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Polymerization of hexamethylene diisocyanate in solution and a 260.23 *m/z* $[M+H]^+$ ion in exposed human cells

Adam V. Wisnewski*, Jian Liu, Carrie A. Redlich, Ala F. Nassar

Department of Internal Medicine, Yale University, New Haven, CT 06520, United States

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ABSTRACT

Hexamethylene diisocyanate (HDI) is an important industrial chemical that can cause asthma, however pathogenic mechanisms remain unclear. Upon entry into the respiratory tract, HDI's N=C=O groups may undergo nucleophilic addition (conjugate) to host molecules (e.g. proteins), or instead react with water (hydrolyze), releasing CO_2 and leaving a primary amine in place of the original N=C=O. We hypothesized that (primary amine groups present on) hydrolyzed or partially hydrolyzed HDI may compete with proteins and water as a reaction target for HDI in solution, resulting in polymers that could be identified and characterized using LC-MS and LC-MS/MS. Analysis of the reaction products formed when HDI was mixed with a pH buffered, isotonic, protein containing solution identified multiple $[M+H]^+$ ions with *m/z*'s and collision-induced dissociation (CID) fragmentation patterns consistent with those expected for dimers (259.25/285.23 *m/z*), and trimers (401.36/427.35 *m/z*) of partially hydrolyzed HDI (e.g. ureas/oligoureas). Human peripheral blood mononuclear cells (PBMCs) and monocyte-like U937, but not airway epithelial NCI-H292 cell lines cultured with these HDI ureas contained a novel 260.23 *m/z* $[M+H]^+$ ion. LC-MS/MS analysis of the 260.23 *m/z* $[M+H]^+$ ion suggest the formula $C_{13}H_{29}N_3O_2$ and a structure containing partially hydrolyzed HDI, however definitive characterization will require further orthogonal analyses.

Introduction

Hexamethylene (and related) diisocyanate compounds are widely used and are among the best-recognized chemical causes of occupational asthma [1]. Reactivity of diisocyanate upon entry into the human body is unclear, but likely central to pathogenic mechanisms leading to asthma [2]. Inhaled diisocyanate may react with host molecules (e.g. proteins or peptides) [3–8], or water [9–11]. Research to date has focused largely on diisocyanate reactivity with host molecules, as this process can cause structural (neo-epitopes) or functional changes that stimulate the host immune system [12–14]. Relatively less is known about the reactivity of diisocyanates with water in vivo, although its occurrence is supported by limited data [11,15–17].

Much of our understanding of diisocyanates' reactivity with water has been inferred from studies with corresponding monoisocyanates, or in relation to its industrial use [9,18,19]. Isocyanate reactivity with water yields unstable carbamic acids that rapidly decompose, releasing carbon dioxide and leaving behind a primary amine group in place of the original N=C=O [9]. Under laboratory conditions, isocyanate reactivity with water can be catalyzed via acid, base, and organometallic compounds [9,15,18–20]. Proteins, salts, and other organic compounds have been suggested to similarly influence isocyanate reactivity with water in vivo [10].

Under physiologic conditions, hydrolyzed or partially hydrolyzed diisocyanate may itself serve as a reaction target for unreacted N=C=O groups, resulting in polymers of the urea or oligourea type. We used LC-MS and LC-MS/MS to evaluate this hypothesis through in vitro experiments with HDI in a model physiologic solution, a pH-buffered, isotonic suspension containing albumin protein. Under these conditions, LC-MS and LC-MS/MS describe the formation of new $[M+H]^+$ ions with *m/z*'s and fragmentation patterns upon CID consistent with those expected for dimers and trimers of partially hydrolyzed HDI (e.g. urea/oligourea). LC-MS and LC-MS/MS were used to further assess the HDI urea's biological activity in vitro and lead to discovery of a novel 260.23 *m/z* $[M+H]^+$ ion in exposed human cells.

Abbreviations: BPC, base peak chromatogram; CID, collision induced dissociation; HDI, hexamethylene diisocyanate; PBMCs, peripheral blood mononuclear cells

* Corresponding author. Department Internal Medicine/Section General Medicine, Program in Occupational & Environmental Medicine, Yale School of Medicine, 300 Cedar Street/PO Box 208057, Building: TACS420, New Haven, CT 06520-8057, United States.
E-mail address: adam.wisnewski@yale.edu (A.V. Wisnewski).

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Materials and methods

Reactivity of HDI in a physiologic solution

HDI was reacted in a model physiologic solution containing normal saline, pH buffering ions (phosphate), and protein at a concentration roughly equivalent to that of airway fluid [21]. HDI or 1,6 diisocyanatohexane (CAS Number: 822-06-0) was obtained from Sigma-Aldrich (St Louis, MO) and was of ≥99% purity by gas chromatography, with a refractive index (n20/D) = 1.453, and a density of 1.047 g/mL at 20 °C. Briefly, 100 μL of HDI was introduced dropwise with stirring, into 25 mL of a 0.5% (w/v) solution of sterile filtered (0.2 μm, Merck Millipore Ltd; Bellencia, MA) endotoxin-free albumin (Sigma) in tissue-culture grade phosphate buffered saline (PBS) pH 7.2 (Gibco; Grand Island, NY), and mixed end-over-end for 2 h at 37 °C. The reaction conditions, containing 20 mM HDI and 73 μM albumin, have previously been shown to yield products that induce human innate immune responses in vitro [13]. At this molar ratio of HDI:albumin (~325:1), we estimate ~18−fold molar excess of N=C=O to protein reaction sites, assuming each albumin molecule contains at most 37 $-NH_2$ groups capable of reacting with (toluene) diisocyanate [22], and each HDI molecule contains 2 N=C=O groups. The reaction products were centrifuged at 1000 × g to pellet precipitated material, sterile (0.2 μm) and further filtered through a 3 kDa molecular weight cut-off spin column (Amicon Ultra 0.5 mL Centrifugal Filters Ultracel 3K) from Merck Millipore Ltd. Control reactions without HDI were performed and identically processed in parallel.

Liquid chromatography coupled mass spectrometry

LC-MS and LC-MS/MS were performed on an Agilent G6550A Q-TOF system coupled to an Agilent 1290 Infinity LC system, using a rapid resolution HT Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm) from Agilent Technologies (Santa Clara, CA). Samples filtered through a < 3 kDa spin column were mixed 1:10 (for HDI reaction products in physiologic solution and U937, NCI-H292 cell lysates) or 1:1 (for PBMC cell extracts, see below) in buffer A (water containing 0.1% formic acid) before 5 μL was loaded and eluted over a 6 min period starting at time 0 with 5% buffer B (acetonitrile containing 0.1% formic acid), increasing to 20% buffer B between 0 and 3 min, 60% buffer B between 3 and 4 min, 98% buffer B between 4 and 5 min and back to 5% buffer B from 5 to 6 min. LC-MS studies with PBMCs used a slightly steeper elution rate, going from 5 to 60% buffer B between 0 and 5 min, up to 90% buffer B at 7 min, 95% buffer B at 9 min and 98% buffer B at 10 min. Positive electrospray ionization (ESI) was performed using the following parameters: gas temp- 280 °C, gas flow- 11 l/min, nebulizer-40 psig, sheath gas temp- 350 °C, sheath gas flow-11, Vcap-4000 V, nozzle voltage-2000 V, fragmentor voltage– 175 V, skimmer voltage 65 V, octopole RF peak voltage 750 V. The *m/z* values of all ions present in the mass spectra were corrected against two reference ions (purine, $[M+H]^+$ *m/z* 112.9856 and 1H, 1H, 3H tetra(fluoropropoxy)phosphazine, $[M+H]^+$ *m/z* 922.0097). The data acquisition range, for LC-MS was from 110 to 1700 *m/z*. For MS/MS analyses, the collision energy was automatically set using Agilent MassHunter Acquisition software according to the formula, slope x (*m/z*)/100 + offset; with the slope of 5 and offset of 2.5. MS/MS data were obtained for the 5 most intense ions, in some experiments with preference given to species of interest with masses of 285.23, 401.36, 259.25, 427.34, 402.34, 260.23 or 143.12 ± 100 ppm. Data were acquired and analyzed using Mass Hunter Workstation software from Agilent.

In vitro cultures and processing

Peripheral blood was obtained from N = 3 subject by venipuncture and mononuclear cells were purified by density gradient centrifugation as previously described [23]. Human monocytic (U937) and airway epithelial cell lines NCI-H292 were obtained from the American Type Culture Collection (Rockville, MD). Human PBMC cultures were initiated with 2 × 10^6 cells/mL in RPMI 1640 media (Gibco; Grand Island, NY) supplemented with 10% autologous serum. U937 cultures were initiated with 5 × 10^5 cells/mL, and NCI-H292 cell cultures were initiated at 30% confluence in RPMI 1640 media supplemented with 10% fetal bovine serum (Gibco). Following 48 h of incubation with a 1:10 dilution of either HDI ureas (< 3 kDa fraction of HDI reaction products in physiologic solution) or control reaction products (identically generated and processed without HDI), cells were washed 3 times with tissue culture grade PBS, and pelleted in a microfuge tube at 10,000 × g. Pellets of 5 × 10^6 PBMCs or 5 × 10^7 U937 or NCI-H292 cells were lysed by sonication in 500 μL of HPLC-MS grade water (Fisher Scientific; Fairlawn, NJ) and the soluble intracellular contents were filtered through a 3 kDa molecular weight cut-off spin column (Amicon Ultra 0.5 mL Centrifugal Filters Ultracel 3K) from Merck Millipore Ltd. The study was approved by the Human Investigations Committee of Yale University and written informed consent was obtained from all participants.

Results

LC-MS and LC-MS/MS characterization of HDI polymerization in physiologic solution

Initially LC-MS analysis was performed on HDI following reactivity in a physiologic pH buffered, isotonic solution of albumin, under conditions previously shown to generate products that induce innate

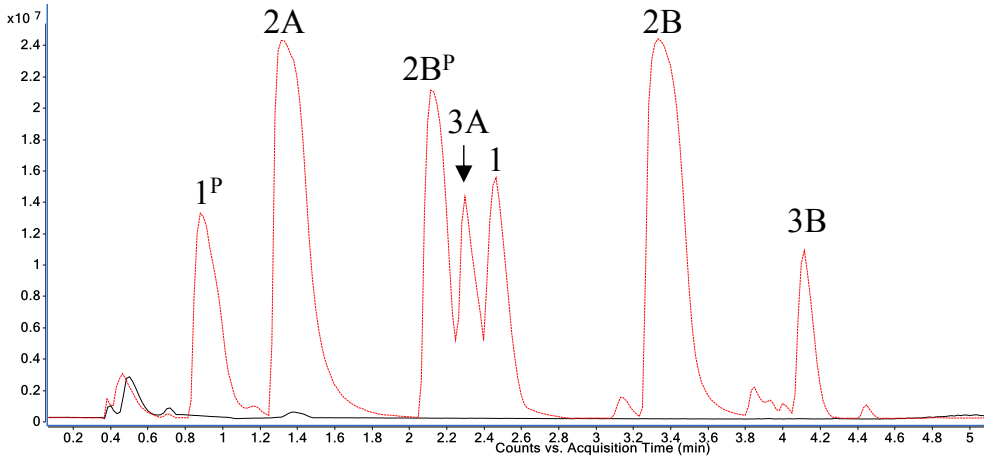

Fig. 1. LC-MS analysis of the low molecular weight (< 3 kDa) products formed when HDI reacts in physiologic solution. LC-MS BPCs of the < 3 kDa fraction of HDI reaction products (red dashed line) vs. control reaction products (black solid line). Y-axis depicts relative ion intensity and X-axis depicts elution time. The dominate $[M+H]^+$ ions comprising the major peaks eluting at specific retention times (RT) in minutes are listed in Table 1. *indicates saturating levels for given ions. Likely phosphate adducts (+98 kDa/H_3PO_4) and doubly charged (z = 2) species are observed for some ions. See Fig. 2 for MS and MS/MS data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
HDI hydrolysis products and polymers.

*RT	Peak #	Major ions & charge (z)
2.5	1	143.12 [z = 1]
0.9	1^P	*143.12 [z = 1]
		*241.10 [z = 1] H_3PO_4 adduct?
1.3	2A	*130.13 [z = 2]
		259.25 [z = 1]
3.4	2B	*285.23 [z = 1]
2.1	$2B^P$	*383.21 [z = 1] H_3PO_4 adduct?
		285.23 [z = 1]
		143.11 [z = 2]
2.3	3A	201.18 [z = 2]
		401.36 [z = 1]
4.1	3B	*427.34 [z = 1]
		214.17 [z = 2]

*RT = Retention time in minutes.

immune responses [13] and compared to a control sample reaction without HDI. Overlapping base peak chromatograms (BPCs) of the low molecular weight (< 3 KDa) reaction products shown in Fig. 1 highlight prominent new $[M+H]^+$ ions observed when HDI is introduced into a physiologic solution. Table 1 lists the retention time, *m/z* values, and charge state (z) of the most intense $[M+H]^+$ ions observed at different elution times.

Further MS/MS characterization of the new ions formed upon incubation of HDI in physiologic solution (Fig. 2) revealed CID fragmentation patterns expected for partially hydrolyzed HDI, and urea-like polymers (dimers and trimers) of partially hydrolyzed HDI as modeled in Fig. 3. MS/MS of peak 1 revealed limited fragmentation of the predicted cyclized HDI reaction product (with itself), aside from loss of ammonium (−17 Da) yielding the 126.09 *m/z* $[M+H]^+$ fragment ion. MS/MS analysis of peaks 2A and 2B (dimeric HDI ureas) highlight the prominence of CID $[M+H]^+$ ions consistent with those expected for

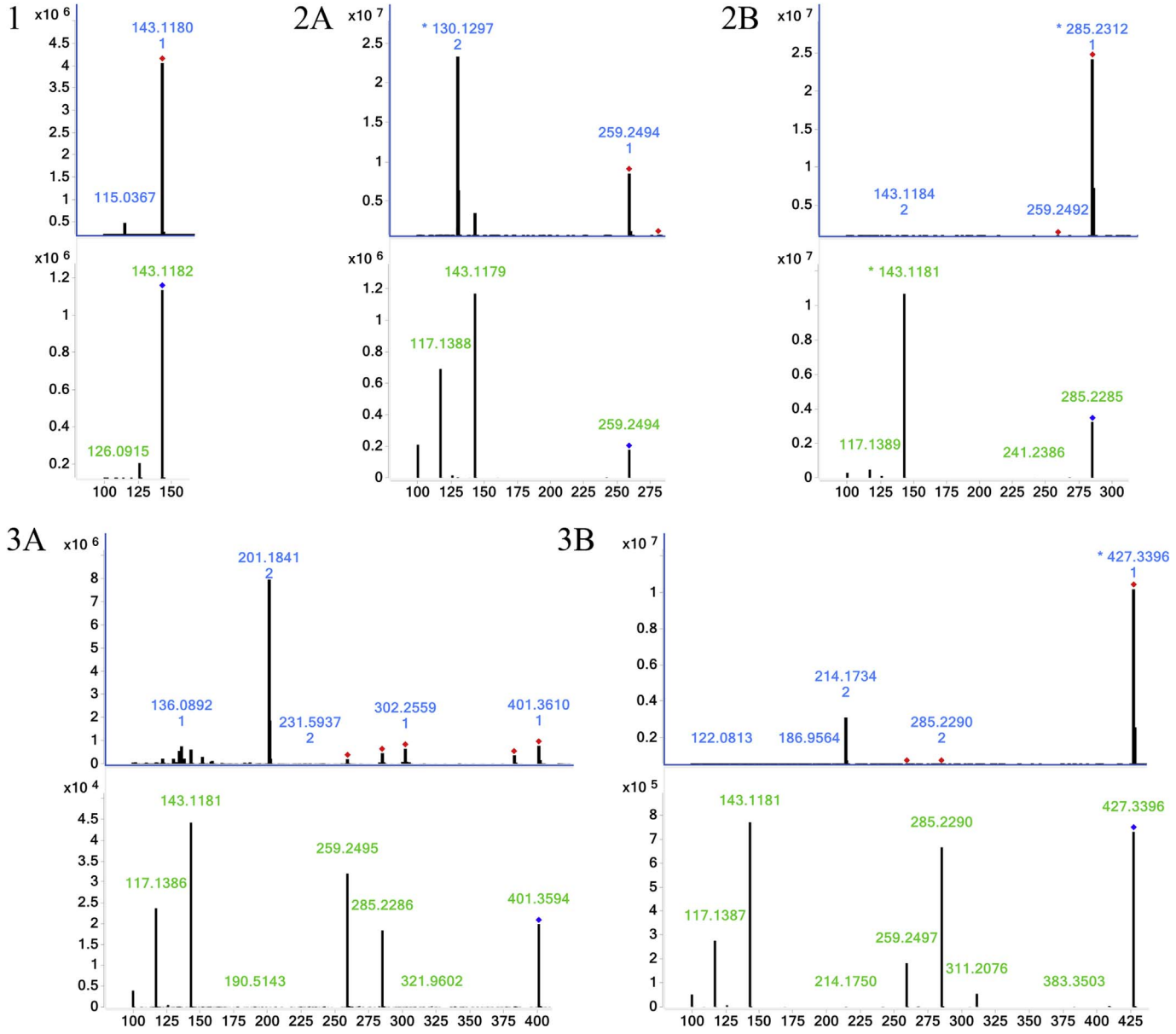

Fig. 2. LC-MS and LC-MS/MS analysis of major low molecular weight (< 3 kDa) products formed when HDI reacts in physiologic solution. For each of the major peaks labeled in Fig. 1, MS and MS/MS analyses are shown for the precursor/parent $[M+H]^+$ ions (top panels) and the fragments produced upon CID (bottom panels). The data are consistent with the predicted structures shown for partially hydrolyzed HDI (peak 1), dimers of partially hydrolyzed HDI (peaks 2A and 2B) and trimers of partially hydrolyzed HDI (peaks 3A and 3B). Note dominance of doubly charged species for the 259.25 and 401.36 *m/z* $[M+H]^+$ ions, (peaks 2A and 3A) which are predicted diamines, and the limited change in the 143.12, 285.23 and 427.34 *m/z* $[M+H]^+$ ions' (peaks 2B and 3B) intensity following CID, consistent with a cyclized structure. The charge state (z) is noted in the MS plots under the *m/z* value.

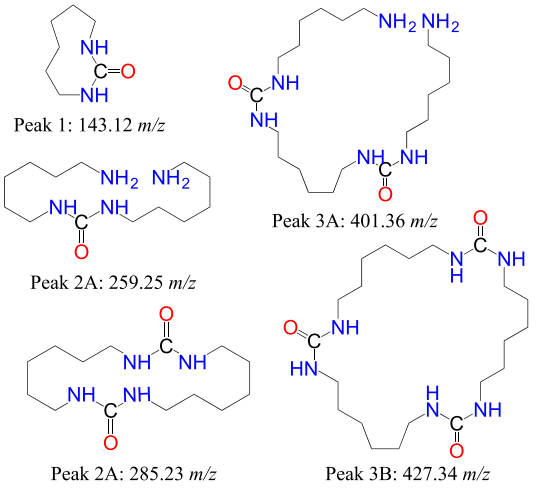

Fig. 3. Proposed structures for reaction products of HDI in physiologic solution. Models are provided for the new $[M+H]^+$ ions formed when HDI is introduced into a physiologic solution, based on LC-MS and LC-MS/MS analyses shown in Figs. 1 and 2.

partially hydrolyzed HDI (143.12 *m/z*), completely hydrolyzed HDI (117.14 *m/z*), and further loss of ammonium from hydrolyzed HDI (100.11 *m/z*). Analysis of peaks 3A and 3B (trimeric HDI oligoureas) reveal these same CID $[M+H]^+$ ions as well as those expected for dimeric HDI ureas (259.25 and 285.23 *m/z*).

The proposed diamines (peaks 2A and 3A) are observed predominately as doubly charged (z = 2) ions, consistent with their containing two functional groups protonated under acidic conditions. Cyclized structures proposed for the 142.12, 285.23, and 427.34 *m/z* $[M+H]^+$ ions are supported by their limited fragmentation upon CID (note scale of Y-axes), as they should retain their *m/z* despite cleavage of any single bond. Additional base peaks with a "P" superscript (1^P and $2B^P$) contain 241.10 and 383.21 *m/z* $[M+H]^+$ ions, which are likely phosphate adducts (+98 Da) of partially hydrolyzed HDI (peak 1) or dimers of partially hydrolyzed HDI (peak 2B), formed from buffer (sodium phosphate) under acidic LC-MS/MS conditions as previously described [24]. As expected, these phosphate adducts elute earlier than the corresponding $[M+H]^+$ ions and are completely abolished upon CID (see Supplemental Materials Figs. S1 and S2). Thus, when HDI enters a physiologic protein containing solution it can partially hydrolyze and polymerize into multimers of partially hydrolyzed HDI (e.g. ureas and oligoureas).

LC-MS and LC-MS/MS identifies a unique 260.23 m/z $[M+H]^+$ ion in human cells cultured with HDI ureas

We next used LC-MS to evaluated the potential biological effect of the HDI ureas that form when HDI is introduced into a pH buffered, isotonic protein containing (e.g. physiological) solution. Preliminary studies were performed with PBMCs from N = 3 human subjects, and focused on potential changes in intracellular metabolites. Subtraction analyses of LC-MS BPCs of cellular extracts obtained after 48 h of exposure lead to discovery of an HDI urea-induced increase in an $[M+H]^+$ ion with a 260.23 *m/z* and a retention time ~2.4 min, as highlighted in Fig. 4. Increases in a 259.25 *m/z* $[M+H]^+$ ion with the same retention time (~1.3 min) as that of dimeric HDI urea (peak 2A in Figs. 1 and 2) were also noted, although its corresponding doubly charged (z = 2) ion was not present, possibly due to differences in sample preparation and pH (PBMC lysates were mixed 1:1 with water/0.1% formic acid, while HDI samples were diluted 1:10 with water/0.1% formic).

LC-MS analyses of human cell lines cultured with HDI ureas also identified the 260.23 *m/z* $[M+H]^+$ ion in monocyte-like U937 cells but not in an airway epithelial derived cell line (NCI-H292), as shown in comparative BPCs and extracted ion chromatograms (Fig. 5). A 259.25 *m/z* $[M+H]^+$ ion (and its doubly charged species) with the same retention time (~1.3 min) as the above described dimeric HDI urea (peak 2A) was also increased in U937 cells, but not NCI-H292 cells. Of note, NCI-H292 cells incubated with HDI ureas contained increased amounts of a 274 *m/z* $[M+H]^+$ ion (noted with asterisk in Fig. 5) also present in control NCI-H292 cells, and found in U937 cells without change upon culture with HDI ureas (data not shown).

MS/MS analysis of the HDI urea induced 260.23 *m/z* $[M+H]^+$ ion from U937 cells (Fig. 6) produced CID fragments consistent with a structure of the formula $C_{13}H_{29}N_3O_2$, containing partially hydrolyzed HDI covalently attached to a 6 carbon molecule via an N- or O-linkage, as shown in Fig. 7. The 100.11 *m/z* $[M+H]^+$ ion likely results from loss of water (−18 Da) from the 118.12 *m/z* $[M+H]^+$ CID fragment.

MS/MS analysis (Fig. 8) also identified a 260.23 *m/z* $[M+H]^+$ ion as a major CID fragment of a larger 402.34 *m/z* $[M+H]^+$ ion that eluted at a later time point (retention time ~3.4 min). Notably, the mass difference between the 402.34 parent $[M+H]+$ ion and the 260.23 daughter $[M+H]^+$ fragment (~142.11 amu) is consistent with that expected for 1 partially hydrolyzed HDI molecule. The CID fragmentation pattern of the 402.34 *m/z* $[M+H]^+$ ion is consistent with the structure proposed in Fig. 9, containing the 260.23 *m/z* $[M+H]^+$ ion attached to partially hydrolyzed HDI.

Discussion

The present study used LC-MS and LC-MS/MS to evaluate the ability of HDI to polymerize in physiologic solution. The data demonstrate the capacity for hydrolyzed, or partially hydrolyzed HDI, to compete with water and protein for reactivity with unreacted HDI in solution. Polymerized HDI reaction products were characterized as soluble HDI ureas and oligoureas, essentially dimers and trimers of partially hydrolyzed HDI, possessing either 2 amine groups (diamines) or cyclized structures. When human PBMCs and monocyte-like U937 cells were cultured with low molecular weight oligomers of partially hydrolyzed HDI, LC-MS and LC-MS/MS analysis of their intracellular contents identified a novel 260.23 *m/z* $[M+H]^+$ ion, which we hypothesize possesses the formula $C_{13}H_{29}N_3O_2$ and a structure containing partially hydrolyzed HDI. Thus, LC-MS and LC-MS/MS proved useful for characterizing low molecular weight polymers of aliphatic hexamethylene diisocyanate that form in physiologic solution. The technique offers multiple advantages over prior methods used to investigate isocyanate reactivity in water, which have sometimes relied upon indirect measurements (CO_2 release), or require extensive work-up (hydrolysis/derivatization) before gas chromatography-MS analysis [9,10]. LC-MS and MS/MS were also applicable in the present study as a discovery science tool and identified a previously undescribed 260 *m/z* $[M+H]^+$ ion within human cells exposed to HDI ureas.

The precision of LC and MS make them uniquely suited for studying chemical reactions relevant to adverse health outcomes (asthma, hypersensitivity pneumonitis) due to diisocyanate exposure. Diisocyanate reactivity can be monitored based upon unique mass increases resulting from nucleophilic addition of chemical or partially hydrolyzed chemical, and changes in LC retention time due to the chemical's innate hydrophobicity. The present data are in good agreement with prior studies suggesting the formation of low molecular weight ureas from aliphatic isocyanates in aqueous phase [9,10,18]. Our data suggest such ureas can form despite the presence of functional groups (primary amines) on proteins, sodium, chloride, and phosphate ions present in solution. The present study was performed with a model solution of albumin at a concentration roughly equivalent to that predicted to exist in airway fluid [21]. In vivo however, proteins other than albumin, and non-protein targets (amines, thiols) may also react with HDI, and the ratio of HDI:reactants is uncertain. Further LC-MS/MS studies varying the concentration of HDI vs. protein/non-protein targets and altering buffer composition (e.g. surfactant, glutathione, bicarbonate) may

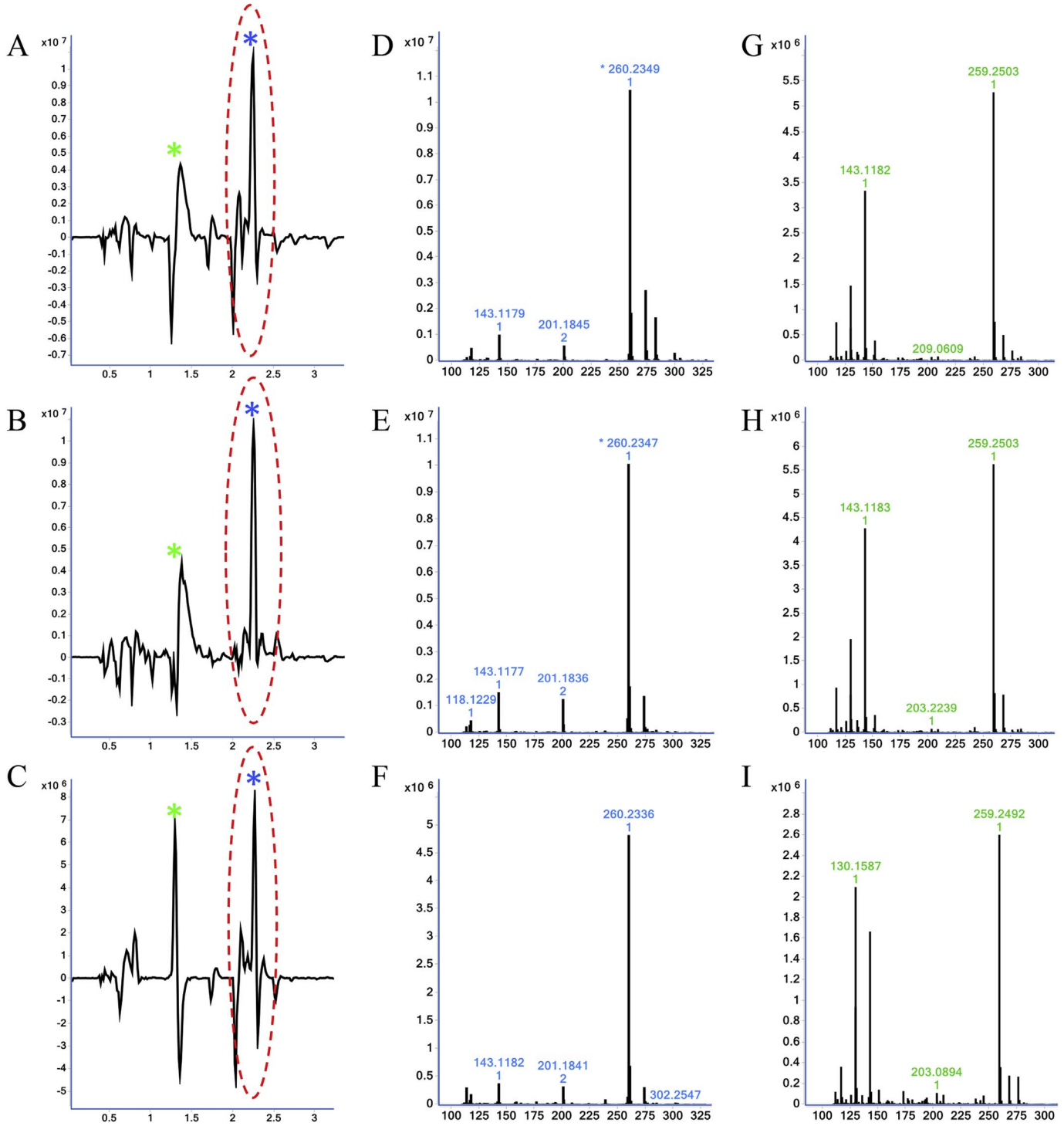

Fig. 4. A 260.23 *m/z* $[M+H]^+$ ion present in PBMCs upon culture with reaction products of HDI in physiologic solution. Human PBMCs from 3 different subjects were cultured for 48 h in the presence of HDI ureas, or control reaction products. Panels A, B, and C show subtraction plots of the LC-MS BPC for extracts from HDI urea exposed cultures – control cultures for each subject. Panels D, E and F show MS data for exposed samples eluting ~2.4 min (peak circled in red dashed line with blue asterisk), the time of maximal difference between exposed and control cultures, and highlight a dominant 260.23 *m/z* $[M+H]^+$ ion. Panels G, H and I show MS data for exposed samples eluting ~1.3 min (peak with green asterisk) and highlight a dominant 259.25 *m/z* $[M+H]^+$ ion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

better define the polymerization of HDI (and other diisocyanates) as they occur inside the body.

By utilizing LC-MS in discovery mode, we were able to readily identify a novel 260.23 *m/z* $[M+H]^+$ ion in human cells exposed to soluble HDI polymers formed in physiologic solution. Further LC-MS/MS studies suggest the 260.23 *m/z* $[M+H]^+$ ion is a long chain aliphatic amino-alcohol possessing the formula $C_{13}H_{29}N_3O_2$; however, the data cannot rule out the unlikely possibility that the ion is an ether containing diamine, as shown in Fig. 3 (see hypothesis below on possible derivation of the molecule). Greater than 500 chemicals [25] are known to possess the chemical formula $C_{13}H_{29}N_3O_2$; however, we could not identify any among these that would yield the LC-MS/MS CID fragmentation patterns we observed. Similarly no chemical structures could be found in the MolPort database [27] that matched our proposed

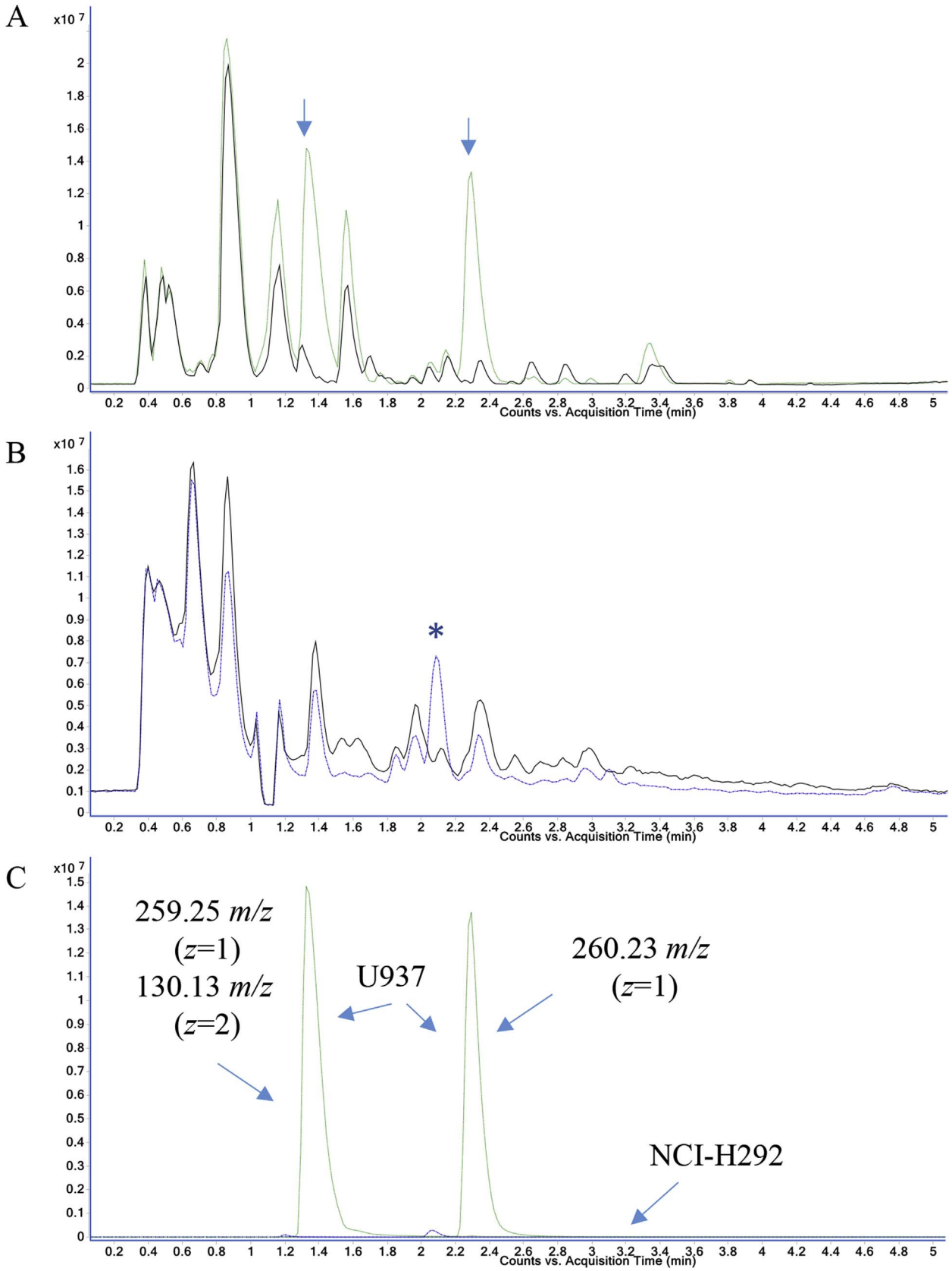

Fig. 5. Novel 260.23 *m/z* $[M+H]^+$ ion observed in human monocytic (U937) cell line cultured with HDI reaction products (ureas). LC-MS BPC of the < 3 kDa fraction of U937 cells (A) or NCI-H292 cells (B) incubated for 48 h with HDI (blue or green dashed lines) or control reaction products (black solid lines). Prominent distinct peaks (highlighted with arrows) in U937 cells cultured with HDI reaction products contain $[M+H]^+$ ions with same 260.23 *m/z* observed in experiments with PBMCs shown in Fig. 4, and the 259.25 *m/z* $[M+H]^+$ ion that comprises peak 2A in Fig. 1. Panel C shows extracted ion chromatograms for the 260.23 *m/z* $[M+H]^+$, and 259.25 *m/z* $[M+H]^+$ (and its corresponding doubly charged ion) from exposed U937 cells (green line) and NCI-H292 cells (blue base line) as labeled. Y-axis depicts relative ion intensity and X-axis depicts retention time in minutes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

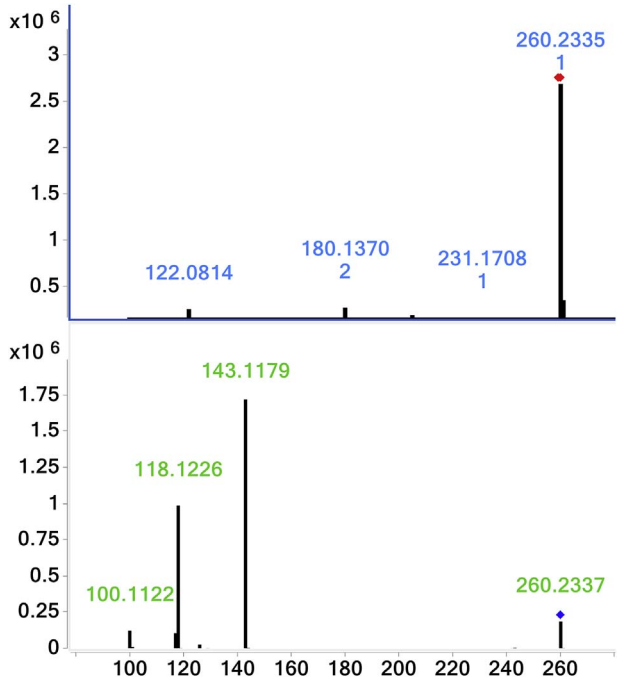

Fig. 6. MS and MS/MS of the 260.23 *m/z* $[M+H]^+$ ion observed in U937 cells incubated with HDI ureas. Top pane shows MS at time of maximal elution (~2.4 min) of the 260.23 *m/z* $[M+H]^+$ ion based on extracted ion chromatogram. Bottom pane shows $[M+H]^+$ ion fragments of the 260.23 *m/z*$[M+H]^+$ ion produced upon CID during MS/MS.

(N- or O-linked) structure for the 260.23 *m/z* $[M+H]^+$ ion. Attempts to further characterize the 260.23 *m/z* $[M+H]^+$ ion by nuclear magnetic resonance (NMR) were unsuccessful. ^{1}H NMR of the 260.23 *m/z* $[M+H]^+$ ion was inconclusive as the purified molecule was insoluble in chloroform and contained exchangeable hydrogen atoms in sites key to structural determination (differentiating N- vs. O-linkage). Limited sample sizes of the present investigation precluded ^{13}C-NMR analysis. Further studies, beyond the scope of this initial discovery, will be necessary to validate our predicted structure for the newly described 260.23 *m/z* $[M+H]^+$ ion.

As noted in the Results Section (3.2.2), a 260.23 *m/z* $[M+H]^+$ ion is also a major fragment of a larger 402.34 *m/z* $[M+H]^+$ ion present in cells cultured with HDI ureas. Its CID fragmentation pattern and the mass difference between 402.34 and 260.23, equivalent to that of partially hydrolyzed HDI, suggest the 402.34 *m/z* $[M+H]^+$ ion might represent a structure analogous to the 260.23 *m/z* $[M+H]^+$ molecule, with the addition of another partially hydrolyzed HDI.

The source of the newly described 260.23 and 402.34 *m/z* $[M+H]^+$ ions remains unclear. We hypothesize their derivation by metabolism of "dimeric" or "trimeric" HDI ureas of the diamine type, by oxidative deamination and reduction, as described for other xenobiotics [26–28], rather than reactivity of partially hydrolyzed HDI with a 6-carbon amino alcohol. Other long chain aliphatic diamines are well recognized substrates for amine oxidase [29–31] and influence histamine activity in vitro and in vivo [32].

The strengths and weaknesses of the present study are important to highlight when evaluating the potential biological relevance of the present findings. As mentioned above, the precision of LC-MS/MS for separating different molecules, calculating their molecular mass, and developing structural models based on CID fragmentation patterns is excellent. The major weakness of the study is the reductionist approach, evaluating the reactivity of HDI in vitro using a model physiologic solution and potentially saturating amounts of N=C=O relative to protein reaction targets. Our original analysis was focused on cell uptake of HDI-albumin reaction products, given their link to occupational exposure, immune responses and asthma and thus, did not include analysis of (a) HDI reaction products in buffer without albumin, (b) extracellular medium or activation markers, or (c) measurements of hexamethylene diamine. Ongoing studies in our lab have since used LC-MS/MS to analyze reaction products of HDI in phosphate-buffered saline (PBS) without protein and have found qualitatively similar but quantitatively higher total ion chromatograms, with relative increases in higher molecular weight polymers of partially hydrolyzed HDI (see Supplemental Materials Figs. S3-S5). Human U937 cells incubated with the < 3 kDa fraction of HDI reaction products in buffer (no protein) similarly contained the novel 260.23 *m/z* $[M+H]^+$ ion described herein (see Supplemental Materials Fig S6). Future studies comparing the ureas generated from HDI in the presence/absence of varying

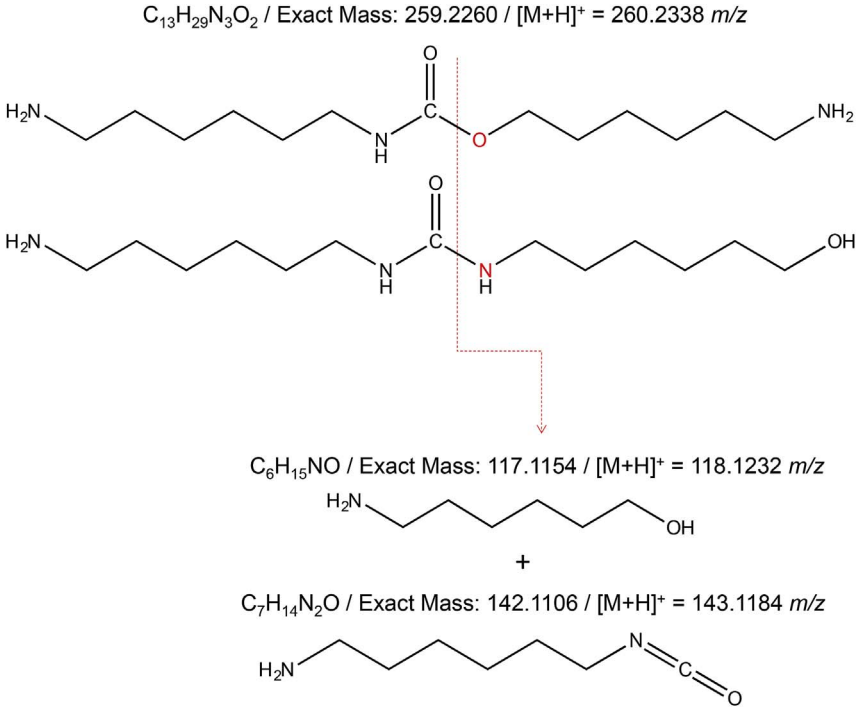

Fig. 7. Proposed structures for the 260.23 *m/z* $[M+H]^+$ ion from human cells incubated with HDI ureas. The expected mass and fragmentation pattern observed following CID (see Fig. 6) are consistent with a structure possessing the formula $C_{13}H_{29}N_3O_2$ and containing partially hydrolyzed HDI.

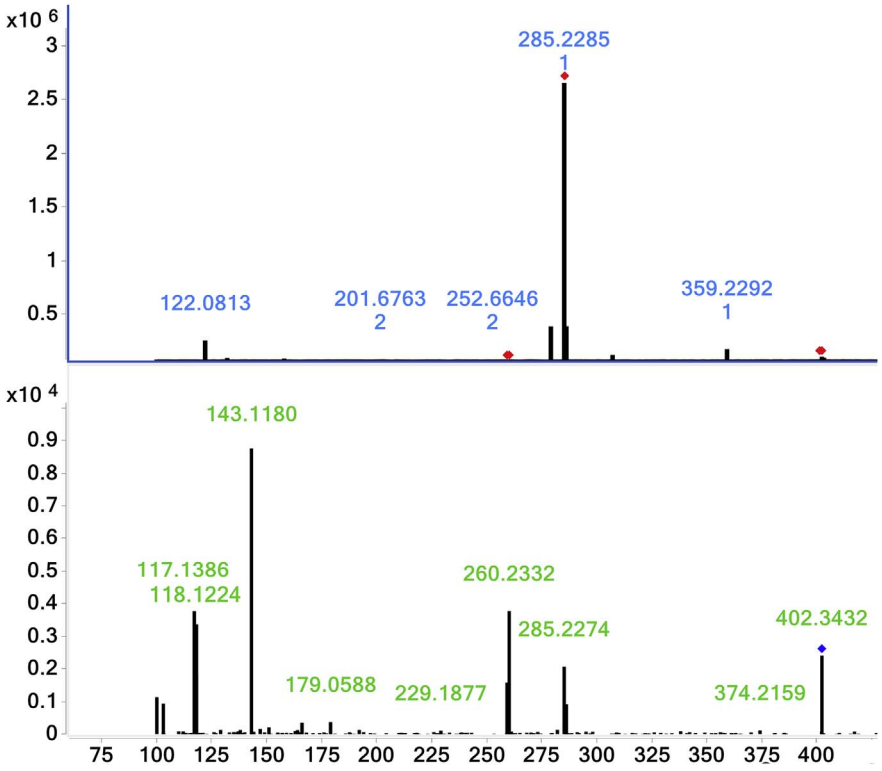

Fig. 8. MS and MS/MS of the 402.34 *m/z* $[M+H]^+$ ion observed in U937 cells incubated with HDI ureas. Top shows MS from the time point (~3.4 min) of maximal elution for the 402.34 *m/z* $[M+H]^+$ ion. Bottom MS/MS data shows $[M+H]^+$ ion fragments of the 402.34 *m/z* $[M+H]^+$ ion produced upon CID.

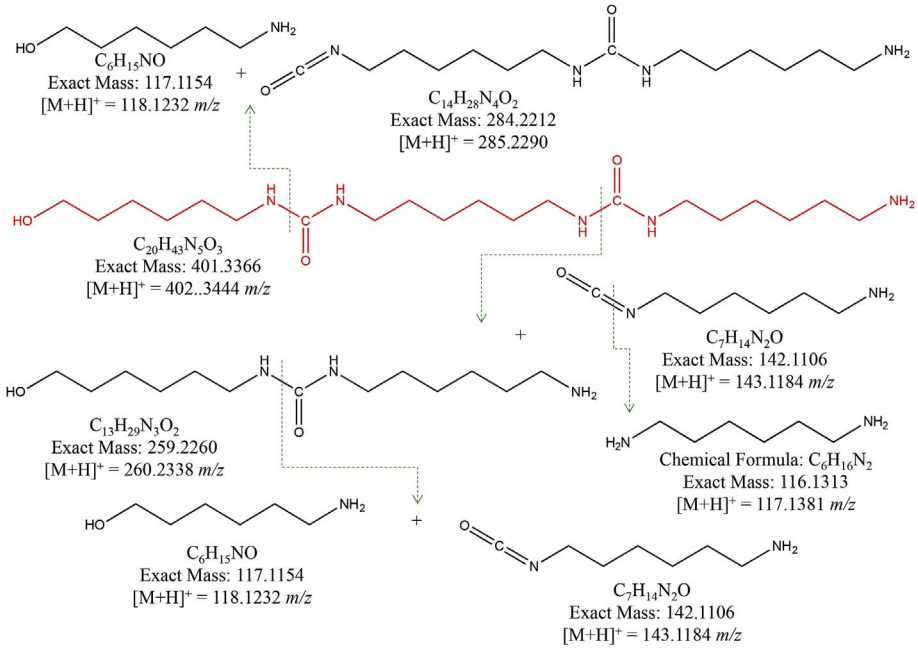

Fig. 9. Proposed structure(s) for the 402.34 *m/z* $[M+H]^+$ ion from human cells incubated with HDI ureas. The expected mass and fragmentation pattern observed following CID (see Fig. 8) are consistent with a structure (in red with *) possessing the formula $C_{20}H_{43}N_5O_3$ and containing partially hydrolyzed HDI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

amounts of protein or other reactants (as mentioned above), and their effects on intracellular as well as extracellular molecules should provide a better assessment of their biological relevance.

In summary, we utilized LC-MS and MS/MS techniques to characterize polymers of the aliphatic diisocyanate, HDI, an occupational asthma-causing chemical, that form in physiologic solution. The techniques permitted direct characterization of diisocyanate polymerization without reliance upon indirect assessment (e.g. CO_2 evolution) or complex sample workup (e.g. acid hydrolysis at high temp followed by derivatization and gas chromatography) [9,10]. The data identified dimers and trimers of partially hydrolyzed HDI, with distinct properties (LC elution time, *m/z*, doubly vs. singly charged ionization, and MS/MS fragmentation patterns). When these soluble low molecular weight HDI polymers were incubated with human cells, LC-MS and LC-MS/MS data readily identified a novel 260.23 *m/z* $[M+H]^+$ ion, and suggest the molecule contains partially hydrolyzed HDI and possesses the formula $C_{13}H_{29}N_3O_2$. Further studies will be necessary to confirm the newly described 260.23 *m/z* $[M+H]^+$ ion's structure and its relevance to human occupational HDI exposure.

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Declaration of interests

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2017.11.017.

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