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Electrochemistry/liquid chromatography/mass spectrometry to demonstrate irreversible binding of the skin allergen *p*-phenylenediamine to proteins

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RATIONALE: *para*-Phenylenediamine (PPD) is a potent and well-known allergen, which is commonly used in hair or fur dyes and can cause severe allergic contact dermatitis. In this work, the skin-sensitizing potential of PPD with respect to the conjugation of proteins was evaluated using an approach without animal testing.

METHODS: Electrochemistry (EC) coupled offline to liquid chromatography (LC) and electrospray ionization mass spectrometry (ESI-MS) was employed to convert the pre-hapten PPD into its reactive hapten analogs. A previous study had already shown that this purely instrumental method is suitable for accelerating and simulating the various oxidation processes, which PPD may undergo, and that the emerging products are prone to react with soft thiol groups of small nucleophiles like glutathione and cysteine.

RESULTS: This investigation was extended by successfully demonstrating adduct formation between EC-generated PPD oxidation products and the three proteins β -lactoglobulin A (β -LGA), human serum albumin and human hemoglobin. A tryptic digest of modified β -LGA provided evidence for irreversible protein binding of monomeric PPD, a PPD dimer and the PPD trimer known as Bandrowski's Base. It was shown that the main oxidation product *p*-phenylene quinone diimine, and the reactive oligomerized species, primarily attack the free thiol function of proteins rather than other nucleophilic amino acid residues.

CONCLUSIONS: The pre-hapten PPD was efficiently activated upon EC oxidation and the resulting species were further reacted with different proteins leading to diverse hapten-protein complexes. Thereby, problems related to the complex matrix present in conventional *in vitro* or *in vivo* methods could effectively be avoided. Copyright © 2012 John Wiley & Sons, Ltd.

Allergic reactions eventually leading to contact dermatitis are most often caused by the haptenation of proteins by low molecular weight (LMW) compounds. This well-accepted theory is based on early findings from Landsteiner and Jacobs, who postulated by reference to tests with guinea pigs, that "simple chemical compounds", especially *p*-phenylenediamine (PPD), probably combine with proteins rather than act as antigens by themselves.^[1,2] Thus, the molecular weight of most chemical allergens is below 500 enabling an easy penetration through the lipid-rich stratum corneum of the skin where they irreversibly bind to available proteins such as keratin or collagen.^[3,4] As sensitizing agents are apparently too small to cause an immune response on their own, this reactivity toward skin proteins plays a key role in understanding immunological mechanisms and, consequently, allows the skin-sensitization potential of such substances to be assessed. However, not all the known allergens induce hapten-protein complexation directly. Some can only form such a macromolecular immunogen after being activated

either chemically via spontaneous oxidation on air exposure (pre-haptens) or metabolically via enzyme-mediated processes in the epidermis (pro-haptens).^[5–7] Hence, the activation, which is necessary for approximately one-third of sensitizers, might happen either prior to or upon cutaneous absorption.^[5,8]

PPD is a strong skin sensitizer and commonly used as a permanent hair dye, but it is also present in many other products like printing ink, rubbers and plastics or even in henna tattoo mixtures, where it is used to increase color intensity.^[9,10] Since it is highly unstable and liable to autoxidation in aqueous media, PPD is classified as a pre-hapten. It has been shown that the immunogenicity of this substance derives from the formation of a reactive quinone diimine intermediate (PPQD) on exposure to air oxygen or other oxidizing conditions for simulation purposes (e.g. ferrocyanide or peroxidase-peroxide oxidation systems).^[11,12] This electrophilic hapten further undergoes self-conjugation reactions leading to the sequential generation of various oligomers. A major product among these PPD oligomers is the so-called Bandrowski's Base (BB), a rearrangement product of the PPD trimer, which can itself serve as a hapten and modify important biomolecules.^[13–15] Hence, diverse activation pathways are conceivable for PPD: autoxidation in the presence of air oxygen, oxidation

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in skin via CYP enzymes of keratinocytes, or acetylation by *N*-acetyltransferases, where the latter results in detoxification with respect to sensitization.^[16]

Established methods to assess the skin-sensitizing potential of LMW compounds mainly include animal tests (e.g. with guinea pigs or mice in *in vivo* experiments)^[17,18] or the incubation of isolated cells, cell extracts or enzymes from human or animal tissue (e.g. with T lymphocytes or dendritic cells in *in vitro* experiments).^[19] Although developments like the Local Lymph Node Assay (LLNA)^[20–24] achieved a reduction in the number of animals required compared with guinea pig tests, the necessity to develop alternative non-animal test methods such as the recently developed Direct Peptide Reactivity Assay (DPRA)^[8,25] remains urgent. This is of particular importance in view of the European Union ban on *in vivo* testing of cosmetic and toiletry ingredients (European Commission Regulation No. 1223/2009).^[26] One major challenge in the development of novel assays for hazard identification and risk evaluation of skin-sensitizing chemicals will be the incorporation of an activation or metabolism simulation step in order to address those molecules that need to be (bio)activated before acting as haptens.^[27,28] A promising approach was introduced by Gerberick and co-workers who tried to overcome this problem by combining the DPRA method with a horseradish peroxidase-hydrogen peroxide oxidation system.^[12]

In this work, electrochemistry (EC) coupled to liquid chromatography (LC) and electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) was used to evaluate the skin-sensitizing potential of the pre-hapten PPD by conducting protein-binding studies subsequent to activation via electrochemical oxidation. During the last few years, the EC/(LC)/MS system has become a valuable and versatile tool, especially with respect to the mimicry of oxidative metabolism reactions of drugs.^[29–33] Furthermore, online EC/ESI-MS was used by van Berkel's group to study the electrochemical polymerization of aniline. Oligomers of aniline with up to ten monomeric units were detected in different redox states, proving this method to be a valuable tool in identifying the initial products of electropolymerization.^[34] In terms of protein modification, Lohmann *et al.* used a simple flow-through cell to electrochemically oxidize the pharmaceuticals paracetamol, amodiaquine, as well as clozapine, and successively trapped the emerging reactive metabolites online with β -lactoglobulin A (β -LGA) or human serum albumin (HSA), respectively.^[35] As shown only recently by our group, the autoxidation of PPD can easily be accelerated and, concurrently, a possible oxidative metabolic activation of this allergen in skin can be simulated by applying the electrochemical approach.^[36] The generation of PPQD, a PPD dimer, and BB along with other oxidation products was successfully achieved with EC/ESI-MS. Moreover, adduct formation of these three reactive species with nucleophiles was confirmed for glutathione (GSH) and cysteine (Cys). Building on these results, the aim of the present study was to further demonstrate the applicability of this instrumental approach for the generation of hapten–protein complexes. Therefore, the conjugation of electrochemically oxidized PPD with the three different proteins β -LGA, HSA and human hemoglobin (Hb) was investigated.

EXPERIMENTAL

Chemicals

p-Phenylenediamine (PPD), β -lactoglobulin A from bovine milk (β -LGA), human serum albumin (HSA), human hemoglobin (Hb), trypsin from bovine pancreas, guanidine hydrochloride, ammonium acetate (NH₄Ac) and ammonia solution (25%) were from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Acetic acid (HAc) and formic acid (FA) were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was acquired from ABCR GmbH & Co. KG (Karlsruhe, Germany) and acetonitrile (ACN) for HPLC was obtained in gradient grade quality from BDH Prolabo, VWR (Darmstadt, Germany). All other chemicals and solvents were ordered in the highest quality available. Purified water was generated by an Aquatron A4000D distillation system (Barloworld Scientific, Nemours Cedex, France). Disposable PD MidiTrap and PD-10 desalting columns (both Sephadex G-25 M) were obtained from GE Healthcare (Little Chalfont, UK).

Instrumentation

All ESI-MS measurements were performed on a time-of-flight (TOF) mass spectrometer (micrOTOF, Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. The instrument was controlled via micrOTOFControl 1.1 software and data processing was carried out by DataAnalysis 3.3 software (both from Bruker Daltonics). For LC/ESI-MS measurements, the micrOTOF instrument was coupled to an ALEXYS LC system from Antec Leyden (Zoeterwoude, The Netherlands) containing two LC 100 pumps, an OR 110 organizer rack with a degasser and a pulse dampener, an AS 100 autosampler and a ROXYTM column oven. The LC system was operated by ALEXYS software (Antec Leyden).

Electrochemical oxidation of PPD and protein modification

Electrochemical oxidation was accomplished in an amperometric thin-layer cell equipped with a boron-doped diamond (BDD) working electrode, a Pd/H₂ reference electrode and a counter electrode made of graphite-doped Teflon (Reactor-CellTM, Antec Leyden). Potentials were applied using either the electrochemical detector system ROXYTM (Antec Leyden) controlled by DialogueTM software (Antec Leyden) or a home-made system consisting of a potentiostat and appropriate software.

To study adduct formation between oxidized PPD and the three proteins β -LGA, HSA and Hb, the EC cell was operated offline with subsequent LC/ESI-MS detection of the samples. During this experiment, the BDD working electrode of the cell was kept at a constant potential of 1700 mV, while a 100 μ M dilution of PPD in 10 mM NH₄Ac (pH 7.4) containing 30% ACN (to reduce adsorption on the electrode surface) was introduced by a syringe pump model 74900 (Cole Parmer, Vernon Hills, IL, USA) at a flow rate of 10 μ L/min. The electrochemical oxidation was carried out over a time period of 50 min (total volume of oxidized PPD solution: 500 μ L). The effluent of the EC cell was trapped in 500 μ L (1:2 dilution) of a 20 μ M solution of the respective protein in 6.5 M aqueous guanidine, which was employed for denaturing the macromolecules. Prior to the first collection of effluent

after turning on the thin-layer cell, the EC unit was left to equilibrate for 5 min to ensure a reproducible number and concentration of oxidation products. The generated mixtures of oxidized PPD (fivefold molar excess based on the amount of protein) and β -LGA, HSA or Hb were briefly homogenized using a vortex mixer and incubated at 37 °C for 10 min. For purification of these reaction mixtures, PD MidiTrap desalting columns were utilized based on the supplier's protocol (sample volume: 1 mL, eluting solvent: 0.01% FA). Negative control samples were prepared by incubating and desalting the respective 20 μ M protein solution mixed with 10 mM NH_4Ac (pH 7.4) containing 30% ACN (1:2 dilution). All samples were finally analyzed by LC/ESI-MS.

Tryptic digest of PPD-modified β -LGA

In order to investigate the type of adduct formation between PPD metabolites and β -LGA and to determine the location of the covalent binding between these species, oxidation of PPD was carried out as previously described. However, fivefold higher concentrations of the skin allergen (500 μ M in buffer solution) and the protein (100 μ M in 6.5 M guanidine) were employed in this case. After oxidation of PPD in the EC cell, the effluent was collected in a vial containing β -LGA (1:2 dilution, fivefold molar excess of PPD compared with the amount of protein). Subsequently, the sample was briefly homogenized using a vortex mixer and incubated at 37 °C for 10 min. After the incubation time, excess reagent and guanidine were removed on a PD-10 desalting column according to the supplier's protocol, using 0.01% FA as mobile phase and a sample volume of 2 mL. Reduction of the disulfide bridges was achieved by adding a ninefold molar excess of TCEP (100 mM solution in 0.3 M aqueous NH_3) per oxidized S-atom in the protein to the purified mixture and incubating at 37 °C for 30 min. The reduction was terminated by passing the solution again through a PD-10 desalting column. Digestion with trypsin was performed by adding 5 μ L of a stock solution of 2.3 mg of trypsin in 1 mL of water to 1 mL of the reduced PPD-protein mixture containing approximately 16 μ M of modified protein and 50 mM of ammonium bicarbonate. After 90 min at 37 °C, the digest was terminated by adding 500 μ L of ice-cold ACN containing 0.1% FA. A negative control experiment was again carried out by mixing a 100 μ M β -LGA solution with 10 mM NH_4Ac (pH 7.4) containing 30% ACN (1:2 dilution) and applying the same preparation steps. The samples were then finally analyzed by LC/ESI-MS.

LC/ESI-MS conditions

LC/ESI-MS analyses of the proteins and the protein adducts were carried out using a Discovery BIO Wide Pore C5 column (150 $\times$ 2.1 mm i.d., 5 μ m particle size from Supelco (Sigma Aldrich) with the column oven temperature set to 40 °C. A volume of 5 μ L of sample was injected. The mobile phase consisted of aqueous 0.1% FA as eluent A and ACN as eluent B, at a total flow rate of 250 μ L/min. The applied gradient profile for the studies with intact proteins (unmodified and after adduct formation with oxidized PPD) was as follows: 0.0–2.0 min isocratic at 25% B, 2.0–6.0 min linear increase from 25–60% B, 6.0–9.0 min isocratic at 60% B, 9.0–12.0 min linear decrease to 25% B and 12.0–15.0 min re-equilibration at 25% B. The gradient conditions used for separation of the tryptic peptides after digest of unmodified and modified β -LGA with trypsin were: 0.0–2.0 min isocratic at 10% B, 2.0–12.0 min linear increase from 10–80% B, 12.0–15.0 min isocratic at 80% B, 15.0–7.0 min linear decrease to 10% B and 17.0–20.0 min re-equilibration at 10% B. Mass spectrometric detection of intact proteins was carried out in positive ionization mode ESI(+) on the micrOTOF instrument under the following conditions: Nebulizer gas (N_2) pressure: 1.5 bar; drying gas (N_2) flow rate: 9.0 L/min; drying temperature: 190 °C; end plate offset: –450 V; capillary: –3.8 kV; capillary exit: 120 V; skimmer 1: 40 V; skimmer 2: 25 V; hexapole 1: 26 V; hexapole 2: 21.4 V; hexapole RF: 300 Vpp; lens transfer time: 70 μ s; pre-pulse storage time: 25 μ s; lens 1 storage: 40 V; lens 1 extraction: 21.1; lens 2: 7.4 V; lens 3: 22 V; lens 4: 0.3 V; lens 5: –27.5 V; m/z range: 50–4000. For the ESI-MS measurements of the tryptic peptides, the same conditions were used except for the following parameters: skimmer 2: 23 V; pre-pulse storage time: 12 μ s.

RESULTS AND DISCUSSION

In order to investigate adduct formation between reactive PPD oxidation products and different proteins, offline coupled EC/LC/ESI-TOFMS experiments were performed (Fig. 1). The conditions for oxidative activation of PPD in the electrochemical flow-through cell were selected on the basis of previously reported results.^[36] Subsequent conjugation of the emerging products with β -LGA, HSA and Hb, respectively, was achieved by directly mixing the EC cell effluent with the protein of interest. Figure 2 provides a concise overview of the reactive oxidation products of PPD upon EC activation and their postulated adduct formation

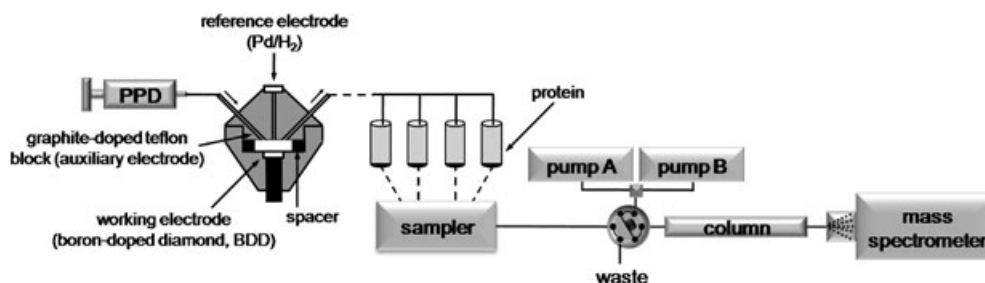


Figure 1. Instrumental set-up used for the EC/LC/ESI-TOFMS experiments. PPD solution was oxidized offline at 1700 mV vs. Pd/H₂ and trapped with the protein of interest prior to LC/MS analysis. For unmodified proteins the EC unit was omitted.

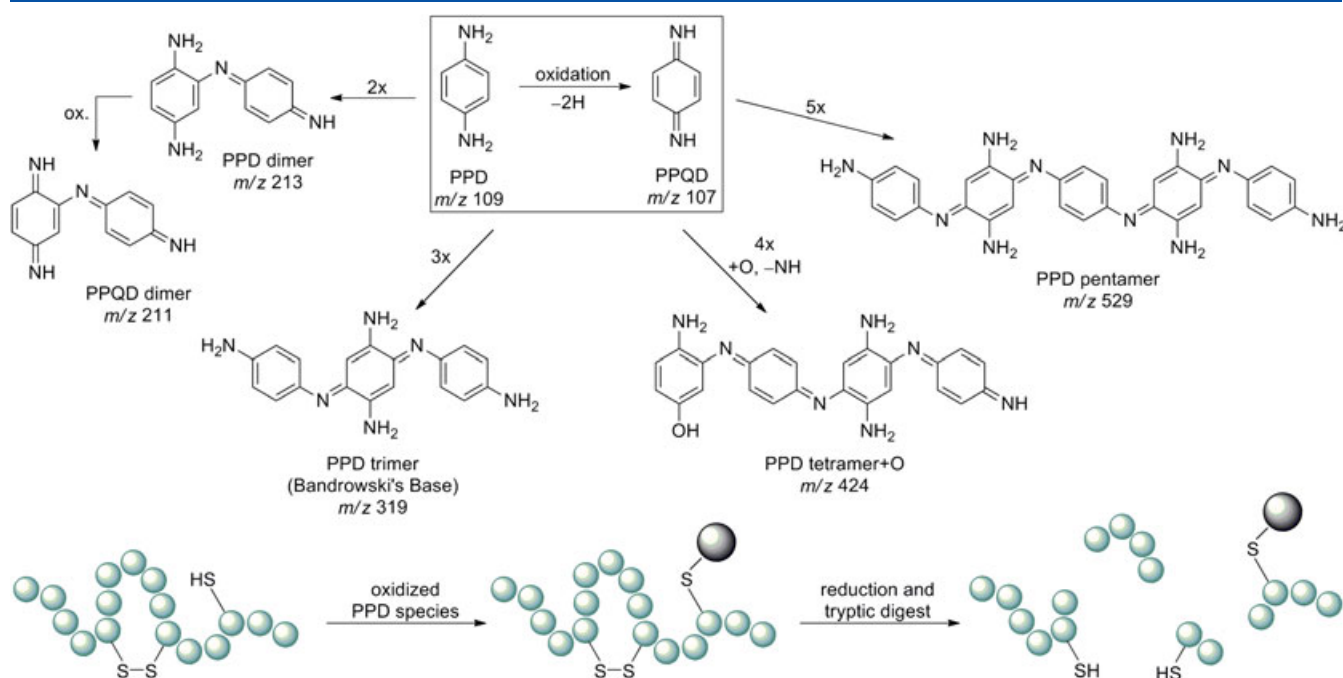


Figure 2. Overview on the reacting electrophilic oxidation products of PPD generated in the EC cell along with the proposed adduct formation pathway with unblocked Cys residues of different proteins. The m/z data refer to the $[M + H]^+$ ions of the partly oligomeric oxidation products.

Table 1. Comparison of calculated and experimental average neutral masses for the different protein adducts and the unmodified proteins as well as the resulting mass gains. Section (a) includes the data for β -LGA, (b) for HSA, (c) for the α -chain of Hb, and (d) for the β -chain of Hb. Furthermore, the type of modification with respect to molecular formulae is listed

Protein form	Experimental average neutral mass [Da]	Calculated average neutral mass [Da]	Mass increase [Da]		Type of modification
			Experimental	Calculated	
a) β -LGA unmodified	18363.2	18363.1	—	—	—
β -LGA + PPD	18468.8	18469.2	105.6	106.1	$C_6H_6N_2$
β -LGA + PPD dimer	18573.7	18573.4	210.5	210.3	$C_{12}H_{10}N_4$
β -LGA + BB	18680.1	18679.5	316.9	316.4	$C_{18}H_{16}N_6$
β -LGA + PPD tetramer + O	18784.7	18784.6	421.5	421.5	$C_{24}H_{19}N_7O$
β -LGA + PPD pentamer	18891.1	18889.7	527.9	526.6	$C_{30}H_{26}N_{10}$
b) mHSA (SH) unmodified	66439.7	66437.5	—	—	—
nHSA (S – S) unmodified	66557.8	66556.6	—	—	—
mHSA + PPD	—	66543.6	—	106.1	$C_6H_6N_2$
mHSA + PPD dimer	66646.7	66647.7	207.0	210.2	$C_{12}H_{10}N_4$
mHSA + BB	—	66753.8	—	316.3	$C_{18}H_{16}N_6$
c) Hb α -chain unmodified	15126.2	15126.2	—	—	—
Hb α -chain + PPD	15232.0	15232.3	105.8	106.1	$C_6H_6N_2$
Hb α -chain + PPD dimer	15335.6	15336.4	209.4	210.2	$C_{12}H_{10}N_4$
Hb α -chain + BB	15442.8	15442.6	316.6	316.4	$C_{18}H_{16}N_6$
Hb α -chain + PPD tetramer + O	—	15547.7	—	421.5	$C_{24}H_{19}N_7O$
d) Hb β -chain unmodified	15865.5	15867.1	—	—	—
Hb β -chain + PPD	15969.9	15973.2	104.4	106.1	$C_6H_6N_2$
Hb β -chain + PPD dimer	16075.3	16077.3	209.8	210.2	$C_{12}H_{10}N_4$
Hb β -chain + BB	16184.4	16183.4	318.9	316.3	$C_{18}H_{16}N_6$
Hb β -chain + PPD tetramer + O	—	16288.5	—	421.4	$C_{24}H_{19}N_7O$

pathways with the studied proteins. A summary of experimental and theoretical data concerning the hapten–protein complexes observed in this work is included in Table 1. The details of these studies are discussed below.

Adduct formation with β -LGA and tryptic digest analysis

The whey protein β -LGA was chosen to trap the EC-generated oxidative species of PPD in this study because of its structural homogeneity and comparably small size (162 amino acids, 18.4 kDa). In addition to having one free thiol group, it can be regarded as an ideally suited model protein, which is easy to handle and allows for an immediate recognition of changes in the charge distribution after reaction with electrophilic species. Figures 3(a) and 3(b) display the mass spectra of the unmodified protein, showing the mass distribution between charge states +16 and +12 (Fig. 3(a)) next to the deconvoluted mass spectrum (Fig. 3(b)) with an average neutral mass of 18363.2 Da. This is in good accordance with the theoretical value of 18363.1 Da calculated from the amino acid sequence. After having been incubated with oxidized PPD solution, the charge state distribution of β -L is notably shifted to higher masses and additional signals of lower intensities are even detected in a regular pattern next to the main ions (Fig. 3(c)). Deconvolution reveals the formation of five new peaks, which can be viewed in Fig. 3(d), indicating an almost quantitative conversion of the protein, as unmodified β -LGA can hardly be found in the mixture anymore. The highest new signal with a neutral mass of 18468.8 Da

corresponds to a mass gain of 105.6 Da that can be assigned to the formation of an adduct between β -LGA and the reactive PPQD intermediate (calculated average neutral mass 18469.2 Da, theoretical difference 106.1 Da). The other signals were analyzed in the same way by comparing the theoretical with the experimental values regarding protein adduct masses and their differences from unmodified β -LGA. The resulting data is summarized in Table 1(a). From these calculations it was deduced that the second novel protein signal (deconvoluted neutral mass 18573.7 Da) correlates well with the addition of a PPD dimer to β -LGA, whereas the third peak (deconvoluted mass 18680.1 Da) is in agreement with the formation of a β -LGA–BB adduct. Thus, conjugation of the three major reactive PPD oxidation products could not only be demonstrated for the amino acid Cys and the tripeptide GSH, but now also for the model protein β -LGA. Moreover, the higher oligomeric species that were generated from PPD in the EC cell also showed reactivity toward β -LGA since the addition of the deaminated and hydroxylated PPD tetramer (deconvoluted neutral mass 18784.7 Da) as well as of the PPD pentamer (deconvoluted neutral mass 18891.1 Da) could be confirmed.

Considering the preceding studies with GSH, Cys, lysine and KCN, in which only the first two nucleophiles underwent adduct formation, it can be assumed that the different oxidative PPD metabolites react with the whey protein via its free thiol group.^[36] However, it is still possible that, instead of a reaction with dimeric or trimeric PPD species, two or more single PPQD molecules add to the protein at different

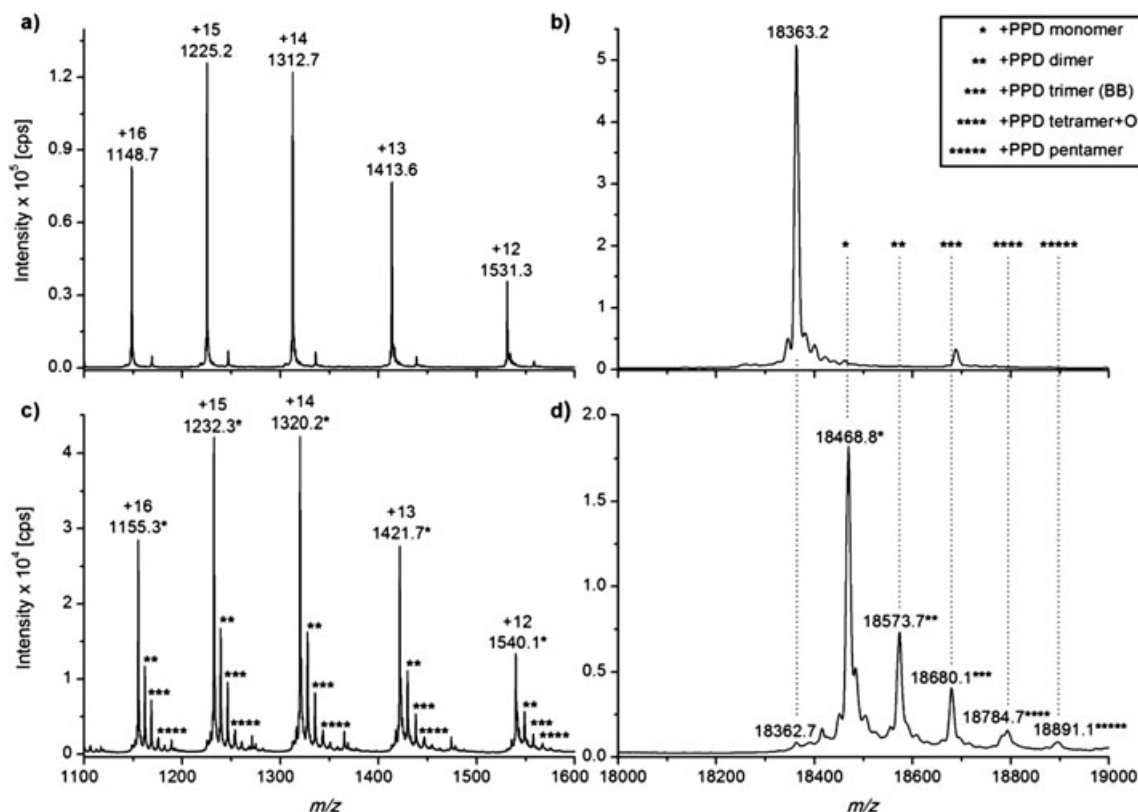


Figure 3. Mass spectra of (a) unmodified β -LGA, (b) unmodified β -LGA upon deconvolution, (c) β -LGA after reaction with oxidized PPD, and (d) modified β -LGA upon deconvolution. Asterisks indicate which type of protein adduct has been formed by assigning the different PPD species to the according protein signals.

positions including the free cysteine sulfhydryl group. Consequently, a tryptic digest of modified β -LGA with trypsin was performed in order to elucidate the actual nature of the protein modification and its location. Moreover this experiment could be used to demonstrate the covalent protein binding of oxidized PPD. Trypsin specifically cleaves proteins at the C-terminal site of lysine and arginine and a digest of the unmodified whey protein comprises, in addition to 17 other peptides, the T13 fragment (sequence: YLLFCMENSAE-PEQSLVCQCLVR, amino acid residues 102–124). This peptide is of major interest as it includes the only free thiol function of β -LGA at Cys-121, but also two further Cys residues which both had been part of different disulfide bridges before digest. Besides, the T13 peptide does not contain the basic amino acids lysine or histidine that might be able to bind reactive electrophilic compounds via nitrogen as a nucleophilic site. In Figs. 4(a) and 5(a), the triply charged ion of the unmodified T13 fragment is illustrated with its m/z value for the monoisotopic signal of the isotope pattern (the first isotope peak in this case). Interestingly, a comparison of the calculated monoisotopic ion at m/z 892.416 for $[T13 + 3H]^{3+}$ with the experimental monoisotopic ion at m/z 891.736, and of the corresponding isotope patterns, unveils a significant deviation. However, when considering a loss of two hydrogen

atoms, the calculated monoisotopic ion m/z 891.744 correlates well with the experimental value, leading to the assumption that a new disulfide bridge between Cys-6 and Cys-19 must have been formed within the peptide upon digest with trypsin.

Analyzing the T13 peptide of β -LGA after modification with EC-oxidized PPD by LC/MS resulted in the mass spectrum shown in Fig. 4(b). Instead of the unmodified $[T13 + 3H]^{3+}$ ion, which has vanished almost completely, three novel signals are observed with nearly equidistant m/z values. These triply charged ions can be designated to the T13 peptide having added one PPQD molecule, dimeric PPD or trimeric PPD (BB), respectively. This hypothesis is confirmed by comparing calculated and experimental mass spectra for all three adducts, while taking the presence of a disulfide bridge within the T13 fragment into account. As can be seen from Figs. 5(b)–5(d), the corresponding isotopic patterns and monoisotopic masses are consistent with each other. Furthermore, none of the other detected tryptic peptides containing various nucleophilic functionalities carried a PPD modification (recovery rate: 72%, 13 out of 18 fragments were identified, partly in two different charge states). This finding confirms the work of Jenkinson *et al.* who did not detect modified lysine, tryptophan or any other nucleophilic amino acid residues, excluding Cys, in PPD-bound HSA either,^[37] although previous studies from Eilstein *et al.* had shown that this reaction pathway is possible.^[38–40] It was concluded by Jenkinson *et al.* that covalent modification of Cys residues predominates over alternative conjugation pathways in the protein. Hence, the reactivity of oxidative PPD metabolites toward the single arginine residue in the T13 peptide can be considered of negligible importance because of the presence of the free Cys-121 thiol group. This leads to the conclusion that modification of β -LGA must proceed via attack at its free Cys-121 thiol group (Michael addition) and, thus, that oligomeric PPD species, rather than several monomeric molecules of the allergen, are indeed binding irreversibly to this protein. Moreover, the present study extends this knowledge by proving the addition not only of a single PPQD molecule to the protein, but also of further secondary oxidation products, including BB. Specifically, the protein binding of BB was also observed by Farrell *et al.* in mice.^[14]

A closer look at the conjugation product between T13 and dimeric PPD reveals an experimental mass difference of 208.2 Da from the unmodified T13 peptide instead of the expected theoretical value of 210.1 Da. This observation suggests the covalent attachment of a doubly dehydrogenated PPD dimer (labeled as 'PPQD dimer', Fig. 2) to the tryptic peptide (considering the presence of a disulfide bridge) as was observed for GSH and Cys in the preceding study.^[36] In this case, the theoretical mass difference is calculated to be 208.1 Da, which is in good agreement with the measured value. These findings differ from the results with intact β -LGA (discussed above), where adduct formation with a formal PPD dimer was confirmed. A possible explanation might be the additional oxidation of oligomeric PPD species or rather their protein adducts in the ESI interface. In addition, extended autooxidation of the PPD intermediates via atmospheric oxygen can be envisaged as this process was accelerated when applying the offline coupled EC approach.^[36]

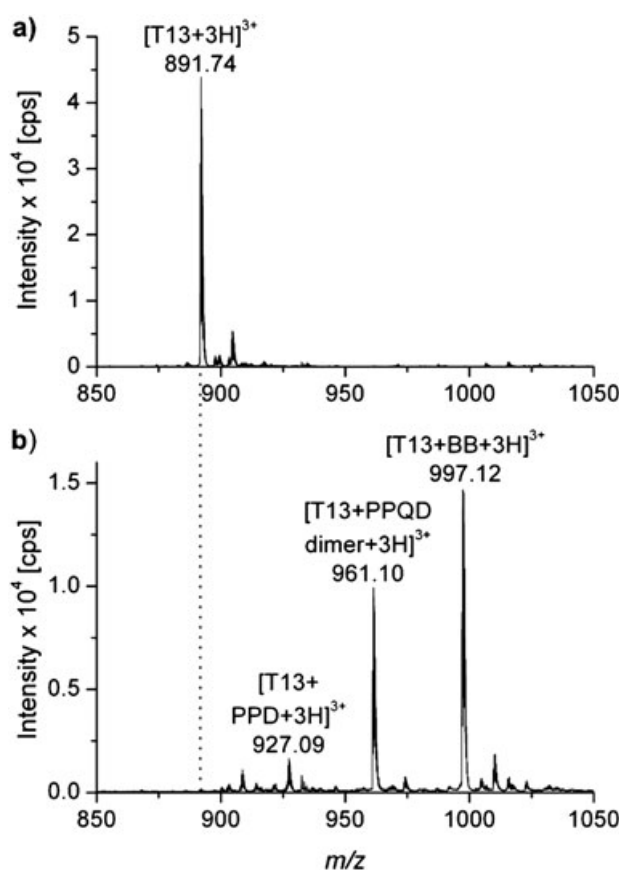


Figure 4. Experimental mass spectra of the tryptic T13 peptide after digest of (a) unmodified β -LGA and (b) β -LGA following modification with EC-generated PPD oxidation products. The signals illustrated refer to the monoisotopic masses of the $[M + 3H]^{3+}$ ions of the peptide which contains the amino acid residues 102–124 of β -LGA.

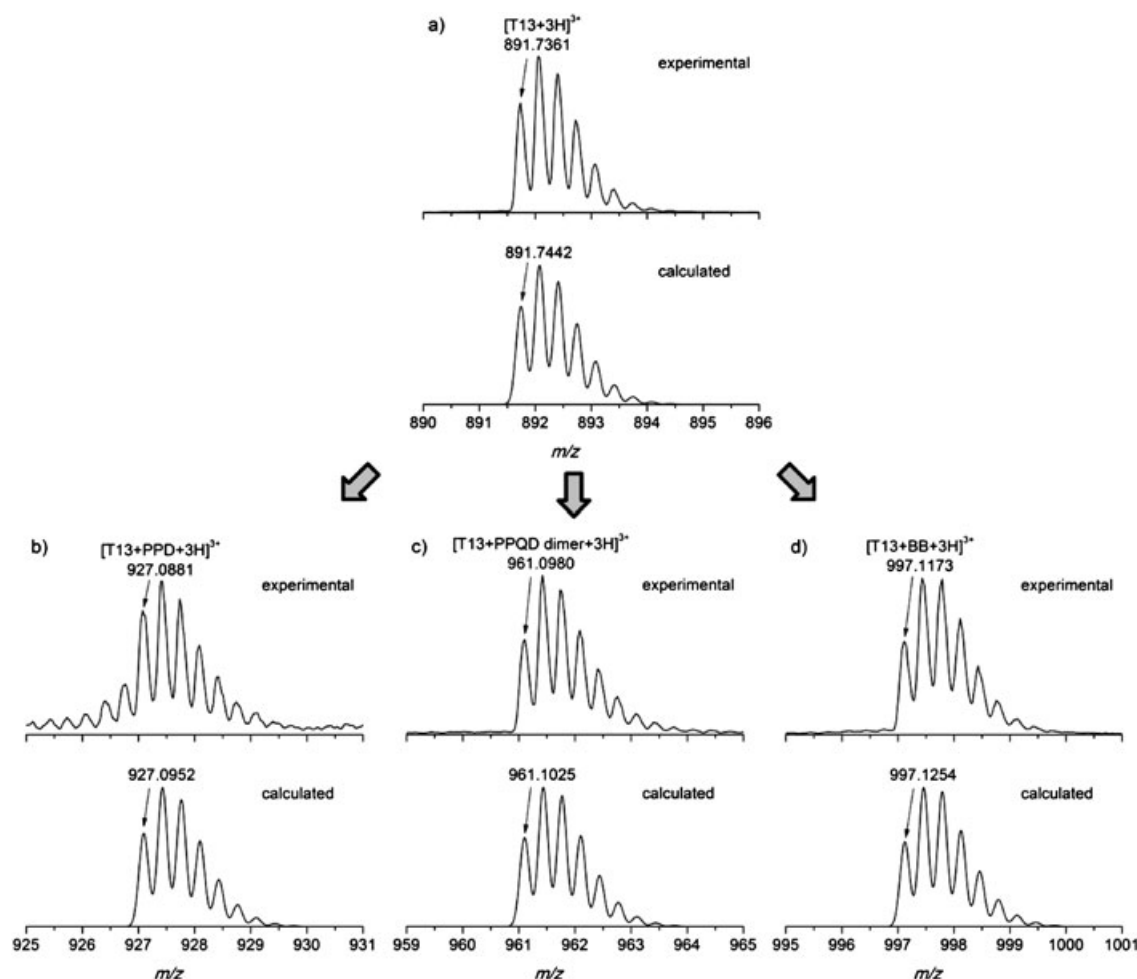


Figure 5. Comparison between calculated and recorded mass spectra of the tryptic T13 peptide containing the amino acids 102–124 of β -LGA. Isotope patterns of the $[M + 3H]^{3+}$ ions are depicted for (a) the unmodified protein, (b) the adduct between β -LGA and monomeric PPD, (c) the β -LGA–PPQD dimer adduct, and (d) the β -LGA–BB adduct. The m/z data refer to the corresponding monoisotopic masses.

Adduct formation with HSA

In a second approach, adduct formation between EC-oxidized PPD and HSA (585 amino acids, 66.5 kDa) was investigated. Since it has been shown previously by Brander *et al.* that hapten–HSA complexes possess a T-cell stimulatory capacity,^[41] HSA was selected as a more complex and more relevant protein in the present investigation with the skin sensitizer PPD. Commercially available, unmodified HSA is microheterogeneous (Figs. 6(a) and 6(b)) and comprises the two isoforms: mercaptalbumin (mHSA), which features one free thiol group at Cys-34, and non-mercaptalbumin (nHSA), in which this thiol function is blocked due to a mixed disulfide bridge with an additional small molecule like Cys or GSH.^[42] In Fig. 6(a), the charge-state distribution of the untreated protein between the charge states +53 and +50 is depicted. The two isoforms of HSA are clearly distinguished in this mass spectrum, confirming the integrity of the protein. mHSA was detected with a deconvoluted neutral mass of 66439.7 Da, whereas the deconvoluted neutral mass of nHSA was 66557.8 Da (Fig. 6(b)). These data correlate with the calculated average molecular masses of 66437.5 Da for mHSA and 66556.6 Da for nHSA, respectively. The charge-state

distribution of HSA after the addition of an electrochemically oxidized PPD solution to the protein can be viewed in Fig. 6 (c). The main noticeable information on this mass spectrum is the disappearance of the signals related to the mHSA isoform, suggesting a quantitative reaction with PPD oxidation products. Furthermore, no mass gain is observed for nHSA which meets the expectations that adduct formation between oxidative PPD species and the protein is unlikely to occur, if no free thiol functions are available. Deconvolution leads to the spectrum presented in Fig. 6(d) and confirms the initial assumptions as no signal is now obtained for mHSA, whereas nHSA is still detected with a similar deconvoluted molecular mass (66557.3 Da) to that prior to treatment with oxidized PPD. Although mHSA is assumed to have been modified by reactive PPD-related electrophiles, neither in the charge-state distribution nor in the deconvoluted mass spectrum were additional signals observed. The calculated average molecular mass of a simple mHSA–PPD adduct would be 66543.6 Da (compare Table 1(b)) which is close to the determined deconvoluted molecular mass of 66556.6 Da for unmodified nHSA. The MS analysis of large macromolecules such as intact proteins with extremely high masses always involves a certain unavoidable

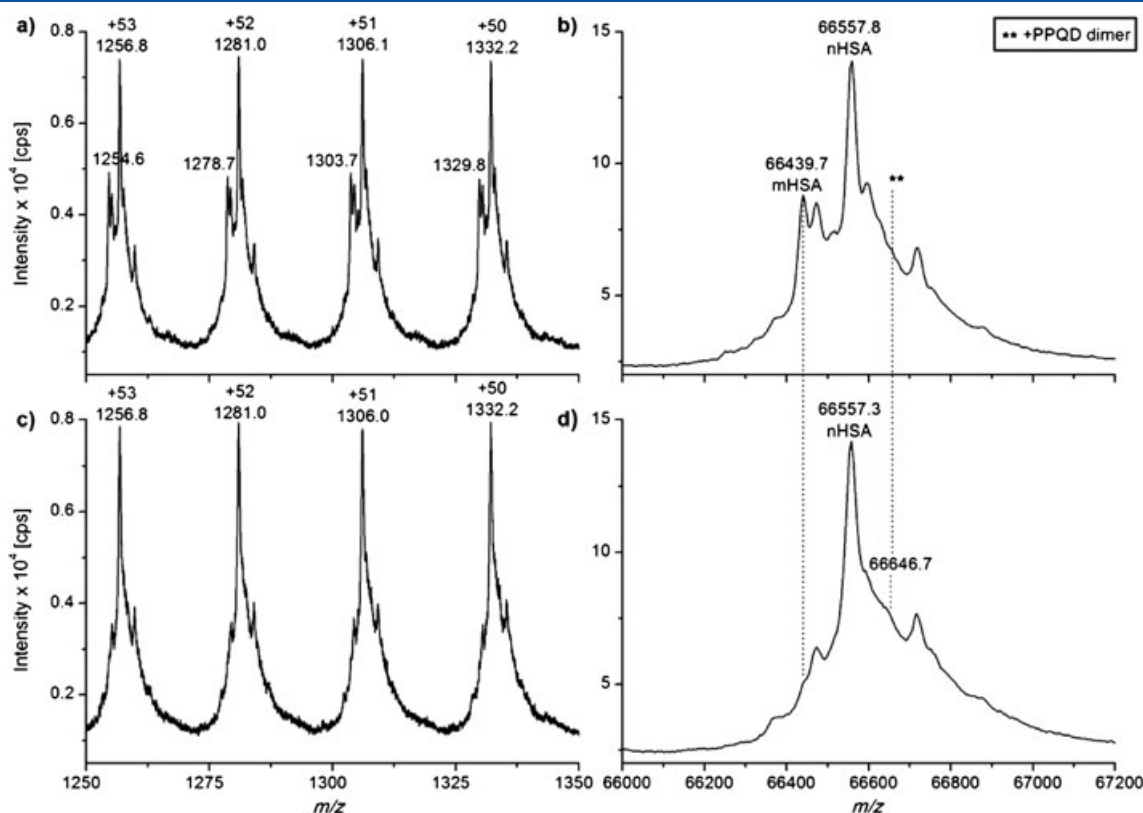


Figure 6. Mass spectra of (a) unmodified HSA, (b) unmodified HSA upon deconvolution, (c) HSA after reaction with oxidized PPD, and (d) modified HSA upon deconvolution. Although mHSA was quantitatively depleted following reaction with oxidized PPD, a single asterisk cannot be assigned to the respective signal of a mHSA–PPD adduct, which probably lies underneath the nHSA peak. The double asterisk highlights the potential formation of a mHSA–PPQD dimer conjugate.

mass inaccuracy. However, compared with the previous results with β -LGA, where only minor deviations between the calculated and experimental data were found, this mass difference still appears to be significant. A hypothetical explanation is adduct formation between a hydroxylated PPQD compound and mHSA (calculated average neutral mass 66559.6 Da), although such an oxidation product of PPD had not been observed in the preceding studies. It can be supposed that a mHSA–PPD adduct or possibly a mHSA–PPD–OH adduct has indeed been formed, but that the corresponding signal is located underneath the nHSA peak, making individual identification difficult.

Concerning the reactivity of secondary PPD oxidation products toward the plasma protein, a small, not well-resolved signal at 66646.7 Da can be observed in the deconvoluted mass spectrum (Fig. 6(d)), possibly indicating addition of a dimeric PPD species to mHSA (mass increase of 207.0 Da). As previously mentioned, the calculated average molecular mass better correlates with an added PPQD dimer (calculated average neutral mass 66645.7 Da, theoretical difference 208.2 Da) than with an unchanged PPD dimer (calculated average neutral mass 66647.7 Da, theoretical difference 210.2 Da). However, taking the mass inaccuracy and the low signal intensity into account, evidence for the existence of an adduct between dimeric PPD and mHSA remains ambiguous. In contrast to the results with β -LGA, no further hapten–protein complexes of higher oligomerized PPD species such as BB could be detected during the investigation with HSA, which

agrees once more with the findings of Jenkinson *et al.*^[37] One possible explanation might be steric hindrance due to the more heterogeneous structure of the large plasma protein. In summary, albumin primarily reacts with EC-generated PPQD only, which is probably covalently bound to the free thiol group at Cys-34 of the mHSA isoform. This deduction, concerning the reactivity pathway, is supported by findings from the preceding studies with β -LGA (discussed above) as well as from experiments with GSH, Cys, lysine and KCN.^[36]

Adduct formation with Hb

Apart from being the predominant protein in the red blood cells of almost all vertebrates, Hb is one of the main chromophores in human skin (next to the pigment melanin) and it resides in the microvascular network of the lower skin layer (dermis).^[43] Several studies have shown that Hb adducts can be useful biomarkers for the estimation of exposure to chemical substances.^[44] Hagmar *et al.*, for example, investigated adducts of Hb with the skin and airway irritant, acrylamide, in tunnel workers.^[45] Adduct formation between Hb and organic acid anhydrides, like the particularly strong sensitizer hexahydrophthalic anhydride, has also been characterized.^[46,47] Consequently, Hb represents another important protein in humans for which the investigation of its adduct formation with EC-generated PPD metabolites was intended. Human Hb consists of two α - and two β -subunits (each binding a prosthetic heme *b* group) which results in a

total molecular weight of 64.4 kDa. One α -chain of the metalloprotein includes 141 amino acids with one free thiol group (Cys-104), whereas one β -chain contains 146 amino acids with two free thiol functions (Cys-93 and Cys-112). The calculated average molecular masses of these chains are 15126.2 Da for one α -subunit and 15867.1 Da for one β -subunit, which is in agreement with the experimentally determined deconvoluted masses of the unmodified protein (15126.2 Da and 15865.5 Da, respectively) given in Fig. 7(b) and Table 1(c). The corresponding mass distribution between charge states +16 and +12 for the α -chain and +17 and +13 for the β -chain is illustrated in Fig. 7(a). Treatment of Hb with the EC-oxidized allergen, however, leads to significant changes in the mass spectrum, illustrated in Fig. 7(c). Similar to the study with β -LGA, several additional signals are detected next to the variably charged ions of the two protein chains, which are still observed in their unmodified form as well. Deconvolution (Fig. 7(d)) reveals the formation of three different adducts involving the α -chain with neutral masses of 15232.0 Da, 15335.6 Da and 15442.8 Da. Considering the previous results and the availability of just one single free thiol group in the Hb α -chain, these adducts can readily be assigned to monomeric, dimeric and trimeric PPD being bound to the Cys-104 function. Evaluation of the respective calculated average molecular masses (15232.3 Da, 15336.4 Da and 15442.6 Da), which correlate well with the measured ones, and comparison of the experimental and calculated differences from the unmodified α -subunit strongly support this inference (Table 1(c)). Moreover, it is noticeable that the

PPD dimer seems to be present in its original form without being further dehydrogenated after adduct formation with the α -chain of the metalloprotein.

Due to the two free sulfhydryl functions contained within the β -chain of Hb, interpretation of the deconvoluted mass spectrum is not as straightforward in this case. Three novel peaks are detected next to the signal of the unmodified β -subunit with deconvoluted neutral masses of 15969.9 Da, 16075.3 Da and 16184.4 Da (Fig. 7(d)). These signals can clearly be distinguished from those in the same mass region, which are already being monitored before the addition of the EC effluent (compare Fig. 7(b)), since the molecular masses significantly differ from each other. Again, the peak pattern initially suggests the generation of three different hapten–protein complexes, which could be induced by the addition of PPQD, a PPD dimer, and BB to one free thiol group of the Hb β -chain (Cys-93 or Cys-112 modified). However, the addition of a single PPQD molecule to each of the two free sulfhydryl functions (Cys-93 and Cys-112 modified), for example, can also be imagined. Further combinations involving the different reactive PPD species are possible. The calculation of the average molecular masses given in Table 1(d) is based on the modification of only one thiol group to better compare the differences from the unmodified β -subunit with the results from the investigations with β -LGA and HSA. Thus, the deconvoluted neutral mass of 15969.9 Da (mass increase of 104.4 Da) can unambiguously be assigned to a reaction of only one thiol function with PPQD, although

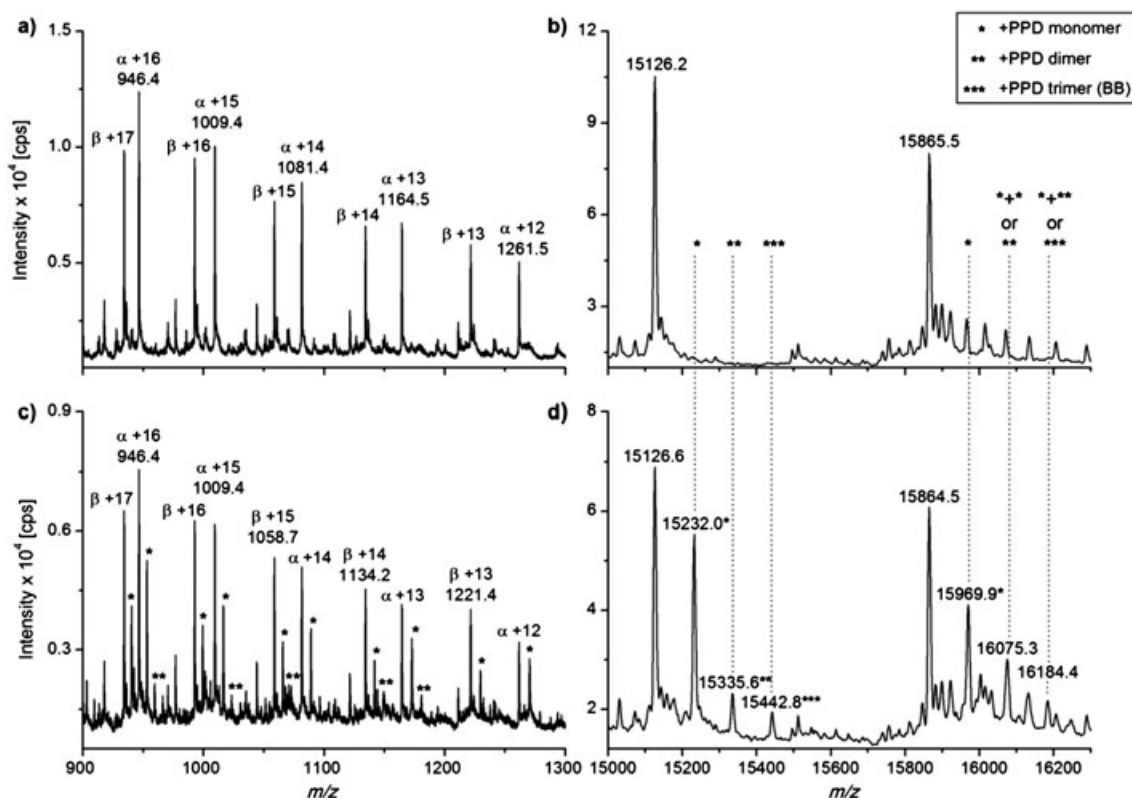


Figure 7. Mass spectra of (a) unmodified Hb, (b) unmodified Hb upon deconvolution, (c) Hb after reaction with oxidized PPD, and (d) modified Hb upon deconvolution. Asterisks point out which type of protein adduct has been formed by assigning the different PPD species to the according signals. For the Hb β -chain, which contains two free thiol groups, two identifications are conceivable for the conjugates at 16075.3 Da and 16184.4 Da each.

it is not possible to predict to which Cys residue the electrophile is attached without carrying out further investigations such as a tryptic digest. The deconvoluted neutral mass of 16075.3 Da exhibits a mass gain of 209.8 Da compared with the untreated β -chain so that both the formation of two individual Cys–PPD bonds and the formation of a single Cys–PPD dimer bond are conceivable. Finally, the signal at 16184.4 Da with a mass difference of 318.9 Da from unmodified Hb indicates the addition of one PPQD molecule and a PPD dimer to two different sulfhydryl groups or reaction of BB with one Cys function. In résumé, it can be stated that EC oxidation of PPD and subsequent conjugation with Hb led to a successful modification of the unblocked Cys residues for both the α - and the β -subunit, whereas the exact adduct pattern remains to be elucidated for the latter. Furthermore, no cross-linkage between the four different protein chains was observed which could have occurred upon autooxidation of a thioether and addition to a neighboring thiol group.

CONCLUSIONS

The potent skin sensitizer PPD was oxidized in an electrochemical flow-through cell and the arising products (detailed discussion in a previous publication^[36]) were effectively trapped by different proteins using an offline coupled EC/LC/MS set-up. Irreversible protein binding of reactive electrophilic PPD species could be demonstrated for all three model proteins under investigation. Specifically, the whey protein β -LGA showed extensive adduct formation and was quantitatively converted into five new conjugates resulting from the addition of monomeric up to pentameric PPD intermediates. This was confirmed for PPQD, dimeric PPD and BB by digesting the modified protein with trypsin and analyzing the T13 peptide. The tryptic digest further revealed that oxidized PPD selectively binds to the free thiol group of Cys-121 as none of the other detected peptides carried a modification. Additionally, HSA and Hb were studied with respect to the generation of corresponding hapten–protein complexes. Whereas for HSA only reaction with the monomeric quinone diimine PPQD could be assured, the α -chain of Hb showed a similar peak pattern to β -LGA upon modification. The same is true for the β -chain of Hb, although a combination of different conjugates has to be assumed here, since this subunit contains two free thiol groups which are both equally liable to electrophilic attack. Thus, EC was shown to be well suited for the quick and uncomplicated generation of hapten–protein adducts from pre- or pro-haptens that would otherwise have to be activated via more complex and time-consuming methods. In particular, the reactivity of PPQD and BB toward proteins is already well documented as being the major reason for the skin-sensitizing properties of PPD.^[11,13,37] Consequently, the applicability of this simple and purely instrumental system for assessing the skin-sensitizing potential of the LMW compound PPD could nicely be illustrated with this protein-binding study. The forthcoming ban on animal testing for toiletry and health care products reinforces the need to search for alternative methods and EC/(LC)/MS could play an important role as a complementary technique to address this challenge.

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REFERENCES

- [1] K. Landsteiner, J. Jacobs. Studies on the sensitization of animals with simple chemical compounds. *J. Exp. Med.* **1935**, *61*, 643.
- [2] K. Landsteiner, J. Jacobs. Studies on the sensitization of animals with simple chemical compounds. II. *J. Exp. Med.* **1936**, *64*, 625.
- [3] C. K. S. Pease, D. A. Basketter, G. Y. Patlewicz. Contact allergy: the role of skin chemistry and metabolism. *Clin. Exp. Dermatol.* **2003**, *28*, 177.
- [4] J. D. Bos, M. M. H. M. Meinardi. The 500 Dalton rule for the skin penetration of chemical compounds and drugs. *Exp. Dermatol.* **2000**, *9*, 165.
- [5] C. K. Smith, S. A. M. Hotchkiss. *Allergic Contact Dermatitis: Chemical and Metabolic Mechanisms*, Taylor & Francis, London, **2001**.
- [6] J. P. Lepoittevin. Metabolism versus chemical transformation or pro- versus pre-haptens? *Contact Dermatitis* **2006**, *54*, 73.
- [7] J. P. Lepoittevin. Chemical bases of contact allergy. *Rev. Fr. Allergol.* **2011**, *51*, 208.
- [8] M. Aleksic, E. Thain, D. Roger, O. Saib, M. Davies, J. Li, A. Aptula, R. Zazzeroni. Reactivity profiling: covalent modification of single nucleophile peptides for skin sensitization risk assessment. *Toxicol. Sci.* **2009**, *108*, 401.
- [9] M. C. Di Prisco, L. Puig, A. Alomar. Contact dermatitis due to para-phenylenediamine (PPD) on a temporal tattoo with henna. Cross reaction to azoic dyes. *Invest. Clin.* **2006**, *47*, 295.
- [10] S. Benomar, N. Ismaili, M. Rmili, L. Benzekri, B. Hassam. Black henna tattoo: beyond the contact dermatitis. *Rev. Fr. Allergol.* **2009**, *49*, 376.
- [11] P. Aebly, T. Sieber, H. Beck, G. F. Gerberick, C. Goebel. Skin sensitization to p-phenylenediamine: the diverging roles of oxidation and N-acetylation for dendritic cell activation and the immune response. *J. Invest. Dermatol.* **2009**, *129*, 99.
- [12] G. F. Gerberick, J. A. Troutman, L. M. Foertsch, J. D. Vassallo, M. Quijano, R. L. M. Dobson, C. Goebel, J. P. Lepoittevin. Investigation of peptide reactivity of pro-hapten skin sensitizers using a peroxidase-peroxide oxidation system. *Toxicol. Sci.* **2009**, *112*, 164.
- [13] M. Krasteva, J. F. Nicolas, G. Chabeau, J. L. Garrigue, H. Bour, J. Thivolet, D. Schmitt. Dissociation of allergenic and immunogenic functions in contact sensitivity to para-phenylenediamine. *Int. Arch. Allergy Immunol.* **1993**, *102*, 200.
- [14] J. Farrell, C. Jenkinson, S. N. Lavergne, J. L. Maggs, B. K. Park, D. J. Naisbitt. Investigation of the immunogenicity of p-phenylenediamine and Bandrowski's base in the mouse. *Toxicol. Lett.* **2009**, *185*, 153.
- [15] J. M. L. White, P. Kullavanijaya, I. Duangdeeden, R. Zazzeroni, N. J. Gilmour, D. A. Basketter, J. P. McFadden. p-Phenylenediamine allergy: the role of Bandrowski's base. *Clin. Exp. Allergy* **2006**, *36*, 1289.
- [16] H. F. Merk, J. M. Baron. The effect of low molecular weight substances on the human skin. Molecular mechanisms and their consequences. *Hautarzt* **2004**, *55*, 1125.
- [17] E. V. Buehler. Delayed contact hypersensitivity in the guinea pig. *Arch. Dermatol.* **1965**, *91*, 171.

- [18] B. Magnusson, A. M. Kligman. The identification of contact allergens by animal assay. The guinea pig maximization test. *J. Invest. Dermatol.* **1969**, 52, 268.
- [19] S. F. Martin, P. R. Esser, S. Schmucker, L. Dietz, D. J. Naisbitt, B. K. Park, M. Vocanson, J. F. Nicolas, M. Keller, W. J. Pichler, M. Peiser, A. Luch, R. Wanner, E. Maggi, A. Cavani, T. Rustemeyer, A. Richter, H. J. Thierse, F. Sallusto. T-cell recognition of chemicals, protein allergens and drugs: towards the development of in vitro assays. *Cell. Mol. Life Sci.* **2010**, 67, 4171.
- [20] E. V. Warbrick, R. J. Dearman, L. J. Lea, D. A. Basketter, I. Kimber. Local lymph node assay responses to paraphenylenediamine: intra- and inter-laboratory evaluations. *J. Appl. Toxicol.* **1999**, 19, 255.
- [21] G. F. Gerberick, C. A. Ryan, I. Kimber, R. J. Dearman, L. J. Lea, D. A. Basketter. Local lymph node assay: validation assessment for regulatory purposes. *Am. J. Contact Dermat.* **2000**, 11, 3.
- [22] C. A. Ryan, G. F. Gerberick, L. W. Cruse, D. A. Basketter, L. Lea, L. Blaikie, R. J. Dearman, E. V. Warbrick, I. Kimber. Activity of human contact allergens in the murine local lymph node assay. *Contact Dermatitis* **2000**, 43, 95.
- [23] Guideline No. 429. Skin sensitisation: local lymph node assay. *OECD Guidelines for Testing of Chemicals*, Organisation for Economic Cooperation and Development (OECD), Paris, **2010**.
- [24] Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Report. The murine local lymph node assay: a test method for assessing the allergic contact dermatitis potential of chemicals/compounds. *NIH Publ.* **1999**, No. 99-4494.
- [25] G. F. Gerberick, J. D. Vassallo, R. E. Bailey, J. G. Chaney, S. W. Morrall, J. P. Lepoittevin. Development of a peptide reactivity assay for screening contact allergens. *Toxicol. Sci.* **2004**, 81, 332.
- [26] European Commission (EC). Regulation (EC) No. 1223/2009 of the European Parliament and of the council of 30 November 2009 on cosmetic products. *Off. J. Eur. Union* **2009**, L 342, 71-72.
- [27] G. F. Gerberick, C. A. Ryan, P. S. Kern, R. J. Dearman, I. Kimber, G. Y. Patlewicz, D. A. Basketter. A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. *Contact Dermatitis* **2004**, 50, 274.
- [28] G. F. Gerberick, C. A. Ryan, P. S. Kern, H. Schlatter, R. J. Dearman, I. Kimber, G. Y. Patlewicz, D. A. Basketter. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis* **2005**, 16, 157.
- [29] A. Alvarez-Lueje, S. B. Dragnic. Stability and drug metabolism assessed by electrochemical methods. *Comb. Chem. High T. Scr.* **2010**, 13, 712.
- [30] A. Baumann, U. Karst. Online electrochemistry/mass spectrometry in drug metabolism studies: principles and applications. *Expert Opin. Drug Metab. Toxicol.* **2010**, 6, 715.
- [31] T. A. Getek, W. A. Korfmacher, T. A. McRae, J. A. Hinson. Utility of solution electrochemistry mass-spectrometry for investigating the formation and detection of biologically important conjugates of acetaminophen. *J. Chromatogr.* **1989**, 474, 245.
- [32] W. Lohmann, U. Karst. Simulation of the detoxification of paracetamol using on-line electrochemistry/liquid chromatography/mass spectrometry. *Anal. Bioanal. Chem.* **2006**, 386, 1701.
- [33] S. M. van Leeuwen, B. Blankert, J. M. Kauffmann, U. Karst. Prediction of clozapine metabolism by on-line electrochemistry/liquid chromatography/mass spectrometry. *Anal. Bioanal. Chem.* **2005**, 382, 742.
- [34] H. T. Deng, G. J. Van Berkel. Electrochemical polymerization of aniline investigated using on line electrochemistry/electrospray mass spectrometry. *Anal. Chem.* **1999**, 71, 4284.
- [35] W. Lohmann, H. Hayen, U. Karst. Covalent protein modification by reactive drug metabolites using online electrochemistry/liquid chromatography/mass spectrometry. *Anal. Chem.* **2008**, 80, 9714.
- [36] S. Jahn, H. Faber, R. Zazzaroni, U. Karst. Electrochemistry/mass spectrometry as a tool in the investigation of the potent skin sensitizer *p*-phenylenediamine and its reactivity toward nucleophiles. *Rapid Commun. Mass Spectrom.* **2012**, 26, 1453.
- [37] C. Jenkinson, R. E. Jenkins, M. Aleksic, M. Pirmohamed, D. J. Naisbitt, B. K. Park. Characterization of *p*-phenylenediamine-albumin binding sites and T-cell responses to hapten-modified protein. *J. Invest. Dermatol.* **2010**, 130, 732.
- [38] J. Eilstein, J. P. Lepoittevin, E. Gimenez-Arnau, D. Duche, F. Rousset. Synthesis and reactivity toward nucleophilic amino acids of 2,5-[C-13]-dimethyl-*p*-benzoquinonediimine. *Chem. Res. Toxicol.* **2006**, 19, 1248.
- [39] J. Eilstein, J. P. Lepoittevin, E. Gimenez-Arnau, D. Duche, F. Rousset. Mechanistic studies on the lysine-induced N-formylation of 2,5-dimethyl-*p*-benzoquinonediimine. *Chem. Res. Toxicol.* **2007**, 20, 1155.
- [40] J. Eilstein, E. Gimenez-Arnau, D. Duche, N. Cavusoglu, G. Hussler, F. Rousset, J. P. Lepoittevin. Sensitization to *p*-amino aromatic compounds: study of the covalent binding of 2,5-dimethyl-*p*-benzoquinonediimine to a model peptide by electrospray ionization tandem mass spectrometry. *Bioorgan. Med. Chem.* **2008**, 16, 5482.
- [41] C. Brander, D. Maurihellweg, F. Bettens, H. Rolli, M. Goldman, W. J. Pichler. Heterogeneous T-cell responses to beta-lactam-modified self-structures are observed in Penicillin-allergic individuals. *J. Immunol.* **1995**, 155, 2670.
- [42] T. P. King. On the sulfhydryl group of human plasma albumin. *J. Biol. Chem.* **1961**, 236, PC5.
- [43] G. Zonios, J. Bykowski, N. Kollias. Skin melanin, hemoglobin, and light scattering properties can be quantitatively assessed in vivo using diffuse reflectance spectroscopy. *J. Invest. Dermatol.* **2001**, 117, 1452.
- [44] M. Ogawa, T. Oyama, T. Isse, T. Yamaguchi, T. Murakami, Y. Endo, T. Kawamoto. Hemoglobin adducts as a marker of exposure to chemical substances, especially PRTR class I designated chemical substances. *J. Occup. Health* **2006**, 48, 314.
- [45] L. Hagmar, M. Törnqvist, C. Nordander, I. Rosen, M. Bruze, A. Kautiainen, A. L. Magnusson, B. Malmberg, P. Aprea, F. Granath, A. Axmon. Health effects of occupational exposure to acrylamide using hemoglobin adducts as biomarkers of internal dose. *Scand. J. Work Environ. Health* **2001**, 27, 219.
- [46] C. H. Lindh, B. A. G. Jönsson. Human hemoglobin adducts following exposure to hexahydrophthalic anhydride and methylhexahydrophthalic anhydride. *Toxicol. Appl. Pharm.* **1998**, 153, 152.
- [47] M. H. Kristiansson, B. A. Jönsson, C. H. Lindh. Mass spectrometric characterization of human hemoglobin adducts formed in vitro by hexahydrophthalic anhydride. *Chem. Res. Toxicol.* **2002**, 15, 562.