Synthesis of Novel Biodegradable Quaternary Amine-based Cross-linked Poly(β-amino ester) and Its Self-assembly/disassembly with Plasmid DNA

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In recent years, various cationic polymers constructed using ester bonds, phosphoester bonds or disulfide bonds have been synthesized to create environmentally-responsible biodegradable delivery systems.[1-3] Among them, a cross-linked type polymeric gene delivery carrier was reported to show enhanced transfection efficiency due to its three-dimensional structure leading endosome buffering effect, and self-assemble with plasmid DNA giving more stable polyplexes due to its slow degradation in aqueous media, in contrast to linear type carriers.[4]

Several quaternary amine-based polymeric gene delivery carriers were reported and they showed considerable gene delivery potencies.[5-8] Moreover, a primary amine-based biodegradable polymeric carrier was known to have potentially rapid self-destructive tendency due to the attack of its own primary amines.[9] Because quaternary amines have no nucleophilicity, quaternary amine-based biodegradable polymeric carriers have no possibility of 'self-destruction'. So, we designed a novel quaternary amine-based cross-linked poly(β-amino ester) polymer (Q-CLPAE).

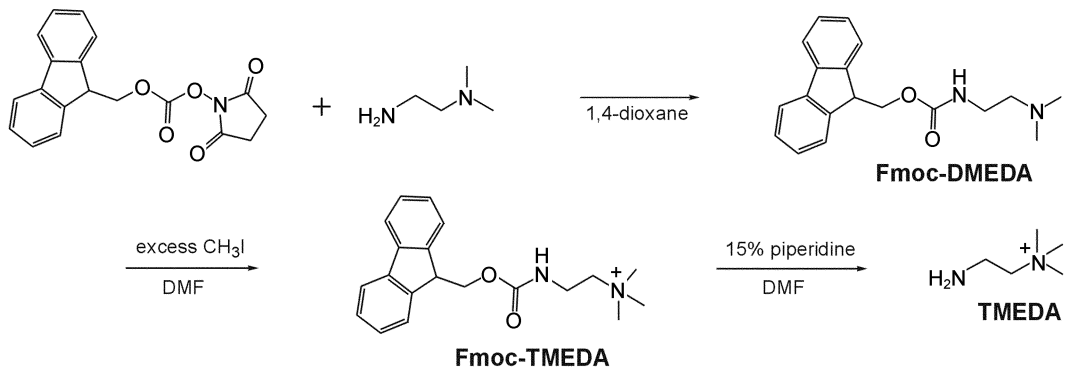
Here, we report the synthesis, characterization of Q-CLPAE. In addition, the self-assembly of polymers with plasmid DNA and in vitro disassembly of plasmid DNA from polyplexes are presented.

Q-CLPAE was synthesized by using a Michael reaction of a quaternary amine monomer and a triacrylate monomer according to Lynn *et al.*[10] First, a quaternized amine monomer was synthesized through the following steps (Scheme 1).

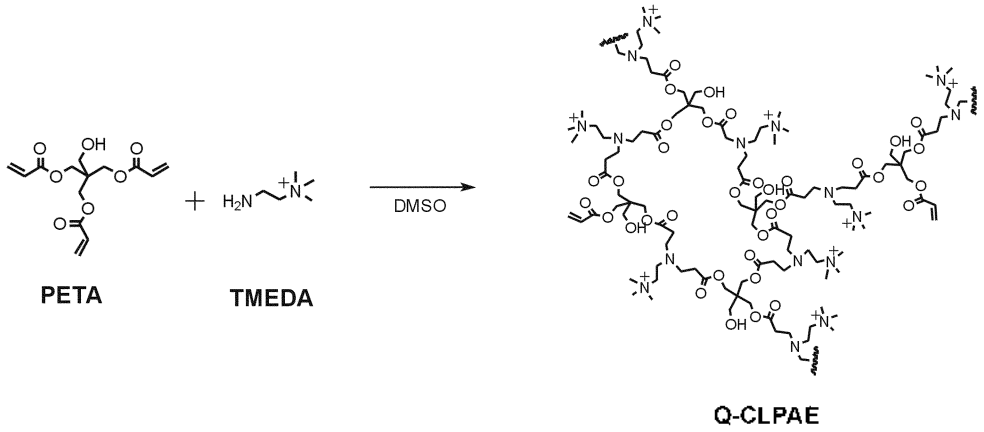
Fmoc blocking reaction of N,N-dimethylethylenediamine (DMEDA). Fmoc-OSu (1 g, 2.96 mmol, Novabiochem, Laufelfingen, Switzerland) was dissolved in 1,4-dioxane. DMEDA (0.488 mL, 4.45 mmol, Sigma-Aldrich, St. Louis, MO) was dissolved in 1,4-dioxane and added to an Fmoc-OSu solution dropwise, keeping it at r.t. overnight. Then, the reaction mixture was poured into diethyl ether and extracted with water to remove the residual DMEDA. The ether layer was extracted again with 0.1 M HCl solution. At this time, the acidified product was transferred to the HCl layer and the residual Fmoc-OSu was left in the ether layer. The HCl layer was basified to ~pH 11 with 1 N NaOH solution and the hydrophobic product in the aqueous layer was extracted with diethyl ether. Finally, after evaporation of the ether layer, a purified product, N-(9-Fluorenylmethoxycarbonyl)-N',N'-dimethylethylenediamine (Fmoc-DMEDA) was left as a white solid.

Methylation of Fmoc-DMEDA. Fmoc-DMEDA (195 mg, 0.598 mmol) was dissolved in DMF, 3.72 mL of iodomethane (d = 2.28, 59.8 mmol, Sigma-Aldrich) was added to an Fmoc-DMEDA solution, keeping it at r.t. overnight. The product was precipitated with diethyl ether three times to remove the unreacted materials, leaving a light yellow solid, N-(9-Fluorenylmethoxycarbonyl)-N',N',N'-trimethylethylenediamine (Fmoc-TMEDA).

Fmoc deblocking of Fmoc-TMEDA. Fmoc-TMEDA (101 mg, 0.296 mmol) was dissolved in DMF and mixed with an equal volume of 30% piperidine solution (DMF). After stirring at room temperature, the product was pre-



Scheme 1. Synthesis scheme of quaternized amine monomer.



Scheme 2. Synthesis scheme of Q-CLPAE.

Table 1. Average sizes (Z_{ave}) and zeta-potentials of Q-CLPAE polyplexes. Measured values were presented as the average values of 5 runs

	Wr 1	2	5	10	20
Z_{ave} (nm)	229.3 ± 10.0	106.1 ± 5.4	343.5 ± 22.7	293.6 ± 13.2	277.6 ± 30.7
Zeta-potential (mV)	−29.3 ± 0.1	−13.2 ± 1.6	4.9 ± 0.5	20.1 ± 0.3	26.5 ± 2.6

Wr: Weight ratio of the polyplexes

cipitated with cold diethyl ether to remove the residual piperidine, leaving a white solid, N,N,N-trimethylethylenediamine (TMEDA). All syntheses were confirmed by 300 MHz ^{1}H NMR (Bruker DPX-300).[11]

Then, Q-CLPAE polymerization was performed in DMSO (Scheme 2). TMEDA (18.1 mg, 0.173 mmol) and pentaerythritol triacrylate (PETA, 25.9 mg, 0.087 mmol, Sigma-Aldrich) were dissolved in DMSO. To synthesize cross-linked polymers, PETA was used as a trivalent linker and chosen because it had the shortest carbon backbone length among analogous acrylate monomers and so its polymer would possess the high charge density, which is known to generally lead to high transfection in cells. Moreover, it has a hydroxyl group, which would enhance the water solubility of the polymer. TMEDA solution was added to the triacrylate solution, keeping it at 50 °C. After 5 days, the reaction mixture was cooled to room temperature and evaporated under vacuum. The product was dissolved in DMF to separate the DMF-insoluble TMEDA and precipitated into diethyl ether 2 times, leaving a light yellow solid, Q-CLPAE.

The average molecular weight of the polymer was determined by multi-angle laser light scattering (MALLS) in combination with size exclusion chromatography (SEC).[12] The M_w of Q-CLPAE was measured to be 8.43×10^3 Da (PDI = 1.28, dn/dc = 0.104).

The sizes of Q-CLPAE polyplexes were measured by light scattering to confirm the self-assembly of Q-CLPAE with pCN-Luci plasmid DNA giving nano-sized polyplexes, and the zeta-potentials of each polyplex were determined at various weight ratios (Table 1).[13] The average size of Q-CLPAE polyplexes was 344 nm at the weight ratio of 5 and decreased to 278 nm at 20. Considering the zeta potential values of polyplexes together, Q-CLPAE was thought to self-assemble with plasmid DNA to form partially condensed and negatively charged polyplexes intermediate below the weight ratio of 5. Above the weight ratio of 5, Q-CLPAE showed that it could self-assemble with plasmid DNA to form positively charged and stable polyplexes in the size or zeta potential values.

In general, linear type cationic ester polymers were reported to be degraded in aqueous media within a few hours and be unable to self-assemble with plasmid DNA any longer.[1,9] Considering the harsh *in vivo* condition including a fast blood stream, enzyme attack and low pH, the stability of polyplexes is important for therapeutic purposes. Therefore, the disassembly pattern of plasmid DNA from Q-CLPAE polyplexes was examined by agarose gel retardation assay (Figure 1).[14] The stability of Q-CLPAE polyplexes increased with increasing weight ratio where the polyplex remained intact over 2 days at a weight ratio of 20. We can conclude that the stability of Q-CLPAE polyplexes is enhanced compared to that of linear biodegradable polymer/DNA complexes.

In summary, biodegradable quaternary amine-based cross-linked poly(β-amino ester) (Q-CLPAE) was synthesized for

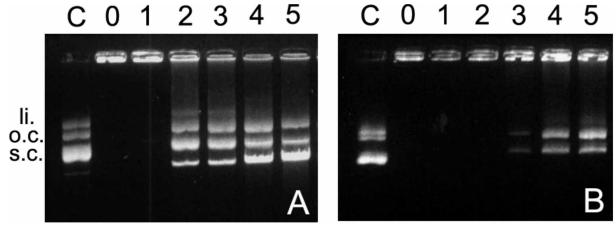


Figure 1. Gel Retardation assay. (A) weight ratio = 5. (B) weight ratio = 20. C: DNA only. Numbers represent the incubation days of the polyplexes. s.c. = super coiled DNA, o.c. = open circular DNA, li. = linear DNA.

gene delivery system. Q-CLPAE is able to self-assemble with plasmid DNA to create positively charged stable polyplexes from the weight ratio of 5, and the size of these polyplexes were estimated to be 278 nm at the weight ratio of 20. Fulfilling our expectations, Q-CLPAE polyplexes showed prolonged disassembly patterns with plasmid DNA and an increased stability of the polyplexes, which may be due to cross-linked structure and non-nucleophilc quaternized amines of Q-CLPAE. Transfection assay and other cell-based experiments are in progress.

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11. **Fmoc-DMEDA** ($CDCl_3$): δFmoc (aromatic protons) = 7.26-7.78; δDMEDA (-CONHC**H**$_2$-) = 3.28 (q); δDMEDA (-CH_2C**H**$_2$N-) = 2.42 (t); δ DMEDA (-CH_2N**Me**$_2$) = 2.24 (s). **Fmoc-TMEDA** (MeOD): δ Fmoc (aromatic protons) = 7.20-7.80; δ TMEDA (-CONHC**H**$_2$-) = 3.41 (q); δ TMEDA (-CH_2C**H**$_2$N-) = 3.36 (t); δ TMEDA (-CH_2N**Me**$_3$) = 3.05 (s). **TMEDA** (MeOD): δ TMEDA (NH_2C**H**$_2$$CH_2$-) = 2.99 (t); δ TMEDA (-CH_2C**H**$_2$N-) = 3.29 (t); δ DMEDA (-CH_2N**Me**$_3$) = 3.08 (s).
12. The SEC system consisted of a P680 HPLC pump from Dionex Corp. (Sunnyvale, CA). Polymer sample was detected by a 3-angle laser light scattering detector (miniDAWN® Tristar™, 30 mW GaAs laser, 690 nm) and an interferometric refractometer (Optilab DSP™) from Wyatt Technologies (Santa Barbara, CA). DMF was used as an eluent after being filtered through a 0.22 μm filter and degassed before use. A Styragel® HR 3 column from Waters (Milford, MA) was used.
13. (a) 2 mL of polyplex solutions containing 5 μg of DNA were prepared at various weight ratios ranging from 1 to 20. After 30 min incubation, polyplex sizes were measured using a Zetasizer 3000HS (5 mW HeNe laser, 633 nm, Malvern Instruments, UK). Scattered light was detected at a 90° angle. (b) Polyplex solutions were prepared in same method as above. Polyplex solutions were diluted to 10 mL of the final volume prior to measurements. Zeta-potential measurements were carried out using a Zetasizer 3000HS at 25 °C.
14. Polyplexes at weight ratios of 5 and 20, were prepared in Hepes buffered saline (HBS, 20 mM Hepes, 150 mM NaCl, pH 7.4). The samples were collected for assay at various times ranging from the 1st to the 5th day, electrophoresed on a 0.7% (w/v) agarose gel and then stained in a 1% (w/v) ethidium bromide solution.