Molar-Mass Analysis of Dendrigraft Poly(l-lysine) (DGL) Polyelectrolytes by SEC-MALLS: The “Cornerstone” Refractive Index Increment

Barbara Maret, Agnès Crépet, Clément Faye, Laurent Garrelly,* Catherine Ladavière*

Dendrigraft poly(l-lysine) (DGL) polyelectrolytes, obtained by iterative polycondensation of N-trifluoroacetyl-l-lysine-N-carboxyanhydride, constitute very promising candidates in many biomedical applications. In order to get a better understanding of their structure–property relationships in these applications, their absolute average molecular weights have to be accurately measured. Size-exclusion chromatography coupled to a multi-angle laser-light-scattering detector (SEC-MALLS) is known to be the most appropriate analytical tool. These measurements require the determination of the refractive index increment, $dn/dc$, of these highly branched polycationic macromolecules in aqueous solution. This optical property has to be measured in the same aqueous conditions as SEC-MALLS eluents. Consequently, data are determined and discussed as a function of different aqueous SEC-MALLS eluents, as well as different counter-ions of the many ammonium groups of DGL (generation 3, DGL-3, used as a model herein). The resulting number-average molecular weights, $M_n$, are found to be very dissimilar when the measured $dn/dc$ values are directly considered. In contrast, very close $M_n$ values are obtained (average $M_n = 18 700$, standard error of 1110 g mol$^{-1}$ with a low coefficient of variation for such data (ca. 6% for six analyses), when the $dn/dc$ are corrected by the exact lysine amount (measured by the total Kjeldahl nitrogen method).

1. Introduction

The architecture of synthetic polymer chains can be linear, branched, cross-linked, or even dendritic according to the polymerization chemistry used. Among the dendritic architectures, the dendrigraft polymers present a less controlled structure than dendrimers but similar properties at a much lower synthesis cost. Moreover, their synthesis can be efficiently conducted on a multi-gram scale as shown for the dendrigraft poly(l-lysine)
(DGL) polyelectrolytes, which are obtained by iterative polycondensation of N-carboxyanhydride (NCA) groups in a weakly acidic aqueous medium. Their synthesis involves a multiple-generation process by adding N-trifluoroacetyl-L-lysine-N-carboxyanhydride onto the previous generation DGL-(n-1) (Scheme 1). The first-generation DGL-1, used as macrorinitiator, was prepared by spontaneous NCA polycondensation. The subsequent alkaline removal of trifluoroacetyl (Tfa) protecting groups affords DGL-(n) generation, directly used in the synthesis of the next DGL-(n+1) generation.

The biocompatibility and non-immunogenicity demonstrated for these flexible polycationic structures hold great promise for their use in many biomedical applications such as i) vectors for drug delivery applications, for brain-targeting gene delivery, ii) MRI contrast agents, and iii) supports to facilitate the membrane penetration.

The physicochemical characterization of dendrimers and particularly DGLs is crucial for these applications as their macromolecular dimension and behavior in aqueous solution determine their biomedical efficiency. Their average molecular weights (connected to their average degrees of polymerization, DP) are also important for establishing a structure–property relationship. For example, Hofman et al. have evaluated the transfection abilities of four DGL generations with special emphasis about a systematic description of how generation affects the transfection efficacy. These DGL data are also essential to calculate the repeat unit or end-group number in order to realize their chemical modification (conjugation with drugs, proteins, fluorophores).

Size-exclusion chromatography (SEC) is a powerful analytical tool currently used for determining the average molecular weights ($\bar{M}_w$, $\bar{M}_n$) and dispersities ($D$) of polymers. The principle of SEC is based on the limited accessibility for the polymer chains of pore volume within the SEC column packing particles. Hence, these polymer chains are separated according to their hydrodynamic volume (with the larger size chains eluted before the smaller ones). Based on this separation principle, the determination of molecular weights is carried out either from this relationship between residence time in the column and hydrodynamic volume (previously defined with a series of narrow standard polymers to obtain relative average molecular weights), or by coupling molecular weight sensitive detectors (to get absolute average molecular weights).

However, the first case is an approximation since the conformation in solution, or the hydrodynamic volume, of a polymer chain is noticeably dependent on its chemical structure, which can be dissimilar to that of a standard. This can lead to considerable differences as for the dendrimers, which present underestimated average molecular weights via the SEC findings. Indeed, their highly branched dendritic globule resides longer in the pores than the statistical coil of a linear polymer with the same molecular weight in the same mobile phase and the same column.

Consequently, with the development of molecular-weight-sensitive detectors such as the multi-angle laser light scattering (MALLS) detector, the SEC-MALLS combination is today preferred for determining the absolute average molecular weights of polymers, and notably for dendrimers. The resulting molecular weights depend only on MALLS and differential refractive index (dRI) detector findings, not on the elution volume. In a MALLS detector, the scattered light of a laser beam passing through a cell is measured at angles different from zero, and the intensity, the excess Rayleigh ratio $R(\theta)$ of the scattered light at an angle $\theta$, is related to the weight average of molecular weight, $\bar{M}_w$, of the dissolved macromolecules as shown in Equation 1:

$$K^* C/R(\theta) = 1/\bar{M}_w P(\theta) + 2A_2 C$$

where $C$ is the concentration of the polymer, $A_2$ is the second virial coefficient, and $P(\theta)$ describes the scattered
light angular dependence. In this Equation 1, \( K^* \) is an optical constant containing Avogadro’s number \( N_a \), the wavelength of the laser beam \( \lambda_0 \), the refractive index of the solvent \( n_0 \), and the refractive index increment \( dn/dc \) (Equation 2):

\[
K^* = 4\pi n_0^2 (dn/dc)^2 / \lambda_0^2 N_a
\]

(2)

The refractive index increment, \( dn/dc \), is consequently a crucial parameter to get absolute average molecular weights and dispersities of polymers. Its exactness is essential since \( \bar{M}_n \) is proportional to the inverse of this value squared. It is intrinsically dependent on the solvent, analysis temperature, laser wavelength, but also the presence of additives in solution. In the case of poly- electrolytes, and here of DGLs, a usual difficulty for their characterization is the presence of additives in solution. In the case of poly- electrolytes, and here of DGLs, a usual difficulty for their analysis in aqueous media is to know if the counter-ions, associated to their numerous amine charged groups, have to be involved in the \( dn/dc \) determination. These counter-ions may result from the synthesis process, but also from chromatographic eluents composed of salts required for the SEC separation in aqueous media. Indeed, this separation has to be only based on the polymer hydrodynamic volume, and implies neither electrostatic adsorption nor electrostatic repulsion between sample and stationary phase. It is well known that these harmful secondary interactions can be eliminated by increasing the ionic strength of the eluent.

Cottet’s team was the first to carry out such a physicochemical characterization of DGL synthesized by following the chemical process described before. To determine the DGL molecular weights (by SEC coupled to a differential viscosimeter, a MALLS and a dRI detector), the authors used different \( dn/dc \) values. In their first work,\(^{[23]}\) a value equal to 0.185 mL g\(^{-1}\) was employed in agreement with a typical \( dn/dc \) value obtained for proteins whereas 0.134 mL g\(^{-1}\) (in a Na\(_2\)HPO\(_4\) solution at 50 g L\(^{-1}\) pH 4.5) was used for DGL-3 in their second study.\(^{[3]}\) Because of this variation in \( dn/dc \) value, which is the “cornerstone” of an absolute molar mass determination, a specific study about this parameter as a function of DGL counter-ions and SEC-MALLS eluents seemed necessary to us.

In this context, the main aims of this paper were to perform this DGL refractive index increment study, and to determine resulting DGL absolute average molecular weights by SEC-MALLS. Several eluents with anionic counter-ions owning different molecular masses (carbonate, trifluoroacetic acid, chloride, and phosphate ions) were tested. To conduct this investigation, we chose a generation 3 of DGL as model (DGL-3, named DGL-3/CO\(_3\), DGL-3/TFA, DGL-3/Cl, DGL-3/PO\(_4\), respectively).

2. Experimental Section

2.1. Synthesis of Dendrigraft Poly(L-lysine) with a Generation 3 (DGL-3)

DGL-3 was synthesized in water at 4 °C, pH 6.5 by an iterative process. Briefly, the first step of this synthesis consisted in the polymerization of the N-trifluoroacetyl-L-lysine-N-carboxyanhydride (Isochem, Vert Le Petit France, France) without any initiator in aqueous carbonate buffer 0.1 M pH 6.5 leading to an insoluble product. Trifluoroacetyl-L-lysine and soluble low molar mass oligomers were eliminated by centrifugation and by washing twice the precipitates with water. The N-trifluoroacetyl protecting group was removed (by hydrolyzing Tfa-acetamide lysine bond) by adding NH\(_3\) in a methanol/water mixture at 40 °C for 15 h leading to the first generation of soluble DGL (DGL-1). The unprotected polymer was evaporated under reduced pressure to remove ammonia and further freeze-dried. By using this DGL-1 as the macroinitiator, the first step was reproduced to give the next generation. The Tfa protecting group was then removed to obtain the second generation of soluble DGL, DGL-2. Finally, DGL-3 was obtained using the same method (Scheme 2).

2.2. Determination of the Intergeneration Purity of Dendrigraft Poly(L-lysine) DGL-3 by HPLC

HPLC analyses were used to determine the intergeneration purity. They were carried out on a Waters Alliance system with an E2695 separation module and a 2998 photodiode array detector, and with a C18 silica-based BEH300 RP-HPLC column (50 × 4.6 mm, 3.5 μm, Waters). The mobile phase was a gradient of water and acetonitrile during 20 min at a flow rate of 0.8 mL min\(^{-1}\) (see Table 1). Trifluoroacetic acid (TFA) at 0.1 wt% was added to water and acetonitrile as ion pairing reagent to improve the retention of DGL on the solid reverse phase.\(^{[24]}\) The hydrophobic anions form ion pairs with polycationic DGL through electrostatic interactions, thus passing their own hydrophobicity onto the DGL species.\(^{[24]}\) DGL samples were prepared at 5 mg mL\(^{-1}\) in water, and 10 μL were injected.

The HPLC chromatogram displayed the high purity of DGL-3/TFA, since only traces of DGL-2 are present in the DGL-3/TFA sample (<3 wt%, see chromatogram in the Supporting Information).

2.3. Preparation of Dendrigraft Poly(L-lysine) DGL-3 Bearing Different Counter-ions

At the end of the DGL-3 synthesis, the counter-ions associated to dendrigraft ammonium groups are TFA anions since the deprotection of DGL-3 bearing Tfa as protecting group by ammonia adding leads to the hydrolysis and cleavage of all the acetamide bonds, and the consequent formation of TFA ions (\( F\_3\_C\_–\_C\_═\_O\_^-\)). Then, DGL-3/TFA was eluted on an automated preparative SEC to provide DGL-3/CO\(_3\). To this end, 10 mL of 100 g L\(^{-1}\) DGL-3/TFA was injected on an AKTA purifier 100 system (General Electric) equipped with an UV-900 detector (elution monitored at \( λ = 210 \) nm), using a sephadex G25 250 10/300GL column. Running buffer was NH\(_4\)HCO\(_3\) 0.1 M with a flow rate of 12 mL min\(^{-1}\).
The chromatogram shows two peaks, the first one corresponding to DGL-3/CO$_3$ which was collected, and the second one, the salt peak. From this DGL-3/CO$_3$ solution, DGL-3/Cl was obtained by decreasing pH at 6.5 with an aqueous solution of HCl 3 M, and directly freeze-dried. Finally, DGL-3/PO$_4$ was prepared by mixing DGL-3/CO$_3$ with an aqueous solution of H$_3$PO$_4$ 1 M (pH 6.5), and directly freeze-dried.

2.4. Characterization of Dendrigraft Poly(l-lysine) DGL-3 by NMR Spectroscopy Analyses

For all spectroscopic analyses, a Bruker AM 300 Avance apparatus at 300 MHz was used, and 25 mg of DGL-3/TFA, DGL-3/PO$_4$, or DGL-3/CO$_3$ were dissolved in 0.6 mL D$_2$O. $^1$H NMR spectroscopic analyses (D$_2$O, 300 MHz, 298 K) of DGL-3/TFA: $\delta = 1.13$–$2.11$ ppm (6H, m, H$\beta$-H$\delta$-H$\gamma$ of Lys); DGL-3/CO$_3$: 2.70–3.56 ppm (2H, m, H$\epsilon$ of Lys); DGL-3/PO$_4$: 3.85–4.48 ppm (1H, m, H$\alpha$ of Lys). $^{19}$F NMR spectroscopic analyses (D$_2$O, 300 MHz, 298 K) of DGL-3/TFA: $\delta = -75.31$ ppm, and DGL-3/CO$_3$: $\delta = -75.38$ ppm (residual quantity of TFA $< 1$ wt% by the PULCON method developed by Bruker).

2.5. Determination of Average Molecular Weights and Dispersities of Dendrigraft Poly(l-lysine) DGL-3

The number–average molecular weight ($M_n$) and the molar-mass dispersity ($\tilde{D}$) were measured by using a SEC column (superose 12, GE Healthcare, i.d. = 7.8 mm, and L = 300 mm) coupled with a differential refractometer (Optilab T-rEX, Wyatt Technology) with a laser at $\lambda = 658$ nm, thermostated at 25 $^\circ$C, and a MALLS instrument (Dawn EOS, Wyatt Technology) equipped with a laser operating at $\lambda = 690$ nm. The flow rate was 0.5 mL min$^{-1}$. Degassed and filtered (on 0.1 μm membrane) phosphate (NaH$_2$PO$_4$/Na$_2$HPO$_4$, 0.5 M, pH 4.5), carbonate (NH$_4$HCO$_3$, 0.1 M, pH 8.0) buffers, and 0.3 M NaCl solution (pH 6.0) were used as eluents. These eluents were also used as solvent of samples, and the resulting solutions were filtered on a 0.45 μm membrane before injection. Finally, 100 μL of each sample at 2 mg mL$^{-1}$ was injected. The data have been exploited thanks to the ASTRA 6.0.6 software (Wyatt Technology).

2.6. Determination of Refractive Index Increments of Dendrigraft Poly(l-lysine) DGL-3

Refractive index increments ($d_n/dc$) were determined in batch by using a differential refractometer (Optilab T-rEX, Wyatt Technology) with a laser at $\lambda = 658$ nm, and thermostated at 25 $^\circ$C. The solvents were filtered on 0.1 μm membrane. For each $d_n/dc$ determination, six concentrations (e.g., 0.1, 0.2, 0.4, 0.6, 0.8, 1 mg mL$^{-1}$) of DGL-3 were prepared, and injected (ca. 1 mL) without filtration to prevent any DGL loss on the membranes. The data have been exploited thanks to the ASTRA 6.0.6 software (Wyatt Technology).

2.7. Assay of Nitrogen by Total Kjeldahl Nitrogen (TKN) in Dendrigraft Poly(l-lysine) DGL-3 Sample

TKN analysis measures the organic form of nitrogen in DGL-3 sample. The analysis starts by an acid digestion of the sample, converting organic nitrogen to ammonium sulfate salt. This requires boiling the sample in concentrated sulfuric acid, potassium sulfate, and with a selenium catalyst. The role of potassium sulfate in this step is to increase the boiling point of the medium (from 337 to 373 $^\circ$C). Then, once the acidic digestion is performed, the sample pH must be raised to 9.5 with the addition of concentrated sodium hydroxide. Then, the resulting mixture is distilled (at this pH, ammonia is transferred by distillation into the acidic trapping/absorbing solution). Finally, the ammonia amount, and

<table>
<thead>
<tr>
<th>Flow [mL min$^{-1}$]</th>
<th>Time [min]</th>
<th>%A (water/TFA 0.1 wt%)</th>
<th>%B (acetonitrile/TFA wt 0.1%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.8</td>
<td>1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.8</td>
<td>5</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>0.8</td>
<td>20</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>
thus the nitrogen amount, is determined by titration with an HCl solution at 0.02 M.

### 3. Results and Discussion

In SEC, the separation should be exclusively governed by size exclusion, and non-exclusion effects can cause severe errors. More particularly in aqueous SEC of polymers, because of the presence of polar (often anionic) groups in the SEC column stationary phases, the mobile phase must be carefully chosen to repress “polymer/SEC gel” interactions. A classic way to screen the electrostatic interactions is the addition of an electrolyte in the chromatographic eluent. In order to understand the influence of this added electrolyte on the refractive index increment value, and consequently on the average molecular weights of DGL polyelectrolytes, different chromatographic eluents (NaCl solution, carbonate or phosphate buffer) were used to analyze DGL-3. Furthermore, in parallel with this investigation, the role of DGL counter-ions in mass analyses was examined by associating non-covalently different counter-ions to DGL-3 at the end of the synthesis process (scheme 2). A large and well-distributed mass range for the anionic counter-ions was chosen (chloride, Cl\(^-\), 35 g mol\(^{-1}\), carbonate, HCO\(_3\)^-, 61 g mol\(^{-1}\), phosphate, H\(_2\)PO\(_4\)^-, 97 g mol\(^{-1}\), and trifluoroacetic acid, F\(_3\)C(=O)O\(^-\), 113 g mol\(^{-1}\), and DGL-3/X with X = Cl, CO\(_3\)^-, PO\(_4\)^-, TFA, respectively). The chemical modification process used to exchange the counter-ions associated to DGL-3 is firstly described hereafter.

#### 3.1. Chemical Modification Process of Counter-ions Associated to DGL-3

At the end of the DGL synthesis, the counter-ions associated to DGL-3 ammonium groups were trifluoroacetic acids (DGL-3/TFA) due to the ammonia deprotection step of Tfa protecting groups. Hence, the successive preparations of DGL-3/CO3, DGL-3/PO4, and DGL-3/Cl derived of this species (Figure 1), and for the sake of characterization comparison, a same batch of DGL-3/TFA was used for all non-covalent chemical modifications. Briefly, the second DGL-3 type, the carbonate DGL-3 (DGL-3/CO\(_3\)) was obtained by eluting DGL-3/TFA samples in a SEC preparative column with a 0.1 M ammonium carbonate (NH\(_4\)HCO\(_3\)) buffer at pH 8.4 as eluent, at a flow rate of 12 mL min\(^{-1}\). After that, residual ammoniac was removed by evaporation. It was checked thanks to a spectrophotometric method of NH\(_4^+\) determination (the Nessler method) that there was no any NH\(_4^+\) ion left in DGL-3/CO\(_3\) and DGL-3/TFA samples. From this resulting DGL-3/CO\(_3\) solution, DGL-3/Cl was obtained by decreasing pH at 6.5 with an aqueous solution of HCl. Finally, the addition of a H\(_3\)PO\(_4\) solution at pH 6.5 to DGL-3/CO\(_3\) led to DGL-3/PO\(_4\) species (Figure 1).

#### 3.2. Experimental Determination of Refractive Index Increments of DGL-3 Samples

The determination of the optical property of DGL-3, the refractive index increment value, requires a differential refractometer with a cell composed of two compartments (one for solvent and the other for solution) separated by a transparent glass wall. Light passing through the cell deflects a tiny amount converted into a displacement proportional to the difference of refractive index between the solution and the solvent (\(\Delta n = n - n_0\)). In practice, aliquots of known concentration of DGL-3-counter-ions were injected into the refractometer at a given temperature. Each concentration corresponds to one refractive index measurement plateau, as shown in an example presented in Figure 2A. After defining baselines and setting peak regions, \(dn/dc\) value is obtained by plotting the refractive index of the
DGL-3/counter-ions solution versus DGL-3/counter-ions concentration (example in Figure 2B). For the example given in Figure 2, the value of $dn/dc$ is equal to 0.1905 ± 0.0012 mL g$^{-1}$. This procedure was repeated for all the different DGL-3/counter-ion samples (Table 2).

### 3.3. Influence of the Eluent Nature on the Refractive Index Increment Value of DGL-3

Table 2 displays the impact of the eluent nature on the refractive index increment of DGL-3 measured at a same temperature (25 °C), by using six concentrations of a same DGL-3 batch but bearing different counter-ions. All the simple linear regressions of refractive indexes as a function of growing DGL-3/Cl concentrations display satisfactory coefficients of determination ($R^2 > 0.9940$). These coefficients show that a simple linear regression fits very well all the data.

These values of refractive index increments are highly disparate (from 0.1149 to 0.1905 mL g$^{-1}$, difference = 0.0756 mL g$^{-1}$) according to DGL counter-ions and SEC eluents used. As a general trend, a higher $dn/dc$ value is obtained when the counter-anion mass is lower (i.e., Cl < CO$_3$ < PO$_4$ < TFA). Concerning DGL-3/TFA, the pH effect, which would seem to exist at pH 8.0, could be explained by a more condensed conformation of polyelectrolyte due to a smaller protonated α-amino group amount (reducing the interchain repulsions).

These different refractive index increments of DGL-3/counter-ions values were used to determine the average molecular weights and dispersities of DGL-3 by SEC-MALLS. For that purpose, each DGL-3/counter-ion was dissolved at 2 mg mL$^{-1}$ in each considered eluent and 100 μL of the resulting sample was injected in the SEC-MALLS system at a flow rate of 0.5 mL min$^{-1}$. All the obtained chromatograms are displayed in Figure 3. The first remark is that the species were eluted two by two (DGL-3 bearing the same counter ion as the used eluent, plus DGL-3/TFA in the same eluent) according to the eluent nature. The second remark highlighted by Figure 3 concerns the chromatographic elution order.

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**Table 2.** Determination of refractive index increments ($dn/dc$) of DGL-3/counter-ions as a function of chromatographic eluents (solvents here), and corresponding values of determination coefficients of simple linear regressions.

<table>
<thead>
<tr>
<th>DGL-3/counter-ions</th>
<th>SEC eluent</th>
<th>Refractive index increment$^a)$ $dn/dc$ [mL g$^{-1}$]</th>
<th>Determination coefficient$^b)$ $R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGL-3/PO$_4$</td>
<td>Phosphate buffer pH 4.5</td>
<td>0.1307 ± 0.0025</td>
<td>0.9981</td>
</tr>
<tr>
<td>DGL-3/CO$_3$</td>
<td>Carbonate buffer pH 8.0</td>
<td>0.1858 ± 0.0064</td>
<td>0.9941</td>
</tr>
<tr>
<td>DGL-3/Cl</td>
<td>NaCl solution pH 6.0</td>
<td>0.1905 ± 0.0012</td>
<td>0.9998</td>
</tr>
<tr>
<td>DGL-3/TFA</td>
<td>Phosphate buffer pH 4.5</td>
<td>0.1163 ± 0.0014</td>
<td>0.9993</td>
</tr>
<tr>
<td>DGL-3/TFA</td>
<td>NaCl solution pH 6.0</td>
<td>0.1149 ± 0.0010</td>
<td>0.9996</td>
</tr>
<tr>
<td>DGL-3/TFA</td>
<td>Carbonate buffer pH 8.0</td>
<td>0.1309 ± 0.0005</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

$^a)$The accuracy provided herein comes from the difference between the simple linear regression and the experimental values; $^b)$Simple linear regression of the differential refractive index as a function of DGL-3 concentrations.
which follows the decrease of counter-ion volume (phosphate > carbonate > chloride). This could be in agreement with the SEC separation principle based on the hydrodynamic volume as above-mentioned. According to the chromatograms, the decreasing order of hydrodynamic volume of DGL-3 with its counter-ions would be DGL-3/PO₄ > DGL-3/CO₃ > DGL-3/Cl.

Table 3 shows high discrepancies between the \( M_n \) values by using the \( dn/dc \) measured in different eluents and with different counter-ions associated to DGL-3 (Table 2). The values vary from ca. 21 500 to 38 700 g mol\(^{-1}\) (difference = 17 200 g mol\(^{-1}\)). This dissimilarity is highly unexpected since all the samples come from the same initial batch of DGL-3. Nevertheless, given the Equation 2 indicating that \( M_n \) is inversely proportional to the square of \( dn/dc \) according to the counter-ions (between 0.1149 and 0.1905 mL g\(^{-1}\), \( dn/dc \) ratio: 1.66), the divergence of \( M_n \) values is not so surprising.

Since a correlation between the \( dn/dc \) values of DGL-3 and the masses of associated counter-ions appears in Table 2 (i.e., a correlation between \( dn/dc \) decrease and counter-ion mass increase), the DGL-3 concentrations used in the \( dn/dc \) measurement should probably be considered without the mass contribution of counter-ions. Hence, the next step of this investigation was to take into account only the DGL-3 mass (and not the counter-ion contribution) in the concentration calculations. However, the direct determination of the amount of counter-ions (carbonate, trifluoroacetic acid, chloride, or phosphate ions) in samples is difficult by classical analytical and chemical assays. Consequently, the lysine mass

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**Table 3.** Number-average molecular weights \((M_n)\) and dispersities \((D)\) of DGL-3/counter-ions by using refractive index increments \((dn/dc)\) presented in Table 2.

<table>
<thead>
<tr>
<th>DGL-3/counter-ions</th>
<th>SEC eluent</th>
<th>Refractive index increment, (dn/dc) [mL g(^{-1})]</th>
<th>Number-average molecular weight(^{a)} (M_n) [g mol(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGL-3/PO₄</td>
<td>Phosphate buffer pH 4.5</td>
<td>0.1307</td>
<td>36 125 ± 710 (1.122)</td>
</tr>
<tr>
<td>DGL-3/CO₃</td>
<td>Carbonate buffer pH 8.0</td>
<td>0.1858</td>
<td>21 460 ± 260 (1.138)</td>
</tr>
<tr>
<td>DGL-3/Cl</td>
<td>NaCl solution pH 6.0</td>
<td>0.1905</td>
<td>22 260 ± 429 (1.222)</td>
</tr>
<tr>
<td>DGL-3/TFA</td>
<td>Phosphate buffer pH 4.5</td>
<td>0.1160</td>
<td>38 720 ± 825 (1.163)</td>
</tr>
<tr>
<td>DGL-3/TFA</td>
<td>NaCl solution pH 6.0</td>
<td>0.1149</td>
<td>36 560 ± 920 (1.202)</td>
</tr>
<tr>
<td>DGL-3/TFA</td>
<td>Carbonate buffer pH 8.0</td>
<td>0.1309</td>
<td>32 455 ± 860 (1.154)</td>
</tr>
</tbody>
</table>

\(^{a)\)The uncertainty given for the number average molecular weight was obtained from the ASTRA software with the Zimm fitting model; the numbers in brackets represent the polydispersity.
Table 4. Lysine amounts determined by TKN in each sample and subsequent correction of refractive index increments of DGL-3/counter-ions.

<table>
<thead>
<tr>
<th>DGL-3/counter-ions</th>
<th>Lysine amount determined by TKN [wt%]</th>
<th>SEC eluent</th>
<th>Corrected refractive index increment(^a) (dn/dc) [mL g(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGL-3/PO(_4)</td>
<td>60.75</td>
<td>Phosphate buffer pH 4.5</td>
<td>0.2151</td>
</tr>
<tr>
<td>DGL-3/CO(_3)</td>
<td>77.00</td>
<td>Carbonate buffer pH 8.0</td>
<td>0.2413</td>
</tr>
<tr>
<td>DGL-3/Cl</td>
<td>70.70</td>
<td>NaCl solution pH 6.0</td>
<td>0.2694</td>
</tr>
<tr>
<td>DGL-3/TFA</td>
<td>54.78</td>
<td>Phosphate buffer pH 4.5</td>
<td>0.2123</td>
</tr>
<tr>
<td>DGL-3/TFA</td>
<td>54.78</td>
<td>NaCl solution pH 6.0</td>
<td>0.2097</td>
</tr>
<tr>
<td>DGL-3/TFA</td>
<td>54.78</td>
<td>Carbonate buffer pH 8.0</td>
<td>0.2390</td>
</tr>
</tbody>
</table>

\(^a\)The \(dn/dc\) correction corresponds to \(\frac{100 \times (dn/dc \text{ of Table 2})}{\text{corresponding lysine amount}}\).

proportion was rather assessed in all DGL-3/counter-ion samples. Afterward, this lysine mass ratio was taken into consideration to correct the total DGL-3/counter-anion mass.

3.4. Determination of Lysine Amounts in the DGL-3 Samples and Consecutive Correction of Refractive Index Increments

The mass amount of lysine in each sample was measured by the total Kjeldahl nitrogen (TKN) method for all the DGL-3/counter-ion samples (Table 4). After that, the refractive index increments were corrected of these lysine amount values to obtain the actual concentrations of DGL-3 (i.e., without the salt linked to ammonium groups, Table 4).

Note that this mass correction reduces the difference between the experiments performed with various counter-ions (Figure 4). Assuming that these results are normally dispersed around an average value, it is possible to calculate the mean value and the coefficient of variation. The average corrected refractive index increment is significantly higher than the non-corrected ones (0.2311 mL g\(^{-1}\) versus 0.11 to 0.19 mL g\(^{-1}\) in Table 2), and a low coefficient of variation (10%) (Table 4) is found to be very satisfactory for a same initial DGL-3 batch. The variance seems to be due to random part of the uncertainty.

This average \(dn/dc\) value of 0.2311 mL g\(^{-1}\) is close to the \(dn/dc\) value of 0.2450 ± 0.002 mL g\(^{-1}\) found for linear poly(\(L\)-lysine) hydrochloride (with nitrogen content determined by the micro-Kjeldahl method) in 1 M NaCl by Applequist and Doty.\(^{26}\) Nevertheless, note that this \(dn/dc\) value was measured at 436 nm whereas the value presented in our work was determined at 658 nm. Refractive index increment is wavelength-dependent according to Cauchy’s dispersion relation \((dn/dc = A + B \lambda^{-2})\) where \(A\) and \(B\) are constants for a given substance. This is the reason for which the \(dn/dc\) must be measured at the same (or closest possible) wavelength \((\lambda_{\text{Optilab T-rEX} \text{ of DRI} = 658 \text{ nm in our work}})\) of the laser used for light scattering \((\lambda_{\text{Dawn EOS} \text{ of MALLS} = 690 \text{ nm in our work}})\). Nevertheless, a difference below 5–10% for \(dn/dc\) was expected between 436 and 658 nm. As a result, the coherency of \(dn/dc\) value found in our study with the literature one is satisfactory. Unfortunately, there are no other study to the best of our knowledge in the literature about the determination of the \(dn/dc\) value for linear poly(Lysine) samples in aqueous media to be compared.

Given that the differences between \(dn/dc\) of Table 4 are low and likely due to uncertainties of measurement in the TKN method and \(dn/dc\) determination, we have considered that the average \(dn/dc\) was the value the most reliable to calculate average molecular weights (Table 5).

3.5. Determination of Average Molecular Weights of DGL-3 by Using Refractive Index Increments Corrected with Lysine Amounts

The number-average molecular weights \((M_n)\) and dispersities \((D)\) of DGL-3 by using the average refractive index increment (0.2311 mL g\(^{-1}\), Table 4) are presented in Table 5. The average \(M_n\) value of DGL-3 was found to be 18 700 g mol\(^{-1}\), which corresponds to a degree of polymerization of DGL-3 equal to ca. 146 (\(= 18 700/128\) g mol\(^{-1}\)).

Figure 5 enables to easily compare the \(M_n\) values before and after correction of lysine amount. It underlines...
the interest to correct the refractive index increments with the value of the lysine amount to obtain absolute and reliable number-average molecular weight. In these conditions, the histogram representation of $M_n$ values confirms a much lower variation (gray vs black histogram in Figure 5). The relative standard error is also low and very satisfactory for SEC data (ca. 6%, Table 5). The standard error of the mean of 450 g mol$^{-1}$ is very

![Figure 4. Different refractive index increments of DGL-3 given in Table 2 (black line, without correction of lysine amount in sample), and Table 4 (gray line, with correction of lysine amount).](image)

![Table 5. Number-average molecular weights and dispersities of DGL-3/counter-ions by using the average refractive index increments after the correction of the lysine amount ($dn/dc = 0.2311$ mL g$^{-1}$).](table)

<table>
<thead>
<tr>
<th>Sample/counter-ions</th>
<th>Eluent</th>
<th>Number-average molecular weight$^{a,1)}$ $M_n$ [g mol$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGL-3/PO$_4$</td>
<td>Phosphate buffer pH 4.5</td>
<td>20 430 ± 400 (1.122)</td>
</tr>
<tr>
<td>DGL-3/CO$_3$</td>
<td>Carbonate buffer pH 8.0</td>
<td>17 250 ± 210 (1.138)</td>
</tr>
<tr>
<td>DGL-3/Cl</td>
<td>NaCl solution pH 6.0</td>
<td>18 350 ± 345 (1.222)</td>
</tr>
<tr>
<td>DGL-3/TFA</td>
<td>Phosphate buffer pH 4.5</td>
<td>19 440 ± 415 (1.163)</td>
</tr>
<tr>
<td>DGL-3/TFA</td>
<td>NaCl solution pH 6.0</td>
<td>18 180 ± 460 (1.202)</td>
</tr>
<tr>
<td>DGL-3/TFA</td>
<td>Carbonate buffer pH 8.0</td>
<td>18 385 ± 490 (1.154)</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>18 700</td>
</tr>
<tr>
<td>Standard error</td>
<td></td>
<td>1110</td>
</tr>
<tr>
<td>Relative standard error (coefficient of variation)</td>
<td></td>
<td>6%</td>
</tr>
<tr>
<td>Standard error of the mean (standard error/$\sqrt{n}$)</td>
<td></td>
<td>450</td>
</tr>
</tbody>
</table>

$^{a}$The uncertainty given for the number average molecular weight was obtained from the ASTRA software with the Zimm fitting model; the numbers in brackets represent the polydispersity values.
acceptable too. Note that a similar average $M_n$ value would have been found (18 900 g mol$^{-1}$) if each $dn/dc$ would have been kept to calculate $M_n$ value instead to use the average $dn/dc$. But the relative standard error would have been higher (ca. 14%) probably due to the uncertainty brought by the TKN method (ca. 20%).

This decrease in molecular weight variation can be also detected in Figure 6 which plots the cumulative weight fraction as a function of the molar mass. The cumulative weight fraction of DGL-3/counter-ion samples in different eluents is much more dispersed before the $dn/dc$ correction by the lysine amount than ones after the correction (leading to tightened curves).

In conclusion of this work, the more reliable $dn/dc$ of DGL-3 corresponds to corrected values with the DGL amount, without taking into account salts of diverse counter-ions. Note that this is almost close to the constraining theory of light scattering of a polyelectrolyte in a salt solvent,\cite{27} which implies a $dn/dc$ measurement at constant chemical potential (requiring a dialysis of each solution against the eluent during 24 h, up to the osmotic equilibrium) and not at constant chemical concentration to obtain true and not apparent values of refractive index increments and molecular weights. Here, the refractive index increment seems to be a signal only dependent on DGL, and not on counter-ions associated to DGL in aqueous solution. These counter-ions would be “invisible” towards the dRI signal. However, they significantly “contribute” to the total mass of sample, and the polymer concentrations must be corrected as shown in this study. This “dRI transparency” of counter-ions could be explained as follows. In the first case, where the polymer counter-ions are the same as those of SEC eluent (e.g., DGL-3/PO$_4$ eluted in phosphate buffer, or DGL-3/Cl eluted in NaCl solution), the difference between the dRI signal of counter-ions potentially associated to DGL-3, and the dRI signal of same counter-ions constituting the eluent would be null.

In the second case, where DGL-3/TFA was analyzed in different eluents, all the TFA counter-ions are dissociated from DGL-3, as demonstrated by SEC elution profiles and $^{19}$F NMR analysis of the DGL-3 peak collected at the end of SEC column (i.e., no corresponding $^{19}$F NMR peak). These TFA counter-ions were probably replaced by SEC eluent counter-ions, and the dRI difference with the eluent counter-ions is null as in the first case. Concerning the determination of the DGL-3/TFA $dn/dc$ values in different solutions using the differential refractometer in batch mode, the TFA mass amount in refractometer cell is negligible compared with the mass amount of the counter ions of the solution (e.g., CO$_3$/TFA mass ratio = 13 for the maximal $dn/dc$ concentration of DGL3-TFA of 1 mg mL$^{-1}$ and a carbonate buffer concentration of 0.1 M). Furthermore, the TFA refractive index (ca. 1.285 at 20 °C) is close

![Figure 5. Comparison of number-average molecular weights of DGL-3 given in Table 2 (without correction of lysine amount in samples, black histogram), and Table 5 (with correction of lysine amount in samples, gray histogram).](image)

![Figure 6. Plots of cumulative weight fraction as a function of molar mass of different of DGL-3/counter-ions and different eluents without $dn/dc$ correction by lysine amount in samples (top graph), and with $dn/dc$ correction (bottom graph).](image)
to refractive indexes of phosphate, carbonate, and chloride solutions.

4. Conclusion

The aim of this investigation was to determine the absolute average molecular weights, and so the refractive index increments of dendrigraft poly(L-lysine) polyelectrolytes. The work was achieved with a generation 3 of DGL (used as model), and by varying the counter-ions associated to the polyelectrolyte, as well as the SEC aqueous eluents. The number-average molecular weight data obtained by using the refractive index increment values measured directly on the DGL solutions were found to be very dispersed. Consequently, the sample concentrations employed to determine dn/dc were corrected by the mass of lysine amount assayed by the TKN method in each sample (i.e., by removing the mass contribution of salt). Thanks to the analytical methodology developed in this work, the dn/dc values were found to be much less dispersed and the consequent $M_n$ values were relatively close, as demonstrated by a low relative standard error of 6% ($\sigma_{M_n} = 18700 \pm 1110$ g mol$^{-1}$). This variation coefficient value is satisfactory for SEC-MALLS measurements.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author. An HPLC chromatogram showing the intergeneration purity of dendrigraft poly(L-lysine) DGL-3 is provided.

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