Direct Mass Spectrometry Analysis of Complex Mixtures by Nano-Electrospray with Simultaneous Atmospheric Pressure Chemical Ionization and Electrophoretic Separation Capabilities

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Supporting Information Placeholder

ABSTRACT: Accuracy and rapid analysis of complex microsamples are challenging tasks in translational research. Nano-electrospray ionization (nESI) is the method of choice for analyzing small sample volumes by mass spectrometry (MS) but this technique works well only for polar analytes. Herein we describe a versatile dual non-contact nESI/nAPCI (nano-atmospheric pressure chemical ionization) source that allows simultaneous detection of both polar and nonpolar analytes in microliter quantities of samples under ambient conditions and without pre-treatment. The same device can be activated to enable electrophoretic separation. The non-contact nESI/nAPCI MS platform was applied to analyze different samples, including high sensitive direct analysis of biofluids and the efficient detection of proteins in buffers with high concentration of nonvolatile salts. Excellent linearity, accuracy and limits of detection were achieved for compounds with different chemical properties in different matrices. The high sensitivity, universality, simplicity and ease of operation make this MS technique promising for use in clinical and forensic applications.

One of the challenges of translational research is the availability of reliable quantitative assays that have the required sensitivity and accuracy to evaluate complex biological samples in a high throughput fashion. Mass spectrometry (MS) has shown great promise in this respect, allowing quantification of both biomolecules and small organic compounds.[1-3] It is well recognized that sample preparation is the slowest step of the chemical analysis process and so the recent introduction of ambient ionization methods, which integrate analyte extraction and ionization into a single step, represents an important advancement for achieving rapid analysis. Aside from quantitation and high throughput requirements, another important merit in the biomedical field is the analysis of microsamples (<50 μL) with minimal dilution. Nano-electrospray ionization (nESI) MS is one of the most efficient methods for small volume analysis.[4,5] With appropriate modifications, it can be used to analyze untreated complex mixtures.[6,7]

An unmet need is for the integrated, robust, and versatile nESI system that can quantitatively and rapidly ionize polar and non-polar organic compounds, and large biomolecules in various matrices. Herein, we describe a simple integrated approach that combines non-contact nESI, a novel nAPCI (nano-atmospheric pressure chemical ionization, explained below), and electrophoretic separation in one complimentary analytical tool to make possible a more complete analysis of structurally diverse compounds from small complex mixtures. The results indicate that a non-contact mode nESI allows the application of >4 kV spray voltage without damaging the tip of the glass emitter. In the presence of an auxiliary electrode (e.g., Ag), placed in close proximity to the emitter tip, the applied high voltage induces corona discharge in ambient air, which allows for simultaneous formation of protonated $(M+H)^+$ and molecular ions $(M^{\bullet+})$ via atmospheric pressure chemical ionization (APCI). We suggest referring to this ionization process as nAPCI because it ionizes gas-phase species delivered at a ~10 nL/mL flowrate from a conventional direct infusion nESI setup. Unlike the traditional APCI method, the low flowrates in the nAPCI process allows effective desolvation and molecular ion generation without the use of nebulizer gas. The elimination of heated nebulizer gas in turn enabled a more simplified and compact experimental setup, with the corona discharge needle (i.e., the auxiliary Ag electrode) coaxial with the direction of the nanodroplets. Corona is not formed at low spray voltages (≤3 kV), hence protonated $(M+H)^+$ or deprotonated $(M-H)^-$ ions are predominantly generated enabling the detection of large biomolecules like proteins by the same ion source.

Many studies have investigated various aspects of the nESI setup, including (i) the mode by which the analyte solution is electrically charged (i.e., contact versus non-contact),[8] (ii) the source/nature of the electrical energy (e.g., piezoelectric discharge, triboelectric nano-generator, pulsed DC/AC voltage and square-wave potential),[9-12] (iii) flowrate manipulation to control ion suppression and sample consumption (e.g., via the use of smaller tip size[13,14] or on-demand pulsed charges[11]), (iv) reduction of electrical current (via the use of high input ohmic resistance) to avoid destructive corona discharge phenomenon when electrospraying under high voltage conditions,[15,16] and (v) the use of other operational tricks like step voltage and polarity reverse applications.[17,18] None of these methods are completely adequate, especially for simultaneous generation of different ion types. There appear to be one case where the alternative operation of independent nESI and APCI sources allowed for the generation of ions of opposite polarities for ion/ion reactions. Unlike our proposed approach, that study used the conventional contact-mode nESI and two separate HV power supplies.[19] Hybrid ESI and APCI ion sources have been developed[20,21] but they are designed for larger volumes of samples, and often combined with liquid chromatography and/or electrophoresis; the

corona discharge is not applied to the mixture of gas analytes derived directly from droplets as is done here. In addition to the integration of experimental steps and small sample consumption, the proposed dual non-contact nESI/nAPCI method is capable of *in-situ*, in-capillary liquid/liquid extraction,[6] which enables part-per-trillion (pg/mL) level sensitivity for cocaine and part-per-billion (ng/mL) detection limit for a non-polar β-estradiol analyte, all from untreated whole human blood. The adapted in-capillary liquid/liquid extraction procedure facilitates microsamples (5 µL) processing and analysis by MS, compared with offline extraction procedures that require sample dilutions and additional transfer steps. Analysis of the dilution sample certainly will require a more sensitive instrument, one that might not be readily available. The potential applications of this non-contact nESI/nAPCI MS approach may include omics fields, in various steps of drug development (pre-clinical research) and in clinical applications (e.g., newborn screening, forensic toxicology, therapeutic drug monitoring, etc.), all requiring minimally invasive microsampling.

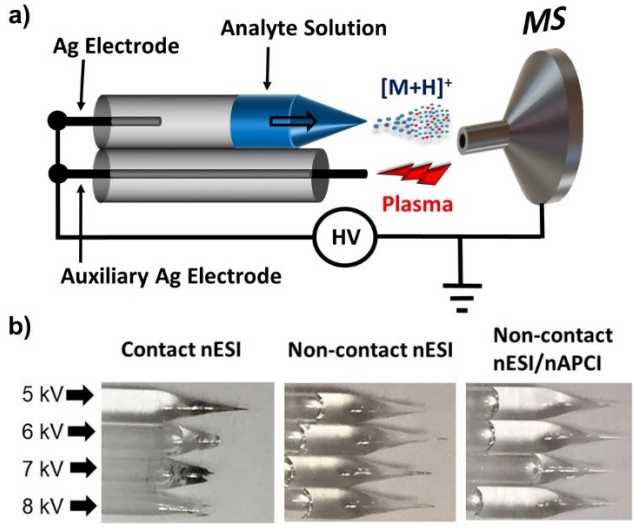


Figure 1. a) Schematic diagram of non-contact nESI/nAPCI platform for the analysis of homogeneous solutions. **b**) Photographs showing the effect of Joule heating on stability of emitter tip (filled with water) for contact nESI, non-contact nESI, and non-contact nESI/nAPCI sources.

EXPERIMENTAL SECTION

Non-contact nESI/nAPCI Apparatus. The dual non-contact nESI/nAPCI experimental setup is as shown in Figure 1a, and is capable of three spray modes: a) Non-contact nESI in which the analyte solution present in a disposable glass capillary (ID 1.2 mm; ≤5 µm pulled tip, Fig. S1) is electrically charged through electrostatic induction.[22,23] That is, the Ag electrode on which the DC high voltage (HV) is applied is not in physical contact with the analyte solution. Instead, a ~1 cm air gap is created, and as little as 1 kV applied voltage is able to induce electrostatic charging, which causes the release of charged droplets from the capillary tip that are sampled by the mass spectrometer. b) Non-contact nESI/nAPCI mode, where both charged droplets and plasma are simultaneously produced when potentials above the breakdown voltage (4 kV) of air are applied. Here, the presence of auxiliary Ag electrode placed in a collimating glass capillary (ID 1.2 mm) allows the exposure of the resultant solvated/gas-phase ions to corona discharge. Note: a single HV power supply (available from the MS instrument) is used, plus no further modification of the conventional nESI source is required except for the attachment of the auxiliary Ag electrode, which does not obstruct nESI performance at low spray voltages. c) Electrophoretic separation spray mode in which polarity reversing (from negative to positive voltage) enables detection of highly resolved multiply-charged protein ions under high voltage conditions in the presence of concentrated inorganic salts.

Instrumentation. Sample analysis was carried out with a Velos Pro LTQ mass spectrometer using Xcalibur 2.2 SP1 software (Thermo Fisher Scientific, San Jose, CA, USA). Applied MS parameters were as follows: 400 °C capillary temperature; spray voltage (2kV for nESI, 6 kV for non-contact nESI/nAPCI, and alternate from -5 kV to 2 kV for electrophoretic separation); 60% S-lens voltage; 5 mm distance from an ion source to MS inlet; 3 microscans; 100 ms ion injection time; 30 s spectra recording time. Analytes were identified by tandem MS with collision induced dissociation (CID).

Chemicals, Reagents, and Samples. Caffeine (99.0%), 3,5-dimethyl-1-hexyn-3-ol (surfynol 61, 98%), docosanoic acid (99%) ergocalciferol (vitamin D2, 98%), β-estradiol (98%), ethyl acetate (99.8%), 5-fluorouracil (99%), lauric acid (99.5%), linoleic acid (99%), methanol (99.9%), oleic acid (99%), stearic acid (95%), and thymol (98.5%) were all purchased from Sigma Aldrich (St. Louis, MO, USA). Phenol (99%), and stereo 2X-4X microscope (S 15019975) was provided by Fisher Scientific (Pittsburgh, PA, USA). 1.0 mg/mL standard solution of cocaine and 100 µg/mL of cocaine-D3 were acquired from Cerilliant (Round Rock, TX, USA). Dichloromethane (99.9%) was supplied by Acros Organics (Geel, Belgium). Both single donor human blood and single donor human serum were obtained from Innovative Research (Novi, MI, USA). Bovine blood was provided by LAMPIRE Biological Laboratories (Pipersville, PA, USA). 18.2 MΩ water was used for water solutions (Milli-Q water purification system, Millipore, Billerica, MA, USA). Borosilicate capillaries (ID 1.17 mm) were provided by Sutter Industries (Novato, CA, USA).

RESULTS AND DISCUSSION

Stability of Glass Tip at High Voltages. Figure 1b compares tip stability under different spray conditions. Joule heating[24] (among other factors) generated after applying 5-8 kV to an electrode in contact with analyte solution (conventional nESI) is mainly responsible for the tip burning/breakage observed here. For example, the electric field at the emitter apex is a key parameter in controlling electrospray current, which in turn determines the intensity of heat generated. Therefore, heating can be expected to be more effective at the tip of the glass capillary. To investigate the effect of field strength on heat generation in our experiment, we monitored the extent of tip damage/burning as a function of spray distance (1 – 50 mm) from the glass tip to the inlet of the mass spectrometer while keeping the spray voltage constant at 6 kV in the contact mode nESI experiment. As expected, we observed a reduced burning effect as the spray distance was increased (Fig. S2a). For 1 – 25 mm distances, ion signal was stable for ~30 s, after which an obvious boiling of the aqueous analyte solution occurred (indicated by visible bubble formation due to extensive Joule heating; see Video S1). This period was followed immediately by the burning of the glass tip, resulting in concomitant termination of ion signal

(Fig. S2b; also see Video S2). Additional close examinations indicated that tiny bubbles begin to form at ~3 s following the application of voltage (Video S1) and bright glow (electrical discharge) formed at the tip follows after 10 s later. Physical inspections of the tip revealed partial cracks at ~14 s of electrospray under the contact mode conditions. Based on these empirical observations we propose the following mechanism: boiling of analyte solution happens first, followed by burning, which leads to partial breakage (cracking) of the glass tip. These events reduce solvent flow at the tip and cause the observed electrical discharge while Joule heating exacerbates. The combined effects of discharge and heating result in visible damage of the glass tip. Glass tips remained stable at 50 mm spray distance but signal intensity decreased by two orders of magnitude due to inefficient ion transfer. Joule heating significantly reduced in the non-contact spray mode due to the presence of the air gap (resistivity of air is >1.3 x 10^{16} Ω at 200 °C), which led to much more stable tips at the same applied voltages. Interestingly, the glass tips became remarkably stable in the presence of the proximal auxiliary Ag electrode (non-contact nESI/nPACI, Fig. 1b). In this case, the well-known cooling effects of corona discharge further reduces Joule heating by inducing rapid movement of air/droplets around the tip area.[25,26] As will be shown, the ability of non-contact nESI/nAPCI setup to tolerate high voltages could be a useful feature in MS analysis.

Detection of Polar and Nonpolar Compounds. Figure 2a shows positive-ion mass spectrum recorded after the analysis of a methanol solution containing equimolar (200 μM) mixture of 5-fluorouracil (1), caffeine (2), β-estradiol (3), cocaine (4), and vitamin D2 (5) using the conventional contact mode nESI source at an applied voltage of 2 kV. As can be observed, only the polar cocaine analyte with high proton affinity (930 kJ/mol) was detected at m/z 304. Caffeine (MW 194), another polar analyte was significantly suppressed despite having relatively high proton affinity (914 kJ/mol). Not surprisingly, detectable ion signal was not observed for 1, 3 and 5, even from individual solutions (i.e., in the absence of other analytes) at 10 ppm concentration levels (Fig. S3). Similarly, protonated cocaine ions were predominantly detected when the mixture was analyzed by non-contact nESI operated using 2 kV spray voltage in the absence of corona discharge (data not shown). Upon increasing the voltage from 2 to 6 kV, corona discharge was induced on the auxiliary Ag electrode, expecting the ionization of both polar and non-polar compounds delivered by the spray plume. The corresponding non-contact nESI/nAPCI positive-ion mass spectrum is shown in Figure 2b, which confirms the presence of all five analytes. Compounds 1, 2, and 4 were observed as protonated $(M+H)^+$ ions at m/z 131, 195, and 304, respectively. Like conventional APCI experiment,[27] dehydration reactions involving (pseudo) molecular ions were also observed with β-estradiol (MW 272) registering as $[M+H-H_2O]^+$ species at m/z 255. Radical species $M^{\bullet+}$ and $(M-H_2O)^{\bullet+}$ were also detected for vitamin D2 (MW 397) at m/z 397 and 379, respectively. Other nonpolar compounds (carminic acid, thymol, surfynol 61, phenol), which could not be detected by conventional nESI at 1 ppm level, were also successfully characterized by non-contact nESI/nAPCI tandem MS (Table 1). These results establish the integrated non-contact nESI/nAPCI MS platform as efficient method to simultaneously ionize both polar and nonpolar compounds simply by increasing spray voltage from 2 to 6 kV.

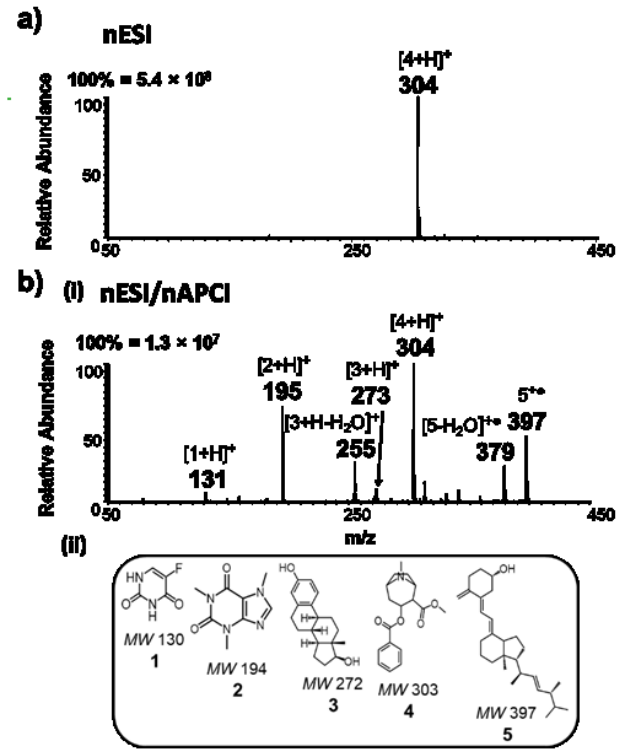


Figure 2. Analysis of 200 μM equimolar mixture of 5-fluorouracil (1), caffeine (2), β-estradiol (3), cocaine (4), and vitamin D2 (5) in methanol by **a**) conventional nESI and **b**) non-contact nESI/nAPCI methods operated at 2 and 6 kV spray voltages, respectively.

Direct Biofluid Analysis. These encouraging results motivated us to explore direct biofluid analysis. Here, 3 μL of ethyl acetate was first placed in the sharp tip of the disposable glass capillary. The organic ethyl acetate solvent was chosen because it is immiscible in water, and prior studies[6,23] have shown it to have high solubilizing power for a wide range of organic compounds and it is suitable for electrospray. A small volume (5 μL) of the biofluid sample spiked with a selected analyte (Table 2) was then introduced on top of the ethyl acetate solvent (Fig. 3a), followed by a short shake (e.g., 1 – 3 strokes) to initiate liquid-liquid extraction in the capillary as well as to remove air bubbles that may be present at the capillary tip. Ion signal for extraction analytes was not significantly affected by different number (n>3) of strokes (data not shown). Note that the three strokes of shaking employed here form part of the regular nESI MS analysis, and do not add extra steps to the analytical process. Often, the shaking process resulted in the disintegration of the biofluid into smaller compartments (Fig. 3b), which facilitated efficient extraction via increased interfacial contact with the extracting organic solvent. The high buoyancy of the less viscous ethyl acetate solvent (density 0.9 g/mL) draws the clean extract to the sharp tip of the glass capillary for easy analysis by non-contact nESI/nAPCI MS. Moreover, since the Ag electrode is not in direct contact with sample/solvent, extraction equilibrium is not disturbed; a contact mode experiment where the electrode is pushed through the biofluid will reintroduce contaminants into the extract, which may cause matrix effects during analysis. The pure extract present at the tip of the glass capillary typically offered a stable 1 min spray time (Fig. 3c), which is sufficient for complete MS analysis, including tandem MS (MS/MS). Unless otherwise stated, internal standard (IS) used for analyte quantification was added to the biofluid. The

optimal amount of extraction solvent (3 µL) was used to compromise between spray

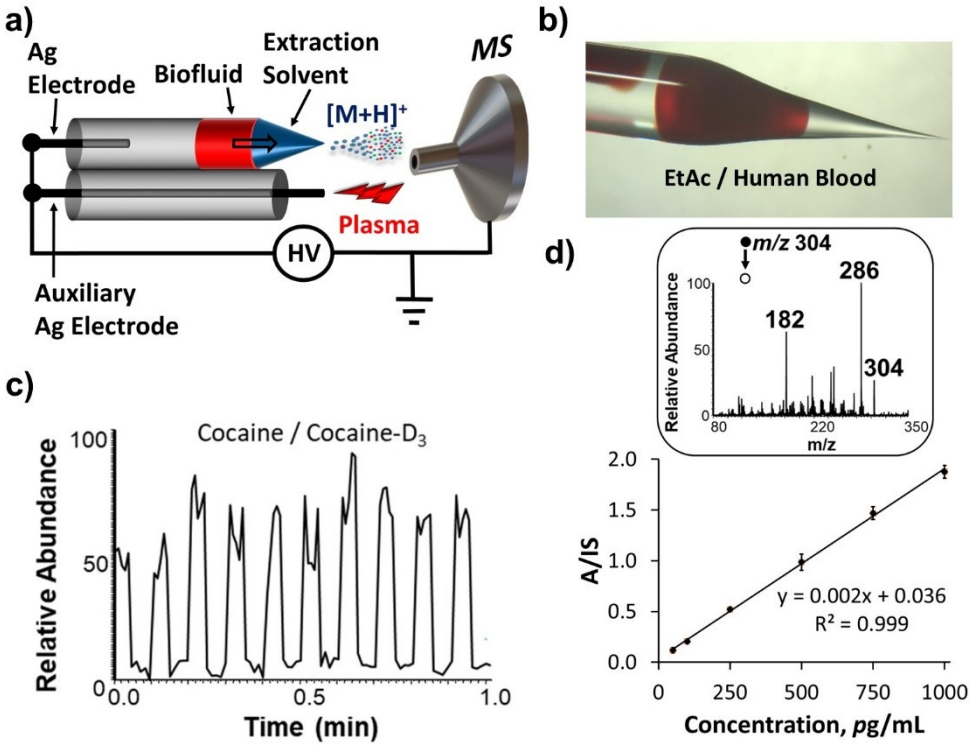
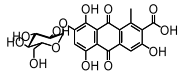
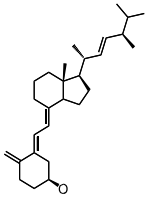
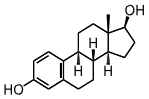
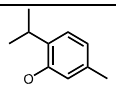
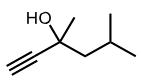


Figure 3. **a**) Experimental setup enabling the application of non-contact nESI/nAPCI MS platform for direct biofluid analysis. **b**) Photograph showing in-capillary liquid/liquid extraction of cocaine from whole human blood (5 µL) by ethyl acetate. **c**), **d**) Quantification of blood samples spiked with cocaine (50 – 1000 *p*g/mL) and 500 *p*g/mL cocaine-D3 as IS using non-contact nESI/nAPCI MS^2 with MRM (transitions *m/z* 304 → 182 and *m/z* 307 → 185 for the analyte and IS, respectively). Insert in 3d shows MS^2 of cocaine at 50 pg/mL level.

Table 1. Detection ability of non-contact nESI/nAPCI MS/MS mode

#	Compound	Structure	MW	Observed Ion(s)	MS/MS Transition(s) by CID
1	Carminic acid		492	$[M+H_2+H]^+$	495 → 453, 439, 411, 383
2	Vitamin D2		397	$M^{+\bullet}$	397 → 379, 369, 351, 327, 271
				$[M+H]^+$	398 → 380, 370, 328, 272
3	*β*-Estradiol		272	$[M+H-H_2O]^+$	255 → 237, 199, 173, 159, 145, 133, 109
				$[M+H]^+$	273 → 255, 159, 135
4	Thymol		150	$[M+H]^+$	151 → 133, 123, 109, 93, 81
				$[M+H-C_3H_6]^+$	109 → 81, 67
5	Surfynol 61		126	$[M+H]^+$	127 → 109, 99, 85
				$[M+H-H_2O]^+$	109 → 81, 67
6	Phenol		94	$M^{+\bullet}$	95 → 77, 67
				$[M+H]^+$	94 → 79, 66

Compounds were analyzed in MeOH at 1 ppm level by non-contact nESI/nAPCI. Conventional nESI failed to produce detectable signal at 1 ppm for all compounds, in both full MS and MS/MS modes.

Table 2. Limit of detections for compounds analyzed in different biofluids employing non-contact nESI/nAPCI MS/MS

Analyte	Sample	Voltage (kV)	LOD (*n*g/mL)
Cocaine	Serum	2	0.5×10^{-3}
	Blood		1.2×10^{-2}
	Urine	6	0.01
β-Estradiol	Blood	6	10
Caffeine	Blood	6	15

time and signal intensity. For instance, applying 3 µL versus 5 µL of ethyl acetate increased analyte to internal standard (A/IS) signal ratio for cocaine extracted from serum by a factor of 10 (here, IS was spiked into the extraction solvent to keep the amount of IS constant, Fig. S4). This increase in signal might be due to a number of related factors, which include: i) pre-concentration effects, arising from the large K (partitioning coefficient = 794 in chloroform, which is similar by polarity to ethyl acetate) value for cocaine between water and the organic solvent. In absolute terms, the amount of cocaine extracted into 3 µL solvent is expected to be ~2X more than that extracted into 5 µL µL; ii) it was observed that the volume of clean extract pooled to the emitter tip (Fig. 3b), after mixing with 5 µL biofluid, was comparable regardless of the initial volume of organic solvent used. For higher volumes (e.g., 5 µL), significant amount of the excess extract was found trapped at the back of the biofluid that cannot be accessed during electrospray. This effect decreases the concentration of analyte in the extract residing at the capillary tip for larger solvent volumes and hence reduced A/IS ratio; iii) effective saturation of organic solvent with water in smaller volumes. We determined that ethyl acetate saturated with water (2% maximum) is a much better electrospray solvent than the pure organic solvent (see SI, Fig. S5, for details). Therefore, we expect the doping of water from the biofluid into 3 µL volume to be more efficient during the liquid/liquid extraction process, which can contribute to the enhanced signal observed. Initial volumes of solvent lower than 3 µL result in decreased amount of extract pooled to the tip of the glass capillary, which in turn reduced spray times (< 1 min).

Representative product ion spectrum for 50 pg/mL cocaine spiked in undiluted blood (5 µL) is shown as insert in Figure 3d, which registered the diagnostic fragment ion at m/z 182 in high abundance. Figure 3d shows a calibration curve derived from comparing the product ion (m/z 182) intensity at different concentrations of cocaine analyte (50 – 1000 pg/mL) to that of internal standard (IS, cocaine-d3, 500 pg/mL) spiked into the blood sample. Excellent linearity (R2 = 0.999) and limit of detection (LOD) of 12 pg/mL were achieved. LODs for other analytes are shown in Table 2, which include 0.5 pg/mL sensitivity for cocaine in serum. Aside from high extraction efficiency (controlled by pre-concentration effect), high ionization efficiency, and minimal matrix effects, additional enhancing effect may arise from the smaller initial droplets of ethyl acetate expected from the low flowrate (~10 nL/min) non-contact mode nESI experiment. (Comparable tip size of µµm yields the typical ~30 nL/min flowrate in traditional nESI,[28] Figs. S6 – S8). The smaller flowrates derived from the highly volatile ethyl acetate spray solvent facilitated analyte introduction into the gas phase for efficient APCI ionization. On the contrary, we observed a markedly reduced ionization efficiency for aqueous-based samples (Fig. S9) presumably because of limited solvent evaporation from the aqueous droplets, which reduced the efficiency of ion formation via chemical ionization. Flowrates (Fig. S7) for non-contact nESI/nAPCI at high voltages (≥4 kV; Fig. S2) were surprisingly similar to those for non-contact nESI recorded at low voltages (≤2 kV). Although this requires further investigation, it might be related to differences in the shape of the electric field at the emitter tip, when comparing the presence (high voltage) and absence (low voltage) of corona discharge from the auxiliary electrode. Another factor influencing ionization efficiency, and hence sensitivity, is our ability to generate different ion types simply by using higher spray voltages. For example, the weakly polar and high eluent strength (0.58) properties of ethyl acetate is expected to result in high extraction efficiency for steroid analytes such as β-estradiol (MW 272 Da). However, analysis by contact mode nESI MS often yields low sensitivity due to low proton affinity. Derivatization reactions are typically used to overcome this limitation.[6,29] A 10 ng/mL LOD (Table 2) was observed for β-estradiol in whole human blood by utilizing an optimized spray voltage of 6 kV, which enable the detection of $(M+H–H_2O)^+$ ion in tandem MS (m/z 225 → 199; see Fig. S10a at the limit of detection) mode without derivatization reactions. For caffeine (MW 194 Da) in whole blood, the measured LOD was 15 ng/mL (see Table 2 and Fig. S10b) in MS/MS analysis of $(M+H)^+$ species (i.e., m/z 195 → 177). Vitamin D2 and phenol were detected as molecular ions ($M^{\bullet+}$; Table 1).

The fact that the non-contact dual non-contact nESI/nAPCI source is operated without the assistance of nebulizer gases, and in the presence of limited solvent molecules under the nL/mL flowrate conditions suggests highly reactive ionic species [e.g., $H^+(H_2O)_n$; where n = 1 or 2][30,31] might be involved in the ionization process compared with the conventional APCI experiment, which employs N_2 gas and high solvent flowrates (µL/mL). Evidence for the presence of low molecular weight water clusters is seen in the fragmentation of protonated species, which could be a result of the large difference in proton affinities (PA) between analytes (e.g., β-estradiol; PA 814 kJ/mol)[32,33] and the reagent ion, H_3O^+. Aside from gas-phase chemical reactions, we believe the present configuration of the non-contact nESI/nPACI setup (co-axial) may allow surface-assisted ionization processes. This is exemplified by the detection of $[M+H_2+H]^+$ species from methanol solution of carminic acid (Table 1), which was recently characterized to involve the hydrogenation of C=O bond for analytes adsorbed at the surface of the corona discharge electrode.[34] The presence of surface-assisted reactions has profound implications on the mechanism of $(M+H)^+$ and $M^{\bullet+}$ ions formation, which may include field ionization and field-induced proton transfer reactions ($M^{\bullet+}_{(surf)} + H_2O \rightarrow [M+H]^+ + HO^{\bullet}$). Another unique feature of the proposed setup is that biosamples can be reanalyzed by repeated cycles of in-capillary extraction and ionization. Here, similar ratios of cocaine signal to that of IS were observed in serum for seven cycles of analyses performed in the same day (Fig. S11), and for four cycles of analyses conducted over a period of one month for a sample storage in freezer (Fig. S12).

The in-capillary extraction methodology adapted here does not only provide a simple means to effectively process small volume of biofluids, but it also affords a way to contain the biofluid in the enclosed environment of the glass capillary preventing possible unexpected exposure of biofluid to analyst, something that is difficult to achieve with other direct MS analysis platforms such as desorption electrospray ionization and paper spray. The use of ethyl acetate spray solvent further minimizes safety risks due to its antimicrobial activity.[35] In addition, ethyl acetate is immiscible in aqueous samples and thus extracts only the organic components, leaving behind the bulk of the blood matrix in the glass capillary for safe disposal. Like all other spray-based atmospheric pressure ion sources, additional protective casing can be used to restrain the charged aerosols.

Electrophoretic Separation. The last integrated application examined for the new ion source was electrophoretic desalting and detection of proteins in concentrated salt solutions. As demonstrated by others,[36,37] we also employed polarity-reversing on our non-contact nESI/nAPCI platform where a step potential was used starting from negative to positive high voltage polarities. A unique capability provided by our experimental setup is the fact that large step voltage differences (e.g., from –5 kV to +2 kV) can be used without damaging the disposable glass tip due to reduced Joule heating. Figure 4 shows real-time separation of cytochrome c in 1X phosphate-buffered saline solution (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4 and 1.8 mM KH_2PO4) obtained after applying –5 kV for 10 s followed by the application of +2 kV (see insert of Fig 4a; 0.1% of formic acid was added to the buffered protein solution). Figures 4a-c show three distinct time domains during the mass analysis at +2 kV: highly charged protein species were detected first (between 10 – 31 s). After this initial spray time, a broad range of protein charge states emerged within 31 – 88 s. All the denatured bulky proteins were exhausted after 88 s of continuous spray at which point only low charge state proteins were detected for the remaining 3.7 min spray time. Overall, the solution with depleted salt lasted for about 5 min, which is sufficient for complete MS analysis. Similar desalting effect was observed for ubiquitin using –5 kV to +2 kV step voltage conditions with 2.5 min of total spray time (Figs. S13). The differences in protein charge state distribution observed at different spray times may be related to a number of factors, including changes in protein conformation during the time of ionization, which could be caused by changes in solvent composition. Protein unfolding can also occur during the 10 s electrophoretic separation period, followed by refolding events in the ionization process. It is also possible that different conformations of protein may exist in solution prior to the electrophoretic experiment, which can be separated based on differences in electrophoretic mobilities between the bulky unfolded versus folded proteins. Note: without polarity reversing, proteins could not be detected in 1X PBS buffer employing either our setup or the regular contact mode nESI source. With polarity reversing, our setup offered acceptable separation in real-time not only for the temporal desalting of biomolecules but also the spatial separation of different conformations of a single protein. The later effect has not been reported before in all other polarity-reversing experiments. In some cases, the separation can be achieved without adding acid (i.e., ubiquitin, Fig. S13).

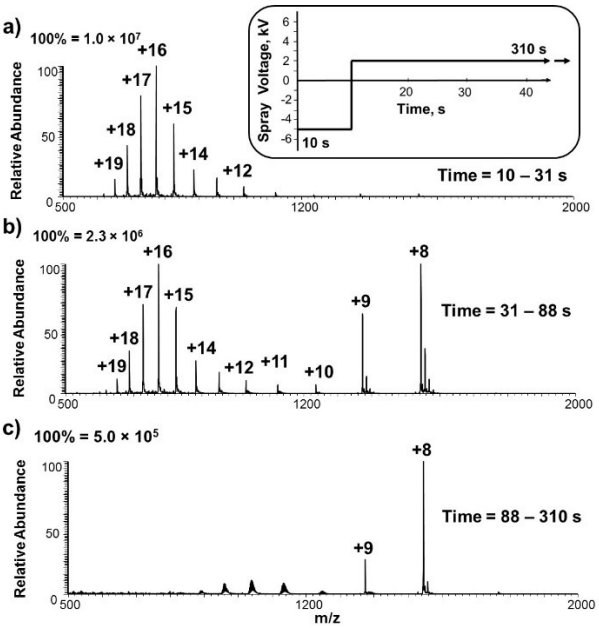


Figure 4. Electrophoretic desalting of 45 uM of cytochrome c in PBS (1X) solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4 and 1.8 mM KH_2PO4) in the presence of 0.1% of formic acid using the non-contact nESI/nAPCI setup, with a step voltage function starting with –5 kV for 10 s before switching to +2 kV for 5 extra minutes (see the insert in a) where mass spectra were recorded. **a-c** show selected mass spectra at different time domains namely 10 – 31 s, 31 – 88 s and 88 – 310 s, respectively.

CONCLUSIONS

In conclusion, a new non-contact nESI/nAPCI ion source is described that can be activated to operate in electrospray, atmospheric pressure chemical ionization, and/or electrophoretic separation modes. The integrated dual ionization capabilities are achieved simultaneously on a single device allowing the detection of polar and non-polar organic compounds as well as large biomolecules. The technique is highly sensitive, reliable and fast enabling the direct analyses of (i) various biofluids via an in-capillary liquid/liquid extraction process and (ii) different proteins in high concentrated salt solutions through an *online* electrophoretic separation method. The robustness, simplicity, and ease of operation make this method very attractive for ultra-small complex mixture analysis, with vast implications in translational and biomedical research.

ASSOCIATED CONTENT

Supporting Information

Additional material including, materials, instrumentation, full experimental details, additional Figures S1 – S13 and Videos S1 – S2 are available free of charge at the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interests.

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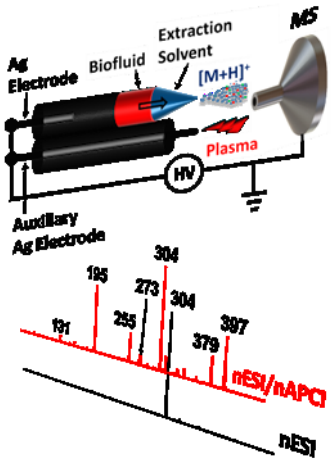
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