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Communications

Synthesis and Characterization of Poly(L-lysine-*co*-L-proline) as a Non-viral Gene Delivery Vector

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Introduction

Gene therapy, the expression of genetic materials with therapeutic activity, has been considered as an encouraging approach to heal life threatening diseases with genetic deficiency.[1] Since direct delivery of the DNA in the absence of a carrier experiences rapid degradation by nuclease and generally exhibits poor cellular uptake, it is one of the main challenges facing today's gene therapy to establish safe and efficient vectors for delivering therapeutic genes to specific cells.[1]

Gene delivery systems investigated so far include viral and non-viral vectors. Viral vectors are intensively used in clinical applications because they efficiently integrate their genetic information into the host chromosomes; however, toxic immunological reactions and recombination events into virulent products still remain as major drawbacks.[2] Non-viral vectors, including liposomes and cationic polymers, display nonimmunogenicity, low acute toxicity, and flexibility to design a carrier with well-defined structures and chemical properties, while they have major problems in transfection efficiency which is limited as only few percentage of that by viral vectors.[1,3]

Among non-viral vectors, poly(L-lysine) (PLL) has been widely used due to the reasonable efficiency and biocompatible nature of peptide bonds in the backbones. However, transfection efficiency of PLL is not so high enough that modification of PLL, such as introduction of targeting ligands or endosomal escape moieties, has been investigated for the use of PLL *in vivo*.[4]

Proline is the only amino acid with a cyclic structure containing a secondary α-amino group and this structural feature gives unique stereochemical and biological properties to the peptides containing proline residue. Water soluble proline-rich peptides have been reported as naturally occurring cell-permeant peptides, which are able to break the cell membrane and deliver the attached carriers into the cells without causing lethal membrane disruption.[5]

In this communication, we report the synthesis of lysine and proline based amino acid copolymers. Characterization of the copolymer and the effect of proline content on the formation of copolymer/DNA complex, *in vitro* cytotoxicity and transfection efficiency were investigated to examine the possibility of the synthesized copolymers as a polymeric gene delivery carrier.

Results and Discussion

Lysine and proline based copolymers were successfully synthesized by ring-opening polymerization of corresponding α-amino acid *N*-carboxyanhydride (NCA)s with hexylamine as an initiator.[6] The synthetic scheme of copolymerization is illustrated in Scheme I. We are employing the short sample code for the copolymers, for example, PK9. P refers to a copolymer and K9 to a composition of the copolymer with 90 mol% lysine and 10 mol% proline. Molecular weights, degree of polymerization, and compositions of copolymers were measured by ^{1}H NMR and GPC. Compositions in the copolymers, 9/1 and 7/3 molar ratio of lysine/proline, were well agreed with the feed ratio and the molecular weights were controlled to be around 17,000. Even though all the molecular weights were not in the same range, the difference was small enough for eliminating the effect of molecular weight on *in vitro* experiment. Degree of polymerization, controlled by the initial concentration ratio between NCA monomers and the initiator, was calculated using ^{1}H NMR based on the integration ratio of the characteristic peak from hexylamine at 0.83 ppm to the peaks from amino acid in the repeating units. Since primary aliphatic amines are more nucleophilic than the active chain ends, the rate of initiation is faster than that of propagation, which

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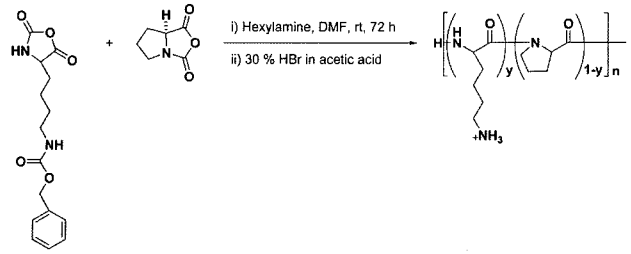

Scheme I. Synthetic scheme of lysine and proline copolymerization.

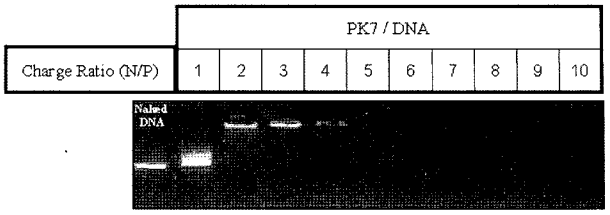

Figure 1. Gel retardation of PK7/DNA complexes at different charge ratios (+/-).

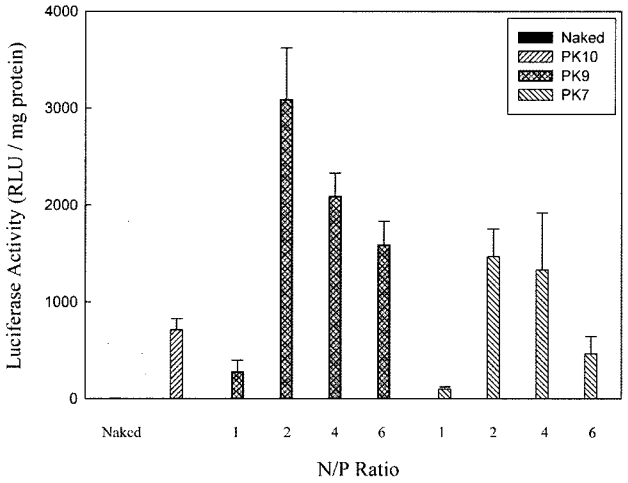

Figure 2. *In vitro* transfection efficiencies of polymer/DNA complexes at different charge ratios on 293T cell line (n=3).

gives narrow molecular weight distribution of copolymers around 1.2 from GPC measurements.

The ability of synthesized copolymers to complex pCMV-luc plasmid DNA was demonstrated by the gel retardation assay and the result for PK7 is shown in Figure 1. Complete neutralization of the complex was achieved at the copolymer/DNA charge ratio (N/P) between 1 and 2, regardless of the copolymer compositions. Considering the fact that PLL homopolymer is reported to form neutralized complex with DNA at the charge ratio of 1, the complex formation of synthesized copolymers with DNA was not so efficient as PLL probably due to the stiffened backbone of the copolymers by the introduction of rigid cyclic proline residues. The result of gel retardation assay was confirmed by zeta potential measurement. The zeta potentials of copolymer/DNA complexes formed at the charge ratio of 0.5 were slightly negative around -10 mV, which means that the copolymers formed complexes with plasmid DNAs to some extent, but not with all the DNAs present. Complete neutralization was achieved at the charge ratio between 1 and 2, which corresponded to the results of agarose gel assay. The obtained value of zeta potential reached a plateau around +20 mV at charge ratio above 2. Net positive charges of the complex play an important role in endocytosis by the ionic interaction with negatively charged cell membrane.

For the transfection of targeted cells, the copolymers must self-assemble plasmid DNA into a size small enough to enter a cell by endocytosis. For the most cell lines, the size limitation is on the order of 200 nm or less. The ability of the copolymers to condense the plasmid DNA into nano-sized complexes was determined by dynamic laser light scattering. At the composition where the efficient complex formation was not achieved, the particle size exceeded 1,000 nm, probably due to the presence of free plasmid DNA in the solution. With increasing the charge ratio, the size of complex gradually decreased to the value around 170 nm regardless of copolymer compositions.

Transfection efficiency of copolymer/DNA complex was measured by luciferase reporter gene assay on 293T cell line. The results are shown in Figure 2 with PLL homopolymer having molecular weight of 20,700 as a control, which is the PLL most commonly used in gene delivery. Transfection of the copolymers reached a maximum value at the copolymer/DNA charge ratio of 2 and decreased as the charge ratio increased. Regardless of the copolymer compositions, transfection efficiency followed the same trend with noticeable differences in the maximum efficiency. PK9 displayed the transfection efficiency four times higher than that of PLL homopolymers, while the difference was two times in the case of PK7. Increase in transfection efficiency was believed to be from the introduction of hydrophobic and cell-penetrating proline residues that caused additional interaction between the carriers and the cell membranes. It is difficult to clearly address the reason why PK9 showed higher transfection efficiency than PK7 at this moment; however, a maximum transfection efficiency depending on the molar ratio of introduced cell penetrating or endosomal escape moieties are often observed and mainly affected by the stability of complex formation.[4] To determine the cytotoxicity of the proline-containing copolymers in comparison with PLL, MTT assay on 293T cell line was performed with increasing charge ratio from 2 to 6.[7] The results are shown in Figure 3. The cytotoxicity of copolymer/DNA complex at charge ratio of 2, which was the charge ratio used in the

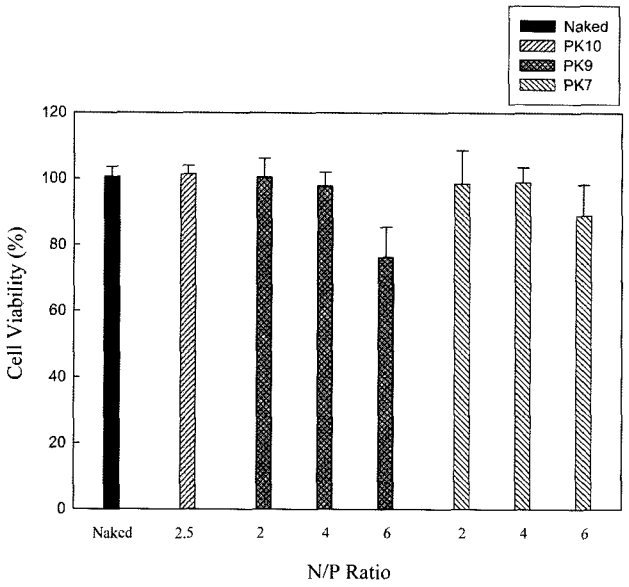

Figure 3. Cytotoxicity evaluation by MTT assay of polymer/DNA complexes at different charge ratios on 293T cell line (n=8).

previous transfection experiments, was almost negligible with 100% cell viability.

In summary, to develop a non-viral gene delivery carrier, copolymers based on lysine and proline were synthesized by the ring-opening polymerization of α-amino acid *N*-carboxy-anhydrides (NCAs). Molecular weights of the copolymers were around 17,000 by ^{1}H NMR regardless of copolymer compositions, which were lysine to proline molar ratio of 9/1 and 7/3. Synthesized copolymers effectively condensed pCMV-luc plasmid DNA to the size around 170 nm at the charge ratio above 2. The size of the complexes and zeta potential were dependent on the charge ratio between the copolymers and DNA rather than the composition of the copolymers. Copolymers displayed improved transfection efficiency up to four times higher than that of PLL homopolymer with the molecular weight of 20,700. Cytotoxicity of the copolymers was almost negligible at the charge ratio used in the transfection efficiency measurement and slightly increased as the amount of proline residues in the copolymers increased. Based on those results, synthesized copolymers of lysine and proline are believed a good candidate for an efficient non-viral gene delivery carrier.

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