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PII: S0003-2670(16)31286-7

DOI: 10.1016/j.aca.2016.11.001

Reference: ACA 234866

To appear in: *Analytica Chimica Acta*

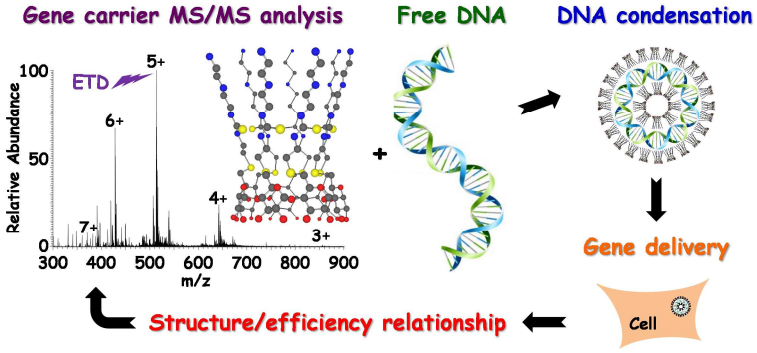
Received Date: 21 August 2016

Revised Date: 2 October 2016

Accepted Date: 4 November 2016

Please cite this article as: C. Przybylski, J.M. Benito, V. Bonnet, C.O. Mellet, J..M. García Fernández, Toward a suitable structural analysis of gene delivery carrier based on polycationic carbohydrates by electron transfer dissociation tandem mass spectrometry, *Analytica Chimica Acta* (2016), doi: 10.1016/j.aca.2016.11.001.

Graphical Abstract



Toward a suitable structural analysis of gene delivery carrier based on polycationic carbohydrates by electron transfer dissociation tandem mass spectrometry

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ABSTRACT

Polycationic carbohydrates represent an attractive class of biomolecules for several applications and particularly as non viral gene delivery vectors. In this case, the establishment of structure-biological activity relationship requires sensitive and accurate characterization tools to both control and achieve fine structural deciphering. Electrospray-tandem mass spectrometry (ESI-MS/MS) appears as a suitable approach to address these questions. In the study herein, we have investigated the usefulness of electron transfer dissociation (ETD) to get structural data about five polycationic carbohydrates demonstrated as promising gene delivery agents. A particular attention was paid to determine the influence of charge states as well as both fluoranthene reaction time and supplementary activation (SA) on production of charge reduced species, fragmentation yield, varying from 2 to 62%, as well as to obtain the most higher both diversity and intensity of fragments, according to charge states and targeted compounds. ETD fragmentation appeared to be mainly directed toward pending group rather than carbohydrate cyclic scaffold leading to a partial sequencing for building blocks when amino groups are close to carbohydrate core, but allowing to complete structural deciphering of some of them, such as those including dithioureidocysteaminyl group which was not possible with CID only. Such findings clearly highlight the potential to help the rational choice of the suitable analytical conditions, according to the nature of the gene delivery molecules exhibiting polycationic features. Moreover, our ETD-MS/MS approach open the way to a fine sequencing/identification of grafted groups carried on various sets of oligo-/polysaccharides in various fields such as glycobiology or nanomaterials, even with unknown or questionable extraction, synthesis or modification steps.

Keywords (6 max): polycationic, carbohydrates, ETD, cyclodextrin, ESI-MS/MS, gene delivery,

1. INTRODUCTION

Polycationic polysaccharides can be encountered alone or conjugated to other biomolecules.[1-3] They can be obtained directly after extraction/purification steps from natural matrix as chitosan [4, 5] or by chemical or enzymatic modification of neutral or anionic carbohydrates such as alginate, dextran, pullulan, cellulose, cycloamylose, carboxymethylcellulose or also hyaluronic acid.[6-10] Their applications are various ranging from agro-alimentary to medicine. A particular attractive field which has constantly gained interest is the capacity of some of them to efficiently compact polynucleic acids such as DNA/RNA acting as potential non-viral gene delivery agents.[8, 9, 11-14] Nevertheless, synthesis of such architectures requires several time consuming steps and the use of often heterogeneous starting materials. One relevant solution consists to use molecules with both well-defined structure and size to efficiently delineate a structure/activity relationship. In this sense, oligosaccharide based scaffolds such as cyclodextrins (CDs) after modification to enhance their basicity properties are attractive candidates for efficient gene delivery.[15-20] Nevertheless, even with such edifices, a straightforward structural control of the end products in term of size and number of grafted functions must be achieved. Nowadays, few analytical techniques are available to address those requirements.

Since two decades, mass spectrometry (MS) with its emblematic ionization mode, electrospray (ESI) has emerged as a forefront suitable technique to efficiently portray the solution content. Due to its gentle process and sensitivity features, ESI-MS allows the study of a wide variety of compounds even at trace levels and/or carrying labile moieties. These two essential criteria allowing a low sample consumption and access to a structural deciphering *via* multistep fragmentation, make to ESI-MS^n a major tool for oligo-/polysaccharide characterization. Nevertheless, CDs as other neutral oligosaccharides present an intrinsic preference for alkali metals than protons.[21,

22] Consequently, the widely used collision induced dissociation (CID) fragmentation mode using multi-protonated molecules can be limited to finely characterize some moieties grafted on such oligosaccharides.[23] More recently, an efficient MS fragmentation mode named electron transfer dissociation (ETD), has been introduced by Hunt, Coon, Syka and coworkers.[24-26] ETD induces fragmentation of multiply charged molecules subsequently to electron transfer from an anionic reagent to cations. Initially, ETD has been developed to preserve labile post translational modifications of proteins and furthermore to increase fragmentation efficiency of multiply charged whole protein during ESI-MS^n analysis.[25, 26] Nevertheless, ETD potential has been sporadically applied to other molecules such as synthetic polymers,[27] combinatorial chemistry end products,[28] pyridinium-based amino acid analogs (desmosine and isodesmosine),[29] crosslinking elastin[29], or also glycerophosphocholine lipids.[30] Few studies report the use of ETD for carbohydrate analysis and concerning exclusively neutral structures like milk oligosaccharides,[31] maltodextrin pentamer[32] or lacto-*N*-fucopentaose/difucohexaose.[33] To obtain dicharged species, their ionization was assisted by coordination with ammonium or more specifically with cations of group IA (Na^+, Li^+, K^+ Rb^+, Cs^+), group IIA (Ca^{2+}, Mg^{2+}, Be^{2+}, Sr^{2+}, Ba^{2+}) and group IIB (Zn^{2+}, Hg^{2+}).[31-34] Parameters affecting ion/ion reactions leading to optimal fragmentation efficiency of peptide such as the identity of the charge-bearing, cation charge state, the choice of the reagent, the reagent reaction time, the precursor/reagent ratio, and the Mathieu Q parameter have been thoroughly investigated.[35-37] All these studies have unambiguously demonstrated their influence on the ETD efficiency. Among them, it clearly appeared that the reaction time is the most important factor which directly correlates to the charge states as well as the location of charge sites i.e. the fine structure.

In this paper, we investigated the behaviour of a library of cyclic polycationic carbohydrates upon ETD-MS/MS. A particular attention was paid to delineate the effect of reagent reaction time both for various charge states of a given compound or for the same charge state of different compounds. Moreover, both the fragments and the charges of the reduced species content resulting from ETD, as well as the usefulness of supplementary activation were estimated. Considering an identical scaffold (cyclodextrins; CD), carrying various structural elements with protonation sites, the study herein aimed to establish particular reaction time/charge states/side modifications relationship occurred. ETD fragmentation efficiency was finally compared with CID only for the structural deciphering of the various molecules.

2. MATERIALS AND METHODS

2.1 Reagents.

Methanol (MeOH) used for sample preparation was of HPLC grade and was purchased from VWR (West Chester, PA, USA). Water was of ultrapure quality, obtained from a MilliQ apparatus (Millipore, Milford, USA).

2.2 Samples.

Synthesis of per-6-modified-β-CD (Figure 1, Compounds **1**-**5**) were realized as previously described.[38, 39] Samples were prepared at 1 mg/mL in water/methanol 1/1 (v/v)

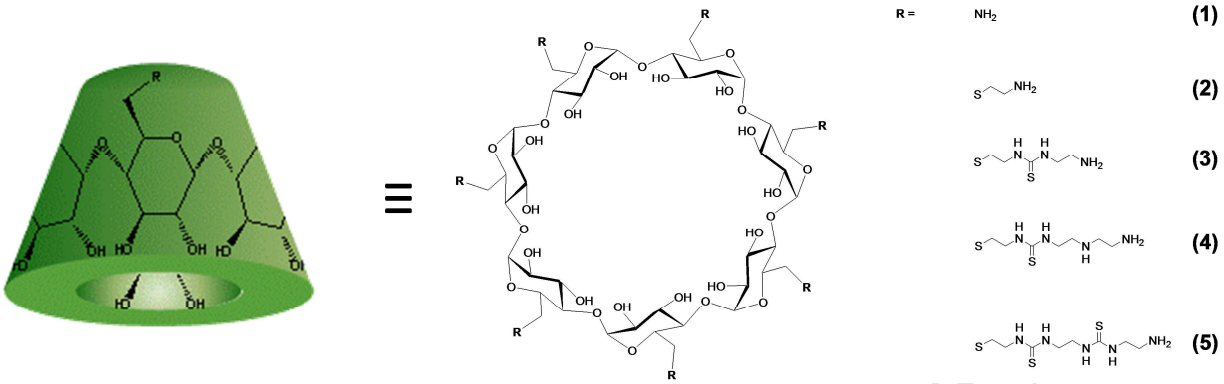

Figure 1. Structure of the polycationic carbohydrates library studied here (value in bracket indicates the compound's number). Compounds are (**1**) Per-6-amino-β-CD (monoisotopic mass: 1127.4817 g/mol and average mass: 1128.0912 g/mol), (**2**) Per-6-cysteaminyl-β-CD (monoisotopic mass: 1547.5053 g/mol and average mass: 1548.9253 g/mol), (**3**) Per-6-thioureidocysteaminyl-β-CD (monoisotopic mass: 2261.6814 g/mol and average mass: 2264.0398 g/mol), (**4**) Per-6-aminoethylthioureidocysteaminyl-β-CD (monoisotopic mass: 2562.9768 g/mol and average mass: 2565.5147 g/mol) and (**5**) Per-6-dithioureidocysteaminyl-β-CD (monoisotopic mass: 2975.8576 g/mol and average mass: 2979.1544 g/mol).

2.3 Mass spectrometry.

ESI-MS experiments were carried out using a LTQ-Orbitrap XL from Thermo Scientific (San Jose, CA, USA) and operated in positive ionization mode, with a spray voltage at 3.7 kV. A water/methanol 1/1 (v/v) mixture was continuously infused using a 500 µL syringe at 3 µL/min flow. Applied voltages were 31 and 115 V for the ion transfer capillary and the tube lens, respectively. The ion transfer capillary was held at 275°C. Resolution was set to 60 000 (at m/z 400) for all studies, and the m/z ranges were set to 200-2000 (normal mass range) or 200-4000 (high mass range) in profile mode during full scan experiments. Spectra were analyzed using the acquisition

software XCalibur 2.0.7 (Thermo Scientific, San Jose, CA, USA), without smoothing and background subtracts. During MS/MS scans, collision induced dissociation (CID) fragmentation occurred in the linear ion trap analyzer and detection in Orbitrap with centroid mode. For CID fragmentation, an activation Q value of 0.25 and an activation time equal to 30 ms were used. Normalized collision energy (NCE) between 0-40% was used. The automatic gain control (AGC) allowed accumulation up to 1.10^6 ions for FTMS scans, 2.10^5 ions for FTMSn scans and 1.10^4 ions for ITMSn scans. Maximum injection time was set to 500 ms for both FTMS and FTMSn scans and 100 ms for ITMSn scans. For all scan modes, 1 µscan was acquired. The precursor selection window was 2 Da. For ETD reagent (fluoranthene), the AGC was set to 2×10^5, and reaction time was varied from 0.03 to 250 ms with or without supplemental activation (SA) from 0 to 20% as specified in the text.

3. RESULTS AND DISCUSSION

3.1 Charge state/ETD reaction times relationship

The effect of charge state and the reagent reaction time on the dissociation rate of polycationic carbohydrates upon electron transfer from a radical anion issued from fluoranthene was investigated on a series of five molecules exhibiting a number of theoretical protonable primary and secondary amino groups ranging from 7 to 35 or 14 considering that thiourea can be potentially protonable or not (Figure 1). Nevertheless, the observed charge states were always lower than these maximum possible values (Figure 2 and see the Supporting Information Figure S1). Indeed, in our experimental conditions, the mass spectra revealed charge states ranging from 1+ to 7+ according to compound features (Figure 2 and see the Supporting Information Figure S1).

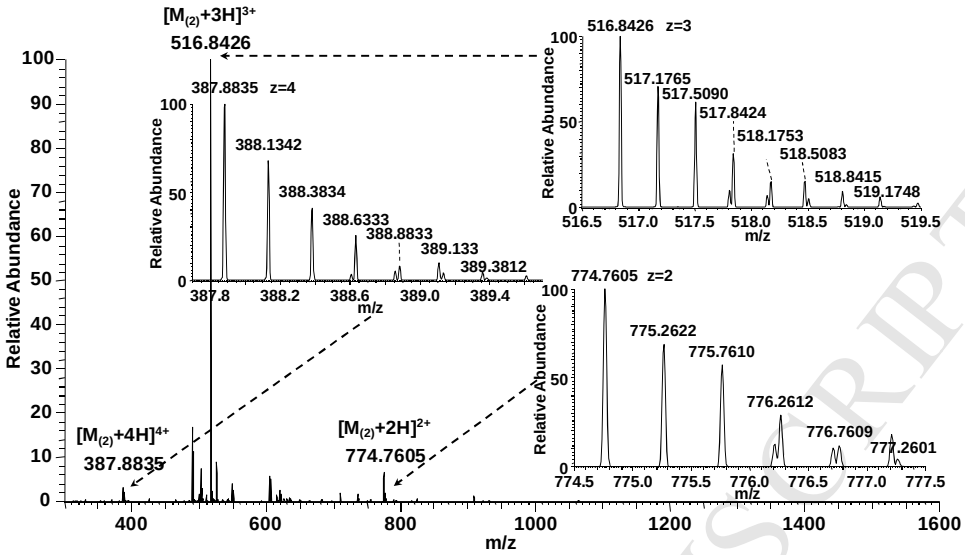

Figure 2. MS spectrum of per-6-thioureidocystenaminyl-β-CD (Compound **2**). Insets indicate isotopic clusters.

As example, the MS spectrum of per-6-thioureidocystenaminyl-β-CD (Compound **2**) was dominated by an ion at m/z 516.8426 (monoisotopic theoretical: 516.8429; 0.6 ppm) assigned to $[M+3H]^{3+}$.Two other ions were also detected at 774.7605 (monoisotopic theoretical: 774.7605; 0 ppm) and 387.8835 (monoisotopic theoretical: 387.8841; 1.6 ppm) corresponding to $[M+2H]^{2+}$ and $[M+4H]^{4+}$, respectively (Figure 2).

Defining an optimal reagent reaction time according to charge states and structural features are a major primer as evidenced elsewhere for peptide ETD fragmentation.[35] For this, we have plotted the absolute ion intensity as function of reaction time for the five studied carbohydrates and various charge states. The total ion current can be decoupled from three of these components namely the precursor signal, the resulting reduced charge species (CRS) and the part corresponding to fragment ions. Analysing the isotopic distribution of the CRS, it allows to delineate the

contribution of two common channels: the hydrogen abstraction from the proton transfer reaction ($[M+(n)H]^{n+}$) and the electron transfer without dissociation ($[M+(n+1)H]^{n+\bullet}$). Similarly, the dissociation events can be observed both consecutively to proton transfer reaction (PTR) and by electron transfer dissociation (ETD). As example, for the doubly protonated ion of Compound **2**, the maximal ion intensity is obtained at 66 and 100 ms for the fragments and the reduced charge species, respectively (Figure 3A). This slight shift suggests that increasing reaction time from 66 to 100 ms leads to more conversion of precursor to reduced charge species while fragments diminish dramatically. The main consequence of longer reaction time is the further decrease of reduced charge species and fragments as well as the total ion current (Figure 3A).

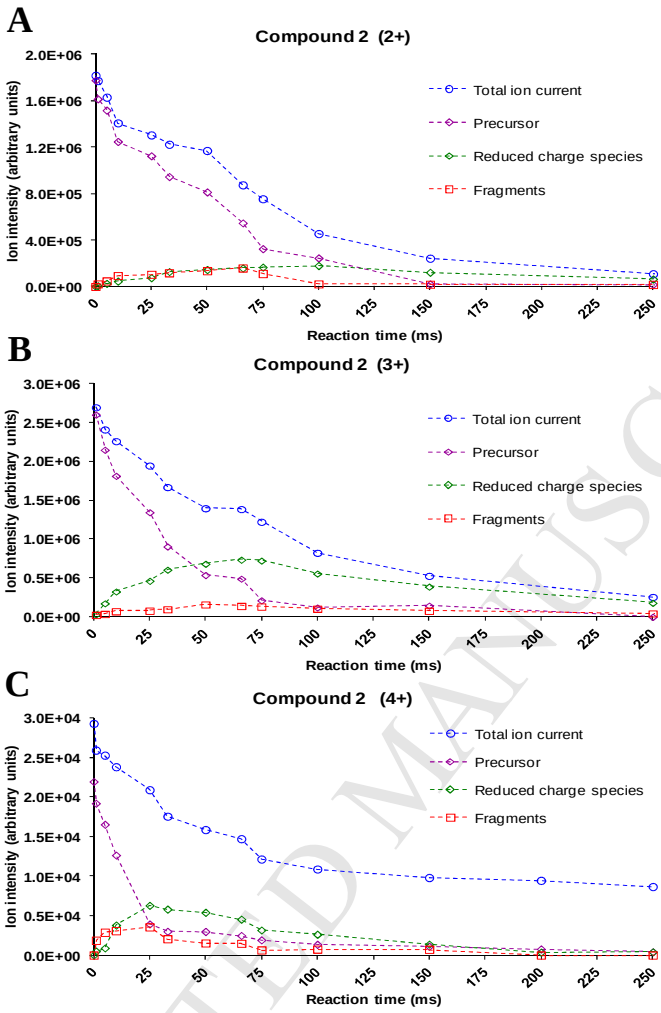

Figure 3. Variation of some ion currents from ETD-MS/MS experiments on per-6-thioureidocystenaminyl-β-CD (Compound 2) as function of reagent reaction time for 2+ (A), 3+ (B) and 4+ (C) charge states.

An identical behaviour was obtained for the 3+ precursor, with optimal values of 50 and 66 ms, for the fragments and the reduced charge species, respectively (Figure 3B). Concerning the 4+ precursor, the optimal reaction time was 25 ms giving priority to both reduced charge species and fragment intensities (Figure 3C). This result indicates that the charge localisation is kinetically

favourable to a fast conversion of precursor to fragment as well as CRS until 25 ms (Figure 3C). To memory, it was demonstrated that ion/ion reaction rates increase proportionally to the square of the charge.[26] Moreover, this phenomenon could be also emphasized by intramolecular electron transfer like a close Rydberg shell promoting electron attachment in peptides, as previously described by Simons.[40, 41] According to the various screened reaction time, we determined that the charge states yielding to both the widest diversity and the most intense fragments were 3+ and 4+ for Compounds 1/2 and 3/4/5, respectively. It can be quoted out that such attributed charge states did not necessarily correspond to the most intense ions obtained by full MS scan. Therefore, as the privileged criteria was the fragmentation yield, the optimal reaction time values were 66 and 50 ms for the 2+ and 3+ Compound **2** precursor (Table 1). Optimal reaction times were between 10 to 150 ms, according to both charge states and compound structural features (Table 1).

Table.1 Optimal fluoranthene reaction times as function of the charge states for the five studied compounds.

Compound	Reagent reaction time (ms) / charge states					
	2+	3+	4+	5+	6+	7+
1	150	66	33	-	-	-
2	66	50	25	-	-	-
3	100	100	66	33	12.5	ND
4	ND	66	50	33	12.5	ND
5	-	66	33	12.5	10	ND

ND: no fragment detected.

Further examination of results unambiguously shows a gradual decrease of required reaction time by 1.5-3 folds as charge state increases, whatever the compound. More intriguing is the no negligible variation at a given charge state *i.e.* 66-150, 66-100, 25-66 and 12.5-33 ms, for 2+, 3+, 4+ and 5+, respectively. However, closer values were obtained for 6+ (10-12.5 ms) for Compounds **3** to **5**. It was also observed that ETD fragmentation of some low intensity ions e.g. 2+ of Compound **4** and 7+ of Compounds **3** to **5**, did not give fragments. Such differences for 2+ to 5+ highlighted that optimal reaction time are not only depending on the number of possible charges but also on the structural features. Some variations could originate from the proton affinity (PA) of the various functions contained in CD grafted chains. Among them, we delineate methylamine (PA: 899.0 kJ/mol) for Compound **1**, ethylamine (PA: 912.0 kJ/mol) for Compounds **2** to **5**, one dimethylamine (PA: 929.5 kJ/mol) for Compound **4** and one or two *N*,*N* dimethylthiourea moieties (PA: 926.0 kJ/mol) in Compound **3** and **4**/**5**, respectively.

Another factor to take into account is the gradual extension of the grafted arm length enhancing flexibility, although the thiourea ethers are rather rigid involving a lesser flexibility on compound **5** as compared to compound **4**. Nonetheless, a wide distribution of amino groups can potentially promote intra-chain proton transfer processes. Such influence of the structure is quite similar to those observed for peptides where identity and position of basic residues (Arg, Lys and His) within backbone, play a key role in the ETD efficiency.[36, 37] In addition, we noted significant differences as compare to values observed in literature for other molecules with fluoranthene. Indeed, the most wide reaction time used for ETD carbohydrates and peptide analysis is 120-160 ms[42] and 50-150 ms, respectively,[31, 43] where for peptides mostly selected independently of the charge states or applying an empirical formula like reaction time (ms): 100 × 2/z, with z being

the precursor charge state.[44, 45] Meanwhile, literature reported that 100 ms were required for doubly sodiated glycerophosphocholine based lipids,[30] or doubly protonated (iso)desmosine,[29] 120-160 ms for doubly cationizated neutral oligosaccharides[31] or 100 and 150 ms for doubly and triply protonated chiral pentenoic amides.[28] Finally, we conclude that reaction times were lower for 3+ to 6+ with our cyclic polycationic carbohydrates than those commonly set for other described compounds.

3.2 Delineating of PTR, ETD and ETnoD contribution

It is well known that during an ETD reaction, several channels can be obtained involving concomitantly or not the transfer of an electron from reagent to the precursor inducing its fragmentation (ETD) or not (ETnoD), or also the abstraction of one hydrogen from precursor (PTR). Due to numerous amino groups of our carbohydrates, a not negligible part of channels other than ETD occurs as evidenced by ETD-MS/MS spectra of 4+, 3+ and 2+ precursors from Compound **2** in Figure 4. It was previously demonstrated on peptides, that partitioning of the different channels can be affected by identities of the reagent and the locations of the charge carrying sites.[24, 37, 46] Each channel contribution can be estimated in percentage after division by the total ion current. As example, analyzing of the one fold reduced charge isotopic pattern resulting from dissociation of 2+, 3+ and 4+ precursor of Compound **2** (insets Figure 4A-C), unambiguously highlighted the occurrence of simultaneous PTR (81, 62 and 52%, respectively), while ET is divided in ETD (5,17 and 31%, respectively) and ETnoD (14, 21 and 17%, respectively); McLuckey and others have clearly evidenced that proton transfer is favoured over electron transfer for a multiply protonated molecules such as peptides, and that competition occurred between the two channels only when the species from which the negative ion is formed have a low electron affinity and the

anion has favourable Frank-Condon factors associated with the transition from anion to neutral.[37, 47] Here, we observed that for a given compound, the higher the charge state, the higher ETD process occurred as compared to ETnoD and PTR reaction. Indeed, the number of charges strongly affects energy surfaces and consequently the products partitioning during the reaction.[36]

Similarly, for a given charge state, ETD is also depending on the compounds and especially, more promoted by the increase number of amino groups within (see the Supporting Information, Table S1). These results were consistent with some observations obtained elsewhere for a large collection of peptides exhibiting charge states from 2+ to 7+,[36] and from a given charge state to the next to examine the roles of the sites of protonation.[37] Efficient fragmentation can be diminished leading to poor structural information with dominant charge reduction process as ETnoD, which considerably reduces the ETD fragmentation efficiency.

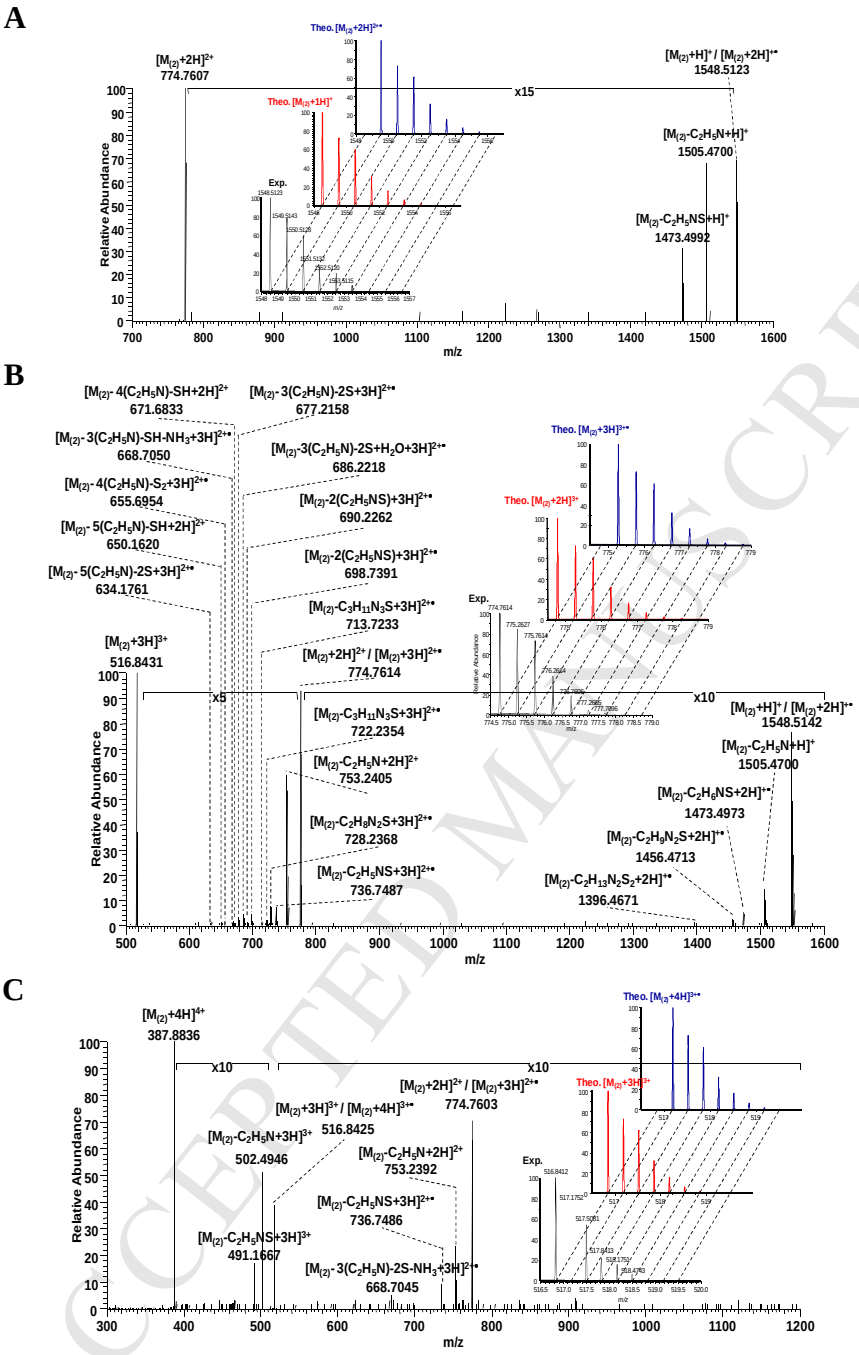

Figure 4. ETD-MS/MS spectra of $[M+2H]^{2+}$ (A), $[M+3H]^{3+}$ (B) and $[M+4H]^{4+}$ (C) ions from per-6-thioureidocystenaminyl-β-CD (Compound **2**). Insets indicate experimental (black) and theoretical both charge reduced precursor (red) and electron-transfer no dissociation (blue), isotopic clusters.

Such process, which readily occurred with peptides, was also preponderantly observed with our molecules. To overcome this shortcoming and promote more extensive fragmentation, a particular attention must be paid to limit such side reaction, by subsequently activating analyte during reaction *via* SA. Several means have been employed to reach its goal with peptides or also oligonucleotides.[42, 48-51] However, the most easily available on the marketed instrument is the CID.[31, 42, 50] It must be kept in mind that in contrast to CID based SA applied to peptide and generating a mixture of fragments characteristic of both modes (b and y and c and z-type products), here fragments corresponding to CID and ETD are present even without SA. Consequently, the main goal for our polycationic carbohydrates is to obtain more fragments with better intensity and thereby increasing the performance of ETD.[24, 25, 42, 51]

3.3 ETD with CID based supplementary activation (SA)

As aforementioned, the important level of PTR and species are an impediment for the more complete structural deciphering of our compounds. To diminish them, SA was varied from 0 to 20% by step of 5% for each optimal reaction time previously defined. For example, Figure 5 represents the influence of SA values on the triply charged ions precursor, reduced charge species and detected fragments of the five compounds studied. As previously observed for the variation of ETD reaction time, increasing of SA by deposited CID energy constantly decreases both the total ion current (data not shown) and the precursor intensity (Figure 5A). According to structural features, different behaviours were observed upon SA. At 5% SA, abundance of precursor of Compound **1** remains unaffected while others were slightly reduced (losses of 3-10%). In the same time, the CRS were diminished by 25, 6 and 43% for Compounds **1**, **2** and **3**, respectively, while those of Compounds **4** and **5** were slightly enhanced by 4 and 1%, respectively. It seems that a

small part of CRS (+2 to 9%) was converted to detectable fragments as revealed by homogeneous increases for all studied Compounds (Figure 5C). The doubling of SA i.e. 10% led to precursor decrease with a minimal loss for Compound **1** (-5%), intermediate for Compounds **3** to **5** (-11 to -15%) and drastic for Compound **2** (-32%).

In parallel, CRS of all structures were diminished, ranging from -8/14%, -31/40% and 73% for Compounds **4**/**5**, **1**/**2** and **3**, respectively. We noted an increase of fragments abundance equal to 9, 52, 19, 18 and 29% for Compound **1** to **5**, respectively. Taking into account the sum of precursor and CRS intensities in one hand and fragment intensities on the other hand, we determined that only one quarter of the losses of precursor and CRS are converted into fragments for Compounds **1** and **3**. Meanwhile, almost three quarters and entirety are converted for Compounds **2** and Compounds **4**/**5**, respectively. Further increase to SA 15% accentuated the decrease of both precursor

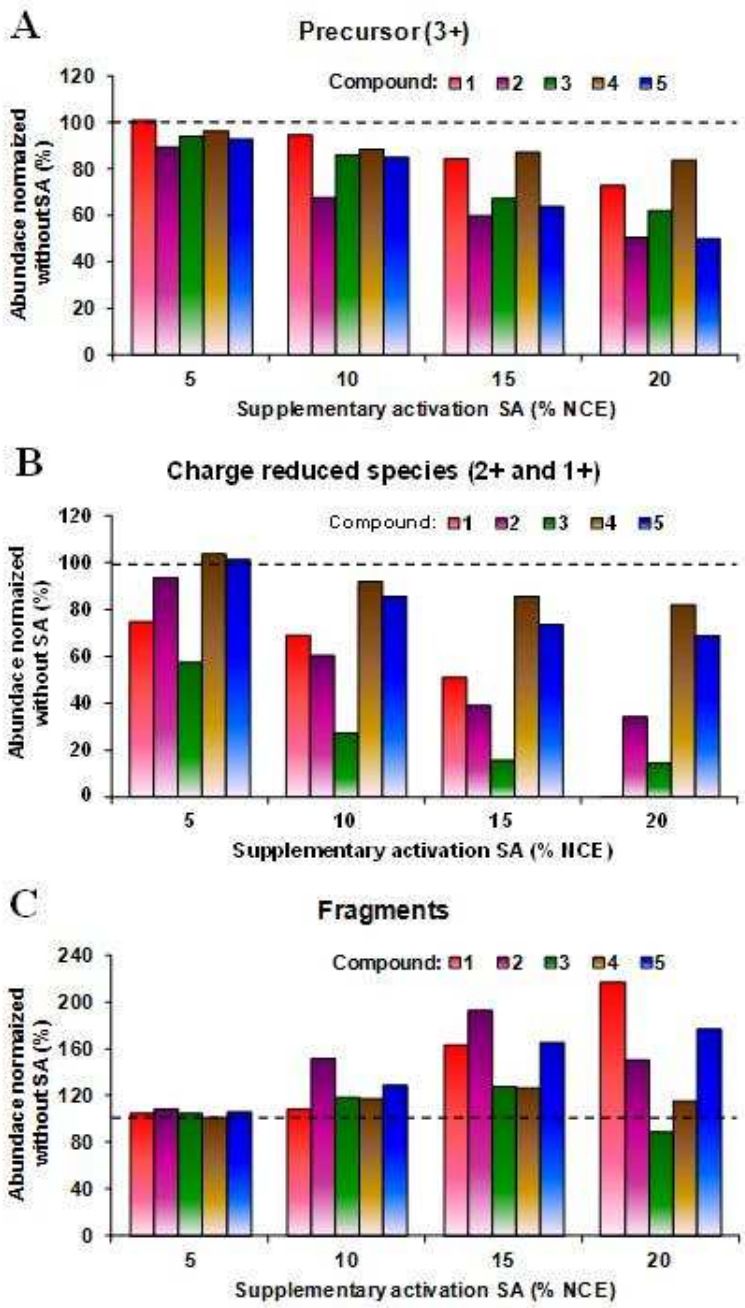

Figure 5. Effects of supplementary activation (SA) on the ion abundance of 3+ precursor (A), charge reduced species (B) and fragments (C), obtained after normalization from ETD-MS/MS experiments on Compounds **1** to **5**. Standard deviation (3.2-5.1%) from five replicates was omitted for clarity.

and CRS intensities for all compounds. However, some subtle differences occurred, since compared to SA 10%, precursor of Compound **1**, **2** and **4** are weakly affected with moderate decrease of 10, 8 and 1%, respectively, while for Compounds **3** and **5**, -19 and -21% were readily observed. Similarly, additional reduction of CRS levels by 6 to 21% was also observed as compared to SA 10%. Moreover, except for Compound **3** where only one fourth of precursor and CRS ions are converted into fragments, the losses of such ions corresponding to Compounds **1**, **2**, **4** and **5** were almost all quantitatively ascribed to their yielded fragments. Application of a maximal value of 20% SA led to a minor precursor diminishment from -3 to -14% compared to SA 15%, and reaching 16 to 50 %, considering those obtained without SA. Concerning CRS, only 2 to 5% of additional signal reduction occurred from SA 15 to 20% for Compounds **2** to **5** while a complete signal extinction was obtained for Compound **1** (Figure 5B).

3.4 Comparison of CID versus ETD with or without SA

Upon CID fragmentation mode, triply charged precursor of Compound 1 mainly yields to glycosidic cleavages detected only under lower charge states like $[C_7/Z_n+2H]^{2+}$ with n=6, 5, 4 and 3 at *m/z* 484.2148, 403.6793, 323.1456 and 242.6111, respectively and $[C_7/Z_n+H]^{+}$ with n=4, 3, 2 and 1 at *m/z* 645.2829, 484.2148, 403,6793, 323,1456 and 162.0764, respectively (Figure 6A). Thorough examination of isotopic pattern allows to unambiguously reveal the simultaneous presence of 1+/2+ ions for some m/z. Several water losses were also observed (-18.011 mass units according to corresponding charge states, ♦), as well as minor losses of NH_3 (-17.027 mass units, ◊) present under triply charged forms. Corresponding ETD MS/MS spectrum showed charge reduced species with PTR ($[M+nH]^{n+}$) and ETnoD ($[M+nH]^{n-1+\bullet}$) at *m/z* 564.7488 and 1128.4882 (Figure 6B). Three minor ions resulting from ammoniac or from low water losses at m/z

556.7389 and 370.8318/466.2140, respectively, were also detected. Only one fragment corresponding to glycosidic bond rupture was obtained at *m/z* 484.2147 ($[C_7/Z_6+2H]^{2+}$). Unfortunately, applying the maximal permitted value of 20% SA, it only led to more ammoniac or water losses and except for a series of ions starting from *m/z* 532.2240 attributed to the loss of one short exocyclic arm $CHNH_2$ ($[M-2H_2O-CHNH_2+2H]^{2+}$), it did not improve the sequence coverage by increasing number of glycosidic or cross-ring cleavages (Figure 6C). Hence, ETD-MS/MS with or without SA yields relatively poor spectra as compare to those obtained upon CID for compounds 1 (Figure 6) and 2 (data not shown). Nonetheless, a different trend was quoted out from compound 3 to 5. Indeed, it was previously observed that the higher the grafted arm length, the lower the CID fragmentation yield.[23] Such CID reduced fragmentation efficiency results in either exclusive grafted arms sequencing (Compound 3), or both absence of carbohydrate cleavage with only a partial identification of cationic chain (Compound 4 and 5). [23] Such detrimental effect of CID fragmentation on the sequence coverage was especially impressive for Compound 5 (Figure 6D). Indeed, the fragmentation yield reached only ≈ 15-20% and spectrum includes some ammoniac losses (*m/z* 736.4581 and *m/z* 732.2039 for $[M-2NH_3+4H]^{4+}$ and $[M-3NH_3+4H]^{4+}$, respectively) and loss of $CS(CH_2)_2(NH)_2$ with or without ammoniac loss (*m/z* 953.2758 or *m/z* 719.4658 for $[M-CS(CH_2)_2(NH)_2-NH_3+3H]^{3+}$ and $[M-CS(CH_2)_2(NH)_2+4H]^{4+}$) (Figure 6D). The ETD MS/MS spectrum still exhibits several ammoniac losses and partial chain sequencing as already observed during CID or consecutive to electron capture, such as *m/z* 959.2876 and 1438.4275 for $[M-CS(CH_2)_2(NH)_2+4H]^{3+\bullet}$ and $[M-CS(CH_2)_2(NH)_2+3H]^{2+\bullet}$, respectively. Two fragments issued from the electron transfer dissociation process were detected at *m/z* 944.9402 ascribed to $[M-CSNH(CH_2)_2(NH)+4H]^{3+\bullet}$ and 925.2792 which could be attributed to loss of either $CSNH(CH_2)_2NH$ group from two distinct end arms or one unique

$CSNH(CH_2)_2NHCSNH(CH_2)_2NH_2$ from only grafted moiety. The last choice was preferred, since no ion was detected corresponding to two simultaneous $CSNH(CH_2)_2NH$ losses, supporting the fragmentation of a unique chain (Figure 6E). These results highlight a significant improvement as compared to CID, where only the thiouredido moiety at the end of dithioureidocysteaminyl group have been sequenced (Figure 6D).[23]. Applying SA to the prior ETD activation, it strongly increases the fragmentation efficiency, allowing to completely sequence the dithioureidocysteaminyl chain thanks to two supplementary fragments at *m/z* 910.9309 and 898.5949 ascribed to $[M-(CS)_2(CH_2)_6(NH)_4+4H]^{3+\bullet}$ and $[M-SH_2\ (CS)_2(CH_2)_6(NH)_4+4H]^{3+\bullet}$, respectively. Moreover, the carbohydrate containing building block can be also identified by some ions such as the triply charged one at *m/z* 845.2491 corresponding to one glycosidic cleavage as $[C_7/Z_6+4H]^{3+\bullet}$ (Figure 6F).

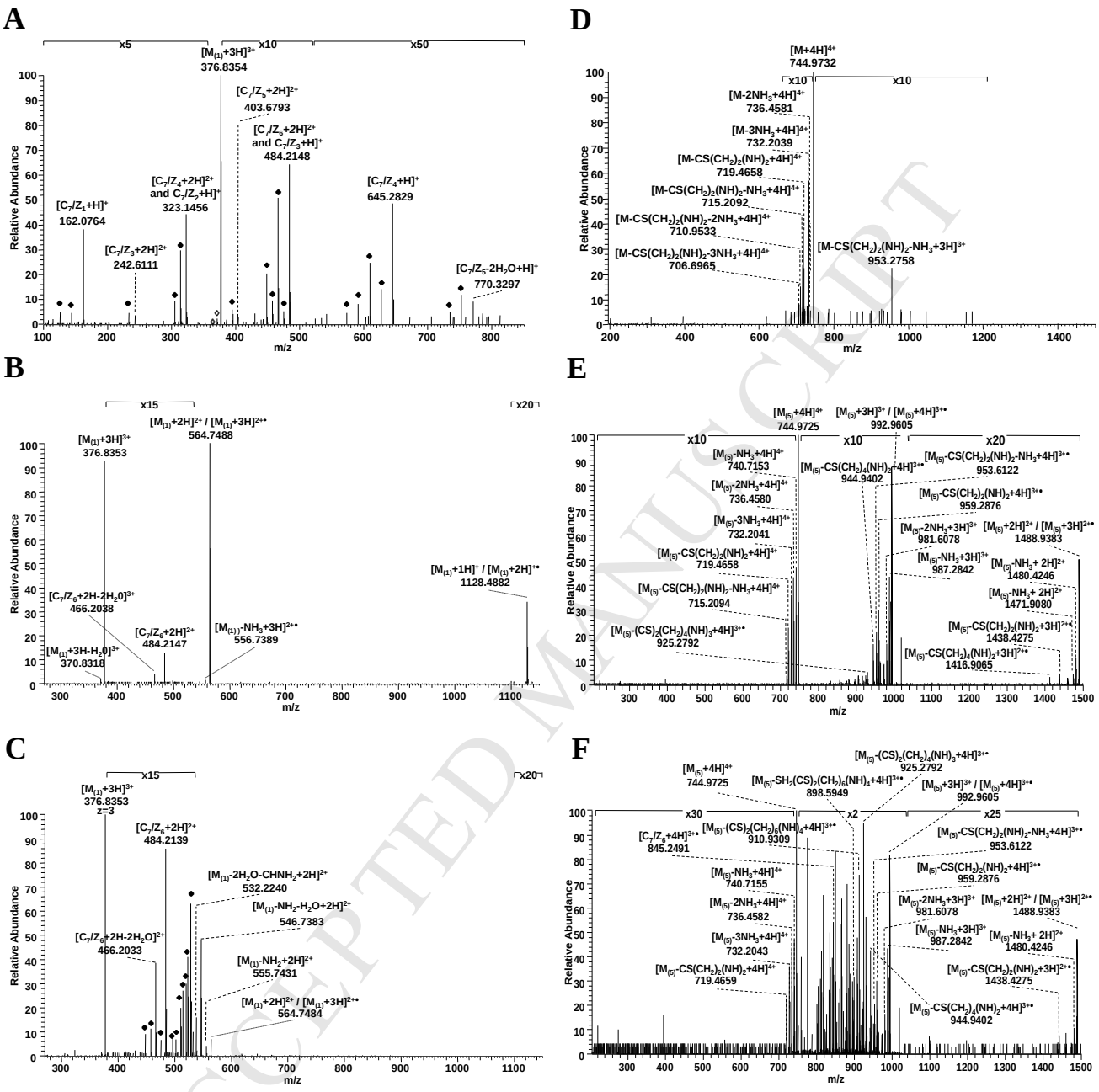

Figure 6. MS/MS spectra of 3+ and 4+ precursor from Compound **1** and **5**, respectively by CID (A and D), ETD without SA (B and E) and ETD with CID supplementary activation at 20% (C and F). H_2O loss at the precursor (◊) and lower (♦) charge states were not systematically indicated on spectrum and some ions were deliberately omitted for clarity.

As regards the CRS, the decrease reaches 31% to complete signal extinction at SA 20% compared to ETD experiments without SA. The CID based supplemental activation targeting the non dissociated electron transfer product species (CRS) allowed to considerably improve the ETD efficiency for our molecules. Notwithstanding, it clearly appears that a fine tuning of SA value is required, according to precursor charge states and type of chain grafted on the carbohydrate scaffold, to obtain the higher fragmentation efficiency. Here, the best results were obtained with SA 15 and 20% for Compounds **2**/**3**/**4** and **1**/**5**, respectively. According to the minimal level of energy required to obtain the maximal fragmentation on one hand and the maximal precursor and CRS ions remaining on the other hand, an order of the susceptibility to fragmentation for the 3+ can be established such as: cysteaminyl-β-CD (Compound **2**) > thioureidocysteaminyl-β-CD (Compound **3**) > amino-β-CD (Compound **1**) > dithioureidocysteaminyl-β-CD (Compound **5**) > aminoethylthioureidocysteaminyl-β-CD (Compound **4**) while considering 4+, the ranking is reversed as Compound **5** > Compound **4** > Compound **3** > Compound **2** > Compound **1**.

4. CONCLUSIONS

The ETD has been successfully applied, for the first time, to the direct analysis (without metal adduction) of a series of cyclic polycationic carbohydrates. Reagent reaction times have to be carefully tuned according to charge states and structural features. Moreover, we demonstrated that ETD with CID based supplementary activation strongly decreases the formation of undesirable charge reduced species while it improves both the variety and the intensity of generated fragments. According to the structures and charge states, optimal ETD fragmentation efficiency varied from 2 to 62%. Nevertheless, due to the high proton affinity of branched moieties, ETD fragmentation appeared to be mainly directed toward pending group rather than cyclic scaffold im-

pairing a complete sequencing for amino group close to carbohydrate core such as per-6- amino-β-cyclodextrin. However, the longer the polycationic chain, the higher the ETD fragmentation efficiency, allowing the complete structural deciphering of a complete building block of per-6-dithioureidocysteaminyl-β-cyclodextrin which was not possible with CID only.

Consequently, it can be judicious to use ETD in combination with other more usual dissociation modes such a CID or higher-energy collision dissociation (HCD). Using such complementary dissociation modes open up future prospects to a detailed characterization of a wider variety of oligosaccharides with more complexity for example exhibiting amphiphilic features or providing a linear or linear/branched mixed scaffold. It appears also of interest to assay alternative ETD reagents such as azobenzene,[35] since it was proved that the efficiency of electron transfer from fluoranthene is in the order of ≈40%.[52] In any event, ETD analysis of our polycationic carbohydrates represents key milestone to answer to some bottleneck for the fine characterization of efficient highly basic carbohydrate based scaffold, because such edifices exhibit an indubitably increasing gain for the DNA/RNA compaction during gene delivery.

However, ETD with CID supplementary activation is self-evident as a definitively suitable MS/MS key tool for the analysis of polycationic carbohydrate based molecules aimed in various fields such as for example glycobiology or nanomaterials.

ACKNOWLEDGMENTS

The authors thank Genopole-France and the Conseil Régional d'Ile-de-France are acknowledged for generous financial support.

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Highlights

* The first ETD-MS/MS characterization of polycationic carbohydrate based non-viral gene delivery agents.

* Suitable selection of charge states and fluoranthene reagent time improves ETD fragmentation efficiency.

* ETD with SA can complete structural deciphering of some building blocks which is not possible with CID only

* ETD based fragmentation is more efficient with long grafted polycationic arms.

* MS/MS results can be used to correlate nitrogen/phosphorus ratio (N/P) in DNA compaction.