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Comparative analysis of aromatic diisocyanate conjugation to human albumin utilizing multiplexed tandem mass spectrometry

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ABSTRACT

Diisocyanates are industrially important chemicals that serve as polymerizing agents in a variety of polyurethane products. In addition to their many industrial uses, diisocyanates have been implicated as causative agents of occupational allergic respiratory disease, although the specific mechanism(s) by which these diseases occur remains unknown. In this study the sites of conjugation of the two most industrially important monomeric diisocyanates, methylene diphenyl diisocyanate (MDI) and toluene diisocyanate (TDI) on human serum albumin are identified utilizing multiplexed tandem mass spectrometry on a quadrupole time-of-flight mass spectrometer. Analysis of human albumin reacted with MDI and TDI over the range of 1:1–40:1 (isocyanate:protein) mol ratio reveals that MDI and TDI react with a maximum of 20 and 37 residues, respectively. Conjugation of diisocyanates to albumin proceeds in a concentration-dependant manner with MDI and TDI reacting at a preferred subset of 5 and 10 residues, respectively, in the limiting case of a 1:1 mol ratio. MDI reacts at fewer residues than does TDI, and is not observed to react with any residues exclusive of TDI. These results cannot be explained on the basis of simple sterics or hydrophobicity, but rather on the basis of increased reactivity of one TDI isocyanate moiety due to electron withdrawing character of the second isocyanate moiety. Furthermore, reaction of diisocyanates with albumin in a phosphate buffered saline (PBS) solution provides three additional reactive sites that are not observed in ammonium bicarbonate buffer. Two lysine residues, Lys^{199} and Lys^{525}, are observed to be reactive to both diisocyanates at all concentrations and in all solvent systems employed in this study. This study presents a comprehensive conjugation map of MDI and TDI on human albumin. The results suggest that several albumin residues are reactive toward both MDI and TDI and may be useful for biomonitoring of diisocyanate exposures.

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1. Introduction

Diisocyanates (dNCOs) are widely used as crosslinking agents in the polyurethane industry for the manufacture of such products as flexible and rigid foams, fibers, paints and varnishes. These products are used in the furniture and automotive industry, and increasingly, as spray-in building insulation material. Methylene diphenyl diisocyanate (MDI) and toluene diisocyanate (TDI) account for greater than 90% of the global diisocyanate market, and annual production exceeds 4 million tons [1]. The reactive isocyanate moiety (–N=C=O) is capable of undergoing nucleophilic addition with a variety of active hydrogen species including alcohols, phenols, amines and thiols [2]. Occupational dNCO exposures are associated with adverse health effects, including sensitization and asthma, contact dermatitis, and hypersensitivity pneumonitis [3]. Diisocyanate-induced asthma in occupationally exposed worker populations is estimated to range from 5 to 30% [3–6]. Furthermore, some occupational tasks may result in significant dermal exposure [7,8] which can also lead to immunological sensitization and subsequent development of asthma-like symptoms with respiratory tract exposure.

It is generally believed that dNCOs act as haptens that react with protein carriers; however, the form(s) of the dNCO-conjugated proteins that function as allergens are, as yet, unknown. The diverse functional groups present in proteins including amines, amides, thiols, alcohols and carboxylic acids present a large number of potential reaction sites for the isocyanate. However, previous studies have suggested that under physiological conditions, these are limited to N-terminal α-amines, the sulfhydryl group of cysteine, the hydroxyl groups of serine and tyrosine, the ε-amine of lysine and the secondary amine of the imidizole ring of histidine [9]. Several protein targets of diisocyanates *in vivo* have been identified, most notably, serum albumin [10–15] and albumin-specific MDI

Abbreviations: dNCO, diisocyanate; MDI, methylene diphenyl diisocyanate; TDI, toluene diisocyanate; HSA, human serum albumin; UPLC, ultra-performance liquid chromatography.

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adducts have been identified in both rats and humans [16,17]; however, these studies did not identify sequence-specific diisocyanate adducts of serum albumin.

Identifying the reaction products formed when dNCOs, such as MDI and TDI, react with model proteins is critical to understand the mechanisms by which these chemicals affect living systems. Human serum albumin (HSA) is an ideal model protein because its sequence and three dimensional structure have been well defined, it is naturally abundant (35–50 mg/mL in serum), and – most importantly – it has been identified as a target of diisocyanate *in vivo* [10–15]. Tandem mass spectrometry is particularly well suited for the analysis of modified proteins. Peptides derived from enzymatic digest may be fragmented and mass analyzed with high mass accuracy over a wide dynamic range. Because reaction of a chemical hapten with a particular amino acid residue results in a change in mass, sequence analysis by tandem mass spectrometry allows unambiguous determination of both the chemistry and site of protein haptenation. To that end, our laboratory has begun examining the reaction products formed between diisocyanates and biological macromolecules by tandem mass spectrometry [18,19]. In particular, we have chosen to employ ultra-performance liquid chromatography (UPLC) coupled to a quadrupole time-of-flight (qTOF) mass spectrometer operated in a multiplexed MS/MS or "MS^e" mode [20]. Briefly, alternating mass spectra are acquired; the first spectrum is acquired at low collision energy and allows high mass accuracy precursor ion mass measurement, whereas the second spectrum is acquired at high collision energy and allows high mass accuracy fragment ion mass measurement. The fragment ion spectra are temporally correlated with precursor spectra post-run. This method of data acquisition allows all precursor ions to be fragmented and analyzed, rather than so called "data dependent acquisition" methods that require real-time decisions to be made on which ions to select for fragmentation, and which frequently miss low-abundance precursor ions, such as isocyanate–conjugated peptides.

To date, there have been few MS/MS based studies on hapten reactivity to albumin. Kristiansson and coworkers [21] determined that at a ten-fold molar excess, hexahydrophthalic anhydride bound to thirty-seven sites on human serum albumin, including the N-terminal aspartic acid and thirty-six lysine residues. Wisnewski and coworkers [22] examined the reaction products between MDI and HSA by HPLC–MS/MS. Their data indicated that for an exposure of approximately 50:1 mol ratio (MDI:protein) in phosphate buffered saline (PBS), MDI reacts with 14 sites on albumin, including 12 lysine and 2 asparagine residues. Furthermore, these authors suggested that the four "dilysine" (Lys–Lys) motifs in human serum albumin are important conjugation sites, and that MDI shows reactive specificity for the second lysine. Recently, our laboratory completed an extensive analysis of the conjugation sites of TDI on human serum albumin [19]. At high (40:1 dNCO:protein) ratios, near-stoichiometric reactivity was observed; TDI conjugates thirty-seven residues on the protein, including the N-terminal amine on aspartic acid at position one and the side chain of thirty-four lysine residues. At lower conjugation ratios (1:2 dNCO:protein), a subset of these thirty-seven residues are conserved, with conjugation observed at the N-terminus and four lysine residues, suggesting these residues are preferred sites of conjugation for TDI.

The aims of this study are to comprehensively map the reactive sites on human serum albumin for MDI utilizing multiplexed tandem mass spectrometry, and to compare the reactivity of MDI and TDI on human serum albumin *in vitro*. In addition, the concentration dependence of MDI and TDI conjugation on albumin is characterized in both ammonium bicarbonate (pH 7.9) and PBS (pH 7.4) solutions.

2. Experimental

2.1. Chemicals and reagents

Caution: *The following chemicals are hazardous and should be handled carefully*: 2,4- and 2,6-toluene diisocyanate, p-tolyl isocyanate, methylene diphenyl diisocyanate. Isocyanates should be handled in a chemical exhaust hood, and appropriate personal protective equipment (e.g., chemical resistant protective gloves, protective clothing, and safety goggles) should be utilized. 2,4-TDI, 2,6-TDI, p-tolyl isocyanate, MDI and formic acid (98%, for mass spectrometry) were acquired from Aldrich (St. Louis, MO). Human serum albumin (>99%, globulin free, lyophilized), iodoacetamide, tributylphosphine, and porcine trypsin were acquired from Sigma Chemical (St. Louis, MO). Acetone (HPLC grade), acetonitrile (HPLC grade) and 3 Å molecular sieves were acquired from Fisher Scientific (Pittsburgh, PA). [Glu]^1-Fibrinopeptide B (EGVNDNEEGFFSAR) was acquired from Protea Biosystems (Morgantown, WV). 18 MΩ distilled deionized water was produced in the laboratory by a Millipore Synthesis A-10 (Billerica, MA).

2.2. Preparation of isocyanate–albumin conjugates

Stock solutions of human serum albumin (0.5 mg/mL) were prepared in either 25 mM NH_4HCO_3 (pH 7.9) or 10 mM phosphate buffered saline (0.138 M NaCl; 0.0027 M KCl; pH 7.4). Dry acetone was prepared by incubation of HPLC-grade acetone on 3 Å molecular sieves. Stock solutions of 2,4- and 2,6-TDI, MDI, and p-tolyl isocyanate were prepared at a concentration of 7.6 nmol/μL in dry acetone. Aliquots (0–40 μL) of the isocyanate stock solutions were added to 1 mL of HSA stock solution, vigorously mixed for 5 s, and incubated at 37 °C with shaking (500 rpm) for 1 h. 100 μL aliquots of each conjugate and control were taken for analysis. Disulfide bonds were reduced in both conjugates and control samples by reaction with tributylphosphine for 30 min at room temperature, followed by alkylation with iodoacetamide for 1 h at room temperature. Alkylation was quenched by further addition of tributylphosphine for 15 min at room temperature. Samples were twice dialyzed against 3 L of 25 mM NH_4HCO_3 using 3500 MWCO mini dialysis units (Slide-A-Lyzer, Thermo Scientific, Waltham, MA). Porcine trypsin was suspended in 25 mM NH_4HCO_3 and added to each aliquot at a 40:1 (protein:trypsin) ratio. Samples were incubated overnight at 37 °C with shaking (400 rpm). Samples were centrifuged at 14,000 × *g* in a microcentrifuge (MiniSpin, Eppendorf, Hamburg, Germany) to pellet any insoluble material.

2.3. Ultra-performance liquid chromatography

Enzymatic peptides were separated on a Waters (Milford, MA) nanoACQUITY ultra-performance liquid chromatography (UPLC) system. Aliquots (1 μL) of the digest mixture were injected and trapped/desalted on a 5 μm SymmetryC_{18} (180 μm × 20 mm) trapping column with 99.5/0.5 A/B (A: 0.1% formic acid; B: 0.1% formic acid in acetonitrile) at a flow rate of 15 μL/min for 1 min. Separation was performed on a 1.7 μm BEH130C_{18} (100 μm × 100 mm) analytical column utilizing gradient elution at a flow rate of 400 nL/min and a gradient of 99/1–60/40 A/B over 60 min.

2.4. Tandem mass spectrometry

The eluent from the UPLC system was directed to the nanoelectrospray source of a Waters SYNAPT MS quadrupole time-of-flight (qTOF) mass spectrometer. Positive ion nanoelectrospray was performed utilizing 10 μm PicoTip (Waters) emitters held at a potential of +3.5 kV. The cone voltage was held constant at +40 V for all experiments. Dry N_2 desolvation gas was supplied to the

instrument via a nitrogen generator (NitroFlowLab, Parker Hannifin Corp., Haverhill, MA). [Glu]^1-Fibrinopeptide B (100 fmol/μL in 75/25 A/B) was supplied to an orthogonal reference probe and the $[M+2H]^{2+}$ ion (m/z = 785.84265 u) measured as an external calibrant at 30 s intervals. Ultra-high purity (UHP) argon was used as collision gas. Spectra were acquired in an "MS^e" fashion [20]. Alternating 1-s mass spectra were acquired. The collision energy was set to 6 eV (1 s low energy scan) and a 15–30 eV ramp (1 s high energy scan).

2.5. Data analysis

Data were analyzed with BioPharmaLynx v. 1.2 (Waters), a software program for analysis of peptide mass maps and identification of sites of modification on known protein sequences. Default peptide mass map analysis criteria of 30 ppm mass error in both low and high collision energy mode were specified. Trypsin was specified as the digestion enzyme, and 2 missed cleavages were allowed. The submitted protein sequence was taken from P02768, "serum albumin precursor, homo sapiens" (www.uniprot.org/uniprot/P02768) and the signal and propeptides (residues 1–24) removed. Custom modifiers were created for each isocyanate. For both TDI and MDI, two modifiers were necessary. The first (TDI: $C_9H_6N_2O_2$, m/z = 174.0429 u; MDI: $C_{15}H_{10}N_2O_2$, m/z = 250.0742 u) represents TDI or MDI with both isocyanate moieties bound to a peptide via urea bonds. The second (TDI*: $C_8H_8N_2O$, m/z = 148.0637 u; MDI*: $C_{14}H_{12}N_2O$, m/z = 224.0950 u) represents one isocyanate moiety bound to a peptide via a urea bond, while the second isocyanate moiety is hydrolyzed to the primary amine. For p-tolyl isocyanate, only one bound species is possible (pTI: C_8H_7NO, m/z = 133.0528 u). Identification of an isocyanate conjugation site proceeded via a rigorous procedure that involved the following steps: (1) observation of a potential peptide–isocyanate conjugation product with less than 30 ppm $m/\Delta m$ mass error in the analyte peptide mass map. (2) Comparison of analyte and control peptide mass map from unmodified human serum albumin shows that observed m/z and chromatographic retention time are unique to analyte. (3) MS/MS data contains b_n- and y_n-type ions consistent with the assigned sequence and modifier.

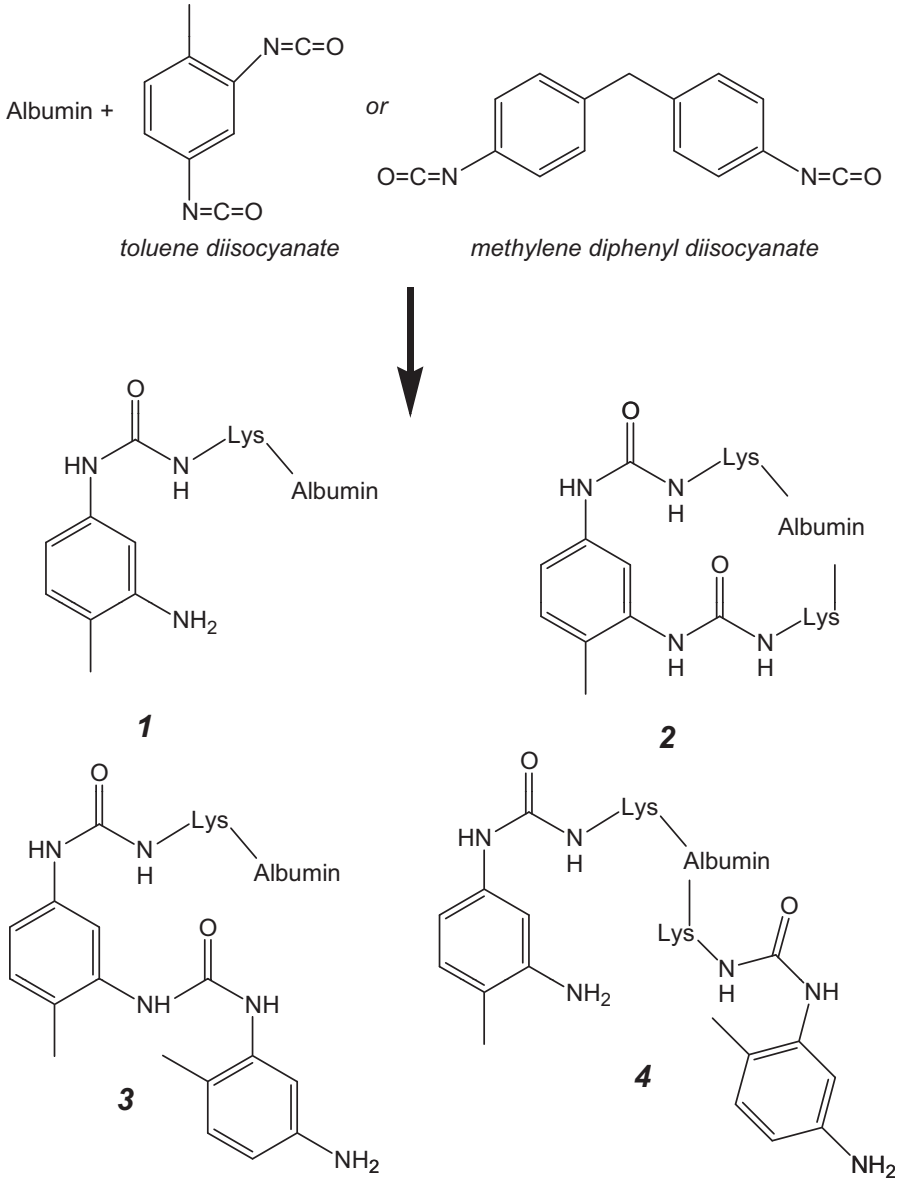
3. Results

Human serum albumin was exposed to MDI and TDI at mol ratios varying from 1:1 up to 40:1 (dNCO:protein) and the resultant dNCO–protein complexes digested with trypsin and analyzed by multiplex tandem mass spectrometry. The resulting tryptic peptides were analyzed with high sensitivity, resolution, and mass accuracy, allowing the conjugation sites of the respective diisocyanates to be determined with high confidence. MS and MS/MS data were acquired for each peptide isocyanate conjugate, with an average mass error ($m/\Delta m$) of less than 10 ppm. A complete listing of the mass spectrometry data for the peptide–diisocyanate conjugates identified across all experiments described herein is available as Supporting Information.

Reaction of diisocyanates with protein in aqueous solution leads to the formation of a variety of products (see Scheme 1). Diisocyanates readily hydrolyze to diamines in aqueous solution, although the kinetics of this reaction is slow compared to the reaction of diisocyanates with amines (to form ureas) or free thiols (to form thiocarbamates) on the protein. TDI and MDI are expected to have only transient existence in water solution, and reaction rates with amines are on the order of 10^4 times faster than hydrolysis [23]. Diisocyanate–protein thiocarbamates are not observed in the tandem mass spectrometry of digested isocyanate–protein conjugates, as these species readily regenerate isocyanate and react with primary amines on proteins. Thiocarbamates have a half-life of ~30 s–5 min at pH 7.4 [24]. Competitive hydrolysis results in the loss of diisocyanate, as well as the formation of mixed hydrolysis products where one isocyanate moiety is hydrolyzed to the amine, while the second isocyanate is bound via urea bond to a primary amine on the protein (Scheme 1, Structure 1). In addition, diisocyanates may form inter- and intra-molecular crosslinked species (Scheme 1, Structure 2), where both isocyanate moieties react with amines on the protein. This may occur between two nearby residues on one albumin molecule, or may occur between two albumin molecules. In general, the isocyanate is observed to react with primary amines such as the N-terminal α-NH_2 and the ε-NH_2 of the side chain of lysine residues [18,19], although at high dNCO:protein ratios occasional reactivity with asparagine [22] or glutamine [19] has been reported. Conjugation of the diisocyanate to a lysine residue results in failure of trypsin to cleave at that site, therefore tryptic peptide–isocyanate conjugates include one or more missed cleavage sites. Observed tryptic peptides generally have the form $[M+dNCO^*+H]^+$ (from Structure 1) or $[M+dNCO+H]^+$ (from Structure 2), where $[M+dNCO^*+H]^+$ represents conjugation of a diisocyanate with one isocyanate moiety hydrolyzed to an amine and $[M+dNCO+H]^+$ represents reaction of both isocyanate moieties with functional groups within the peptide (intra-peptide crosslinking). Other species less commonly observed include $[M+poly\text{-}dNCO^*+H]^+$ (polymerization of the diisocyanate upon a hydrolyzed NCO group; Scheme 1, Structure 3) and $[M+ndNCO^*+H]^+$ (adduction of n hydrolyzed diisocyanates at n sites on the peptide; Scheme 1, Structure 4).

Examination of the tandem mass spectra of the tryptic peptides allows unambiguous assignment of the conjugation sites on serum albumin. For example, Fig. 1 presents the tandem mass spectra of two MDI-adducted human serum albumin tryptic fragments. The tandem mass spectrum of the HSA fragment 411–428 (YTKKVPQVSTPTLVEVSR) $[M+MDI+H]^+$ peptide ion is presented in Fig. 1A. This conjugated peptide has a measured mass of 2282.2307, in good agreement with the theoretical mass of 2282.2179 ($m/\Delta m$ = 5.6 ppm). The adduct mass of +250.07 u corresponds to MDI with both isocyanate moieties covalently bonded to the peptide (e.g., intramolecular crosslinking). Further evidence for intramolecular crosslinking can be obtained from the tandem mass spectrum, via the absence of a b_3^* or y_{15}^* ion, which would require breaking both the peptide backbone linkage as well as an MDI–Lys bond. The b_5^* and b_7^* ions are adducted, appearing 250.07 u higher in mass than the theoretical b_5 and b_7 ions, indicating the MDI is covalently bound N-terminal of the valine at position 5. Similarly, the y_n-type ions y_2–y_{14} all appear at the correct theoretical mass, indicating that MDI is bound N-terminal of the valine at position 5. The tandem mass spectrum of the HSA fragment 65–81 (SLHTLFGDKLCTVATLR) $[M+MDI^*+H]^+$ peptide ion is presented in Fig. 1B. This peptide has a measured mass of 2156.1567 u in good agreement with the theoretical mass of 2156.1321 u ($m/\Delta m$ = 5.7 ppm). The adduct mass of +224.10 u corresponds to an MDI* molecule with one isocyanate moiety hydrolyzed to an amine. The MDI* is covalently linked at the lysine at position 9, as evidenced by the unmodified b_2–b_8 ions and modified b_9^* and b_{10}^* fragment ions. Assignment of all of the TDI, TDI*, MDI and MDI* conjugation sites on human serum albumin may be made in similar fashion.

Table 1 summarizes the human serum albumin conjugation sites observed for both MDI and TDI at mol ratios varying from 1:1 to 40:1 (dNCO:protein). At a low conjugation ratio (1:1) only 5 and 10 residues are observed conjugated to MDI and TDI, respectively. Of these sites, Lys^{199}, Lys^{413}, Lys^{414}, and Lys^{525} are common to both MDI and TDI, with Lys^{199} being the most abundant shared residue (based on absolute intensity of the observed peptide–dNCO ions). As the concentration of diisocyanate is increased, new conjugation sites are identified, however, all sites observed at 1:1 are conserved



Scheme 1. Reaction of human serum albumin with diisocyanates. Methylene diphenyl diisocyanate reaction products similar to toluene diisocyanate products shown.

at higher mol ratios, suggesting that these sites are the kinetically favored reactive sites on albumin for the respective diisocyanate. Seventeen residues are observed to react with TDI but not MDI; three in domain I, eight in domain II, and six in domain III, including the dilysine motif at Lys^{573}–Lys^{574}. The serum albumin conjugation site data is illustrated in Fig. 2 for both MDI (Fig. 2A) and TDI (Fig. 2B). Many of the residues that are observed to be reactive toward TDI but not MDI are located at the surface of the molecule and are highly solvent accessible, suggesting that reactive specificity of a particular amino acid residue for TDI over MDI is not based on steric hindrance or hydrophobicity. Furthermore, p-tolyl isocyanate, a structural monoisocyanate analog of TDI, showed similar reactivity to MDI, rather than TDI (Table 2).

The effect of the solvent environment in which the protein–MDI adduct formation occurs was investigated by performing the reaction for each mol ratio in both ammonium bicarbonate buffer (pH 7.9) and phosphate buffered saline (pH 7.4). The results of these experiments are summarized in Table 3. The reactive sites identified for each mol ratio in the two buffers were very similar. The most abundant MDI conjugation sites, Lys^{199}, Lys^{413}, Lys^{414}, Lys^{525} and Lys^{536} were observed in both bicarbonate and PBS. Three sites (Lys^{73}, Lys^{351}, and Lys^{444}) were observed at low (1:1 or 5:1) mol ratios in PBS, but required higher (40:1) ratios in bicarbonate buffer. Three residues were unique to high ratio (10:1 or 40:1) PBS exposure, Lys^{212}, and the Lys^{573}–Lys^{574} dilysine motif.

4. Discussion

The analysis of MDI and TDI conjugated human serum albumin by multiplexed tandem mass spectrometry allows the sites of diisocyanate conjugation to be determined with high sensitivity and confidence without prior purification or enrichment of isocyanate–conjugated tryptic peptides. Because each precursor ion is analyzed by both MS and MS/MS as it elutes from the UPLC, greater sensitivity for low-abundance diisocyanate-containing precursors is achieved over conventional data-directed analyses. In this study, we present a comprehensive binding map of human serum albumin conjugated to both MDI and TDI *in vitro*.

The N-terminal amine of human serum albumin has long been hypothesized to be a target of diisocyanate *in vivo*. Stark

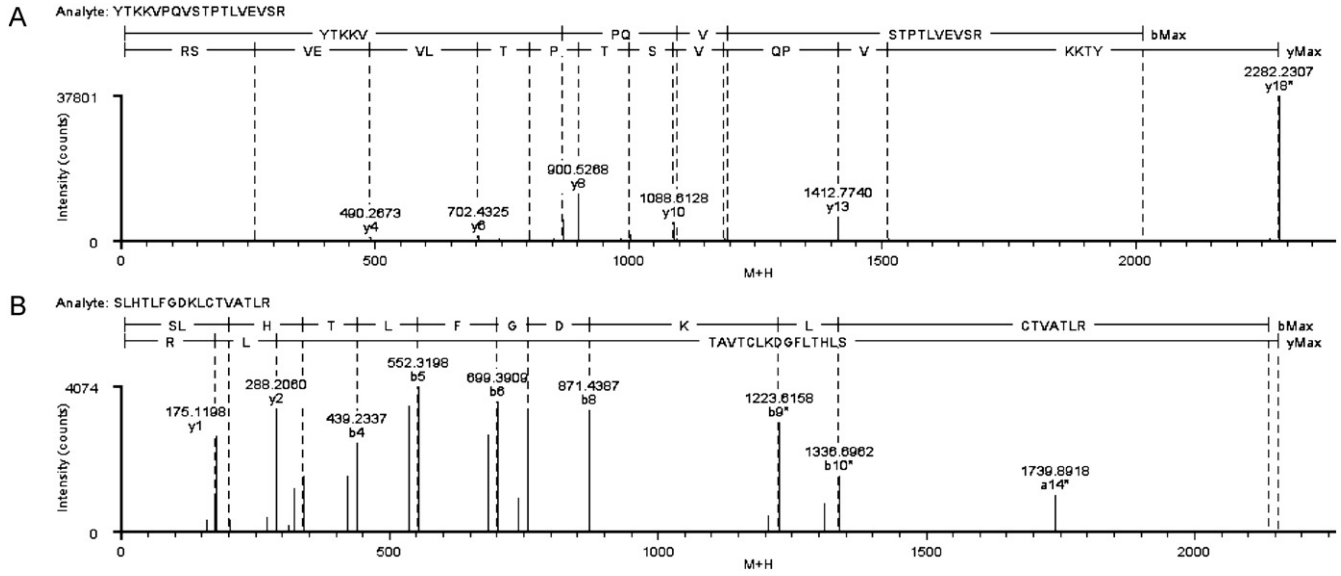


Fig. 1. Tandem mass spectra of two MDI-conjugated human serum albumin tryptic peptides. (A) HSA fragment 411–428 (YTKKVPQVSTPTLVEVSR) $[M+MDI+H]^+$; (B) HSA fragment 65–81 (SLHTLFGDKLCTVATLR) $[M+MDI^*+H]^+$.

completed a study of the kinetics of isocyanate reactivity with protein functional groups in 1965 [25], determining that at pH 7, reaction with an N-terminal amine should proceed approximately 100 times faster than the ε-amine of the lysine side chain. The difference is due to the relative pK_a of the two functional groups (α-NH_3^+ $pK_a \sim 9$ versus Lys ε-NH_3^+ $pK_a \sim 10.5$), as dNCO conjugation proceeds through the neutral $-NH_2$ rather than the charged $-NH_3^+$ species. This finding correlates nicely with the results of studies

Table 1
Comparison of conjugation sites on human albumin observed for MDI and TDI at varying dNCO:protein ratios.

Residue	1:1		5:1		10:1		40:1	
	MDI	TDI	MDI	TDI	MDI	TDI	MDI	TDI
Asp^{1}		X	X	X	X	X	X	X
Lys^{4}		X	X	X	X	X	X	X
Lys^{12}						X	X	X
Lys^{73}					X		X	X
Gln^{104}								X
Lys^{106}								X
Lys^{136}						X	X	X
Lys^{137}						X	X	X
Lys^{159}								X
Lys^{190}					X	X	X	X
Gln^{196}								X
Lys^{199}	X	X	X	X	X	X	X	X
Lys^{205}								X
Lys^{212}						X		X
Lys^{262}								X
Lys^{274}				X		X		X
Lys^{276}						X		X
Lys^{281}						X		X
Lys^{351}						X	X	X
Lys^{378}				X		X		X
Lys^{402}								X
Lys^{413}	X	X	X	X	X	X	X	X
Lys^{414}	X	X	X	X	X	X	X	X
Lys^{432}			X	X	X	X	X	X
Lys^{436}			X		X	X	X	X
Lys^{439}						X	X	X
Lys^{444}		X		X		X	X	X
Lys^{524}		X	X	X	X	X	X	X
Lys^{525}	X	X	X	X	X	X	X	X
Lys^{534}			X	X	X	X	X	X
Lys^{536}	X		X	X	X	X	X	X
Lys^{541}				X		X	X	X
Lys^{545}				X		X		X
Lys^{557}						X		X
Lys^{560}						X		X
Lys^{573}		X		X		X		X
Lys^{574}		X		X		X		X

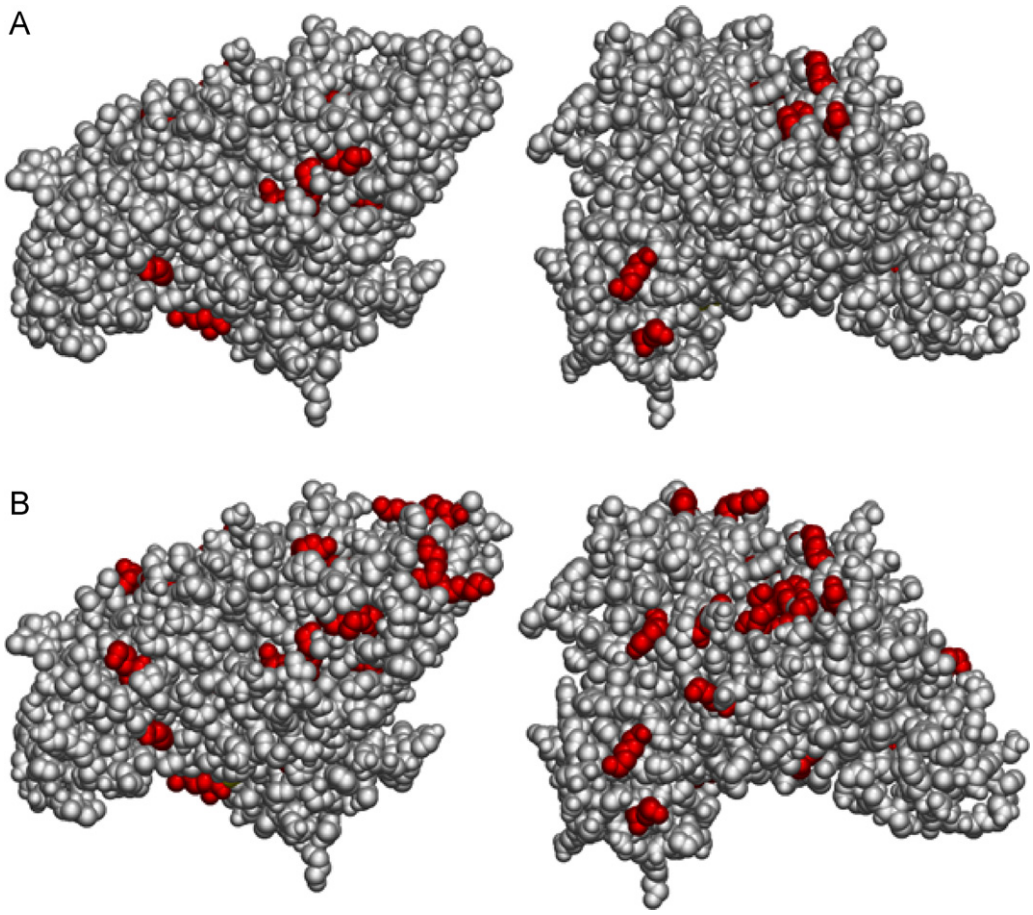


Fig. 2. Front (left) and rear (right) projections of human albumin. Conjugation sites for MDI (A) and TDI (B) are highlighted in red. Visualization based on PDB Structure 1 UOR [32].

on the reactivity of dNCO with small peptides in aqueous solution [18]. Although TDI readily reacts with the N-terminus of HSA at all mol ratios, no evidence of N-terminal MDI conjugation is observed until mol ratios of 5:1 or greater are reached, and even then this constitutes a minor reaction product.

Human serum albumin is a highly charged molecule, in part accounting for its high solubility. A previous study has determined that all 59 lysine residues of HSA are solvent accessible [26]. However, of those 59 Lys residues, only 34 are reactive toward TDI, while 19 are reactive toward MDI. Although it would be tempting to attribute differences in conjugation between TDI and MDI to steric or hydrophobic effects, as illustrated by Fig. 2, lysine residues that are reactive toward TDI, but not MDI, are located in highly accessible regions of the three dimensional structure of the protein. Steric effects are therefore an insufficient explanation for the difference between accessibility and observed conjugation for the two diisocyanates. It is likely that the microenvironment of the residue determines whether or not that residue is reactive toward the isocyanate. We therefore hypothesize that the difference in observed conjugation between TDI and MDI is attributable to the increased reactivity of TDI. The electron withdrawing character of the second N=C=O group on the aromatic ring of TDI significantly increases the reactivity of the first isocyanate. In contrast, the reactivity of the isocyanate functional group(s) on MDI is lower because the *p*-[(4-isocyanatophenyl)methyl] substituent is less electron-withdrawing. This difference is demonstrated by examining the albumin residues reactive with *p*-tolyl isocyanate (see Table 2). Tolyl-isocyanate is a monoisocyanate structural analog of 2,4-TDI, however, because it lacks the electron-withdrawing character of the second isocyanate functional group in the 2-position, the reactivity should be lower. Indeed, although *p*-tolyl isocyanate is approximately the same size as TDI, its reactivity toward albumin more closely resembles that of the larger, but less reactive MDI. These observations are in good agreement with both molecular orbital theory and previous studies of substituted phenyl isocyanates which demonstrate that electron-withdrawing substituents on phenyl isocyanates increase the rate of reaction with active hydrogen species [27].

Lys^{199}, which is known to bind hydrophobic anions such as aspirin and benzyl penicillin, was also determined to be a predominant conjugation site for both TDI and MDI (see Tables 1 and 3). Gerig and Reinheimer [28] determined in 1975 that the pK_a of the aspirin binding site (later determined to be Lys^{199}) of albumin was 7.9. These authors hypothesized based on the reactivity of human serum albumin with dinitrofluorobenzene that there exist two lysines on HSA that have a pK_a as low as 7.9. In addition, Lys^{199} has been shown by molecular dynamics calculations to be predominantly uncharged, undergoing proton transfer with the nearby Lys^{195} [29]. This early observation is in good agreement with the current observation that dNCO is bound to Lys^{199} and not the nearby Lys^{195}. Other residues noted to be abundantly conjugated to diisocyanate, such as Lys^{439} and Lys^{525}, have been observed to undergo nonenzymatic glycosylation *in vivo*. Glycosylation is generally observed to occur at lysine residues located near another amino group, presumed to be charged [30]. Lys^{439} is located in a

Table 2
Comparison of conjugation sites on human albumin observed for p-tolyl isocyanate, MDI and TDI at 40:1 dNCO:protein ratio.

Residue	pTI	MDI	TDI
Asp[1]	X	X	X
Lys[4]	X	X	X
Lys[12]	X	X	X
Lys[73]	X	X	X
Gln[104]			X
Lys[106]			X
Lys[136]	X	X	X
Lys[137]	X	X	X
Lys[159]	X		X
Lys[190]	X	X	X
Gln[196]			X
Lys[199]	X	X	X
Lys[205]			X
Lys[212]	X		X
Lys[262]			X
Lys[274]			X
Lys[276]			X
Lys[281]			X
Lys[351]	X	X	X
Lys[378]			X
Lys[402]			X
Lys[413]	X	X	X
Lys[414]	X	X	X
Lys[432]	X	X	X
Lys[436]	X	X	X
Lys[439]	X	X	X
Lys[444]		X	X
Lys[524]	X	X	X
Lys[525]	X	X	X
Lys[534]		X	X
Lys[536]		X	X
Lys[541]	X	X	X
Lys[545]	X		X
Lys[557]			X
Lys[560]			X
Lys[573]			X
Lys[574]			X

region with two other nearby lysine residues (Lys[432] and Lys[436]) and Lys[525] is part of a dilysine motif.

Wisnewski and coworkers [22] also examined the reaction products between MDI and human serum albumin by HPLC–MS/MS. Their data indicated 14 reactive sites on albumin, including 12 lysine and 2 asparagine residues, whereas we found a total of 20 reactive sites for MDI (Table 1). Of the binding sites identified in that report, we find confirmatory evidence for 11 of 14. We did not observe any evidence of binding to Asn[391], and rather than binding to Asn[429], we attribute binding to the nearby Lys[432]. In addition, these authors suggested that the four "dilysine" (KK) motifs in human serum albumin are important reactive sites, and that MDI shows reactive specificity for the second lysine. As discussed previously, the ability of a lysine residue to transfer its proton to a nearby lysine or histidine residue may, in fact lead to increased reactivity toward diisocyanates. However, as dilysine motifs account for 8 of 36 TDI conjugation sites and 6 of 20 MDI conjugation sites, it is clear that two lysine residues in a "KK" arrangement are not essential for diisocyanate conjugation.

In a previous report, we noted that significant differences in the reactivity of isocyanates with peptides were observed when performed in water versus phosphate buffered saline solution [18]. Here, we examined the residues of human serum albumin reactive toward MDI on serum albumin at varying dNCO:protein ratios in two different buffer systems, 25 mM NH_4HCO_3 (pH 7.9) and 10 mM phosphate buffered saline (pH 7.4); the results are summarized in Table 3. No unique MDI conjugation sites are observed in bicarbonate buffer, whereas there are three unique conjugation sites in PBS (Lys[212], Lys[573], Lys[574]). Furthermore, several conjugation sites are observed at lower MDI concentration in PBS than in bicarbonate buffer (Lys[12], Lys[73], Lys[190], Lys[351], Lys[444], Lys[524], Lys[534], Lys[541]). Because isocyanate–amine nucleophilic reactions proceed via the uncharged $-NH_2$ it could be reasonably anticipated that in the higher pH buffer (NH_4HCO_3, pH 7.9) more lysine side chains would be deprotonated and thus reactive than in the more acidic PBS (pH 7.4), however, this is not what is observed. Both bicarbonate and phosphate have been observed to catalyze hydrolysis of isocyanates and thiocarbamates [24,31]. However, Berode

Table 3
Comparison of conjugation sites on human albumin observed for MDI at varying dNCO:protein ratios in different solvent environments.

Residue	1:1		5:1		10:1		40:1	
	NH_4HCO_3[a]	PBS[b]	NH_4HCO_3[a]	PBS[b]	NH_4HCO_3[a]	PBS[b]	NH_4HCO_3[a]	PBS[b]
Asp[1]			X	X	X	X	X	X
Lys[4][c]			X	X	X	X	X	X
Lys[12]						X	X	X
Lys[73]				X		X	X	X
Lys[136]							X	X
Lys[137][c]							X	X
Lys[190]						X	X	X
Lys[199][c]	X	X	X	X	X	X	X	X
Lys[212][c]						X		X
Lys[351][c]		X		X		X	X	X
Lys[413]	X	X	X	X	X	X	X	X
Lys[414][c]	X	X	X	X	X	X	X	X
Lys[432][c]			X	X	X	X	X	X
Lys[436][c]			X	X	X	X	X	X
Lys[439]							X	X
Lys[444][c]		X		X		X	X	X
Lys[524]		X	X	X	X	X	X	X
Lys[525][c]	X	X	X	X	X	X	X	X
Lys[534]		X	X	X	X	X	X	X
Lys[536]	X	X	X	X	X	X	X	X
Lys[541][c]						X	X	X
Lys[573]								X
Lys[574]								X

[a] 25 mM NH_4HCO_3, pH 7.9.
[b] 10 mM PBS (0.138 M NaCl; 0.0027 M KCl), pH 7.4.
[c] Residues observed to react with MDI at ~50:1 dNCO:protein in Ref [22].

and coworkers demonstrated bicarbonate catalyzes the hydrolysis of hexamethylene diisocyanate (HDI) to 1,6-diaminohexane to a greater extent than phosphate [31]. Therefore, the observation here that the isocyanate is more reactive in PBS than bicarbonate can be explained on the basis of competitive hydrolysis. If hydrolysis of the isocyanate is more rapid in bicarbonate, the effective concentration of isocyanate in solution is reduced and the "onset" of reaction at a specific amino acid is delayed in bicarbonate, relative to PBS.

This report demonstrates the utility of tandem mass spectrometry for the analysis of diisocyanate-haptenated proteins. Comprehensive maps of the human serum albumin residues reactive toward the industrially important chemicals toluene diisocyanate and methylene diphenyl diisocyanate are presented. Diisocyanates may react with proteins *in vitro* to form hydrolysis and cross linked products. The site at which diisocyanates react with albumin depends on a number of factors, including isocyanate concentration, solvent system, and the microenvironment of an individual residue. The results presented here confirm many of the reactive sites presented in earlier reports, as well as demonstrate that two residues, Lys^{199} and Lys^{525} react with both MDI and TDI at all concentrations and under all solvent conditions examined here, suggesting these may be favored conjugation sites that could be of particular interest for future studies of exposure *in vivo*.

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The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention or the National Institute for Occupational Safety and Health.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2011.09.015.

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