Cationic Hyperbranched Poly(amino ester): A Novel Class of DNA Condensing Molecule with Cationic Surface, Biodegradable Three-Dimensional Structure, and Tertiary Amine Groups in the Interior

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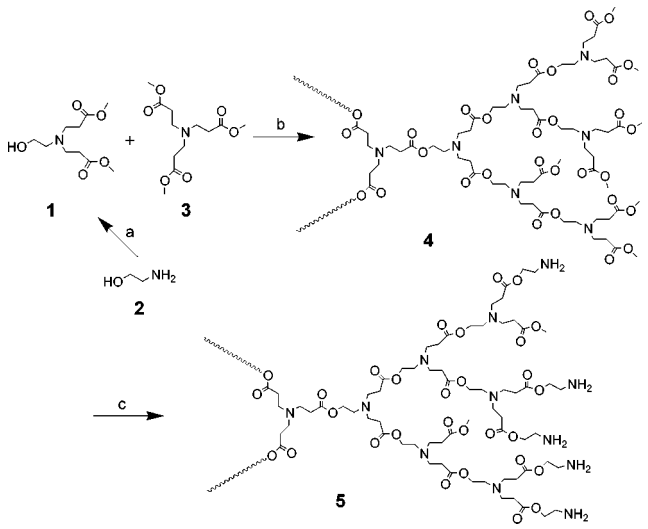
Dendrimers[1] and hyperbrached polymers[2] have become an attractive field of research. Dendrimers have a precisely defined shape and molecular weight, which, however, is achieved at the cost of iterative synthetic steps. In contrast, hyperbranched polymers are synthesized in one-step polymerization resulting in irregular shape and broad molecular weight distribution. Efforts are currently in progress to utilize these highly branched molecules in a variety of applications involving combinatorial chemistry, surface coating, photoactive system, and gene or drug delivery.

Recent advances in nonviral gene delivery have revealed polyethyleneimine (PEI) and starburst PAMAM dendrimers as effective gene transfer molecules.[3] It is generally accepted that the high transfection efficiency of PEI or PAMAM compared to that of other cationic polymers is due to the "endosome buffering" or "proton sponge" effect of the polymers. Tertiary amines in the interior of PEI or PAMAM are protonated at acidic endosomal pH, which disrupts the endosome either directly due to the membrane activity of the cationic polymers or possibly by mechanical swelling or osmotic effects.

It has been reported that biodegradable cationic polymers are nontoxic and condense DNA into compact particles that can transfect mammalian cells.[4] On the basis of these findings, we concluded that biodegradable cationic polymer with three-dimensional structure and interior tertiary amine groups would be a nontoxic and efficient gene carrier through the endosome buffering effect. In this paper, we describe the synthesis and characterization of hyperbranched poly(amino ester) with a novel molecular architecture in that it has a biodegradable, three-dimensional, and interior tertiary amine group containing structure. With a suitable design of AB_2 monomer and surface functionalization, hyperbranched poly(amino ester) could be synthesized conforming to our initial idea (Scheme 1).

Monomer **1**, bearing one hydroxyl group, two methyl ester groups, and one tertiary amine group in the center of the molecule, was synthesized by Michael addition of ethanolamine **2** with methyl acrylate. The polymerization reaction was carried out in the bulk in the presence of ammonia core starburst PAMAM dendrimer −0.5 generation **3** as a core moiety (monomer/core ratio, 200/1) and $Al(O^iPr)_3$ as a catalyst (1 mol %). The core moiety was added to decrease polydispersity and prevent the formation of cross-linked or excessively high molecular weight polymers.[5] In the absence of the core moiety, high conversion resulted in gelation of polymers which were difficult to dissolve in any solvent. It is reported that low molecular weight oligomers are formed in the early stages of polymerization while depleting the monomers.[5a,6] In the absence of the core moiety, the rapid growth of molecular weight via the coupling of existing oligomers in the monomer-starved state should result in gelation.

Scheme 1[a]



[a] Conditions: (a) (i) Methyl acrylate, MeOH, 35 °C. (b) Bulk polymerization under reduced pressure, 140 °C. (c) (i) $C_6H_5CH_2OC(O)NH(CH_2)_2OH$, bulk polymerization under reduced pressure, 140 °C; (ii) H_2, 10% Pd/C, MeOH, $CHCl_3$, room tmeperature.

The reaction was maintained at a relatively low temperature, 140 °C, to minimize the vaporization of monomer and unwanted side reactions. The reaction was driven toward high conversion as the methanol formed was removed continuously under reduced pressure with an optimized reaction time to avoid polymer gelation. Repeated precipitation into diethyl ether resulted in polymer **4** as a viscous oil. Related hyperbranched amine-containing polyester derivatives have been described previously.[7]

It is well-known that size exclusion chromatography (SEC) measurement tends to underestimate the true molecular masses of branched polymers.[8] So it is generally accepted that true molecular masses of branched polymers should be three to five times higher than the values obtained from SEC. To obtain an estimate of the molecular mass of polymer **4** as close as possible to the true value, we used two kinds of standards, polystyrene and half-generation PAMAM dendrimer. Compared to linear polystyrenes, PAMAM dendrimers have branched structures similar to that possessed by polymer **4**. In addition, the structural features of half-generation PAMAM dendrimers are very similar to those of polymer **4**, tertiary amine groups in the interior, and methyl ester groups in the surface. The average molecular masses of polymer **4** obtained with PAMAM standards were about 2.5-times higher than the values obtained with polystyrene standards (Table 1). Since hyperbranched polymers have intermediate structures between linear polymers and perfect dendrimers, the true molecular masses of polymer **4** should be within the range

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Table 1. Characterization of Hyperbranched Polymer **4**[a]

	polystyrene			PAMAM			
entry no.[b]	M_n	M_w	PDI	M_n	M_w	PDI	yield (%)
1	11 400	17 800	1.56	29 700	42 500	1.43	67
2	11 600	17 300	1.50	30 400	42 300	1.39	71
3	9 100	13 200	1.45	22 200	33 400	1.50	66

[a] SEC was performed in THF with polystyrene or half-generation PAMAM dendrimer as molecular weight standards. [b] Polymers were synthesized under the same conditions.

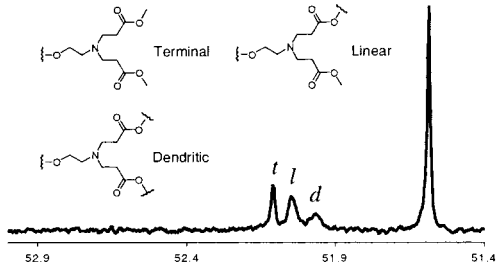

Figure 1. The structures of possible monomer units in the hyperbranched poly(amino ester) and enlarged methylene carbon ($-OCH_2CH_2N-$) region in the 150 MHz INVGATE spectrum of polymer **4**: *t*, terminal units; *l*, linear units; *d*, dendritic units.

of the two values obtained in SEC by using the two kinds of standards. The molecular weights, polydispersity indices, and yields obtained at different batch polymerization reactions under identical conditions showed slight differences, indicating that control over the reaction is possible.

To calculate the degree of branching (DB) values, model compounds representing terminal and linear units of polymer **4** were synthesized. By using unique ^{13}C NMR resonances of the model compounds, we were able to identify the different subunits. The differences were most pronounced in the resonances of methylene carbons ($-OCH_2CH_2N-$), which occurred as three distinct peaks (Figure 1). From the resonances of model compounds, the peaks occurring at 52.11 and 52.04 ppm were found to be the signals of terminal and linear units, respectively. From this result, the other signal occurring at 51.96 ppm could be assigned as dendritic units. For quantification, the ^{13}C NMR spectrum of polymer **4** was obtained by using an inverse-gated broadband decoupled technique (INVGATE) with a relaxation delay of 20 s ($T_1 < 10$ s). The DB of polymer **4** was calculated by using the formula[9]

$$\mathrm{DB} = (N_d + N_t)/(N_d + N_l + N_t)$$

The results indicated that the DB values were 0.62 (entry no. **1**), 0.58 (entry no. **2**), and 0.60 (entry no. **3**), which were synthesized in a different batch under identical conditions. Usually, the DB was found to approach around 0.5 for hyperbranched polymers derived from AB_2 monomers.[5a] The DB values obtained for polymer **4** are close to 0.5 and similar to other hyperbranched polyesters prepared by batch polymerization.

For polymer **4** to be able to condense negatively charged DNA, the surface of the polymer should be functionalized with amine groups that are protonated at near neutral pH. Surface functionalization of polymer **4** was accomplished in two steps (Scheme 1). Polymer **4** was transesterified with 1 equiv of benzyl *N*-(2-hydroxyethyl)carbamate (*N*-cbz-ethanolamine) relative to methyl ester groups in the bulk at 140 °C under reduced pressure. The progress of the reaction was monitored by ^1H NMR at various stages of the reaction. The reaction was stopped when the majority of methyl ester groups in polymer **4** were transesterified by *N*-cbz-ethanolamine. The resulting polymer was precipitated into a large excess of diethyl ether. Characterization by the INVGATE spectrum showed near 80% substitution of methyl ester groups by *N*-cbz-ethanolamine, as judged from the peak intensity ratio of phenyl (3°) carbons of *N*-cbz-ethanolamine and methyl carbons of polymer **4**. The degree of polymerization measured by INVGATE spectrum analysis of end groups was 67. After removing cbz groups using catalytic hydrogenolysis with H_2 over Pd/C, the polymer **5** was obtained containing primary amine groups at the surface.

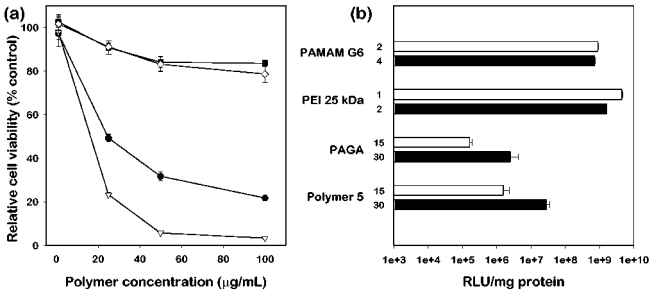

Figure 2. Cytotoxicity profiles (a) and transfection efficiencies (b) of PAMAM G6, PEI 25 kDa, PAGA, and polymer **5**: polymer **5** (■), PAGA (◇), PAMAM (●), and PEI (▽). Numbers indicate the polymer to DNA weight ratios.

The toxicity of polymer **5** in 293 cells was tested by using an MTT assay (Figure 2a). The result indicated that polymer **5** was minimally toxic. Although transfection of the cells with polymer **5** was not as effective as those of PAMAM or PEI, it was increased over 10-fold compared with that of PAGA (Figure 2b). PAGA is a biodegradable cationic polymer that does not possess endosome buffering moiety.[4a,b] Chloroquine, known to disrupt the membrane of the endocytic vesicle, was not used in this experiment to investigate the endosome buffering effect of polymer **5**. This finding suggested that the endosome escape function in polymer **5** should result in the increased transfection. But further developments in transfection efficiency in terms of molecular size and/or functionality are necessary.

In conclusion, hyperbranched poly(amino ester) has an interesting molecular architecture and functionality in that it has biodegradable ester backbone linkages, tertiary groups in the interior, and primary amine groups in the exterior. The polymer showed efficient transfection and minimal toxicity, which would make the polymer a promising nonviral gene carrier.

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Supporting Information Available: Synthetic details and experimental protocols (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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