Editorial for the Free Radical Reseach Special Issue on
"Analytical methods for the detection of oxidised biomolecules and antioxidants"

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The concept of oxidative stress and damage to biomolecules is now well-established, and there is extensive evidence for oxidative damage to proteins, lipids and nucleic acids in a variety of diseases, especially those involving inflammation [1-4]. Consequently there has been considerable interest in the potential of oxidized biomolecules as markers of disease conditions. The benefits of antioxidants and their use in preventative strategies have also been areas of enthusiatic research, although often they have not lived up to the hype, probably because the mechanisms are much more complex than originally anticipated. More recently it has been recognized that protein redox reactions also play a role in several physiological signalling processes [5]. Oxidized lipids and phospholipids are known to have a plethora of bioactive effects [4], and more recently nucleotide oxidation products have also been shown to have signalling effects [6]. Understanding the relationship between oxidative modifications, signalling and disease depends on the ability to determine accurately the levels of oxidized biomolecules and antioxidants in a variety of biological samples, and therefore development of analytical technology is critical. This Special Issue brings together 12 invited reviews on the latest methods for detection of oxidised biomolecules and antioxidants, covering proteins, DNA, carbohydrates (as glycosaminoglycans), lipids and related biomolecules, and antioxidants.

Analysis of oxidative post-translational modifications of proteins is a highly challenging field that relies on advanced techniques such as mass spectrometry as well as reagents or labels for specific oxidation products. Artemenko et al. [7] review the characterization of oxidised proteins by mass spectrometric techniques with either matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI), and consider both label-free and label-dependent approaches. They also discuss the bioinformatics tools that are widely used to help interpret mass spectrometry data. The authors identify a number of areas for rapid development, including algorithms for oxidation prediction; fast search for oxidative modifications; development of tools to discover oxidation motifs and development of enrichment tools for fast, global analysis.

The article by Boronat et al. [8] complements the review of Artemenko et al. [7] by providing a practical-orientated guide to current mass spectrometry-based methods for analysis of protein oxidation. They focused on label-dependent analysis for reversible modifications of cysteine, and irreversible carbonyl formation on amino acid side chains as potential biomarkers. Reversible cysteine oxidation is of great interest owing to its role in protein redox signalling, and Boronat et al. discuss the advantages of various new isotope-coded affinity tag (ICAT) reagents for the identification and quantification of all types of reversible Cys oxidation, building on the principles presented by Artemenko et al.

In a wide-ranging study of the role of redox processes in both normal and pathological ageing by Calabrese et al [9], the exploitation of recent advances in this field of research is reviewed extensively. All types of biological markers of oxidative stress are included in the review but there is a particular focus on the potential of proteomic approaches as innovative tools for monitoring the extent of protein oxidative insult and identification of the targeted proteins. As in the protein reviews above [7,8], this overview of the different proteomic technologies emphasises the contribution of mass spectrometry to both gel and non-gel based approaches to the characterization of oxidized proteins. Although proteomics has already contributed relevant insights in the field of aging research, the authors suggest that reference mapping of oxidized proteins in healthy ageing human subjects is an important direction for future research.

Mass spectrometry is again the dominant technique described in the comprehensive review by Dizdaroglu et al. on the measurement of oxidatively-damaged DNA and its repair [10]. Both gas chromatography-mass spectrometry or liquid chromatography-mass spectrometry in single or tandem versions have been used for the measurement of numerous DNA products such as sugar and base lesions, 8,5'-cyclopurine-2'-deoxynucleosides, base-base tandem lesions and DNA-protein cross-links in vitro and in vivo. Also included are the recent developments in the measurement of DNA repair proteins by LC-MS/MS with isotope-dilution. The review addresses the potential for artifacts that may occur, for example at several stages of GC-MS measurements, especially oxidation of intact DNA bases present in acid hydrolysates of DNA during derivatization at high temperature. Alternative approaches to eliminate such artifacts are discussed.

Lipids, including those esterified in phospholipids and triacylglycerols, are susceptible to oxidative attack, and can produce a wide range of different lipid oxidation products [11]. The oxidation of fatty acids by free radical mechanisms is similar in triacylglycerols, phospholipids and non-esterified fatty acids, as described by Zeb in the review on analytical approaches to the structural identification of oxidized triacylglycerols (TAGs) [12]. The review covers analysis of oxidized TAGs primarily by thin layer chromatography, HPLC with UV and ELSD detection and LC-MS. Mass spectrometry was found to be the most used method of detection and includes techniques such as easy ambient sonic spray mass spectrometry (EASI-MS) and transmission-mode direct analysis in real time ionization coupled with high resolution mass spectrometry (TM-DART–HRMS.

Oxylipin is a broad term that refers to diverse oxygenated products of polyunsaturated fatty acids arising either through non-enzymatic (autoxidation) or enzymatic processes. It is most often used for oxidized fatty acids from plants, but is also sometimes used for analogous oxidized lipids in animals, such as eicosanoids and resolvins; these compounds have important physiological and signalling roles both in plants and mammals [13,14]. Griffiths [15] reviews in detail the biosynthetic pathways and available biochemical methods for the identification and quantification of metabolites. In addition to well-established but non-specific techniques such as the TBARS assay, more advanced techniques for the isolation and characterisation of oxylipins and metabolites are described in detail. Mass spectrometric techniques are the methods of choice: lipidomics analysis can be achieved either by the ‘shotgun’ approach (without prior separation), or by interfacing with chromatographic separation, such as “comprehensive lipidomics analysis by separation simplification” (CLASS). The wide variety of ionization methods and mass spectrometers that can be used for oxylipin analysis are described in this review, including LC-ESI-MS/MS, LC-APCI (atmospheric pressure chemical ionization), LC-TOF-MS, and FTICR-MS (Fourier transform ion cyclotron resonance).

The review by Galano et al. [16] concerns the analysis of isoprostanoids, a group of non-enzymatic oxidized lipids from polyunsaturated fatty acids that are commonly used as oxidative damage biomarkers for lipid peroxidation in diseases, especially those related to vascular systems and neurodegeneration. Galano et al. highlight the formation of isoprostanoids from their respective fatty acids and their use as oxidative damage biomarkers *in vivo,* as well as the assessment of plasma and urine levels using mass spectrometry to evaluate the outcome of oxidative stress in human dietary interventions. The review focuses on isoprostanoids other than F2-isoprostanes and the authors argue for the use of specific isoprostanoids in measurements of oxidative status in humans. Again, mass spectrometry (GC-MS and LC-MS/MS) features predominantly for accurate analysis of isoprostanoids, together with less expensive and specific ELISA techniques.

Two reviews in the Special Issue focus on the identification of specific subclasses of phospholipids, specifically plasmalogens and cardiolipin. Both are known to be susceptible to oxidation, though by different mechanisms, and their oxidation products have important biological effects. Plasmalogens are a unique class of glycerophospholipids (GPLs) containing a fatty alcohol

linked by a vinyl-ether moiety at the sn-1-position of the glycerol backbone. At the sn-2 position there is normally a polyunsaturated fatty acyl residue. Plasmalogens represent up to 20% of the total membrane GPLs in humans and deficiencies have been associated with several human disorders, e.g. Zellweger Syndrome. In the review by Fuchs [17], current methods of analyzing plasmalogens and their oxidation products are described. These include chromatographic methods, (31P) nuclear magnetic resonance (NMR) spectroscopy and soft ionization mass spectrometry (MS) techniques such as electrospray ionization (ESI) or matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF) MS. Shotgun lipidomics with ESI intrasource separation has proved to be an important strategy by which individual molecular species of most major and many minor lipid classes can be fingerprinted and quantitated directly from biological lipid extracts without the need for prior chromatographic separation. This approach together with multi-dimensional MS techniques has, for example, helped to identify new roles for plasmalogens in cerebral function.

Similarly, oxidative lipidomics have been used to assess oxidation of the mitochondria-specific phospholipid, cardiolipin, as described by Tyurina et al [18]. Exposure of animals to rotenone can be used as a model for selective degeneration of dopaminergic neurons in substantia nigra (SN) and development of neuropathological features of Parkinson's disease. As rotenone is an inhibitor of mitochondrial respiratory complex I and leads to increased oxidative stress, there is a significant reduction of oxidizable PUFA-containing cardiolipin molecular species. Detection of cardiolipin and its oxidation products can best be achieved using LC-MS in negative mode, as cardiolipin carries negative charges in the phosphates. Analysis of cardiolipin oxidation products in SN revealed unusual features of rotenone-associated oxidation, specifically the exclusive accumulation of oxygenated linoleoyl chains in sn-1 rather than sn-2 position, and selective generation of mono-oxygenated CL species.

The free-radical induced oxidation of carbohydrates is reviewed by Parsons [19] and is restricted to glycosaminoglycan (GAGs), in particular to hyaluronan and proteoglycans, which are significant components of the extracellular matrix (ECM). The ECM plays a key role in the regulation of cellular behaviour and alterations to it can modulate both the development of human diseases, as well as controlling normal biochemical processes such as cell signalling and pro-inflammatory responses. The characterization of GAGs, and hence studies of their mechanism of fragmentation, has involved a wide range of techniques including viscometry, electron spin resonance, electrophoresis, size exclusion chromatography combined with light scattering detection, kinetic spectroscopic techniques such as laser flash photolysis and pulse radiolysis, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF), non-isothermal chemiluminometry, differential thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC), and immunological techniques using mouse mAbs against native protein epitopes and oxidatively-modified protein epitopes. These techniques have allowed the mechanisms of fragmentation by reactive oxygen species such as hydroxyl radicals, superoxide, carbonate radicals and HOCl to be determined. Evidence is presented for a significant role for GAG chloramide derivatives (probably formed *in vivo* during inflammatory processes involving HOCl) in directing highly efficient site-specific fragmentation.

In order to achieve a balanced view of redox status, it is essential to be able to measure antioxidants in biological samples and assess their reductive ability. Amorati and Valgimigli, in their review on antioxidants [20], focus on a representative range of antioxidant assays, selected for either for their soundness or for their popularity. There is an emphasis on "chemical" testing methods, with a shorter discussion dedicated to "cell-based" assays, as these have also been the subject of other recent reviews [21,22]. The chemically-based tests include oxygen uptake, iodometic assay, TBARS, Rancimat technique, ORAC, carotene bleaching, crocin bleaching, spin trapping, luminol test, DPPH assay, TEAC assay, galvinoxy assay, FRAP assay and Folin-Clocateu assay. The authors conclude their review with a preference for inhibited autoxidation studies,

initiated under controlled conditions by an azo-compound, which they regard as the “gold standard” in antioxidant testing, because they provide information on the kinetics and stoichiometry of the reaction with peroxyl radicals, as well as provide direct evidence of the ability of test compounds to act as antioxidants. Although there is a growing awareness that many “antioxidant” compounds can act *in vivo* by switching on Phase II defence mechanisms, for example via the Nrf2-KEAP1 system [23], assays for antioxidant potential are nevertheless valuable for many purposes, including food biochemistry and development of improved man-made products.

There has been considerable controversy over the the benefits of otherwise of antioxidants as nutritional supplements, especially vitamin C, vitamin E and β-carotene [24]. One possible reason for some detrimental effects of β-carotene, a precursor of vitamin A with free radical scavenging properties, is that it can be oxidized to generate a variety products, some of which have deleterious effects [25]. An extensive review of this topic is presented by Stutz et al. [26]. The authors critically discuss different preparation strategies of standards and treatment solutions including their protection from oxidation. Additionally, *in vitro* oxidation strategies for the generation of oxidized model compounds are surveyed. Extraction strategies are discussed for volatile and non-volatile cleavage products individually. Gas chromatography, (ultra)high performance liquid chromatography, and capillary electrochromatography are reviewed as analytical tools for final analysis, and for confirmation of the identity of analytes, mass spectrometry is seen to be indispensable.

It can be seen that analytical methods for identification and quantification of oxidized biomolecules and antioxidants are central to many studies that are attempting to unravel the role of redox signalling and oxidative damage in nutrition, health, and disease. It is interesting that while many different methods are available to measure these compounds, mass spectrometry stands out as a key technology, owing to its potential to provide specific structural information. The 12 articles in this Special Issue provide an excellent overview of modern approaches and developments in the analytical field, and we are sure they will prove a valuable resource for readers of Free Radical Research.

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