Time-resolved mass spectrometry

Yu-Chie Chen, Pawel L. Urban

Mass spectrometry (MS) offers advantages over conventional spectroscopic assays because it enables structural determination of reactants while preserving temporal resolution. The ability to detect short-lived reaction intermediates or labile metabolites makes time-resolved MS (TRMS) measurements an enabling tool for studies of chemical reactions, chemical kinetics and biochemical dynamics. High temporal resolution requires careful optimization of the interface, which would enable quenching of the chemical process, ionization and rapid transfer of the analytes into the mass analyzer.

We review recent advances in TRMS, and outline the prospects for future developments and applications.

Keywords: Biochemical dynamics; Chemical reaction; Fast process; Kinetic measurement; Mass spectrometry; Reaction intermediate; Reaction monitoring; Structural determination; Temporal resolution; Time-resolved mass spectrometry

Abbreviations: APCI, Atmospheric pressure chemical ionization; C-API, Contactless atmospheric pressure ionization; CE, Capillary electrophoresis; DART, Direct analysis in real time; DESI, Desorption electrospray ionization; DIOS, Desorption ionization on silicon; EESI, Extractive electrospray ionization; ELDI, Electrospray-assisted laser desorption/ionization; ESI, Electrospray ionization; ESSI, Electrosonic spray ionization; FPOP, Fast photochemical oxidation of proteins; FT, Fourier transform; HT, Hadamard transform; ICR, Ion cyclotron resonance; IT, Ion trap; LDI, Laser desorption/ionization; LC, Liquid chromatography; LTP, Low-temperature plasma; MALDI, Matrix-assisted laser desorption/ionization; MS, Mass spectrometry; nanoDESI, Nanospray desorption electrospray ionization; PESI, Probe electrospray ionization; TOF, Time-of-flight; TRMS, Time-resolved mass spectrometry; UASI, Ultrasonication-assisted spray ionization; V-EASI, Venturi easy ambient sonic-spray ionization

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1. Introduction

Sensitivity and mass resolution of mass spectrometry (MS) have attracted much interest of chemists during the past decades. There exist MS techniques that provide ''high masss resolution'' [e.g., Fourier transform MS (FT-MS)] and others that provide ''high lateral resolution'' [e.g., secondary ion MS (SIMS)]. However, it is still challenging to carry out MS analyses with ''high temporal resolution'', which would potentially provide information on short-lived chemical species and fast-changing chemical composition in reaction chambers.

Various optical spectroscopic methods (e.g., vibrational spectroscopy) enable monitoring of ultrafast chemical and physical processes reaching the femtosecond (fs) time scale [1–3]. Infrared (IR) and Raman spectroscopies offer advantages in the monitoring of fast processes. However, due to their limited selectivity, these methods provide limited information on the reaction systems involving multiple and/or unknown compounds. Considering its enabling potential in organic chemistry and biochemistry, time-resolved MS (TRMS) can undoubtedly bring considerable advantages to researchers because the occurrence of transient chemical states can directly be observed in the mass spectra that are easy to interpret. For example, MS offers an alternative detection methodology in enzymatic bioassays: the possibility of monitoring the conversion of substrates to products and its overall versatility make MS an adequate tool for the study of enzyme-catalyzed processes in real time [4].

The temporal resolution of MS systems depends on various factors encompassing the route of an analyte from its origin (test tube, reactor), through ion source, ion guides, analyzer, to the detector. Utilization of rapid mixing devices positioned in the proximity of mass spectrometers can give access to the initial stages of reactions, thus providing valuable data on reaction mechanisms [5]. As noted by Vikse *et al.* [6], although many previous investigations have focused on the structural identification of short-lived or low-concentration intermediates, in some recent studies the intensities of intermediates or reactants and products were monitored over time [7,8].

Ambient ionization techniques have the advantage that they do not require, or

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require minimal, sample preparation [9]. This makes them promising candidates for monitoring chemical and biochemical processes in the time-resolved manner, and much attention has already been paid to the development of ion sources and interfaces to accommodate rapid sampling. The way of sampling, the ion source, and the reaction microenvironment itself can hardly be separated from one another when it comes to the fast monitoring of chemical processes. Decreasing the dead volume and the dead time between the reaction and the ionization zones is the key to improving temporal resolution, and can gain access to the time range that has not been accessible to conventional analysis systems. The data-acquisition rate limitations imposed by MS (in particular, the mass analyzer) cannot be neglected: in other words, slow instruments will not catch up with the quickly changing concentrations of a reaction mixture. However, smart strategies to control and to limit the reaction times have been introduced, and they can indirectly overcome the temporal resolution limits imposed by the design of mass spectrometers.

2. Ion sources

TRMS is closely related with the attempts to couple sample-preparation schemes with mass spectrometers. In particular, when coupling continuous-flow separation platforms [e.g., liquid chromatography (LC) or capillary electrophoresis (CE)], it is necessary to ensure fast, quantitative transfer of effluents containing analyte molecules to the ion source of a mass spectrometer. Efficient transfer of mobile phase to the entrance of the mass spectrometer removes the constraint on the speed of chromatographic separation, so fast chromatographic runs can be executed readily. Electrospray ionization (ESI; discussed in section 2.1) is the ion source of choice for combining LC, CE and microfluidic chips with MS. Coaxial sheath-flow [10], liquid junction [11], microdialysis junction [12], electroosmotic pumping [13] and sheathless [14] interfaces have been proposed. Interfaces for coupling gas chromatographs and liquid chromatographs with mass spectrometers are also well established and manufactured by several companies. They provided a good matching of the separation selectivity and detection sensitivity with minimum ion suppression due to partly overlapping analyte bands.

Batycka *et al.* [15] demonstrated tandem MS scanning – compatible with narrow chromatographic peaks (full width at half maximum: <10 s) – in conjunction with monolithic column LC. Using this system, up to 68% sequence coverage was achieved for 500 fmol bovine serum albumin [15]. LC-ESI-MS is commonly used in the analysis of discrete biological samples, but the temporal resolution of such discrete analyses is generally limited to minutes (cf. [16]). In order to achieve high temporal resolution, other approaches – normally excluding the separation step – need to be employed. As we show below, applications of fast MS – using a variety of ion sources – go beyond the area of separation science. Based on our survey of current literature, we think that ion source is the key part to TRMS, so we further discuss representative examples of studies in which the ion source was important for collecting MS data with temporal resolution.

2.1. Electrospray ionization

2.1.1. Reaction monitoring. ESI – coupled with MS by Fenn [17] – enables efficient transfer of liquid-phase molecules into the gas phase at atmospheric pressure. Outstanding features and advantages of ESI-MS make it one of the most suitable tools for the fast screening of intermediates directly from solution [18]. In early studies, Arakawa *et al.* [19,20] used an on-line ESI-MS system, in which a flow-through photoreaction cell was attached to an ESI interface. The researchers detected intermediates of photochemical reactions of transition-metal complexes that have a lifetime of a few minutes. They showed that on-line ESI-MS is a powerful tool for the study of photochemical reactions [19]. In another representative study, Zechel *et al.* [21] used time-resolved ESI-MS to measure the pre-steady state kinetics of an enzymatic reaction accurately by monitoring a transient enzyme intermediate. The temporal resolution of the apparatus used in this work was limited to tens of ms, so the method is applicable to enzymes with k_{cat} values less than ~10/s [21]. Importantly, this study showed the possibility of collecting MS data within the sub-second time scale.

Hinderling and Chen [22] reported on the use of ESI-tandem MS (ESI-MS/MS) and gas-phase reactions for the rapid screening of Brookhart-type Pd(II) olefin polymerization catalysts. As noted, the combination of sensitivity, speed, direct assay, and versatility shows that one can already envision screening large (n >>100) libraries of combinatorially-generated catalysts [22].

Brum *et al.* [23] used hydrolysis of isatin to examine the effects of different ionization scenarios with regard to monitoring reactant consumption and product formation. First-order hydrolysis kinetics data obtained using negative-ion MS were compared with data acquired via an optical technique [23].

The Heck reaction with arene diazonium salts was also successfully investigated by ESI-MS [24]. Enquist *et al.* [7] applied ESI-MS to detection of reaction intermediates in the air-promoted and ligand-modulated oxidative Heck reaction. ESI-MS and subsequent MS/MS analyses were conducted to detect directly Pd-containing cationic reaction intermediates in a ligand controlled Pd(II)-catalyzed oxidative Heck arylation. However, the reaction took hours [7], so this study cannot be classified as fast reaction monitoring.

Santos *et al.* [25] investigated the mechanism of the Baylis-Hillman reaction by ESI-MS. The proposed intermediates for the catalytic cycle of this reaction have been recorded and structurally characterized [25].

Recently, Schade *et al.* [8] proposed charged tags as probes for analyzing organometallic intermediates and monitoring cross-coupling reactions by ESI-MS. In this study, zero-valent Pd complexes, Zn, and In reacted with ammonium-tagged organic iodides under C–I bond insertion. The consumption of the latter could be monitored by ESI-MS. The detection of the resulting organometallic intermediates by ESI-MS provided detailed information on their molecular nature. In this study, the total monitoring time was longer than 500 s [8]. Based on the examples mentioned above, one may conclude that ESI can be regarded as a universal ionization technique, and can readily be applied to long-term monitoring of organic reactions, and to detect putative intermediate steps. Overall, the examples outlined above show that ESI-MS is suitable for the monitoring of a variety of chemical processes; in some cases, even in the sub-second time regime. For a review on applications of ESI in high-throughput screening of homogeneous catalysts, readers are referred to the article by Chen [26].

2.1.2. Other applications. A beautiful example of the application of TRMS is the study of the evolution of protein native structure with electrospray into the gas phase [27]. The authors proposed that the temporal (picoseconds to minutes) evolution of native protein structure during and after transfer into the gas phase can involve side-chain collapse, unfolding, and refolding into new, non-native structures (Fig. 1). This work contributes to the discussion on the possibility of preserving structure of biomolecules during the ESI process, and on their way to the mass analyzer.

ESI-MS also finds application in the monitoring of complex biochemical systems, involving multiple enzyme-catalyzed reactions, which fulfill the requirements of modern synthetic chemistry applications. Bujara *et al.* [28] presented the development and the application of a new *in vitro* real-time analysis method for the comprehensive investigation and rational programming of enzyme networks for synthetic tasks. In their experimental set-up, the enzyme was retained in a reactor by means of a membrane, and the products of the reaction – which diffused through the membrane – were instantly transferred to an ESI source [28]. That strategy has some resemblance to the so-called ''membrane-inlet MS'', which was earlier used to monitor metabolism of yeast (*Saccharomyces cerevisiae*) in real time [29]. In fact, MS monitoring involving the application of ESI interface has become an enabling tool for microbial metabolomics studies (cf. [30]).

The usefulness of ESI-MS reaches beyond time-resolved monitoring of chemical and biochemical processes. Recently, our team disclosed a method for the monitoring of convection waves in liquid media [31]. The temporal resolution provided by the proposed system was of the order of seconds.

2.2. Ion sources derived from electrospray ionization

2.2.1. Electrosonic spray ionization. There are a number of ion sources, which hold some resemblance to ESI. They often offer additional advantages, compared with ESI, and find applications in particular areas. For example, in the work by Chen *et al.* [32], electrosonic spray ionization (ESSI) was used as a proton source to promote atmospheric-pressure thermal activation of organic reactions.

In another work [33], researchers obtained neutral fragment mass spectra via ambient thermal dissociation of peptide and protein ions, and observed that a much higher fragmentation yield was observed using a coiled tube compared to a straight tube under the same experimental conditions (tube temperature and length). This suggests that the turbulent gas flow in the coiled tube increases the residence time of the peptide precursor ions, and is consistent with few wall collisions being expected in the straight tube arrangement at the near-supersonic velocities achieved in ESSI; a residence time of about 1 ms was calculated for a carrier gas with a velocity of 300 m/s [33].

Conventionally, gas-phase ion/molecule reactions have been studied extensively in the past century for obtaining gas-phase basicity and kinetic information. Formerly, most reactions were studied in one of the mass analyzers in a tandem mass spectrometer [34–36]. These reactions can be carried out in a very short time, and the product species can immediately be detected by the second mass analyzer. Gas-phase basicity of specific analytes and thermal dynamic data can be probed through such reaction-aided assays.

Touboul *et al.* [37] studied deprotonation reactions of multiply-charged protein ions by introducing volatile reference bases at atmospheric pressure between an ESSI source and the inlet of a mass spectrometer. In this case, ion/molecule reactions in gas phase took place at ambient conditions: They introduced a fast, sensitive MS measurement of the GBapp of peptides and proteins based on ESSI-MS [37]. In their set-up, vapor of reference bases (volatile amines and alcohols) reacted with all the charge states of the protein generated by ESSI at atmospheric pressure, before they were sampled into the mass spectrometer. Due to a high reference base pressure, and a sufficient reaction time – estimated to a few hundreds of μs – a high collision rate was achieved [37].

2.2.2. Desorption electrospray ionization. Desorption ESI (DESI)-MS was introduced by Graham Cooks and coworkers in 2004 as a tool for sampling at ambient conditions for MS [38]. In this ingenious approach, a

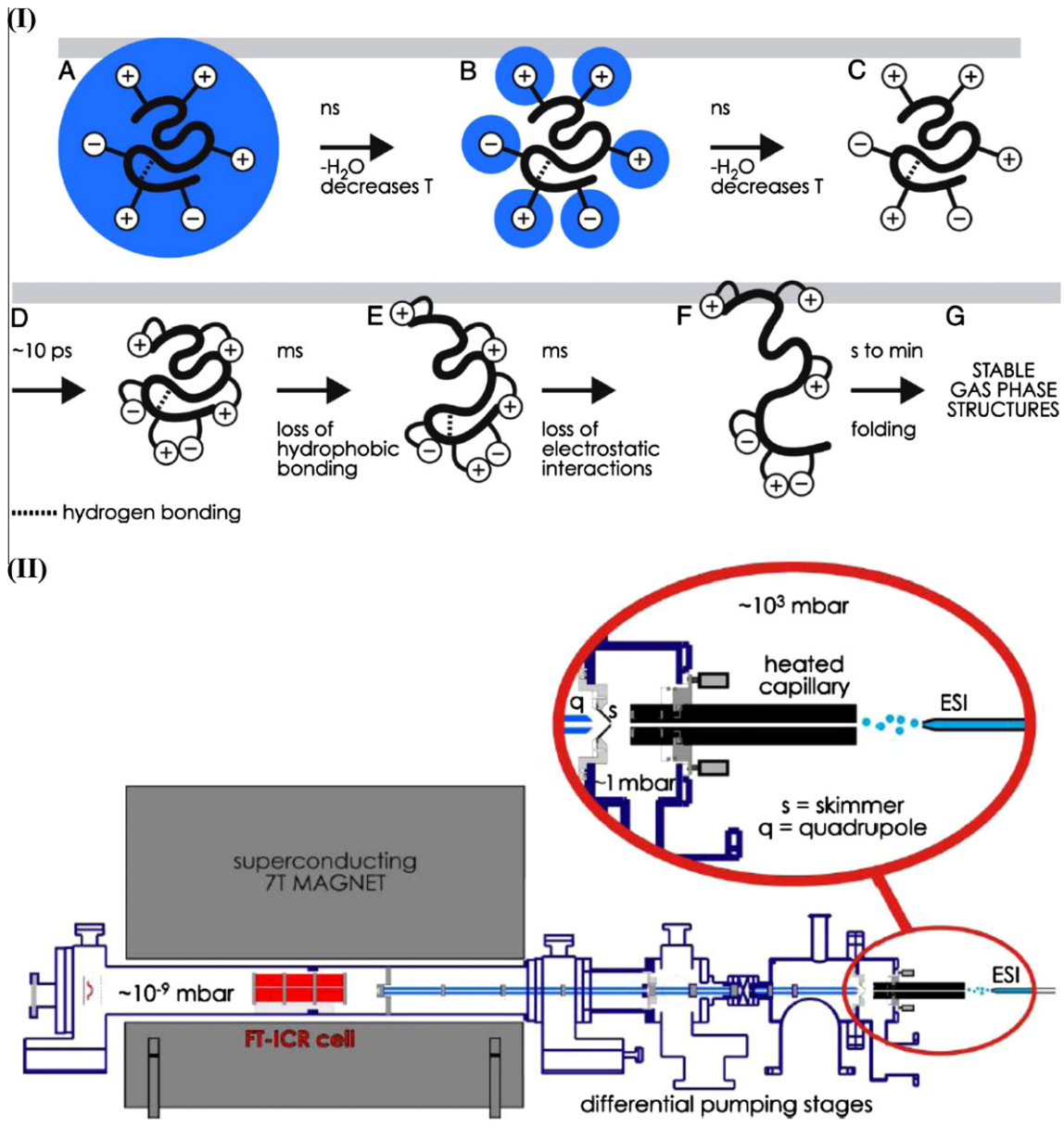

Figure 1. (I) Stepwise evolution after ESI of the structure of a globular protein (e.g., cytochrome *c*, ubiquitin). (*A*) Native protein covered with a monolayer of H_2O, followed by nanosecond H_2O loss and concomitant cooling. (*B*) Native protein with exterior ionic functionalities still hydrated undergoes ns H_2O loss and cooling. (*C*) The dry protein undergoes ≈10-ps collapse of its exterior ionic functionalities. (*D*–*G*) The exterior-collapsed ''near-native'' protein undergoes thermal re-equilibration (*D*), ms loss of hydrophobic bonding (*E*), and ms loss of electrostatic interactions (*F*); the transiently unfolded ions form new non-covalent bonds in seconds, folding to more stable gaseous ion structures (*G*); these stabilize to energy-minima conformers in minutes. (II) Electrospray of a protein solution (from right) at atmospheric pressure with ≈1-kV acceleration of ESI droplets into a heated capillary. Exiting ions are accelerated (≈50 V) into a lower pressure (≈1 mbar) region to undergo variable low energy (<1 eV) collisions, pass through a skimmer into a lower pressure region for possible higher energy (>1 eV) collisions, and are conducted into and trapped inside the measurement cell (≈10^{-9} mbar) of the Fourier-transform mass spectrometer. {Reproduced from [27] © 2008, National Academy of Sciences, USA}.

stream of charged droplets and/or gas-phase ions hits a surface of the sample, and the charged microdroplets containing analyte (or gas-phase ions) – which are desorbed from the sample surface – are projected towards the orifice of mass spectrometer.

Soon after the introduction of the technique, researchers realized that it is straightforward to add reagents to the DESI spray solvent to mediate the interfacial reactions of solution-phase reactants with condensed-phase analytes to yield gas-phase ions. Using DESI-MS, ion-molecule reactions are commonly studied in the ion-source regions, especially at ambient conditions. These experiments (referred to as the ''reactive DESI'') add chemical selectivity to the DESI experiments

[39]. The solution for reactive DESI can be varied to fit the needs of the target species. In general, complexations and adduct formations occur during the fusion of the reactive DESI spray with the sample on the solid support. As a consequence, the ionization efficiency of target species can be improved.

Notably, Perry *et al.* [40] demonstrated that DESI can intercept reactive intermediates formed in the secondary microdroplets on the ms timescale. It is concluded that DESI-MS allows chemical reactions that occur in microscale volumes (droplet volume of approximately 4 mm^3) to be assayed in real time. As discussed, the short timescales of desorption and ionization allow detection of reaction intermediates that have lifetimes of the order of ms with high sensitivity (picomole quantities) [40].

Reactions can greatly be accelerated in highly-concentrated, highly-charged droplets, or very fast reactions can be monitored by droplet fusion during the DESI process [41].

In another work, Miao *et al.* [42] presented the development of a sub-ms TRMS method based on DESI for direct, fast ionization of a high-speed liquid jet stream (Fig. 2). In the experiment carried out, two reactant solutions underwent rapid mixing to produce a free liquid jet, which was ionized by DESI at different positions corresponding to different reaction times. Due to the high velocity of the liquid jet, high time resolution could be achieved. In this study, the fast reduction reaction of 2,6-dichlorophenolindophenol (DCIP) and L-ascorbic acid was chosen as an example to demonstrate this concept, and the reaction rate constant was successfully measured with a time resolution of 300 μs [42].

It should be noted that the environment in the evaporating charged microdroplet differs from that of the bulk [43]:

(1) the pH of the solution moves towards the extremes;
(2) the concentrations of the reagents increase;
(3) the relative surface area increases; and,
(4) collision frequencies increase [43].

We therefore suggest that DESI cannot be regarded as a universal ionization technique for the monitoring of the native characteristics of chemical reactions, and further studies on the mechanism of this novel ionization technique need to be carried out.

A technique related to DESI – nanospray DESI (nanoDESI) – was successfully applied in the monitoring of electrochemical reactions [44]. In the nanoDESI set-up, two capillaries are used:

(1) the first capillary delivers solvent onto the sampling zone; and,
(2) the second (sampling) capillary collects the liquid sample and delivers it to the ionization area, which is similar to that of nano-electrospray.

A limitation of nanoDESI in the monitoring of fast processes is the dead time due to the transfer of liquid-phase analytes along the sampling capillary towards the spray zone.

2.2.3. Other ESI-related ion sources. Extractive ESI (EESI)-MS can also be considered a useful technique for the on-line monitoring and characterization of chemical reactions in real time by quickly sampling the chemicals emerging from a running reaction mixture [45]. McCullough *et al.* [46] used an EESI device for on-line reaction monitoring. The EESI apparatus incorporates a secondary, grounded nebulizer to produce an analyte aerosol and a Venturi pump is then used to transfer a sample of the aerosol to an electrospray source where it is ionized. Notably, one of more recent developments in the monitoring of chemical reactions by MS encompasses the use of Venturi easy ambient sonic-spray (V-EASI) ionization [47].

Recently, an ambient ionization technique, so-called ultrasonication-assisted spray ionization (UASI) [48], was used in monitoring chemical reactions in real time [49]. UASI comprises two components – an ultrasonicator (frequency, ~40 kHz) and a tapered capillary (Fig. 3) [49]. The inlet of a capillary was put in the reaction vial, which was placed in the ultrasonicator. There was no electrical connection or any external accessories attached on the outlet of the capillary. The capillary outlet was positioned close (~3 mm) to the inlet of a mass spectrometer operated at a high voltage (1.5 kV), leading to the polarization occurring on the capillary effluent, and resulting in the formation of gas-phase ions [48,50]. The reaction substrates were readily delivered to the tapered outlet with the assistance of ultrasonication and capillary action, and give rise to gas-phase ions in the UASI spray. Furthermore, the chemical reaction was accelerated because the reaction vial was placed in the ultrasonicator [49]. Chemical reactions can further be accelerated using an ultrasonicator with MHz frequency.

The technique was further simplified (ultrasonicator removed), and named ''contactless atmospheric pressure ionization'' (C-API) [51]. C-API incorporates a tapered capillary as the sampling tube and spray emitter, which is put in the proximity (~1 mm) of a mass spectrometer operated at a high voltage (~3 kV). Because of the simplicity of the C-API set-up, the reaction species can easily be sampled from the reaction vial, and transferred to the MS orifice.

Chemical reactions have successfully been monitored in real time using the C-API-MS approach [52].

A dissimilar strategy was demonstrated by Yu *et al.* [53], who conducted reaction monitoring by implementing probe-ESI (PESI)-MS, where the capillary for sampling and spraying is replaced by a solid needle. High tolerance to salts and direct ambient sampling are major advantages of PESI compared with conventional ESI. Real-time PESI-MS monitoring can give direct

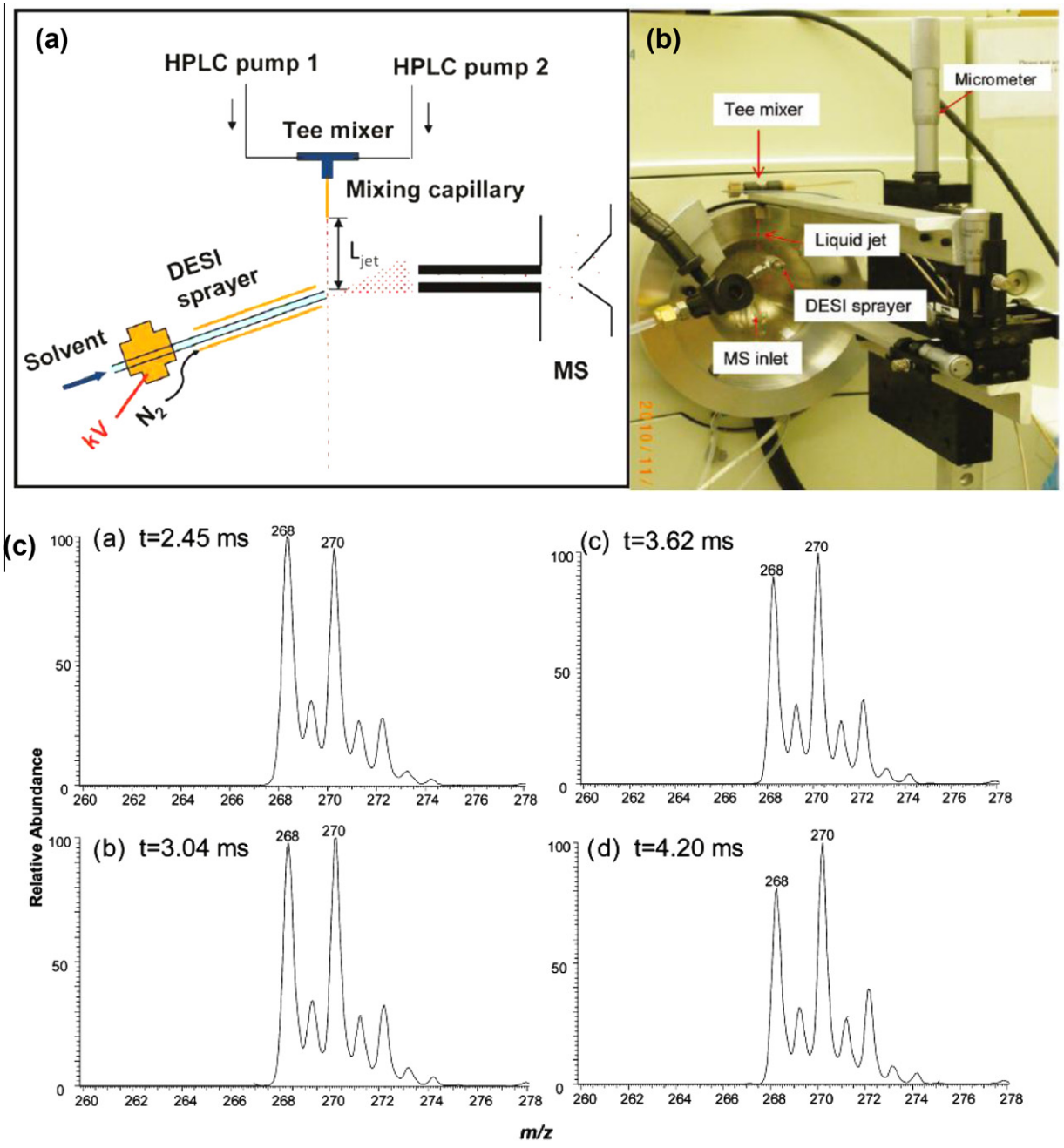

Figure 2. Sub-ms time-resolved mass spectrometry using desorption electrospray ionization. (a) Scheme and (b) real image showing the apparatus of the sub-ms time-resolved DESI-MS. (c) Time-resolved DESI MS spectra of the reaction between 0.1 mM oxidized DCIP and 2.0 mM ascorbic acid (pH = 2.0) sampled at (a) 2.45 ms, (b) 3.04 ms, (c) 3.62 ms, and (d) 4.20 ms, respectively. {Reprinted with permission from [42] © 2011, American Chemical Society}.

information on each chemical species taking part in reactions. Real-time reaction monitoring of protein denaturation, peptide hydrogen/deuterium exchange, and Schiff base formation have been done by PESI-MS [53]. Certainly, this approach may help to gain insights into reaction kinetics of unknown heterogeneous chemistries, which can facilitate the optimization of reaction parameters.

2.3. Laser-aided ionization

Hatakeyama *et al.* [54] presented an ingenious method for sub-µg-scale testing of reaction conditions in solution using nL plugs in microfluidics with detection by matrix-assisted laser desorption/ionization (MALDI)-MS [55]. This approach is off-line, but, as demonstrated by Brivio *et al.* [56], reactions can also be conducted in microflu-

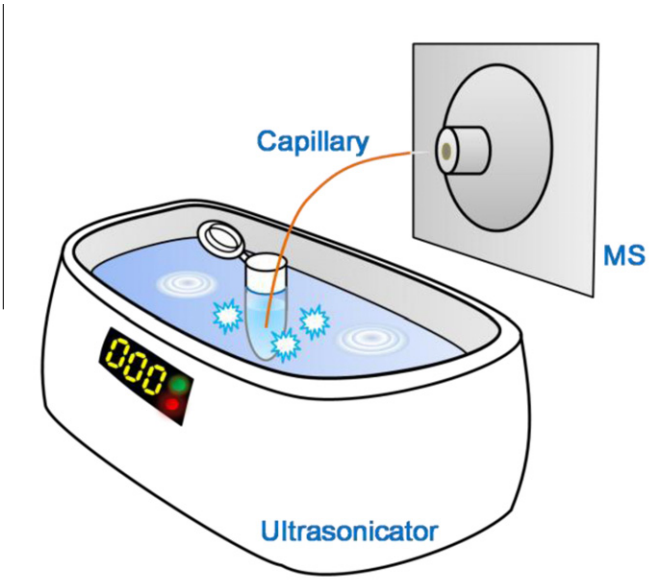

Figure 3. The configuration for on-line monitoring of chemical reactions using UASI-MS. {Reproduced from [49] by permission of The Royal Society of Chemistry}.

idic chips, and, at the same time, monitored ''on-line'' by MALDI-MS. In this case, the reaction product formed in the microreactor channel was identified in real time through a small hole fabricated in the chip (''monitoring window''). It has been concluded that this strategy can facilitate kinetic studies by MALDI-MS, by opening multiple monitoring windows along the microchannel [56].

A device developed by Nichols and Gardeniers [57] incorporated an electrohydrodynamic mixing scheme, and may enable studies of pre-steady-state kinetics [57].

In another study, Nichols *et al.* [58] followed enzyme kinetics in a porous silicon microfluidic channel by combining an enzyme and substrate droplet, allowing them to react and deposit a small amount of residue on the channel walls, and then analyzing this residue using a MALDI-MS instrument. The MS-signal intensity of substrate residue correlated with relative concentrations, and position in the microchannel correlated with time, thus allowing determination of kinetic parameters. It was concluded that the system was especially suitable for initial reaction-velocity determination. The authors suggested that this microreactor is broadly applicable to time-resolved kinetic assays as long as at least one substrate or product of the reaction is ionizable by desorption ionization on silicon (DIOS)-MS [58].

In one of the early studies related to laser MS, IR-laser photodesorption of ammonia from Cu(100) was studied with μs-range resolution [59]. Laser MS was also used to measure the thermal desorption of Ga and of Ga_2O with ps time resolution [60]. A goal of these TRMS studies was to understand the mechanism of laser desorption [61]. Nowadays, TRMS can also be helpful for understanding the mechanism of the MALDI process [62]. For example, using a 400-nm pump pulse, anthracene molecules were excited, and the excitation energy was transferred to a tetracene molecule [62]. Tetracene in the S_1 state was then ionized by a time-delayed probe pulse ($\lambda = 266$ nm), and the resulting tetracene ions were monitored using a TOF mass spectrometer [62]. This TRMS set-up enabled monitoring of events that occurred on the ps time scale.

Despite the mechanistic studies outlined above, one should state that the methods based on laser desorption/ionization (LDI) are mostly off-line, and they can find mainly niche applications. This is due to technical complications imposed because most MALDI instruments are equipped with ion sources maintained under high vacuum. Time-resolved scans are also important for high-throughput chemical mapping using modern MALDI mass spectrometers. The fast analysis of ion plumes formed in the ion source are quickly sent to the mass analyzer (typically TOF), and the motorized XY-stage moves the sample by a short distance to enable analysis of an adjacent location of the two-dimensional sample. Due to the implementation of high-repetition-rate lasers, a sampling frequency of 2 kHz can already be achieved.

Cheng *et al.* [63] used electrospray-assisted laser desorption/ionization (ELDI)-MS for continuous monitoring of chemical reactions in organic or aqueous solution under ambient conditions. They applied this

technique to monitor the progress in several ongoing reactions:

(1) the epoxidation of chalcone in ethanol;
(2) the chelation of ethylenediaminetetraacetic acid with copper and nickel ions in aqueous solution;
(3) the chelation of 1,10-phenanthroline with iron(II) in methanol; and,
(4) the tryptic digestion of cytochrome *c* in aqueous solution [63].

Furthermore, they introduced so-called ''reactive-ELDI'', which supports chemical reactions during the ELDI process. Preliminary data for on-line disulfide-bond reduction using dithiothreitol on oxidized glutathione and insulin show reactive-ELDI to be effective. As suggested by the authors, the laser-desorbed particles merge with the ESI-generated charge droplets to effect chemical reactions prior to on-line MS detection [64].

2.4. Other types of ion source

Meyer *et al.* [65] described a method, with which transient radical cations could be detected in a radical cation chain reaction under conditions comparable to those of the preparative reaction. The reactions were studied by on-line coupling of a microreactor system to atmospheric pressure chemical ionization (APCI)-MS and ESI-MS. This method could be applied to detect transient species in any reaction in solution, presuming that these intermediates are ionic or can be ionized. Two substrate solutions were injected with the same flow rate with a dual syringe pump into an effective micromixer that was coupled directly to the ion source of the mass spectrometer, and the reacting solution was pumped continuously into the MS. While the microreactor was connected directly to the spray capillary, reaction times of 0.7–28 s can be covered [65].

In another study, a single-stage membrane-based interface was developed for real-time MS monitoring of the starting materials and products of a highly-concentrated pharmaceutical-process reaction mixture [66]. The interface was directly connected to the APCI source of a quadrupole mass spectrometer. The reaction was monitored throughout its course, allowing the endpoint to be determined based on the relative concentrations of the reaction precursors and products. The device required minimal analyst intervention, reducing sample preparation and handling prior to on-line real-time MS analysis. However, it took 60 min for the product-ion peak to become the dominating species in the mass spectrum [66]. Coupling APCI-MS with a flow-injection analysis system provided means to automate the monitoring of organic reactions [67]; however, the temporal resolution was limited due to the implementation of discrete sampling.

TRMS employing electron ionization is also applicable to the monitoring of inorganic processes. For example, Zhou *et al.* [68] characterized heterogeneous nanocomposite reactions of Al/CuO, Al/Fe_2O_3 and Al/ZnO using a T-Jump/TOF mass spectrometer equipped with an electron gun for ionization. Low-ms-scale reactions could be monitored using this approach [68].

It should be emphasized that some other ionization techniques [e.g., low-temperature plasma (LTP) ionization or direct analysis in real time (DART)] are also good candidate platforms for fast MS detection and analysis. LTP-MS has been employed for real-time monitoring of some model chemical reactions (e.g., acetylation of ethylenediamine with acetic anhydride, Schiff base formation reaction, and esterifications) [69]. However, in the case of DART-MS, the ''real-time'' speed rather refers to analytical throughput provided by this technique, and its wide applicability in the monitoring of fast chemical processes with high temporal resolution is yet to be proved in future studies.

3. Mass analyzers for time-resolved mass spectrometry

Clearly, the ion source sets the first barrier on the time resolution of MS analysis. For example, it has been estimated that the desolvation of ions in ESI is normally completed in about 1 ms [70]. The next barrier is the mass analyzer – the heart of every mass spectrometer. Modern instruments incorporate one or more mass analyzers. The advantage of having two mass analyzers is the possibility of fragmenting gas-phase ions while taking advantage of high mass resolution of the second mass analyzer. The available mass analyzers include: quadrupoles, time-of-flight (TOF) analyzers, ion traps (ITs), FT ion cyclotron resonance (ICR) analyzers and orbital ITs (Table 1).

High TRMS scans are normally required for coupling high-performance separation systems (CE, ultra-high pressure or nanoflow LC). In these cases, widths of chromatographic peaks are often narrower than 5 s, which imposes the requirement for high scan rates. Fast scans are also required for multiple reaction monitoring, when conducting quantitative analyses on the chromatographically separated analyte zones. Modern mass analyzers already accommodate analysis speed above 10 000 amu/s, so, in most cases, it is the sample interface that limits the temporal resolution of MS analysis.

As a side note, ion-molecule reactions involving gas-phase ions entering the mass analyzers have been used to solve increasingly complex problems, ranging from the determination of the sites of protonation in multi-charged proteins to the development of new ways to characterize the structures of drugs [71]. For example, ion-molecule reactions can be carried out in a tandem the mass spectrometer by using one of mass analyzers as a reaction cell, and then the product ions can be readily characterized by the second mass analyzer [71].

Table 1. Common mass analyzers and their typical performances

Analyzer	Mode	Duty cycle*	Remarks
Triple quadrupole (MRM)	Continuous	15 ms	One-unit mass resolution
Ion trap	Batch	<300 ms	Low to medium budget, multiple fragmentations possible. Very short duty cycles (1 ms) can be achieved at the expense of mass range, mass resolution, and sensitivity
Time-of-flight (with MALDI source)	Batch	<1 ms	Speed related to the laser repetition rate. The high speed is needed for fast MALDI-MS imaging at a high lateral resolution
Quadrupole – time-of-flight	Batch	~20 ms	TOF used for separation of ions; quadrupole used to transfer ions to the TOF tube in the MS mode. Medium budget, medium to high mass resolution
Fourier transform – ion cyclotron resonance	Batch	~1 s	Expensive, bulky, high mass resolution
Orbital ion trap	Batch	~1 s	Expensive, high mass resolution

*These values are rough estimations for some of the commercially available mass spectrometers operated with typical settings. Various instruments (even those with the same analyzer type) will provide different durations of the duty cycle.

Triple-quadrupole instruments were found to be suitable for this kind of experiment [72].

Single-stage mass analyzers are characterized with inherent simplicity, and they are a more economical choice than multiple-stage analyzers. It is helpful to divide mass analyzers into two groups (Table 1):

(1) continuous mass analyzers; and,
(2) batch mass analyzers.

The former separate ions continuously, while the latter separate discrete ''aliquots'' of ions. Magnetic and electric field sectors as well as quadrupoles can separate ions continuously by sweeping magnetic field or electric potential. Sector instruments are nowadays used only in specific applications, while quadrupole instruments are often used in a tandem (two quadrupoles, quadrupole and TOF analyzer, quadrupole and IT). ITs, and FT-ICR analyzer and orbital IT separate discrete batches of ions, so their operation is discontinuous in time. Table 1 indicates typical duty cycles of popular mass analyzers.

Clearly, separation of ions delivered by the ion source is very fast, and recording a narrow m/z-range spectrum can be accomplished within less than 1 ms, even in the case of batch-type analyzers (e.g., IT). In some mass spectrometers (e.g., TOF), acquisition speed and mass resolution are not connected to one another. TOF-MS instruments are convenient for monitoring processes initiated by pulsed laser photolysis [73]. In ITs, acquisition speed can be increased at the expense of mass resolution. Thus, depending on the experimental conditions and requirements, speed and mass resolution have to be adjusted to match research targets. In addition, on ITs, shorter sampling times yield lower sensitivity, and it is up to the experimenter to reach a compromise, taking into account various figures of merit.

In general, the duty cycles of high-mass resolution instruments (FT-ICR-MS and orbital IT) seem to be longer than those of other instruments, so one may conclude that high mass resolution comes at the expense of temporal resolution. Although mass analyzers set a limit on temporal resolution in continuous process monitoring, there are strategies that can overcome this limitation, and gain access to superior temporal resolutions (i.e. <1 ms) (e.g., section 2.2.2, Fig. 2, and [42]).

It should also be noted that using special data-treatment approaches [e.g., Hadamard transform (HT)] can contribute to the development of high-speed instruments [74–76]. HT, a generalized class of FT, has been used in TOF-MS [75]. Unlike conventional TOF-MS approach, HT-TOF-MS can analyze several ion packets traveling simultaneously in the flight tube. These ion packets are encoded by a Hadamard matrix. After detection, all the ion packets can be decoded and deconvoluted. In this way, the time for analysis is shortened and the signal-to-noise ratio can be improved by conducting multiplexed analysis. That is, HT-TOF-MS can provide a faster storage speed than conventional TOF-MS, so it may also be suitable for TRMS that requires fast data acquisition [74].

If a mass spectrometer is equipped with two mass analyzers, both analyzers are used to separate ions when tandem MS is conducted. Otherwise, one of the mass analyzers is used as an ion guide whilst the other one is used for the separation of ions. For example, in popular hybrid mass spectrometers incorporating quadrupole in the first stage and TOF in the second stage, in the MS^1 mode, only the TOF analyzer is used for the separation of ions. In this case, the quadrupole is used only to guide the ions to the pusher of the TOF tube. If both analyzers are continuous analyzers (e.g., quadrupole type), then the hybrid mass spectrometer provides continuous analysis of the supplied ions. However, if the first mass analyzer is a continuous type (e.g., quadrupole) and the second one (used for separation of ions) is a batch analyzer (e.g., TOF), then the operation of the whole system is batch type. Even though the length of the ion path is longer in a hybrid mass analyzer, the overall duty cycle is not significantly longer than that of the batch-type analyzer used for the separation of ions.

4. Sample delivery and treatment

It should be noted that TRMS can be carried out in two ways:

(1) products of the studied process are quickly analyzed by MS; or,
(2) the studied process is preserved in time (e.g., via quenching or continuous reaction at a high speed), and the detection of products at a regular speed follows.

The first option is conceptually straightforward, but it requires high-speed detection. The second option requires closer attention to the interface, which should preserve the momentary chemical composition of a dynamically changing microenvironment. A simple way to achieve such conditions is to take advantage of continuous flow microreactors, which – when operated at high flow rates – can generate samples corresponding to the early stages of chemical reactions.

Another important issue in TRMS is making the sample compatible with both the reaction of interest and the MS detection. For example, some reactions may require salts or other additives that would not work well with direct analysis by MS. This is especially relevant to enzymatic reactions, which may require the use of buffered solutions with adequate pH and ionic strength, as well as the presence of cofactors. In such cases, a compromise has to be reached between the optimal reaction environment and the sample-matrix composition that is compatible with the MS detection.

In most practical applications of TRMS, the investigated process takes place before the mass analyzer, upstream from the ion source. This requires the compartment with the studied process to be coupled to the ion source. As outlined in Section 2, and highlighted in literature [77], it is straightforward to couple an ion source (e.g., ESI) with a batch reactor. In the simplest case, it can be a glass flask filled with the reaction mixture. Examples of coupling batch reactors with MS involve the UASI [49] and C-API [52] techniques, described in sub-section 2.2.3. In these cases, the contents of the reaction mixture are transferred to the orifice of mass spectrometer via a thin capillary (cf. Fig. 3). These strategies are very simple and compatible with many protocols for organic syntheses. Self-pumping of the reaction mixture can also readily be achieved by implementing the recently introduced V-EASI technique [47]. Various arrangements of the (flow-through) reaction systems have been presented over the years to accommodate on-line MS detection. Some of the early studies focused on the combination of flash photolysis with TRMS [78–80] {e.g., I_2 formation due to thermolecular atom recombination in the presence of NO could be followed with μs resolution [80]}. Reactions initiated by a pulsed photolysis laser can nowadays be probed by time-resolved TOF mass spectrometers in the μs time scale [81].

Paiva *et al.* [82] developed a method for detection and characterization of enzyme intermediates on the sub-s time scale using a ''pulsed flow'' method which employs a direct interface between a rapid-mixing device and ESI-MS (Fig. 4).

A more recent example of continuous mixing of reactants involves the rapid mixing at the DESI-MS interface for sub-ms monitoring of a chemical reaction [42] (Fig. 2).

The continuous flow technique can also be used in monitoring structural changes in protein molecules by MS. For example, Konermann *et al.* [83] reported a method for studying the folding kinetics of proteins. This method combined ESI-MS with a continuous flow mixing technique. The reaction time was controlled by the length of the reaction capillary between the mixing point and the ESI source. The method was used to study cytochrome *c* folding kinetics with the time resolution of ~0.1 s [83].

In another study, Hogenboom *et al.* [84] developed a system for continuous-flow analytical screening using ESI-MS. This enabled measurement of the interaction of biologically-active compounds with soluble affinity proteins. The reaction time was 10–20 s, and it depended on the binding constant of reporter ligand-affinity protein complex. The reaction time was chosen in a way that the association of free affinity protein molecules with the reporter ligand was favored, whereas the dissociation of the analyte affinity protein complex was negligible [84].

Griep-Raming *et al.* [85] investigated reactive intermediates of radical chain reactions in solution by ESI-MS. The reaction times could be varied by the flow rate and/or the length of the transfer capillary between the mixer and the ion source. Direct connection of the mixer with the spray capillary allows reaction times of 0.7–28 s. Longer reaction times are easily possible by using a fused-silica transfer capillary of variable length between the mixer and the spray capillary. Mass spectra were measured at reaction times of ~1 s up to several minutes [85].

Flow can also be used for studying gas-phase reactions by MS {e.g., Ludwig *et al.* [86] determined the rate constant for the radical-radical reaction of C_2H_5 and HO_2 at room temperature using a laser-photolysis/flow-reactor combination}.

An outstanding demonstration of an MS method taking advantage of continuous sample delivery is the study by Chen *et al.* [87] (Fig. 5). Unlike many others, this study used an indirect method to follow fast reactions by MS. The strategy coupled a temperature jump with fast photochemical oxidation of proteins (FPOP), whereby folding/unfolding was followed by changes in oxidative modifications by OH radical reactions. This break-

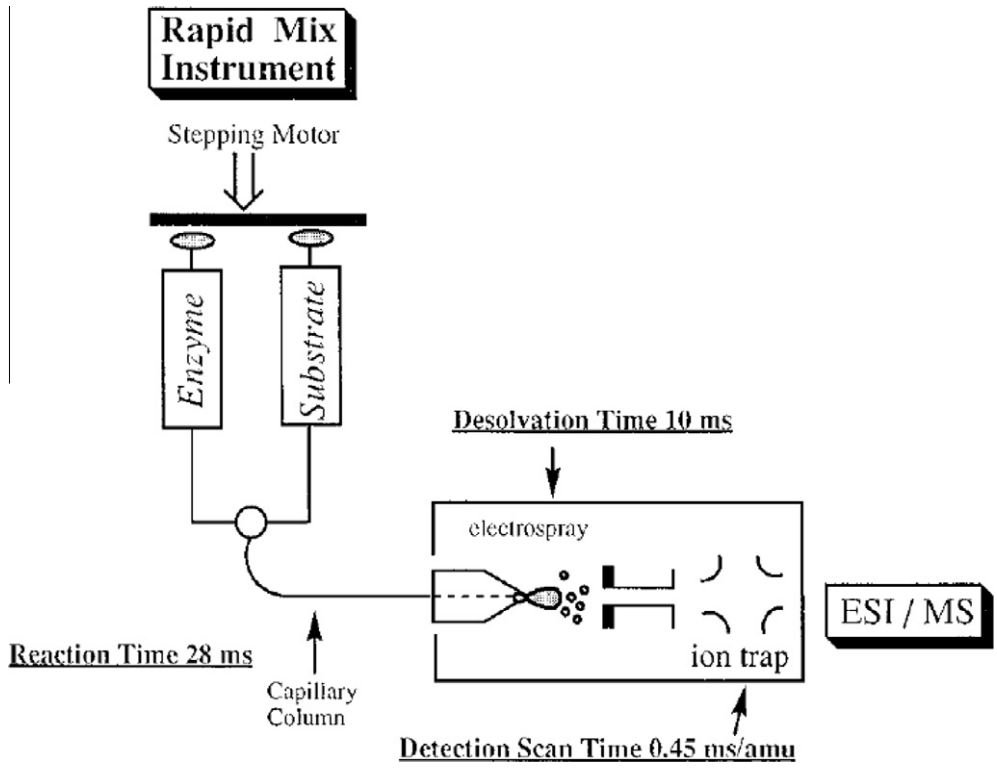

Figure 4. The rapid mix, pulsed-flow electrospray ionization instrument. {Reprinted with permission [82] © 1997, American Chemical Society}.

through offered the promise that the sites and kinetics of folding/unfolding can be followed on the sub-ms time scale [87,88].

Due to the rapid development of microfluidics, it has become possible to couple microfluidic chips with mass spectrometers [89,90]. In addition, microscale ESI (or nano-ESI) emitters can readily be integrated into the polymer block of microchips. Van den Heuvel *et al.* [91] developed a fast, direct method for monitoring enzymatic DNA hydrolysis using ESI-MS. They incorporated a robotic chip-based ESI source for increased reproducibility and throughput. This method enabled detection of DNA fragments and intact non-covalent protein-DNA complexes in a single experiment [91].

Over the past few years, microreactors have been increasingly important in the field of proteomics [92] [e.g., in the study by Liuni *et al.* [93], a microfluidic reactor was used to conduct fast proteolysis (residence time <4 s) followed by ESI-MS detection}. A microfluidic reactor connected to an ESI mass spectrometer also enabled studying H/D exchange in the sub-s regime [94]. Microfluidic systems also enable the monitoring of reactions taking place in microdroplets by MS [95].

Fritzsche *et al.* [96] demonstrated the feasibility of using a microchip as the microreactor for screening catalysts in asymmetric synthesis followed by on-line MS detection. Importantly, they showed a combination of enantioselective organocatalysis, enantiomer separation, and MS detection on a single microchip. In this work, nano-electrospray was used as the interface to couple the microchip with a mass spectrometer. This microchip system was successfully applied to screen catalysts in asymmetric synthesis. The authors suspected that further optimization of the chip layout, by shortening channel lengths, pressure assistance, and the utilization of fast mass spectrometers, should enable detection and identification of short-lived species [96].

In conclusion, continuous-flow reactors – when coupled to mass spectrometers – generally provide better access to fast chemical processes. This is because the residence time of reactants in the reaction zone can be controlled precisely and minimized by adjusting the flow rates of the reaction substrates.

However, fluidic systems coupled with mass spectrometers have applications beyond the monitoring of chemical reactions. Kennedy and co-workers [97] demonstrated the possibility of using segmented flow to transfer samples collected in mouse brain to the ESI emitter. This provided temporal resolution of several seconds. In this case, the segmentation of the flow was important to prevent hydrodynamic dispersion of the collected samples during their transfer to mass spectrometer. The coupling of segmented flow with ESI incurred technical problems, which were elegantly solved.

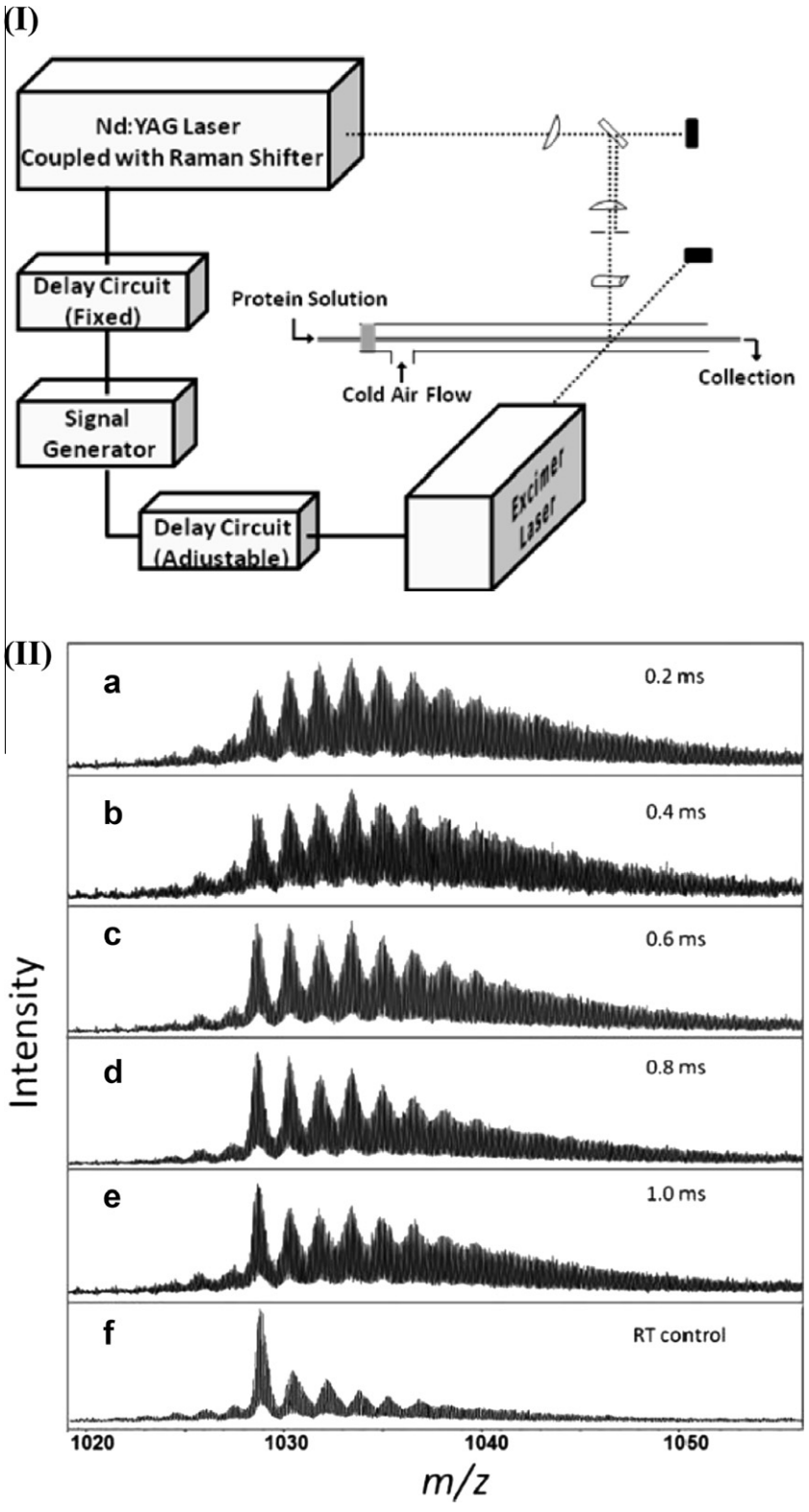

Figure 5. (I) The flow system intersected by two laser beams at a window in the tube, as previously described for fast photochemical oxidation of proteins (FPOP) [101]. The time between the two laser pulses is adjustable with the ''delay circuit''. (II) (a–e) Representative mass spectra of the barstar post-FPOP as a function of the time between the heating pulse and the FPOP probe. (f) Mass spectrum of the barstar post-FPOP at room temperature as a control. {Reprinted with permission from [87] © 2010, American Chemical Society}.

5. Concluding remarks and prospects

As previously noted by Rob and Wilson [98], a primary advantage of TRMS is the ability to detect virtually all reactive species simultaneously. From our literature survey, it appears that the older work (conducted in the 1980s) focused mainly on the study of chemical processes involving low-molecular-weight species. In the past few years – due to the significant technological development (e.g., ion sources) – interest has shifted towards monitoring processes involving larger molecules (>100 Da), including proteins. From the examples presented above, it is evident that TRMS has already found a number of applications, e.g.:

(1) investigation of reaction kinetics and protein folding;
(2) studies on reaction mechanisms;
(3) detection of short-lived intermediates; and,
(4) probing fast biochemical processes *in vitro* and *in vivo*.

However, TRMS also finds applications in less obvious areas, including the monitoring chemical processes in propagating flame [99] or recording convection waves [31].

Despite the high scan rates of modern mass spectrometers, MS techniques are usually applied to monitor chemical reactions with relatively low temporal resolution, typically counted in minutes or seconds; few protocols are available for time resolutions of ms. However, in many cases, the fastest MS methods only enable fingerprinting of the fast processes (e.g., observation of short-lived reaction intermediates).

When it comes to monitoring chemical processes in a continuous fashion, the time resolution is normally much less, and it is typically limited to seconds and tens of seconds. Among the interfaces developed to date, ESI-MS remains the leading one, while DESI-MS is emerging as an interface that can provide extremely high resolution, but its applicability is currently limited to specific cases.

There is still a long way to go in the development of delay-free MS interfaces for reaction monitoring. Although it is feasible to accommodate fast scan rates for the separated zones of analytes with relatively high local concentrations, it is much more difficult to sample unpurified and unseparated mixtures of dilute analytes that are applied for a short period of time. Efficient transfer of samples probed in the reaction vessel to the ion source should be addressed in future studies.

Hydrodynamic dispersion and diffusion, which occur in long transfer lines, effectively decrease temporal resolution of the MS-based monitoring. This problem could be addressed to some extent by implementing segmented flow systems and microfluidic devices. It should also be noted that commercial mass spectrometers are usually designed for coupling separation, not direct infusion, so, when coupling reaction systems with MS instruments for time-resolved studies, possible contamination of the early-stage ion-transfer line is a big challenge to tackle.

Another important issue to address is quantification of analytes in the presence of sample (reaction) matrix, while preserving temporal resolution. It may partly be addressed by using internal standards (e.g., isotopically-labeled analogs of the analyzed molecules). Isotopic labeling prior to the analysis of samples by MS is very convenient in studies of metabolic dynamics [100].

In addition, some research groups currently attempt to miniaturize mass spectrometers in order to make them portable. However, the performance of these ''hand-held'' mass spectrometers needs to be improved to make possible their implementation in TRMS analysis.

Acknowledgements

We recognize that not all the articles relevant to the topic of TRMS could be cited in this review due to space limitations, so we apologize to the authors of the excellent reports that we have not presented in this short review, and acknowledge their important contributions. We appreciate the comments of the anonymous reviewer that helped us improve the manuscript. Y.-C.C. and P.L.U. also acknowledge the support of the National Science Council, Taiwan.

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