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Preparation of CpG ODN-encapsulated Anionic Poly(amino acid) Nanoparticles for Gene Delivery

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A novel method for preparation of CpG ODN-encapsulated anionic poly(amino acid) nanoparticles (NPs) was developed for gene delivery purposes. CpG ODN was successfully encapsulated into the NPs using self-assembly of amphiphilic poly(amino acid), and the amount of encapsulated CpG ODN was dependent on the complex ratio of CpG ODN and polycation. The DNA-encapsulated anionic NPs may be useful as a gene delivery and DNA vaccine system with low toxicity.

Polymeric nanoparticles (NPs) have been widely explored as carriers for the controlled delivery of therapeutic agents such as proteins, peptides, DNA, or RNA.[1] For the purposes of non-viral gene delivery, cationic polymer, such as poly(L-lysine) and polyethyleneimine (PEI) have been used as carriers for complexing gene vectors into polyplexes.[2] A polyplex can be easily formed when the oppositely charged DNA and polycation are mixed in aqueous solution and interact via electrostatic interactions. These polyplexes result in an increased net positive charge of the complexes and promote cellular uptake and transfection efficiency. However, the in vivo applications of polyplexes are limited by low gene expression and toxicity due to their cationic nature.[3] Several studies have attempted to avoid toxicity by including poly(ethylene glycol) (PEG)[4] or polyanions[5] into the polyplexes.

In a previous study, we prepared NPs composed of poly(γ-glutamic acid) (γ-PGA) as the hydrophilic backbone and L-phenylalanine (Phe) as the hydrophobic segment (γ-PGA–Phe).[6] The γ-PGA–Phe formed NPs due to their amphiphilicity in water. The size of the γ-PGA–Phe NPs could be easily controlled (30–200 nm) by changing the preparative conditions, and the NPs showed a highly negative ζ potential (−25 mV) due to the ionization of the carboxyl groups of γ-PGA located near the surface.[7] Moreover, both anionic and cationic proteins were successfully encapsulated into the NPs,[8] and the NPs showed great potential as vaccine delivery carriers and adjuvants.[9] In spite of the anionic nature of the γ-PGA–Phe, the NPs could encapsulate anionic proteins or peptides. Therefore, we speculated that this system could provide a novel DNA carrier in the development of gene delivery and DNA vaccines.

In this study, we developed a novel method for the preparation of CpG oligodeoxynucleotide (ODN)-encapsulated anionic γ-PGA–Phe NPs. It is expected that the complexes of CpG ODN and cationic agents are to be more stabilized by hydrophobic interactions of γ-PGA–Phe compared to another anionic ternary polyplexes. CpG ODN is a synthetic DNA (20 mer) containing unmethylated CpG sequences. CpG ODN exhibits potent adjuvant effects for vaccines, but its rapid degradation and ineffective intracellular delivery are major obstacles to improving its efficacy.[10] Several approaches for CpG ODN delivery using cationic polymers, liposomes, or micro/nanoparticles have been developed to enhance its immunostimulatory efficacy.[11]

The encapsulation of CpG ODN into the γ-PGA–Phe NPs was carried out with both free CpG ODN and polyplexes consisting of CpG ODN/polycations. At first, we tried the encapsulation of CpG ODN in the absence of polycations. To prepare CpG ODN-encapsulated γ-PGA–Phe NPs, γ-PGA–Phe (10 mg mL^{-1} in DMSO) was added to the same volume of FITC-labeled CpG ODN (125 µg mL^{-1} in 0.2 M NaCl) to yield a translucent solution. In this experiment, γ-PGA with 53% degree of Phe grafting was used. The resulting solution was centrifuged at 14000 × *g* for 15 min and repeatedly rinsed to remove un-encapsulated CpG ODN. The amount of CpG ODN encapsulated into the NPs was then measured by fluorescence spectrometry. In spite of the repulsion of anionic charges between the CpG ODN and γ-PGA–Phe, the CpG ODN could be encapsulated into the NPs (0.4 µg mg^{-1} NPs).

To enhance the amount of encapsulated CpG ODN, three biocompatible polycations were used to condense the CpG ODN. As a polycation, spermidine ($M_w = 145$), poly(ε-lysine) (ε-PL) ($M_w = 4.7 \times 10^3$), and protamine ($M_w = 4.3 \times 10^3$) were mixed with CpG ODN to encapsulate the cationic polyplexes into the anionic γ-PGA–Phe NPs. The formation processes of the CpG ODN-encapsulated NPs with polycations are shown in Figure 1. The CpG ODN solution and each polycation solution were mixed at various N/P molar ratios. The charge ratio (N/P) was expressed as the ratio of the amino groups (N) on the polycations and the phosphate groups (P) on the CpG ODN. γ-PGA–Phe was added to polyplexes consisting of CpG ODN and polycations. The concentration of CpG ODN was fixed at 125 µg mL^{-1}. The results of the encapsulation of CpG ODN in the presence of polycations are shown in Figure 2a. According to the increase in the N/P ratios, the amount of CpG ODN encapsulated into the NPs increased. However, at high N/P ratio (more than 5), the aggregation of polyplex-encapsulated NPs was found. These results suggest that the excess of polycation disturbs the formation of stable NPs by γ-PGA–Phe. The amount of encapsulated CpG ODN was different between

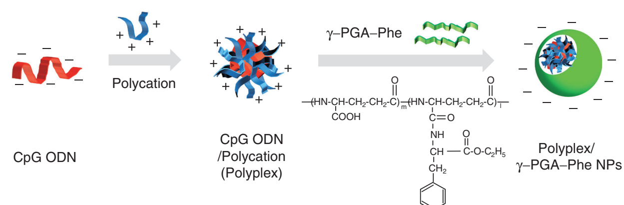

Figure 1. Preparation of CpG ODN-encapsulated γ-PGA–Phe NPs with polycations.

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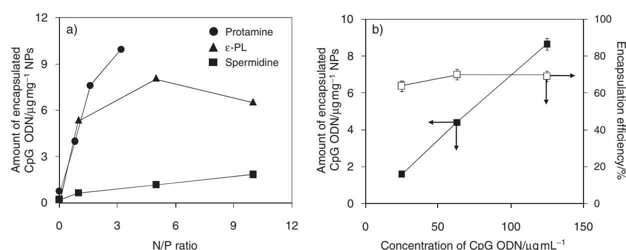

Figure 2. a) Effect of the N/P ratio on the amount of CpG ODN encapsulated into γ-PGA–Phe NPs in the presence of polycations. b) Effect of the CpG ODN/ε-PL (N/P = 2) concentration on the amount of CpG ODN encapsulated into the NPs (■) and the encapsulation efficiency (□). The encapsulation efficiency was calculated as (total encapsulated CpG ODN weight/initial CpG ODN weight) × 100. The results represent means ± S.D. ($n = 3$).

Table 1. Particle size and ζ potential of CpG ODN/ε-PL polyplexes (N/P = 2) and NPs

	Size[a] /nm	PDI[b]	ζ-potential[a] /mV
CpG ODN/ε-PL	111.5 ± 0.7	0.16 ± 0.02	+35.7 ± 0.4
CpG ODN/ε-PL /γ-PGA–Phe	165.0 ± 2.8	0.16 ± 0.03	−26.9 ± 0.8
γ-PGA–Phe NPs	271.0 ± 14.1	0.28 ± 0.02	−25.9 ± 0.8

[a]The size and ζ potential of NPs were measured by Zetasizer nano. The results represent means ± S.D ($n = 3$). [b]PDI represents polydispersity index.

each polycation. When compared to low M_w spermidine, the NPs could efficiently encapsulate CpG ODN in the presence of ε-PL or protamine (pI = 13.8).[12] The pK_a values of the primary amine groups derived from ε-PL or spermidine are almost the same (pK_a = 7–8).[13] These results indicate that the M_w and charge density of the cationic agent influence the formation of polyelectrolyte complexes. When the N/P ratio was fixed at 2, it was confirmed that the CpG ODN loading content was increased in accordance with the concentration of CpG ODN/ε-PL (Figure 2b). Moreover, the encapsulation efficiency of CpG ODN was about 70%, irrespective of the CpG ODN concentration.

Table 1 shows the sizes and ζ potentials of the CpG ODN/ε-PL polyplexes (N/P = 2) and NPs. The polyplexes showed a positive ζ potential and about a 100 nm particle size. In contrast, the CpG ODN/ε-PL/γ-PGA–Phe NPs showed a negative charge, and the size of the NPs was increased to 165 nm. These results suggest that the CpG ODN/ε-PL polyplexes were not absorbed onto the NP surfaces but encapsulated into the NPs or coated by γ-PGA–Phe. The size of the CpG ODN/ε-PL/γ-PGA–Phe NPs was decreased as compared to the γ-PGA–Phe NPs alone. It seems that the electrostatic interactions between the CpG ODN/ε-PL and the γ-PGA–Phe affected the size of the NPs.

In conclusion, the encapsulation of CpG ODN into γ-PGA–Phe NPs was successfully achieved in the absence and presence of polycations. In the presence of relatively high M_w polycations, ε-PL or protamine, the γ-PGA–Phe NPs could efficiently encapsulate the polyplexes by electrostatic interactions. After encapsulation, the ζ potential of polyplexes was inverted from a positive to a negative charge. We have previously demonstrated that the γ-PGA–Phe NPs efficiently delivered loaded proteins from the endosomes to the cytoplasm in dendritic cells.[14] Therefore, the NP itself has several advantages as a DNA carrier, and the system developed in this study may be useful for DNA vaccine delivery and adjuvants. Further studies, including cellular uptake and immunization with DNA-encapsulated NPs, are in progress for the development of DNA vaccines.

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