# DNA Polyplexes Formed Using PEGylated Biodegradable Hyperbranched Polymers

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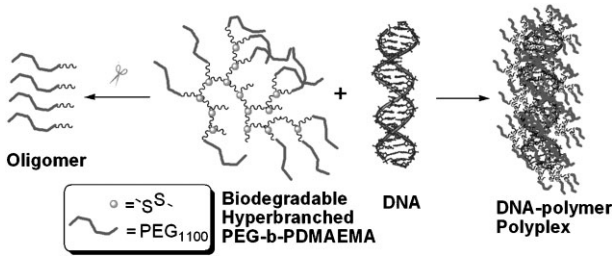
A novel PEGylated biodegradable hyperbranched PEG-*b*-PDMAEMA has been synthesized. The low toxicity, small molecular weight PDMAEMA chains were crosslinked using a biodegradable disulfide-based dimethacrylate (DSDMA) agent to yield higher molecular weight hyperbranched polymers. PEG chains were linked onto the polymer surface, masking the positive charge (as shown by Zeta potential measurements) and reducing the toxicity of the polymer. The hyperbranched structures were also cleaved under reducing conditions and analyzed, confirming the expected component structures. The hyperbranched polymer was mixed with DNA and efficient binding was shown to occur through electrostatic interactions. The hyperbranched structures could be reduced easily, generating lower toxicity oligomer chains.




## Introduction

Many human diseases, such as cancer and cystic fibrosis, have genetic origins.[1–3] "Gene therapy," insertion of a targeting gene into cells to replace or override defective genes, may well be a promising therapeutic approach. Direct administration of naked deoxyribonucleic acid (DNA) is unviable as poor transfer efficiency and enzymatic degradation negate effective therapeutic implementation. Viral (recombinant viruses) and non-viral (synthetic materials) have been proposed as vehicles for DNA.[4–8] Despite their efficient delivery ability, viral vectors have many drawbacks, such as cargo capacity, resistance to repeat infection and safety concerns, limiting their application.[9–11] Cationic polymers have attracted a lot of research interest as potential non-viral vectors as they have the potential to complex with negative charged DNA or RNA, generating neutral or positive charged polyplexes, with the ability to cross the negative charged cell membrane.[12] There are two main obstacles to the use of cationic polymers, polyplex unpacking, and cytotoxicity. It is known that increase in the positive charge on a polymer improves cellular uptake and transfection efficiency, but the accompanying detrimental toxicity effect originating from the destabilization and loss of integrity of the cell membrane is also enhanced, leading to a narrow operating window between efficiency and severe toxicity.[13,14] To reduce the cytotoxicity stemming from positive charge, neutral, non-toxic, and biocompatible polymers, such as poly(ethylene glycol) (PEG) and dextran, have been conjugated with DNA polyplexes to mask the positive charge, reducing their non-specific binding capacity, improving solubility and stability, and minimizing toxicity in vivo and in systemic delivery.[15–17] Another promising approach to improve synthetic polymer gene delivery is by exploiting biodegradable cationic polymers.[18–22] Many low molecular weight polymers possess low toxicity compared to their higher molecular weight counterparts, but are not suitable for forming stable polyplexes for in vivo administration.[23–25] Therefore cross-linking low molecular weight polymers through biodegradable linkages might prove to be an efficient route to effective gene delivery

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vectors, via high molecular weight cationic polymers. After gene delivery, the polymers can undergo degradation, resulting in the reduction of positive charges per molecular and unspecific interactions. Additionally, the degradation products are also more amenable to unpack and release free DNA in the cell. For example, Forrest et al. crosslinked PEI (≈800, essentially non-toxic but inefficient for gene delivery), with diacrylates to generate poly($\beta$-amino esters). Compared to commercial 25 K PEI, the biodegradable polymers were essentially non-toxic (at 14–30 K), and could deliver DNA, mediating gene expression 2–16-fold more efficiently.[26] Recently, biodegradable linear poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) was evaluated as a gene delivery agent and displaying significantly reduced cytotoxicity in a range of cell lines.[27] Centre for Advanced Macromolecular Design (CAMD) recently developed biodegradable hyperbranched PDMAEMA as a potential gene delivery agent,[28] as part of a synthetic research program into producing novel approaches to biodegradable, well-defined polymeric structures.[29–32] In this paper, we describe the synthesis of PEGylated biodegradable hyperbranched PDMAEMA and its use for DNA binding. This work represents an advance on our previous work as PEGylation has been used to shield the charge of the hyperbranched polymer as demonstrated through masking of positive charge via Zeta potential measurements. In previous work we have shown that the masking of charge by PEGylation also leads to reduced protein fouling of metal nanoparticles,[33–35] and therefore improved biocompatibility. Reversible addition–fragmentation chain transfer (RAFT) technology allows control of the polymerization process to yield low molecular weight oligomers with narrow dispersity chain lengths and low toxicity. We describe the use of a cross-linking agent, disulfide-based dimethacrylate (DSDMA), 1,2-bis(2-(3-methylbuta-1,3-dien-2-yloxy)ethyl) disulfane, to link PEGylated-oligomers into biodegradable hyperbranched structures. To our knowledge, this is the disclosure of a synthesis strategy to create biodegradable PEG-PDMAEMA hyperbranched copolymers for DNA polyplex formation.

## Experimental Part

### Materials

*N*,*N*′-dicyclohexylcarbodiimide (DCC, 99%, Sigma), 4-(dimethylamino) pyridine (DMAP, 99%, Aldrich), PEG 1 100 monomethyl ether (Fluka), and DNA sodium salt from salmon testes (DNA, Sigma) were used as purchased. 4-Cyano-4-(ethylthiocarbonothioylthio) pentanoic acid was prepared as previously described,[36] 1,2-bis(2-(3-methylbuta-1,3-dien-2-yloxy)ethyl) disulfane was prepared as previously described.[37] 2,2′-Azoisobutyronitrile (AIBN, 98%, Sigma–Aldrich) was recrystalized twice from acetone, dichloromethane (DCM, 99%, Ajax) was stored over calcium hydride and distilled before using.

Abbreviations: deoxyribonucleic acid (DNA); polyethylene glycol (PEG); chain transfer agent (CTA); dichloromethane (DCM); molecular weight (MW); polydispersity index (PDI); 2,2′-azoisobutyronitrile (AIBN); *N*,*N*-dimethylacetamide (DMAc); 4-(dimethylamino)pyridine (DMAP); gel permeation chromatography (GPC); molecular weight cut-off (MWCO); phosphate buffer (PB); phosphate buffered saline (PBS).

### Measurement

Gel permeation chromatography (GPC) analyses of polymers were performed in *N*,*N*-dimethylacetamide (DMAc) (0.03% w/v LiBr, 0.05% BHT stabilizer) at 50 °C (flow rate: 0.85 mL · min$^{-1}$) using a Shimadzu modular system comprising a DGU-12A solvent degasser, an LC-10AT pump, a CTO-10A column oven, and an RID-10A refractive index detector. The system was equipped with a Polymer Laboratories 5.0 mm bead-size guard column (50 × 7.8 mm$^2$) followed by four 300 × 7.8 mm$^2$ linear PL columns ($10^5$, $10^4$, $10^3$, and 500). Calibration was performed with narrow polydisperse polystyrene standards ranging from 500 to $10^6$ g · mol$^{-1}$.

$^1$H NMR spectra were obtained using a Bruker AC300F (300 MHz) Spectrometer or a Bruker DPX300 (300 MHz) Spectrometer. Multiplicities were reported as singlet (s), broad singlet (bs), doublet (d), triplet (t), quad (q), and multiplet (m). Agarose gel electrophoresis was carried out with ReadyAgrose mini Gel (BioRad).

## Methods

### PEG Trithiocarbonate (CTA, 1)

4-Cyano-4-(ethylthiocarbonothioylthio) pentanoic acid (1.00 g, 3.8 mmol), PEG 1 100 (3.84 g, 3.2 mmol), and DMAP (0.10 g) were dissolved in dry DCM (50 mL), DCC (0.99 g, 4.8 mmol) was added under nitrogen atmosphere. The mixture was stirred at 22 °C for 16 h, and filtered to remove white solid. The solvent was dried over $MgSO_4$ and distilled under vacuum to yield a yellow residue. The crude was purified by column chromatography on silica gel [methanol in DCM (1.0–8.0%)] to yield the product as a yellow waxy solid (3.31 g, 76.9%). $^1$H NMR (300.18 MHz, $CDCl_3$): $\delta$ (ppm) = 4.27–4.24 (m, 2H, $COOCH_2$), 3.89–3.52 (m, 48.7H, PEG unit), 3.37 (s, 3H, $OCH_3$), 3.34 (q, 2H, J = 7.4 Hz, $SCH_2$), 2.68–2.32 (m, 4H, $CCH_2CH_2$), 1.87 (s, 3H, $CCH_3$), 1.35 (t, 3H, J = 7.4 Hz, $SCH_2CH_3$). IR (cm$^{-1}$): 2 880, 1 736, 1 466, 1 343, 1 280, 1 111, 843.

### Synthesis of Hyperbranched PEG-*b*-PDMAEMA

A typical polymerization is described as follows:

DMAEMA (316 mg, 2.0 mmol), CTA (135 mg, 0.1 mmol), DSDMA (87 mg, 0.3 mmol) and AIBN (4.9 mg, 0.03 mmol) were dissolved in DMAc (3.0 mL). Aliquots were transferred to four different vials that were then sealed with rubber septa. Each vial was deoxygenated by purging with

nitrogen for 30 min prior to placement in a preheated water bath at 70 °C. The vials were taken out at 4, 8, 22 and 29 h. Immediate cooling with ice-water bath and exposure to air quenched the polymerizations. The polymer solutions were subjected to direct GPC analyses. The polymers were collected after precipitation from DCM to hexane and then dried under vacuum.

### Static Light Scattering (SLS) Analysis of the Hyperbranched PEG-*b*-PDMAEMA

A Brookhaven BI-9000AT Digital Autocorrelator was used for SLS measurements which applies vertically polarized laser light of wavelength 632.8 nm. In SLS, measurements were carried out at a temperature of 25 °C (unless otherwise stated) to determine the weight-average molecular weight ($\overline{M}_w$) of the hyperbranched copolymer in DMAc solution from Zimm plots according to the relation;[38,39]

$$\frac{Kc}{\Delta R_\theta} = \frac{1}{\overline{M}_w}\left[1 + \frac{q^2 R_g^2}{3}\right] + 2A_2 c \quad (1)$$

where $K$ is the optical constant, which depends on the refractive index increment of the polymer solution ($K = 4\pi^2 n^2 (dn/dc)^2 / N_A \lambda^4$). Here, $c$ is the concentration of the polymer solution, $n$ the refractive index of the solvent, $\theta$ the angle of measurement, $\lambda$ the wavelength of laser light, $\Delta R_\theta$ the excess Rayleigh ratio [$\Delta R_\theta = R_\theta$ (solution) $- R_\theta$ (solvent)], $dn/dc$ the refractive index increment of the copolymer solution, and $N_A$ is the Avogadro's constant. The scattering angles ranged from 50° to 130° at 10° intervals while the copolymer concentration ranged from 1 to 10 mg · mL$^{-1}$. A plot of $Kc/R_\theta$ versus [$\sin^2(\theta/2) + kc$] (where $k$ is a plotting constant) can be used to determine the molecular parameters. A simultaneous extrapolation to zero angle and concentration yields an intercept, that is the inverse of the $\overline{M}_w$.

The refractive indices of copolymers in DMAc were measured by using a BI-DNDC differential refractometer at a wavelength of 620 nm to determine the refractive index increment ($dn/dc$) of each solution. The instrument was primarily calibrated with potassium chloride (KCl) in aqueous solution and the $dn/dc$ value was found to be 0.150 ± 0.011 mL · g$^{-1}$ for PEG-*b*-PDMAEMA in DMAc.

### Protonation of the Hyperbranched Polymer

Hyperbranched copolymer (30 mg) was dissolved in HCl aqueous solution (5.0 mL of 0.01 M). After removal of excess acid by centrifugation filtration [molecular weight cut-off (MWCO): 10 000], water was removed by a freeze drying process to yield polymer as a yellow powder.

### Deoxyribonucleic Acid (DNA) Binding by the Protonated Hyperbranched Polymer

Deoxyribonucleic acid (DNA, 0.2–1.0 μg · μL$^{-1}$) was dissolved in 5% glucose aqueous solution. A series of protonated PEG-*b*-PDMAEMA solutions (0.1–1.0 μg · μL$^{-1}$) in 5% glucose aqueous were added slowly to the DNA solution. The amount of the PEG-*b*-PDMAEMA added was calculated based on the chosen positive/negative ($P/N$) ratio of the polymer to DNA. The solution was incubated at 25 °C for 30 min with gentle stirring, and then the solution was used directly for gel electrophoresis.

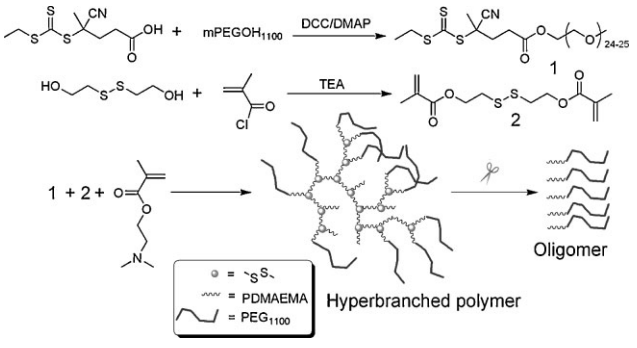
### Analysis of Particle Size and Zeta Potential of Polyplexes

The determination of hydrodynamic diameters and zeta potentials of the DNA polyplexes (10 μg · mL$^{-1}$ DNA and 2–500 μg · mL$^{-1}$ protonated PEG-*b*-PDMAEMA in 5% glucose aqueous solution) was performed at 25 °C by a Zetasizer Nano dynamic light scattering detector (Malvern Instruments, He–Ne laser 633 nm). The mean diameter was obtained from the arithmetic mean using the relative intensity of each particle size.

## Results and Discussion

### Preparation of Biodegradable Hyperbranched PDMAEMA

PEGylated chain transfer agent (**1**) (Scheme 1) was prepared using the reaction between commercial methyl ether PEG (≈1 100) and 4-cyano-4-(ethylthiocarbonothioylthio) pentanoic acid in the presence of DCC using DMAP as a catalyst. The $^1$H NMR spectrum (Figure 1a) of the CTA exhibited signals at 4.25 and 3.37 ppm corresponding to the ester group and the methyl ether moiety, respectively. The integration ratio of these two peaks is 2/3.08,




*Scheme 1.* Synthesis of biodegradable hyperbranched PEG-*b*-PDMAEMA and the subsequent cleavage reaction.



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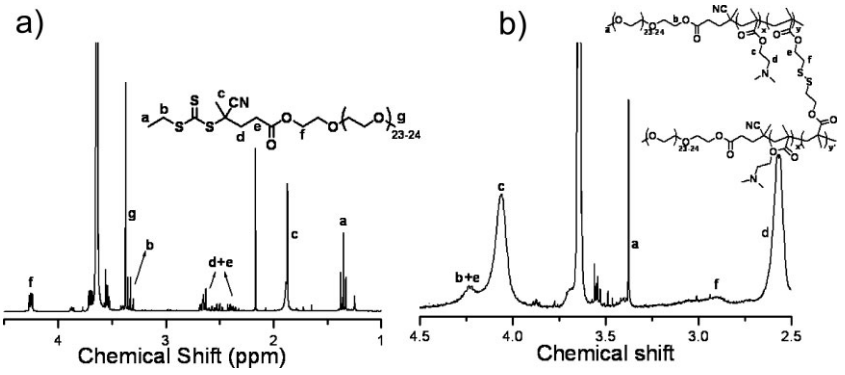


*Figure 1.* $^1$H NMR spectra of (a) CTA, (b) hyperbranched PEG-PDMAEMA copolymer.

close to the predicted 2/3, indicative of complete esterification. This PEGylated CTA was employed to synthesize polymer from DMAEMA, adding DSDMA as crosslink agent to increase the degree of polymer branching whilst avoiding undissolvable gel formation.[37,40–42] The final polymer was prepared with a ratio, [CTA]/[DSDMA]/[monomer] = 1:3:20. The $^1$H NMR spectrum of the final copolymer (Figure 1b) displayed signals at 4.06 and 3.37 ppm corresponding to the ester group of PDMAEMA and methyl ether group from the CTA moiety. The integration ratio of these two peaks was ≈42/3.0, close to the predicted value of 40/3, confirming that the oligomer chains have predetermined chain lengths. The signal at 2.86–3.00 ppm was attributed to the methylene group adjacent to the disulfide, confirming that the cross-linking agent was incorporated into the polymer structure.

The polymerizations were monitored over time as shown in Figure 2. As expected both the molecular weight and branched polymer ratio increased with increase in reaction times. As GPC was calibrated with polystyrene standards it could not be used to quantitatively analyze the polymers. Consequently, SLS was employed to characterize the "true" molecular weight yielding a weight average molecular weight of $\overline{M}_w \approx 342$ K.

## Cleavage of the Biodegradable Hyperbranched Polymer

As every branched oligomer is linked through a disulfide bond, the polymer should be cleavable in the presence of reductants. Consequently, the polymer was treated with DTT (0.1 M) in DMAc solution for 16 h. The GPC chromatogram (Figure 3) indicated that the post-reduction polymer possessed a much lower molecular weight [$\overline{M}_n$ (GPC) ≈11 600] than the original polymer. The narrow polydispersity of the cleaved polymer (PDI ≈ 1.18) also indicated uniformity, consistent with RAFT polymerization control, demonstrating a design control capacity over the final degraded polymer via the original synthesis protocol.

## Deoxyribonucleic Acid (DNA) Binding to the Biodegradable Hyperbranched PDMAEMA

Deoxyribonucleic acid (DNA) sodium salt from salmon testes was used as model DNA. The hyperbranched degradable PEG-PDMAEMA was protonated prior to DNA binding. The protonated polymer was mixed with DNA having different *P/N* ratios, and the binding was monitored by agarose gel electrophoresis. The negative signal from DNA decreased with an increase in *P/N* ratio, as shown in Figure 4a. When the ratio reached 1:1, the DNA was neutralized perfectly and there was no DNA complex

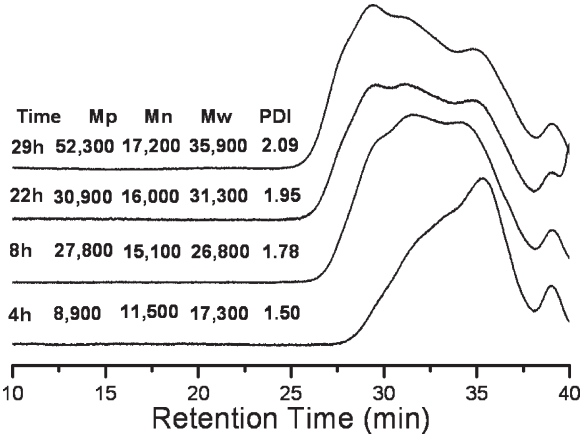


*Figure 2.* Gel permeation chromatography (GPC) traces of hyperbranched polymers generated from [Monomer]/[CTA]/[DSDMA]/[initiator] ratio of 20:1:3:0.3 at 70 °C.

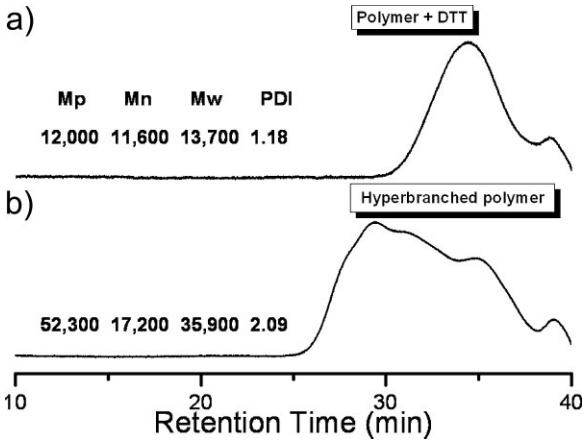


*Figure 3.* Polymers (a) before and (b) after incubating with DTT.

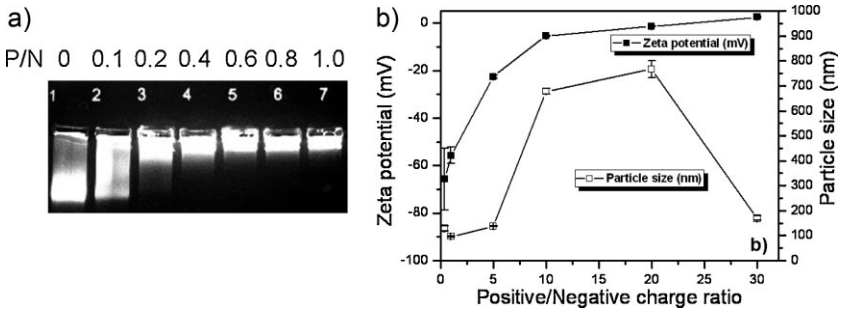


*Figure 4.* (a) Agrose gel electrophoresis; (b) zeta potential and particle size analysis of the DNA bonded by hyperbranched polymer under different *P/N* ratio.

observed via the gel, consistent with the effective formation of a polymer/DNA complex.

The particle sizes and zeta potentials of the polyplexes were also measured. As shown in Figure 4b, when the *P/N* ratio of polymer to DNA is less than 10, the sizes of polyplexes were below 200 nm. The small particle sizes can be attributed to the surface charges (–22.57 mV, Figure 4b), preventing aggregation of the polyplexes. When the *P/N* ratio reached 20:1, the polyplex possessed a nearly neutral surface charge (−1.42 mV), leading to the formation of larger particles (≈770 nm) due to aggregation. When the *P/N* ratio reached 30:1, the surface charge of the polyplex became positive (2.49 mV), and the particle size decreased significantly (≈170 nm), a result we attribute to the surface stabilized with PEG chains promoting the separation of aggregated particles.

## Conclusion

We have described a straightforward methodology to synthesize new hyperbranched bio-degradable PEG-PDMAEMA copolymers via RAFT polymerization. The positively charged, hyperbranched polymer core is covered by PEG chains, masking the positive charge and reducing the toxicity of the polymer. The polymeric structures are inherently biodegradable producing oligomers with narrow dispersity chain lengths on reduction. This makes it possible to optimize the synthetic protocol to minimize the toxicity of the oligomers during their excretion phase. Cleavage tests under reducing condition confirmed that the branched chains were well-defined (consistent with RAFT control). This design strategy for shielded cationic polymers yields DNA polyplexes via multivalent electrostatic interactions, enabling their use as non-cytotoxic gene delivery agents.

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