Gene Delivery

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Significance of Branching for Transfection: Synthesis of Highly Branched Degradable Functional Poly(dimethylaminoethyl methacrylate) by Vinyl Oligomer Combination**

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Abstract: *A series of degradable branched PDMAEMA copolymers were investigated with the linear PDMAEMA counterpart as gene-delivery vectors. The branched PDMAEMA copolymers were synthesized by controlled radical cross-linking copolymerization based on the "vinyl oligomer combination" approach. Efficient degradation properties were observed for all of the copolymers. The degree of branching was found to have a big impact on performance in transfection when tested on different cell types. The product with the highest degree of branching and highest degree of functionality had a superior transfection profile in terms of both transfection capability and the preservation of cell viability. These branched PDMAEMA copolymers show high potential for gene-delivery applications through a combination of the simplicity of their synthesis, their low toxicity, and their high performance.*

The notion that the macromolecular structure of nonviral gene vectors alters their transfection efficacy has inspired numerous novel designs of 3D polymeric structures, such as globular dendrimers,[1] micelle block copolymers,[2] and star-shaped,[3] randomly branched,[4] or cyclized[5] copolymers, for both transfection enhancement and cytotoxicity reduction. Although the "gold standard", branched poly(iminoethylene) (PEI), generally shows high transfection, its associated high cytotoxicity ($IC_{50} \approx 30\ \mu g\,mL^{-1}$)[6] is a major drawback and has been a driving force for PEI modification[7] and the exploration of other vectors.

With a buffering capacity ($pK_a = 7.5$) and lower cytotoxicity than that of PEI,[6] poly(dimethylaminoethyl methacrylate) (PDMAEMA) is a promising gene-delivery system, as its design and precise synthesis from the vinyl monomer *N*,*N*-dimethylaminoethyl methacrylate (DMAEMA) is relatively simple. Controlled molecular weights, well-defined chain ends, and different macromolecular architectures (such as block, star, graft, and knot) were readily accessible by the use of techniques of controlled/living radical polymerization (CRP), such as atom-transfer radical polymerization (ATRP)[8] and reversible addition–fragmentation chain transfer (RAFT).[9] However, most designs of PDMAEMA have led to the formation of long nondegradable carbon–carbon chains during chain-growth polymerization. Therefore, the cytotoxicity of PDMAEMA-based vectors is maintained at a relatively high level,[10] and modifications aiming at reducing cytotoxicity, such as PEGylation, are usually accompanied by a loss of transfection.[11] The introduction of a hydrophobic segment (i.e. polycaprolactone) significantly improved the transfection efficiency;[12] however, multiple steps were required for the preparation of the copolymer, and the transfection level was still much lower than that of commercial transfection agents. Whereas there have been numerous studies on linear or block PDMAEMA, the study of 3D branched PDMAEMA is still comparatively rare.[4] Although a branched DMAEMA/ethylene glycol dimethacrylate (EGDMA) copolymer showed comparable transfection capability to that of commercial agents and a much higher level than its linear counterpart,[4a] the effect of the PDMAEMA branching structure on transfection efficiency has not yet been fully understood and explored. Moreover, the study did not address issues such as degradation or functionalization.

As a potential alternative to dendrimers, hyperbranched (HB) polymers have the advantages of simpler and more cost-effective synthesis as well as a high range of functionality. Recently, a new strategy, so-called "vinyl oligomer combination", has been developed for the preparation of HB polymers from multi-vinyl monomers (MVMs) through chain-growth polymerization.[13] By kinetic control and statistical manipulation, HB structures consisting purely of extremely short primary -C–C- chains were obtained. We predicted that this strategy could, in principle, be applied to a copolymerization system. We report herein the preparation of a series of highly branched DMAEMA/bis(2-acryloyl)oxyethyl disulfide (BADS) copolymers by "vinyl oligomer combination" through in situ deactivation enhanced ATRP (in situ DE-ATRP). We used high initiator/DMAEMA ratios (1:8–1:32) to supply a high concentration of initial short primary chains, which could enhance intermolecular combination and thus

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lead to a highly branched structure. The logic for hypothesizing that this highly branched structure would lead to greater performance is twofold: first, by the creation of a 3D branched structure with multiple functional groups for DNA interaction, and second, by efficient intracellular cleavage of the disulfide bond[14] for low cytotoxicity.

The aim was therefore to assess whether the degradable branched PDMAEMA–BADS copolymer would be superior to linear PDMAEMA in terms of both transfection capability and lower toxicity. Commercially available transfection vectors (25 k bPEI, Xfect, and polyamidoamine (PAMAM) dendrimer) were also used for comparison. Branched PDMAEMA–BADS polymers with similar molecular weights but varying degrees of branching were synthesized. After end capping of the vinyl groups with functional molecules, these polymers were studied on different cell types, and it was found that highly branched PDMAEMA–BADS was superior to linear PDMAEMA.

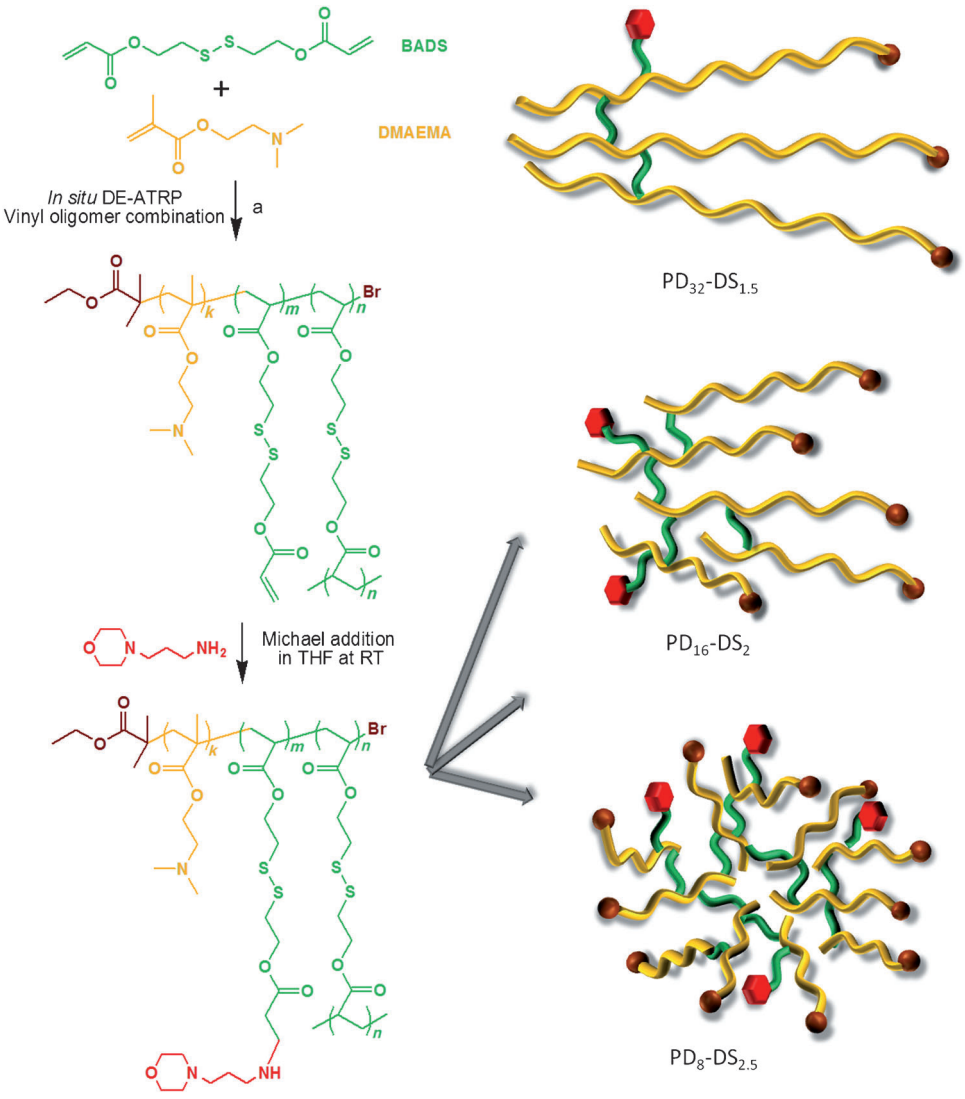

Figure 1. Left: Controlled radical cross-linking copolymerization through in situ DE-ATRP, followed by a postfunctionalization process. Reaction conditions: a) ethyl 2-bromoisobutyrate, $CuCl_2$, PMDETA, L-ascorbic acid, THF, 50°C. Right: Graphical representation of structures with different degrees of branching. The efficacy of functionalization depends on the content of the pendent vinyl groups.

Figure 1 outlines the design and synthesis of the $PDMAEMA_x$–$BADS_y$ polymers (termed PD_x–DS_y below). The initial initiator/DMAEMA/BADS molar ratio was set at 1:*x*:*y*. Varying of the component ratios enables a range of degrees of branching. The reaction system in this study could be classified as controlled radical cross-linking copolymerization (CRCC, a controlled/living form of the "Strathclyde synthesis"), in which a single vinyl monomer is copolymerized with a multivinyl cross-linker by controlled/living methods.[15] However, there are two significant features of our reaction system which distinguish it from the traditional reaction system. First, the initial molar ratio of cross-linker to initiator is more than 1. In the traditional CRCC, the amount of cross-linker is usually lower than that of the initiator, since it is widely accepted that critical gelation happens when the average number of cross-linkages (cross-linker in which both vinyl groups have reacted) per primary chain exceeds unity if the primary chains are uniform. This hypothesis has been confirmed in various experiments.[16] According to this hypothesis, the reaction systems described herein will eventually lead to cross-linked networks before complete monomer conversion is reached. However, if the monomer conversion is kept incomplete and some of the divinyl cross-linker does not fully react, or is consumed by intramolecular cyclization, gelation may not be observed. The benefit of a higher ratio of the cross-linker is that much more highly branched structures could be synthesized, and pendent vinyl groups could be reserved in the product as universal chemical groups for functionalization in various ways. The acrylate-based cross-linker was used in this study for the ease of Michael addition to the amine groups on the functional molecules.

The second feature is that the molar ratio of the initiator to the vinyl monomer is relatively high (1:8–1:32) as compared to that in the traditional protocol (1:50–1:100).[16b] This high ratio was adopted for the purpose of shortening the primary chains and enhancing their intermolecular combina-

tion, by which highly branched structures are favored. If the linkages between primary chains are cleavable in a specific environment, the polymer product will have the potential to fragment into small pieces of oligomers, which are more readily removed or processed by cells and the organism.

The reaction process was followed by the analysis by size-exclusion chromatography (SEC) of samples extracted during the reaction (Table 1). Figure 2 shows typical SEC traces for the synthesis of the three polymers. The traces show similar evolution of molecule growth in the reaction system. Highly symmetrical and unimodal peaks at 2 h indicate the controlled nature of growth. The molecular-weight distributions were also narrow for all polymers at 2 h ($M_w/M_n < 1.4$), thus indicating the formation of predominantly linear chains with rare branching. As the reaction progressed, both molecular weight and polydispersity increased gradually because of the increased participation of divinyl BADS at higher monomer conversion. The peaks at 5 h were slightly asymmetrical with the left side spread a little, thus indicating the formation of molecules with moderate branching. The right sides of the peaks at 5 h were parallel to those of the corresponding peaks at 2 h, thus manifesting the living nature of the reaction. The peaks at 20 h were spread dramatically owing to the large degree of intermolecular branching between primary chains when the critical overlap concentration is reached. Above this conversion threshold, the barriers to intermolecular reaction are significantly reduced; thus, branching becomes a priority. After precipitation, the small linear chains and the monomers were removed to leave only the highly branched products. In this way, the interference of linear molecules was removed.

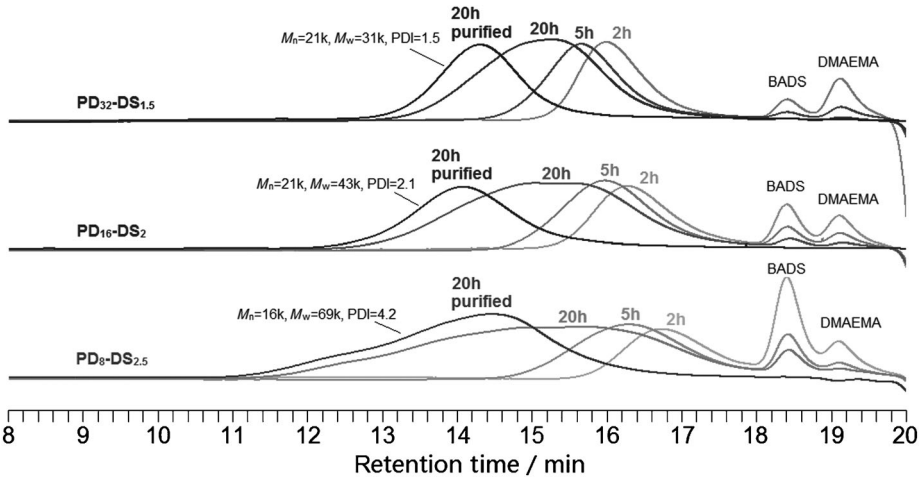

Figure 2. Time dependence of the composition of the polymerization mixture for the three PD_x–DS_y syntheses, as monitored by SEC with a refractive-index detector. The traces show subtly different reaction pathways.

Table 1: Polymerization conditions and molecular-weight characteristics of the polymers with different monomer feed ratios.

	t [h]	M_n[e] [kDa]	M_w[e] [kDa]	M_w/M_n[e]	Conversion[f] [%]
PD_{32}–$DS_{1.5}$[a]	2	3.2	4.4	1.36	74.8
	5	4.2	6.8	1.60	90.3
	20	6.8	15.6	2.29	98.3
	purified[d]	20.9	31.1	1.49	–
PD_{16}–DS_2[b]	2	2.3	3.1	1.35	69.2
	5	3.1	5.1	1.67	83.8
	20	5.4	18.1	3.36	96.0
	purified[d]	20.9	43.0	2.06	–
PD_8–$DS_{2.5}$[c]	2	1.5	2.0	1.34	49.8
	5	2.3	4.0	1.78	75.6
	20	4.4	35.7	8.16	90.8
	purified[d]	15.7	69.4	4.42	–

[a] Reaction conditions: I/DMAEMA/BADS/$CuCl_2$/PMDETA/AA (1:32:1.5:0.16:0.16:0.032), THF (solvent), [DMAEMA] = 2 mol L^{-1}, 50 °C. I (initiator) = ethyl 2-bromoisobutyrate, PMDETA = 1,1,4,7,7-pentamethyldiethylenetriamine, AA = L-ascorbic acid. [b] Reaction conditions: I/DMAEMA/BADS/$CuCl_2$/PMDETA/AA (1:16:2:0.08:0.08:0.016), THF, [DMAEMA] = 2 mol L^{-1}, 50 °C. [c] I/DMAEMA/BADS/$CuCl_2$/PMDETA/AA (1:8:2.5:0.04:0.04:0.008), THF, [DMAEMA] = 2 mol L^{-1}, 50 °C. [d] For its purification, the polymer was precipitated three times from hexane/diethyl ether (10:7, v/v) and then passed twice through an Al_2O_3 column. [e] The values M_n, M_w, and PDI were determined by SEC with a refractive-index detector. [f] The conversion was calculated by the integration of peaks in the SEC trace.

There were also some subtle differences in the evolution of molecular weight when the molar ratio of initial reactants was varied. A lower molar ratio of the initiator to the vinyl monomer (PD_{32}–$DS_{1.5}$) led to a faster molecular-weight increase in the linear-growth period (Figure 2). However, the higher content of the divinyl cross-linker in PD_8–$DS_{2.5}$ promoted the intermolecular combination reaction and thus resulted in a stronger exponential increase in molecular weight during the later stage of the reaction.

To minimize the interference of the effect of molecular weight (well-known to affect the performance of transfection),[3] we stopped the reactions after different time periods (24 h for PD_{32}–$DS_{1.5}$, 20 h for PD_{16}–DS_2, and 18 h for PD_8–$DS_{2.5}$ with a conversion of 98.5, 96.0, and 87.1 %, respectively, as determined by SEC) to obtain similar molecular weights (see Figure S1 in the Supporting Information for the SEC traces and ^{1}H NMR spectra of the final PD–DS products). The weight-average molecular weights were 42, 43, and 45 kDa for PD_{32}–$DS_{1.5}$, PD_{16}–DS_2, and PD_8–$DS_{2.5}$, respectively. The ratios of components in the products were calculated from the ^{1}H NMR spectra (Table 2). The amounts of the DMAEMA and BADS units were basically proportional to their initial feed ratio for all the three polymers. The DMAEMA units had a slightly higher ratio, thus indicating a higher reactivity of the methacrylate derivative as compared to the acrylate derivative. This difference in reactivity may lead to the different rate of incorporation into the chains. The resulting branched polymer is therefore probably a structure

Table 2: Variation of the composition of the PD_x–DS_y polymers by adjustment of the monomer feed ratio of the simple "one-pot" reaction.

	Initiator [%]	DMAEMA [%]	BADS [%]	Branch unit /total [%]	Vinyl unit /total [%]	Vinyl conc. [mmol g^{-1}]
PD_{32}–$DS_{1.5}$	3.0	92.7	4.3	2.7	1.6	0.10
PD_{16}–DS_2	5.8	85.4	8.8	5.3	3.5	0.21
PD_8–$DS_{2.5}$	10.9	70.1	19.0	9.4	9.6	0.53

of heterogeneously distributed branches[17] or more like a branched core decorated with PDMAEMA short hairs. Determination of the polymer structure by ^{1}H NMR spectroscopy also showed that these polymers possess a high degree of branching, which is highest for PD_8–$DS_{2.5}$ (9.4%; Table 2). This value indicates that one branching unit exists for every 20 atoms in a -C–C- chain. The initiator content in the polymer products is higher than the content of the branching unit. This result indicates that one connection per primary chain is still not reached, and thus the reaction conversion is still under the gelling point. The high conversion could be attributed to the fact that the divinyl cross-linker does not fully react, or is inevitably consumed by intramolecular cyclization at later stages.[18] Free vinyl groups were also left within the polymer structure (Table 2; see also Figure S1), thus providing an opportunity for a range of postsynthetic functionalization reactions.

All three purified polymers were end capped with 3-morpholinopropylamine (MPA) through a Michael addition between the unreacted acrylate moieties on those polymers and the primary amine of MPA. The morpholino structures were recently introduced into a poly(β-aminoester), and the resulting polymer exhibited good transfection performance.[19] The ^{1}H NMR spectra of PD_8–$DS_{2.5}$ before and after end capping (see Figure S2) showed that all vinyl groups were consumed; the typical peaks for the MPA chemical structure appeared after end capping, thus indicating a good connection of MPA to the polymer.

The highly branched structures were also confirmed by cleavage of the disulfide bonds, which underwent fast reduction upon the addition of 20 mM glutathione. If a typical highly branched structure was formed, exposure of the polymer to glutathione would result in cleavage of the branching units, including those used as intermolecular links, thus resulting in the production of small fragments of the polymer (Figure 3a). It can be imagined that a polymer comprised of shorter primary chains and with a higher degree of branching would be cleaved into smaller pieces. The three PD–DS polymers were analyzed for their M_w value and polydispersity index (PDI) before and after treatment with glutathione for 1 h. A significant decrease in the molecular weight occurred for all polymers (Figure 3b), thus confirming that this structure is comprised predominantly of short primary chains. PD_8–$DS_{2.5}$, synthesized with the highest ratio of the initiator to the monomer and the highest amount of the disulfide cross-linker, showed the most

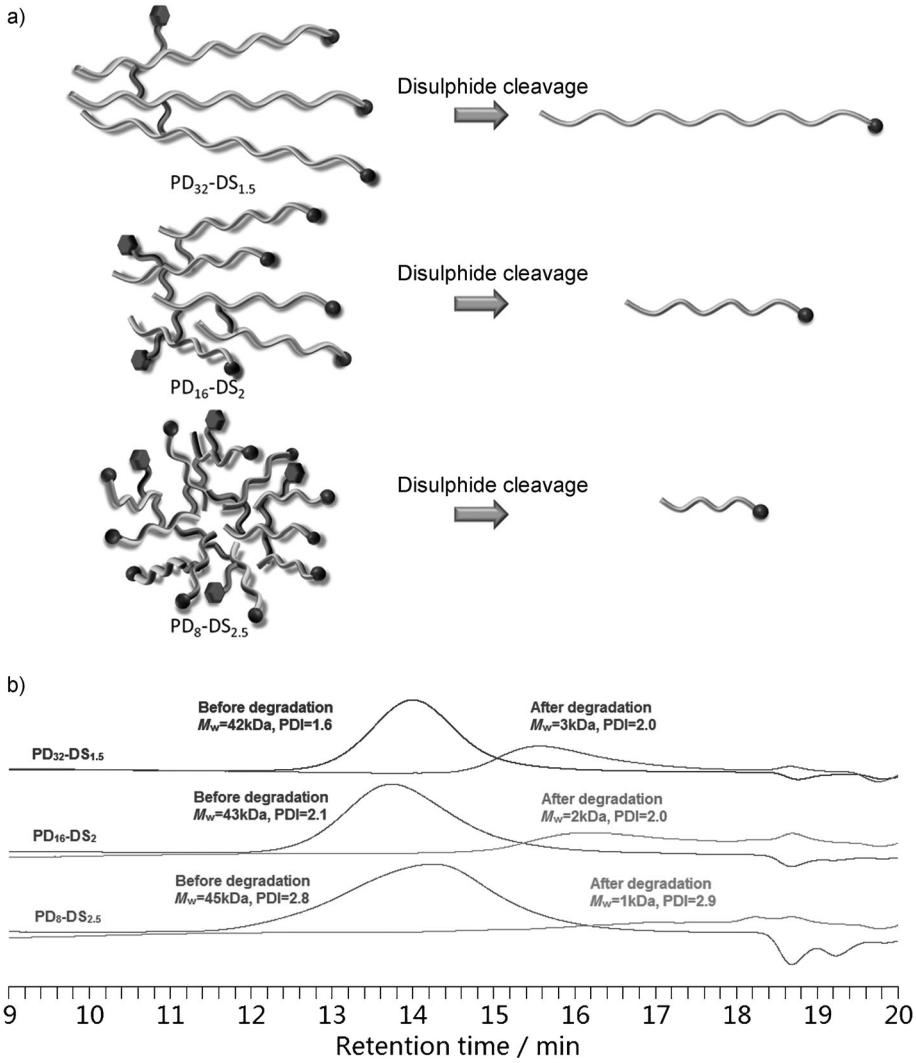

Figure 3. Degradation of the three PD_x–DS_y polymers. a) Graphical representation of the degradation of structures with different degrees of branching. b) SEC trace of the three PD_x–DS_y polymers before (darker curve) and after treatment (lighter curve) with 20 mM glutathione for 1 h. A significant decrease in the molecular weight was observed.

significant decrease in the M_w value from 45 to 1 k and thus showed the desired behavior with respect to the designed shortening of the primary chains. This efficient degradation capability is expected to have potential in reducing cytotoxicity, since high cytotoxicity is usually induced by high-molecular-weight foreign molecules, which are too difficult for cells to metabolize or exocytose.

The polymer/DNA interaction (polyplex) was first characterized by agarose gel electrophoresis (see Figure S3 a–c). The weight ratios for critical complex formation for PD_{32}–$DS_{1.5}$, PD_{16}–DS_2, and PD_8–$DS_{2.5}$ were below 0.4, 0.6, and 0.8, respectively. The different ratios could be attributed to the differences in the DMAEMA content and the resulting differences in the positive charge density of these polymers. Another possible reason is that less branched molecules possess higher flexibility and thus need a lower entropy change for DNA binding as compared to rigid highly branched molecules. For the characterization of polyplex size and charge (see Figure S3 d,e), we chose three polymer/plasmid weight ratios of 1:1, 3:1, and 5:1 for the formation of polyplexes. All polyplexes formed by the PD–DS polymers exhibited a decrease in size and an increase in surface potential as the polymer/DNA weight ratio increased. The particle sizes ranged from 50 to 200 nm, whereby the PD_8–$DS_{2.5}$ polyplexes showed the largest sizes, possibly because of the increasing hydrophobicity caused by the higher amount of BADS units. The surface potential of the polyplex particles was positive in all cases. Interestingly, the surface potential of the polyplexes increased at different rates (the PD_8–$DS_{2.5}$ polyplexes grew fastest), thus indicating different manners of DNA binding between the polymers and DNA.

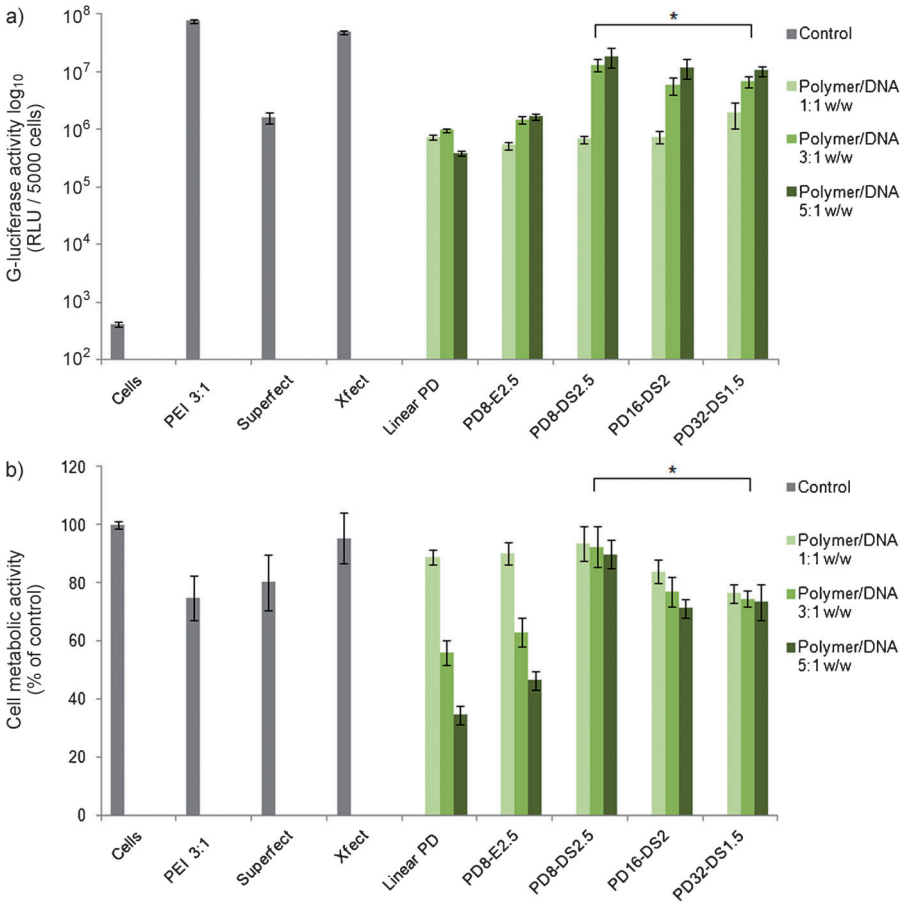

Figure 4. Analysis of the G-luciferase transfection of HeLa cells and cytotoxicity after incubation for 48 h. The branched PD_x–DS_y polymers manifest more favorable transfection properties in terms of a) transfection ability and b) cytotoxicity than linear PDMAEMA ($n = 6$, 5000 cells and 1 μg of pDNA per well, error bars indicate ± standard deviation, and asterisks indicate a significant difference from linear PDMAEMA). RLU = relative light units.

The transfection capability of the three polymers was assessed by the secreted G-luciferase protein assay on HeLa cells (Figure 4 a). In each G-luciferase study, three polymer/plasmid weight ratios of 1:1, 3:1, and 5:1 were tested for each of the PD–DS polymers, together with linear PDMAEMA (44 kDa) and nondegradable branched $PDMAEMA_8$–$EGDMA_{2.5}$ (labeled as PD_8–$E_{2.5}$ in Figure 4) for comparison. To estimate the real transfection capability of the highly branched PD–DS polymers and their commercial prospects, commonly used polymer transfection agents, such as PEI, Superfect, and Xfext, were used as the positive control throughout these studies, according to the protocol of the manufacturers. The subsequent effect on cell viability of those commercial agents and polymers with different weight ratios was also plotted (Figure 4 b). The alamarBlue reagent was used to measure any reduction in cell metabolic activity, which was normalized to indicate cell viability by plotting as a percentage of the control cells.

Highly branched PD–DS polymers exhibited far higher transfection capabilities than that of linear PDMAEMA when the polymer/plasmid weight ratio was above 3 (Figure 4 a). Meanwhile, the transfection capability increased with branching from PD_{32}–$DS_{1.5}$ to PD_8–$DS_{2.5}$, thus showing the strong effect of changing the degree of branching on the transfection performance. Despite complete complexation of the plasmid at polymer/plasmid weight ratios below 1, highest transfection was observed at weight ratios above 3, possibly because of the smaller particle size observed at the higher ratio, and sufficient positive charge to aid membrane translocation. Degradable PD_8–$DS_{2.5}$ also showed higher transfection capability than did the nondegradable PD_8–$E_{2.5}$ polymer with a similar degree of branching. A contributing factor could be the higher cytotoxicity associated with the nondegradable PD–E polymers, as shown in Figure 4 b. In fact, PD–E may not be the best candidate for comparison, as the similar

reactivity of the methacrylate groups in DMAEMA and EGDMA could result in a homogeneous branch distribution different from that of the PD–DS polymers. Cell metabolic activity was analyzed by the use of HeLa cells exposed to the polymers at different concentrations for 48 h. As the concentration was increased (by increasing the polymer/plasmid ratio), reduced cell viability was seen for all polymers. On the other hand, PD–DS polymers showed a much lower adverse effect on cells. PD_8–$DS_{2.5}$ showed cell metabolic activities above 90% even at the high weight ratio of 5. We also performed the cytotoxicity test on other cell lines (normal human keratinocyte, NHK, and human adipose stem cells, hADSC) and found a similar trend in the influence of the polymers on the cells (see Figure S4). This result is extremely promising, since a high transfection capability and low cytotoxicity could be achieved at the same time by introducing a high degree of branching into a PDMAEMA polymer, along with the ability to undergo efficient degradation.

Fluorescence microscopy imaging of the expression of green fluorescent protein (GFP) was also used to confirm the transfection of different cell types, including HeLa and hADSC (see Figures S5 and S6). The GFP expression of HeLa cells is qualitatively in accordance with their expression of G-luciferase. Large areas of cells could be transfected, and widespread GFP expression was observed throughout the cell cytoplasm, when PD_8–$DS_{2.5}$ was used as the transfection agent. Meanwhile, most of the cells remained alive after transfection for 48 h without changing the medium posttransfection, thus revealing low cytotoxicity. Good cell compatibility of PD_8–$DS_{2.5}$ was also observed for hADSC cells, although the transfection efficacy was much lower for this cell type.

In conclusion, highly branched degradable PD–DS copolymers consisting of short primary-chain molecules were synthesized and proved to be effective in vitro gene-delivery agents. The special structure and components of PD–DS copolymers provide the possibility of postfunctionalization or labeling as well as the capability of efficient degradation. The highly branched copolymers also offer different patterns of interaction between the polymer and plasmid DNA, and lead to a general profile of transfection capability that is comparable to that of the leading commercial transfection agents. By adjusting the degree of branching and the length of primary-chain molecules, the cytotoxicity of the polymers could be lowered, thus rendering the PD–DS copolymers a more attractive alternative to linear PDMAEMA. This strategy towards highly branched degradable structures, previously inaccessible theoretically and experimentally, will open new avenues for the field of gene delivery.

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