Dendritic Polymers

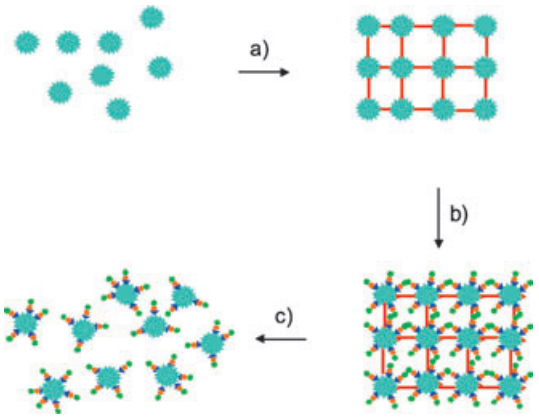
Reversible Cross-Linking of Hyperbranched Polymers: A Strategy for the Combinatorial Decoration of Multivalent Scaffolds**

*Michael Barth, Rainer Fischer, Roland Brock, and Jörg Rademann**

Functional polymers in the nanometer range have been reported to control biomedical processes including immunization, transfection, adhesion, and drug delivery.[1] Multivalent scaffolds such as linear or branched polymers including dendrimers have been especially useful in these applications.[2] They have served for the multiple presentation of single effective ligands in order to amplify low-affinity binding.[3] In addition, dendritic structures have been found to be significantly more stable towards proteolysis in vivo than the respective monovalent ligands.[4]

Whereas the plain, nondecorated polymers are easily accessible,[5] the generation of chemically modified polymers with specific biological activity is much more demanding. Synthesis on soluble polymers has been studied extensively[6] and has been been inefficient in most cases due to tedious workup procedures and low yields. The one practical method to date is the conjugation of the polymer with preformed small molecules. Conjugation, however, is restricted to few coupling reactions and does not allow a flexible variation of the polymer decoration and loading. For structure–activity studies for example, during the biological optimization of polymer drugs, the preparation and variation of decorated multivalent scaffolds with increased throughput by parallel or combinatorial methods will be necessary.

Herein, we present a strategy that eliminates the problems of polymer modification and facilitates flexible access to complex decorated scaffolds (Scheme 1). Branched polymers were cross-linked to yield a swellable resin which served as a robust support for subsequent solid-phase synthesis (Scheme 1, top). Making use of easy-to-perform polymer-supported protocols for the multistep synthesis, we could assemble multiple copies of a target molecule on each macromolecule. Finally, the cross-linking units of the polymer support were cleaved, yielding the decorated scaffolds (Scheme 1, bottom).



Scheme 1. Reversibly cross-linked hyperbranched polymers facilitate the multivalent decoration macromolecular structures. a) Highly branched PEI is cross-linked to yield a swellable resin that serves as a robust support in polymer-supported synthesis. b) Complex molecules can be constructed by multistep solid-phase synthesis. c) Finally, disintegration of the resin is effected by cleaving the reversible cross-linking.

Highly branched polyethylene imine (PEI) was selected as the ideal starting material for the preparation of a reversibly cross-linked resin.[16,17] In several biological appli-

[*] Prof. Dr. J. Rademann
Medizinische Chemie
Forschungsinstitut für Molecular Pharmakologie
Robert-Rössle-Strasse 10, 13125 Berlin (Germany)
Fax: (+49) 30-9479-3280
E-mail: rademann@fmp-berlin.de
and
Institut für Chemie
Freie Universität Berlin
Takustrasse 3, 14195 Berlin (Germany)
Dipl.-Chem. M. Barth
Institut für Organische Chemie
Eberhard Karls Universität Tübingen
Auf der Morgenstelle 18, 72076 Tübingen (Germany)
Dipl.-Biochem. R. Fischer, Dr. R. Brock
Interfakultäres Institut für Zellbiologie
Eberhard Karls Universität Tübingen
Auf der Morgenstelle 15, 72076 Tübingen (Germany)

[**] This research was supported by the DFG (Graduiertenkolleg "Chemie in Interphasen, Projekt 6", fellowship for M.B.). We thank Dr. Volker Braig, BASF AG, Ludwigshafen for conducting the gel permeation chromatography.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/anie.200460559

cations including in vivo transfection,[7] PEI served as an efficient hyperbranched structure. Recently, it was demonstrated that various PEIs are well suited for the construction of ultrahigh-loaded polymer supports ("Ultraresins") useful in organic synthesis and for polymer reagents.[8–10]

To obtain a robust solid support constructed of reversibly cross-linked hyperbranched polymers, the cross-linker must be cleaved orthogonally. Dialdehyde **2**, which contains a dialkoxysilane tether, was chosen for this purpose. Reversibly cross-linked resins were obtained by polycondensation of highly branched PEI **3** ($M_n = 10000$; $M_w = 25000$, polydispersity $= 2.5$) with **2** (Scheme 2). For homogeneous cross-linking in high yields, the concentrations of PEI and of the cross-linker **2** were critical. Resin **4** was characterized by ^{1}H MAS NMR spectroscopy, FT-ATR-IR spectroscopy, and elemental analysis (MAS = magic angle spinning, ATR = attenuated total reflection). Disintegration of resin **4** could be effected by cleaving the silicon–oxygen bonds in the cross-linker with acid (50% trifluoroacetic acid in dichloromethane, 2 h) or fluoride (1M tetrabutylammonium fluoride in THF, 2 h). Completion of the disintegration of **4** was proven by gel-permeation chromatography (GPC) yielding a PEI product with an M_n value identical to that of the starting polymer (Figure 1).

Resin **4** was employed in solid-phase synthesis (Scheme 3). Peptide synthesis could be conducted directly on the secondary amines of resin **4** to yield resin **5.** Alternatively, the peptide sequences were assembled on the 4-hydroxymethyl benzoic acid linker (HMBA) (→resin **6**). This base-labile linker can be used such that orthogonal cleavage of the protected peptides from resin **6** does not affect resin integrity. In addition, the linker enables the cleavage of deprotected peptides from the released multivalently decorated scaffolds **8**–**16** as required for analytical purposes or as desirable for specific in vivo applications. As a third option,

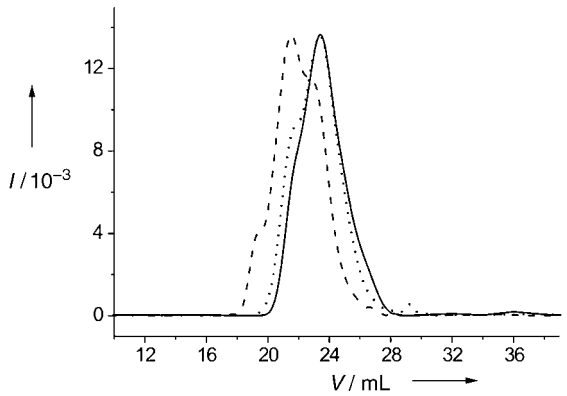
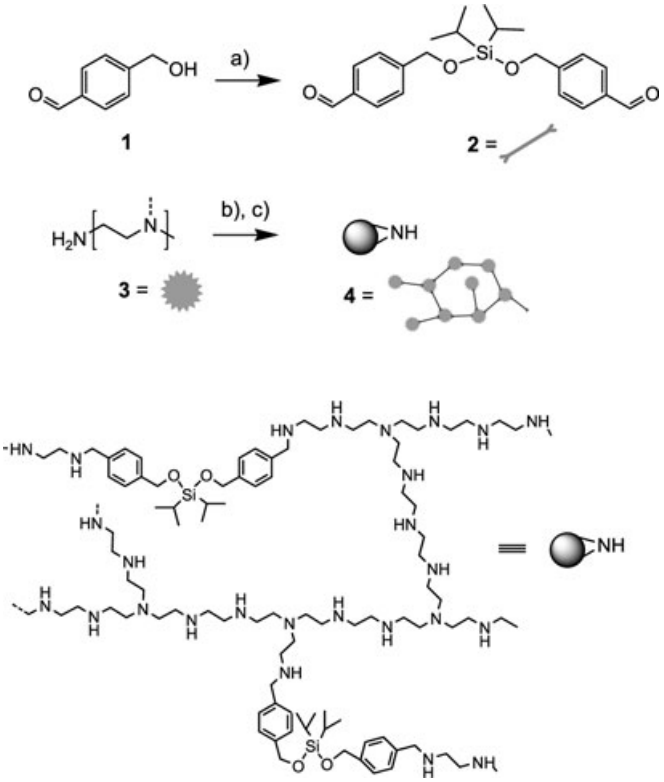
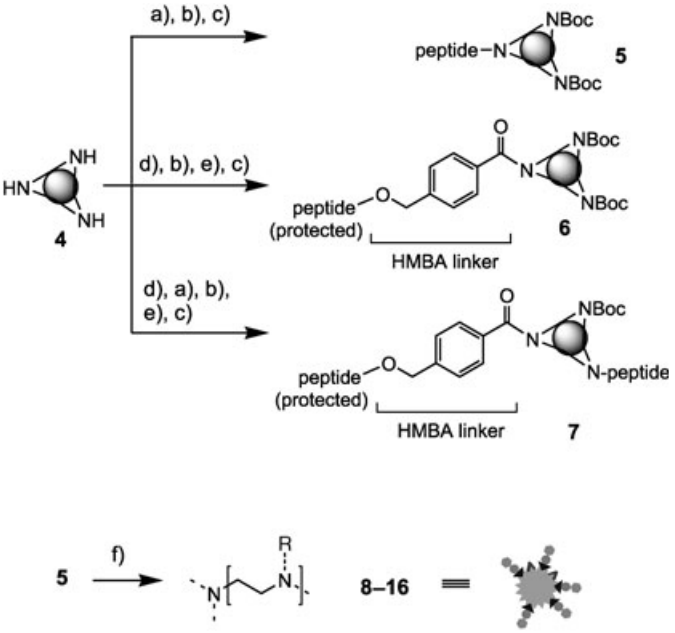


Figure 1. GPC of a) starting PEI **3** ($M_n = 10000$, $M_w = 25000$, continuous line), b) PEI obtained by decomposition of reversibly cross-linked resin **4** (dotted line), and c) peptide-decorated PEI polymer **8** ($M_n = 24000$, $M_w = 75000$, dashed line). V = elution volume, I = intensity of the detector signal.



Scheme 2. Cross-linker **2** was constructed from **1** and employed for construction of polymer support **4**. a) Diisopropyldichlorosilane, pyridine, 1 h, 60°C; b) **2**, THF, 4 h, RT; c) Sodium borohydride THF/MeOH 2:1, 16 h, RT.



Scheme 3. Reversibly cross-linked resin **4** was employed in the synthesis of decorated multivalent scaffolds. a) Fmoc-AA, TBTU, HOBT, DIPEA, DMF; b) di-*tert*-butyl dicarbonate, DIPEA, DMF, 2×2 h, RT; c) peptide synthesis following the Fmoc strategy; d) 4-[(acetyloxy)methyl]benzoic acid, TBTU, HOBT, DIPEA, DMF, 4 h, RT; e) NaOMe 0.1M in MeOH, 0.5 h, RT; f) 95% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% water, 4 h, RT. R = unprotected peptide, Boc = *tert*-butyloxycarbonyl, Fmoc = 9-fluorenylmethoxycarbonyl.

only a small fraction of free amines were coupled with the HMBA linker in order to allow for the analytic monitoring of peptide synthesis by partial cleavage (→resin **7**).

To adjust the peptide content of the resulting multivalent decorated scaffolds, a substoichiometric amount of the linker or the first amino acid was coupled on resin **4**. The remaining secondary amines of the polymer backbone were capped by reaction with Boc-anhydride. Benzotriazolyltetramethyluranium tetrafluoroborate (TBTU) was used to activate the Fmoc-amino acids. The success of the synthesis could be monitored by employing the Kaiser test or by cleavage and deprotection to give the final peptide product.

To demonstrate the feasibility of the concept, a selection of potentially bioactive decorated multivalent scaffolds was prepared (Table 1). The selected peptides include sequences for intracellular targeting (decorated scaffolds **8** and **9**),[11] B and T cell epitopes for vaccination (**10**–**14**), and a peptide described to disrupt molecular interactions involved in the regulation of apoptosis inside the cytoplasm (**15**).[12] This selection represents a broad spectrum of biological and biomedical applications.

Resins **5**, **6** and **7** were decomposed under acidic conditions (95% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% water, 4 h), and the standard side-chain protection groups of amino acids were also removed in the same step. For workup of the decorated polymers, protocols routinely used in peptide synthesis could be employed. Repeated precipitation of the peptide-functionalized polymers in cold diethyl ether furnished pure decorated polymer scaffolds and removed the nonvolatile residues from the trityl and the 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl (Pbf) protecting groups as determined by NMR spectroscopy (see the Supplementary Information).

To evaluate the biological applicability of the peptide-decorated polymer scaffolds obtained by the novel strategy, cellular uptake was investigated by confocal microscopy of living cells. The decorated scaffold **8** bearing the fluorescein-labeled nuclear localization sequence Fluo-PKKKRKV was selected for this purpose. HeLa cells were incubated with the scaffold at a concentration of 400 nM for 2 h at 37 °C. Both a distinct vesicular staining and a homogeneous cytoplasmic and nuclear localization were observed (Figure 2). The vesicular staining colocalized with high-molecular-weight

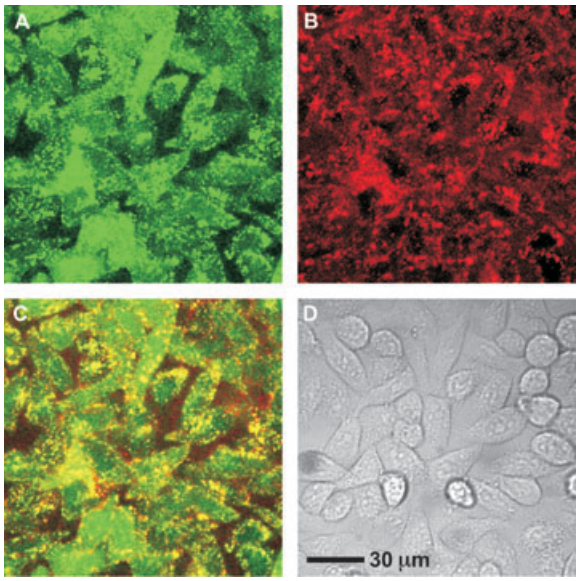


Figure 2. HeLa cells were incubated with serum-free medium containing fluorescein-labeled dendrimer (400 nM) and AlexaFluor 647-dextran (10 μM) for 2 h, washed, and analyzed by multichannel confocal laser scanning microscopy. A) fluorescein fluorescence, B) AlexaFluor 647-dextran fluorescence, C) superposition of both fluorescence channels, D) transmission picture.

(10000 Da) dextrans, which are internalized by fluid-phase endocytosis.[13] The observed subcellular distribution is indicative of uptake by endocytosis and subsequent escape from endocytic compartments. The cytoplasmic and nuclear localization could be inhibited by incubation with bafilomycin A1, a highly potent and selective inhibitor of vacuolar-type H^+-ATPases (see the Supplementary Information).[14] The homogeneity of the uptake on the level of a population of cells and the cellular toxicity of the nanoscale constructs was studied by flow cytometry. A low concentration of **8** (50 nM) was sufficient for homogeneous loading of the cell population.[15] In addition, no toxicity was observed at concentrations up to 1 μM (data are given in the Supplementary Information).

Reversibly cross-linked resins prepared from hyperbranched polymers are powerful new tools for the generation of decorated multivalent scaffolds. By creating a transition from solution to solid-phase methods, the concept combines the advantages of polymer-supported synthesis with the ease of conventional reaction monitoring, including on-bead and off-bead analysis. With these resins, the repertoire of combinatorial methods including parallel synthesis, automation, and split-and-mix operations is applicable to the decoration of macromolecular structures. Furthermore, the multivalent scaffolds we obtained are suitable for cellular applications. The entire cell population was affected homogeneously by scaffold **8**. In future studies the concept will be used for the development and optimization of biologically active polymers targeting the cytoplasm or specific organelles of eukaryotic cells.

Table 1: Synthesis of decorated multivalent scaffods with potentially bioactive peptide sequences.

Decorated scaffold	Sequence	Resin	Loading [mmol g^{-1}]	M_p [g mol^{-1}][a]	Purity (214 nm) [%]	M_n [g mol^{-1}][b]	Copies per PEI molecule
8	Fluo-PKKKRKV	**5**	1.6	–	–	30000	16
9	ANWTGPKKKRKV	**5**	1.6	–	–	32000	16
10	EQPRKFG	**5**	3.0	–	–	35000	40
11	MAYPRISVNNG	**6**	1.8	1234.6	82.5	32000	18
12	QSQPQPPHPTPYWIG	**6**	1.8	1745.8	84.3	41000	18
13	KVSTLPAITLKLGGKG	**6**	1.8	1595.9	82.5	38500	18
14	QSQPQPPHPTPYWIG	**7**	2.2	1745.8	88.3	48000	22
15	AVPIAQKK(Fluo)G	**7**	2.2	1088.7[c]	88.3[c]	38000	22
16	KQAIPVAK(Fluo)G	**7**	2.2	1088.7[c]	86.6[c]	38000	22

[a] Mass of the peptide methyl ester. [b] Mass of the product. [c] Mass and purity of AVPIAQKK(Dde)G-OMe and KQAIPVAK(Dde)G-OMe. Fluo = 5(6)-carboxyfluorescein.

Experimental Section

Synthesis of **4**: Polyethylene imine ($M_n = 10000$, $M_w = 25000$, 1.25 g) was dissolved in THF (6.1 mL), and a solution of **2** (0.495 g, 1.29 mmol) in THF (4.6 mL) was added rapidly. After one minute the stirring bar ceased rotating. After 4 h the polymer was crushed, washed with THF, and suspended in THF/MeOH (2:1, 24 mL). Sodium borohydride (0.097 g, 2.56 mmol) was added, and the suspension was shaken for 16 h at RT. The polymer was washed with THF and MeOH, pressed through a sieve (400 μm pores), washed again with MeOH and CH_2Cl_2, and dried in vacuo to give resin **4** (1.5 g, 89%). Elemental analysis: C 55.6, H 9.7, N 21.0; ^{1}H MAS NMR (400 MHz, MeOD, rotation frequency 4500 Hz): $\delta = 0.9$–1.2 (m, isopropyl, rel. integration 16.7), 2.2–3.0 ppm (m, PEI-CH_2, 100), 3.72 (br.s, *sec*-N-CH_2-aryl, 2.52), 4.56 (br.s, *tert*-N-CH_2-aryl, 0.53) 7.2–7.5 ppm (br.s, aryl-H, 6.51); FT-ATR-IR: $\tilde{\nu} = 815$, 1063, 1090, 1461, 1572, 2815, 2932, 3277 cm^{-1}.

General procedure for the synthesis of peptide-decorated polymer scaffolds: Fmoc-glycine (446 mg, 1.5 mmol) was coupled with TBTU (482 mg, 1.5 mmol) *N*-hydroxybenzotriazole (HOBt; 230 mg, 1.5 mmol) and *N*,*N*-diisopropylethyl amine (DIPEA; 257 μL, 1.5 mmol) in DMF to resin **4** (100 mg). After 4 h the resin was washed with DMF and CH_2Cl_2 and dried in vacuo. The resin was capped using di-*tert*-butyl dicarbonate (1.1 g, 5 mmol) and DIPEA (1.7 mL, 10 mmol) in DMF (2 × 2 h) at RT. The absence of primary and secondary amines was indicated by the Kaiser test and the chloranil test, respectively. The loading of the resin was determined photospectrometrically by cleaving the Fmoc group from the resin.

Peptides were synthesized by the Fmoc strategy using four equivalents of amino acid (based on the loading with first amino acid), TBTU, HOBT, and DIPEA in DMF for 90 min. The Fmoc group was cleaved by treatment with 20% piperidine in DMF (2 × 8 min). Completion of the acylation was determined by the Kaiser test.

Decomposition together with removal of the amino acid side-chain protection of the peptide-decorated resin was performed by using 95% trifluoroacetic acid (TFA), 2.5% H_2O, and 2.5% triisopropylsilane for 4 h at RT. The solution was filtered, and the filter was washed with TFA. Collected solvents were evaporated. After precipitation with cold diethyl ether (4 ×) the soluble peptide-decorated polymer scaffold was lyophilized (*tert*-butanol/water 4:1).

Received: May 5, 2004
Revised: August 23, 2004
Published online: February 2, 2005

Keywords: combinatorial chemistry · drug delivery · peptides · polymers · solid-phase synthesis

[1] a) R. Duncan, *Nat. Rev. Drug Discovery* **2003**, *2*, 347–360; b) U. Boas, P. M. H. Heegaard, *Chem. Soc. Rev.* **2004**, *33*, 43–63; c) M. J. Cloninger, *Curr. Opin. Chem. Biol.* **2002**, *6*, 742–748; d) S.-E. Stiriba, H. Frey, R. Haag, *Angew. Chem.* **2002**, *114*, 1385–1390; *Angew. Chem. Int. Ed.* **2002**, *41*, 1329–1334.

[2] J. P. Tam, *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 5409–5413.

[3] M. Mammen, S.-K. Choi, G. M. Whitesides, *Angew. Chem.* **1998**, *110*, 2908–2953; *Angew. Chem. Int. Ed.* **1998**, *37*, 2755–2794.

[4] L. Bracci, C. Falciani, B. Lelli, L. Lozzi, Y. Runci, A. Pini, M. G. De Montis, A. Tagliamonte, P. Neri, *J. Biol. Chem.* **2003**, *278*, 46590–46595.

[5] a) G. R. Newcome, C. N. Moorefield, F. Vögtle, *Dendrimers and Dendrons*, Wiley-VCH, Weinheim **2001**; b) *Dendrimers and Other Dendritic Polymers* (Eds.: J. M. J. Fréchet, D. A. Tomalia), Wiley, Chichester, **2001**.

[6] In classic organic synthesis on dissolved polymers, various polyols were employed: a) M. Mutter, E. Bayer, *Angew. Chem.* **1974**, *86*, 101–102; *Angew. Chem. Int. Ed. Engl.* **1974**, *13*, 88–89; b) E. Bayer, A. Geckeler, *Justus Liebigs Ann. Chem.* **1974**, 1671–1674; c) R. Haag, A. Sunder, A. Hebel, S. Roller, *J. Comb. Chem.* **2002**, *4*, 112–119.

[7] K. Aoki, S. Furuhata, K. Hatanaka, M. Maeda, J.-S. Remy, J.-P. Behr, M. Terada, T. Yoshida, *Gene Ther.* **2001**, *8*, 508–514.

[8] M. Barth, J. Rademann, *J. Comb. Chem.* **2004**, *6*, 340–349.

[9] J. Rademann, M. Barth, *Angew. Chem.* **2002**, *114*, 3087–3090; *Angew. Chem. Int. Ed.* **2002**, *41*, 2975–2978.

[10] M. Barth, S. T. Ali Shah, J. Rademann, *Tetrahedron* **2004**, *60*, 8703–8709.

[11] D. A. Jans, C.-Y. Xiao, M. H. C. Lam, *Bioessays* **2000**, *22*, 532–544.

[12] C. R. Arnt, M. V. Chiorean, M. P. Heldebrant, G. J. Gores, S. H. Kaufmann, *J. Biol. Chem.* **2002**, *277*, 44236–44243.

[13] C. Plank, B. Oberhauser, K. Mechtler, C. Koch, E. Wagner, *J. Biol. Chem.* **1994**, *269*, 12918–12924.

[14] a) E. J. Bowman, A. Siebers, K. Altendorf, *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 7972–7976; b) T. Merdan, K. Kunath, D. Fischer, J. Kopecek, T. Kissel, *Pharm. Res.* **2002**, *19*, 140–147.

[15] For comparison, in a recent publication 50 μM of a fluorophore-labeled trimeric peptide was required for detectable incorporation in HeLa cells: J. Fernandez-Carneado, M. J. Kogan, S. Castel, E. Giralt, *Angew. Chem.* **2004**, *116*, 1847–1850; *Angew. Chem. Int. Ed.* **2004**, *43*, 1811–1814.

[16] The fragmentation of a macroscopic polymer to give a modified, multivalent structure as described in this article must be clearly distinguished from the numerous works on enzymatically or chemically cleavable dendrimers. Cleavable dendrimers are fragmented into monomeric building blocks, and thereby the multivalent character is lost. Two recent examples on triggered dendrimer cleavage are: a) F. M. H. de Groot, C. Albrecht, R. Koekkoek, P. H. Beusker, H. W. Scheeren, *Angew. Chem.* **2003**, *115*, 4628–4632; *Angew. Chem. Int. Ed.* **2003**, *42*, 4490–4494; b) J. R. Amir, N. Pessah, M. Shamis, D. Shabat, *Angew. Chem.* **2003**, *115*, 4632–4636; *Angew. Chem. Int. Ed.* **2003**, *42*, 4494–4499.

[17] Polyethylene imine was selected as the most inexpensive commercially available starting polymer. In principle, the protocol should be applicable for other branched polyamines including amine dendrimers.

