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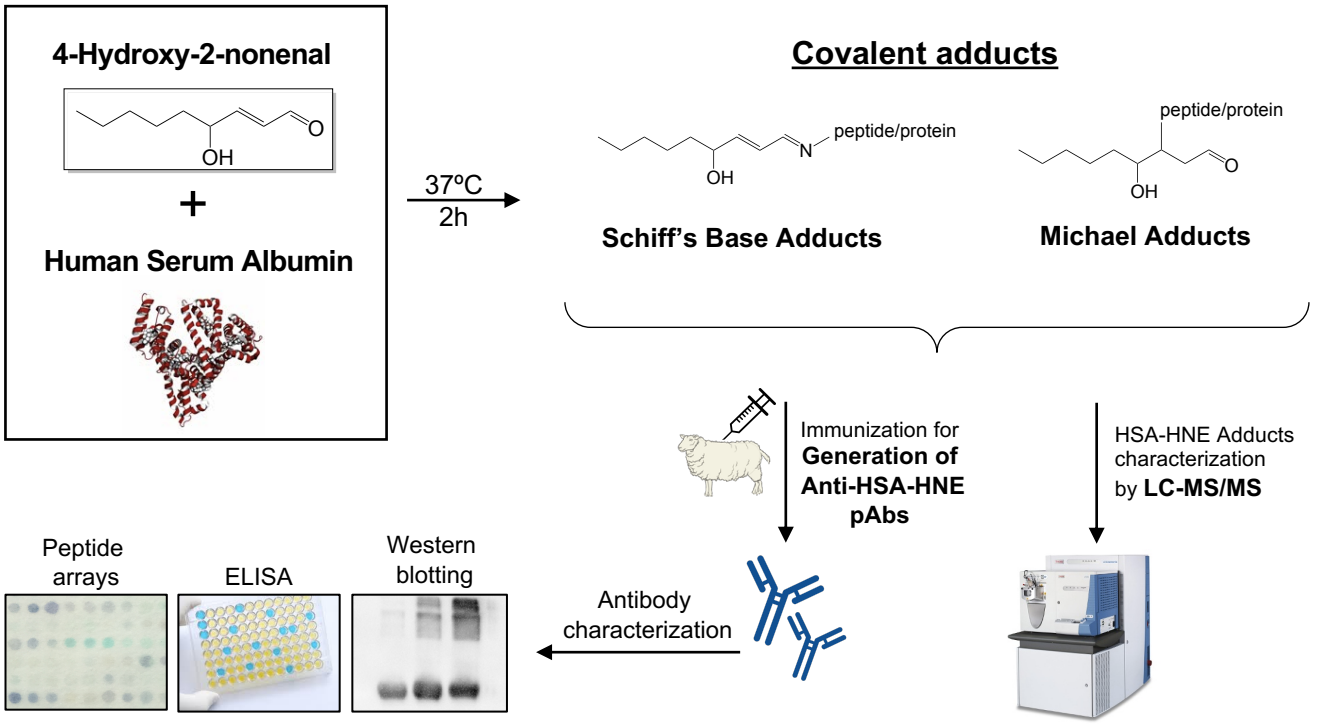
4-Hydroxy-2-nonenal
OH
O
+
Human Serum Albumin
37ºC
2h
Covalent adducts
peptide/protein
N
OH
Schiff's Base Adducts
peptide/protein
OH
O
Michael Adducts
Immunization for
Generation of
Anti-HSA-HNE
pAbs
HSA-HNE Adducts
characterization
by LC-MS/MS
Peptide
arrays
ELISA
Western
blotting
Antibody
characterization

Epitope mapping and characterization of 4-hydroxy-2-nonenal modified-human serum albumin using two different polyclonal antibodies

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Key words: Lipid peroxidation; protein lipoxidation, mass spectrometry, diagnostics, antibodies, peptide arrays, epitope mapping.

Abstract:

Lipids are susceptible to damage by reactive oxygen species, and from lipid oxidation reactions many short chain lipid peroxidation products can be formed. 4-Hydroxy-2-nonenal (HNE) is one of the most abundant and cytotoxic lipid oxidation products and is known to form covalent adducts with nucleophilic amino acids of proteins. HNE-modified proteins have value as biomarkers and can be detected by antibody-based techniques, but most commercially available antibodies were raised against HNE-keyhole limpet hemocyanin. We used HNE-treated human serum albumin (HSA) to raise sheep antiserum and report for the first time the use of covalently modified peptide arrays to assess epitope binding of antibodies (Abs). Peptide arrays covering the sequence of HSA and treated post peptide synthesis with HNE were used to compare the different binding patterns of a commercial polyclonal antibody (pAb) raised against HNE-treated KLH and an in-house anti-HNE enriched pAb. The results were correlated with analysis of HNE-modified HSA by high-resolution tandem mass spectrometry. Both anti-HNE pAbs were found to bind strongly to eight common peptides on the HNE-treated HSA membranes, suggesting that HNE adducts *per se* induced an immune response in both cases even though different immunogens were used. Both antibodies bound with the highest affinity to the peptide ^{365}DPHECYAKVFDEFKPLV^{381}, which contains K378 and was also shown to be modified by the mass spectrometry analysis. Overall, the commercial anti-HNE pAb showed better specificity, recognizing nine out of the eleven adducts found by MS/MS, while the in-house enriched pAb only recognizes six. Nevertheless, the in-house pAb recognized specific peptides that were not recognized by the commercial pAb, which suggests the presence of clones uniquely specific to HNE adducts on HSA.

Abbreviations:

A1AT, alpha-1 anti-trypsin; Abs, Antibodies; BSA, Bovine serum albumin; CAM, carbamidomethylation; CID, collision induced dissociation DCM, dichloromethane; DDA, data dependent acquisition; DEA, diethylacetal; DIC, 1,3-diisopropylcarbodiimide; DMA, dimethylacetal, DMF, dimethylformamide; DTT, Dithiothreitol; ECL, enhanced chemiluminescence; ELISA, Enzyme linked immunoassay adsorbent assay; ESI, electrospray ionisation; Fmoc, Fluorenylmethyloxycarbonyl chloride; FASP, Filter Aided Sample Preparation; HNE, 4-hydroxy-2-nonenal; HOBt, 1-hydroxybenzotriazole; HRP, Horseradish peroxidase; HSA, Human serum albumin; IgG, Immunoglobulin; KLH, Keyhole Limpet Hemocyanin; LFA, Lateral flow assay; MA, Michael adduct; mAb, Monoclonal antibody; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight; MS, Mass spectrometry; NHS, N-hydroxysuccinimide; NMP, 1-methyl 2-pyrrolidinone; OVA, Ovalbumin; pAb, Polyclonal antibody; PBS, Phosphate-buffered saline; PBST, Phosphate-buffered saline, with 0.1% Tween 20; pNPP, p-Nitrophenylphosphate; SB, Schiff's base; SDS-PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBST, Tris-buffered saline, with 0.1% Tween 20; TFA, trifluoracetic acid; TIPS, triisopropylsilane; TMB, 3,3',5,5'-tetramethylbenzidine; UPLC, ultra-performance liquid chromatography.

1. Introduction

It is now becoming well accepted that redox imbalance occurs in a number of diseases involving inflammation, and contributes to their pathology, even if it is not their cause [1]. One of the many consequences of increased levels or production of free radicals, reactive oxygen species (ROS) or reactive nitrogen species (RNS) is oxidative damage to biological macromolecules, which causes dysfunction of cells, tissues, and organs [2]. Lipids, in particular the polyunsaturated ones, are susceptible to oxidative damage and undergo lipid peroxidation to generate lipid peroxyl radicals and hydroperoxides, which can be further rearranged or fragmented to yield a wide variety of esterified and non-esterified lipid oxidation products [3]. Some of these products, specifically those containing carbonyl groups, are electrophilic and react with nucleophilic molecules to form covalent adducts, a process called lipoxidation [4,5]. One of the most toxic lipid peroxidation products is 4-hydroxy-2-nonenal (HNE), a γ-substituted α, β-unsaturated aldehyde that is derived from the oxidation of *n*-6-polyunsaturated fatty acids, such as arachidonic and linoleic acids [6]. It is particularly reactive due to the fact that the aldehydic C=O function is conjugated to an unsaturated C=C double bond at the alpha, beta position (**Fig. 1**), making it bifunctional and capable of cross-linking reactions. HNE can form Schiff's base adducts between its carbonyl group and the free amino groups of lysine and arginine, and/or Michael adducts between its β carbon and the nitrogen lone pair of the histidine and lysine, or the sulfhydryl group of cysteine [7]. Because Michael adducts can form a hemiacetal structure that is more stable, these adducts are easier to detect, as opposed to Schiff's bases. Studies have shown that Cys residues exhibit the highest reactivity towards HNE, followed by His and Lys, but His–HNE adducts can be more stable than Cys-HNE adducts due to the poorer leaving group ability of the imidazole over the thiol under neutral conditions. In any case, Michael adducts, and Schiff's bases adducts are both reversible covalent adducts [4,8,9].

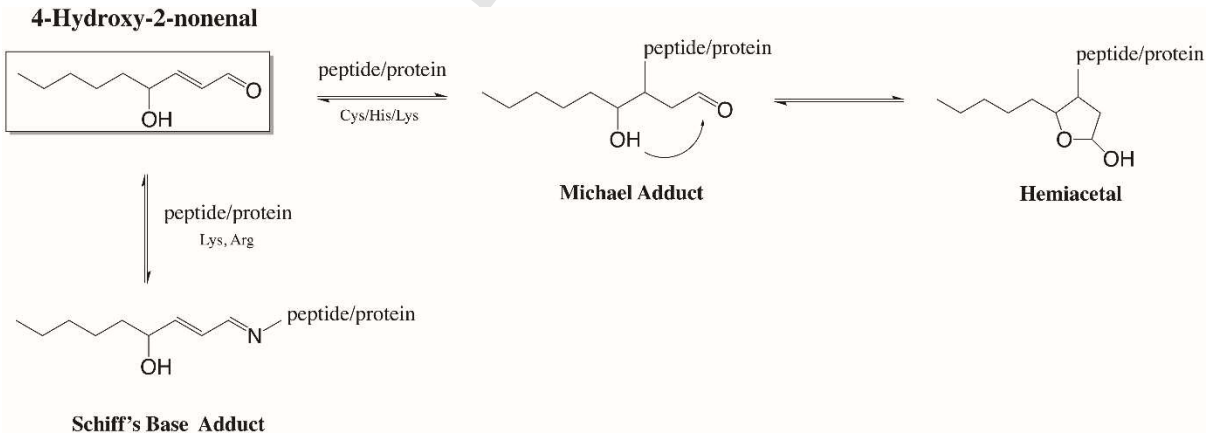


Fig. 1. Chemical structure of 4-hydroxy-2-nonenal (HNE). The functional groups, particularly the C=C double bond and the C=O carbonyl group present a very high reactivity towards nucleophilic residues of peptides/proteins. Michael adducts can be formed with cysteine, lysine or histidine containing peptides/proteins and Schiff's base adducts with lysine containing peptides/proteins.

Physiologically, HNE levels are in the range of 0.1-1 μM, but under certain inflammatory conditions such as severe rheumatological diseases or systemic lupus erythematosus, the steady state concentration of HNE can be 3-10-fold higher than the normal concentrations [10,11]. According to the literature, 1-8% of the HNE formed is covalently bound to proteins [12], and the accumulation of HNE-modified macromolecules, and lipoxidation adducts in general, is strongly associated with inflammation and oxidative stress related pathologies. For example, increased amounts of HNE have been seen in chronic liver diseases, including alcoholic hepatitis, hemochromatosis and primary biliary disease [10], and HNE adducts have been found in Alzheimer's disease [13], systemic lupus erythematosus [14], cancer [12,15], cardiovascular diseases, diabetes, and Parkinson's disease [2,16]. Lipoxidation adducts are also implicated in the modulation of inflammation and immune responses, via binding to the receptor for advanced glycation end products (RAGE), which activates the pro-inflammatory transcription factor NF-κB [17].

In view of the evidence for the role of HNE in cellular dysfunction and as an indicator of disease severity [18,19], rapid and accurate methods for the detection and quantification of lipoxidation adducts are highly desirable for diagnostic applications. While mass spectrometry approaches to identifying lipoxidation adducts are being developed and applied [20–22], they have the limitation of requiring expensive specialist equipment and being challenging to perform. In contrast, antibody-based methods such as immunoassays have the advantage of high sensitivity, relative simplicity and accessibility, and an ultimate goal in terms of healthcare diagnostics is the development of lateral flow assays that are additionally extremely rapid. However, the development of immunoassays depends on the availability of polyclonal or monoclonal antibodies with suitable specificity and sensitivity.

The generation of antibodies against HNE-adducts was started in the late 1980s, when Palinski *et al*. (1989) and Jurgens *et al*. (1990) produced antibodies against HNE-treated LDL [23,24]. Subsequently other groups adopted different approaches and raised antibodies against alternative immunogens, such as HNE-treated keyhole limpet hemocyanin (KLH) [25–27] a synthetic peptide Gly_3-His-HNE-Gly_3 conjugated to KLH [28], or HNE-modified HSA [14,29]. The majority of these were polyclonal sera, able to recognize HNE adducts on a variety of proteins, but some monoclonal antibodies were also produced [26,27]. In some cases, specificity of a particular modified residue or adduct type was reported, e.g. histidine adducts [26,27] or cysteine adducts [30], while other antisera showed broader specificity. Several commercial antibodies for detection of HNE-adducts are available, ranging from non-labelled, horseradish peroxidase- or alkaline phosphatase-labelled polyclonal antibodies to monoclonal antibodies, and their applications include western blotting, ELISAs, immunofluorescence, and immunocytochemistry [31]. However, the majority of these antibodies have been raised against HNE-KLH, and moreover adduct recognition is not always consistent from antibody to antibody; this can be an issue in the development of clinical assay platforms and point-of-care diagnostic tests. Thus, a better understanding of the epitopes recognized by anti-HNE antibodies and the dependence on the underlying peptide sequence recognized within proteins would be extremely useful.

Human serum albumin (HSA) is a highly abundant serum protein that has already excited interest as a site of oxidative modifications, including lipoxidation, and a potential biomarker for inflammatory

diseases [14,22,32–34]. The development of immunoassays for HNE-adducts of HSA therefore also has commercial potential, but currently anti-HNE-HSA antibodies are not widely available or affordable. Partially purified polyclonal antisera to HNE-HSA have been produced recently and used to investigate the occurrence of HNE-modified albumin in systemic lupus erythematosus (SLE) [14] and rheumatoid arthritis [32], but many questions remain about the specificity for modified HSA and the sites of modification recognized by the antisera. We therefore used a similar procedure but with additional purification steps for the production of anti-HNE-HSA antibodies, and characterization of the immunogen by high resolution mass spectrometry. In order to investigate the epitope recognition of the in-house polyclonal serum, we adopted a novel approach using peptide arrays treated with HNE and compared it with a commercial polyclonal antibody raised against HNE-KLH.

2. Experimental

2.1. Reagents

Human serum albumin (A1653) and albumin from chicken egg white (A5503) were obtained from Sigma-Aldrich. Imject™ mcKLH (in PBS) was purchased from Thermo Fischer Scientific (UK). 4-hydroxynonenal diethylacetal (HNE-DEA) was generously supplied by Prof. Giancarlo Aldini from University of Milan. HNE was prepared from HNE-DEA by 1 mM HCl hydrolysis (1 hour at room temperature) and quantified by UV spectroscopy (λ_{max} = 224 nm; ε=13750 L $mol^{-1}cm^{-1}$). Fluorenylmethyloxycarbonyl chloride (Fmoc) protected amino acids were purchased from Intavis (20x 1 cartridge of 0.5 mmol of Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu) OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln (Trt)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(OtBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH). 1-methyl 2-pyrrolidinone (NMP), dimethylformamide (DMF), 1,3-diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazole (HOBt) acetonitrile, formic acid, dichloromethane (DCM), and ethanol, were purchased from Rathburn chemicals Ltd (Walkerburn, Scotland). Trifluoracetic acid (TFA), triisopropylsilane (TIPS), Piperidine ≥99.5 was obtained from Sigma. Commercial goat polyclonal antibody anti-HNE (Ab46544) was purchased from Abcam, and 3, 3', 5, 5'-tetramethylbenzidine (TMB) and HRP-labelled anti-goat IgG were purchased from Sigma Aldrich (UK). All solvents were of LC-MS grade and all solutions were prepared using ultra-pure Milli-Q water. All other reagents were of analytical grade.

2.2. Generation of HSA-HNE adducts

HSA-HNE adducts were prepared by treating of 5 mg of HSA with HNE at a molar ratio of 1:10 in 5 mL of phosphate buffer saline buffer (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) for 2 hours at 37 ºC.

2.3. LC-MS/MS of HSA tryptic digested peptides modified by HNE

HNE-modified HSA samples (50 µg) were digested with trypsin according to the Filter Aided Sample Preparation (FASP) protocol [35]. Briefly, proteins were reduced with dithiothreitol (DTT) (100 mM, 1 h, RT), transferred onto a Microcon centrifugal filter (30 KDa cut-off) by centrifugation at 14 000 x g, alkylated in situ with iodoacetamide (50 mM, 20 min in the dark), washed two times with urea buffer (8 M Urea, 100 mM Tris-HCl, pH 8.5) and two times with ammonium bicarbonate buffer (50 mM) before digestion with trypsin (1:25 enzyme to protein ratio w/w) overnight at 37 °C in a humidified environment. Peptides were recovered in ammonium bicarbonate buffer (50 mM), dissolved at 250 ng/µL in 3%(v/v) acetonitrile in water and used for LC-MS analysis using a nano-ACQUITY UPLC system (Waters GmbH, Eschborn, Germany) coupled online to an LTQ Orbitrap XL ETD mass spectrometer equipped with a nano-ESI source (Thermo Fischer Scientific, Bremen, Germany). Eluent A was formic acid (0.1% v/v) in water and Eluent B was formic acid (0.1% v/v) in acetonitrile. Samples (10 µL injection) were diluted in Eluent A and loaded onto the trap column (nano Acquity Symmetry C18; internal diameter 180 µm, length 20 mm, particle diameter 5 µm) at a flow rate of 10 µL/min. The separation was performed using a BEH 130 column (C18 column, internal diameter 75 um, length 100 mm, particle diameter 1.7 µm) at a flow rate of 0.4 µL/min, with a linear gradient from 3% to 30% of eluent B over 18 min and then to 85% of eluent B over 1 min. The transfer capillary temperature was set to 200 ºC and the tube lens voltage to 110 V. An ion spray voltage of 1.6 kV was applied to a PicoTip online nano-ESI emitter (New Objective, Berlin, Germany). The precursor ion survey scans were acquired on an Orbitrap resolution of 60,000 at *m/z* 400 across a *m/z* range from 400 to 2000. CID tandem mass spectra (isolation width 2.00, activation Q 0.250, normalized collision energy 35.0%, activation time 30.0 ms) were recorded in the linear ion trap by data-dependent acquisition (DDA) for the top six most abundant ions in each survey scan with a dynamic exclusion of 60 s using Xcalibur software 3.0 (Thermo Fischer Scientific, Bremen, Germany).

2.4. Identification of HSA tryptic peptides modified by HNE using the Sequest search engine

HNE modifications were identified using the Sequest search engine (Proteome Discoverer 1.4, Thermo Scientific) against Homo sapiens (Human) database, allowing up to two missed cleavages and a mass tolerance of 10 ppm for precursor ions and 0.8 Da for product ions. Oxidation of methionine and cysteine, carbamidomethylation (CAM) of cysteine, HNE Michael adducts (on cysteine, lysine and histidine) and Schiff's base adducts (on lysine) were used as variable modifications and results were filtered for rank 1 peptides and score vs. charge states corresponding to Xcorr/z 2.0/2, 2.25/3, 2.5/4, 2.75/5.

2.5. Identification of HNE modifications on HSA by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) MS

The analysis of native HSA and HNE modified HSA samples by MALDI-TOF MS was performed on a Bruker Autoflex Speed (Bruker, MA, USA) using sinapinic acid (10 mg/ml in 70:30 (v/v)

water/acetonitrile with 0.1% TFA) as matrix in positive mode. A 20 µL aliquot of each sample was desalted using a mixed bed ion exchange resin (Amberlite MB-9L from Sigma-Aldrich), and 1 µL of desalted sample was spotted onto the MALDI plate and allowed to dry naturally. Finally, 1 µL of matrix was spotted onto the dry sample and allowed to dry naturally before analysis.

2.6. Generation of anti-HSA-HNE polyclonal antibodies

Aliquots of 1 mg/mL and 0.25 mg/mL of HNE-modified HSA were used to immunize sheep SA369 by Orygen (Edinburgh, UK). Before immunization, a pre-immune bleed was collected for further control analysis. The first immunization was carried out with 1 mg of antigen and repeated every 28 days for three months with an additional injection of 0.25 mg of antigen. Seven days after each injection a bleed (~300 mL) was collected.

2.7. Selective capture of anti-HSA-HNE antibodies

Selective enrichment of anti-HSA-HNE antibodies from crude serum of sheep SA369 was carried out by following two different affinity purification approaches. The first approach comprised a first affinity chromatographic step using a HiTrap™ NHS activated column modified with HNE-treated HSA (1 mL) for purification of the mixture of anti-HSA and anti-HSA-HNE Abs, and a second affinity purification with a HiTrap™ NHS activated column modified with HSA (1 mL) for depletion of the anti-HSA Abs. Anti-HSA-HNE pAbs were collected from the unbound fraction of the second purification run.

The second approach consisted of a first purification step with a HiTrap™ Protein G column (1 mL) for purification of the whole population of antibodies, and later, for the specific purification of anti-HNE antibodies a HiTrap™ NHS activated column modified with HNE-treated KLH was used. For both approaches, the columns were equilibrated with 5 column volumes of PBS pH 7.4, prior to loading of 10 mL of crude serum (previously centrifuged and filtered). Washing for removal of non-specific binding compounds was achieved by increasing the salt concentration to 1 M NaCl for 2 column volumes. After re-equilibration into PBS, the elution of bound antibodies was triggered by decreasing the pH to 2.5, using a 0.1 M Glycine-HCl aqueous buffer. Column flow through and eluate were continuously collected as 5 and 2 mL fractions, respectively, in a FRAC 950 fraction collector from GE Healthcare. The collected samples containing the antibody of interest were combined neutralized by adding 60 µl of 1M Tris-HCl pH 9.0, per each 5 mL, and then placed in dialysis tubing overnight in 2 L of PBS. After dialysis, samples were loaded onto the second NHS modified column (KLH-HNE or HSA-modified) for depletion of anti-HSA antibodies. The same elution and dialysis methods were applied. The chromatography runs with both columns were performed on an Äkta™ Purifier 10 system from GE Healthcare. The data collection and processing were accomplished using Unicorn 5.1 software. Chromatographic parameters such as conductivity and UV absorbance at 280 nm were continuously measured.

The concentration of the eluate samples from both columns was calculated by measuring the absorbance at 280 nm using a spectrophotometer, and then dividing that value by 1.4 (extinction coefficient of IgG).

2.8. Design and synthesis of peptide arrays

The sequence of HSA, including the 18-residue signal peptide and 6-residue pro-peptide (accession number P02768) and ovalbumin (OVA) (accession number P01012) was divided into 15-mer peptides with sequential peptides overlapping by 13 amino acids, and each peptide was synthesised on commercially available derivative membranes (amino-PEG500-UC540 Sheets) based on conventional Fmoc chemistry, using the Intavis ResPep SL peptide synthesiser (INTAVIS, Köln, Germany). Peptides were cellulose-coupled to the C-termini and the principle of the peptide arrays is depicted in **Supplementary Fig. 1**.

The membranes were initially washed with DMF and ethanol, the coupling of the amino acids was achieved by pre-activation in 0.5 M amino acid (in NMP), 1.1 M activator (HOBt) and 1.1 M of DIC in DMF. Any unreacted free amino groups were blocked by washing the membranes with 10% acetic anhydride in DMF (capping solution). The peptides were built one amino acid at a time, starting from the C-terminus to the N-terminus. In the last coupling cycle, the capping step was omitted and the final Fmoc-deprotection was carried out with 20% piperidine in DMF. The HSA and OVA sequences of each synthesised peptide and their localization on the peptide array are shown in **Supplementary Table 1** and **Supplementary Table 2**, respectively. Once the synthesis was completed, the membrane was treated with a mixture of TFA/TIPS/H_2O (18:1:1) for 1-2h for final side-chain deprotection, and then washed with DCM for 30 min. Finally, the membranes were washed with ethanol and water, in cycles of 5 minutes and once dried they were stored in a sealed container at RT.

In the text, HSA residues are numbered from the start of the mature protein sequence, without the signal and pro-peptides.

2.9. Epitope mapping on peptide arrays

The cellulose membranes were initially washed 3 times for 10 mins with PBS to rehydrate it and for the HNE adduct formation, the membranes were then incubated with 0.02 mM HNE in PBS for 2 hours at 37ºC (for negative controls this step was omitted). The membranes were then washed twice with phosphate buffered saline containing 0.1% (v/v) Tween 20 (PBST) for 30 min, and blocked with 20 mL of blocking solution (5% skimmed milk powder in PBST) for 1 hour. The blocking reagent was then discarded and a 20-mL aliquot of 100 ng/mL of HRP-conjugated antibody in blocking agent was added. After a one-hour incubation the membranes were washed with 3 volumes of PBST (3 x 5 min) and incubated with 5 mL of TMB for 15-30 min, until the signal was completely developed. The membranes were then washed with two volumes of water, allowed to drip dry and then scanned using a GS-800^{TM} Calibrated Densitometer from Bio-Rad (CA, USA). The regeneration of the membranes was accomplished by incubation with 20 mL of 1% SDS in 100 mM Tris-HCl, pH 6 + 140 µl of β-mercaptoethanol.

2.10. SA369 Anti-HSA-HNE Antibody Screening by direct ELISA

The collected bleeds were screened for antibody binding by direct ELISA. 100 µL aliquots of HNE treated ovalbumin (1:10 HNE:protein molar ratio) were added to wells in 96-well microtiter plates (Corning, MA, USA) and incubated overnight at 4□C. Following aspiration of the antigen solution, the plate was washed three times with tris-buffered saline, 0.1% Tween 20 (TBST), and the wells were blocked with 120 µL of 1% (w/v) BSA/PBS (v/v) for 1h at RT. After another three-wash step with TBST, 100 µL aliquots of each bleed were added to the wells in 4-fold dilution factors in 1% (w/v) BSA/PBST (1:400, 1:1600, 1:6400, 1:25600 and 1:102400) and the plate was incubated for 1 hour with shaking at RT. The wells were once again washed three times with TBST and the plate was incubated with 100 µL of 1:25000 dilution of mouse anti-sheep IgG conjugated to alkaline phosphatase in 1% (w/v) BSA/PBST, for 1h with shaking at RT. The detection was done using pNPP (Thermo Fisher Scientific, USA) and the absorbance was read at 405 nm using a microplate reader.

2.11. SDS-PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed to evaluate the profile of the modified and non-modified proteins and also to characterise the SA369 polyclonal serum and the commercial anti-HNE antibody. Samples were prepared in Laemmli loading buffer and heated at 100^{o}C for 5 min. Samples were loaded onto a 12% acrylamide gel, prepared from 40% acrylamide/bis stock solution (29:1) from Bio-Rad, and run at 100 mV using 192 mM glycine, 25 mM Tris–HCl and 0.1% SDS, pH 8.3. Gels were stained with Coomassie Brilliant Blue (Pharmacia, Uppsala, Sweden). Images were acquired with the Gel Doc EZ System from Bio-Rad (CA, USA).

2.12. Western Blotting using the SA369 Polyclonal Antibody (pAb)

For the western blot analysis, samples were run on 12% SDS-PAGE gels and separated following the conditions described before. Proteins were transferred from the gel to PVDF membranes using the Trans-Blot Turbo Transfer System (Bio-Rad, CA, USA). The membranes were blocked overnight at 4□C with AdvanBlock-PF blocking solution (Advansta, Inc, CA, USA) and then incubated with the primary antibodies (either the in-house enriched anti-HNE pAb or the Abcam anti-HNE pAb, dilution 1:10000), for 1h at RT. The membranes were washed with AdvanWash washing solution (Advansta, Inc, CA, USA) three times for 10 minutes and incubated with the secondary antibody (HRP-labelled anti-sheep and anti-goat, dilution 1:10000). Detection was performed using the WesternBright Sirius ECL reagent (Amersham, UK), and images acquired using the ChemiDoc™ XRS+ System (Bio-Rad, CA, USA).

2.13. Sandwich ELISA for HNE adducts

A sandwich ELISA using the SA369 pAb as capture antibody and the Abcam anti-HNE pAb as detection antibody was developed for the detection of HNE adducts. 96-well ELISA plates were coated overnight at 4ºC with 100 µL per well of the capture antibody (SA396 anti-HSA-HNE pAb) solution at 1 µg/mL. Plates were washed three times with TBST (20 mM Tris, 137 mM NaCl 0.05% Tveen-20 pH 7.6) and blocked with 120 µL of 1% BSA in PBS (137 mM sodium chloride, 2.7 mM potassium chloride, 8 mM disodium hydrogen phosphate, 1.5 mM potassium dihydrogen phosphate, pH 7.5), for one hour at room temperature. Plates were once again washed for three times with TBST, and then incubated with 100 µL of the antigen (KLH, HSA or OVA previously treated with HNE at a protein:HNE 1:10 molar ratio for 2h at 37ºC and prepared in 1% BSA/PBST at a range of 0 to 20 µg/mL) for one hour with shaking. After another three wash steps with TBST, the plates were incubated with 100 µL of the detection pAb (Abcam anti-HNE HRP-labelled (dilution: 1/2000)) for one hour. TMB substrate solution (100 µL) was used for detection and its reaction was stopped after 15-30 min by addition of 50 µL of stopping solution (1 M HCl). Absorbance was read at 450 nm.

3. Results

3.1. Confirmation of HNE adducts formation by LC-MS/MS

Prior to initiating the immunization protocol, the structural modifications of HSA by HNE were characterized by digesting the native HSA and HNE-modified HSA samples with trypsin and analysing the generated peptides by LC-MS/MS. The Sequest search engine provided 81% protein sequence coverage, from which 15 peptides containing HNE adducts were identified, of which 8 were Michael adducts and 3 were Schiff's Bases (**Table 1**). The HNE-modified peptides were identified based on their *m/z*, which were determined with mass accuracy ≤5 ppm, and an increase of the retention times over the unmodified peptides. Due to the treatment with iodoacetamide during the digestion protocol, most of the unmodified cysteine-containing peptides were identified as carbamidomethylated (CAM). The exact location of some of the HNE modifications on the peptide sequence could not be determined because of the facile neutral loss of the HNE moiety during MS/MS fragmentation. Examples of this are His105 and Lys106 (peptide ^{99}NECFLQHKDDNPNLPR^{114}); however, the manual annotation of the MS spectra, the *m/z* of the modified peptides and the differences observed in retention times between the modified and equivalent unmodified peptides allowed the formation of an HNE adduct on these three peptides to be confirmed (**Table 1**). Longer retention times for HNE-modified peptides are typical as HNE molecules increase the overall hydrophobicity of a peptide, therefore increasing the retention time of the peptide on the HPLC column. It should be noted that the LC-MS/MS data report on the total variety of modifications detected, and that any individual protein molecule is likely to contain a subset of these adducts.

Table 1. Tryptic peptides of HNE-modified HSA identified using a Sequest database search.

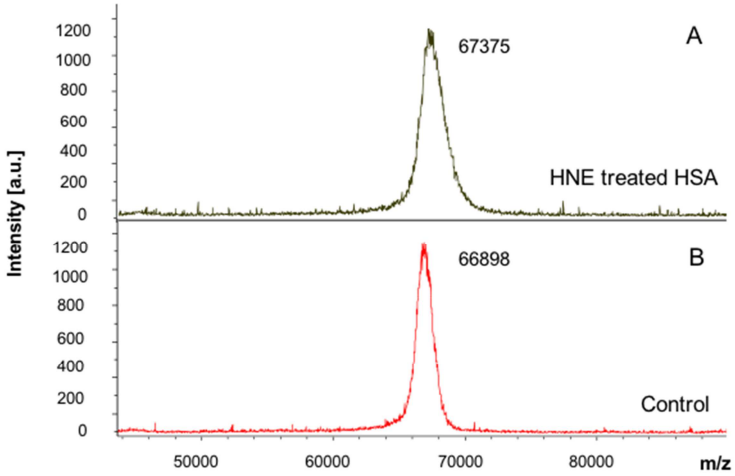
HSA Peptide Sequence	*Modifications* [a]	*Theo. MH^+ [Da]* [b]	*z*	*m/z [Da]*	*Δ ppm*	*t_R (min)*
^{52}*TCVADESAENCDKSLHTLFGD*^{73}	2xCAM C53; C62; 1xHNE MA H67	2653.2120	3	885.0750	0.37	35.0
	2xCAM C53; C62	2497.0970	3	833.0372	-0.28	27.4
^{65}*SLHTLFGDK*^{73}	1xHNE MA H67	1173.6514	2	587.3284	1.42	35.3
	-	1017.5364	2	509.2706	2.08	22.6
^{52}*TCVADESAENCDKSLHTLFGDKLCTV ATLR*^{81}	3xCAM C53; C62; C75; 1xHNE MA H67/K73	3567.7128	4	892.6862	-3.04	39.2
	3xCAM C53; C62; C75;	3411.5978	3	1137.8713	-0.67	33.8
^{94}*QEPERNECFLQHKDDNPNLPR*^{114}	1xCAM C101; 1xHNE MA H105	2792.3421	3	931.4533	-1.39	27.8
	1xCAM C101	2636.2270	3	879.4126	1.22	20.2
^{99}*NECFLQHKDDNPNLPR*^{114}	1xCAM C101; 1xHNE MA H105/K106	2153.0444	3	718.3517	1.57	28.8
	1xCAM C101	1996.9294	2	998.9680	0.17	20.9
^{115}*LVRPEVDVMCTAFHDNEETFLK*^{136}	1xCAM C124; 1xHNE MA H128	2806.3790	3	936.1309	0.15	40.5
	1xCAM C124	2650.2640	3	884.0940	-1.51	35.3
^{115}*LVRPEVDVMCTAFHDNEETFLKK*^{137}	1xCAM C124; 1xHNE MA H128	2934.4740	4	734.3732	0.70	38.3
	1xCAM C124	2778.3590	3	926.7914	-0.43	33.1
^{226}*AEFAEVSKLVTDLTK*^{240}	1xHNE SB K233	1788.9993	2	895.0037	-0.55	47.3
	-	1650.8949	2	825.9522	-1.54	39.3
^{241}*VHTECCHGDLLECADDR*^{257}	3xCAM C245; C246; C253; 1xHNE MA H242	2242.9526	3	748.3223	-0.11	26.1
	3xCAM C269; C270; C277	2086.8375	3	696.2834	0.65	19.2
^{241}*VHTECCHGDLLECADDRADLAK*^{262}	3xCAM C245; C246; C253; 1xHNE MA H242	2741.2328	3	914.4163	-0.74	26.7
	3xCAM C245; C246; C253	2585.1177	3	862.3770	0.27	22.7
^{241}*VHTECCHGDLLECADDRADLAKYICE NQDSISSK*^{274}	2xCAM C245; C246; 1xOxidation C265; 1xHNE MA H247	4067.8089	4	1017.7064	1.05	27.0
	2xCAM C245; C246; 1xOxidation C265	3911.6939	4	978.6787	0.02	23.5
^{373}*VFDEFKPLVEEPQNLIK*^{389}	1xHNE SB K378	2183.1998	2	1092.1036	-0.21	48.4
	-	2045.0954	2	1023.0520	-0.80	38.3
^{501}*EFNAETFTFHADICTLSEK*^{519}	1xCAM C514; 1xHNE MA H510	2416.1377	2	1208.5740	-1.34	42.1
	1xCAM C514	2260.0227	2	1130.5161	-1.13	35.7
^{501}*EFNAETFTFHADICTLSEKER*^{521}	1xCAM C514; 1xHNE MA H510	2701.2814	3	901.1012	-3.03	39.8
	1xCAM C514	2545.1664	2	1273.0881	-1.13	33.7
^{542}*EQLKAVMDDFAAFVEK*^{557}	1xHNE SB K545	1979.0194	2	990.0121	1.15	47.3
	-	1840.9150	2	920.9618	-0.88	41.7

[a] For each peptide, top line (white background) is the modified peptide and the bottom line (grey background) is the non-modified peptide.
[b] Theoretical mass

3.2. Confirmation of HNE adducts formation by MALDI-TOF-MS

As MALDI-TOF-MS allows the analysis of large proteins, the formation of HNE adducts on HSA (sample treated in a 1:10 protein:HNE molar ratio) could also be confirmed by this method. The data

in **Fig. 2A** shows that the HNE-modified HSA was shifted by +477 Da compared to native HSA (**Fig. 2B**), which corresponds approximately to the addition of 3 molecules of HNE per HSA molecule on



average, assuming the adducts were formed via Michael addition (+156 Da). Nevertheless, this number could be slightly higher if there were a mixture of different adducts being formed on HSA, as suggested by the MS/MS data described above.

Fig.2. MALDI-TOF-MS spectra of HSA treated with HNE at a 1:10 protein:HNE molar ratio (**A**) and native HSA (**B**). The difference in mass between the peak representing the modified protein and the peak representing the native form correspond to the addition of ~3 molecules of HNE *per* HSA.

3.3 SA369 Anti-HSA-HNE antibody screening

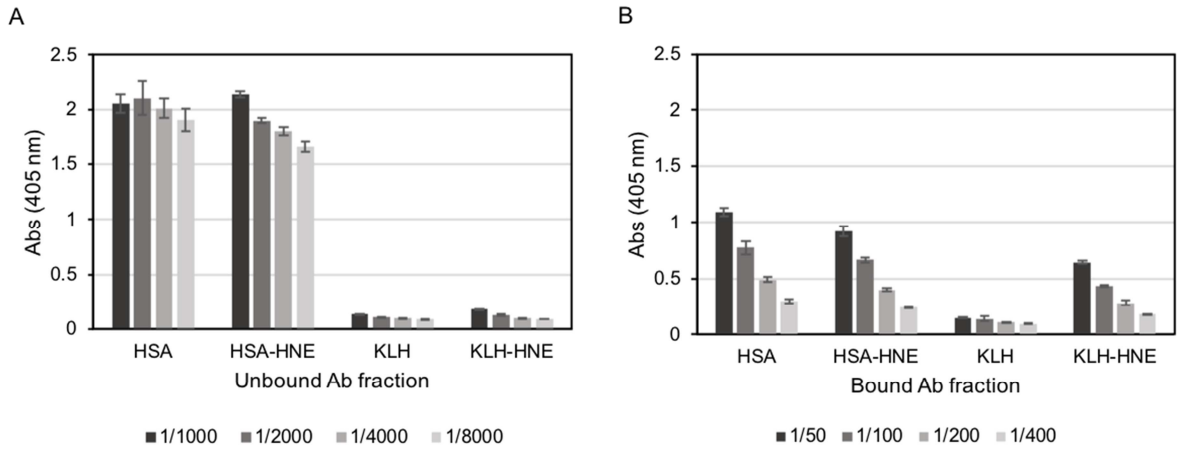
The screening of the polyclonal antibodies in each of the collected bleeds (and in the pre-immune bleed, as control) was performed by an ELISA assay with HNE-modified OVA coated plates. As the HNE is a small molecule (MW 156 Da) and does not bind effectively to the microplate wells, the assay was performed using a protein other than HSA to discriminate the anti-hapten specific antibodies (anti-HNE) from the anti-carrier specific antibodies (anti-HSA). OVA was chosen as the carrier protein because, despite its name, it does not have any sequence similarity to HSA, but it has a similar molecular weight. ELISA assays with non-modified OVA were also performed as a control.

All the four bleeds collected after the immunization exhibited a good immune response against HNE-modified OVA (**Supplementary Fig. 2**), as revealed by antibody titers >1/20,000 achieved by direct binding ELISA. It was also observed that, surprisingly, the strength of the antibody response seemed to decrease with time post inoculation.

3.4. Enrichment of SA369 Anti-HNE Antibodies

The polyclonal antibodies (pAb) of interest (anti-HNE and, possibly anti-HSA-HNE antibodies) were purified from the crude serum using two different approaches. The first strategy involved an initial purification step using an HSA-HNE coupled resin for recovery of all anti-HSA and anti-HSA-HNE

antibodies, followed by a second purification step with an HSA-coupled resin, for the depletion of antibodies with affinity for unmodified HSA. The enrichment of anti-HSA/HSA-HNE antibodies in the first chromatography step gave a strong peak, but most of these antibodies were depleted on the second column, indicating that the majority of antibodies recognized HSA (**Supplementary Fig. 3A**). The small amount of enriched SA369 pAb mixture obtained had stronger affinity for HSA-HNE than for unmodified HSA, although there was still significant reactivity to the latter. The enriched pAb preparation also recognized OVA-HNE, but showed very low binding to unmodified OVA, indicating that the pAb mixture contained antibodies with affinity for HNE but not for an unrelated protein (**Supplementary Fig. 3B**). However, as cross-reactivity to unmodified HSA was always observed and the yield and concentration of the enriched antibodies achieved was not high enough for further experiments, an alternative strategy was tested. This involved a first purification of all the IgGs from the sera using a protein G resin, followed by a second purification with an NHS-activated sepharose column coupled to HNE-modified keyhole limpet hemocyanin (KLH) for recovery of all the anti-HNE and potentially anti-HSA-HNE antibodies and depletion of the anti-HSA antibodies (**Supplementary Fig. 4**). KLH was chosen as carrier protein as it is a large protein (390 kDa) and has a high content of histidine and lysine residues (219 and 151, respectively), and is therefore a good target for HNE modifications. The purity of the enriched SA369 anti-HNE pAb fraction was assessed by SDS-PAGE electrophoresis (**Supplementary Fig. 5**). Traces of serum albumin and immunoglobulins of higher molecular weight (possibly IgM) were still observed in the gel, so the SA369 anti-HNE antibody



cannot be considered completely purified, but rather enriched. The bound and unbound pooled fractions collected from the second chromatography step with the KLH-HNE conjugated resin were evaluated by an ELISA assay to assess the binding to two different HNE modified proteins, and their unmodified controls.

Fig. 3. The unbound (A) and bound (B) IgG material collected from the KLH-HNE affinity chromatography step was analysed by direct ELISA to assess the affinity towards HNE modified and non-modified HSA and KLH. Different dilutions ranges were applied to bound and unbound fractions as the antibody concentration was different and are indicated by the symbols under the graphs. All the samples were analysed in triplicate.

It was expected that this alternative enrichment strategy would select antibodies that recognized HNE with less cross-reactivity to HSA. **Fig. 3** shows that the unbound fraction (that did not bind to the KLH-HNE modified resin) had a very high binding towards both HSA and HSA-HNE but showed little reactivity to KLH or HNE-treated KLH, indicating that substantial depletion of the anti-HSA antibodies was achieved with the second chromatography step. However, the bound fraction had similar binding to HSA, HSA-HNE, and HNE-modified KLH, but low affinity for native KLH. The cross-reactivity to native HSA suggests that some of the epitopes recognized by the SA369 enriched antibodies include not only the HNE moiety of the adducts on HSA or KLH, but also regions of the HSA, to which they can bind even in the absence of HNE.

In order to investigate further the specificity of the SA369 enriched antibodies, HSA, OVA or alpha-1 anti-trypsin (A1AT) were incubated with HNE in three different molar ratios, and the binding of the pAb preparation to these different HNE-modified proteins was assessed by Western blotting (**Fig. 4A**. A1AT was tested as an additional plasma protein of comparable size with clinical relevance. The same western blot was also performed with a commercial anti-HNE pAb, as a positive control, which demonstrated treatment concentration-dependent binding to all three HNE-modified proteins but not to the unmodified controls, reflecting the recognition of HNE adducts regardless of the protein carrier (**Fig 4B**).

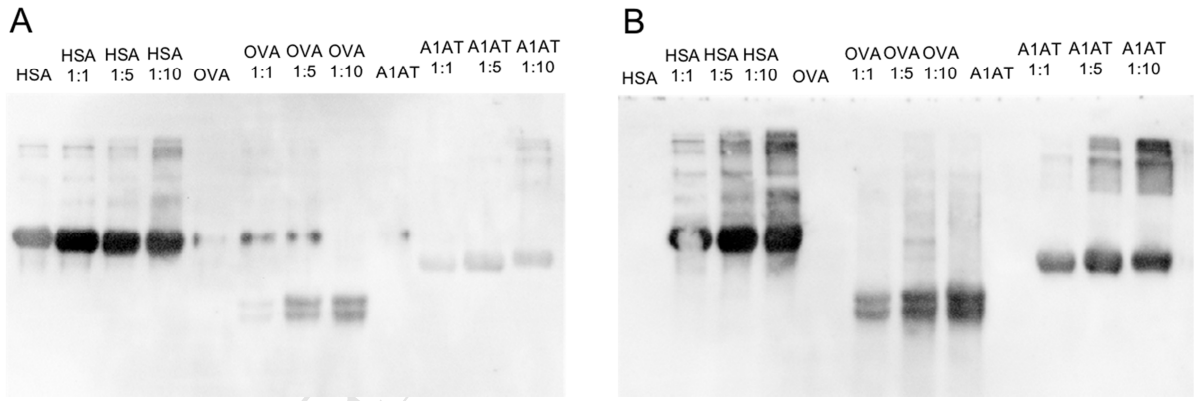


Fig. 4. Human serum albumin (HSA), ovalbumin (OVA) and alpha 1-antitrypsin (A1AT were treated with HNE at 1:1, 1:5 and 1:10 protein:HNE molar ratios, and their binding to the Abcam anti-HNE pAb (A) and SA369 anti-HNE enriched pAb (B) was determined by western blotting. Both membranes were visualized under identical conditions, so the binding strength would be comparable.

The enriched SA369 anti-HNE pAb showed strong concentration-dependent binding to HSA-HNE, but also bound weakly to unmodified HSA (**Fig. 4B**), confirming the results obtained by the ELISA assay. In contrast, the OVA-HNE and A1AT-HNE were recognized more weakly, both in comparison to the HSA-HNE and the binding of the commercial antibody, and there was little detection of native OVA or A1AT. This suggests there is additional combined specificity to the carrier (HSA) in the purified SA369 pAb preparation. The decrease in signal at the MW corresponding to the native proteins with increasing HNE:protein molar ratio in both blots may be explained by the formation of higher molecular weight forms and aggregates seen in the gels.

3.5. Epitope mapping

In view of the apparent recognition of both HNE and epitopes from HSA in the SA369 pAb preparation, we wanted to investigate further the epitopes recognized. Therefore, SPOT peptide array technology was used to identify and compare the binding sites of the commercial anti-HNE pAb (generated against HNE-modified KLH), and the in-house SA369 enriched pAb (generated against HNE-modified HSA), using peptide arrays designed with 15-mer peptides covering the whole sequence of HSA and OVA. One set of arrays were incubated with HNE prior to the incubation with each individual antibody, and the analysis was performed with control replicate membranes that were not treated with HNE.

Fig. 5 shows that both antibodies are able to recognize many epitopes in the HNE-treated arrays, as indicated by the darker spots. For the commercial pAb, twelve main peptides on the array stand out in terms of the intensity of signal: A27-29, C1-3, C16, D2-4, E11-16, E21-24, F2-F7, F16-20, G12-17, G25-28, I1-4 and I26-29 (**Fig. 5A** and **Suppl. Table 1** for the peptide sequences), but binding to HSA control membrane was very weak (**Fig. 5C**). There was substantial overlap in the set of HNE-modified peptides recognized by the in-house SA369 pAb (**Fig. 5B**), indicated by the red boxes, but there was also binding to a number of other peptides, and it can be seen that the background binding to the control HSA membrane (**Fig. 5D**) was substantially stronger than that of the commercial pAb. Similarities between the SA369 pAb binding to the HSA-HNE and HSA control membranes are indicated by yellow boxes. The OVA-HNE arrays showed clear similarities between the binding of the commercial pAb (**Fig. 5E**) and the SA369 pAb preparation (**Fig. 5F**), although the non-specific background staining was higher for the latter. Five regions were recognized by both pAbs (A5-9, B5-9, B26-30, F8-16 and G3), while the peptides D8-11 were only recognized by SA369 pAb and only the commercial pAb bound to C1.

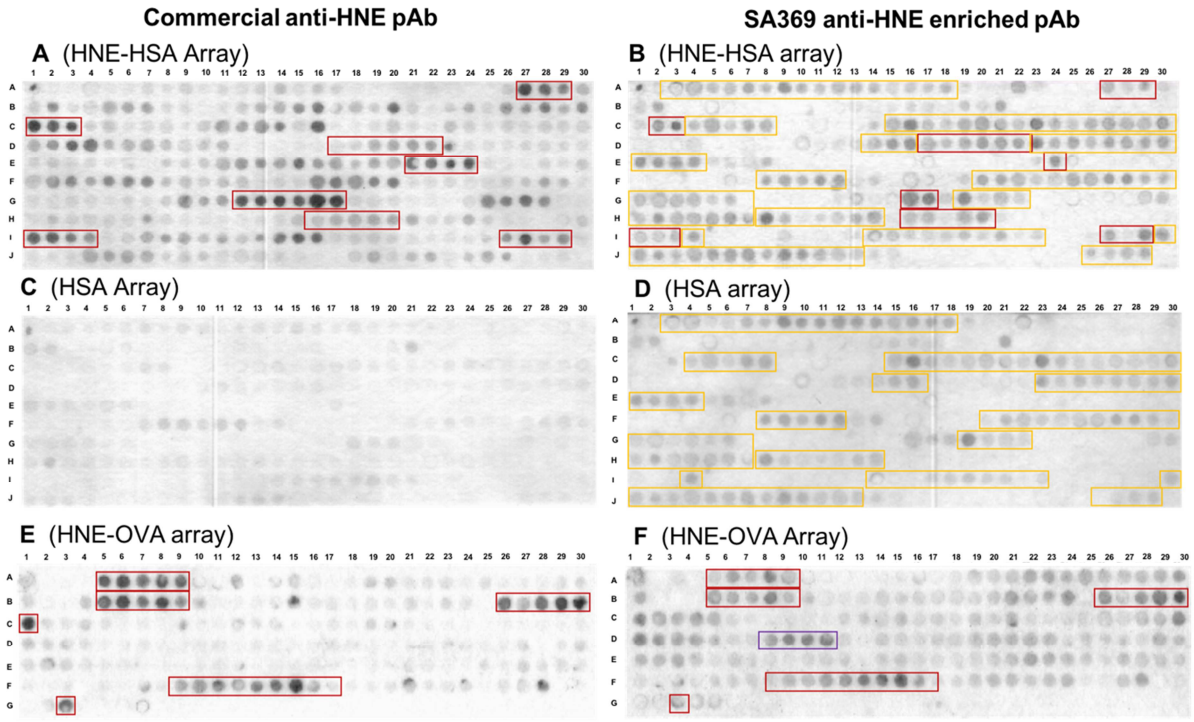


Fig. 5. Comparison of pAb binding to HNE-treated and untreated peptide arrays. A and B are arrays of HSA peptides (15-mer) treated with HNE; C and D are the equivalent arrays without HNE treatment; E and F are arrays of ovalbumin peptides (15-mers) treated with HNE. Binding of the commercial Abcam anti-HNE pAb is shown in the lefthand column (panels A, C, E) and binding of the SA369 enriched pAb is shown in the righthand column (panels B, D, F). Peptides recognized by both polyclonal antibodies are boxed in red and cross-reactivity to HSA peptides recognized by the SA369 enriched pAb are boxed in yellow. The single purple peptide represents an OVA peptide recognized by the in-house pAb but not by the commercial pAb. All experiments were carried in duplicate and representative blots are shown.

3.6. Anti-HNE Sandwich ELISA assay

Despite the polyclonality of both the commercial antibody anti-HNE and the SA369 enriched pAb raised against HSA-HNE, it was clear that both contain individual clones that are able to recognize HNE adducts when covalently bound to a protein but have some diversity in the epitopes recognized. From the translational point of view, these pAbs would be most useful if they could be combined in a sandwich assay (either ELISA or a lateral flow assay (LFA)), as these are up to 5 times more

sensitive than a direct ELISA and suitable for analysis of complex samples. Consequently, a paired sandwich ELISA assay was developed by using the enriched SA369 pAb as a capture Ab, and the Abcam anti-HNE pAb was linked to horseradish peroxidase (HRP) for use as the detection Ab. The ELISA was evaluated for its ability to recognize HNE adducts on different HNE-treated proteins, and **Fig. 6A** shows that the signal from the HNE-modified proteins increased with increasing concentrations of the capture pAb, reaching a plateau at approximately 2 µg/mL for KLH, while non-modified proteins were not strongly recognized. For equivalent protein antigen concentrations, KLH-HNE showed by far the strongest response, followed by OVA-HNE, with HSA-HNE giving the weakest signal (filled triangles in Fig. 6), although this was still significantly higher than the negative controls. The strong KLH response is in line with the large number of nucleophilic amino acids it contains (52 Cys, 219 His and 151 Lys) compared to the other proteins, and therefore the number of potential adducts present in each protein. Subsequently, an optimum capture antibody concentration of 1 µg/mL was chosen and the analysis of HSA-HNE was investigated in more depth; it can be seen a strong specificity for HSA-HNE over unmodified HSA was achieved with essentially negligible cross-reactivity to HSA (**Fig. 6B**). The response was linear until 2-3 µg /mL of antigen, but above this was saturated.

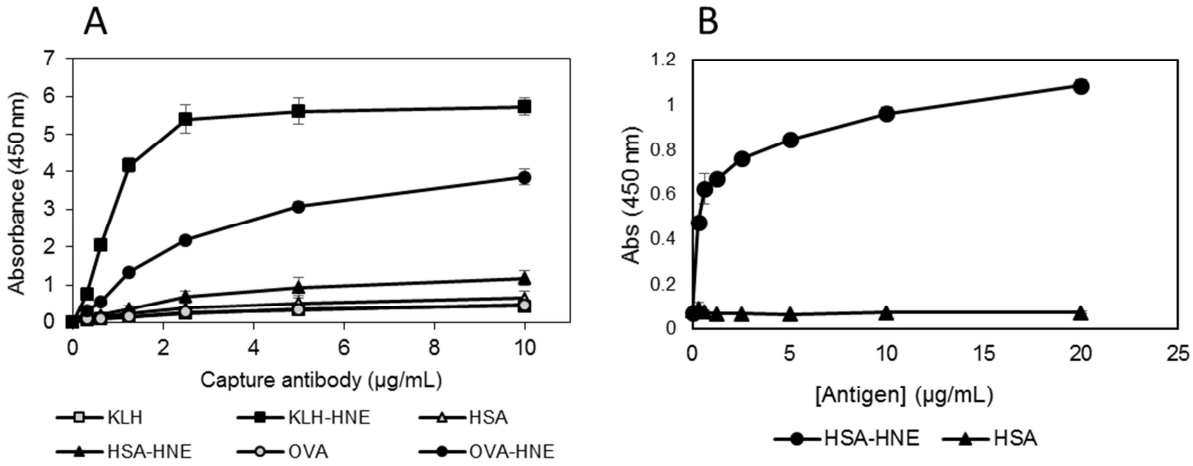


Fig. 6. Sandwich ELISA combining the enriched SA369 pAb as the capture Ab with HRP-linked Abcam anti-KLH-HNE at the detection Ab for analysis of HNE adducts. (A) Analysis of HNE-treated KLH, HSA and OVA by direct ELISA using the enriched SA369 pAb. Proteins were incubated individually with HNE at a 1:10 protein-HNE molar ratio, and non-HNE-treated protein tested as negative control. (B) Anti-HNE ELISA using a fixed concentration of the capture antibody (1 µg/mL) and a fixed concentration of the detection pAb (0.5 µg/mL). The antigen was added in a range of concentrations from 0 to 20 µg/mL. The assays were performed in triplicate and data is presented as mean ± SEM.

4. Discussion

There is substantial interest in HNE as a reactive lipid peroxidation breakdown product that can modify proteins and can be considered as a biomarker oxidative damage, as HNE-protein adducts

have been studied in a variety of chronic diseases [36,37]. Antibodies that recognize HNE-modified proteins are widely used in such studies; those that are commercially available have been raised against KLH as an antigenic carrier protein. Our aim was to produce an antiserum against HNE-modified HSA, as albumin is known to be susceptible to lipoxidation and there is great interest in oxidized forms as biomarkers [33,38–40]. Human rather than ovine albumin was used for the immunization process in order that the immune response could be significantly triggered by the modified protein alone; the HNE modification of ovine albumin may not have been sufficient in itself to evoke an immune response. In addition, we were interested to see if some selectivity for modified albumin, rather than just the modification itself, could be induced in the antibodies generated, potentially providing selectivity in diagnostic assays.

As HNE-protein adduct formation is potentially reversible, we initially immunised two sheep with HNE treated HSA and two other sheep with HNE treated HSA that had then been $NaBH_4$ reduced, which stabilizes the adduct (at the cost of changing the structure slightly). However, in the initial screening of the bleeds collected from the four sheep for binding to HNE modification, only SA369 immunised with non-reduced HNE treated HSA showed reactivity towards HNE modifications. We focused on this source as, from the application point of view, reduction of the HNE adducts does not occur *in vivo,* so the antibodies generated should recognise the HNE adducts that are likely to be formed *in vivo.*

The modifications to the HNE-treated HSA were confirmed by LC-MS/MS, and 15 unique peptides containing HNE adducts were found. Interestingly, Cys34, which is known to be the only free thiol in HSA, was not found to be modified by HNE and was normally observed in the carbamidomethylated form. However, owing to the reduction-alkylation protocol used, this does not preclude the presence of reversible modifications of cysteine, including mixed disulfides such as cysteinylation, that could have prevented further modification by HNE.

The antiserum obtained was enriched using 2 different two-step approaches and showed binding to several HNE-modified proteins but also to unmodified HSA in direct ELISAs and western blotting, although in these assays the binding to HNE-HSA was stronger than to other HNE-modified proteins. In contrast, the commercial pAb raised against HNE-modified KLH (a very large protein containing a high number of lysines and prone to modification by HNE) had much lower cross-reactivity to unmodified proteins and showed less carrier protein dependence. A novel approach that compared binding of pAb preparations to native or HNE-treated peptide arrays showed that the commercial pAb and the in-house enriched SA369 pAb overlapped in their recognition of epitopes in HSA-HNE and OVA-HNE, but there were also differences and the in-house pAb had a higher background recognition of HSA. Nevertheless, when a sandwich ELISA was established using the in-house SA369 pAb for antigen capture and the commercial pAb for detection, improved selectivity for modified protein over unmodified protein was achieved, including for HSA.

Some differences in the binding of the KLH-HNE column enriched SA369 pAb were apparent between the different assays used. The direct ELISAs showed relatively poor ability to discriminate between native and modified HSA, whereas western blotting indicated a substantially stronger binding to HSA-HNE, which was more in line with the findings from the peptide arrays. This is most likely because in a direct ELISA the antigen is in a folded and 3-dimensional conformational, therefore

representing a structural epitope, whereas both the peptide arrays and western blotting involve linear epitopes [41]. However, overall the data from all three methods supported the conclusion that the commercial pAb had specificity for HNE adducts regardless of the carrier protein, and lower cross reactivity to unmodified proteins, compared to the in-house pAb.

Thus, the data yielded generally consistent but somewhat unexpected results. The original aim was to purify or enrich antibodies that recognized HNE adducts on HSA, but did not cross react with unmodified HSA, and so the initial approach involved selecting antibodies that bound to a resin crosslinked to HSA-HNE, following by depletion of antibodies that bound to unmodified HSA-resin. This was partially successful, but the yield was extremely low and some cross-reactivity to native protein remained. The extensive depletion in the 2nd step suggested that most of the proteins that were enriched in the first step actual recognized HSA, yet the enriched fraction bound to HNE adducts on OVA without a strong recognition of native OVA. The alternative approach selected IgGs in the first step and should have selected only pAbs with affinity for HNE-adducts without any carrier specificity in the second step, as KLH-HNE cross-linked to resin was used. Nevertheless, cross-reactivity to native HSA was still observed; this inability to separate the reactivities suggests that instead of the polyclonal pool containing some antibodies with specificity for adducts and others with specificity for HSA, it appears to contain a significant fraction of antibodies that recognize epitopes containing structural features of both HSA and HNE, as they cannot be readily deconvoluted. An alternative explanation could be that, as the commercial HSA used for the majority of this study was purified from plasma, it may already contain some oxidative modifications, as has been described previously [42]**.** To investigate this, the reactivity of the SA369 pAb against recombinant HSA expressed in *Saccharomyces cerevisiae* was tested by ELISA. The recognition of recombinant or plasma purified HSA was indistinguishable in the ELISA, with the SA369 binding to both plasma purified and recombinant HSA, whereas the commercial anti-HNE pAb from Abcam showed no binding. (**Supplementary Fig. 6**). This suggests there is recognition of both protein and modification in the epitope.

It is also possible that reactivity to HNE on other proteins such as A1AT might result from reversibility of HNE-adducts, especially Schiff's bases, leading to transfer of HNE to other proteins within the host. In contrast, in two previous studies anti-HNE antibodies raised against HNE-treated HSA were purified only with a protein A resin and the authors reported strong binding to the immunogen and almost negligible reactivity to unmodified HSA, which is surprising in the absence of a relevant depletion step [14,29]. This discrepancy emphasizes the need to understand in more detail which peptide epitopes of HNE-modified HSA triggered B-cell clonal expansion during immunization and are recognized by the resulting immunoglobulins.

The use of HNE-treated peptide arrays provided detailed information on the linear epitopes recognized by the in-house SA369 pAb raised against HSA-HNE, and supported the concept that it contains a diversity of antibodies, some of which are able to recognize specifically the HNE part of the adduct (e.g. on OVA-HNE), while others bind to HNE combined with residues from HSA. Examination of the peptides recognized strongly by both pAbs indicated that they contain at least two-three nucleophilic amino acids in the sequence that are susceptible to attack by HNE (Cys, His or Lys) [43],

although for HSA it is well established that only Cys34 is in the thiol form and susceptible to attack by HNE; all other cysteines are disulfides [33,44,45]. It was clear that the profile of peptide recognition by the two pAbs was similar for OVA, with relatively few reactive peptides, but distinct for HSA. One limitation that is important to note is that peptide array screening is not a quantitative assay; rather, it is semi-quantitative as the amount of peptide in each spot on the array varies due to difference in the synthetic yield and peptide purity [46] . However, arrays were prepared in duplicate and gave comparable results, suggesting that this was not a major issue.

To understand the relationship between the HSA-HNE epitopes detected and the structure of the protein, peptides found to be HNE-modified by LC-MS/MS were mapped onto the crystal structure of HSA and compared with those identified in the peptide arrays (**Fig. 6**). As the epitope arrays only indicate which peptide sequences were recognized by the Abs, the exact location of the HNE modification could not be pinpointed and therefore the possible modified residue was only labelled for the cases where the peptide detected matched the peptide identified by MS/MS. It can be seen that several peptides identified as HNE-modified by LC-MS/MS (**Fig. 7A**) corresponded with epitopes detected in the arrays (**Fig. 7B**). In particular, three HNE-modified peptides/residues detected by both pAbs (His105, His367 and His510), and two others detected only by the commercial pAb (His128 and His288), were found to be contain HNE adducts. It is notable that the majority of epitopes contained histidines, reflecting the higher stability of this adduct despite the greater reactivity of cysteine [8,9]. The peptide that showed the highest binding intensity for both pAbs had the sequence ^{365}DPHECYAKVFDEFKPLV^{381}, and contained the potential target amino acids His367, Lys372 and Lys378. It is worth noting the residue numbering does not include the signal peptide (1-18) and pro-peptide (19-24) of HSA.

These are part of an alpha helix and a loop located at the surface of the protein, which might play an important role in immunogenicity. The amino acid position within the peptide sequence seemed to be critical for the modification and/or the binding of the pAbs, as some peptides differing only by two neutral amino acids at one end of the peptide were consistently not recognized. Steric hindrance might explain this absence of binding, as adjacent bulky amino acids can hinder the access of HNE to the target amino acid, and thus slowing the chemical reaction. Alternatively, it is important to note that the isoelectric point, and consequently charge state of a peptide, as well as neighbouring-group participation effects, have a significant effect on the chemical reactivity of functional groups of individual amino acids. These are likely to be different even between adjacent, overlapping peptides on the array and may also account for the differences in modification observed for the same residue in different peptides. Another possible explanation that synthetic linear peptides are not always authentic structural mimics of epitopes on globular proteins, where protein folding gives a distinct 3-dimensional structure [47]. This can lead to underestimation of affinity of the antibody binding. Detailed examination of the three-dimensional structure of HSA indicated that most of the epitopes recognized by the pAbs are alpha helix-containing loops, which might suggest that certain secondary structures and loops, in particular, might be more immunogenic [48].

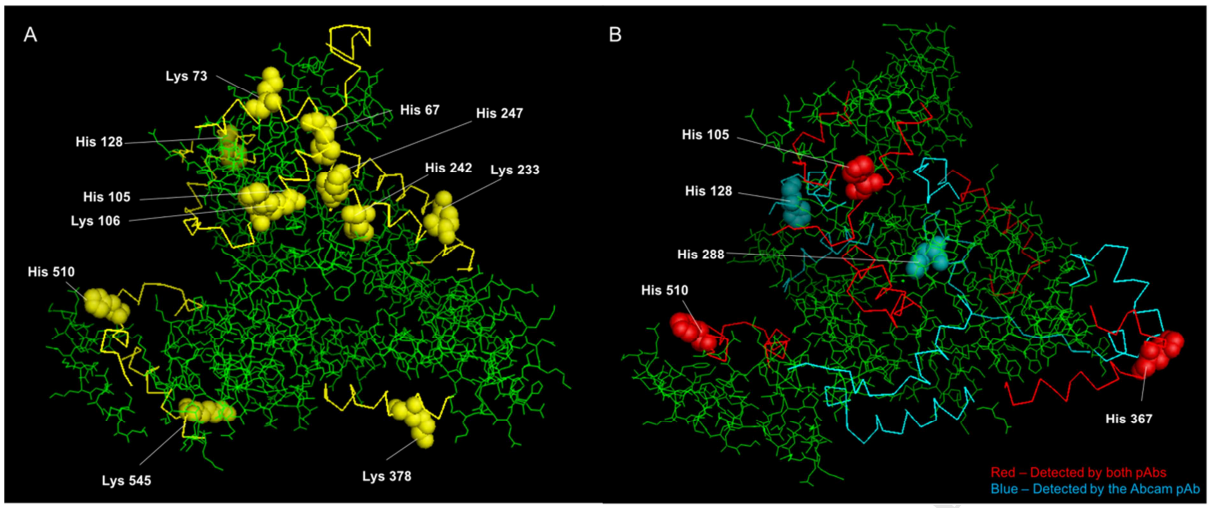


Fig 7. 3D structure of HSA (PDB:1AO6) depicting HNE-modified peptides identified using the Sequest database search (A), and HNE-modified peptides detected by the commercial anti-HNE pAb (blue) and the in-house SA369 enriched pAb (red) on the HNE-treated HSA peptide arrays (B). The peptides recognized are indicated by backbone structure unless the nucleophilic residue likely to be responsible within the peptide could be identified. The residue numbering does not include the signal peptide (1-18) and pro-peptide (19-24) of HSA, in contrast to the arrays which did include these peptides (Supplementary Table 1).

In the development of diagnostic assays for lipoxidation of HSA by HNE, cross-reactivity to HSA would be a major drawback. However, the sandwich ELISA assay developed by pairing the in-house pAb as capture antibody and the commercial pAb as the detection antibody was shown to discriminate well between HSA-HNE and native HSA, and was sensitive to concentrations as low as 1 μg/mL of antigen. The availability of a supply of enriched SA369 pAb would make economically viable further development and testing of assays, including lateral flow assays, to analyse HSA-HNE and investigate its potential as an inflammatory marker. Alternatively, hybridoma technology or phage display for selection of single clones might offer a good approach for the future to overcome cross-reactivity issues and improve specificity.

Acknowledgements

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Epitope mapping and characterization of 4-hydroxy-2-nonenal modified-human serum albumin using two different polyclonal antibodies

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Highlights

- 15 HNE-modified peptides on human serum albumin were identified by LC-MS/MS
- Antiserum generated against HSA-HNE showed binding to several HNE-modified proteins
- A novel HNE-peptide array approach was designed to test antisera binding
- HNE-modified peptides identified by LC-MS/MS were confirmed by peptide arrays
- Different epitope binding was seen between the in-house pAb and a commercial pAb