight of PEG was 5000. (S) indicates substrate grafted polymer, while (A) indicate negative control peptide pendant polymer where serine residue was replaced with alanine.



Gene regulation ability was investigated by cell-free expression experiment and *in vitro* expression using cancer cell. Luciferase-encoding plasmid was used as reporter gene. For bPEI-PEG, luciferase expression was compared at pH 7.4 and 6.5. For in vivo experiment, polyplexes with various charge ratios were injected into tumor or normal subcutaneous tissue in tumor bearing mice.

Results: PKCα is known to be hyper activated in various cancer cells. Actually, PPC polymer showed tumor specific gene expression *in vitro* and *in vivo* due to the high PKCα activity. However, its enhancement was 10 folds at best. In contrast, LPEI(S) showed nearly 500 folds enhancement in gene expression with PKCα phosphorylation (R. Toita, J. Am. Chem. Soc. 2012: 134: 15410-15417). Cell experiment using various inhibitors indicated that this improved signal responsibility was caused by higher endosomal escape activity of polyethyleneimine moieties due to its proton sponge effect. Colloidal stability was also improved in LPEI(S). In the case of PPC(S), the polymer/DNA complex was extremely destabilized in blood so that it could not be applied HepG2 bearing mice even in direct injection into tumor, because the tumor had rich blood vessels. On the other hand, LPEI(S)/DNA still showed enhancement of gene expression in the tumor comparing with that in normal tissue. However, the stability was still insufficient for *i.v.* injection. Then, PEG was introduced into polymer backbone. Obtained NPAK-PEG revealed enhanced colloidal stability with DNA in serum. However, gene suppression was attenuated with increasing of PEG content, probably due to the steric effect of PEG which prohibits the effective condensation of DNA strand (A. Tsuchiya, J. Biomed. Mater. Res. Part A, 2012: 100: 1136-1141). To overcome this PEG dilemma, we tried introducing PEG chains in pH-labile manner, because 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. In this carrier, PEG was introduced into polymer through benzoic imine linker. This moiety is known to be hydrolyzed at pH below 6.8, which is often observed near tumor tissue. Following figure 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). 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. This carrier will achieve 1500 times activation of transgene in cancer cell comparing with normal organs.

Conclusions: PKCα-responsive system showed high cancer cell specificity. In this research, we realized stable polyplex with PKCα-responsive carrier. The carrier will be able to apply to *i.v.* administration.