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Review Article

Radical Reactions in the Gas Phase: Recent Development and Application in Biomolecules

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This review summarizes recent literature describing the use of gas phase radical reactions for structural characterization of complex biomolecules other than peptides. Specifically, chemical derivatization, in-source chemical reaction, and gas phase ion/ion reactions have been demonstrated as effective ways to generate radical precursor ions that yield structural informative fragments complementary to those from conventional collision-induced dissociation (CID). Radical driven dissociation has been applied to a variety of biomolecules including peptides, nucleic acids, carbohydrates, and phospholipids. The majority of the molecules discussed in this review see limited fragmentation from conventional CID, and the gas phase radical reactions open up completely new dissociation channels for these molecules and therefore yield high fidelity confirmation of the structures of the target molecules. Due to the extensively studied peptide fragmentation, this review focuses only on nonpeptide biomolecules such as nucleic acids, carbohydrates, and phospholipids.

1. Introduction

The innovation and implementation of novel ion dissociation methods remain the forefront field of mass spectrometry research due to the demand to generate structural informative fragmentation patterns for better identification of a diverse array of molecules. The fundamentals of all activation methods are quite similar, in a way that they all involve depositing energy into an ion to induce bond cleavages, the fragments of which are then used to deduce the structural or sequence information of the precursor molecules. As the mass spectrometric methodologies and technologies for biological and biotechnology problems continue to rapidly evolve and improve, more versatile tools are introduced for characterization of molecules of higher complexity [1, 2].

The way ions fragment in the gas phase is largely determined by the ion structures, binding energies, and conformations [3–9]. The most commonly used collisional based dissociation method involves accelerating an ion to a high velocity, letting it collide with an inert gas and resulting in conversion of kinetic energy into internal energy, which then leads to fragmentation of the ion [10, 11]. Due to the robustness and ease of implementation of the collision-induced dissociation (CID) or collision-activated dissociation (CAD), this technique became an integral part of almost every commercial tandem mass spectrometer [12, 13]. However, as the size and complexity of the analyte continue to increase, the need for alternatives to CID has led to the development of many other dissociation techniques, including electron capture dissociation (ECD) [14], electron transfer dissociation (ETD) [15], ion-ion reactions [16, 17], and photodissociation (PD) [18–20]. Most of these techniques involve the generation of a radical site on the parent ion, and the fragmentation patterns follow an odd electron dissociation mechanism. Comparing to the common even electron fragmentation method like the CID, the radical mediated bond dissociation presents complementary structural information of the analyte of interest in many cases. For instance, ECD or ETD generates a radical site through an exothermic electron attachment process and is widely used in preserving posttranslational modifications of the peptides and proteins during large scale bottom-up or top-down proteomics applications [21].

Recently, the radical mediated dissociation technique has been expanded to fields other than peptide and proteins.

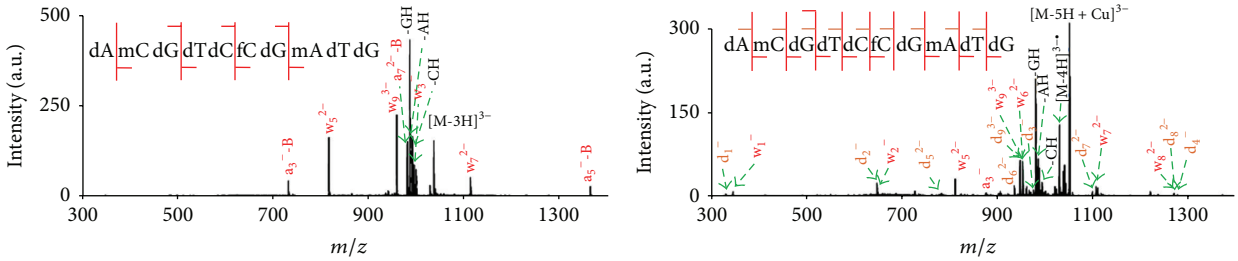


Figure 1: MS/MS product ion spectra of 2′-(F, H, OMe) mixmer. (a) Ion trap CID of $[M\text{-}3H]^{3-}$ and (b) ion trap CID of $[M\text{-}4H]^{3-\bullet}$ formed from negative electron transfer reaction between $[M\text{-}4H]^{4-}$ and $Cu(phen)_2^{+}$. Reprinted with permission from [28]. Copyright 2012 John Wiley & Sons, Ltd.

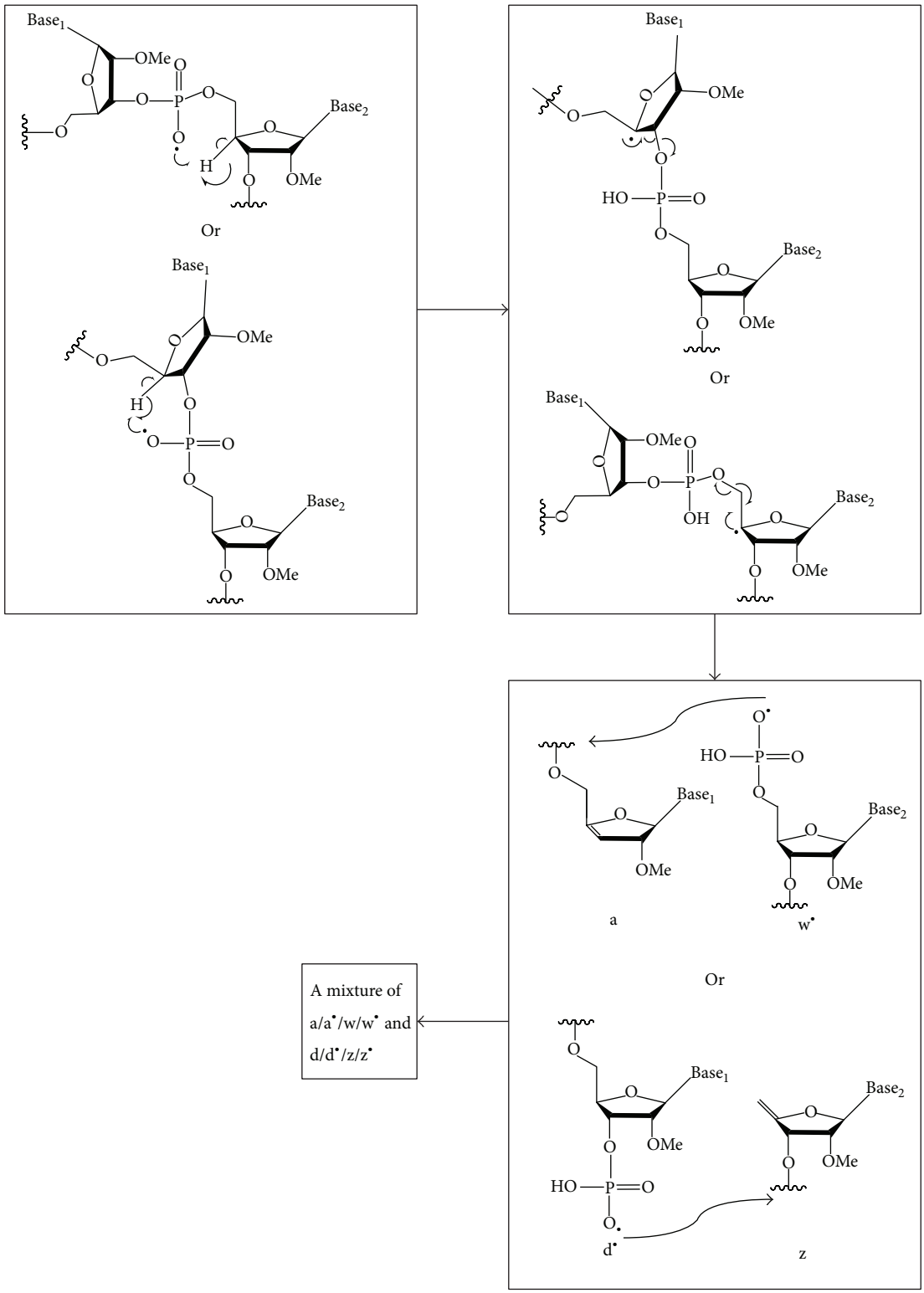
The booming of this technique suggests the wide application of gas phase radical chemistry in biomolecular structural elucidation, including proteins, nucleic acids, glycans, and lipids. The present review is devoted to summarize the development and application of the radical mediated fragmentation and gas phase radical reaction in recent years and demonstrate their capability in biological and biotechnology researches. The focuses of the review are on nucleic acids, oligosaccharides, and lipids. Results using radical-driven dissociation on peptides were recently reviewed by Oh and Moon [22]. Most of the nonconventional ways of generating radical ions in the gas phase discussed in the review require specially modified instrument that is not commercially available, which provides directions for future instrumentation development that can better facilitate the biological researches.

2. Radical Reaction of Nucleic Acids

Structural elucidation of oligonucleotides using mass spectrometry took off ever since the introduction of electrospray [23]. CID has been the most commonly used technique in obtaining sequence informative fragments of the ion of interest. It had proved powerful and robust until recently the focus moved to chemically modified oligonucleotides [24–27], which have better pharmaceutical potentials.

Gao and McLuckey [28] reported that 2′-substituted oligonucleotide has different fragmentation pattern compared to nonmodified DNA or RNA single strands. The gas phase stability of 2′-substituted nucleotide is higher than the DNA or RNA moieties, and thus the 2′-substituted moieties are inert to collision activation when there are DNA or RNA moieties in the same strand. Specifically, oligonucleotide "mixmers" that do not have the same 2′-modifications result in "cleavage gaps" due to significant differences in the energetic requirements associated with cleavages of the adjacent phosphodiester backbones. For instance, the presence of DNA moieties in the sequence, along with residues with 2′-OMe and 2′-fluoro substituents, can result in a situation where all of the backbone decomposition occurs via the low energy channels associated with DNA, whereas little or no structurally diagnostic cleavages take place on the 3′-sides of the 2′-O-methyl and 2′-fluoro substituted residues (Figure 1(a)). The gas phase stability of the 3′-side phosphodiester bond follows the order: 2′-fluoro > 2′-OMe > 2′-OH > 2′-H according to a systematic study of a series of chemically modified 21- and 23-mers [29]. The McLuckey group further investigated the possibility of using radical mediated fragmentation to obtain a more universal fragmentation pattern [30]. They used copper(II) phenanthroline complex as an electron accepting reagent, which can abstract an electron from the multiply charged oligonucleotide anion during ion/ion reaction, and formed an odd electron species that bear a radical on the phosphodiester linkage (Scheme 1). Subsequent collisional activation was applied and led to further dissociation of the oligonucleotide radical anion, resulting in uniform fragments of d/w-ions (Figure 1(b) and Scheme 1).

In addition to radical generation via electron transfer, high energy electrons or high energy photon is also used as an approach to generate radicals on oligonucleotides. Electron detachment dissociation (EDD) takes place with higher kinetic energy electrons (>10 eV) which cause the detachment of one or several electrons from the analytes. While ECD is typically used for multiply protonated analytes, EDD is dedicated to multiply deprotonated analytes like nucleic acids [31, 32]. Nguyen et al. [33] combined double resonance and EDD/SORI-CID experiments on thymine-rich oligonucleotides and illustrated that fragment ions originate concomitantly from both consecutive dissociation in EDD and directly from the precursor ion without electron detachment (electron induced dissociation, EID). In their work, the loss of thymine is favored by a radical process whereas it is very difficult to achieve with CID due to its lower proton affinity. Since the loss of nucleobases is the first rupture leading to the a-B/w-ion series, thymine-rich DNA can yield incomplete sequence information. EDD allows the observation of w-ions from thymine-rich oligonucleotides, which are not detected in CID spectra. Ultraviolet photodissociation (UVPD) at 193 nm [34] and electron photodetachment dissociation (EPD) [34–36] at 260 nm have been applied to multiply charged oligonucleotide anions. Gabelica et al. [35, 36] studied the electron photodetachment of oligonucleotide anions using 250–285 nm laser and found that laser irradiation at 260 nm results in minimal fragmentation of the DNA anion, generating predominant charge-reduced radical anions. CID of the charge-reduced radical anions arising from the irradiation produced complementary a/w- and d/z-fragment ions. Smith and Brodbelt [34] examined UVPD and



Scheme 1: Proposed pathways for phosphodiester bond cleavage following initial electron removal from the phosphodiester linkage. Reprinted with permission from [28]. Copyright 2012 John Wiley & Sons, Ltd.

EPD of a series of modified oligonucleotides (6- to 20-mer) at 193 nm and observed all types of backbone fragments and internal fragments. EPD also resulted in abundant sequence ions in terms of a/(a-B)/w- and d/z-products, and it appeared even more informative than UVPD in providing similar or more complete series of fragment ions thus extending the sequence coverage up to 20-mer [35].

Generally, electrons can be removed from a multiply deprotonated oligonucleotide via electron transfer to a cation, or photodetachment. Due to the limited data published to date, it is not feasible to draw any firm conclusions regarding any differences in initial sites of radical formation or how such differences might lead to differences in structural information generated by a subsequent activation step. However, the present data do show the value of the laser photodissociation in promoting extensive arrays of w-, z-, a-, and d-type product ions. The introduction of such hybrid techniques (e.g., activated-electron photodetachment (activated-EPD) [35]) that combine the generation of a new ion type (radical) with another structural probe has opened up new possibilities for obtaining more sequence informative fragments that can help deduce the structural information. Such hybrid methods are promising for improving the state of the art in the primary structural elucidation of nucleic acids by tandem mass spectrometry and also need further development in the near future.

3. Radical Reaction of Oligosaccharides

The development of mass spectrometry for oligosaccharides is relatively slow comparing to that of protein and nucleic acid analysis, largely because oligosaccharides are a more challenging set of targets for structural elucidation [37]. Conventional CID typically generates glycosidic bond cleavage [38, 39], whereas the more recently developed ExD techniques (ECD [40], EDD [41], and ETD [42]) provide extensive and complementary fragment information of oligosaccharide structures. A few studies have shown the application of ECD for positively charged slightly basic oligosaccharide [39, 43], whereas EDD is used primarily in the negative ion mode for analysis of acidic glycosaminoglycans (GAGs) [39], neutral, sialylated, and chloride-adducted glycans [44, 45]. Similarly, ETD can be applied in both polarities by using different electron transfer reagents [42, 45].

The application of chloride anion attachment to analyze oligosaccharide was first reported by Harvey for N-linked glycans [46]. The anion attachment was implemented to simultaneously analyze neutral and acidic oligosaccharide without changing the instrument polarity. Kornacki et al. [44] employed EDD and CID for the structural elucidation of underivatized maltoheptaose bearing a chloride anion adduct. The results suggested that EDD of underivatized chloride-adducted carbohydrates yields complementary structural information to the EDD of the corresponding doubly charged species. It was noted that complete isolation of the doubly sialylated oligosaccharide was not achieved and led to the lack of unique fragment ions observed. Thus, this approach may be limited for the case in which oligosaccharides bear two or more acidic protons.

Quite a few mechanisms have been proposed for ExD, all of which involve a radical mediated fragmentation process followed by complex hydrogen migration and rearrangement [41, 43]. The major fragmentation channels are glycosidic bond cleavages and cross-ring cleavages. The major fallback of these techniques is the fragmentation efficiency as well as the consistency of the cross-ring cleavages, largely due to the absence of well-defined sites of radical generation [47]. Gao and coworkers have recently demonstrated an approach that uses chemical derivatization of the reduced terminus for proton-catalyzed glycan sequencing (PRAGS) free radical-activated glycan sequencing (FRAGS), and this has been successfully established with a range of glycans to produce systematic and predictable cleavage processes [47] (Figure 2). This method has advantage of easy derivatization and low instrumentation requirement and can be more easily implemented to most research labs comparing to the ExD technique that would require more sophisticated instrument.

In addition to ExD, photon-based activation techniques have also been used to generate and activate oligosaccharide radical ions. Racaud et al. [48] examined the dissociation behavior of deprotonated heparin-derived di- to tetrasaccharides under UV irradiation at 220 nm. Two fragmentation pathways, photon-induced dissociation and electron detachment, can be observed, and the competition can be controlled by manipulating the ionization state of carboxylic groups on the oligosaccharide anions. Their work demonstrates that directed UVPD at 220 nm and activated-electron photodetachment (activated-EPD) can lead to more comprehensive structural analysis of oligosaccharide anions, thus complementing the conventional collisional activation modes. Oligosaccharide radical ions can also be generated by photodissociation of iodide derivatives. Zhang and Julian [49] covalently or noncovalently attached a radical-generating reagent to an oligosaccharide ion, and a radical can be generated by homolytic bond cleavage of specific carbon-iodine bonds in protonated systems by UVPD followed by radical transfer from the reagent to the analyte. Subsequent activation of the radical species generates information-rich spectra including a variety of cross-ring fragments. Their work demonstrates the advantage of radical chemistry over other approaches for the characterization of oligosaccharides, especially the identification of positional isomers of various sizes.

4. Radical Reaction of Lipids

Structural characterization of lipids is almost always limited to conventional tandem mass spectrometry, which is sufficient for determining lipid class and identifying the number of carbons as well as the degree of unsaturation. However, the more detailed structural information, including double bond position, chain branching, and cyclic structures, is beyond the capability of conventional ion trap CID and beam type CID. The dissociation of even electron lipid ions does not generate product ions from internal fragmentation of the fatty acyl chains. In most cases, the fatty acyl chains "fall off" as neutrals and are therefore not detected by the mass spectrometer. Previous studies have shown that intrachain

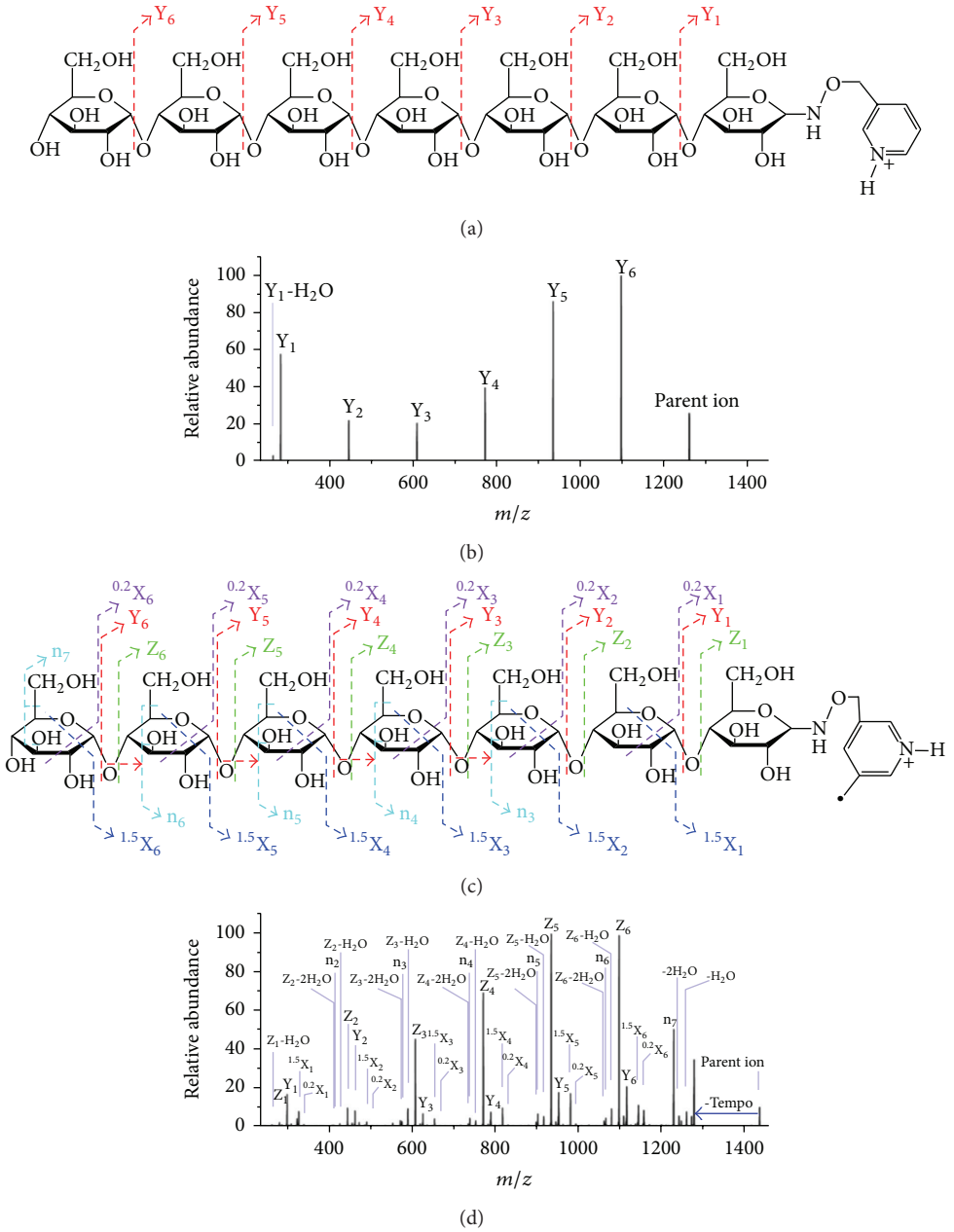


Figure 2: Fragmentation patterns observed following CID of singly protonated PRAGS-derivatized (a) and FRAGS-derivatized (c) maltoheptaose and CID spectra of singly protonated PRAGS-derivatized (b) and FRAGS-derivatized (d) maltoheptaose. Reprinted with permission from [47]. Copyright 2013 American Chemical Society.

fragmentation of lipids can be achieved by either high energy CID (>keV) or multistage CID on ion-trap platforms. The former requires using sectors or TOF/TOF instrument [50, 51], whereas the latter takes place on ion-trap platforms [52, 53]. The broader implementation of these techniques has been limited primarily due to the incompatibility of the high energy platforms with ESI and the low sensitivity of the MS^n experiments [54]. Castro-Perez and coworkers [55] demonstrated the use of time-aligned-CID spectra on an ion-mobility device coupled to a quadrupole time-of-flight mass spectrometer (IM-qTOF MS) to improve the sensitivity of the multistage CID.

Alternatively, selective ion-molecule reactions in the gas phase (e.g., covalent attachment chemical ionization [56] and ozone induced dissociation [57]) have been utilized to pinpoint the double bond positions in complex lipids. Dobson and Christie [58] found that CID of radical ions produced by traditional electron impact ionization (EI) resulted in extensive intrachain fragmentation in fatty acids and their derivatives. However, the use of EI is limited to complex lipids due to their high vapor pressure and thermal instability. In order to take advantage of the radical mediated fragmentation without being limited to EI, electron transfer reactions have been applied to lipids [59, 60] analogous to

the contemporary methods used in the proteomics community. These approaches are also limited in lipidomics because most lipids produce predominantly singly charged ions.

Pham and coworkers [54] took an alternative way to generate radical ions by attaching a photocaged radical precursor to the analytical target and then "uncage" the radical by photon irradiation in the mass spectrometer. The subsequent CID of the nascent radical ion induced radical mediated dissociation of the precursor. Two photocaged radical precursors (4-iodobenzoate (IB) and 4-iodoaniline (IA)) have been investigated as they bear a UV-labile carbon-iodine bond and a para-substituted functional group that can bind noncovalently to complex lipids. The radical mediated dissociation spectra (Figure 3) show a variety of fragments arising from acyl chain backbone cleavages. The application of radical mediated dissociation is also demonstrated for the biologically derived lipid extracts.

Instead of UV irradiation inside a mass spectrometer, Ma and coworkers [61] induced the radical reaction in the ESI source (Figure 4(a)). They demonstrated an approach that couples tandem mass spectrometry with the unique chemistry of Paternò-Büchi (P-B) reaction towards C=C bond for lipid structure elucidation. The online P-B reaction was conducted using acetone as the reagent and reacting with oleic acid. The UV irradiation at the source resulted in a mass increase of 58.0423 Da relative to deprotonated oleic acid, corresponding to C_3H_6O, the elemental composition of acetone (Figures 4(b) and 4(e)). CID of the C_3H_6O adducted precursor resulted in all retro P-B pathways, which revealed the location of the double bonds in oleic acid. This approach was also investigated in the context of complex phospholipids and successfully yielded diagnostic ions for double bond positions. This technique has simple experimental setup and does not need MS instrument modifications, which makes it accessible and attractive to many laboratories.

5. Conclusion

The development of mass spectrometry instrumentation is always challenged by the fast growing demand from the biological researches. The complexity of the target molecule and the level of structure information require not only more sensitive and accurate MS instrument, but also more specific probes that can "tease out" the structural information in a predictable manner. Collision-induced dissociation, although being the most powerful and robust tool, starts to see its limitations in many different fields due to its inherent energy deposition mechanism and the gas phase fragmentation chemistry of biological ions. As the only information that comes out of a mass spectrometer is the *m/z*'s of fragment ions, the ultimate task for researchers is to obtain as many types of predictable fragment ions as possible. This inevitably makes any single probe (collision with the inert neutral particles) not feasible. The invention of the electron based techniques significantly improves the amount of information that a mass spectrum can provide. However, they suffered from low efficiency and sometimes unpredictable fragmentation pattern. Extensive researches have been done on various ways of adding or substracting an electron from the target ion using a wide range of deposit energies. The fragmentation pattern largely depends on how much energy is put into the system, how much energy is converted into internal energy, and how fast the conversion takes place. Then photodissociation was developed to make faster and more accurate deposition of the energy. Of all the new techniques, the generation of radical is almost always being the key in the fragmentation process. Observations made by many research groups over the past decades have been broadly self-consistent. However, some inconsistencies in the literature have been observed, which may arise from the differences in instrument platforms and variations in conditions. For example, different correlations between electron photodetachment efficiency and nucleobase identification have been reported.

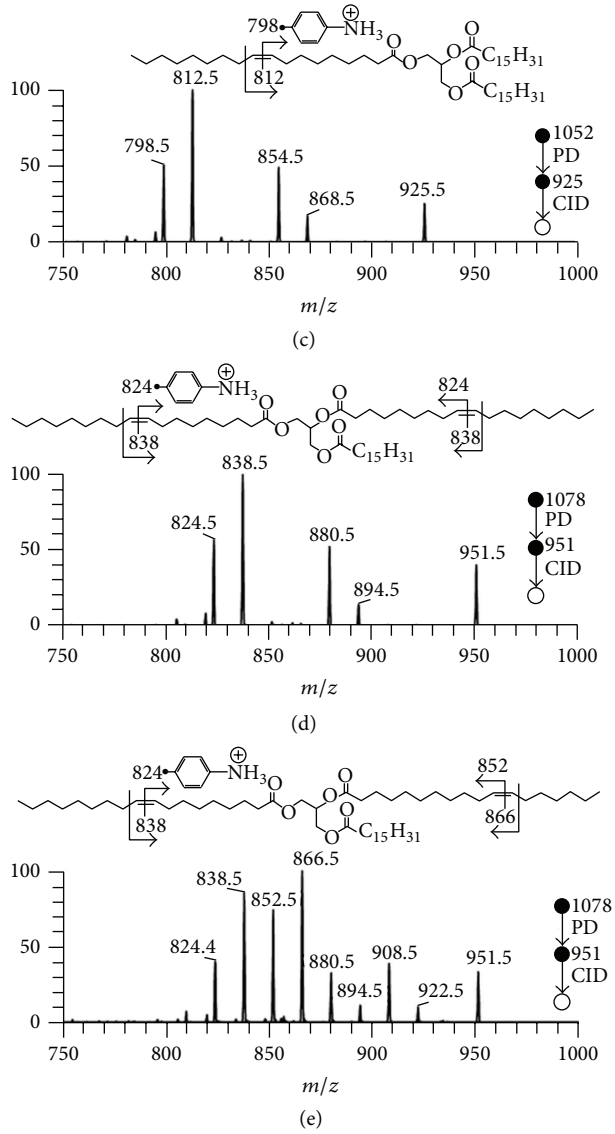


Figure 3: Radical mediated dissociation spectra obtained from $[TG + IA]^+$ complexes for (c) TG(16:0/16:0/9Z-18:1), (d) TG(16:0/9Z-18:1/9Z-18:1), and (e) TG(16:0/11Z-18:1/9Z-18:1). Adapted with permission from [54], (a) and (b) not included. Copyright 2012 American Chemical Society.

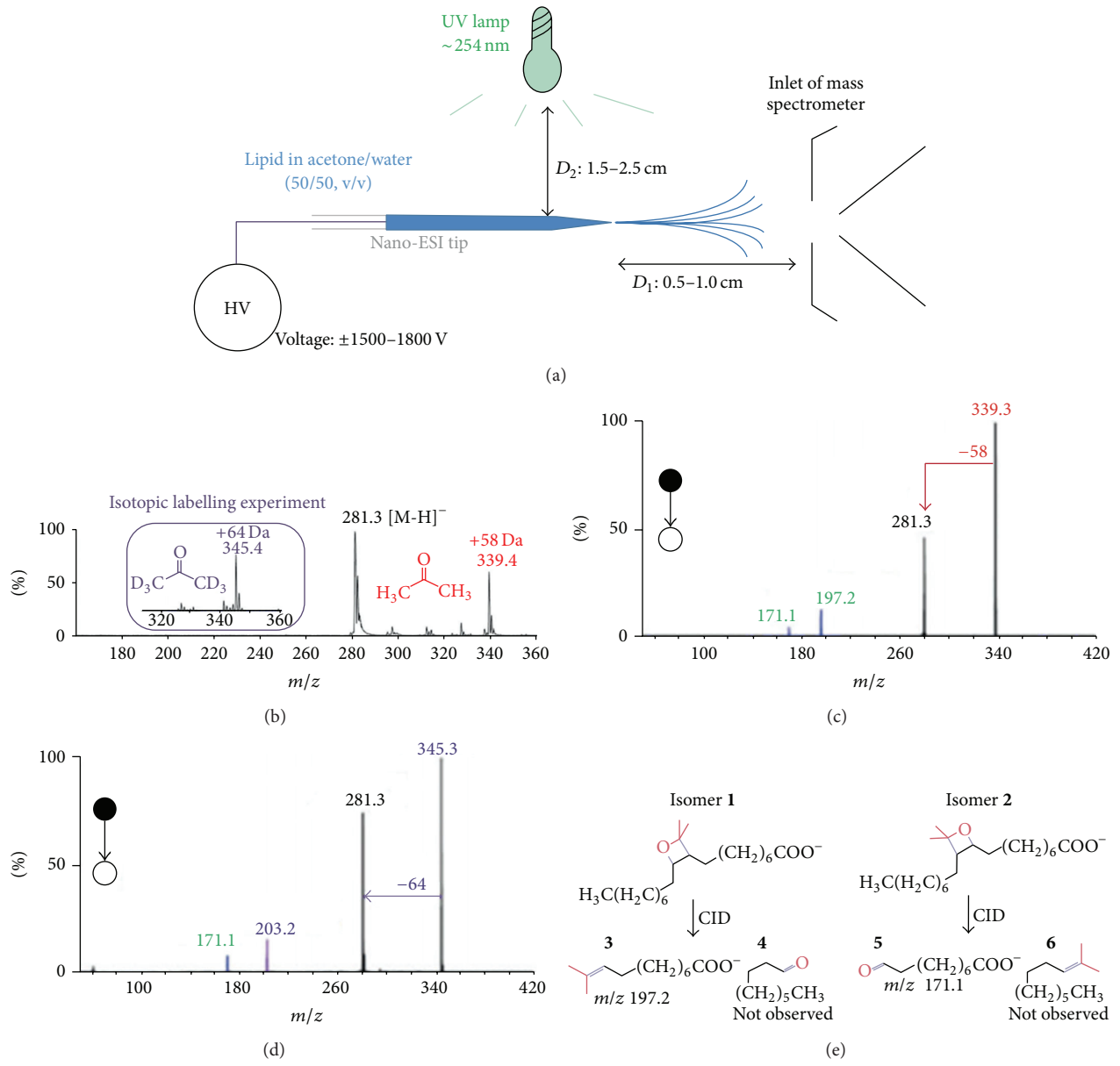


FIGURE 4: Online coupling of P-B reactions with MS for lipid analysis. (a) Experimental setup. (b) P-B reaction mass spectrum of oleic acid and acetone induced by UV irradiation of nano-ESI. Inset: P-B reaction spectrum using D6-acetone (C3D6O). MS2 CID of the P-B reaction products at (c) *m/z* 339.3 and (d) *m/z* 345.3. (e) Fragmentation scheme of P-B reaction product isomers. Reprinted with permission from [61]. Copyright 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

As a tool for the structural characterization of biomolecules, tandem mass spectrometry continues to evolve and versatile applications are being developed to fit the needs of various analyses.

This review mainly focuses on different ways of generating radical ion precursors in the gas phase and the application of radical reactions in structural elucidation of biomolecules. The contents cover solution phase derivatization, in-source derivatization, and gas phase ion/ion reactions. These approaches involve different levels of protocol complexity and require various types of instrument modification. Regardless of immediate application to the field, the methods presented all yield extensive structural information and have unique potential applications for specific analytes. Nevertheless, the sample preparation protocols/new instrumentation need to be further optimized to fit the general demand for the larger community. For instance, the solution derivatization of glycans needs simpler preparation procedures that can ideally be designed as a kit. Moreover, the in-source P-B reaction needs better parameter control to be able to fit the quantitation needs of an LC-MS platform. Similarly, ideal electron transfer reagent needs further investigation for modified oligonucleotides. With increasing discoveries made on the fundamental gas phase radical

reactions, more applications will be developed and better fidelity and specificity will be achieved.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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