Electron Capture and Collisionally Activated Dissociation Mass Spectrometry of Doubly Charged Hyperbranched Polyesteramides

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Electron capture dissociation (ECD) of doubly protonated hyperbranched polyesteramide oligomers (1100–1900 Da) was examined and compared with the structural information obtained by low energy collisionally activated dissociation (CAD). Both the ester and amide bonds of the protonated species were cleaved easily upon ECD with the formation of odd electron (OE^+) or even electron (EE^+) fragment ions. Several mechanistic schemes are proposed that describe the complex ECD fragmentation behavior of the multiply charged oligomers. In contrast to studies of biomolecules, the present results indicate that consecutive cleavages induced by intramolecular H-shifts are significant for ECD and of less importance for low energy CAD. The capture of an electron by the ionized species results in fragmentation associated with a redistribution of the excess internal energy over the products and the subsequent bond cleavage. Low energy, multiple collision CAD is found to be a more selective dissociation method than ECD in view of the observation that only amide bonds are cleaved for most of the hyperbranched polymers examined with CAD in this study. ECD appears not to provide complementary structural information compared to CAD in the study of hyperbranched polymers, even though a significantly more complex ECD fragmentation behavior is observed. ECD is shown to be of use for the structural characterization of large oligomers that may not dissociate upon low energy CAD. This is a direct result of the fact that ECD produces ionized hyperbranched oligomers with a relatively high internal energy. (J Am Soc Mass Spectrom 2003, 14, 332–341) © 2003 American Society for Mass Spectrometry

Dissociative recombination (DR) has been studied for over 50 years in the physics community for which mainly di- and tri-atomic molecules were used [1, 2]. It was serendipitously discovered by McLafferty and coworkers [3] that recombination of electrons and large multiply protonated molecules also leads to dissociation. This process is termed electron capture dissociation (ECD). Both a direct bond cleavage (similar to DR) and a hot hydrogen atom rearrangement have been proposed as possible dissociation mechanism. In contrast to dissociation techniques like collisionally activated dissociation (CAD), blackbody infrared dissociation (BIRD), infrared multiphoton dissociation (IRMPD) and surface induced dissociation (SID), ECD is said to be a non-ergodic dissociation process. Large biomolecules have been studied (>10 kDa) with ECD and the results demonstrate that more sequence specific product ions and fewer internal fragment ions are formed compared to the other dissociation methods [4–11]. Internal fragment ions are defined as ions that originate from consecutive cleavages of two or more bonds leading to a product ion that does not contain one of the endgroups of the parent ions. CAD, BIRD, and IRMPD often lead to dissociation pathways that are of the lowest in energy. These fragmentation pathways are generally sequence unspecific and involve the loss of H_2O or the competing losses of H_3PO_4 and HPO_3 from phosphorylated ions [8].

An advantage of the non-ergodic nature of ECD is that the secondary and tertiary structure of gas-phase

biomolecules can be studied [9, 12]. ECD can be a powerful tool for the determination of the primary and secondary structure of synthetic polymers as well. Cerda et al. used ECD for the characterization of the secondary structure of poly(ethylene glycol) [13]. Successful ECD was obtained with doubly protonated, ammoniated and sodiated species. In contrast to biomolecules, the ions formed by ECD of doubly charged oligomers are in their case not odd-electron species $OE^{+\cdot}$, (e.g., no $PEG_x + 2H)^{+\cdot}$, but are EE^+ ions (e.g., $PEG_x + H)^+$ generated by the loss of a hydrogen atom, NH_3, or a sodium atom. Single cleavages of the backbone with the formation of EE^+ ions were the only dissociation products, whereas fragment ions with an additional H_2O loss were formed by CAD. The fragmentation behavior of the doubly sodiated oligomer indicated that the secondary structure of the oligomers was correct and in agreement with results by Bowers and co-workers obtained by employing molecular mechanics and dynamics calculations in combination with ion mobility analysis [14–17]. The primary structure (sequence) of the copolymer PEG-block-PPG-block-PEG was analyzed successfully with ECD and CAD. The ECD results indicated that approximately 80% of the isomeric structures have a di-block rather than a tri-block structure. Only ~70% of the isomeric structures are di-block when measured with CAD. This discrepancy was attributed to internal fragmentation in the CAD process, which is absent upon ECD [18].

A complication for the determination of the primary structure of polymers with CAD is the presence of oligomers with different sequences in the same sample. The resulting CAD mass spectra can only indicate whether the copolymer has a block or random sequence. A partially block/random sequence cannot be distinguished [19]. In addition, information about partially block/random sequences can be obtained only if bonds can be cleaved with a high specificity. Another complication is the presence of isomeric structures with different functional groups, as is the case for hyperbranched polyesteramides [20]. Gas-phase H/D exchange experiments have been used recently to successfully separate isomeric structures of hyperbranched polyesteramides based on a difference in their gas-phase basicity [20]. Different isomeric structures could also be distinguished by using the MS^2/MS^3 approach "dissociation of depleted ion populations" (DoDIP) [20].

Most ECD studies that have appeared in the literature thus far deal with single, often biomolecular, compounds of which the amino acid sequence is well known. Analyzing an ECD fragmentation mass spectrum of such compounds is relatively easy because the m/z values of all possible fragments can be calculated beforehand and compared with the m/z values of the fragments measured. In this paper, ECD is used for the characterization of hyperbranched polyesteramides, a potential alternative for expensive dendrimers, to examine whether primary structural information can be obtained from the ECD spectra, in spite of the fact that the exact isomeric composition of the hyperbranched polyesteramides is not known. ECD is a complementary tool to low energy CAD for the determination of the primary structure for several types of biomolecules. ECD produces more cleavages for polypeptides than CAD, and could thus be thought to provide more structural information of the hyperbranched polymers. It is, for example, of interest to investigate whether ECD can be used to distinguish between isomeric structures that contain different functional groups. It is also hypothesized here that differences in ECD behavior may be used to distinguish between branched or linear hyperbranched polyesteramides, which was not possible with CAD [20]. The polymers are made by the polymerization of a di-functional anhydride and a tri-functional di-isopropanolamine. These hyperbranched polymers do not allow the study of ions with a single isomeric structure since already the smallest oligomer possible, consisting of one anhydride and one di-isopropanolamine, can contain two isomeric structures [28]. Moreover, it would be difficult to obtain doubly charged ions of such small molecules. One possibility to study single isomeric structures is by adding chain stoppers during the polymerization reaction. This allows to synthesize molecules that are isomeric pure but have structural differences with regular hyperbranched polyesteramides. Whether these structural differences influence their fragmentation behavior is discussed in this paper.

Experimental

The experiments described in this paper were performed with a modified 7T Bruker (Fällanden, Switzerland) Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS) with an electrospray ionization (ESI) source constructed at the FOM Institute in Amsterdam [21–23]. Ions were isolated with SWIFT isolation pulses [24] using an in-house constructed arbitrary waveform generator [25]. The ECD experiments were performed with an electrically heated Rhenium filament (10×3 mm) on axis in the ICR cell. The open cell was also constructed at the FOM Institute [26]. The duration of the ECD event and kinetic energy of the electrons was chosen such that the intensity of the fragment ions was maximal. In our experiments, the best ECD performance was obtained by exposure of the ions for 1–3 s with approximately 100 nA of electrons. The kinetic energy of the electrons depends on the potential drop over the Rhenium filament and corresponds to electrons with a kinetic energy ranging from 0.4 to 2.2 eV.

Low energy CAD experiments were performed with on-resonance CAD. Argon was used as collision gas ($P_{Ar} = 5.2 \times 10^{-6}$ mbar). The geometry factor α of the open cell necessary for the calculation of the kinetic energy of the ions was estimated to be 2.26 based on a comparison between room temperature breakdown di-
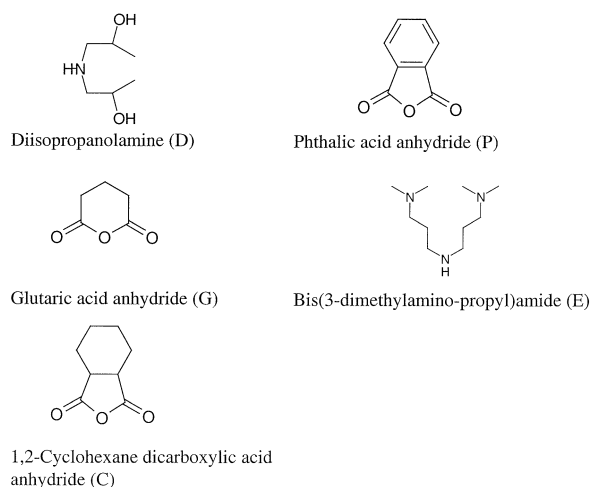
Figure 1. Structure of the di- and tri-functional monomers for the synthesis of the hyperbranched polyesteramides.

Diisopropanolamine (D)

Phthalic acid anhydride (P)

Glutaric acid anhydride (G)

Bis(3-dimethylamino-propyl)amide (E)

1,2-Cyclohexane dicarboxylic acid anhydride (C)

Figure 2. Low energy CAD of $[P_6D_7 + 2H]^{2+}$ ($E_{kin,lab}$ = 158 eV, m/z 856.90). P and D denote the phthalic acid and di-isopropanolamine units.

agrams of several peptides measured in different ICR cells. One of the ICR cells had a known geometry factor that was used for calibration purposes. The peak-to-peak voltage of the RF excitation signal was 17.6 V in all CAD experiments.
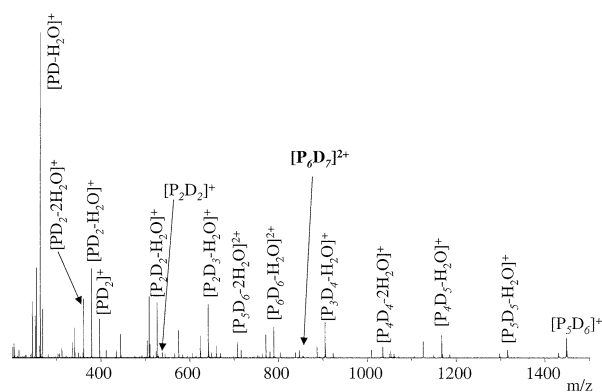
The hyperbranched polyesteramides are synthesized by the polycondensation of the trifunctional di-isopropanolamine (D) and a di-functional anhydride of phthalic acid (P), glutaric acid (G) or 1,2-cylohexane dicarboxylic acid (C), see Figure 1 for their structures.

The polymer that contains only one isomeric structure was made of D and C with bis(3-dimethylamino-propyl)amide (E) endgroups. The synthesis of the polymers has been described in more detail elsewhere [27]. The polymers were sprayed in a 0.1–0.5 mg/ml solution of 74:24:2 methanol (Merck, Darmstadt, Germany): H_2O:HOAc (Biosolve, Valkenswaard, The Netherlands).

Results and Discussion

CAD

The CAD fragmentation behavior of the singly charged protonated hyperbranched polyesteramide ions has been discussed elsewhere [20, 28, 29]. The present study is concerned with the ECD behavior of hyperbranched polymers, compared with their CAD behavior. In view of the fact that ECD requires multiply charged ions, both the CAD and ECD results of doubly charged hyperbranched oligomers are presented and discussed. The three hyperbranched polyesteramides studied here differ only in the structure of the di-acid, i.e., phthalic, glutaric, and 1,2-cyclohexane dicarboxylic acid, allowing studies of the influence of the di-acid structure on the CAD and ECD behavior. A fourth hyperbranched polyesteramide that contains only one isomeric structure is included in this study, in order to ensure that all dissociation products originate from one type of parent ion. This should in turn result in a more facile interpre-

tation of the CAD and ECD spectra. It will also allow the investigation of the usefulness of ECD for structural studies on these polyesteramides.

CAD behavior of the phthalic acid based polymer. The CAD studies were performed with ions that have a high abundance in the mass spectrum. Figure 2 shows the CAD mass spectrum of $[P_6D_7 + 2H]^{2+}$. A detail of the oligomeric structure with three phthalic acids and four di-isopropanolamines is presented in Figure 3a. To obtain one of the possible isomeric structures of P_6D_7, three R-groups must be replaced by PD units connected by ester bonds. The other three R groups must be replaced with hydrogen atoms.

The collision energy was chosen such that almost the complete amount of the parent ions is fragmented. This procedure was chosen because the dissociation of the different types of ionized isomers are associated with different critical energies as described elsewhere [20]. Using this relatively high collision energy ensured that all isomeric species are fragmented. Singly and doubly charged fragment ions were observed of which only a few have been labelled in Figure 2. All fragment ions can be explained by amide cleavages (Scheme **1**) to yield protonated fragments or by amide cleavages followed by an additional loss of one H_2O molecule and the formation of oxazolonium ions as discussed else-
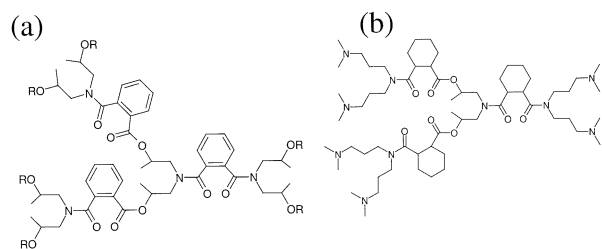
Figure 3. Detail of a possible isomeric structure of a phthalic acid containing oligomer (**a**) and the structure C_3DE_3 (**b**). P, D, C, and E denote phthalic acid, di-isopropanolamine, 1,2-cyclohexane dicarboxylic acid, and bis(3-dimethylamino-propyl)amide units. R can be replaced by other PD units or hydrogen to obtain other isomeric structures.
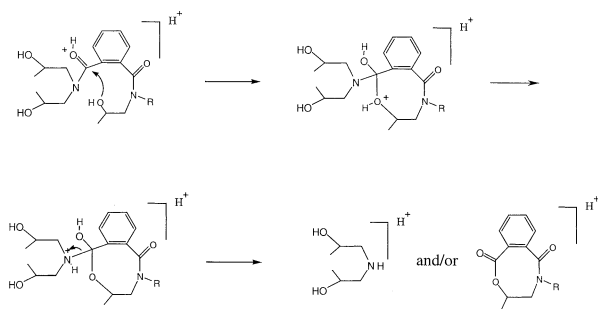
Scheme 1. Proposed cleavage of the amide bond of a doubly protonated oligomer under low energy CAD conditions.

where [20, 28, 29]. Fragment ions due to the loss of one and two molecules of H_2O were also observed. The m/z values of the fragment ion series are shown in Table 1.

CAD behavior of the glutaric and 1,2-cyclohexane dicarboxylic acid based polymers. Precursor ions of glutaric and 1,2-cyclohexane dicarboxylic acid were chosen for the CAD study that have a similar size and m/z value as $[P_6D_7 + 2H]^{2+}$ ($m/z = 856.9$), so that their CAD behavior can be compared. The selected ions for CAD were $[G_7D_8 + 2H]^{2+}$ and $[C_6D_7 + 2H]^{2+}$ with m/z 869.52 and 875.05, respectively, and a $E_{kin,lab}$ of 155 eV (spectra not shown). The structure of these oligomers is similar to the structure of the oligomer presented in Figure 3a with the exception that the phthalic acids are replaced with glutaric acid and 1,2-cyclohexane dicarboxylic acid, respectively (see Figure 1 for their structures). Table 2 shows the m/z values of the fragment ions in a concise manner. The values in Table 2 are the combined masses of the endgroup (M_{end}) and a proton. The masses of the fragment ions can be calculated by summing $n*M_{mon}$ and the mass given in Table 2 where M_{mon} is the monomer mass and n the degree of polymerization (between brackets).

The fragment ions that are observed are similar to

the ions formed by CAD of $[P_6D_7 + 2H]^{2+}$. Ion series $[X_nD_n + H]^+$ is formed for the 1,2-cyclohexane dicarboxylic acid anhydride based polymer (X = C) but not for the glutaric acid based polymer (X = G). Ion series $[P_nD_n + H]^+$ is also formed in the CAD experiments with $[P_6D_7 + H]^+$ as revealed in Figure 2. These ion series cannot be explained by cleavage of the amide bond of an alternating oligomer because this leads to the ion series $[X_nD_n - H_2O + H]^+$. However, results based on gas-phase H/D exchange experiments described elsewhere demonstrated that the oligomer series $P_nD_n^{+1}$ and $C_nD_n^{+1}$ consist of alternating and non-alternating oligomers [20]. The origin of the $[C_nD_n + H]^+$ and $[P_nD_n + H]^+$ ions can be explained by a cleavage of an amide bond according to the mechanism in Scheme **1** of a non-alternating oligomer.

CAD of the 1,2-cyclohexane dicarboxylic acid based polymer with bis(3-dimethylamino-propyl)amide endgroups. The three hyperbranched polyesteramide oligomers included in this study have in common the feature that multiple isomeric structures can exist, but the exact isomeric composition is unknown. Less complicated spectra can be obtained if an oligomer of a hyperbranched polyesteramide with only one isomeric structure is selected for the CAD experiments. As discussed in the introduction, isomeric pure compounds cannot be obtained for the phthalic acid, 1,2-cyclohexane dicarboxylic acid and glutaric acid based polymers under normal polymerization conditions. Isomeric pure compounds can, however, be obtained by adding a chain stopper during the polymerization reaction to the reaction vessel. Figure 3b shows the structure of the hyperbranched polyesteramide oligomer C_3DE_3 based on 1,2-cyclohexane dicarboxylic acid (C) and di-isopropanolamine (D) with bis(3-dimethylamino-propyl)amide (E) endgroups that has only one possible isomeric structure. The bis(3-dimethylamino-propyl)amide was

Table 1. Fragment ions formed by low energy CAD of $[P_6D_7 + 2H]^{2+}$ ($E_{kin,lab} = 158$ eV, $m/z = 856.90$)

	$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$
1+ fragments						
P_nD_{n+1}[a]	397.233	660.348	923.472	1186.582	1449.695	—
P_nD_{n+1}-H_2O	379.224	642.336	905.454	1168.570	1431.655	—
P_nD_{n+1}-$2H_2O$	361.213	624.328	887.443	1150.459	—	—
P_nD_n	282.128	545.239	808.370	—	—	—
P_nD_n-H_2O	264.123	527.239	*790.354[b]	1053.477	1316.583	—
P_nD_n-$2H_2O$	246.113	509.230	772.344	1035.438	1298.576	—
2+ fragments						
P_nD_{n+1}-H_2O	—	—	—	584.796	716.348	847.897
P_nD_{n+1}-$2H_2O$	—	312.667	444.225	575.785	707.343	838.904
P_nD_{n+1}-$3H_2O$	—	303.661	435.231	566.779	—	—
P_nD_n-H_2O	—	—	—	—	—	*790.354[b]
P_nD_n-$2H_2O$	—	—	—	—	649.789	781.347
Total number of fragments	41					

[a]P and D denote the phthalic acid and di-isopropanolamine units and n the degree of polymerisation.
[b]The fragment ions highlighted with * appear at the same m/z and are therefore not distinguishable. Ions $[P_nD_n + H]^+$ contain amine endgroups. Accuracy of the mass measurements is 0.001 Da.
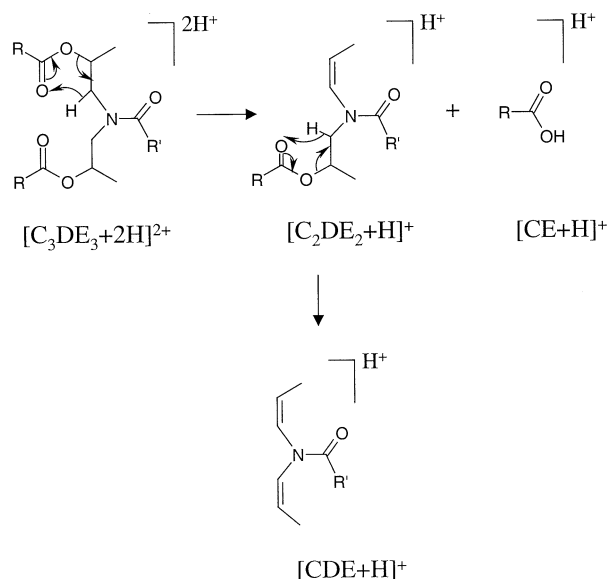
Table 2. Fragment ions formed by low energy CAD of $[G_7D_8 + 2H]^{2+}$ ($E_{kin,lab}$ = 156 eV, m/z = 869.52) and $[C_6D_7 + 2H]^{2+}$ ($E_{kin,lab}$ = 155 eV, m/z = 875.05)

	$[G_7D_8 + 2H]^{2+}$	$[C_6D_7 + 2H]^{2+}$
+1 fragment ions		
X_nD_{n+1}	134.116 (n = 1, 2, 6)	134.124 (n = 1–5)
X_nD_{n+1}-H_2O	116.108 (n = 1–6)	116.112 (n = 1–5)
X_nD_{n+1}-2H_2O	98.097 (n = 1–6)	98.101 (n = 1–4)
X_nD_n	—	288.184 (n = 0–4)
X_nD_n-H_2O	230.139 (n = 0–5)	270.173 (n = 0–4)
X_nD_n-2H_2O	212.129 (n = 1–5)	252.169 (n = 1–4)
+2 fragment ions		
X_nD_{n+1}-H_2O	58.551 (n = 4–6)	58.574 (n = 4–6)
X_nD_{n+1}-2H_2O	49.551 (n = 2, 4–6)	49.555 (n = 2–6)
X_nD_{n+1}-3H_2O	40.544 (n = 3–6)	40.554 (n = 3–5)
X_nD_n-H_2O	—	—
X_nD_n-2H_2O	106.574 (n = 4–6)	126.596 (n = 3–5)
Total number of fragment ions	40	42

[a]The X must be replaced with G or C to obtain the composition of the fragment ions. Printed values include the masses of the endgroup and a proton. The masses of the fragment ions can be calculated by summing $n·M_{mon}$ and the printed mass. M_{mon} and n are the monomer mass and degree of polymerization, respectively (see text). For example, the fragment ion series X_nD_{n+1} observed for the glutaric acid anhydride based polymer, 134.116 (n = 1, 2, 6), consist of the fragment ions $[GD_2 + H]^+$, $[G_2D_3 + H]^+$ and $[G_6D_7 + H]^+$ with m/z values of 363.247, 592.378 and 1508.902. The monomer masses of the glutaric acid and 1,2-cyclohexane dicarboxylic acid containing oligomers are 229.131 and 269.163 Da, respectively. Ions $[C_nD_n + H]^+$ contain amine endgroups. Accuracy of the mass measurments is 0.001 Da.
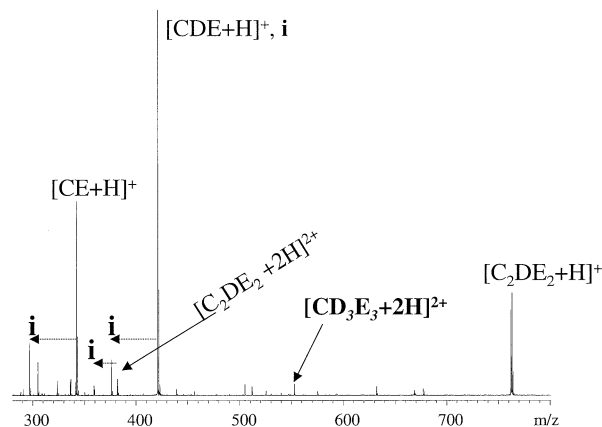
added as a chain stopper. A structural difference between this oligomer and the phtalic acid, glutaric acid, and 1,2-cyclohexane dicarboxylic acid based oligomers is that it also contains amine functionalities and no hydroxyl endgroups.

One of the most important fragmentation pathways is the cleavage of the ester bond shown in Scheme **2**, which leads to fragment ion $[CE + H]^+$ and its counterpart $[C_2DE_2 + H]^+$ (see Figure 4 for the CAD spectrum of $[C_3DE_3 + 2H]^{2+}$). Fragment ion $[CDE + H]^+$ is due to an additional cleavage of an ester group from the primary fragment ion $[C_2DE_2 + H]^+$. Al-

though two covalent bonds have to be broken to obtain this fragment ion, $[CDE + H]^+$ is the base peak in the spectrum. The high abundance of this fragment ion indicates that consecutive cleavages may complicate the determination of the structure of hyperbranched polymers because this implies the occurrence of internal fragment ions, that is, fragment ions without one of the endgroups from the parent ion. That ester bonds are cleaved easily is an interesting result because the fragmentation behavior of the oligomers described in preceeding sections can all be explained by amide bond cleavages.

Ester cleavages are also observed if a relatively low collision energy (47 eV) is used to dissociate the $[C_3DE_3 + 2H]^{2+}$ ion. The $[C_2DE_2 + 2H]^{2+}$, $[CDE + H]^+$, and

Scheme 2. Consecutive cleavage of two ester bonds of doubly protonated C_3DE_3 upon low energy CAD.

Figure 4. Low energy CAD of $[C_3DE_3 + 2H]^{2+}$ ($E_{kin,lab}$ = 162 eV, m/z 552.29). The **i** indicates that the fragment ions are due to consecutive fragmentations. Additional losses of $NH(CH_3)_2$ are indicated with an arrow.

$[CE + H]^+$ fragment ions lose an additional $NH(CH_3)_2$ from the endgroup indicated by an arrow in Figure 4. More fragment ions have been observed that can only be explained by consecutive cleavages. Fragment ions that were attributed to consecutive fragmentations are denoted by an **i** in Figure 4.

Discussion CAD of doubly charged hyperbranched oligomers. The CAD mass spectra of doubly charged oligomer ions $[P_6D_7 + 2H]^{2+}$, $[C_6D_7 + 2H]^{2+}$ and $[G_7D_8 + 2H]^{2+}$ reveal a similar fragmentation behavior as the singly charged ions described in references [20, 28, 29], which was due to amide bond cleavages and additional H_2O losses. The nature of the di-acid does not have a significant influence on the appearance of the CAD MS/MS spectra. In contrast to findings with these oligomers, the ester bonds of oligomer $[C_3DE_3 + 2H]^{2+}$ are also cleaved. In addition, consecutive cleavages have been observed for $[C_3DE_3 + 2H]^{2+}$, thus leading to a less straightforward interpretation of the MS/MS spectra. This is most probably a direct result of the structural difference of the chain stopper compared to the endgroups of P_6D_7, C_6D_7, and G_7D_8. This structural difference has a significant influence on its fragmentation behavior. Consecutive cleavages giving rise to internal fragment ions are not observed for $[P_6D_7 + 2H]^{2+}$, $[C_6D_7 + 2H]^{2+}$, and $[G_7D_8 + 2H]^{2+}$ ions because this should lead to the ion series $X_nD_{n-1}-2H_2O$ (two amide bonds cleaved). This observation implies that CAD is a selective dissociation method for the study of the sequence of hyperbranched polyesteramides. However, branched polymers are not distinguished from linear polymers with the use of CAD.

ECD

The ECD behavior of the same ions as studied by CAD is discussed in the next sections in order to compare the potential of the two dissociation methods for the characterization of hyperbranched polyesteramide oligomers. ECD may lead to more structure-specific fragmentation of large molecules as has been shown for several biomolecules, in particular proteins. Less internal fragmentation, compared with low energy CAD, is expected because of the non-ergodic nature of ECD. The different dissociation reactions induced by ECD compared with CAD may open the possibility for the distinction between isomeric structures, such as branched and linear structures.

ECD of the phthalic acid based polymer. The ECD spectrum of $[P_6D_7 + 2H]^{2+}$ is presented in Figure 5. The ions are exposed to low energy electrons for 2 s. All of the 39 fragment ions that are observed are singly charged, in agreement with the ECD mechanism. The nomenclature of the ECD fragments that is introduced is different from the nomenclature as used for biomolecules. We have chosen to do so because the structures of both parent and fragment ions of hyperbranched polymers
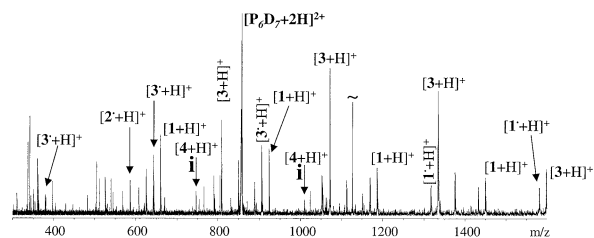
Figure 5. ECD of $[P_6D_7 + 2H]^{2+}$ (2 s) leads to the formation of fragment ions $[1 + H]^+$, $[1^\cdot + H]^+$, $[2^\cdot + H]^+$, $[3 + H]^+$, $[3^\cdot + H]^+$, and $[4 + H]^+$. Schemes **3**, **4**, **5**, and **7** are the proposed fragmentation pathways to obtain these fragment ions. The $\sim$ and **i** denote electronic noise and internal fragment ions, respectively.

and biomolecules are different. The ECD behavior of hyperbranched polyesteramides can therefore be very different from the ECD behavior of biomolecules.

Table 3 gives an overview of all fragment ions that are formed. In Scheme **3**, a proposal is shown for the cleavage of the amide bond with formation of the $[1 + H]^+$ and $[1^\cdot + H]^+$ fragment ions. Amide bonds are also cleaved (Scheme **4**) leading to $[2^\cdot + H]^+$ ions. Its counterpart, the $[2 + H]^+$ ion, is not observed. Fragment ions due to the cleavage of the ester bonds give rise to the most abundant peaks in the spectrum and are likely to arise by the capture of an electron followed by homolytic cleavage of the (CO)O–C bond (Scheme **5**). This pathway leads to two fragment ions indicated by $[3 + H]^+$ and $[3^\cdot + H]^+$. A fragment ion appears in the spectrum with a *m/z* value of 1 lower than the $[3^\cdot + H]^+$ ion. This particular fragment ion can be explained by the loss of an additional R″ from ion $[3^\cdot + H]^+$ to $[3' + H]^+$ via a 1,5 H-shift followed by homolytic cleavage of a (CO)–O bond (Scheme **6**). This results in internal fragment ions. Note that the radical fragment ion $[3^\cdot + H]^+$ becomes an even electron species after secondary fragmentation, as observed elsewhere [30, 31]. The formation of the fragment ion with a *m/z* value of one lower than the $[3^\cdot + H]^+$ ion can also be explained by the loss of H_2O from the $[1 + H]^+$ ion.

Fragment ions $[2^\cdot + H]^+$ and $[3 + H]^+$ also lose an additional H_2O, whereas the $[3^\cdot + H]^+$ ion loses up to two H_2O molecules. A possible explanation for the additional loss of H_2O is that not all energy gained by the ion upon electron capture is consumed in the primary cleavage of a bond. The remaining internal energy is ergodically distributed over the fragments and may lead to a subsequent loss of H_2O from one of the endgroups. Note that this observation is different from results published in the literature, which indicate that such consecutive cleavages do not occur. Another possibility for the formation of these ion series could be the cleavage of an amide bond. However, a loss of H_2O is more likely to be responsible for these ion series because this process can be associated with a lower critical energy than the cleavage of the amide bond. This explanation is in line with the results for collisionally activated dissociation of hyperbranched polyesteramide oligomers described previously [29].

Table 3. Fragment ions formed by ECD of $[P_6D_7 + 2H]^{2+}$, $[G_7D_8 + 2H]^{2+}$, and $[C_6D_7 + 2H]^{2+}$. The masses of the fragment ions can be obtained as described for Table 2
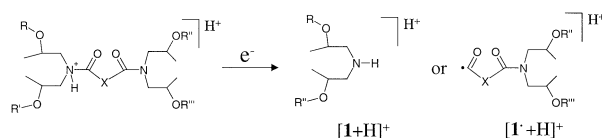
Fragment ion	$[P_6D_7 + 2H]^{2+}$	$[G_7D_8 + 2H]^{2+}$	$[C_6D_7 + 2H]^{2+}$
$[1 + H]^+$	134.116 ($n = 1$–5)	134.097 ($n = 2$–6)	134.124 ($n = 2$–6)
$[1^. + H]^+$	265.133 ($n = 4$, 5)		
$[2 + H]^+$		75.968 ($n = 5$–7)	76.097 ($n = 4$–6)
$[2^. + H]^+$	60.083 ($n = 2$–5)	60.014 ($n = 3$–6)	60.083 ($n = 2$–5)
$[2^. - H_2O + H]^+$	42.073 ($n = 2$–4)		42.084 ($n = 5$)
$[3' + H]^+$	19.019 ($n = 2$–6)	18.985 ($n = 4$–6)	19.023 ($n = 2$–6)
$[3 - H_2O + H]^+$	1.012 ($n = 2$–5)		1.048 ($n = 3$–6)
$[3^. + H]^+$	117.116 ($n = 1$–5)		
$[3^. - H_2O + H]^+$	99.105 ($n = 1$–5)		
$[3^. - 2H_2O + H]^+$	81.091 ($n = 1$–4)		
$[4 + H]^+$	220.144 ($n = 2$, 3)		226.180 ($n = 2$–4)
$[5 + H]^+$		232.015 ($n = 5$, 6)	272.189 ($n = 1$–5)
$[6 + H]^+$			118.117 ($n = 2$–5)
Unknown[a]		146.032 ($n = 5$, 6)	99.114 ($n = 2$–4)
		87.937 ($n = 6$)	186.150 ($n = 2$–4)
Total nr. of fragment ions	39	20	40

[a]Two fragment ion series appeared in the spectrum with an unknown origin.

Finally, a fragment ion is observed that is a direct product of two consecutive cleavages induced by an intramolecular H-shift. Scheme **7** shows the capture of an electron by the amide group followed by a cleavage of a N–CO bond and loss of CO. A 1,5 H-shift precedes the cleavage of the second bond with the formation of an internal fragment ion indicated by $[4 + H]^+$ (see also Figure 5).

The interpretation of the ECD spectra of these doubly protonated oligomers is very complex because several bonds are cleaved by different mechanisms. Four factors influence this complex ECD behavior: (1) The difference in proton affinity of the functional groups over the entire polymer chain is likely to be small. As a result, the proton involved in the capture of the electron is not necessarily localised on a specific site; (2) the polymer is a mixture of different isomeric structures that each will give rise to different ECD reactions; (3) an excess of internal energy after ECD leads to consecutive fragmentations. Such processes are well known from electron impact ionization; (4) ECD gives rise to a very complex ion chemistry that involves free radical chemistry.
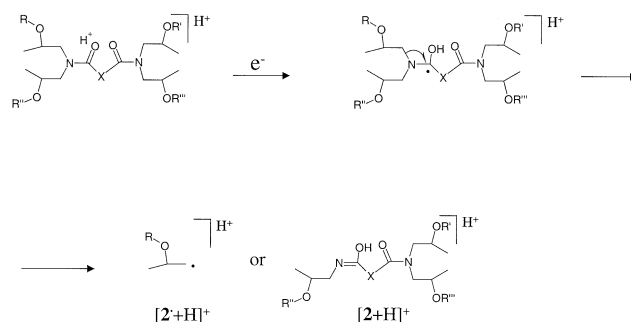
ECD of the glutaric acid based polymer. The ECD spectrum of $[G_7D_8 + 2H]^{2+}$ from which the CAD behavior was described earlier is presented in Figure 6. In total 20 fragment ions of the type $[1 + H]^+$, $[2^. + H]^+$, $[2 + H]^+$,

and $[3 + H]^+$ are observed. The ion $[2 + H]^+$ (Scheme **4**) is formed in this particular system (Figure 6) in contrast to the findings for the phthalic acid based polyesteramide polymer. The $[3^. + H]^+$ and $[2^. + H]^+$ ions are not formed whereas these ions were observed for the phthalic acid based polymer. In addition, fragment ions due to H_2O loss are also not present in the ECD experiments with the $[G_7D_8 + 2H]^{2+}$ ions. It can be thought that ECD of $[G_7D_8 + 2H]^{2+}$ results in fragment ions either with less excess internal energy or a higher critical energy for the consecutive cleavages compared to the phthalic acid containing polymers.

A new type of fragment ion series appears in the spectra that was not observed for the phthalic acid anhydride based polyesteramide. The possible mechanism for the formation and structure of these ions (denoted by $[5 + H]^+$) is presented in Scheme **8**. The positively charged carbonyl carbon captures an electron followed by a 1,4 H-shift and homolytic cleavage of the amide bond. The $[5 + H]^+$ ion with a ketene endgroup is observed whereas its counterpart, the $[5^. + H]^+$ ion, a secondary amine radical cation, is not.
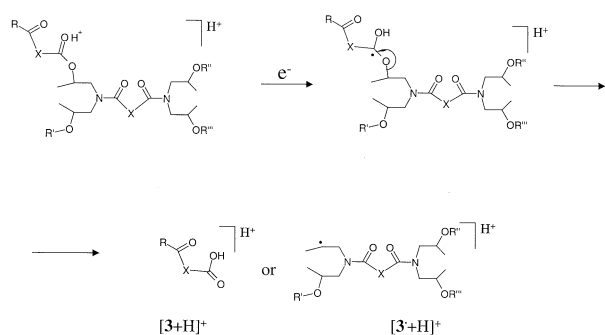

Scheme 3. Proposed scheme for the cleavage by ECD of the amide bond of a doubly protonated oligomer leading to $[1 + H]^+$ and $[1^. + H]^+$ ions. R, R', R'', and R''' can be replaced by hydrogens, PD units, or polyesteramide chains. X is a phenyl group.
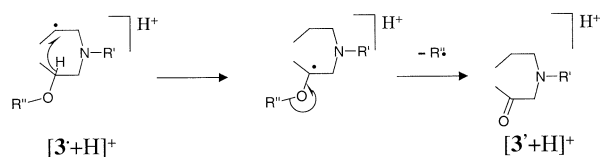

Scheme 4. Proposed scheme for the cleavage by ECD of the amide bond of a doubly protonated oligomer leading to $[2 + H]^+$ and $[2^. + H]^+$ ions.
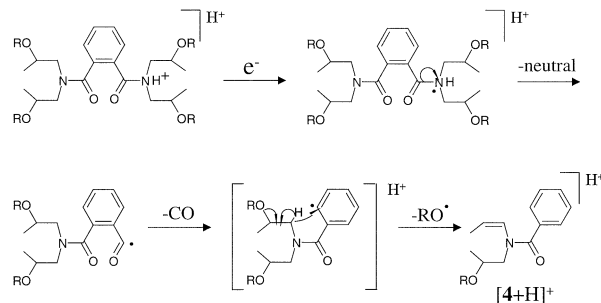
Scheme 5. Proposed scheme for the cleavage by ECD of the ester bond of a doubly protonated oligomer with the formation of fragment ions $[3 + H]^+$ and $[3^{\cdot} + H]^+$.
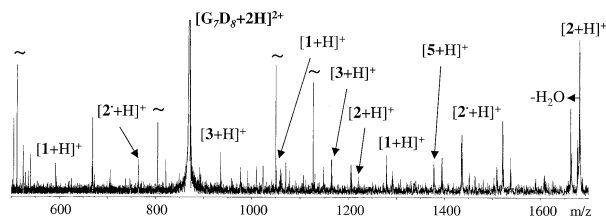

Scheme 7. Proposed scheme for the consecutive cleavage by ECD of two bonds induced by an intramolecular proton shift. This leads to the internal fragment ion $[4 + H]^+$.

ECD of the 1,2-cyclohexane dicarboxylic acid based polymer. ECD was performed with $[C_6D_7 + 2H]^{2+}$ for 2 s (spectrum not shown). Most of the fragment ions that are observed for the other polymers appeared in the ECD experiments with the charged 1,2-cyclohexane dicarboxylic acid based polyesteramide. Fragment ions of the type $[1 + H]^+$, $[2^{\cdot} + H]^+$, $[2 - H_2O + H]^+$, $[2 + H]^+$, $[3 + H]^+$, $[3 - H_2O + H]^+$, $[4 + H]^+$, and $[5 + H]^+$ due to the reactions in Schemes **3**, **4**, **5**, **7**, and **8** are formed. The formation of $[5 + H]^+$ ions can proceed by an opening of the cyclohexane ring. A new type of fragment ion appears in the spectrum that can be explained by a cleavage of the ester bond as shown in Scheme **9**. The formation of these fragment ions ($[6 + H]^+$) involves the capture of an electron by the positively charged carbonyl carbon followed by homolytic cleavage of the C–O bond.

ECD of the 1,2-cyclohexane dicarboxylic acid based polymer with bis(3-dimethylamino-propyl)amide endgroups. The complexity of the ECD spectra described above can be explained partly by the unknown isomeric composition of the parent ion. To investigate the effect of isomers, ECD experiments were performed with C_3DE_3 with only one isomeric structure as described in the CAD section. Figure 7 shows the ECD spectrum of the doubly protonated oligomer $[C_3DE_3 + 2H]^{2+}$. Fragment ions that appear are $[1 + H]^+$ (low abundance), $[2 + H]^+$, $[3 + H]^+$, $[3^{\cdot} + H]^+$, and $[5 + H]^+$. The pathways leading to these ions may be formulated in line with the mechanistic proposals in Schemes **3**, **4**, **5**, and **8**. Other fragment ions arise due the loss of a H atom, and the formation of $[7^{\cdot} + H]^+$ ions from $[1^{\cdot} + H]^+$ ions as a

result of a loss of a CO. A 1,5 H-shift and the consecutive cleavage of an ester bond to fragment ion $[4 + H]^+$ (in analogy with Scheme **7**) has also been observed.

Fragment ions due to the cleavage of two ester bonds are also observed. The first ester bond is cleaved to $[3^{\cdot} + H]^+$ ions followed by the cleavage of the second ester bond leading to a fragment ion with a 3,4-dimethyl pyrrolidine endgroup (see also Scheme **5**). A fragment ion with an m/z 1 lower than the m/z of fragment ion $[3 + H]^+$ is due to the formation of a $[2^{\cdot} + H]^+$ ion followed by propene loss, or due to a fragmentation mechanism outlined in Scheme **9** leading to $[6^{\cdot} + H]^+$ ions. Most interesting is the appearance of an abundant fragment ion ($m/z = 818.589$) that cannot be explained by any of the mechanisms described above but could result from consecutive cleavages of two or even more bonds. An explanation for the relatively high abundance of fragment ions that are due to the cleavage of multiple bonds, compared with the oligomers studied earlier, is that the C_3DE_3 oligomer does not contain OH endgroups. As a result, the fragment ions of $[C_3DE_3 + 2H]^{2+}$ cannot lose H_2O and must therefore consume their excess internal energy by an consecutive cleavage of an ester bond.

The ECD fragment ions of, in particular, the doubly protonated C_3DE_3, give direct evidence that consecutive cleavages occur. This clearly demonstrates that internal fragment ions upon ECD of hyperbranched polyesteramides can be pronounced, thus complicating the interpretation of the ECD spectra.

Scheme 6. Proposed scheme for the cleavage by ECD of an additional ester bond of a doubly protonated oligomer from $[3^{\cdot} + H]^+$ fragment ions with formation of the internal $[3' + H]^+$ fragment ions.

Figure 6. ECD of $[G_7D_8 + 2H]^{2+}$ (1 s) leads to the formation of fragment ions $[1 + H]^+$, $[2 + H]^+$, $[2^{\cdot} + H]^+$, $[3 + H]^+$, and $[5 + H]^+$. Schemes **3**, **4**, **5**, and **8** are the proposed fragmentation pathways to obtain these fragment ions. The ~ is electronic noise.
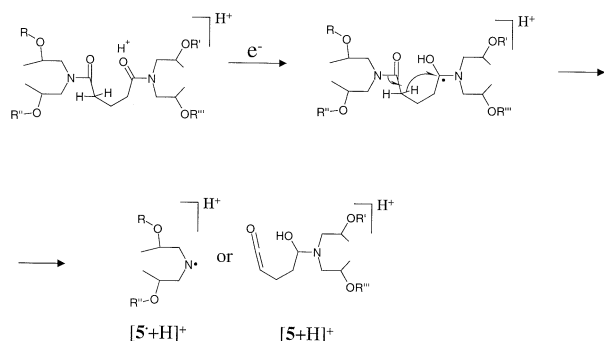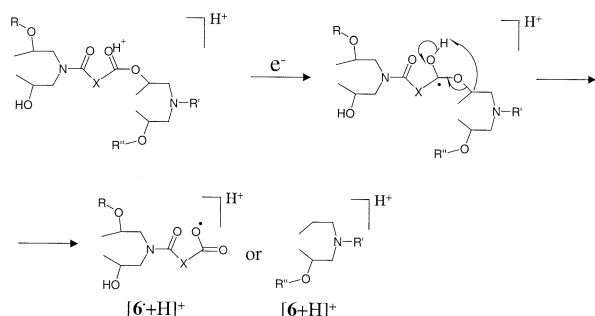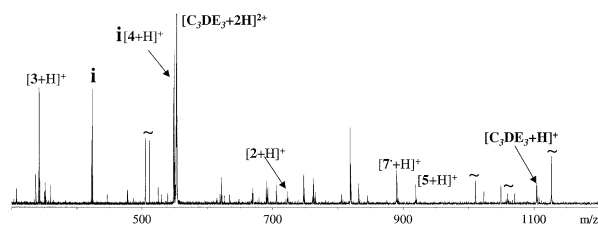
Scheme 8. Proposed scheme for the cleavage by ECD of the amide bond of a doubly protonated oligomer via a 1,4-hydrogen rearrangement leading to $[5 + H]^+$ and $[5^{\cdot} + H]^+$ ions.

Figure 7. ECD of $[C_3DE_3 + 2H]^{2+}$ (3 s) leads to the formation of fragment ions $[2 + H]^+$, $[3 + H]^+$, $[4 + H]^+$, and $[5 + H]^+$. Schemes **4**, **5**, **7**, and **8** are the proposed fragmentation pathways to obtain these fragment ions. Fragment ion $[7^{\cdot} + H]^+$ is proposed to originate from $[1^{\cdot} + H]^+$ as a result of a CO loss. The ∼ and **i** denote electronic noise and consecutive fragmentations, respectively. Note that the origin of most peaks is unknown but they are probably a result of complex internal rearrangements.

Comparison of CAD with ECD of Doubly Charged Hyperbranched Polymers and Concluding Remarks

Both low energy CAD and ECD are successful methods for dissociation of doubly protonated hyperbranched polyesteramide oligomers. Low energy CAD is a more selective dissociation method in view of the observation that only amide bonds are cleaved. ECD of doubly protonated hyperbranched polyesteramides results in different types of cleavages of the ester and amide bond leading to $OE^{\cdot+}$ and EE^+ ions. A significant influence of the nature of the di-acid on the appearance of the ECD spectra is observed, although the structures of the polymers are very similar. This provides complementary structural information on the di-acid in comparison with CAD where only amide bonds are cleaved. Four factors can explain the complexity of the ECD spectra: (1) The proton that is involved in the capture of the electron is not located on a specific site with a higher proton affinity than other sites. The polymers studied here contain the same type of functional groups along the polymer chain, which will have only a small difference in proton or sodium affinity. (2) The polymer is a mixture of different isomeric structures that can have a different ECD pattern. (3) An excess of internal energy after ECD leads to internal fragmentation. This may be due to the energetic and non-ergodic nature of the capture of an electron (∼6 eV) by the parent ion leading

to fragmentation and a distribution of the excess internal energy over the fragments, which is sufficient to cleave an additional bond. This process would lead to internal fragment ions that can complicate the interpretation of the ECD spectra. Such consecutive cleavages are well known from electron impact ionization. (4) ECD gives rise to a very complex chemistry that may involve free radical chemistry. Additionally, the origin of many fragment ions, especially from the C_3DE_3 oligomer, cannot be explained by the seven ECD schemes proposed in this paper and are most probably due to complex internal rearrangements prior to dissociation. The complexity of the ECD spectra and the formation of internal fragment ions can partly be avoided by performing ECD on sodiated oligomers (results not shown).

The results described in this paper demonstrate that ECD is a very useful method to induce cleavages that are not observed upon CAD. Such complementary cleavages can provide structural information for many classes of compounds. Additionally, ECD analysis is ∼10 times faster than the CAD experiments described here. Although ECD provides complementary fragments, for these relatively small oligomers of hyperbranched polyesteramides ECD does not provide complementary sequence information, as is the case for biomolecular analyses [4–11]. A number of limitations of the ECD method for these polymers may be mentioned here: (1) With the exception of C_3DE_3, low energy CAD cleaves the amide bonds selectively, providing the sequence of the mixture of different isomers. The complementary cleavages of the ester bonds and other types of cleavages observed with ECD do not provide additional information about the polymer monomer sequence. (2) Isomers could not be distinguished by ECD only, although this was possible with DoDIP. A combination of CAD with ECD or a combination of different ECD experiments with varying electron capture energy (for example high energy versus low energy ECD) can be a possible approach to obtain additional isomeric information. Note that neither dissociation method is able to distinguish between branched and non-branched oligomers. (3) CAD leads

Scheme 9. Proposed scheme for the cleavage by ECD of the ester bond of a doubly protonated oligomer leading to $[6 + H]^+$ and $[6^{\cdot} + H]^+$ ions.

to smaller fragment ions, which can give more information about the endgroups. (4) ECD leads to more internal fragmentation than CAD.

It will be interesting to extend this research to larger oligomers where CAD does not transfer enough energy to induce fragmentation. ECD will probably be useful for such oligomers because the excess energy must be distributed over many more degrees of freedom leading to a minimum of internal fragment ions. It will also be of interest to study the ECD behavior of polymers charged by different cations since this leads to less fragmentation as was observed for sodiated oligomers (results not shown). In conclusion, more extended studies of hyperbranched polyesteramides that contain different di-acids but only one type of isomeric structure are required to confirm the ECD mechanisms proposed in this paper.

Acknowledgments

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