Stable Isotope Labeling Method for the Investigation of Protein Haptenation by Electrophilic Skin Sensitizers

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ABSTRACT

The risk of contact sensitization is a major consideration in the development of new formulations for personal care products. However, developing a mechanistic approach for non-animal risk assessment requires further understanding of haptenation of skin proteins by sensitizing chemicals, which is the molecular initiating event causative of skin sensitization. The non-stoichiometric nature of protein haptenation results in relatively low levels of modification, often of low abundant proteins, presenting a major challenge for their assignment in complex biological matrices such as skin. Instrumental advances over the last few years have led to a considerable increase in sensitivity of mass spectrometry (MS) techniques. We have combined these advancements with a novel dual-labeling/LC-MS² approach to provide an in-depth direct comparison of human serum albumin (HSA), 2,4-dinitro-1-chlorobenzene (DNCB), 5-chloro-2-methyl-4-isothiazolin-3-one (MCI), trans-cinnamaldehyde, and 6-methyl coumarin. These data have revealed novel insights into the differences in protein haptenation between sensitizers with different reaction mechanisms and sensitizing potency; the extreme sensitizers DNCB and MCI were shown to modify a greater number of nucleophilic sites than the moderate sensitizer cinnamaldehyde; and the weak/non-sensitizer 6-methyl coumarin was restricted to only a single nucleophilic residue within HSA. The evaluation of this dual labeling/LC-MS² approach using HSA as a model protein has also demonstrated that this strategy could be applied to studying global haptenation in complex mixtures of skin-related proteins by different chemicals.

Key words: contact allergy; GeLC-MS/MS; proteomics; sensitizer; skin

Skin sensitization, which leads to the development of allergic contact dermatitis (ACD), is an immune response to commonly harmless chemicals causing significant morbidity without any benefit to those affected (McFadden et al., 2013). It was first proposed over 75 years ago that the key event in the induction of skin sensitization is the covalent modification (haptenation) of skin proteins by low molecular weight chemicals (haptenes), which are too small to be recognized by an immune system (Landsteiner and Jacobs, 1935).

This phenomenon has been studied extensively, utilizing a variety of techniques and models, including peptides and relevant proteins to study haptenation by sensitizers (e.g., Aleksic et al., 2007, 2008; Alvarez-Sanchez et al., 2004a,b; Gerberick et al., 2007). Despite different study designs, these studies conclude that binding of sensitizing chemicals to protein nucleophiles is selective and governed by three main factors: electrophilicity of the sensitizer, nucleophilicity of the target, and steric constraints. Most sensitizers are electrophilic, reacting predomi-
nanty with nucleophilic side chains of lysine and cysteine, less commonly tyrosine, histidine, and arginine (Ahlors et al., 2003).

Many intra and extracellular events can be triggered by sensitization reactivity. For example, reactivity drives glutathione conjugation as well as activation of the NF-κB pathway and thus induction of phase II metabolizing enzymes via covalent binding to Cys residues on Keap1 protein (Migdal et al., 2013). An insight into the dynamic interplay between the detoxification events leading to the removal of an electrophilic chemical and haptenation of proteins, followed by elicitation of an immune response, is sorely missed. Thus an understanding of qualitative and particularly quantitative aspects of skin protein haptenation is one of the major gaps in advancing our knowledge of the adverse outcome pathway of induction (and elicitation) of skin sensitization (OECD, 2012).

Determination of sites of protein modification in mixtures represents a considerable analytical challenge. Technical and instrumental limitations have to date restricted detailed investigations of protein haptenation in complex protein mixtures and tissues including human skin. In skin sensitization research, in parallel to developments elsewhere (reviewed by Liebler, 2008), methods for studying protein haptenation in mixtures range from immunochemistry-based studies, utilizing antibodies specific to the adduct(s) formed (e.g., Elahi et al., 2004), to proteomics and Nuclear Magnetic Resonance (NMR)-based techniques (e.g., Elbayet al., 2013). Some investigators were able to utilize unique features of certain sensitizers (such as fluorescent adducts of monobromobimane) to pinpoint the amino acid site of haptation in human skin (Simonsson et al., 2011); however, such techniques are only applicable to chemicals possessing those unique features.

Further afield from sensitization, researchers have demonstrated ways to overcome the most critical issue of low abundance of modified proteins in cell/tissue extracts. A variety of techniques have been used, ranging from 2D gel-based separations using radiolabeled probes (e.g., Tzouros and Pahler, 2009), affinity capture, and shotgun proteomics strategies utilizing biotin-linked electrophiles (e.g., Denney et al., 2006), to post-labeling click chemistry approaches (Vila et al., 2008). Although very useful, none of the above approaches are ideal and raise additional issues, for example cost of synthesis, radiolabel stability, and the influence of bulky linker and affinity tags on target selectivity of an otherwise small electrophile structure. There is to date no globally applicable methodology to study protein haptenation in complex mixtures.

Instrumental advances have led to a considerable increase in sensitivity of mass spectrometry (MS) techniques recently. Here we combine the gel-based approach GeLC-MS/MS (Schirle et al., 2003) with a novel dual-labeling/liquid chromatography-MS$^6$ approach (LC-MS$^6$) to provide an in-depth direct comparison of human serum albumin (HSA) haptation by classical sensitizers. HSA is well characterized, prevalent in skin, and has been previously used in binding studies (Curry et al., 1998). A number of studies have also shown specific T cell responses after modification with the extreme sensitizer 2,4-dinitrobenzene sulfonic acid (DNBS) (Dietz et al., 2010); via p-phenyleneediamine (PFD) modification at position Cys 34 (Jenkinson et al., 2010); or by transporting nickel(ii) directly to TCR/MHC conjugates (Thierry et al., 2004), suggesting that HSA may play a role as a carrier protein mediating T cell activation in contact allergy. We have studied HSA haptation by well-understood sensitizers dinitrochlorobenzene (DNCB), cinnamaldehyde, and 5-chloro-2-methylisothiazol-3-one (MCI). In addition, we investigated haptation by 6-methyl coumarin, which has been classes as a non-sensitizer in the murine local lymph node assay (Ashby et al., 1995).

Our initial studies have shown that haptenation of proteins is non-stoichiometric in nature, presenting challenges for their analysis. By taking advantage of the MS$^5$ data independent mode of data acquisition (Silva et al., 2006), and combining this with the dual labeling approach, we were able to pinpoint low abundant modified peptides using an isotopic precursor ion signature and confirm the peptide identity and amino acid site of haptation using product ion fragmentation data.

**MATERIALS AND METHODS**

Test chemicals. DNCB (99% purity; MW, 202.55 Da) was obtained from Sigma, UK, and DNCB-D$_3$ (99% purity; MW, 205.57 Da) was obtained from QMX Laboratories.

Trans-cinnamaldehyde (CA) (99% purity; MW, 132.16 Da) and 6-methyl coumarin (99% purity; MW, 160.17 Da) were obtained from Sigma-Aldrich and trans-cinnamaldehyde-D$_3$ (98% purity; MW, 137.12 Da) and 6-methyl coumarin-D$_3$ (99% purity; MW, 163.15 Da) were custom synthesized by Quotient Amersham Radiochemicals.

5-chloro-2-methyl-4-isothiazolin-3-one (MCI) (MW 149.60 Da) and C$^{13}$-labeled MCI (MW 150.8 Da) were synthesized and kindly donated by Professor Jean-Pierre Lepoittevin and Dr Elena Gimenez Arnau, Labaratoire de Dermatochimie, Strasbourg. Exact locations of the stable isotopes for each chemical are shown in Table 1.

HSA sensitzer modification. To study the effect of DNCB haptation with increasing concentration and time, HSA (fraction V, >97% purity, A9511 (lot no. 107K7560), Sigma) was solubilized in PBS (pH 7.4) to a final concentration of 1 mg/ml. DNCB and deuterated DNCB were solubilized in 100% Dimethyl sulphoxide (DMSO). HSA was incubated with either DNCB or deuterated DNCB at a range of concentrations (1500, 750, 150, 15, 7.5 µM; final concentration of DMSO < 1%) for either 1, 6, 24, or 192 h (8 days) at 37°C. The relative amount of protein modification was measured by absorbance at 360 nm for each dose of DNCB at each time point (Kesner et al., 1967). Measurements were background corrected with background calculated as mean of the absorbance of DNCB + PBS control at 360 nm minus mean of buffer absorbance of HSA + PBS control at 360 nm (n = 3).

To maximize the amount of haptended peptides and determine the differences in haptation with a range of chemicals, stock solutions of sensitizers were prepared in 100% DMSO (for DNCB) or 100% ethanol (for cinnamaldehyde, MCI, and 6-methyl-coumarin) containing 50%, by molar concentration, of unlabeled sensitizer and 50% stable isotope labeled sensitizer. HSA (fraction V, >95% purity, 126658 (lot no. D00095847), Calbiochem, UK) was solubilized in 0.1-M TEAB (pH 8.0) at 1 mg/ml prior to modification with a 1:100 molar excess of sensitizer to protein (control samples were prepared in 0.1-M Triethylammonium bicarbonate (TEAB) (pH 8.0) with the addition of 0.2% of the relevant solvent) and incubated at 37°C. Aliquots of sensitizer-modified (or control) HSA were taken at 24 h, 2 weeks, and 4 weeks. Free chemical and salts were removed by resolving the samples through an SDS polyacrylamide gel for 45 min.

**Assessment of the reducing capacity of DNCB.** Bodipy-FL-cysteine (5 µM in PBS, pH 7.4; or 50 mM sodium acetate + 100 mM NaCl, pH 4.5; Life Science Technologies, Paisley, UK) was incubated...
TABLE 1. Structures, Position of Stable Isotope, Potency Category (Including EC3 Value, as Derived from the Local Lymph Node Assay), Δ Mass (Da) Expected Following Haptenation, Reactivity Domain, Variable Modifications of Sensitizers for Database Searching of MS Data Within PLGS

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Structure and position of stable isotope labels (*)</th>
<th>Potency category (% EC3)</th>
<th>Δ mass (Da) expected for unlabeled and (labeled) adduct</th>
<th>Reactivity domain</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-chloro-2,4-dinitrobenzene (DNCB)</td>
<td><img src="image1" alt="Structure of 1-chloro-2,4-dinitrobenzene" /></td>
<td>Extreme (0.05)</td>
<td>+166.0015 (+169.0015)</td>
<td>S_NAr</td>
<td>Cys, His, Lys, Tyr</td>
</tr>
<tr>
<td>Cinnamaldehyde</td>
<td><img src="image2" alt="Structure of Cinnamaldehyde" /></td>
<td>Moderate (3.0)</td>
<td>+114.047 (+119.047)</td>
<td>Schiff base</td>
<td>Arg, Cys, His, Lys</td>
</tr>
<tr>
<td>5-chloro-2-methyl-4-isothiazol-3-one (MCI)</td>
<td><img src="image3" alt="Structure of 5-chloro-2-methyl-4-isothiazol-3-one" /></td>
<td>Extreme (0.0009)</td>
<td>+99.032 (+100.032)</td>
<td>Amide adduct</td>
<td>Cys, Lys, His</td>
</tr>
<tr>
<td>6-methyl coumarin</td>
<td><img src="image4" alt="Structure of 6-methyl coumarin" /></td>
<td>Non-sensitizer</td>
<td>+158.0368 (+161.0368)</td>
<td>Michael adduct</td>
<td>Cys, Lys</td>
</tr>
</tbody>
</table>

* denotes the positions and number of stable isotopes incorporated into the chemicals used in this study.

for 1 h at 37°C with varying concentrations of DNCB (2.5, 5, 250, 500, and 1000 μM) or Dithiothreitol (DTT) (25, 250 μM, 2.5, 5, 10, 15 mM) as a positive control. The fluorescence of the treated Bodipy-FL-cysteine was measured (excitation λ 485 nm, emission λ 530 nm) to determine the extent of reduction.

GeLC-MS/MS. Modified HSA and control samples (20–40 μg) were denatured in final sample buffer (NuPAGE LDS Sample Buffer, Life Technologies, Paisley, UK) containing 10-mM DTT at 70°C for 10 min. The denatured samples were resolved using a NuPage 4–12% Bis-Tris gel (Life Technologies) at 200 V for approximately 45 min. Following staining with Coomassie Blue, the large band at approximately 66 kDa was excised and subjected to in situ trypsin digestion using the method of Shevchenko et al. (1996). The resulting peptides were extracted and ~1–2 μg of sample loaded onto a reverse phase trap column (Xbridge BEH C18 Na-
Database searches. The raw mass spectra were processed using ProteinLynx Global Server Ver 2.4 (Waters) and the data processed to generate reduced charge state and deisotoped precursor and associated product ion peak lists. These peak lists were searched against the HSA sequence (obtained from UniProt) using MassLynx 4.1 and the amino acid site of modification determined, where possible.

Bioinformatic analysis. As the modification of a protein by a sensitizer is non-stoichiometric, modified peptide ions are lower in abundance and often found within the “noise” of a mass spectrum. This combined with the increased search space resulting from the addition of modifications to the database searches leads to a large number of false positive assignments. In this study, we have employed a dual isotope labeling strategy to enable us to eliminate false positives as follows.

Following database searching, precursor ion peak pairs were extracted from the Peptide 3D files generated from processing raw data using PLGS based on the following criteria; ion pairs with a fixed mass difference (corresponding to the number of stable isotopes incorporated into the labeled sensitizer), with similar ion intensity and within a retention time window of 1 min. Extracted peptide pairs were correlated with modified peptide masses identified after database searching using m/z and retention time. Spectra were subsequently manually inspected using MassLynx 4.1 and the amino acid site of modification determined, where possible.

The structure of HSA was imported from Protein Data Bank (PDB, http://www.pdb.org/pdb/home/home.do; structure 1AO6) into the molecular viewer and 3D molecular editor software PyMOL version 0.99 for editing, enabling the modified amino acid residues to be highlighted within the structures.

RESULTS

Initial haptenation studies comparing the intensities of haptenated peptides within complex samples revealed their low abundance compared with unmodified peptides (data not shown). To reduce the bias toward highly abundant peptide species and improve the ability to detect and identify low abundant haptenated peptides, we replaced the Data Dependent Acquisition commonly used for these types of studies, with the data-independent mode of acquisition, MS² (Geromanos et al., 2009; Michalski et al., 2011). Time points for the investigation were chosen by monitoring the rate of binding of DNCB to HSA using ultraviolet (UV) absorbance at 360 nm at four different time points (Fig. 1). The observed levels of haptenation increased as a function of time and concentration, for a low level of HSA autofluorescence.

We used the dual labeling approach as a way of eliminating false positives, whereby samples were haptenated with a 50:50 molar ratio of unlabeled and stable isotope-labeled sensitizer; haptenated peptides were identified by two peptide clusters, the first cluster relating to the modified peptide and the second cluster at a fixed mass difference relating to the peptide modified with the stable isotope-labeled sensitizer. Figure 2 illustrates incorporation of MS² and stable isotope labeling into the sample preparation and MS analysis workflow.

Modified peptides were confirmed where two peptide clusters of fixed Δmass were observed in the MS spectrum. The MS/MS spectrum of the modified precursor ion was subsequently analyzed to determine the exact site of haptenation. An example of MS and MS/MS spectra generated is shown in Supplementary figure 1.

A total of 34 residues were confirmed to be haptenated by DNBC, 33 by MCI, and nine by cinnamaldehyde (Supplementary data). No confirmed modifications were observed for 6-methyl coumarin. For all chemicals tested, we detected a number of potential sites of haptenation which unfortunately could not be confirmed as the fragmentation spectra were of insufficient quality to determine the haptenation location. For example, the most prominent signal for 6-methyl coumarin haptenation was for the peptide ADDKETCFAEGKK, which included two peptide clusters of 1.5 Da apart.

Some common features were observed when comparing data from different chemicals. DNBC and MCI both preferentially haptenate Lys (Supplementary data), possibly due to higher proportion of Lys in HSA compared with other nucleophilic residues. Interestingly, cinnamaldehyde showed high affinity for haptenating Arg residues. Double cinnamaldehyde adducts have previously been observed on the guanidine moiety of Arg side chains (Aleksic et al., 2009), but this was not investigated in the present study due to complexity.

The majority of DNBC-haptenated residues were observed throughout the time course and as early as 24 h suggesting that the same residues susceptible to haptenation continue to be haptenated over time. However, certain residues only appear haptenated by DNBC at later time points (e.g., His 3, Lys 262, and Tyr 263). Similar observations were made for MCI (where His 3, Lys 4, Lys 106, and Lys 181 were found haptenated after 24 h) and cinnamaldehyde (where His 128 was haptenated after 24 h). This may possibly be due to a change in structure of the protein molecule by initial haptenation, thus making new nucleophilic sites susceptible to further haptenation.

Several residues appeared modified at 24 h by DNBC, but could not be detected at the later time points (Lys 12, Cys 34, Lys 64, Cys 75, Lys 93, Cys 124, Cys 177, Lys 432, and Lys 545) (Fig. 3). On further inspection, evidence of possible further modifications such as oxidation and methylation of these modified HSA peptides was observed which would obscure these signals from database searches.

Four HSA lysines are commonly targeted by all tested hapten. These include Lys190, Lys212, Lys475, and Lys574 (Fig. 4).
FIG. 1. The rate of HSA haptenation by DNCB monitored by the increase in absorbance at 360 nm as a function of incubation time. Data from triplicate absorbance readings (n = 3) mean A360 of sample minus background, with background calculated as mean A360 of DNCB + PBS control minus mean A360 of HSA + PBS control.

Conversely, certain nucleophilic residues are exclusively haptenated by a single sensitizer. For example, DNCB exclusively haptenates Cys34, Lys64, Cys75, Lys93, Cys124, Cys177, Tyr263, Cys392, Tyr411, and Lys413; MCI exclusively haptenates Lys51, Lys159, Tyr161, Lys276, Tyr319, Lys323, Lys538, and Lys564; and cinnamaldehyde exclusively haptenates His128, Arg186, Arg218, and Arg428 (Fig. 4).

Contrastingly, there are three regions of HSA where no haptenation sites were found with any of the test chemicals used (residues 287–316, 437–472, and 477–509). Although these regions contain nucleophilic residues, it is likely that the tertiary structure of HSA prevented haptenation as the nucleophilic side chains are facing inward toward the center of the molecule (Fig. 5).
FIG. 3. Schematic drawing of the secondary structure of a single molecule of HSA (downloaded from PDB, 1A06), front and back. Modified nucleophilic residues are highlighted and shown as stick structures, the remaining unmodified nucleophilic residues are highlighted only. This figure was generated using PyMol.

FIG. 4. Circular representation of the sequence of HSA showing which nucleophiles were haptenated after exposure with the test chemicals DNCB, MCI, and cinnamaldehyde. The four amino acids that were found to be haptenated by all three chemicals are outlined with a rectangular box.
DNCB is probably the most studied skin sensitizer and has previously been shown to bind to the Lys, His, Tyr, and Cys via nucleophilic substitution (SNAr), resulting in the addition of a dinitrophenyl adduct with a mass of +166 Da (Aleksic et al., 2007). In our study, DNCB modifies a large number of available nucleophilic residues on HSA (Fig. 3). The degree of dinitrophenylation of HSA, as determined by measuring the absorbance at 360 nm with a range of DNCB concentrations over time, suggests that DNCB concentration has a significant effect on the overall amount of modified protein (Fig. 1). Because no plateau in the rate of modification (Fig. 1) was observed, it is likely that saturation of the protein has not yet occurred by the end of the 1-week time course studied. This may suggest that DNCB haptenation causes exposure of more nucleophilic sites due to structural changes within HSA. In support of this, a number of studies have shown changes in the secondary and tertiary structure of the protein in response to temperature (Wetzel et al., 1980), pH (Bhattacharya et al., 2011), chemical denaturation (Santra et al., 2005), ligand binding (Bertucci and Domenici, 2002), and covalent modification by chemicals (Anraku et al., 2001).

In total, 34 amino acids were confirmed to be haptenated by DNCB. This is in contrast to previous work where it was considered that DNCB modification was limited to only 10 sites (Aleksic et al., 2007). The differences in these findings are most likely due to the increase in sensitivity of the instrumentation, the mode of data acquisition employed (MS²), and the use of stable isotopes. Interestingly, three residues reported as DNCB-haptenated by Aleksic et al. (2007), N-term, His 9, Tyr 140, were absent from these data, although modifications were found within the same peptides at positions His 3, Lys 4, and Lys 137. This could potentially be explained by differences in incubation conditions, particularly because the Aleksic study used a high proportion of organic solvent in the incubation buffer, which could have effects on the conformation of the protein and potentially the reactivity toward DNCB (Griebenow and Klibanov, 1996).

The modification of Cys residues that are reported to be S-S bridged in HSA was repeatedly observed only with DNCB and was confirmed at multiple time points except time zero. It is therefore unlikely that these were generated as a consequence of sample processing. We assessed the reducing capacity of DNCB by incubation with bodipy-FL-cysteine, which fluoresces when reduced, and compared this with the strong reducing agent DTT. No fluorescence was detected after treatment with DNCB confirming that DNCB is unlikely to be capable of reducing the disulphide bridges in HSA. These cysteine modifications were observed with HSA from two sources (Sigma and Calbiochem) suggesting that this is not preparation specific and may be as a result of low levels of free thiols that may have become available for modification, for example through the liberation of a free thiol via release of the nitroso group from an S-nitrosylated cysteine residue. Alternatively, DNCB may be unstable and mobile allowing modification of thiols liberated during the reduction step of the in-gel digestion procedure. Further work will be required to determine why we observe modifications of these cysteines by DNCB which are thought to exist only in disulphide bonds.

We confirmed a total of 33 residues haptenated by MCI. The majority of haptenated residues were Lys and others included...
His and Tyr. Cys 34, the only Cys residue in HSA not involved in an S-S bridge was not found hapttenated by MCI. A study by Alvarez-Sanchez et al. (2004a) found that MCI exclusively modified His and Lys residues and suggested that a lack of cysteine modification may have been due to the limited accessibility of MCI for Cys34. It is likely that MCI requires an initial interaction with a thiol for the reaction to progress through to generation of amide (+99) and thioamide (+115) adducts (Alvarez-Sanchez et al., 2004b), which were the two most commonly observed adducts of MCI in this study (+99 and +115 Da, respectively). This is possibly accomplished by reactivity with glutathione in vivo, however, it appears that a single Cys residue available in HSA is sufficient for this purpose. The numbers of sites hapttenated by MCI are comparable to that by DNBC, both of which are extreme sensitizers.

Cinnamaldehyde is a moderate sensitizer and reacts with protein nucleophiles either by formation of a Schiff base with Lys residues, resulting in a mass increase of +114 Da, or by Michael addition to Cys residues, resulting in a mass increase of +132 Da (Majeti and Suskind, 1977). Both types of adduct were observed with hapttenation of fewer Lys, His, and Arg residues when compared with DNBC and MCI. The data do not show a preference for either mechanism of hapttenation. This is in contrast to the previous assignment of cinnamaldehyde as a Michael acceptor as a preferred reaction mechanism (Roberts et al., 2007).

6-methylcoumarin, a synthetic fragrance material, has been defined both as a non-sensitizer in the murine LLNA and as a photo-contact sensitizer (Ashby et al., 1995; Kato et al., 1985; Maguire and Kaidby, 1982). Because this compound was not photoactivated for the purposes of this study, 6-methylcoumarin is defined as a weak/non-sensitizer. However, low levels of reactivity have been shown in peptide depletion assays with Cys and Lys without photo-activation (Aleksic et al., 2009). In our experiments, no reactivity was observed with HSA after 24 h and 2-week exposure, supporting this compound as a weak/non-sensitizer. Hapttenation of Lys 574 observed after 4-week exposure remains putative due to insufficient quality of fragmenta-
tion spectra to assign the amino acid site of modification (Supplementary data).

DISCUSSION

A cascade of events triggered by covalent modification of skin proteins (hapttenation) ultimately lead to the generation of the adaptive immune response termed skin sensitization. The resulting skin condition, ACD, has an appreciable effect on the quality of life and it affects a significant number of individu-
als. Nonetheless little is known about the true nature of this key molecular initiating event. Due to the experimental and analyti-
cal complexity, our current knowledge of hapttenation mainly comes from studies utilizing surrogates such as model peptides or isolated single proteins (e.g., butylamine, propanethiol, N-acetyl-cysteine, etc. (Alvarez-Sanchez et al., 2003; Franot et al., 1994; Meschkat et al., 2001a), glutathione (Aptula et al., 2006), synthetic peptides (Aleksic et al., 2009; Alvarez-Sanchez et al., 2004b; Gerberick et al., 2004; Natsch and Gfeller 2008; Natsch et al., 2007) to single isolated model proteins (Aleksic et al., 2007, 2008; Alvarez-Sanchez et al., 2004a; Meschkat et al., 2001b)). Thus, much is known about the similarities of individual hapttenations or groups of hapttenations with related reactivity mechanisms. So far, the proteome that is being hapttenated, i.e., the human skin, was largely overlooked. Advances in proteomics and instrument sensitivity as well as greatly increased sample throughput and software capabilities have now allowed detailed investigations of the skin proteome (Parkinson et al., 2014), but the understanding of the extent and specificity of hapttenation in a complex proteome is still lacking.

To further our knowledge of hapttenation, it is necessary to investigate mechanisms and specificity of covalent protein modifications in cells and skin tissue by overcoming the technical and analytical challenge that this possesses. In the present study, improvements in the sensitivity, resolution, and modes of acquisition of modern MS combined with a novel isotopic labeling strategy have allowed us to identify low abundant hapttenated peptides on a single model protein despite the non-stoichiometric nature of hapttenation. In comparison to previous similar studies, major differences were noted and attributed to improved technique and instrumental sensitivity. This novel approach should enable detailed investigation of hapttenation events in complex protein mixtures, cell lines, and ultimately ex vivo topicaly treated human skin.

Understanding the mechanisms and extent of hapttenation in human skin will be essential to understanding the immunogenicity of hapttenated proteins. Misleading conclusions can potentially be drawn from using very simple models, such as nucleophiles and nucleophile containing peptides. For example, cinnamaldehyde is an α, β-unsaturated aldehyde that can react either via Schiff base formation or Michael addition or both. Theoretically, Michael addition reaction is the preferred reaction mechanism for cinnamaldehyde (Roberts et al., 2007), however, both types of adducts were observed when using synthetic peptides (Aleksic et al., 2009). In isolation, cinnamaldehyde reacts with Cys, Lys, His, and Arg residues on single nucleophile peptides. However, our data show that cinnamaldehyde appears to preferentially hapttenate Lys and Arg residues on HSA, which was somewhat unexpected. Basic amino acids Lys and Arg are higher in abundance in the human skin proteome when compared with Cys (Parkinson et al., 2014); therefore, it is more likely that immunogenic entities generated by cinnamaldehyde hapttenation will occur on Lys- and Arg-rich proteins, whereas Cys hapttenation (e.g., to Cys of glutathione) may result in detoxification. It is, however, difficult to speculate about the exact immunological or toxicological implications of the modifications observed on a model protein. Beyond description of Cys54 of keratin 5 as a major (but not the only) target for monobromobimane (Simonsson et al., 2011), little evidence exists to suggest which proteins or indeed which nucleophilic residues may be targeted by hapttenes and how specific hapttenation events could influence immunogenicity. It is likely that chemicals capable of modifying a variety of nucleophilic side chains may be more successful in generating a larger number and variety of antigens than those reactive to a single nucleophile type. Conversely, a single modification of a key residue may still lead to a T cell response, in preference to more abundant modifications, for example proliferation of PPD-specific T cells was observed after stimulation of HSA modified at Cys 34 (Jenkinson et al., 2010). This remains a gap in our knowledge, thus understanding the variety and abundance of hapttenation in human skin is the first step in elucidating molecular initiating events in skin allergy. Our future aim is to use the stable isotope methodology developed here to study hapttenation in relevant complex protein mixtures including cell lines and ex vivo tissue.

The use of data-independent MS acquisition, more sensitive MS instrumentation, and the dual isotope labeling strategy implemented in this study has revealed more about the modifications of HSA by chemical sensitizers than was previously known. The strength of these approaches means that their application
in larger studies looking at the global modification of complex mixtures of skin-related proteins by different chemicals is now possible. Similarly, this approach is likely to find utility in drug and drug metabolite binding studies. In particular quantitative studies of skin protein haptenation may enable estimation of the levels of modified protein which is thought to be a key metric in the induction of skin sensitization (MacKay et al., 2013; Maxwell et al., 2014). Increasing our understanding of the skin proteome, potential target protein(s) and the immunogenicity of the covalent modifications will ultimately enable us to better interpret reactivity data obtained from reactivity studies using simple model nucleophiles.

SUPPLEMENTARY DATA
Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


Franot, C., Roberts, D. W., Smith, R. G., Basketter, D. A., Benezra,


Michalski, A., Cox, J. and Mann, M. (2011). More than 100,000 detectable peptide species elute in single shotgun proteomics runs but the majority is inaccessible to data-dependent LC-MS/MS. J. Proteome Res. 10, 1785–1793.


