Interactions of Dendrimers with Selected Amino Acids and Proteins Studied by Continuous Wave EPR and Fourier Transform EPR

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Interactions of polyamidoamine dendrimers, termed Gn, where n indicates the generation (=number of amidoamine layers), at different protonation levels with selected amino acids and proteins have been investigated by means of continuous wave electron paramagnetic resonance (cw-EPR) and pulsed-EPR (electron spin−echo = ESE) analyses. A low-generation dendrimer (G2) and a high-generation one (G6) were labeled with nitroxides for the EPR measurements. Gly, Glu, Arg, and Leu were selected as representative of neutral(zwitterionic)-polar, acidic, basic, and low-polar amino acids, respectively. The water-soluble proteins α-chymotrypsin and albumin were selected on the basis of a basic and an acidic isoelectric point, respectively. The cw-EPR spectra were analyzed by computing the line shapes to extract information about the dendrimer−biomolecule interactions. In general, dendrimers at a high protonation level interact stronger with amino acids than those at a low level of protonation. However, even for highly protonated dendrimers, a synergistic effect between hydrophilic and hydrophobic interactions promoted the formation of stable Gn−amino acid adducts, as demonstrated by the enhanced interactions with Leu. As expected from acid−base interactions, stable adducts were formed between Arg and highly protonated dendrimers and between Glu and low level protonated dendrimers. The relatively strong dendrimer interactions with the protein chymotrypsin and the poor interactions of dendrimers with albumin demonstrated that the protonated amino groups of the dendrimers are predominantly involved in the interactions with these proteins and indicated a significant role in the interactions with the dendrimers of the hydrophobic external residues of chymotrypsin. Computer-aided analysis of the ESE experiments was consistent with the cw-EPR results and supported the conclusion of a partial complexation of the nitroxides of the dendrimer with Leu and α-chymotrypsin.

Introduction

Polyamidoamine (PAMAM) dendrimers (Chart 1) of different generations (the generation refers to the number of covalently attached amidoamine layers), henceforth termed Gn,[1] where n indicates the generation, that belong to the family of dendritic polymers,[2] have been used in gene therapy to act as drug and gene carriers to selected cells.[3,4] In vitro experiments demonstrated that DNA wraps around the Gn dendrimers. A DNA delivery mechanism in which the DNA/Gn adducts reach the cell membrane and DNA is released in the cells for DNA/RNA replication has been proposed. Experiments conducted using electron paramagnetic resonance (EPR) spectroscopy with spin probe techniques showed that the Gn dendrimers interact well with components of biomaterials, such as the cell membrane (mimed by vesicles)[5,6] and oligo- and polynucleotides.[7,8] Among them, DNA plays the most relevant role in the delivery process. Recently we have shown that different supramolecular structures are formed between DNA and Gn at different Gn/DNA molar ratios, which correspond to different saturation conditions.[7]

Among the biomacromolecules, proteins are protagonists in the process involving the dendrimers as gene or drug carriers. Therefore, it is important to study protein−Gn interactions in order to clarify the mechanisms of protein−dendrimer complexation in such biological processes. The building blocks of proteins and polynucleotides, the amino acids, may interact differently with each other

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Chart 1. Structure of the Dendrimers

G2

(16 surface groups)

29 Å

70 Å

G6

(256 surface groups)

$$\rangle N-CH_2-CH_2-CO-NH-CH_2-CH_2-N\langle$$

8 Å

Gn-T

Chart 2. Amino Acids

Gly
Glycine
(neutral)

Leu
Leucine
(hydrophobic)

Arg
Arginine
(basic)

Glu
Glutamic acid
(acidic)

and with the surface amino groups of G*n* depending on their chemical nature. Therefore, for a better understanding of the protein−G*n* and DNA−G*n* interactions, it is useful to perform a basic analysis of the amino acid−G*n* interactions. The amino acids used in the present study (Chart 2) were selected based on the different chemical properties of the side chains, that is, the neutral glycine (Gly), the base arginine (Arg), the glutamic acid (Glu), and the hydrophobic leucine (Leu).

We selected two water-soluble proteins for investigation on the basis of a basic and an acidic isoelectric point (IP):

α-chymotrypsin (IP = 8.34, henceforth termed Chym) and albumin (IP = 4.9, henceforth termed Alb). Chym also presents hydrophobic residues at the external surface, which are available for interactions with molecules in solution.

The dendrimers selected for our investigation were both an "early" generation prototype (G2) and a "late" generation prototype (G6) (Chart 1). The dendrimer structure is rather flexible for G2 and rather rigid/compact for G6.[9] The G*n* solutions were used at different levels of protonation (through control of pH) of the basic dendrimer amino groups at the surface, since interactions with the biomolecules having different acid/base properties are affected by the degree of protonation of the dendrimer surface. To follow the fate of the dendrimers and to analyze the dendrimer/biomolecule interactions, we used spin-labeled dendrimers, that is, dendrimers with a nitroxide radical covalently attached to the external surface amino groups (Chart 1). These labeled dendrimers, termed G*n*-T, have been successfully used in previous studies in which the interactions of the dendrimers with different molecules and surfaces have been investigated.[5b,6−8] Analysis of the EPR spectra, based mainly on computer-aided computation of the line shapes, provided the parameters descriptive of the localization and distribution of the labels and, consequently, of the attached dendrimers. Both continuous wave EPR (cw-EPR) and pulsed-EPR (electron spin−echo = ESE) were used to provide an overview of the location of the label and the environment of the label.

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Experimental Section

Materials and Sample Preparation. Millipore doubly distilled water was used for the preparation of all the solutions. Purification of the dendrimers was accomplished by ultrafiltration. The purity of the dendrimers was determined by a combination of ^{13}C NMR spectroscopy, mass spectrometry, and gel electrophoresis.[10] Labeling of the dendrimer external surface (synthesis of Gn-T, Chart 1) was accomplished by employing a modified method used by Pauly et al. for labeling DNA-nucleosides[11] and was described previously.[8] The level of labeling was evaluated as 1 label for about 30 external surface amino groups, established by quantitative cw-EPR spectroscopy, employing a nitroxide standard and a flat cell fixed in the EPR cavity.

Water solutions of G2-T and G6-T were prepared at a concentration of 0.1 M in surface amino groups. The solutions were stored under nitrogen in order to avoid oxidative degradation and kept in the refrigerator when not in use. Protonation of the surface amino groups was accomplished by adding controlled amounts of dilute HCl (0.05 M, Merck). The acid−base properties of the Gn have been determined in a previous study.[12] The natural pH of the dendrimer solution is about 8.5 due to the hydrolysis of the amino surface groups. Previous studies[12] demonstrated that, at this pH, the dendrimer external surface is partially protonated and that complete protonation is accomplished at pH = 4.5. We found that the dendrimer solution acts as a buffer solution: indeed the addition of the amino acids or the protein, even if acidic or basic molecules, changed the pH of the solution negligibly. Therefore, we assume that the protonation level of the dendrimers practically does not change after addition of the various biomolecules used in this study.

Water solutions of G2-T and G6-T at a low level of protonation of the external surface (pH = 8.5) (termed Gn-T) or at a high level of protonation of the external surface (pH = 4.5) (termed Gn-T$^+$) were added to the water solutions of the different amino acids and proteins (Sigma, used as received) to obtain final concentrations of the dendrimers of 0.01 and 0.005 M in surface amino groups and 1 mM of amino acids or proteins (in amino acid groups).

To decrease the mobility of the spin labels for cw-EPR experiments, a temperature of 287 K was selected, which allowed a comparison among the different investigated systems. The same procedure was used in previous studies with spin-labeled dendrimers to investigate the interaction with vesicles and polynucleotides in solution.[5b,6−8]

Additional experiments were performed as a function of temperature and concentration. A temperature increase up to 333 K (a higher temperature could cause decomposition of the nitroxide spin probe) gave rise to a progressive (logarithmic) increase in the rotational diffusion mobility of the radicals (measured from computation of the EPR spectra, see below). However, since the analysis of the cw-EPR spectra as a function of temperature did not provide further information on the interactions between the dendrimers and the biomolecules, these additional experiments are not discussed hereafter in this paper. Also, variation in the relative concentrations of biomolecules and dendrimers did not provide further information on the interactions between dendrimers and amino acids or proteins and, henceforth, are not discussed.

For pulsed-EPR measurements, dendrimers, amino acids, and proteins were dried under a vacuum at 37 °C. D$_2$O (98%) solutions at concentrations similar to those of the cw-EPR measurements were prepared under an argon atmosphere; these solutions were then introduced in 4 mm quartz tubes for the measurements.

Instrumentation. The cw-EPR spectra were recorded by means of a Bruker 200D or a EMX-Bruker spectrometer, both operating at X band (9.5 GHz), interfaced to a PC−IBM computer (Stelar software for 200D and Bruker software for EMX) for data acquisition and handling. The temperature was controlled with a Bruker ST 100/700 or a Bruker ST3000 variable-temperature assembly cooled with liquid nitrogen.

ESE experiments were carried out at 4 K using a Bruker ESP380E FT-EPR spectrometer equipped with the liquid helium cooling accessory ER4112HV. On the basis of previous literature, we assumed that the structures of dendrimers, biomolecules, and their supramolecular arrangements were retained at 4 K, under the conditions of rapid freezing.[13,14] Two-pulse (2p-) and three-pulse (3p-) ESE experiments were employed. For 2p-ESE experiments, the pulse sequence was (16−τ−32 ns), and signals were obtained as a function of variation of the time between the pulses. For 3p-ESE, the pulse sequence was (16−τ−16−T−16 ns), and signals were registered by varying T, the time between the second and third pulses. The magnetic field was set at the central EPR peak, that is, 3430 G at 9.656 GHz.

Computation of cw-EPR and ESE Signals. The simulation of the cw-EPR spectra used the method developed by Freed, Budil, and co-workers.[15] The **g** tensor components for the coupling between the electron spin and the magnetic field are assumed to be constant: g_{ii} = 2.009, 2.006, and 2.003; the components of the hyperfine tensor, A_{ii} (estimated accuracy, 3%), for the coupling between the electron spin and the nitrogen nuclear spin are calculated from the frozen spectra of the labeled dendrimers in water solution (at the liquid nitrogen temperature) as A_{ii} = 8, 6, and 37 G. They are also assumed to be constant for all samples.

The main input parameter, changing from one system to another, is the perpendicular component of the correlation time for motion τ_{perp} (estimated accuracy = 3%). This correlation time for motion was obtained by assuming a jump model for the diffusional rotational motion of the label, for which the diffusion coefficient is $D_{perp} = 1/\tau_{perp}$. The label rotates around the main axis corresponding to the direction of the p orbital containing the unpaired electron. Fast rotation of the label at room temperature is guaranteed by the σ bonds at the dendrimer surface level. Variation of τ_{perp} monitors the change in the strength of interaction between the surface groups of the dendrimer and the interacting groups and the biomolecule, for a series of spectra from similar systems (e.g., interaction of the same dendrimer with different amino acids or proteins). The τ_{perp} parameter is related to the effect of the interacting molecules (dipole−dipole, ion−dipole, or electrostatic interactions) on the mobility of the nitroxide groups, and therefore it provides a quantitative evaluation of the interaction itself, in average and on the basis of a comparison among equivalent systems. Of course it is possible that amino acid or protein residues with different ionization states affect differently the microenvironment of the dendrimer, but we expect that probes in different environments provide different EPR components contributing to the total line shape.

The ESE spectra simulation method has been described in ref 16. The modulated decay curve in both 2p- and 3p-ESE experiments is described by the function[17] $V(t) = V_m(t)V_d(t)$. $V_m(t)$ describes the modulation pattern, or electron spin−echo envelope modulation (ESEEM), due to the presence of weakly coupled paramagnetic nuclei in the vicinity of the nitroxide. The modulation depends on the ENDOR frequencies ν_+ and ν_-. $V_d(t)$ is the observed decay function on varying the time interval between the first two pulses and that between the second and the third pulses for the 2p-ESE and the 3p-ESE experiments, respectively. Analysis of the modulation pattern is based on the assumption of isotropic shells with weakly coupled nuclei around

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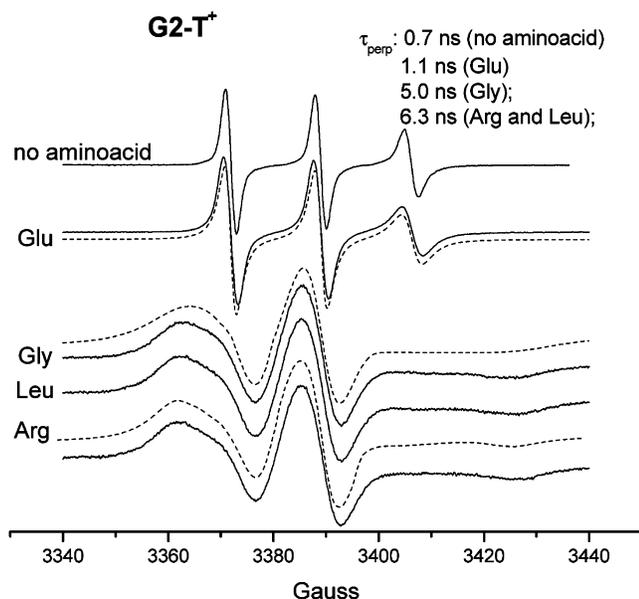(11) Pauly, G. T.; Bobst, E. V.; Bruckmann, D.; Bobst, A. M. *Helv. Chim. Acta* **1989**, *72*, 110.

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Figure 1. cw-EPR spectra of G2-T$^+$ (5 mM in external surface amino groups) in aqueous solutions (pH = 4.5) with various amino acids (1 mM) at 287 K. The dashed spectra show examples of simulations. τ_{perp} values obtained from computation are reported in the figure.

the electron spin distribution at distances below 0.6 nm. This simulation is performed using a best fit procedure in which the parameters are the type and number of nuclei inducing modulation n_i^0, their distance from the paramagnetic center r_i (the accuracy of r_i is 0.01 nm), and their hyperfine coupling constants a_i (the accuracy of a_i is 0.02 MHz). In the case of deuterium, the modulation function $V_m(t)$ was computed by means of the second-order perturbation approach derived by Heming et al.[18] and a quadrupole interaction (Q_i) was included. An exponential dumping function for the modulation with characteristic time τ_d had to be introduced to reproduce the experimental data. The decay function $V_d(t)$ is affected by the concentration of the spins at resonance.[17] For diluted glassy solutions of nitroxide radicals at low temperature, it has been shown that the decay time of the 2p-ESE depends on the vibrational motions of the nitroxide residue,[19] on the concentration of paramagnetic nuclei of the matrix surrounding the paramagnetic center,[16b] and in particular on the presence of methyl groups in the molecules composing the matrix.[20] For 3p-ESE, the decay is mostly affected by spin−lattice relaxation.[21] In general, the ESEEM pattern shows two types of modulation: a low-frequency modulation (with a period of about 500 ns) due to coupling of the electron spin to the deuterium atoms of the D$_2$O solvent molecules surrounding the nitroxide group and a high-frequency modulation (with a period of about 30 ns) due either to matrix (distant) protons belonging to the amino acids or proteins in solution or to near protons of amino acids or proteins interacting with the dendrimer. For paramagnetic nuclei that are located more closely to the electron spin, the modulation depth is normally larger for nuclei with a smaller gyromagnetic ratio and depends both on the number of nuclei and on the strength of the dipolar interaction. Therefore, the appearance and increasing depth of the proton modulation in samples containing a specific kind of biomolecules indicate specific interactions between the dendrimer and these biomolecules in solution, leading to biomolecule protons approaching the unpaired electron site.

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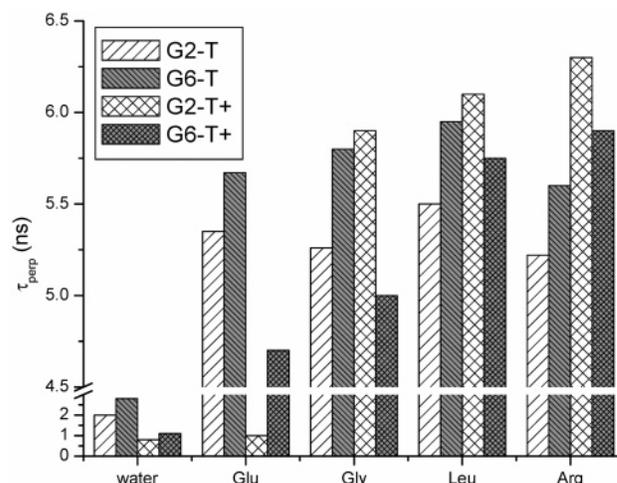

Figure 2. Correlation time for motion (perpendicular component) of the spin-labeled dendrimers at low (pH = 8.5) and high (pH = 4.5) protonation levels in the absence and presence of various amino acids.

Results and Discussion

Amino Acids. Figure 1 shows as examples the cw-EPR spectra at 287 K of G2-T$^+$ in solutions with the various amino acids. The spectra of the other dendrimers are not reported for the sake of brevity. The dashed spectra in Figure 1 are examples of computation (one component) of the spectra. The main parameter, τ_{perp}, obtained from the computation of the spectra is also reported in the figure.

The histogram in Figure 2 compares τ_{perp} of the various, labeled dendrimers in the absence and in the presence of the different amino acids. An increase in τ_{perp} (decrease in mobility of the spin label) indicates an increased interaction of the dendrimers with the substrate. Analysis of the results, shown in Figure 2, indicates a specific *effect of dendrimer protonation*: variation of the mobility parameter among the various dendrimer/amino acid systems is more significant for dendrimers at a high level of protonation than for those at a low level of protonation, indicating a high sensitivity of the protonated dendrimers to the amino acids, facilitating interactions or charge repulsions when each of them takes place.

Analysis of the histograms in Figure 2 indicates the following trends for the dendrimer−amino acid interactions:

strength of interaction for G2-T and G6-T:
    no amino acid < Arg < Gly < Glu < Leu

strength of interaction for G2-T$^+$ and G6-T$^+$:
    no amino acid < Glu < Gly < Leu < Arg

These trends are in agreement with the following conclusions:

Contemporaneous Electrostatic and Hydrophobic Interactions. For all the dendrimers, the interaction with Leu was relatively significant, indicating a synergistic effect between electrostatic interactions of the zwitterionic (carboxylate + ammonium) group of Leu and the polar or charged surface groups of the dendrimers, and hydrophobic interactions of the Leu side chain with the dendrimer sites at low polarity ($-CH_2-CH_2-$ groups). Similar behavior has already been found for charged surfactants with the chain entering the dendrimer structure and anchoring at the interior low polarity sites,

whereas charged groups interact with the charged dendrimer surface.[22]

Acid−Base Neutralization Related to the Ionization State of the Species. The titration of the dendrimer surface as a function of added acids and bases, described in a previous study,[12] allowed evaluation of the pK_a for the deprotonation of the protonated ammonia groups at the external dendrimer surface, that is, $pK_a = 8.5-13$. This provided a protonation extent of about 30% at the natural pH (8.5−9) of the dendrimer solutions used in the present study, and complete protonation of both the internal and external dendrimer amino groups is expected at pH = 4.5. Also, the protonation range of the external $-NH_2$ groups guaranteed the buffering effect of the dendrimers themselves. On the other hand, the pK_a of the $-COOH$ and $-NH_3^+$ of the amino acids is known to be 2 and 10, respectively; furthermore, the basic nature of arginine is demonstrated by its $pK_a = 12.5$, whereas glutamic acid presents a $pK_a = 4$. Therefore, lowly protonated dendrimers, which provide a pH = 8.5−9, are mainly basic and undergo a neutralization reaction with the glutamic acid, which promotes the electrostatic interactions between the two species; conversely, highly protonated dendrimers, at pH = 4.5, are acidic species with respect to the basic Arg and undergo a neutralization dendrimer−Arg reaction, which also promotes the electrostatic interactions between the two species. However, previous studies from this group already demonstrated that the PAMAM dendrimers interact via the protonated amino groups with negatively charged species, such as sodium dodecyl sulfate (SDS) surfactants and their aggregates;[23] this behavior justifies the enhanced interactions of the dendrimers, even at a low protonation level, with the negatively charged amino acids, if compared to the dendrimer solution in the absence of the amino acids. Furthermore, spectroscopic data and pH behavior, reported in the literature,[24] suggested that acidic molecules such as benzoic acid were forming stable ion pairs with the dendrimer's internal, basic tertiary nitrogens. This kind of interaction may also take place between Glu and the dendrimer interior. Finally, the possible encapsulation of the amino acid molecules into the dendrimer structure is also supported by an extensive literature which demonstrated that dye molecules such as rose bengal are included into the dendrimer structure and form stable host−guest compounds.[25−28] However, the EPR results show that acid−base neutralization prevails in promoting the interactions between the dendrimers and the amino acids, since the lowly protonated dendrimers, mainly basic in nature, interact poorly with the basic Arg but well with the acid Glu. The neutral Gly shows an intermediate interacting ability (interactions through the zwitterionic group of the amino acid). The interacting ability is somewhere between those of the acidic Glu and the basic Arg amino acids but less interactive than Leu, which
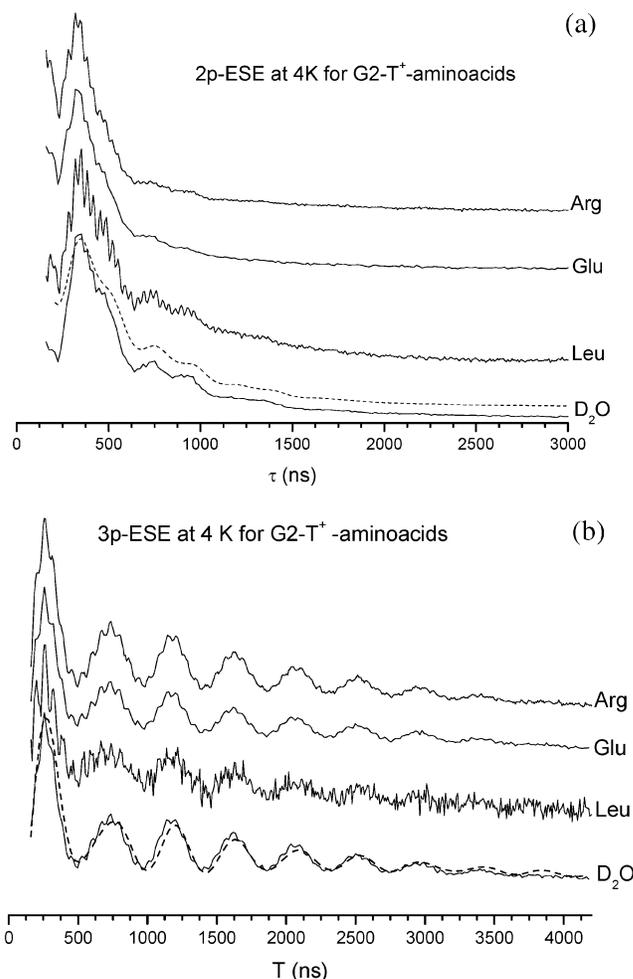
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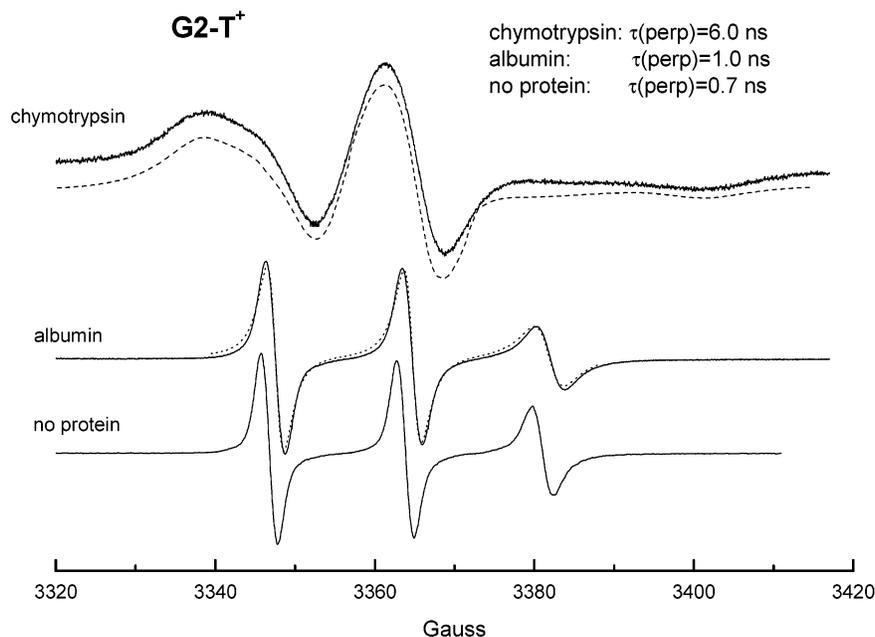

Figure 3. 2p-ESE (a) and 3p-ESE (b) traces of G2-T$^+$ in D$_2$O (4 K) in the absence and in the presence of Arg, Leu, and Glu ($H_0 = 3.43$ kG; $\nu = 9.63$ GHz; for 3p-ESE, $\tau = 160$ ns). Simulations for the G2-T$^+$/D$_2$O spectra are reported (dashed lines) as examples.

Table 1. Parameters for the Computation of the ESE Spectra: n, Number of Deuterium Nuclei; d, Distance of Deuterium Nuclei to Unpaired Electron; a, Hyperfine Coupling; Q, Quadrupole Coupling; τ_i, Decay Times of 3p-ESE

system	n_i	d_i (nm)	a_i (MHz)	Q_i (MHz)	τ_1 (μs)	τ_2 (μs)
G2-T$^+$/D$_2$O	1	0.25	0.5	0.2	0.4	10
	2	0.36	0	0		
G6-T/D$_2$O	1	0.26	0.2	0.4	1	39
	2	0.37	0	0		
G2-T$^+$/Chym	1	0.25	0.3	0.4	1.1	40

^{a} Accuracy of the parameters, 3%.

further indicates the enhanced interaction in the presence of a hydrophobic side chain of the amino acids.

Figure 3 shows as examples the 2p-ESE (a) and 3p-ESE (b) of G2-T$^+$ in D$_2$O in the absence and in the presence of Leu, Arg, and Glu (the spectra with Gly were similar to the spectra of Glu). The spectra in Figure 3 were recorded at 3.43 kG and a frequency of 9.63 GHz; the value of τ for 3p-ESE traces was 160 ns. The dashed line shows the computation of the G2-T$^+$/D$_2$O patterns as an example. The simulation of the ESEEM pattern shows a deep deuterium modulation but only a mildly observable hydrogen modulation. The empirically measured D/H ratio (from the depth of deuterium and proton peaks, after subtraction of the decay pattern) is about 9. The simulation

Figure 4. cw-EPR experimental (full lines) and simulated (dashed lines) spectra obtained for the protonated dendrimer, G2-T$^+$ (5 mM in external surface amino groups) in the presence of Alb and α-Chym (1 mM in amino acids). The spectrum of the labeled dendrimer in the absence of the proteins is also reported for comparison.

was therefore performed by assuming only the presence of nearby interacting deuteriums. The parameters extracted from the computations are reported in Table 1. The presence of one deuterium at a distance of 0.25 nm and two deuteriums at 0.36 nm indicated that the nitroxide site is complexed by at least one water molecule.

Arg, Glu, and Leu provided significant variations in the spectral line shape with respect to the D$_2$O solutions (Figure 3), in particular for the presence of a deeper proton modulation. In detail, the D/H ratio of G2-T$^+$ samples decreased from 9, as indicated above, in the absence of amino acids, to 5.5 for Glu, 5 for Gly, 3 for Arg, and 2 for Leu, which is in line with an increasing interaction between the dendrimer and the amino acids in the following series: no amino acid < Glu < Gly < Arg < Leu. This is almost equivalent to the strength of interaction found by the cw-EPR experiment. In particular, for all dendrimers, a significantly deeper proton modulation was observed for Leu, when compared to the D$_2$O samples and the samples of the other amino acids. This finding is in line with the high number of protons/molecule ($n = 12$). The Fourier transformation of the 3p-ESE proton modulation (experimental results not shown), obtained by subtracting the simulated modulation of the deuterium, provides a strong proton matrix line at the proton Larmor frequency (ν_H) but also a broad band of 4 MHz width at ν_H, which is typical for the presence of interacting Leu hydrogens (at 0.2−0.3 nm distance from the unpaired electron) overlapping the weak band of dendrimer protons. These features are in good agreement with cw-EPR analysis.

The decay patterns are related to the relaxation mechanisms caused by the presence of paramagnetic nuclei, such as protons of the matrix, coupled protons, rotating methyl groups, and different mobility of the nitroxide. Therefore, a lower decay time (faster decay rate) is interpreted as a change in the nitroxide environment that induces a decrease of the nitroxide mobility, which in turn indicates interaction between dendrimers and amino acids. In line with the absence of interactions, the decay time of 2p-ESE (T_M) in the dendrimer solutions without amino acids is high ($T_M = 450$ ns), but it is reduced
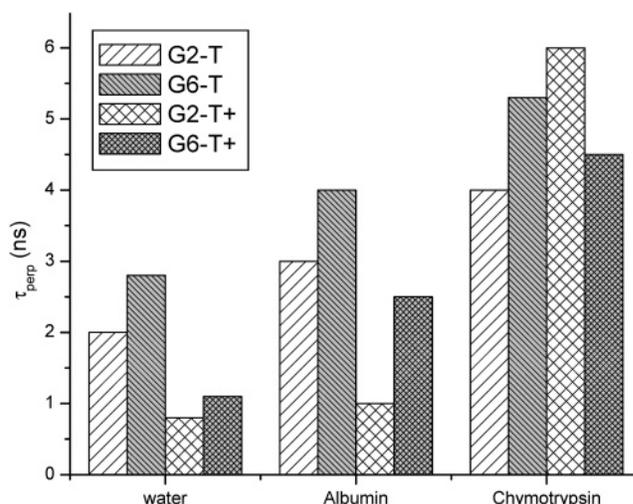

Figure 5. Correlation time for motion (τ_{perp}) (accuracy, 3%) of spin-labeled dendrimers in aqueous solution in the absence and in the presence of the proteins chymotrypsin and albumin.

in the presence of the amino acids: for Gly, $T_M = 420$ ns; for Glu, $T_M = 350$ ns; for Leu, $T_M = 290$ ns; for Arg, $T_M = 230$ ns. The decrease in the T_M values reports increasing interaction of the nitroxide with the amino acids.

Proteins. Figure 4 shows, as examples, the cw-EPR experimental (full lines) and computed (dashed lines) spectra obtained for the protonated dendrimer G2-T$^+$ in the absence and in the presence of α-Chym and Alb.

The histogram in Figure 5 shows the variation of τ_{perp} for the various dendrimer−protein systems. The results are summarized as follows:

The characteristics of α-Chym with respect to its interacting ability with dendrimers are mainly due to the high isoelectric point (8.3, due to basic amino acids such as Arg) and the presence of a hydrophobic pocket (hydrophobic amino acids such as Leu) at the substrate-linking site. As expected, the basic nature of this protein favors interactions with the protonated amino groups of G2-T$^+$ and G6-T$^+$. The presence of hydrophobic sites
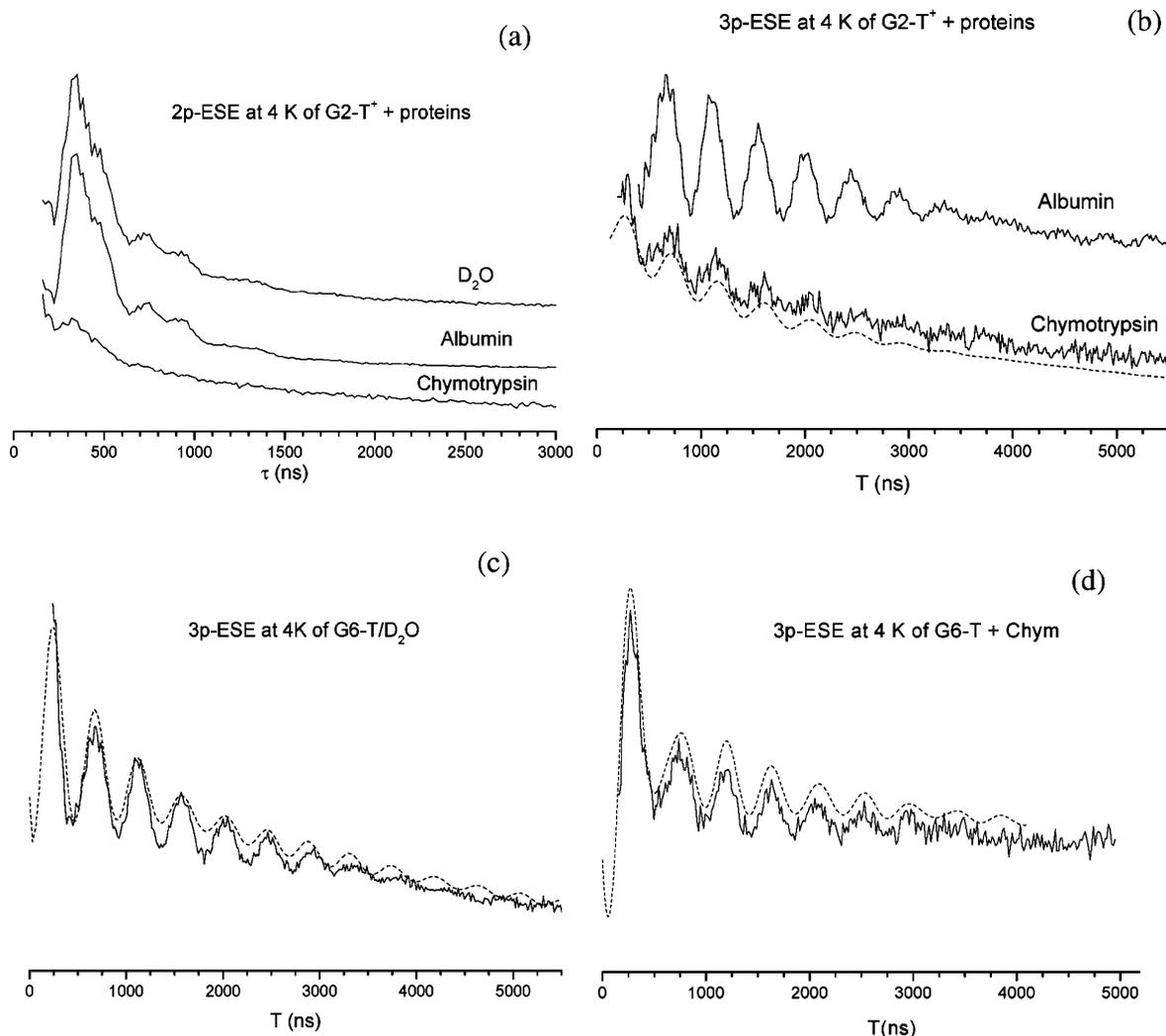
Figure 6. 2p-ESE (a) and 3p-ESE (b) traces of G2-T$^+$ in D$_2$O (4 K) in the absence and in the presence of Chym and Alb ($H_0 =$ 3.43 kG; $\nu = 9.63$ GHz); 3p-ESE traces were recorded with $\tau = 160$ ns for D$_2$O and Alb and 200 ns for Chym. The simulation for the 3p-ESE of the G2-T$^+$/Chym spectrum is reported as a dashed line. (c) 3p-ESE trace of G6-T/D$_2$O and (d) 3p-ESE trace of G6-T/Chym ($\tau = 120$ ns). Simulations of the traces are shown as dashed lines.

available for the interaction surely enhanced the interactions, mainly for the flexible G2 structure.

Alb, due to its acid IP (4.9), interacts better with low protonated (basic) dendrimers than with the highly protonated ones. However, the interactions (as probed by τ_{perp}) are weaker for Alb than for Chym. Several studies reported in the literature describe the interactions of albumin with hydrophobic molecules, indicating a variation in the conformation of the protein which favors a direct interaction of hydrophobic molecules with hydrophobic residues of the protein that comprise the binding pocket in subdomain IIIA or subdomain IA or other hydrophobic residues such as cysteine34, Trp76, bilirubin, or methionines.[29] However other studies have shown that exposure to charged species, like Co(II), favors an A−B transition of Alb from the conformation of weaker affinity for charged species to one of stronger affinity.[30] Therefore,

the EPR results indicate that the less polar sites of the dendrimers are probably not accessible to the albumin hydrophobic residues and the weak dipole−dipole or ion−dipole interactions between the albumin hydrophilic residues and the dendrimer surface do not allow any significant structural adaptation of the dendrimer and Alb macromolecules to one other.

Figure 6 reports the 2p-ESE (a) and 3p-ESE (b) spectra of G2-T$^+$ (selected as an example, since this dendrimer gave the highest variation in mobility from Chym to Alb) in D$_2$O in the absence and in the presence of Chym and Alb. The spectra were recorded at 3.43 kG and a frequency of 9.64 GHz; the value of τ was 160 ns (200 ns for the Chym). In Figure 6b, the simulation for the 3p-ESE of the G2-T$^+$/Chym spectrum is reported. Figure 6c shows, for comparison, the experimental and computed 3p-ESE spectra of G6-T/D$_2$O and G6-T/Chym. The parameters used for computation are reported in Table 1. Analysis of the spectra indicated the following trends:

The Chym 2p-ESE spectrum shows a lower decay time than that exhibited by Alb. Therefore, mobility of the nitroxide is partially impeded by the dendrimer−protein interaction, as was found in the cw-EPR analysis. Moreover, dramatic changes in the modulation pattern were found for the 2p- and 3p-ESEEM of G2-T$^+$. In this case,

(29) (a) Pantoja-Uceda, D.; Shewry, P. R.; Bruix, M.; Tatham, A. S.; Santoro, J.; Rico, M. *Biochemistry* **2004**, *43*, 6976. (b) Rampon, V.; Brossard, C.; Mouhous-Riou, N.; Bousseau, B.; Llamas, G.; Genot, C. *Adv. Colloid Interface Sci.* **2004**, *108−109*, 87. (c) Mao, H.; Hajduk, P. J.; Craig, R.; Bell, R.; Borre, T.; Fesik, S. W. *J. Am. Chem. Soc.* **2001**, *123*, 10429. (d) Moreno, F.; Cortijo, M.; Gonzalez-Jimenez, J. *Photochem. Photobiol.* **1999**, *70*, 695.
(30) Liang, H.; Huang, J.; Tu, C.-Q.; Zhang, M.; Zhou, Y.-Q.; Shen, P.-W. *J. Inorg. Biochem.* **2001**, *85*, 167.

the interaction of the dendrimers with Chym is indicated by the displacement of two outer coordinated D_2O molecules by the protein in the nitroxide environment. In fact, the deuterium 3p-ESEEM profile is fitted best with a single deuterium nucleus at 0.25 nm. Moreover, the coupling constants (a and Q) are rather different from those of the coordinated deuterium in the sample $G2-T^+/D_2O$. This result is peculiar, since it also indicates a significant distortion of the geometry of the first hydration shell of the nitroxide environment. Such a distortion was almost absent (as found from the ESE analysis) for $G6-T^+$ and for the low protonated dendrimers, $Gn-T$ (results not shown). The conclusion is that the $G2-T^+$ dendrimer (flexible structure) adapts well to the Chym external surface, which is in agreement with the cw-EPR results.

The $Alb-G2-T^+$ ESE spectra (Figure 6b) are quite similar to the $D_2O/G2-T^+$ spectra (Figure 3b). Therefore, interaction of the small dendrimer with this protein is very weak, which was also found by the cw-EPR spectra analysis.

Conclusions

Interactions between low-generation and high-generation prototypes of polyamidoamine dendrimers labeled with a Tempo nitroxide (G2-T and G6-T) with amino acids and proteins have been investigated by means of cw-EPR and ESEEM analyses. Gly, Glu, Arg, and Leu were selected as representatives of neutral(zwitterionic)-polar, acidic, basic, and low-polar amino acids, respectively. α-Chym and Alb were selected on the basis of the basic and acidic IP, respectively. G2-T and G6-T were used at high (pH = 8.5) and low (pH = 4.5) levels of protonation of the dendrimer surface.

In the case of the amino acids, variation of the mobility parameters among the various dendrimer/amino acid systems is more significant for the dendrimers at a high level of protonation than for dendrimers at a low level of protonation. Even for highly protonated dendrimers, interaction with Leu was significant, due to a synergistic effect between hydrophilic and hydrophobic interactions. Due to acid−base interactions, Arg (basic) interacts strongest with highly protonated dendrimers, whereas Glu (acidic) interacts stronger with dendrimers at a low

level of protonation. Gly (neutral) shows an intermediate interacting ability, somewhere between those of the acidic (Glu) and the basic (Arg) amino acids. The ESE results supported the conclusions obtained from cw-EPR, indicating the complexation of deuterium from the water surrounding the nitroxide moiety but also the presence of hydrogens, mainly for Leu, in the vicinity of the dendrimer surface at the nitroxide site.

In the case of the proteins, the relatively strong interactions of dendrimers with Chym and the poor interactions with Alb demonstrated that the protonated amino groups of the dendrimers are mainly responsible for the binding process. Hydrophobic residues at the protein surface play a substantial role in the interactions. The results from ESE agreed with the cw-EPR results and supported the finding about a partial complexation of the nitroxide with water molecules and their consequent displacement when the α-Chym surface approached the dendrimer surface.

We conclude that the binding of the dendrimers with proteins and their amino acid components is mainly promoted when both electrostatic and hydrophobic interactions take place. Our results also show that the cw-EPR and pulsed-EPR measurements integrate well with one another to clarify the supramolecular interaction of complex systems such as those presented in this study.