

REVIEW

Oxidation of proteins: Basic principles and perspectives for blood proteomics

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Protein oxidation mechanisms result in a wide array of modifications, from backbone cleavage or protein crosslinking to more subtle modifications such as side chain oxidations. Protein oxidation occurs as part of normal regulatory processes, as a defence mechanism against oxidative stress, or as a deleterious processes when antioxidant defences are overcome. Because blood is continually exposed to reactive oxygen and nitrogen species, blood proteomics should inherently adopt redox proteomic strategies. In this review, we recall the biochemical basis of protein oxidation, review the proteomic methodologies applied to analyse redox modifications, and highlight some physiological and *in vitro* responses to oxidative stress of various blood components.

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1 Introduction

Oxidative modifications to proteins have been for the most part considered as deleterious, irreversible, and ultimately leading to protein inactivation, degradation, and clearance [1, 2]. From decades of molecular studies of protein oxidation, the picture has dramatically evolved, and protein oxidations are now considered as two-faced modifications: on the one hand, oxidation mechanisms take part in many normal regulatory processes (beside energy conversion), such as enzyme activity modulation [3], signalling [4, 5], or gene reg-

ulation [6–9]. On the other hand, oxidative modifications also appear when oxidative stress overcomes antioxidant defences, and are then damaging [10, 11]. The response of living systems to oxidative stress is of primary importance in understanding cellular defence and aging [12–15]. Disorders of normal oxidative metabolism, or damages due to oxidative stress, have also been proven to be key players in a broad spectrum of diseases, from neurodegenerative disorders, such as Alzheimer disease [16–22], to various kinds of cancer [23–26], diabetes [27–29], and atherosclerosis [30–33].

From a molecular point of view, protein oxidation results in a wide variety of chemical modifications, ranging from protein backbone cleavage or protein crosslinking, to amino acid side chain subtle modifications. Moreover, oxidative damage can introduce new reactive chemical groups into proteins, such as aldehyde and ketones, leave nonconventional peptidic ends at both the N- and C-termini. Such an array of modifications is difficult to tackle with a single analytical approach, and large-scale studies of protein oxidations have usually focused on the detection of a single modification, such as cysteine or tyrosine oxidation, or a subclass of

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Abbreviations: DNPB, dinitrophenylhydrazine; ECD, electron capture dissociation; MBLT, methylene blue light treatment; NO, nitric oxide; SOD, superoxide dismutase

oxidation by-products, such as protein carbonyls. Whereas these approaches are compulsory steps on the way to understand oxidation processes and their effect on a large scale, there is no way to date to get a full picture of a proteome oxidative status.

Nevertheless some blood components are inherently subjected to oxidative stress. For example, RBCs are typically exposed to continuous fluxes of ROS due to their function; platelets are exposed to ROS at sites of inflammation, where coagulation happens. Additionally, protein oxidation mechanisms are of particular interest in transfusion medicine, and have been hypothesised to be responsible for the “blood storage lesion” [34–38]. Whether blood product oxidation is due to exposure of blood to oxidizing agents during puncture, handling, and blood product preparation (*e.g.*, pathogen inactivation procedures), or appears only during storage as a result of aging or stress is still unclear. In this review, we give a biochemical overview of protein oxidation processes, discuss the main methodological and instrumental approaches for the study of protein oxidation on a large scale, and present selected examples with relevance to blood analysis.

2 Biochemical overview

The gist of this section is not to give a comprehensive and detailed overview of protein oxidation mechanisms; this has been done elsewhere [39–44]. It is rather to provide an overview of protein oxidation products in terms of diversity and chemical specificity in order to highlight the possible analytical workflows and current challenges in redox proteomics. Oxidative modifications of proteins are due to attacks by ROS such as hydrogen peroxide (H_2O_2), anion superoxide (O_2^-), or hydroxyl radical (OH^\bullet), and reactive nitrogen species (RNS) such as nitric oxide (NO), nitrate (NO_3^-), nitrite (NO_2^-), and peroxynitrites ($ONOO^-$), as shown in Fig. 1 [45]. These species can appear as by-products of oxygen metabolism, or be present in the environment, and their appearance is the result of a complex interplay between the environment, and the cellular enzymatic machinery. The attack of proteins by these highly reactive species can lead to amino acid side chain modifications, cleavage of protein backbone, generation of carbonyl derivatives and formation of crosslinked protein complexes. Some reactions are limited and specific to certain residues, whereas others give rise to widespread and nonspecific modifications. Moreover, reactive oxygen and nitrogen species are also responsible for damages to DNA bases and sugar moieties, and degradation of lipids through peroxidation, the by-products of which can in turn modify proteins.

2.1 Protein backbone oxidation and cleavage

Protein backbone can be attacked by hydroxyl radicals on the α -carbon of amino acids, resulting in the formation of a carbon-centred radical. Under anaerobic conditions, two such

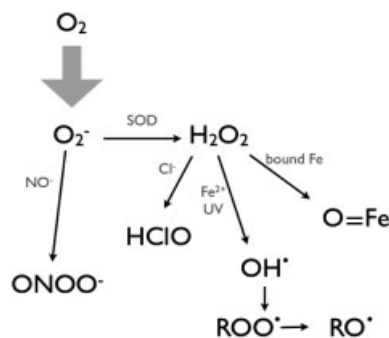


Figure 1. ROS and RNS. SOD stands for superoxide dismutase.

carbon-centred radicals can combine to form an intra- or interprotein crosslinkage (see below). In the presence of oxygen, a hydroxyl group can be added to this carbon-centred radical. The hydroxylated α -carbon can then undergo peptide backbone cleavage at the N–C bond through the α -amidation pathway, which leaves an amide at the C-terminal side of the N-terminal part of the protein, and a α -keto-acyl residue at the N-terminal side of the C-terminal part of the protein [40, 42, 46–48].

The same carbon-centred radical can undergo further attack by O_2 , that induces cleavage of the peptide backbone, at the C–C bond through the diamide pathway, as shown in Fig. 2A. The diamide pathway initially induces one cleavage, leaving a diamide derivative on the C-terminal side of the N-terminal part of the protein, and an isocyanate derivative on the N-terminal of the C-terminal part of the protein, that spontaneously form the derivatives shown in Fig. 2A [40, 46, 47].

Additionally, oxidation of glutamyl and prolyl residues can also result in single backbone cleavage. As shown in Fig. 2B, the cleavage at glutamyl residue leaves an amide at the C-terminal side of the N-terminal part of the protein, and a pyruvyl residue at the N-terminal side of the C-terminal part of the protein, whereas the prolyl oxidation leaves two protein fragments with conventional termini and releases α -amino butyric acid [47].

Lastly, beta-scission can occur through radical attack on the β (C3) position, as shown in Fig. 2C [40, 42, 49]: the release of the side chain as a carbonyl compound leaves a radical on the α -carbon, which is then prone to backbone cleavage through mechanisms similar to that of the diamide or α -amidation pathways.

2.2 Protein carbonyls

Protein carbonyls appear through side-chain oxidation of proline, arginine, and lysine, as shown in Fig. 3 [13, 42, 50]. They can also result from backbone cleavage through the α -amidation pathway or β -scission. Alternatively, they can be introduced into proteins through Michael addition of unsaturated aldehydes produced by peroxidation of lipids (the

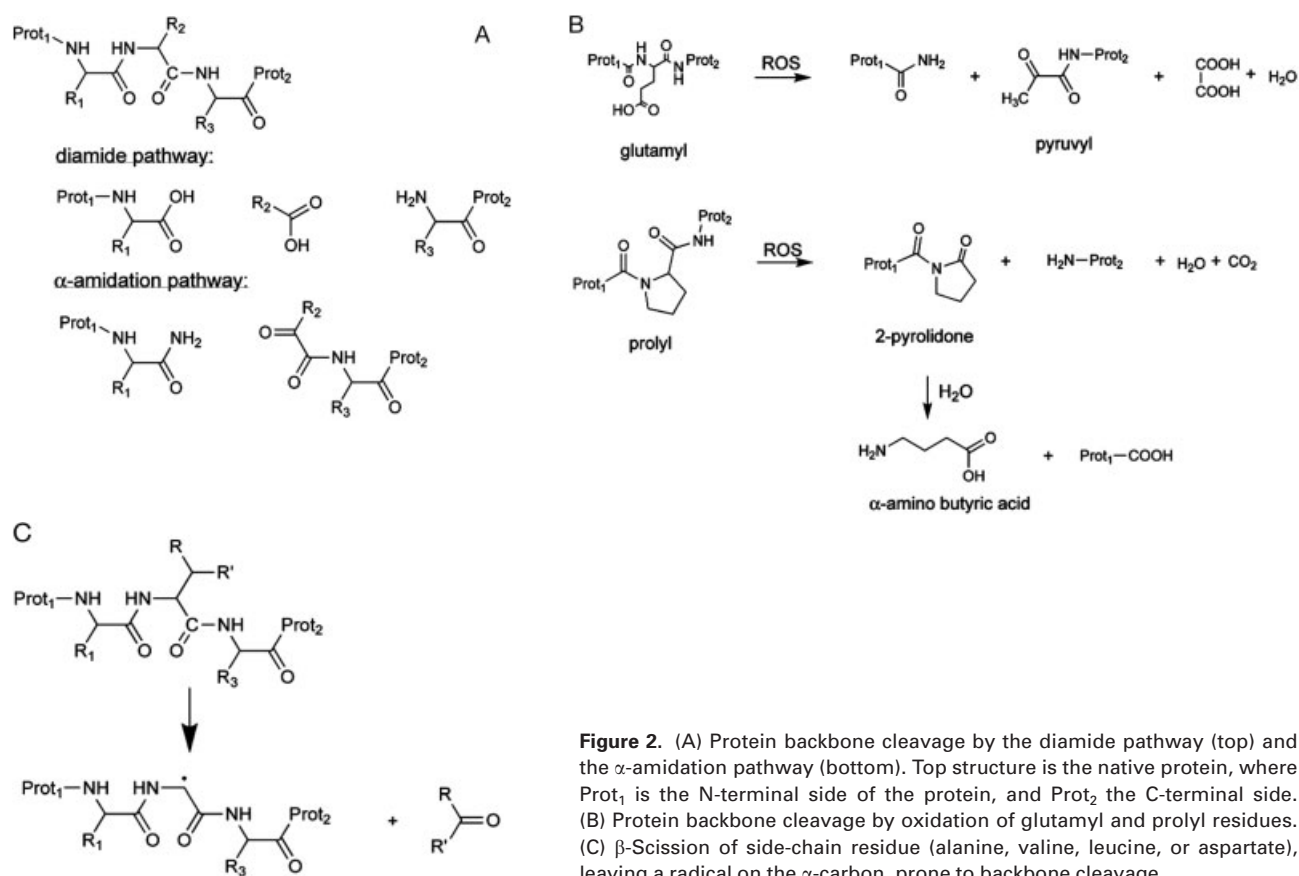


Figure 2. (A) Protein backbone cleavage by the diamide pathway (top) and the α -amidation pathway (bottom). Top structure is the native protein, where Prot₁ is the N-terminal side of the protein, and Prot₂ the C-terminal side. (B) Protein backbone cleavage by oxidation of glutamyl and prolyl residues. (C) β -Scission of side-chain residue (alanine, valine, leucine, or aspartate), leaving a radical on the α -carbon, prone to backbone cleavage.

main product being the addition of 4-hydroxy-2-nonenal on cysteine, histidine, and lysine, as shown in ref. [51–54] and in Fig. 4).

As carbonylation results in the introduction of reactive aldehyde or ketone groups in the protein, they are easily quantifiable (see below) and are indeed considered in practice as reliable markers of oxidative stress [55, 56].

2.3 Protein thiols and thioethers

Cysteinyl thiols can undergo a large array of oxidative modifications, depending on their accessibility in the protein structure, and the species they can contact to. Moreover, as cysteines play a pivotal role in protein structure through the formation of disulphide bonds, their oxidation status is of primary importance for protein function. In the recent years, cysteine oxidation has been more and more recognised as a basal regulation mechanism [57]. Free sulphhydryl groups can undergo direct, reversible oxidation to sulphenic acid, and most often further irreversible oxidation to sulphinic and sulphonic acid, as shown in Fig. 5. Free cysteines can also be nitrosylated [58].

In addition, free sulphhydryl groups can also form disulphide bridges with low molecular weight sulphhydryl compounds present in the protein environment, such as free

cysteine, and glutathione [59–62]. S-glutathionylation is in most cases a permanent modification, except if a second cysteine is present in the close vicinity, and is available for disulphide bridge formation.

Together with cysteine, methionine belongs to the most easily oxidisable amino acid. Its oxidation products are shown in Fig. 6 [63]. The cyclic oxidation–reduction of methionine through NADPH-dependant thioredoxin reductase is an important antioxidant mechanism [64–67]. Age-dependent increase in methionine sulphoxide content of proteins was reported for different tissues, notably erythrocytes [67].

2.4 Nitrotyrosine

Peroxonitrite (ONOO⁻) results from the reaction of superoxide (O₂⁻) with NO (see ref. [45, 68–77] for review). It is a strong oxidant with a short biological half-life. Once formed intravascularly, it can directly undergo oxidation with several biological targets or generate radicals resulting later in oxidation and nitration reactions. Tyrosine nitration occurs *via* a two-step mechanism: (i) a tyrosyl radical is formed, (ii) the tyrosyl radical reacts with the free radical NO to form 3-nitrotyrosine (Fig. 7) [78]. The latter has been revealed as a

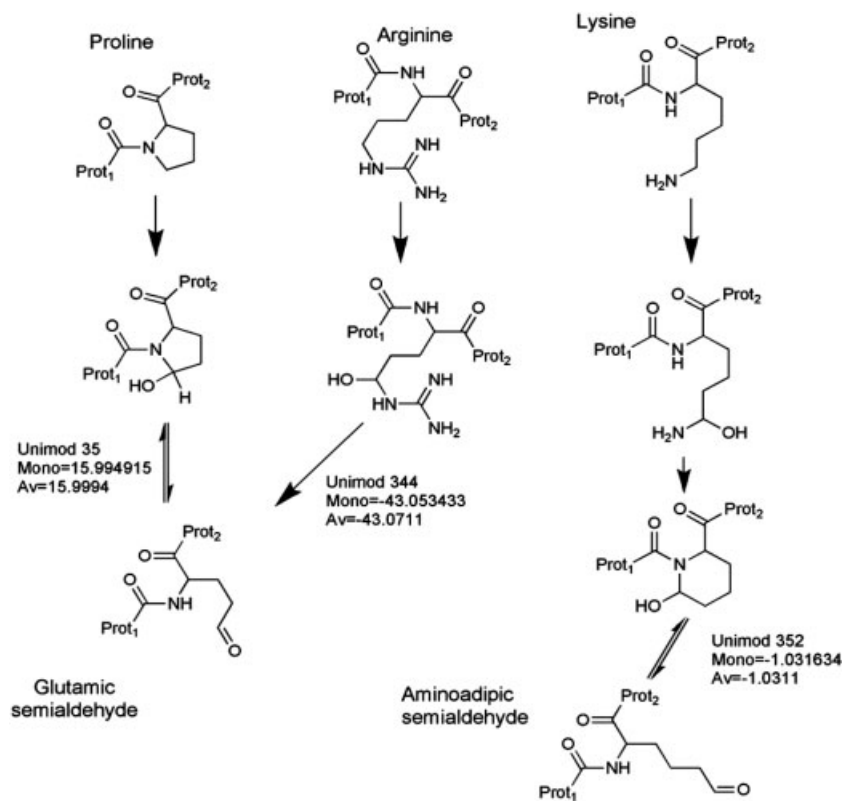


Figure 3. Carbonylation of amino acids side chains. Mass differences are given compared to the unmodified amino acid [196].

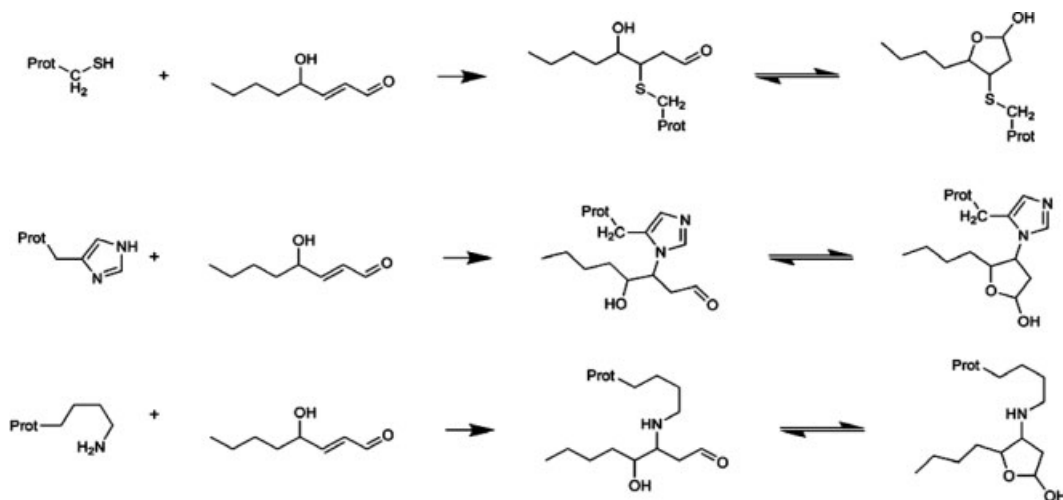


Figure 4. Introduction of carbonyls into proteins through Michael addition of 4-hydroxy-2-nonenal on cysteine (top), histidine (middle), and lysine (bottom) side chains.

biomarker of nitrosative stress and may serve as predictor of coronary artery disease [73, 79].

2.5 Protein crosslinking

As mentioned above, protein backbone can be attacked by hydroxyl radicals on the α -carbon of amino acids, resulting in

the formation of a carbon-centred radical. In the absence of oxygen, two such carbon-centred radicals can combine to form a covalent intra- or interprotein crosslink. Additionally, intra- or interprotein crosslinks can appear through cysteine oxidation *via* the formation of disulphide bridges. Lastly, other crosslinks induced by oxidation of specific residues have been reported, such as dityrosine formation [80], or

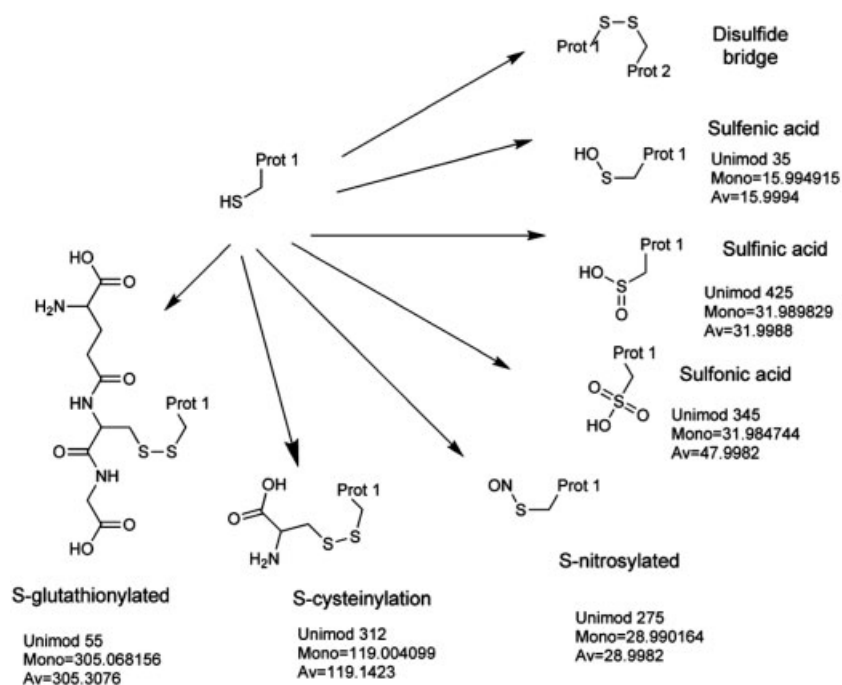


Figure 5. Main oxidation products of cysteine. Mass differences are given compared to the unmodified amino acid.

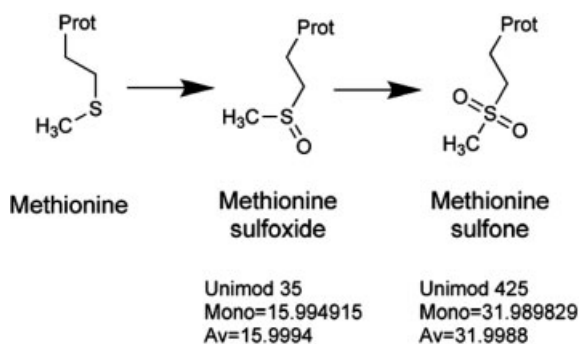


Figure 6. Methionine oxidation products.

sulphur–nitrogen crosslinking (for example, Cys–Lys and Cys–Arg) [81].

3 Methodologies for redox proteomics

The field of redox proteomics inherits a whole armada of methodologies for the analysis of protein oxidation products from classical biochemistry studies. Nevertheless, proteomics aim at analysing the whole proteinaceous content of a given sample, and identifying all modifications present down to the single amino acid level. At the same time, oxidative modifications are nonstoichiometric, and present a large diversity (protein fragments with both conventional and nonconventional termini, hydroxylated protein backbone, carbonyls, oxidised cysteines and methionines, nitrotyrosines, crosslinked proteins, just to name the main mod-

ifications). There is to date no proteomic workflow able to catch up with such a diversity. Most redox proteomic studies thus focus on a particular type of oxidative modification, a few of which are described below.

3.1 Carbonyl detection and quantification

Carbonyls have been long regarded as global markers of protein oxidation. Carbonyl tagging is relatively easy to perform due to the presence of reactive aldehyde or ketone groups. These groups react quantitatively with hydrazine to form hydrazone. Carbonyls can thus be quantified spectrophotometrically by 2,4-dinitrophenylhydrazine (DNPH, the structure of which is shown in Fig. 8). The results are usually expressed in moles of carbonyls *per* gram of proteins [82, 83]. Such spectrophotometric assay is not exempt from biases, such as the presence of excess DNPH [84], or nonprotein carbonyls. Alternatively, ELISA assays have been developed for the quantitation of DNPH-derivatised carbonyls [85–87]. The same chemistry can be used in combination with gel electrophoresis, followed by an immunodetection [88, 89]. Alternatively, Yoo and Regnier [90] have developed a biotinylation strategy for the specific labelling of carbonylated proteins after 2-DE.

3.2 Carbonyl enrichment

Another possible strategy is to use DNPH derivatisation in combination with anti-DNPH antibodies to immunoprecipitate and enrich carbonylated proteins, which has been demonstrated by England and Cotter in the study of ER pro-

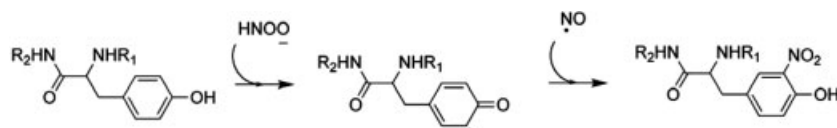


Figure 7. Formation of 3-nitrotyrosine.

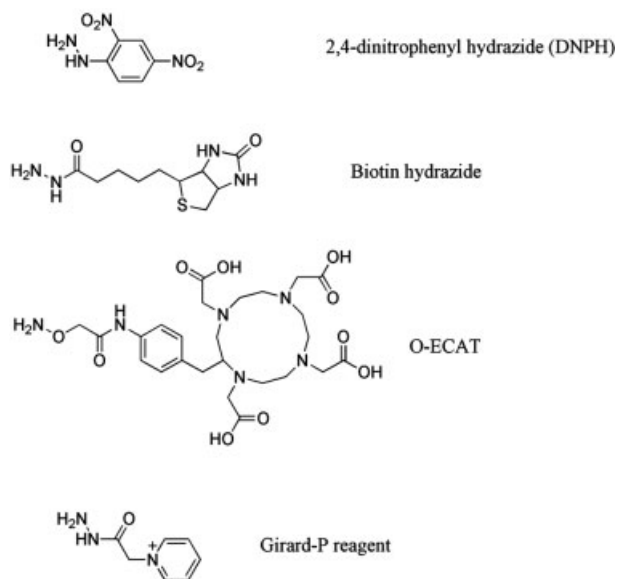


Figure 8. Probes reactive toward the aldehyde and ketone groups of carbonyls. On the O-ECAT reagent, the four nitrogen atoms can coordinate a metal centre (not shown here).

tein susceptibility to oxidation by 2-DE and MALDI-TOF MS [91], and by Kristensen *et al.* [92] in rice leaf mitochondria oxidation study with 2-D LC-MS/MS.

An alternative is to use affinity baits for the specific isolation of carbonylated proteins. For example, one can use biotin hydrazine for the derivatisation of ketones and aldehydes, and avidin columns for specific isolation of derivatised peptides and proteins [93–98]. Interestingly, Mirzaei and Regnier [97] compared three different strategies based on biotin hydrazine tagging of carbonyls, affinity selection, proteolysis, RP-HPLC, and MS, and found that performing the affinity selection and chromatography at the protein level before proteolysis and mass spectrometric protein identification, was more informative because working with intact protein allowed the detection of crosslinked or truncated proteins. Using a similar approach, Roe *et al.* [99] directly derivatised glass beads with a hydrazine group, allowing spin down isolation of carbonylated proteins.

Regnier's group also introduced a different tagging reagent for carbonyls: Girard's P reagent, which bears a hydrazine group, together with a permanent positive charge. Using this tagging reagent in combination with strong cation exchange chromatography, authors were able to enrich carbonylated peptides [100], and quantify them through isotopically labelled Girard's P reagents [101].

Lastly, Lee *et al.* recently introduced the so-called "Oxidation dependent element coded affinity tags" (O-ECAT), which are probes bearing one aminoxy group able to form a covalent bond with aldehydes or ketones, and one metal-chelator moiety. Antibodies against the metal-chelator moiety allow the affinity selection of derivatised peptides and proteins, and the probe can be loaded with various metals prior to any labelling of carbonyls in order to provide relative quantification information in MS [102].

3.3 Probing thiols oxidation

Methods for the study of protein thiols oxidation states have been the subject of recent reviews [44, 103]. Briefly, most techniques lie in the differential labelling of free sulphhydryls and oxidised ones, for example, through maleimide, iodoacetamide, iodoacetate, and thiosulphate chemistries. For example, Baty *et al.* [104, 105] first blocked free cysteines with an alkylating reagent, then reduced the sample to make sulphhydryls previously involved in disulphide bridges and glutathionylation available, and labelled them with a fluorescent probe before 2-DE. Authors were thus able to study the effect of oxidants (such as diamide or H_2O_2) on thiols oxidation state, and therefore identify proteins susceptible to oxidation. Similarly, Laragione [106, 107] used the same method with a probe containing an affinity bait (biotin), and further detected initially thiol-oxidised proteins with a streptavidin–peroxidase conjugate after Western blotting.

The same methodology can be applied for the specific enrichment of oxidised-thiol containing proteins: free thiols are first blocked, oxidised thiols are reduced chemically and further reacted with a probe containing an affinity bait, such as biotin [108–112]. When isotope-coded-affinity-tags (ICAT) reagents are used in this way, relative quantification between two differentially oxidised samples can be obtained [113, 114]. These techniques are useful to identify oxidation-sensitive thiols, but fail to identify the type of oxidation.

More specific is the probing of cysteines susceptible to S-glutathionylation: Brennan *et al.* mimicked a particular oxidative stress by adding biotin-GSSG-biotin to rat tissues; upon excess GSSG, disulphide exchange occurs and glutathionylated proteins can be isolated by avidin columns. Doing so, authors were able to study the proteome of thiols susceptible to S-glutathionylation in heart tissues [115, 116].

Jaffrey *et al.* [117] also targeted a specific cysteine modification (S-nitrosylation, see Fig. 5) by first blocking cysteines, reacting nitrosothiols with ascorbate to leave free

cysteines, and finally reacting these cysteines with biotin-containing reagents for specific enrichment of initially S-nitrosylated proteins.

3.4 Nitrotyrosine

Most studies of tyrosine nitration [118] rely on immunological detection thanks to commercially available anti-nitrotyrosine mAb, whether in the ELISA format [119], in which case it is difficult to distinguish between free circulating 3-nitrotyrosine and protein-bound nitrotyrosine, or in Western blot format after 1-D or 2-DE [120–122]. Immunoprecipitation with antinitrotyrosine antibodies allowed Nikov *et al.* [123] to map nitration sites of HSA by MS.

Interestingly, Zhang *et al.* [124] have recently introduced a methodology for selective isolation of nitrotyrosine-containing peptides: first, primary amines are acetylated, and free sulphhydryls are blocked, nitrotyrosines are then reduced to aminotyrosine, which are then acetylthioacetylated, and the resulting group is deprotected to leave a free sulphhydryl group, which in turn allows specific enrichment of peptides initially containing 3-nitrotyrosine. Doing so, authors were able to dramatically enrich peptides containing 3-nitrotyrosine compared to a direct mass spectrometric analysis.

3.5 Mass spectrometric and bioinformatics challenges

MS is now well established as a central identification technology in proteomics. Nevertheless, the identification and location of PTMs remains a challenge in routine analysis [125]. In the specific context of oxidative modifications, two major difficulties arise due to heterogeneity of possible modifications; first, whereas MS/MS is perfectly suited to the detection and identification of chemical modifications on amino acid side chains, the number of possible modifications (such as the different oxidation states of cysteine or methionine, the presence of nitrotyrosine, to name only the most standard ones) as well as the possibility of non specific peptide cleavages dramatically increase the search space when trying to match tandem mass spectra to peptide sequences in the queried database. This in turn increases the probability of false identification [126].

Even more complex are the cases of backbone cleavages and interprotein crosslinking: because in bottom-up strategies, sequence coverages of identified proteins are intrinsically low, it is very difficult to unambiguously identify a protein fragment or the site of crosslinking (see below for an example about erythrocyte membrane proteins).

Additionally, many oxidised products are chemically unstable, and form adducts with other compounds; for example, one product of the hydroxylation of tyrosine is 3,4-dihydroxyphenylalanine. The latter can be converted to orthoquinone through metal catalysis, and then further undergo Michael addition with a free cysteine [127]. Such nonconventional and unexpected modifications are virtually

impossible to track down with the large-scale tools of MS and bioinformatics.

Another possible complication due to instrumental artefacts is the loss of side-chain modifications during tandem MS. It has been observed that labile modifications are readily lost during gas-phase fragmentation by CID [128]. One promising solution to this problem is the development of “softer” fragmentation techniques such as electron transfer dissociation (ETD) and electron capture dissociation (ECD). For example, Guan *et al.* [129] showed that CID of oxidised methionine containing peptides resulted in the loss of CH₃SOH whereas ECD allowed fragmentation of the peptide backbone while preserving the side-chain oxidation, thus allowing direct location of the oxidised methionine.

Recently, Zhao *et al.* [130] proposed a complete methodology for single protein oxidation mapping based on high resolution, high accuracy MS: they mimicked oxidation of P21Ras by *in vitro* incubation with peroxyxynitrite or GSSH, and analysed both tryptic digests (bottom-up protein MS) and whole proteins (top-down protein MS) by CID and ECD. They were not only able to map the oxidative modifications to the protein, but also compare the reactivity of the different sites susceptible to undergo oxidative modifications. But such studies are possible at the single protein level, and data interpretation is yet hardly amenable to automation for large-scale studies.

4 Perspectives for blood proteomics

4.1 Red blood cells

The RBC proteome has been the subject of extensive efforts, and more and more data accumulate through time [131–141], providing a high quality dictionary of red blood cell proteins. Red blood cells are inherently under continuous oxidative stress, as they pass the lungs once a minute; they contain high levels of O₂ and haemoglobin which auto-oxidises to produce O₂⁻ and H₂O₂. The heme group of haemoglobin can serve as a Fenton reagent to initiate free radical reactions [142]. Additionally, the RBC is often considered as a sink for oxidative species [143–145]: approximately 40% of intravascularly formed peroxyxynitrite diffuses into RBCs: the peroxyxynitrite anion crosses the membrane *via* band 3, a bicarbonate-chloride exchanger, whereas diffusion of peroxyxynitrous acid is passive [146, 147].

The forefront of antioxidant defences has been identified to be superoxide dismutase (SOD), glutathione peroxidase (Gpx), peroxiredoxins (Prdx), and catalase, four enzymes that are highly abundant in red blood cells, as shown in Fig. 9. Superoxide dismutase catalyses the reduction of superoxide to oxygen and hydrogen peroxide through its [Cu–Zn] centre. Glutathione peroxidase catalyses the reduction of hydrogen peroxide to water by the conversion of GSH to GSSG, which can be recycled back to glutathione by the NADPH-dependent glutathione reductase. Catalase directly reduces hydro-

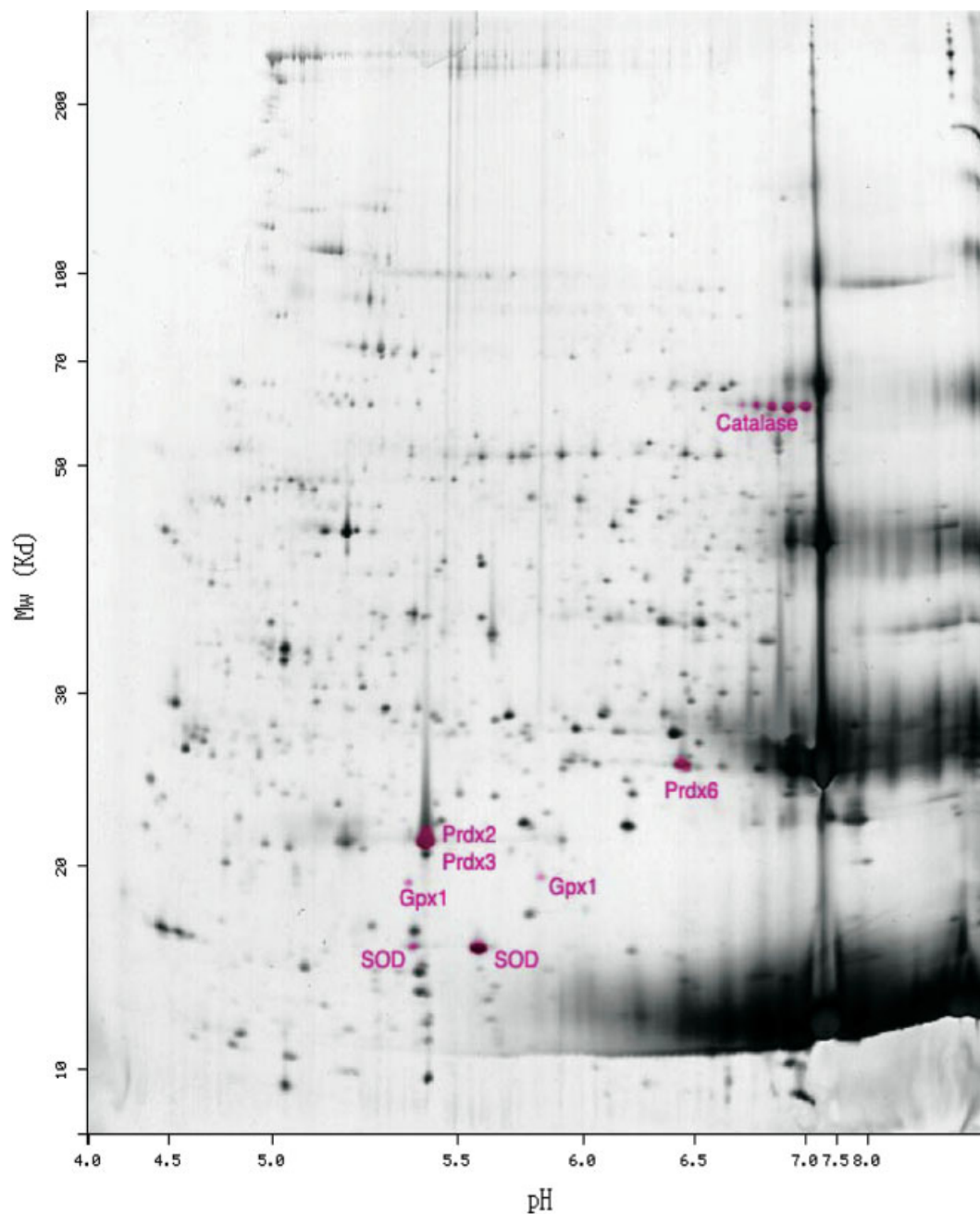


Figure 9. 2-DE of human red blood cells. Annotated in purple are proteins responsible for antioxidant defence (Prdx, peroxidoredoxin; gpx, glutathione peroxidase; SOD, superoxide dismutase). The gel image was obtained from Swiss-2-D PAGE [197].

gen peroxide to water and oxygen in a two-step mechanism thanks to its heme group. Peroxiredoxins catalyse the reduction of H_2O_2 to water by oxidation of one cysteine (the peroxydatic one) to sulphenic acid; another cysteine (for the 2-cys peroxidoredoxin subclass) reacts with the sulphenic acid to form a disulphide bridge. Its regeneration occurs through a thioredoxin /NADPH-dependent thioredoxin reductase system. The overoxidation of the peroxydatic cysteine may abolish the catalytic activity of peroxidoredoxins [148].

The respective role of these enzymes in antioxidant defence is still a matter of debate. Knockout mouse models proved that the lack of peroxidoredoxin I resulted in severe haemolytic anaemia, and appearance of lymphomas, sarcomas, and carcinomas [149]. The lack of peroxidoredoxin II was also shown to result in haemolytic anaemia [150]. On the other hand, patients having hereditary catalase deficiencies were also shown to be victims of oxidative stress and presented a high prevalence of diabetes [151]. Gaetani *et al.* [152]

also demonstrated that catalase is essential for the removal of H₂O₂ from RBCs, having an activity six times higher than glutathione peroxidase. Peskin *et al.* [153] have recently shown that peroxiredoxin 2 and catalase react with H₂O₂ at comparable rates. It was hypothesised that catalase and peroxiredoxin play complementary roles in H₂O₂ detoxification/signalling due to the different recycling mechanisms used [154]; in RBCs, peroxiredoxin 2 was shown to accumulate as a dimer under H₂O₂ challenge, which was only slowly converted back to the active monomer by the thioredoxin system. This behaviour makes peroxiredoxin 2 ideally suited to H₂O₂ sensing at low concentration [155]. When H₂O₂ concentration increases, catalase and glutathione peroxidase are necessary to dispose of excess H₂O₂ [156]. What is unknown is the role of peroxiredoxin 2 dimer and overoxidised forms in H₂O₂ signalling.

Inside RBCs, oxidative stress induces haemoglobin crosslinking to the cytoskeleton [38, 157], which brings the heme centre in close vicinity to the phospholipid membrane where it can act as a local Fenton reagent [142]. Oxidative stress is also associated with protein degradation [158], band 3 clustering [159], phosphatidylserine externalisation [160], activation of caspases [160–162], and down-regulation of glycoproteins [163], some of these being recognised as biomarkers of senescence or “apoptosis”. In particular phosphatidylserine externalisation signals macrophages to recognise and degrade the RBCs.

Recently, D’Amici *et al.* [140] analysed by 2-DE the membrane proteome of RBC during storage, *i.e.*, during hypothesised oxidative stress. They were able to demonstrate dramatic alteration and cleavage of band 4.2, 4.1, band 3 and spectrin through the appearance of numerous spots through time, in addition to slighter modifications (hypothesised to be oxidative side-chain modifications) to numerous cytoskeleton, cytoskeleton-anchored and membrane proteins.

4.2 Platelets

Platelets are responsible for primary haemostasis through adhesion to collagen, release of mediators and aggregation with adjacent platelets. Platelets are exposed to ROS generated by the endothelial cells of the vessel walls; in addition, there is evidence that platelets can themselves produce ROS. Lastly, under inflammatory conditions, platelets are exposed the phagocyte-dependent, acute production of ROS [164].

Throughout haemostasis, the redox environment plays a critical role, notably with respect to platelet integrins. Platelet integrin α IIb β 3, a transmembrane fibrinogen receptor, is under tight regulation by sulphhydryl oxidation: the α IIb subunit contains 18 cysteines while the β 3 subunit contains 56 cysteines. Part of them (located in the extracellular cysteine-rich domain of the β subunit) is present as free sulphhydryls and remains available for redox regulation by extracellular factors. Additionally, the reduction of disulphide bridges

appears to be involved in the conversion of α IIb β 3 to a fibrinogen-binding conformation, a phenomenon commonly referred to as “integrin activation”. This modulation of α IIb β 3 integrin affinity for collagen is the result of an “inside-out” signal following platelet exposure to agonists or to adhesive subendothelial proteins. Fibrinogen binding, among other processes, is mediated by a protein disulphide isomerase (PDI) as well as ERP5, a thiol isomerase protein that is recruited to the cell surface during platelet activation. Similar mechanisms seems to account for other integrins, such as α ₂ β ₁ for example.

4.3 Other blood cells

Ghezzi *et al.* [165] identified two cysteines (52Cys and 62Cys) on cyclophilin A (CypA) as targets of glutathionylation in T lymphocytes and characterised the basis for the reactivity leading to this modification. Glutathionylation of these cysteines might interfere with the formation of the CypA/cyclosporin A complex or the binding of CypA with the HIV-1 capsid protein. It was shown that alterations in the antioxidant defence enzymes contributed to the outcome in diffuse large B-cell lymphoma, the patients with decreased manganese-SOD and thioredoxin inhibitor VDUP1 having the worst prognosis [166]. One explanation for this phenomenon is the modulation of glucocorticoid nuclear receptor function, which is redox sensitive [167].

Regarding blood stem cells, it has been shown that increasing levels of ROS act through defined mitogen-activated protein kinase pathways to limit the life span of cells *in vivo* [168].

4.4 Igs

The oxidation of antibodies increases the hydrophilic nature of the paratopes and increases their tendency to bind to cationic surfaces even without strong surface-to-surface fitting [169]. Studies on mAb during storage revealed clear sites of oxidation [170–172]. Recent findings suggest the existence of “redox-activated” autoantibodies that are not detectable by conventional immunoassays [173, 174]. A possible mechanism responsible for unmasking them may require nitrosylation of tyrosine residues in the hypervariable or complementary determining region of Ig [175]. This concept has potential consequences in the understanding of the antiphospholipid antibodies syndrome, the opsonisation of aging erythrocytes and of their immune elimination [176].

4.5 Fibrinogen

Fibrinogen is a high abundant plasma protein and the major plasma coagulation factor. It consists of two sets of three disulphide-bridged chains (A α , B β , and γ) of 610, 461, and 411 amino acid residues, respectively. Structure, heterogeneity, function and assays were all reviewed else-

where [177–180]. Western blot immunoassay showed that fibrinogen, among other plasma proteins, is highly susceptible to attack by oxidants [181]. Oxidant-induced carbonyl formation in fibrinogen derives largely from amino acid oxidation and not from oxidation of carbohydrate groups [182].

Previous experiments showed that histidine and tryptophan residues in the amino-terminal disulphide knot were affected by methylene blue light treatment (MBLT), which is a photosensitiser used for virus inactivation [183]. Photo-oxidation of an histidine in the B β -chain (16His) located only one amino acid residue away from the thrombin-susceptible bond was shown to impair fibrin polymerisation [184]. Addition of L-histidine, a target of singlet molecular oxygen generated during MBLT, was able to protect fibrinogen from the polymerisation defect in a dose-dependent manner [185]. Measurements of both the release of fibrinopeptide (by HPLC) and the generation of fibrin monomers (by electrophoresis) confirmed that oxidation-induced inhibition of clotting activity derived from an effect on fibrin monomer polymerisation, not from inhibition of thrombin activity [186]. S-nitrosothiols can induce changes in fibrinogen structure by interacting at specific domains rich in aromatic amino acids [187].

Oxidatively modified fibrinogen was also found to modulate blood rheological parameters [188]. Clinical implications of such results need obviously to be investigated further. Oxidised forms of fibrinogen circulating in blood could be interesting in several aspects, for example, for monitoring oxidative stress, controlling coagulation processes and studying protein senescence mechanisms.

4.6 Photoinactivation of blood products

Photoinactivation of blood products involves the addition of an exogenous agent or physicochemical manipulations: its benefits (the inactivation of pathogens) need to be balanced against deleterious effects on cells and plasma proteins [189]. UV irradiation has been proposed for pathogen inactivation of purified plasma proteins and clotting agents used in transfusion medicine. However it has been known for long that UV light exposure can damage proteins through generation of ROS, which can in turn damage proteins [190, 191]. Using 2-D DIGE and MALDI-TOF-MS, Chan *et al.* [192] identified alterations in protein

thiol reactivity, indicative of an oxidative damage. Authors showed modification of various proteins involved in the coagulation cascade, such as kininogen, thrombin, albumin, actin, complement factor 4, serum amyloid P, or retinol binding protein.

MBLT stands as another option for pathogen inactivation of fresh frozen plasma and was also evaluated in the same terms [185, 193–195]. Figure 10 shows modifications of the 2-D pattern of fibrinogen γ chain, transthyretin, and apolipoprotein A-I upon prolonged light exposure in the presence of various concentrations of methylene blue.

5 Conclusion

In this review, we have tried to highlight the complexities of oxidative modifications to proteins, be it part of regulatory processes, responses to oxidative stress or permanent damages induced by exogenous compounds. As far as blood components are concerned, the selected examples described below show that there are complex processes to detect oxidative stress, and eliminate reactive oxygen and nitrogen species. In parallel, reactive oxygen and nitrogen species play physiological roles, such as the modulation of platelet activation for example.

Any blood proteomic approach should thus reveal the presence of oxidised proteins, due to physiological modifications (as is the case in RBC metabolism and platelet activation), and depending on how the sample was punctured, processed, and stored. The fact that most blood proteomic studies do not report such modifications just reflect the difficulty of analysing oxidative modifications to proteins on a large scale, mainly due to their diversity. Targeted strategies to detect and quantify oxidative modifications in blood components exposed various conditions (be it instrumental such as puncture and storage parameters), physiological or biomedical (samples from healthy individuals or with specific diseases) would be highly desirable and perfectly timely to increase our knowledge of blood physiology, give a sound basis for the search of biomarkers in plasma or other blood components, and practical recommendations for the handling, preparation and storage of blood products.

The authors have declared no conflict of interest.

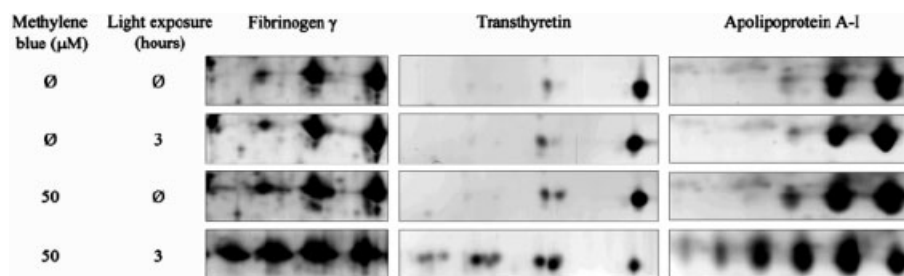


Figure 10. 2-D pattern of fibrinogen γ chain, transthyretin, and apolipoprotein A-I upon prolonged light exposure in the presence of various concentrations of methylene blue. Reproduced from [194] with permission.

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