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Article

Hydrodynamic Behavior of Dendrigraft Polylysines in Water and Dimethylformamide

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Abstract: The four first generations of dendrigraft poly-L-lysine have been studied in dimethylformamide (aprotic solvent) and in 0.2 M NaCl aqueous solutions by isothermal translation diffusion, ^{1}H NMR and viscometry methods. The relationships between diffusion coefficient, intrinsic viscosity and molar mass have been determined for dendrigraft poly-L-lysines, and the scaling index values have been compared to classical trifunctional dendrimers. Dendrimers and dendrigraft poly-L-lysines exhibited similitudes in their hydrodynamic behaviors. Nevertheless, dendrigraft poly-L-lysines displayed a specific behavior in solution. In contrast to dendrimers, a significant change of hydrodynamic dimension of dendrigraft poly-L-lysines according to the nature of the solvent has been observed. In aprotic solvent, the dendrigraft poly-L-lysine dimensions are about two times lower than in aqueous media (*i.e.*, the hydrodynamic volume is contracted by a factor 8 in dimethylformamide), revealing the softness of dendrigraft poly-L-lysine compared to classical trifunctional dendrimers.

Keywords: dendrimer; dendrigraft polylysine; hydrodynamic properties; hydrodynamic radii; diffusion coefficient

1. Introduction

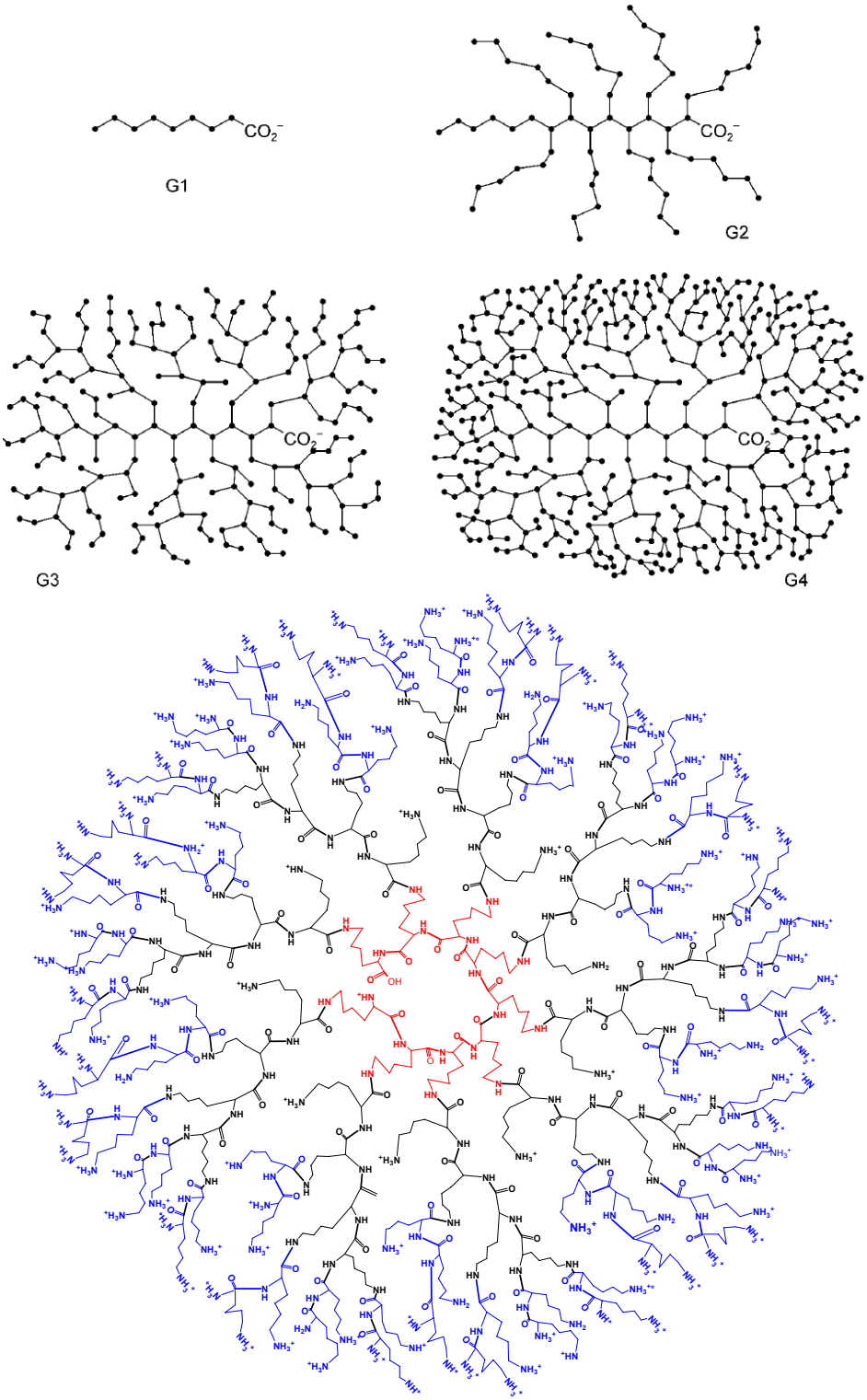
Macromolecules with dendritic architecture have paid more and more attention over the past decade, and well-known polymers have gain interest by studying their dendritic analogues [1–3]. This tendency is related to the advantages of dendrimers in comparison with the linear structure for modern nano- and biotechnology purposes which require a strong control of molecular properties at the nanoscale level [2,3]. In contrast to the linear polymers, dendrimers can be more easily controlled in their structure, molar mass, size, shape and periphery owing to the strategy of synthesis [1]. Synthetic poly(aminoacids) and synthetic polypeptides are polymers of great importance for contemporary medicine, pharmacology, cosmetology and for biochemistry due to their biocompatibility. This class of biopolymers is also concerned by the previously mentioned general trends. Some successful attempts undertaken to design new dendritic polypeptide-like molecules as well as other water soluble dendrimers (for instance well-known poly(amido amine) (PAMAM)) had demonstrated the benefits of turning the shape and surface properties of such structurally ordered macromolecules [2–5].

Among the different classes of dendritic structures, dendrigraft polymers are the most recently discovered subset of dendrimer-like molecules whose solution properties have not been fully investigated. Synthesis of dendrigraft polymers is based on procedure of monomer units grafting onto a polymeric sub-structure using protect-deprotect steps [1]. Dendrigraft polymers are characterized by high molecular weights attained in few synthetic steps. They are considered to stand at intermediate position between the fully controlled well determined dendrimers and the uncontrolled hyperbranched polymers [1]. Within the class of macromolecules having dendritic architecture, dendrigraft polymers are expected to address the challenging issue regarding the industrial development of dendrimer-like structures, due to relatively low production cost while preserving the advantages belonging to dendritic structures.

The present work is devoted to the investigation of structure-properties relationships and to the study of molecular characteristics of dendrigraft poly-L-lysines (DGL, Figure 1), the synthesis of which have been recently reported [6,7]. Contrary to previously described hyperbranched poly-L-lysine synthesis which was performed in organic solvent [8,9], DGL are synthesized by polycondensation of *N*-epsilon-trifluoroacetyl-L-lysine-N-carboxyanhydride in water at pH 6.5 [7]. The spontaneous precipitation of the growing *N*-epsilon-trifluoroacetyl-protected polymer ensures fair control of the molar mass distribution. The subsequent deprotection of the trifluoroacetyl groups leads to water-soluble DGL that can be used as macroinitiator for the synthesis of the next generation. The unique reproducible structure of the DGL is thermodynamically controlled by precipitation in water, and kinetically controlled by steric hindrance during the polymerization. This new synthetic route leads to a straightforward easily scalable process, avoiding time-consuming multiple steps as described for the synthesis of lysine dendrimers [7]. DGLs display an exponential growth of their molar mass with a 2.7 multiplying factor between generations (except between G1 and G2 for which a factor about 6 was obtained). They are non-immunogenic, biocompatible dendritic polypeptides that can be

easily functionalized [10]. They can be used for numerous applications, including drug carriers, gene delivery agents and antimicrobial agents [10,11].

Figure 1. Schematic representation of the four first generations of dendrigraft poly-L-lysines (DGL) and typical chemical structure of DGL G3. Each dot represents a Lys residue. Chemical structure in red corresponds to G1. Chemical structure in red and blue corresponds to G2. The central part of the G3 structure is depicted in a cyclic conformation to facilitate the 2D-representation of the whole structure.



The goal of the present study was to compare the hydrodynamic characteristics of the first four generations of DGL in water (D_2O + 0.2 M NaCl) and in dimethylformamide (DMF, aprotic solvent) using viscometry, isothermal translation diffusion and ^1H NMR methodologies. A second objective was to shed more light on the structural and topological differences between dendrimers and dendrigraft poly-L-lysines. Dendrimers have a constant and maximal branching density value, while for dendrigraft poly-L-lysines the branching density is lower than for dendrimers and passes through a maximum for G4 generation.

2. Experimental Section

2.1. Materials

The first four generations of DGL were provided by COLCOM (Montpellier, France). DGL were synthesized by COLCOM according to a recently published synthetic route [7]. DGL samples have been used without additional preparation and purification. A schematic representation of DGL from generation 1 to generation 4 is depicted in Figure 1 (each dot represents a lysine residue). The first DGL generation (G1) is a linear poly-L-lysine composed of an average of eight lysine residues. G2 is obtained by polymerization of *N*-carboxyanhydride trifluoroacetyl-L-lysine in water at pH 6.5 using G1 as macroinitiator. G2 is a branched poly-L-lysine having an average of 48 lysine residues. G3 is obtained by polymerization using G2 as a macroinitiator and so on. The number-average degrees of polymerization of DGL were previously determined by size-exclusion chromatography coupled to a refractive index and a multi-angle static light scattering detection (see Table 1).

Table 1. Number-average polymerization degree *N*, average diffusion coefficient *D* and hydrodynamic diameter d_h of DGL in different media.

DGL generation	*N*	DMF		D_2O + 0.2 M NaCl		Phosphate solution [a]		
		D [b] (10^{-7} cm^2·s^{-1})	d_h (10^{-9} cm)	D [c] (10^{-7} cm^2·s^{-1})	d_h (10^{-9} cm)	D [d] (10^{-7} cm^2·s^{-1})	d_h [c] (10^{-9} cm)	$d_h(D_2O$+ 0.2 M NaCl)/d_h(DMF)
G1	8	35.4	1.55	17.30	2.10	20. 98	2.06	1.4
G2	48	32.7	1.68	9.92	3.67	11.3	3.92	2.2
G3	123	22.0	2.49	6.23	5.84	7.06	6.12	2.3
G4	365	12.3	4.46	4.77	7.76	5.86	7.38	1.8

[a] 50 g/L $H_2PO_4^-$, Na^+ in water, pH 4.5, 0.61 M ionic strength [6]; [b] Determined by isothermal translation diffusion (DMF); [c] Determined by ^1H NMR (D_2O + 0.2 M NaCl); [d] Determined by Taylor dispersion analysis [6].

Dissolution of DGL samples in DMF and in aqueous solvents was carried out at room temperature just before measurements. All DGL generations were fully and easily dissolved in the two solvents.

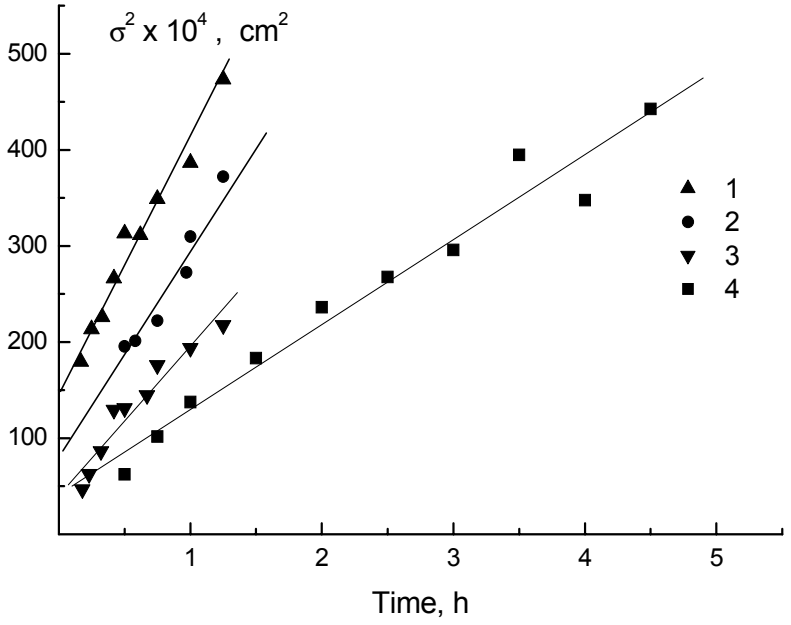
Dimethylformamide (DMF) (Vecton, St. Petersburg, Russia) free from water has been used for the preparation of DGL solutions. DMF possessed the following characteristics at 298 K: density 0.9445 g·cm^{-3}, viscosity 0.796 cP and refractive index 1.4269. Heavy water (D_2O) (deuterium content 99.8%, Institute of Nuclear Physics, St. Petersburg, Russia) having a density of 1.104 g·cm^{-3},

1.18 cP viscosity and 1.3293 refractive index at 298 K, has been used for the characterization of DGL by ^{1}H NMR. Sodium chloride was from Vecton (St. Petersburg, Russia).

2.2. Methods

Different techniques are nowadays available for the determination of translation diffusion coefficient *D* of macromolecules in solutions. In the present study, proton NMR has been applied for estimation of hydrodynamic size of DGL molecules through the determination of their translational diffusion coefficient in D_2O + 0.2 M NaCl. Proton NMR presents some advantages in comparison with the other methods. It requires a relatively small amount of substance with relatively short time of experiment. Nevertheless, it requires pure deuterated solvents for high quality measurements. In this work, classical isothermal translation diffusion has been used for the determination of DGL translation diffusion coefficients in DMF.

Figure 2. Time dependences of the dispersion of diffusion boundary σ^2 for DGL G1 (1), G2 (2), G3 and G4 in DMF at solute concentration of 0.04×10^{-2} g·cm^{-3} and at 298 K. The dispersion values were calculated from the maximal ordinate and the area under the diffusion curve.



Isothermal translation diffusion method is based on the observation of free diffusion by means of the boundary formation between the solution and pure solvent. The installation (Tsvetkov's diffusometer) and cuvette construction used in the present work were described earlier [12]. The diffusion coefficient *D* in this method is determined from the slope of the dependence of dispersion σ^2 of boundary curve with time *t* (Figure 2) according to the relation $\sigma^2 = 2Dt$ [12]. The concentration dependence of *D* with the variation of solute concentration *c* is studied and, finally, the translation diffusion coefficient is determined as $D_o = \lim_{c\to o} D(c)$ by extrapolation at infinite dilution condition c→o. The translation diffusion coefficient is related to the hydrodynamic diameter d_h of molecules by Stokes-Einstein equation [12]:

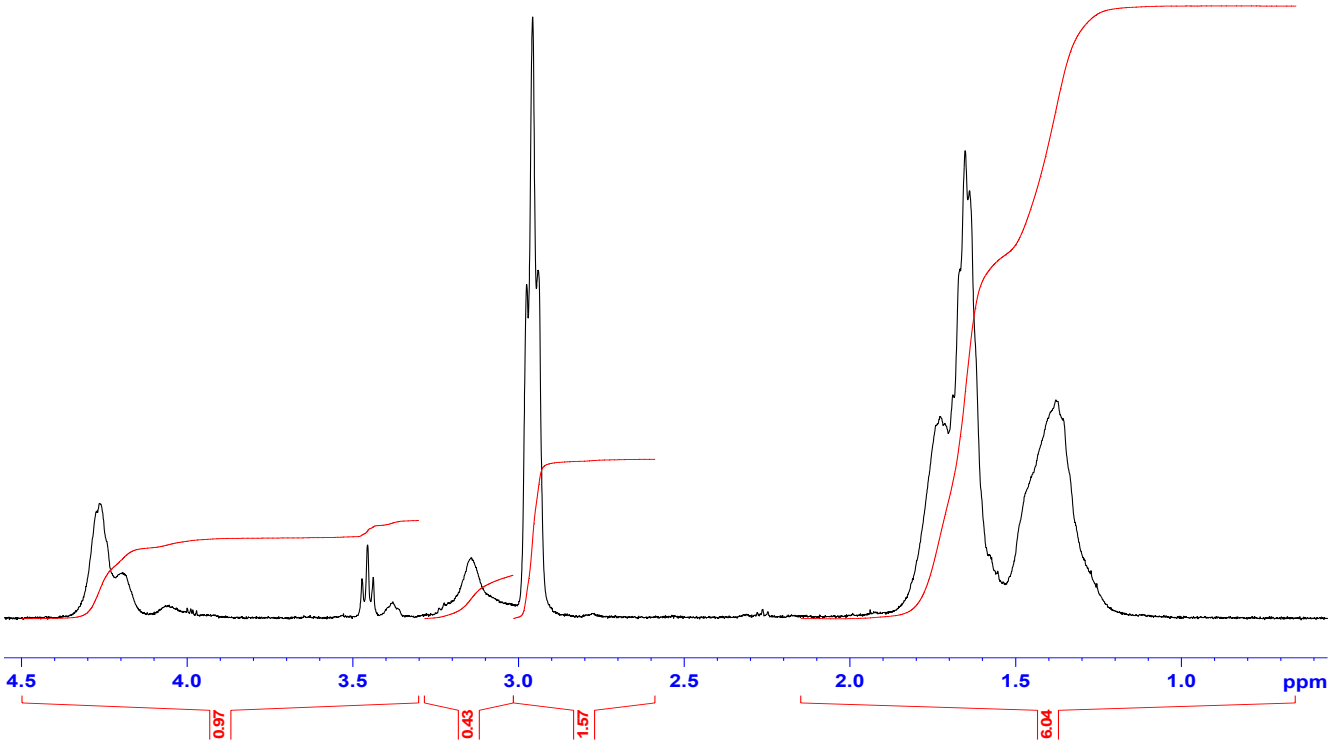
$$d_h = kT/3\pi\eta_o D_o \quad (1)$$

where k is the Boltzmann constant, T is the absolute temperature, and η_o is the solvent viscosity.

Determinations of D for all the generations of DGL have been performed for a concentration range of (0.08–0.04) × 10^{-2} g·cm^{-3}. Concentration dependence of D was not detected for DGL in DMF solution.

Proton NMR in a magnetic field gradient has been applied for the study of translation diffusion coefficients of DGL samples in D_2O + 0.2 M NaCl [13,14]. The measurements were performed using a pulsed Bruker Avance 300 NMR-spectrometer in the temperature interval 298–315 K at solute concentration range (1–0.5) × 10^{-2} g·cm^{-3}. Typical spectrum of one of the DGL samples (G3) is presented in Figure 3. Peak assignment is given in the caption of Figure 3, more details that are not crucial for this work are given in supplementary information of [7]. Temperature dependences of NMR-signal relaxation at 2.93 ppm related to terminal amino-groups and at 4.26 ppm related to the inner amino-groups have been used for the determination of the DGL self-diffusion coefficients D_s [13,14]. Self-diffusion coefficients have been converted into molecular hydrodynamic dimensions using Equation (1). It is generally accepted that self diffusion coefficients determined by NMR are very close to translational diffusion coefficients determined e.g., by isothermal translation diffusion in the case of diluted solutions [15,16].

Figure 3. NMR spectrum of DGL G3 in D_2O containing 0.2 M NaCl. The peak at 4.26 ppm corresponds to protons of –CO–CH–NH– group, the peak at 3.13 ppm corresponds to protons of –CH_2–NH–CO– group, peak at 2.93 ppm relates to –CH_2–NH_3^+ terminal groups, the other peaks correspond to –$(CH_2)_n$–. Temperature dependence of signal relaxation at 2.93 and 4.26 ppm has been used for the determination of the DGL diffusion coefficients.



Ostwald's type capillary viscosimeter has been used for the determination of the intrinsic viscosity value *[η]* of DGL samples in D_2O + 0.2 M NaCl. The measurements were performed at solute concentrations satisfying the relationship $[\eta]\ c \leq 1$ as a required condition for intrinsic viscosity determination [12]. The intrinsic viscosity value was experimentally derived according to the Huggins Equation (2):

$$\eta_{sp}/c=[\eta]+k'[\eta]^2c \quad (2)$$

where $\eta_{sp}/c = (\eta - \eta_o)/\eta_o\ c = (t - t_o)/t_o c$, η and η_o are the viscosities of the polymer solution and the solvent respectively, t and t_o are the times of solution/solvent outflow in the viscosimeter, k' is the Huggins constant. The outflow time of D_2O + 0.2 M NaCl in viscosimeter was 120.5 ± 0.05 s. Intrinsic viscosity values of DGL G1 to G3 in DMF were too small to be determined correctly.

3. Results and Discussion

The linear dependences between dispersion σ^2 of boundary curve and diffusion time t obtained by isothermal translational diffusion method are displayed in Figure 2. The diffusion coefficient D of DGL molecules were determined from the slope of these dependences and converted to d_h (see Equation (1) in the experimental part).

Table 1 contains the experimental data on hydrodynamic diameters of DGL samples obtained by means of isothermal translational diffusion in DMF and by ^{1}H NMR in D_2O + 0.2 M NaCl. DGL are cationic highly ramified polyelectrolytes since their amine groups are protonated in aqueous conditions (see schematic representation of DGL topology in Figure 1). The previous investigation of DGL hydrodynamic behavior by Taylor Dispersion Analysis in phosphate buffers at pH 4.5 and 7 (0.61 M and 0.306 M ionic strength respectively) demonstrated an absence of any significant influence of pH on the DGL dimensions [6]. The present study confirms these results since DGL dimensions determined by ^{1}H NMR in 0.2 M NaCl deuterated water were found to be in good agreement with the previously published values (see Table 1) [6]. Good agreement of DGL molecular size determinations derived from different methods in aqueous solvents may be also considered as an evidence of DGL samples homogeneity.

Nevertheless, according to the data presented in the third column of Table 1, the hydrodynamic diameters d_h of DGL in DMF determined by isothermal translation diffusion strongly differ from those obtained in aqueous solvents. The dimension of DGL is about twice smaller (or compact) in DMF in comparison with water, except for G1 which is only 1.4 smaller in DMF than in water (see the ratio in the last column of Table 1). An obvious reason for the lower DGL dimension in DMF is that DMF is aprotic solvent that should significantly reduce the degree of protonation of the DGL, thus decreasing the repulsion between monomers and between branches that constitute the DGL. Another possible explanation (or mechanism) of DGL compacting in DMF may be related to the conformational state of the linear central backbone constituted of 8 Lys residues (G1). Indeed, in the uncharged state, linear poly-L-lysine tends to adopt a helical conformation. Possible twisting of the central part of DGLs in DMF can contribute to the decrease of overall dendrigraft molecule dimensions.

A peculiar tendency of dendimers bearing charged terminal groups to change their conformations with the variation of charge density was theoretically predicted for dendrimers [17]. In particular, molecular modeling by means of different techniques predicted an essential dependence of size of

PAMAM dendrimers on pH media conditions [18,19]. But experimental methods did not confirm this tendency. For well known dendrimers such as diaminobutane dendrimer (DAB) or PAMAM, there was no detected significant change of the molecular hydrodynamic size with pH or in different solvents. For instance, the invariance of PAMAM dendrimers under variable pH conditions was asserted from small angle neutron scattering data [20,21]. Furthermore, the PAMAM dendrimer dimension was found to vary in the range of 5 to 10%, depending on the solvent quality [22]. One possible reason of such PAMAM behavior under variable pH conditions have been explained in [23]. By means of atomistic molecular dynamics simulation on the fourth generation, it has been shown that the charge density distribution inside the dendrimer can change with the pH conditions, without any swelling.

DGLs may be similar to classical dendrimers regarding their pH behavior since their size just slightly depends on pH in aqueous media [6], but at the same time, DGLs are dendritic macromolecules that behave differently from DAB and PAMAM and display a strong dependence of their size on thermodynamic quality of the solvent, similarly to flexible linear polymer chains [24]. As discussed earlier, this difference can comes from the linear multifunctional core of the DGL compared to the point-like core of PAMAM and DAB dendrimers [1]. In addition, the charged amine end-groups can be situated not only on the outer part of the DGL, but also inside the dendritic structure, in contrast to DAB and PAMAM for which amine end-groups are located only at the periphery. This means that DGL can attract the counter ions into the interior part of the dendritic structure, which is impossible for DAB or PAMAM dendrimers. The transfer of counter ions inside DGL in aqueous buffers (high ionization state) may be additionally responsible for a higher DGL dimension compared to DMF (low ionization state). It can be mentioned also, that the hydrodynamic size of DGL molecules in DMF (Table 1) are close to the size of the same generation number PAMAM in methanol even though molar mass of corresponding DGL generation is more than twice higher [22,25].

Thus, the results of the study of translation diffusion in DMF allow us to conclude that DGL may be considered as one of the most flexible dendritic macromolecules among those currently used or studied for biological applications. This unique property of contraction/swelling of DGL dimension may be advantageously utilized for encapsulation/decapsulation processes.

Figure 4 displays the dependencies of translation diffusion coefficients D and intrinsic viscosity $[\eta]$ *vs.* DGL molar mass M in a double logarithmic scale in water and DMF. Scaling relationships of translation diffusion coefficient for DGL that derived from the plots are the following:

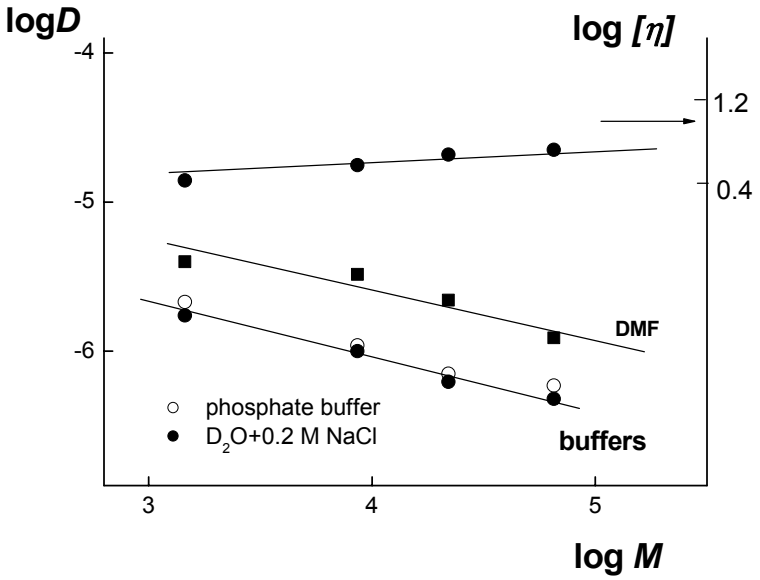
$$D = 4.12 \times 10^{-5} M^{-0.30 \pm 0.07} \text{ (DMF)} \quad (3a)$$

$$D = 2.24 \times 10^{-5} M^{-0.35 \pm 0.03} \text{ (D_2O + 0.2 M NaCl)} \quad (3b)$$

The slopes of the log D = f(log M) dependencies are closed to each other for the two solvents, taking into account the experimental error values. The exponent values in relationships Equations (3a) and (3b) are close to those previously determined in phosphate buffers (0.36–0.37) [6]. The fact that the exponent does not significantly change in the two solvents suggests an homothetic change of the DGL size in DMF and in D_2O + 0.2 M NaCl. The non-variation of this exponent with the solvent, with the ionic strength and with the pH is in great contrast to linear polymers. In the latter case, the scaling exponents are very sensitive to thermodynamic quality of the solvent [24]. Compared to other dendritic

structures, such as PAMAM in methanol [1,25], carbohydrate coated PAMAM [26] and DAB modified by lactose-end groups [27] in aqueous buffers, similar exponents from 0.25 to 0.37 were obtained. Indeed, diffusion scaling indexes for dendrimers and dendrimer-like structures are in the vicinity of 1/3. The latter diffusion coefficient scaling index value was theoretically predicted for homogeneous impenetrable spheres [24]. Thus, in translation diffusion process, DGL behave themselves similar to dense highly ramified structures as for classical dendrimers.

Figure 4. The dependencies of diffusion coefficient *D* and intrinsic viscosity *[η]* with the molar mass of DGL in different conditions. Experimental conditions: D_2O containing 0.2 M NaCl, phosphate buffer pH 4.5 (0.59 M ionic strength) [6] and DMF.



On the other hand, according to the theoretical predictions molecules which hydrodynamic properties correspond to the behavior of homogeneous impenetrable spheres, should have a scaling index $a = 0$ in Mark-Houwink-Kuhn relationship $[\eta] \sim M^a$ [24]. In other words, the intrinsic viscosity that is directly inversely proportional to the density in the macromolecule, becomes independent of the molar mass for impenetrable spheres. In reality, *a* is not equal to zero for many dendrimers and theirs derivatives and the dependence of *[η]* with *M* is often non-monotonous [1]. For DGL in D_2O + 0.2 M NaCl, the following Mark-Houwink-Kuhn equation has been experimentally established for generations 1 to 4:

$$[\eta] = 2.01 \times M^{0.12 \pm 0.01} \quad (4)$$

A clear contradiction appears between the translation diffusion of dendrimers whose behavior can be described by impenetrable spheres (nanoparticles), and the intrinsic viscosity data that does not follow the hard sphere model. This discrepancy is often considered as the manifestation of the dendrimer flexibility and as the evidence that dendrimers are rather macromolecules than nanoparticles [1]. To conclude on that part of the study, it may be asserted that hydrodynamic properties of DGL are similar to charged high molar mass dendrimers, excepted that the DGL dimension is much more affected by the solvent than other dendrimers such as PAMAM. DGL can be therefore considered as soft dendritic structures.

4. Conclusions

This experimental study and the subsequent analysis of hydrodynamic properties of DGL in DMF, also in water, revealed their soft (or highly flexible) nature in comparison with dendrimers of similar chemical structure (DAB and PAMAM cationic trifunctional dendrimers). This special feature was deduced from the significant change of DGL dimension on the solvent properties, which is unusual for other cationic trifunctional dendrimers. The hydrodynamic volumes of DGL (G2 to G4) were found to be contracted in DMF by a factor 8 compared to water. This unexpected behavior is likely related to the non-punctual characteristic of the DGL core and to a more homogenous repartition of the charges and counter-ions into the dendritic structure. Further studies on the effects of solvatation, ionization and counter-ion condensation on the hydrodynamic properties of DGL may contribute to better understand that ability to interact or aggregate in solution (as for polypeptides and proteins).

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