Hyperbranched PEI with Various Oligosaccharide Architectures: Synthesis, Characterization, ATP Complexation, and Cellular Uptake Properties

Dietmar Appelhans,* † Hartmut Komber, † Mohiuddin Abdul Quadir, ‡ Sven Richter, † Simona Schwarz, † Jereon van der Vlist, § Achim Aigner, † Martin Müller, † Katja Loos, § Jürgen Seidel, † Karl-Friedrich Arndt, ‡ Rainer Haag, * † and Brigitte Voit†

Leibniz Institute of Polymer Research Dresden, Hohe Strasse 6, D-01069 Dresden, Germany, Institut für Chemie und Biochemie, Freie Universität Berlin, Takustr. 3, D-14195 Berlin, Germany, Faculty of Mathematics and Natural Sciences, Laboratory of Polymer Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands, Department of Pharmacology and Toxicology, Philipps-University Marburg, School of Medicine, Karl-v.-Frisch-Strasse 1, D-35033 Marburg, Germany, Institute of Physical Chemistry, TU Bergakademie Freiberg, Leipziger Str. 29, D-09596 Freiberg, Germany, and Physical Chemistry of Polymers, Department of Chemistry, TU Dresden, Mommenstr. 4, D-01069 Dresden, Germany

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We present a rapid synthetic method for the development of hyperbranched PEIs decorated with different oligosaccharide architectures as carrier systems (CS) for drugs and bioactive molecules for in vitro and in vivo experiments. Reductive amination of hyperbranched PEI with readily available oligosaccharides results in sugar functionalized PEI cores with oligosaccharide shells of different densities. These core–shell architectures were characterized by NMR spectroscopy, elemental analysis, SLS, DLS, IR, and polyelectrolyte titration experiments. ATP complexation of these polycations was examined by isothermal titration calorimetry to evaluate the binding energy and ATP/CS complexation ratios under physiological conditions. In vitro experiments showed an enhanced cellular uptake of ATP/CS complexes compared to those of the free ATP molecules. The results arise to initiate further noncovalent complexation studies of pharmacologically relevant molecules that may lead to the development of therapeutics based on this polymeric delivery platform.

Introduction

The development of nanosized carrier systems is a rapidly growing research field due to their high potential for application in medicine, biology, and pharmaceutics. 1–5 For that purpose, the generation of materials with specifically designed structural, molecular, and biological properties, which can be used as tools for a specific diagnostic and therapeutic purposes, is of major importance. Besides their successful use as polymer conjugates as anticancer therapeutics in nanomedicines, 2,6 both types of dendritic polymers, dendrimers and hyperbranched polymers with dimensions up to 10 nm, are among the leading carrier molecules for drugs, fluorescent particles, and nucleic acids like RNA or DNA. 1,3–7 With the introduction of DNA in gene therapy as well as with the identification of small RNAs as the key mediators of RNA interference (RNAi), the development of delivery platforms for the systemic application of nucleic acids has gained particular relevance for the establishment of novel therapeutic strategies. 8 The success of dendritic macromolecules is based on the combination of high end-group functionalities and a globular molecular structure. In particular, the properties of dendritic polymers in these applications, for example, with regard to important parameters like solubility, receptor-mediated interaction, or biocompatibility, depend strongly on the nature of the surface groups.

Since the mid-1990s, various dendritic carrier systems have been established and continuously further developed from initial dendritic box via unimolecular micelles to dendritic multishell architectures as universal nanocarriers. 9–17 Recent developments of these carrier systems mainly focused on the enhancement of their biocompatibility. This can be achieved through introduction of water-soluble and biocompatible surface groups, neutral or with anionic charges, in the outer shell of dendritic core macromolecules like poly(propylene imine) (PPI), poly(amide amine) (PAMAM), poly(ethylene imine) (PEI), and other dendritic polyamine scaffolds. 10,11,16–26 For that purpose, poly(ethylene glycol) chains have been preferably used and extensively explored so far. 27–30 However, there is a need to study alternative surface modifications in such core–shell architectures. The coupling of mono- and oligosaccharide units to the outer shell has been successfully introduced in the outer shell as well, and these amphiphilic macromolecules have been explored as carrier systems for drugs 23,31,32 and DNA macromolecules. 33,34 In these systems, in addition to the increase in biocompatibility, the molecular recognition potential of mono- and oligosaccharide units can be used for enhanced and selective cell uptake. 35–40 Recently, PPI dendrimers with a densely organized oligosaccharide shell proved to be promising as antiprion agent. 41

The goal of this work was to establish defined, novel oligosaccharide architectures on dendritic polymer surfaces for...
their use as nanosized carrier systems for drugs, bioactive molecules, or (noble) metal nanoparticles. In other words, multifunctional carrier systems will be realized at which the nature of the oligosaccharide shell, ranging from very dense to very open oligosaccharide shell, exhibits different structural, physicochemical, and biological features and significantly contrary noncovalently interaction properties. For that purpose, hyperbranched PEI was selected as dendritic core macromolecule stimulated by the fact that hyperbranched PEI derivatives have been successfully applied previously as multifunctional nanocarrier for drugs and dyes, including first in vivo imaging studies with a PEI/dye system in tumor tissues. To introduce mono- and oligosaccharide units on PEI surface, one- and multistep approaches in organic solution have been employed in most so far. These oligosaccharide-modified PEI showed already enhanced encapsulation and stabilization of (noble) metal nanoparticles and were used successfully as nonviral transfection agent for DNA macromolecules. However, with both synthetic approaches an only a limited degree of functionalization was realized, not exceeding 35% of the PEI surface, thereby limiting the potential of these modifications. Thus, to the best of our knowledge there is still a need to establish novel synthetic strategies which allow the generation of advanced core—shell architectures for hyperbranched PEI-based polymers.

In this paper, we describe the synthesis of hyperbranched PEI cores with different degrees of oligosaccharide shell functionalizations. For the generation of various oligosaccharide substitution degrees, we establish an easy one-pot approach on the PEI surface (Scheme 1), and we extensively characterize the products, allowing us to distinguish between three different groups with regard to their structure. Thus, this is the first step toward the development of novel dendritic core—shell architectures as nanocarriers which allows the formation of H-bonds and electrostatic interactions as well as hydrophobic—hydrophilic interactions. The oligonucleotide functionalized PEI mediate the complexation/encapsulation of their “payload” followed by enhanced cellular uptake. To analyze this in more detail, isothermal titration calorimetry experiments were carried out using ATP as a model compound, elucidating the complexation behavior between ATP molecules and different PEI derivatives. This allowed us to establish structure—activity relationships between our oligosaccharide-functionalized PEI, as compared to naked PEI, and ATP as a representative molecule. Finally, to test our oligosaccharide-modified PEIs more rigorously, we demonstrate its capability to mediate cellular uptake of ATP in tissue culture by showing enhanced delivery over free ATP molecules.

**Experimental Section**

**General and Materials.** Poly(ethylene imines) [PEI as general abbreviation; Lupasol G20 with Mw 1300 g/mol (PEI-I), Lupasol G100 with Mw 5000 g/mol (PEI-II), and Lupasol WF with Mw 25 000 g/mol (PEI-III)] were received from BASF SE (Ludwigshafen, Germany). Glucose monohydrate (Glc), lactose monohydrate (Lac), maltose monohydrate (Mal), maltotriose (Mal-III), adenosine 5′-triphosphate (ATP), disodium salt hydrate), sodium borate, and borane pyridine complex (BH3·Py complex, 8 M solution in THF) were used as internal calibration (ATP, disodium salt hydrate), sodium borate, and borane pyridine complex (BH3·Py complex, 8 M solution in THF) were used as internal calibration standards.

**Characterization.** The NMR measurements were carried out on a Bruker DRX 500 NMR spectrometer operating at 500.13 MHz for 1H and at 125.75 MHz for 13C using D2O as solvent. Sodium 3-(trimethylsilyl)-3,3,2,2-tetradeteropropionate was added for internal calibration standard. Dynamic light scattering (DLS) experiments were performed at a scattering angle of θ = 90° using a white light goniometer system (model ALV/CGS-8FS/N025). Further details of the equipment, condition for experiments, and calculation of hydrodynamic radii Rg are described elsewhere. Static light scattering (SLS) experiments were performed at a modified FICA 50 (SLS Systemtechnik G. Baur, Denzlingen, Germany) with a He—Ne laser (wavelength of λo = 632.8 nm) in a range of the scattering angle of 15–145°. The refractive index increment (dn/dc) of the polymers was estimated by using a differential refractometer DR 1, (SLS Systemtechnik G. Baur, Denzlingen, Germany) with a He—Ne laser (λo = 632.8 nm) as the light source. Further details of the equipment, condition for experiments, and calculation of weight-average molar mass Mn and radius of gyration Rg are described elsewhere. Charge densities (q) of unmodified and modified PPI dendrimers were determined by polyelectrolyte titration in a particle charge detector (PCD-03, Miiut, Germany) combined with 702 SM Titrisio (Metrohm, Switzerland). Solutions of low molecular weight sodium polystyrene sulfo nate (PSS-NA) or poly(diallyl dimethylammonium chloride) (PDADMAC) were used as titrants for cationic and anionic systems, respectively. q (meq/g) was calculated according to the following formula: q = CtitrantVtitrant/Vm, where Ctitrant is the concentration of titrant (meq/L), V is the volume of titrated solution, Vm is the equivalent titrant volume, and m is the content of polyelectrolyte in titrated solution (g/L).

**Synthesis of Oligosaccharide-Modified PEI.** The following general experimental procedure was applied to obtain the various oligosaccharide-modified PEI: PEI-I, PEI-II, or PEI-III (0.2 g) was taken up in sodium borate solution (0.1 mol in distilled water) followed by the addition of the corresponding mono- or oligosaccharide (Glc, Lac, Mal, Mal-III, or Mal-VII) and BH3·Py complex. The reaction solution was stirred at 50 °C for 7 days. Then, the crude product was purified by dialysis toward double-distilled water for 3 days. The different oligosaccharide-modified PEIs were obtained via freeze-drying. The yields were between 50 and 98%. For the synthesis of the structures A–C, further details of the molar ratio PEI/(oligo)-saccharide are given in the Tables 1, 2, and Table 1, Supporting Information. In this context, the molar amount of (oligo)saccharide and the reduction agent have to be used in an equimolar ratio.

**1H NMR Spectra.** 1H NMR data of the oligosaccharide-modified PEI are given, which present the typical structures A–C of the modified PEI—PEI-III, shown in Scheme 1. To understand the assignment of the 1H signals, the (oligo)-saccharide units were divided into reductive, middle, and terminal units. This assignment is also used for the description of the 13C signals in the Supporting Information. The numbering of the different units always starts from the anomeric carbon atom of the unit. Thus, Glc is described as 1–6 for the reductive unit; Lac is described as 1′–6 for the reductive unit and 1′–5 for the terminal unit; Mal is described as 1–6 for the reductive unit and 1–6 for the terminal unit; Mal-III is described as 1–6 for the reductive unit and 1–6 for the middle unit and 1–6 for the terminal unit; Mal-VII is described as 1–6 for the reductive unit, 1–6 for the middle unit, and 1–6 for the terminal unit. The purity of the oligosaccharide-modified PEIs with respect to unreacted oligosaccharide was proven by 1H NMR. Generally, the 1H NMR spectra of all products show signals in three characteristic regions containing in each case several more or less broadened signals: 2.2–3.3 (PEI protons...
and H-1 or H-1\(^I\) of the reductive unit), 3.3—4.4 ppm (oligosaccharide protons, except the anomeric ones), and 4.4—4.6 ppm [anomeric proton of Lac (H-1\(^{II}\)) or 4.9—5.6 ppm [anomeric protons (H-1\(^{II}\) - H-1\(^{VII}\)) of oligosaccharides Mal—Mal-VII]. Despite the appearance of the spectra (Figure 2 and Figures 1—4 of the Supporting Information) depending on the used oligosaccharide and the obtained structures A—C (Scheme I), significant information concerning the residual primary and secondary amino groups cannot be obtained.

**Isothermal Titration Calorimetry.** Microcal VP-ITC microcalorimeter (MicroCal, LLC, Northampton, MA) was used to carry out the calorimetric experiments. Degassed Millipore water was used to prepare the reagent solutions (using 10 mM HEPES buffer to dissolve ATP and various carrier systems, i.e., PEI-II, PEI-III, and their oligosaccharide-shelled derivatives) and the pH was adjusted to pH 7 and measured prior to individual experiments. A series of test experiments has been done for each of the carrier systems to fix up experimental parameters, including the concentration of the carrier systems and ATP. Thus, for all the systems, the sample cell of the microcalorimeter (1.4 mL) was filled with 1 \(\mu\)M solution of respective carrier molecules, and after baseline equilibration, 0.8 mM of ATP solution was injected in 34 × 8 \(\mu\)L aliquots using the default injection rate. It was found that adding ATP solution from the syringe to the carrier system solution in the cell gives a better representation of the saturation process of the polymers by ATP. The resulting titration curves were corrected for heat of dilution of ATP alone in buffer and analyzed using the Origin 7.0 ITC software supplied by MicroCal, LLC. Experimental parameters for titration experiments were number of injections, 34; cell temperature, 30 °C; stirring speed, 290 rpm; cell volume, 1.43 mL; injection volume,
Table 1. Educt Ratios and Yields for Isolated Hyperbranched PEIs with Different (Oligo-)Saccharide (OS) Surface Groups and Degree of Functionalization (DF), Total Degree of Functionalization (TDF), and Degree of T, L, and D Units of Modified PEI—PEI-III Obtained from Elemental Analysis for Hyperbranched PEI Derivatives with structure A

| Substrate | Educt Ratio PEI/OS | Yield (%) | DF<sub>a</sub>b 2×T + L (%) | TDF<sub>c</sub>d 2×T + L + D (%) | T Unit<sup>b</sup> (%) | L Unit<sup>b</sup> (%) | D Unit<sup>b</sup> (%) |
|-----------|------------------|-----------|-----------------------------|-----------------------------|----------------|-|-|---|
| PEI-I     |                  |           |                             |                             |              |              |              |     |
| 1-Glc<sup>a</sup> | 1:5            | 49.0      | 94                          | 75                          | 36.4          | 36.2          | 27.4          |     |
| 1-Mal<sup>a</sup> | 1:10           | 63.0      | 95                          | 76                          | 36.2          | 43.8          | 20.0          |     |
| 1-Mal-III<sup>a</sup> | 1:5          | 81.3      | 83                          | 66                          | 36.2          | 43.8          | 20.0          |     |
| PEI-II    |                  |           |                             |                             |              |              |              |     |
| 2-Glc<sup>a</sup> | 1:5            | 72.7      | 95                          | 72                          | 36.2          | 43.8          | 20.0          |     |
| 2-Mal<sup>a</sup> | 1:10           | 69.0      | 91                          | 70                          | 36.2          | 43.8          | 20.0          |     |
| 2-Mal-III<sup>a</sup> | 1:5          | 72.1      | 77                          | 58                          | 36.2          | 43.8          | 20.0          |     |
| 2-Lac<sup>a</sup> | 1:5            | 67.0      | 80                          | 61                          | 36.2          | 43.8          | 20.0          |     |
| PEI-III   |                  |           |                             |                             |              |              |              |     |
| 3-Glc<sup>a</sup> | 1:4.5          | 98.0      | 92                          | 71                          | 36.2          | 43.8          | 20.0          |     |
| 3-Mal<sup>a</sup> | 1:25           | 85.9      | 86                          | 66                          | 36.2          | 43.8          | 20.0          |     |
| 3-Mal-III<sup>a</sup> | 1:4.25       | 55.2      | 78                          | 60                          | 36.2          | 43.8          | 20.0          |     |
| 3-Lac<sup>a</sup> | 1:4.25         | 74.0      | ND<sup>b</sup>              | ND<sup>b</sup>              | 36.2          | 43.8          | 20.0          |     |

* Calculation based on elemental analysis; further details are given in Supporting Information. *<sup>a</sup> 2×T means that two oligosaccharides can be coupled on one T unit. L means that one oligosaccharide can be coupled on the L unit. *<sup>b</sup> All branching units are considered for the calculation of functionalization.

Based on converted PEI—PEI-II. Based on converted PEI-III. Not determined.

Table 2. Influence of the Educt Ratio PEI-II: Oligosaccharide (OS) on the Degree of Functionalization (DF) of Converted T and L Units and Total Degree of Functionalization (TDF) of Modified PEI, Obtained from Elemental Analysis, the Determination of the Degree of Branching (DB) by Quantitative 13C NMR, and Degree of T, L, and D Units by Elemental Analysis and Quantitative 13C NMR

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ratio PEI/OS</th>
<th>DF&lt;sub&gt;b,c&lt;/sub&gt; 2×T + L (%)</th>
<th>TDF&lt;sub&gt;b,c&lt;/sub&gt; 2×T + L + D (%)</th>
<th>DB&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>T Unit&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>L Unit&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>D Unit&lt;sup&gt;b&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEI-II</td>
<td></td>
<td>67.4</td>
<td>32.6</td>
<td>36.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>36.7</td>
<td>36.7</td>
<td>36.7</td>
</tr>
<tr>
<td>2-Mal (A)</td>
<td>1:10</td>
<td>91</td>
<td>70</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>2-Mal (A)</td>
<td>1:5</td>
<td>88</td>
<td>67</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>2-Mal (A)</td>
<td>1:2</td>
<td>86</td>
<td>67</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>4-Mal (B)</td>
<td>1:0.5</td>
<td>36</td>
<td>28</td>
<td>65</td>
<td>65</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>6-Mal (C)</td>
<td>1:1</td>
<td>16</td>
<td>14</td>
<td>53</td>
<td>53</td>
<td>53</td>
<td>53</td>
</tr>
<tr>
<td>2-Mal-III (A)</td>
<td>1:5</td>
<td>77</td>
<td>58</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>4-Mal-III (B)</td>
<td>1:0.5</td>
<td>32</td>
<td>25</td>
<td>69</td>
<td>69</td>
<td>69</td>
<td>69</td>
</tr>
<tr>
<td>6-Mal-III (C)</td>
<td>1:0.2</td>
<td>16</td>
<td>14</td>
<td>53</td>
<td>53</td>
<td>53</td>
<td>53</td>
</tr>
<tr>
<td>6-Mal-VII (C)</td>
<td>1:0.5</td>
<td>22</td>
<td>17</td>
<td>59</td>
<td>59</td>
<td>59</td>
<td>59</td>
</tr>
</tbody>
</table>

* Character in brackets presents the structure type for PEI derivative. *<sup>a</sup> Calculation based on elemental analysis; further details are given in Supporting Information. *<sup>b</sup> 2×T means that two oligosaccharides can be coupled on one T unit. L means that one oligosaccharide can be coupled on the L unit. *<sup>c</sup> All branching units are considered for the calculation of functionalization. *<sup>d</sup> Using Fréchet equation: DB = (T + D)/(T + L + D); example of calculation is presented in Table 3. *<sup>e</sup> Determination by quantitative 13C NMR.

Table 3. Structural Parameters of Hyperbranched PEIs Used in this Study

<table>
<thead>
<tr>
<th>Substrate</th>
<th>M&lt;sub&gt;a&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; g/mol</th>
<th>M&lt;sub&gt;&lt;w&gt;0&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; g/mol</th>
<th>DP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>T Unit&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>L Unit&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>D Unit&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>DB&lt;sup&gt;d&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEI-I</td>
<td>800</td>
<td>1300</td>
<td>18.6</td>
<td>36.4 (6.8)</td>
<td>36.2 (6.7)</td>
<td>27.4 (5.1)</td>
<td>63.8</td>
</tr>
<tr>
<td>PEI-II</td>
<td>3600</td>
<td>5000</td>
<td>18.6</td>
<td>36.4 (6.8)</td>
<td>36.2 (6.7)</td>
<td>27.4 (5.1)</td>
<td>63.8</td>
</tr>
<tr>
<td>PEI-III</td>
<td>9600</td>
<td>25000</td>
<td>228.8</td>
<td>29.3 (65.3)</td>
<td>30.7 (25.8)</td>
<td>30.7 (25.8)</td>
<td>67.4</td>
</tr>
</tbody>
</table>

*<sup>a</sup> Given by the supplier (BASF, Germany). *<sup>b</sup> Degree of polymerization estimated according DP = M<sub>a</sub>/M(H<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH) = M<sub>a</sub>/43 g mol<sup>-1</sup>. *<sup>c</sup> The percentages of terminal (T), linear (L), and dendritic (D) units were calculated from 13C NMR signal intensities (Figure 1): T = l<sub>T</sub>; L = 0.5 (l<sub>L</sub> + l<sub>D</sub>); D = 1/3 (l<sub>T</sub> + l<sub>L</sub> + l<sub>D</sub>), with l<sub>T</sub> = 82.5 ppm, l<sub>L</sub> = 65 ppm, l<sub>D</sub> = 65 ppm. The absolute numbers of T, L, and D units are given in brackets and were estimated from DP and the percentages of the according units. *<sup>d</sup> Degree of branching calculated according DB = (T + D)/(T + L + D).
according to \([\alpha] = 100 \, \alpha/(L, C)\) using the units given above. The following specific rotation angle values were received: \([\alpha] = 118.8 \pm 2.6^\circ\) for \(\text{Mal}\), \([\alpha] = 68.8 \pm 0.3^\circ\) for 2-Mal, and \([\alpha] = 62.3 \pm 0.3^\circ\) for 3-Mal. Furthermore, as an approximation we assume, that the chemical coupling of maltose on PEI-II and PEI-III for the synthesis of structure 2-Mal (structure A) and 3-Mal (structure A), respectively, had no further influence on the optical rotation properties of the maltose units compared to the parent Maltose unit.

**Results and Discussion**

**Hyperbranched PEI with Oligosaccharide Architectures.** Poly(ethylene imine) (PEI) offers both terminal (T) primary amino functions (which can react twice to dendritic (D) units) as well as linear (L) secondary amines for postmodification reactions. Previously described synthetic approaches to realize PEI with chemically coupled oligosaccharides focused on the introduction of, at least, one (oligo-)saccharide per T unit. The application of the reductive amination allowed to introduce the complete range of various oligosaccharide architectures from only a low number of converted T units to nearly completely converted T and L units. The tailoring of the substitution degree was achieved using the strong reductive agent borane-pyridine complex in borate buffer at 50 °C for 7 d, converting PEI-I with \(M_w \) 1300 g/mol, PEI-II with \(M_w \) 5000 g/mol, or PEI-III with \(M_w \) 25000 g/mol with minor amount or excess of oligosaccharides (Glc = glucose, Mal =
maltose, Lac = lactose, Mal-III = maltotriose, and Mal-VII = maltoheptaose), as shown in Scheme 1. With this synthetic method, the following oligosaccharide architectures in the outer sphere of the PEI (Scheme 1) were realized.

(1) Structure A with preferred D units over the PEI macromolecule. This means that nearly all T units and also most L units are converted into D units at which the T unit had to react twice with a (oligo-) saccharide unit leading to a dense oligosaccharide shell; (2) Structure B with preferred L units as peripheral groups at which all T units are converted into L units leading to a loose shell; (3) Structure C with a mixture of T and L units as peripheral groups leading to isolated oligosaccharide units in the periphery. The results of the reductive amination of PEI-I–PEI-III are summarized in the Tables 1 and 2 and Table 1, SI. The hyperbranched PEI with oligosaccharide architectures A, B, and C (Scheme 1) were characterized by NMR, elemental analysis, IR, DLS, and SLS.

PEI-I–PEI-III were investigated by 1H and 13C NMR in D2O (Figure 1 and 2A and Table 3). Similar chemical shifts and signal patterns of the T, L, and D units for PEI-I–PEI-III core were received in D2O, as described previously for measurements in CDCl3.53 As one example, the 13C NMR spectrum of PEI-II is presented in Figure 1. One can impressively see in Figure 1 that the T (a and b), L (c and d), and D (f and g) units are separated into two 13C NMR signals where the adjacent structure unit belongs to L units or D units. Furthermore, the L (e) and D (h) units possess also an adjacent T unit. The content of T, L, and D units and the degree of branching (DB) of PEI-I–PEI-III are also presented in Table 3.

The main focus of the NMR characterization of the PEI derivatives was the verification of the oligosaccharide architectures A–C (Scheme 1), presented by the degree of functionalization (DF) and the degree of T, L, and D units (Tables 1 and 2 and Table 1, SI). DF is characterized by the fact that two (oligo-) saccharide units can be coupled to one T unit and one (oligo-) saccharide can be additionally attached to one L unit [DF is presented as 2×T + L (%) in all tables]. The following results were obtained: (1) The synthesis of structure A from PEI-I, PEI-II, and PEI-III is based on the use of excess oligosaccharides. The corresponding DF is in the range between 75 and 95%. Furthermore, the high degree of DF also leads to a high degree of D units (75–95%; Table 1 and Table 1, SI). The structure A was fully supported by NMR investigations (Tables 2 and 3, SI). A typical 1H NMR spectrum of the PEI derivative, 2-Mal, in D2O is presented in Figure 2B in comparison with that of PEI-II (Figure 2A). As further examples, the 13C NMR spectra of 2-Glc and 2-Mal are shown in Figure 3. Generally, the spectra are characterized by broadened signals for the coupled oligosaccharide units and, especially, for the PEI core compared to those received from the unmodified PEI-II (Figures 1 and 2A). One can conclude from 13C NMR spectra in Figure 3 that the D unit (D-CH2-CH2-D) at 53.3 ppm for 2-Glc and at 53.9 ppm for 2-Mal is the dominant structure unit of the PEI core (Table 2, SI). In sharp contrast, the L unit (D-CH2-CH2-L) of the PEI core is certainly the minor component in 2-Glc and 2-Mal. Also, a new structure unit 11, the CH2 group converted carbonyl function of the reductive unit of (oligo-) saccharide, is observed at 59.4 ppm for 2-Glc (Figure 3; top) and at 60.3 ppm for 2-Mal (Figure 3; bottom). The structure unit 11 is always observed after the reductive amination of PEI-I–PEI-III no matter if excess or under stoichiometric amounts of oligosaccharides (Table 3, SI) are used. Finally, the results of 13C NMR confirm the high DF and the content of low L units and high D units, as determined also by elemental analysis (Tables 1 and 2 and Table 1, SI).

(2) Structure B was tailored by varying the molar ratio PEI/oligosaccharide from 1:0.5 to 1:2 (Table 2 and Table 1, SI). The corresponding DF is in the range between 32 and 48%. This is accompanied by a higher degree of L units (51–70%) and a low degree of D units (Table 2 and Table 1, SI). Full NMR analysis is summarized in Tables 2 and 3, SI. The 13C NMR spectrum of 4-Mal is shown in Figure 4 (top) as one typical example for structure B. In contrast to structure A of 2-Mal (Figure 3, bottom), the 13C NMR spectrum of 4-Mal reveals the desired presence of different D units (D-CH2-CH2-D and D-CH2-CH2-L) and L units (L-CH2-CH2-L and D-CH2-
Figure 4. $^{13}$C NMR spectra of 4-Mal (top; structure B) and 6-Mal (bottom; structure C) showing the influence of the substrate ratio PEI-II/Mal 1:0.5 and 1:0.2, respectively.

CH$_2$-L), which were also determined in the parent PEI cores (Figure 1). Also, all T units (Figure 4, top) disappeared after the reductive amination of PEI-II with Mal as the minor component. Further, the structure unit $^1$ in structure B is shifted to high field, overlapped by L and D units in the case of 4-Mal (Figure 4, top).

(3) Structure C was achieved by the use of a molar ratio PEI/oligosaccharide 1:0.2 to 1:0.5 (Table 2 and Table 1, SI). The corresponding DF is in the range between 16 and 50%. Thus, modified PEIs of structure C possess (a) a higher degree of L units (48–67%) than D units and (b) a low content of unreacted T units (2–17%) (Table 2 and Table 1, SI). Full proof of structure C as by NMR investigations can be seen in Tables 2 and 3, SI. The $^{13}$C NMR spectrum of 6-Mal is presented in Figure 4 (bottom) as one typical example for structure C. Beside the expected signal patterns for PEI-II converted with Mal as the minor component, T units are also determinable for structure C (Figure 4, bottom). The degree of branching (DB)$^{54}$ was also calculated by the Fréchet equation (Table 3) after analyzing the T, L, and D units by quantitative $^{13}$C NMR investigations (Table 2). One can conclude that the DB (Table 2) of 81% for 6-Mal, 83% for 6-Mal-III, and 91% for 6-Mal-VII increases compared to the parent PEI-II with a DB of 67.4%.

(4) The tailoring of the oligosaccharide architectures was carefully studied by the conversion of PEI-II with Mal and Mal-III (Table 2). Surprisingly, the desired structures A–C could be realized in both cases. Furthermore, structures A–C on PEI-III surface with higher $M_w$ (up to 25000 g/mol) could also be established (Table 1, SI) when maltotriose (Mal-III) as oligosaccharide was used. Furthermore, we could show that also PEIs with $M_w$ 50000, 250000, and 750000 g/mol could be modified by this method with various oligosaccharides reaching also the structures A–C. The received data and results are not shown here due to high similarity of the received data and results.

(5) The introduction of the oligosaccharides with increasing size (glucose < maltose < maltotriose) was possible by reductive amination aiming for structure A. But, generally the number of oligosaccharide units decreases in the outer sphere of PEI-I – PEI-III with increasing the size of the oligosaccharide (Table 1). This means that the accessibility for larger oligosaccharide units on the PEI surface is reduced at high substitution degree. Further, the reactivity of the disaccharide lactose is higher than that of the disaccharide lactose which results in a higher DF and higher degree of D units for structure A in 2-Mal (PEI/OS with 1:5 in Table 2) compared to 2-Lac (PEI/OS with 1:5 in Table 1).

(6) The most surprising result was the successful introduction of the largest oligosaccharide maltoheptaose (Mal-VII) on the PEI-II surface (Table 2 as 6-Mal-VII). For the ratio PEI/OS 1:0.5, approximate 70% of the T units of PEI-II were converted preferably once with Mal-VII to obtain 6-Mal-III with structure C (Figure 10, SI).

(7) The $^{13}$C NMR investigations of the chemically coupled oligosaccharide units on various PEI macromolecules revealed no further specific information for the characterization of the structures A, B, and C. The reductive amination reaction of the reducing unit of the parent (oligo-)saccharide results in a noncyclic unit partly restricted in mobility by its neighborhood to the PEI scaffold and, therefore, with signals of different broadening, as illustrated by the $^{13}$C NMR spectrum of 2-Glc (Figure 3, top). Additional broadening and splitting should be caused by different stereochemistry and by the substitution pattern of the neighboring amine nitrogen, for example, L or D unit with one or two bonded oligosaccharides. If oligosaccharides were reacted with PEI, this chemically coupled reducing unit connects PEI with retained (oligo-)saccharide moiety. The $^{13}$C chemical shifts of the m and t units are only less affected compared to the parent oligosaccharide and the signals are quite narrow. While confirming the successful coupling of the reductive unit of oligosaccharides and preservation of the residual saccharide rings of the former (oligo-)saccharide units, a detailed signal assignment to substructures failed due to broad and overlapping signals. Nevertheless, for the PEI derivatives with glucose, maltose and maltotriose a general signal assignment independent of structures A, B, and C was obtained by combination of 1D and 2D techniques (Table 3, SI).

For their use as carrier system for biologically active molecules, further studies on the PEI with various oligosaccharide substitution degree were undertaken to determine their hydrodynamic radii ($R_h$), radii of gyration ($R_g$), and weight-average molecular weight ($M_w$) by dynamic and static light scattering experiments. Furthermore, first studies on charge density by polyelectrolyte titration experiments were performed to learn about their properties in water. The results of the light scattering experiments for structure A (2-Glc, 2-Lac, 2-Mal, 2-Mal-III, 3-Mal, 3-Mal-III) and structure B (4-Mal-III) are summarized in Table 4. Also, PEI-III was investigated for comparing with 3-Mal and 3-Mal-III. Within the series of PEI derivatives for structure A based on PEI-II (2-Glc, 2-Lac, 2-Mal, 2-Mal-III) $R_h$ values with unimodal distribution increases with growing size of the oligosaccharide units (from 2 nm for 2-Glc to 4 nm for 2-Lac). Only 2-Mal-III (structure A) shows a tendency to aggregate outlined by a bimodal distribution. Also, within this series for modified PEI-II, 4-Mal-III with structure B reveals a lower $R_h$ value (3 nm) compared to
The following PEI derivatives as carrier system (CS) were selected for this study: 4-Mal (B) and 6-Mal-VII (C) based on PEI-II and 3-Mal-III (A) and 7-Mal-III (C) based on PEI-III. Also, the complexation properties of the PEI derivatives were compared with that of the parent PEI macromolecules, PEI-II and PEI-III. The complexation ratios ATP/CS were determined by isothermal titration calorimetry (ITC) investigations encouraged by the fact that ITC has been proven its efficiency and high level of accuracy for studying thermodynamic and kinetic properties of macromolecular interactions.55,56

Binding isotherms of ATP to PEI-II and derivatives thereof (4-Mal and 6-Mal-VII), as obtained by integration of ITC signals, are illustrated in Figure 6. The isotherm for the ATP interaction with PEI-II shows an exothermic interaction effect. Also, the use of structure B (4-Mal) and structure C (6-Mal-VII) does not change the binding energetic properties to considerable extent. The enthalpy change versus ATP addition followed a fairly similar pattern for all these carrier systems based on PEI-II. Furthermore, one can conclude that the exoergic isomerizations can be mainly attributed to electrostatic interaction between the cationic PEI derivatives and the anionic ATP molecules.

In sharp contrast, the binding isotherms of ATP to PEI-III and its derivatives 3-Mal-III and 7-Mal-III deviated strongly from those of the PEI-II series (Figure 7). Again, a strong exothermic binding region is observed while ATP interacted with the pure PEI-III. However, the shape of the binding curve was completely different from its PEI-II counterpart (Figure 6). The maximum in the binding curves imply that two distinct energetic processes exist during the complexation/encapsulation of the ATP molecules in the presence of PEI-III, indicating a possible two binding site pattern for the interaction between PEI-III and ATP molecules. A simpler exothermic binding process of the ATP molecules in the cases of structure A (3-Mal-III) and structure C (7-Mal-III) is observed. These ITC experiments clearly suggest that the introduction of larger maltotriose units on the PEI surface plays a critical role on the nature of interaction between the high molar mass PEI-III based carrier systems and the ATP molecule.

The binding isotherms of the ATP solution to the various PEI derivatives form the fundamental basis for the calculation of the ATP/CS ratios (using the inflection method). The different ATP/CS ratios are presented in Table 5. From these data, the following conclusions can be extracted: Within the series of PEI-II, a similar ATP/CS ratio (~25) is observed. This means that there is no influence of the degree of attached maltose in structure B and of attached maltotetraose in structure C on ATP/CS ratios compared with the parent PEI-II. Furthermore, the structural parameters (M∞, polydispersity or degree of branching units) of parent and modified PEI-II play obviously no critical role for the interaction between the carrier system and the ATP molecules. In contrast, totally different ATP/CS ratios for the series of PEI-III core were found, from 40 ATP molecules for structure A (3-Mal-III) up to the highest value of about 100 ATP molecules for the parent PEI-III. In this case, one can clearly conclude that there is a marked influence of molar mass and degree of functionalization of the PEI surface with maltotriose unit on the interaction between the carrier systems and ATP molecules.

In summary, the ITC experiments done in this study bring out surprisingly contrary facts regarding the parameters which govern the interaction between the carrier system and the ATP molecules. This allows, however, to fine-tune the ATP/CS ratios and, thus, the interaction by the core macromolecule and the
degree of oligosaccharide substitution. Under defined pH conditions (HEPES buffer at pH 7) we can assume that the complexation behavior of oligosaccharide-shelled PEI macro-molecules toward bioactive molecules is mainly tailored by the predominant effect of electrostatic interaction. In other words, the accessibility of binding sites (primary, secondary, and tertiary amino groups) for ATP interaction is hampered by the maltotriose functionalized PEI-II core in contrast to PEI-II with different but not very dense maltose and maltoheptaose decoration.

Finally, the favorable results regarding the complexation/binding of ATP molecules by parent PEI and its derivatives, which are based on various oligosaccharide architectures, encouraged us to assess whether this complexation also translates into enhanced cellular uptake. Thus, cellular internalization of free nucleotides was compared to nucleotides complexed with 4-Mal (B), and intracellular levels were analyzed by adding traces of radioactively labeled nucleotide. Because ATP is involved in several biochemical reactions which may lead to the rapid loss of the radioactive label, dCTP rather than ATP was used as tracer molecule. However, the fact that cold ATP represented the by far largest amount of nucleotides in the complex and dCTP displays fairly similar physicochemical properties ensured that the complexes used for assessing cellular uptake were comparable to complexes employed in the previous experiments. As shown in Figure 8, the complexation resulted in enhanced time-dependent cellular uptake of ATP as compared to the naked nucleotide (Figure 8, left). In fact, upon 4-Mal complexation a more than 3-fold increased ATP uptake in SKOV-3 cells was observed (Figure 8, right). This is particularly remarkable because transporters have been described which may lead to some “background” internalization as observed in these experiments, thus, rather underestimating the potency of 4-Mal-mediated ATP delivery. This was particularly true for HepG2 cells and may account for the somewhat lower, ∼2-fold, enhancement of ATP uptake in this cell line (Figure 18, SI). Taken together, the significant increase of cellular ATP internalization upon 4-Mal complexation reveals the great potential of oligosaccharide-shelled hyperbranched PEI as a noncovalent carrier for pharmacologically relevant, bioactive molecules.

Conclusions

In summary, reductive amination is a highly efficient working tool to introduce various oligosaccharide architectures in the
outer sphere of hyperbranched PEI with low and high molecular weight. Thus, the establishment of a densely organized oligosaccharide shell with preferred D units (structure A), a PEI surface with preferred L units in the outer shell (structure B) or a PEI surface with a combination of nonconverted T units and of L units (structure C) is possible. The most impressive result was that the oligosaccharides, maltose, lactose, and maltotriose can be used to realize all the structures A–C just by varying the molar ratio PEI to oligosaccharide. The structure of the PEI derivatives were confirmed by detailed NMR investigations as well as elemental analysis. Furthermore, DLS studies revealed that the hydrodynamic radii of the various oligosaccharide-modified PEI increased up to few nm (from 1 to 4 nm on average) compared to those of the parent PEI depending on the nature of chemically coupled oligosaccharide unit and the realized oligosaccharide architecture. Surprisingly, in most cases, the PEI derivatives showed no aggregation in aqueous solution despite the presence of a large number of H-bonding, active oligosaccharide units in PEI derivatives. By the introduction of various oligosaccharide architectures in the outer shell, it was also possible to vary the charge density, which allows the consideration of such multifunctional PEI derivatives as carrier systems for diagnostic and therapeutic application.

The calorimetric results support this concept. Generally, the carrier systems are suited to bind various amounts of ATP molecules depending on the size of the PEI core and its surface modification by various oligosaccharides. Furthermore, the binding of the ATP molecules by the carrier systems is an exothermic process accompanied by distinct types of binding phenomena. One binding type can be assigned to the electrostatic interaction between the negatively charged ATP molecules and the cationic PEI core of the carrier system. Furthermore, the electrostatic interaction can be enhanced by other noncovalent interactions (e.g., hydrophobic interaction, π–π interaction, or OH–π interaction) or a combination of noncovalent interactions. One more challenge for the future is to specify and to differentiate the binding phenomena between the interacting species using well-suited fits and binding models of the calorimetric data.

Finally, our cellular uptake study demonstrated the potential of oligosaccharide-shelled hyperbranched PEI for the delivery of drugs or other bioactive molecules with faster and enhanced cellular uptake. The more detailed analysis of the properties or these carrier systems upon noncovalent complexation of phamacologically relevant molecules may eventually lead to the development of therapeutics based on this polymeric delivery platform.

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Supporting Information Available. NMR data for structures A–C. 1H and 13C spectra and ATR-IR for the structures A–C, additional graph for the heat flow for the binding of ATP molecules with carrier systems possessing PEI–III core, and an additional graph for cellular uptake of the carrier system 4-Mal toward HepG2 hepatocytes. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes