Chapter 1
Introduction

1.1 The Toxicity of Oxygen

Primordial life forms on earth comprised oxygen-sensitive organisms: the anaerobic fermenters and cyanobacteria, which released oxygen as a metabolic by-product, causing the oxygen levels in the atmosphere to rise [1, 2]. Consequently, aerobic respiration processes such as photosynthesis and O_2-dependent electron transport mechanisms, came to predominate in biological systems [1, 2]. The paradox of aerobic respiration is that oxygen, which is vital for life is also a fatal toxin. The consequence of aerobic respiration is the generation of partially-reduced forms of oxygen—reactive oxygen species (ROS), which oxidatively damage important biomolecules such as DNA, lipids and proteins [3, 4]. As a result, highly coordinated and effective antioxidant (AO) defence mechanisms evolved, which counteract and neutralise the toxic ROS [1, 2]. Although well integrated, the AO defences are not infallible [5].

The toxic effects of ROS were first established in 1954 by Gerschman et al. [6] and since then a large volume of evidence has accumulated that demonstrate the role of ROS in a myriad of pathological conditions [7–9]. However, more recently, the role of ROS as signalling molecules with a crucial role in orchestrating specific physiological functions has been realised [5, 10, 11]. Understanding the importance of ROS and AO in health and disease has therefore been an intense area of scientific research. However, the current challenge faced by researchers studying cellular redox state is the design of a robust method that enables real time measurement of redox state in cells [12, 13]. This chapter contains a review of the factors that determine the redox state of a cell and its importance. The advantages and disadvantages of the various tools currently used to study the redox state are discussed with particular focus on fluorescence imaging techniques.

1.1.1 Reactive Oxygen and Nitrogen Species (ROS/RNS)

Most eukaryotic biological systems carry out aerobic respiration in the mitochondria to generate ATP, the energy currency of the cell. This cellular oxidative metabolism is driven by an electron transport reaction, in which O_2 accepts electrons and H^+, and is eventually reduced to water. Although the mitochondrial electron transport chain is extraordinarily efficient, 1–3% of all electrons involved can leak out and be transferred to O_2, resulting in the formation of the superoxide anion radical ($O_2^{\bullet-}$, Eq. 1.1), the primary reactive oxygen species (ROS) [14]. Superoxide can also be produced enzymatically by NADPH oxidase [14].

$$O_2 + e^- \rightarrow O_2^{\bullet-} \tag{1.1}$$

Superoxide is a moderately reactive, short-lived ROS (half-life $\simeq$ 1 µs) and therefore not believed to cause direct oxidation of cellular components (DNA, protein and lipids). Instead it produces secondary ROS and reactive nitrogen species (RNS) (Fig. 1.1), whether directly or via enzyme- and metal-catalysed processes [5]. Secondary ROS include hydrogen peroxide (H_2O_2), the hydroxyl radical ($^\bullet OH$), hypochlorite ion (OCl^-) and singlet oxygen ($^1O_2$), whereas RNS include the nitric oxide radical ($NO^\bullet$) and the peroxynitrite anion ($ONOO^-$) [3].

Superoxide rapidly undergoes dismutation, catalysed by a family of enzymes called superoxide dismutases (SODs), to form H_2O_2 (Eq. 1.2, Fig. 1.1), which is relatively more stable (half-life $\simeq$ 1 ms) [16, 17]. Unlike other ROS, H_2O_2 is a
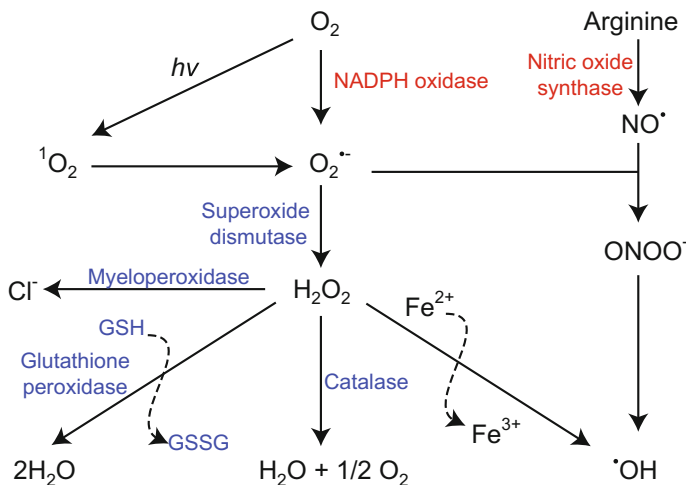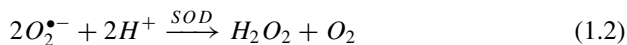

Fig. 1.1 An overview of the production of primary and secondary ROS/RNS. Antioxidants are shown in blue [4, 14, 15]

neutral molecule capable of diffusing across biological membranes. This property allows H_2O_2 to act as a signalling molecule, but at high concentrations it can cause oxidative damage over large distances within the cell [16].

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \tag{1.2}$$

Low levels of H_2O_2 are maintained by the action of a family of enzymes—glutathione peroxidases (GPXs), which transfer electrons from two molecules of glutathione (GSH) to H_2O_2 to form water (Eq. 1.3, Fig. 1.1) [16]. The enzyme catalase can convert six million H_2O_2 molecules per minute to water and oxygen (Eq. 1.4) [16].

$$2GSH + H_2O_2 \xrightarrow{GPX} GSSG + H_2O \tag{1.3}$$

$$H_2O_2 \xrightarrow{Catalase} H_2O + O_2 \tag{1.4}$$
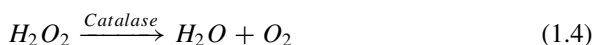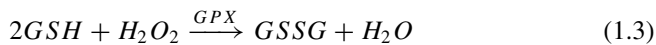
The hydroxyl radical ($^{\bullet}OH$) is formed by Haber-Weiss (Eq. 1.5) catalysed by redox active metals such as Fe and Cu (Fig. 1.1). It is the most reactive among all ROS, tending to react very close to its site of formation. The redox active metals Fe and Cu are closely associated with anionic species such as cell membranes and DNA, hence $^{\bullet}OH$-mediated oxidative damage occurs in close proximity to these species [18]. Furthermore, because the cells lack specific mechanisms to eliminate $^{\bullet}OH$, its excess production can have severe pathological consequences [19].

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Fe^{3+}} {}^{\bullet}OH + {}^-OH \tag{1.5}$$
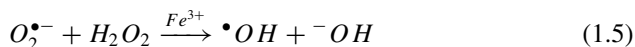
The hypochlorite ion (OCl^-), is formed from the reaction of H_2O_2 with chloride ions (Cl^-), catalysed by the enzyme myeloperoxidase (MPO, Eq. 1.6, Fig. 1.1) [20]. The myeloperoxidase-H_2O_2-Cl^- system is crucial for the proper functioning of phagocytes, giving these cells the ability to kill a wide range of pathogens. Although OCl^- is essential for a proper immune response, uncontrolled production of HOCl within the phagocytes is detrimental to the host tissue [20].

$$H_2O_2 + Cl^- \xrightarrow{MPO} OCl^- + {}^{\bullet}OH + Fe^{3+} \tag{1.6}$$
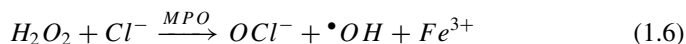
Singlet oxygen ($^1O_2$), unlike other ROS, results from the UV illumination of molecular oxygen (Fig. 1.1), and is moderately stable (half-life $\simeq 3\mu s$). At high concentrations, it diffuses across the nuclear membrane and oxidatively damages the DNA through selective oxidation of guanine to 8-hydroxylguanine. However, $^1O_2$ can be quenched by vitamins A (β-carotene) and E (α-tocopherol).

The primary source of RNS is the nitric oxide radical ($NO^{\bullet}$), which is synthesised endogenously from L-arginine by the enzyme nitric oxide synthase (Fig. 1.1). Following the Nobel prize-winning discovery of nitric oxide as a signalling molecule, the physiological significance of this gaseous radical species has gained great attention

Table 1.1 The sources of ROS

Endogenous sources	**Electron transport chain**—mitochondria, microsomes and chloroplasts.
	Oxidant enzymes—lipoxygenase, monoamine oxidase, indoleamine dioxygenase, tryptophan dioxygenase, xanthine oxidase, galactose oxidase.
	Phagocytic cells—endothelial cells, neutrophils, macrophages, monocytes, eosinophils.
	Autooxidation reactions—adrenalin, thiols, Fe^{2+}
Exogenous sources	**Redox cycling substances**—substances that are reduced in vivo to a radical that then react with oxygen to regenerate the drug and form a ROS for example, paraquat, alloxan, doxorubicin and diquat.
	Other—U.V. radiation, drug oxidation, cigarette smoke, ionising radiation, pollution and heat shock

[21, 22]. $NO^\bullet$ acts as an effector molecule during immune response, as a neurotransmitter and a smooth muscle relaxing factor [23]. It is also implicated in pathological activity such as inflammation, neurodegenerative tissue injury and vasodilation [24]. Direct reaction of $NO^\bullet$ with $O_2^{\bullet-}$ results in the production of peroxynitrite ($ONOO^-$, Eq. 1.7, Fig. 1.1) [25]. With a short half-life ($\simeq 1\,\mu s$), $ONOO^-$ is highly reactive, oxidising tyrosine residues of proteins to form stable 3-nitrotyrosine thereby impacting the structure and functions of proteins [25, 26].

$$O_2^{\bullet-} + NO^\bullet \rightarrow ONOO^- \tag{1.7}$$
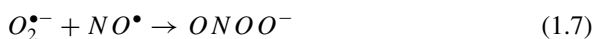
In addition to leakage from the electron transport chain, reactive oxygen and nitrogen species (ROS/RNS) can be produced in the cell from various other endogenous and exogenous sources (Table 1.1) [4, 15].

1.1.2 Scavenging ROS/RNS

Deleterious effects of ROS/RNS make their scavenging all the more essential, and have resulted in the evolution of a series of defence mechanisms mediated by cellular AO (Fig. 1.1). AO act in an altruistic manner, preferentially oxidising themselves in order to protect the cellular components. They can be classified into three groups based on their origin [27].

1. **Endogenous AO** are those that are synthesised naturally by the cells.

   - **Enzymatic** endogenous AO include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPXs), thioredoxins(TRX) and peroxiredoxins(PRXs).

- **Non-enzymatic** endogenous AO are small molecules (L-carnitine, glutathione, α-lipoic acid, uric acid and bilirubin), coenzymes (coenzyme Q) and low molecular weight proteins (metallothionein, coenzyme Q, ferritin and melatonin) [27].

2. **Natural AO** are usually present in daily dietary intake like, ascorbic acid (Vitamin C), tocopherol (Vitamin E) and β-carotene (Vitamin A), lipoic acids, and polyphenol metabolites [27].
3. **Synthetic AO** are the active ingredients of AO supplements. Examples include *N*-acetyl cysteine (NAC), tiron, pyruvate, selenium, propyl gallate, butylated hydroxyltoluene and butylated hydroxylanisole [27].

The cellular repair apparatus comprising AO, DNA repair enzymes and proteases (that degrade damaged proteins) is tightly regulated and is essential in maintaining the cellular redox homoeostasis with low levels of ROS/RNS [28].

1.2 ROS/RNS—Friends and Foe

ROS/RNS are produced in biological systems by normal cellular metabolism. Substantial evidence has been documented that ROS/RNS play a dual role in biological systems—as both beneficial and deleterious species [10, 11, 29]. The levels of ROS/RNS naturally fluctuate in the cells, with well-regulated increases in the production of ROS/RNS ensuring uncompromised physiological functions. On the other hand, uncontrolled increases would result in pathogenesis. It is the AO that play a major role in maintaining healthy levels of these species by scavenging them.

1.2.1 ROS/RNS and Physiology

Transient elevations in the concentrations of ROS/RNS have proved to be essential for many sub-cellular functions ranging from gene expression, enzyme activation and signal transduction to folding of proteins in the endoplasmic reticulum, vascular homoeostasis and apoptosis [30–33]. A widely accepted mechanism of redox signalling involves the reversible oxidation of redox-sensitive thiol residues (in thiols or low molecular weight thioproteins), thereby modulating their function and activity. While the majority of cellular thiols exist in the protonated form (-SH) at physiological pH, a small fraction of them will remain in the form of thiolate anions (-S$^-$), which can also be favoured in a particular local protein microenvironment, increasing the susceptibility to oxidation when compared to the conjugate acid form (-SH) [34, 35]. The oxidation of the thiolate anion by H_2O_2, for example, results in the generation of a sulfenic acid (-SOH), causing allosteric modification of protein structure and function.

This sulfenic acid form enters the signal transduction pathway, in which it is reversibly reduced to the thiolate anion by glutaredoxin (GRX) and thioredoxin (TRX), restoring the original structure and function of the protein [36]. While transient increases in the generation of ROS/RNS bring about a reversible oxidation of thiolate anion to sulfenic acid, chronic elevations can result in further irreversible oxidations, generating sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) residues, rendering the protein structure and function irreparable, and thus resulting in oxidative stress [36]. Other beneficial effects of ROS/RNS include defence against infectious agents and induction of mitogenic response [30]. Concentrations of ROS/RNS higher than those required to perform such functions are immediately scavenged by AO, the cell's defence system.

1.2.2 ROS/RNS and Pathology

Owing to their highly reactive nature and the ability to oxidatively damage vital biomolecules, chronically elevated concentrations of ROS/RNS have been implicated in a variety of pathological conditions [29, 37, 38]. Evidence points to a role of ROS/RNS in the oxidation of purine and pyrimidine bases of DNA and RNA, especially the formation of 8-hydroxylguanine, as well as extensive damage to the deoxyribose backbone [15, 39]. Oxidation of DNA by these reactive species and subsequent genetic damage has been reported to induce aging, mutagenesis and carcinogenesis of cells [15].

ROS/RNS bring about a chain of peroxidative reactions of the polyunsaturated lipids in the biomembranes, consequently increasing the permeability of the cell membrane, leading to cell death. Peroxyl radicals (ROO$^\bullet$), in particular, have been reported to initiate a series of cyclisation reactions resulting in the formation of malondialdehyde (MDA), a potent carcinogen [4]. Functional properties of proteins and enzymes can be drastically altered or completely lost due to the oxidation-induced modification of protein structure by ROS/RNS [10]. Cysteine and methionine residues are the most vulnerable to oxidative damage, which mostly involves thiolation reactions. Thiolation reactions result in the reversible formation of disulfides and involve either intra-molecular reactions—with cysteine residues within the protein or intermolecular reactions with cysteine residues of other proteins or low molecular weight thiols like glutathione (GSH). Owing to the extensive damaging effects of ROS/RNS on vital biomolecules many physiological functions will be impaired, hindered or completely altered thereby resulting in a myriad of pathological conditions (Table 1.2) [5, 27, 40–46].

Transient elevations in the concentrations of ROS/RNS play a crucial role in signal transduction and related physiological functions following which the cellular AO levels are upregulated and the cellular redox homoeostasis is re-established. However, these repair mechanisms are prone to errors and mutations, and in conditions of chronic elevations in cellular oxidative capacity, the repair mechanisms fall short and the cell fails to return to its original state of redox homoeostases,

Table 1.2 Pathological conditions induced by ROS [5, 27, 40–46]

Organ/injury	Pathological conditions
Brain	Parkinson's disease, Lou-gehrig's disease, neurotoxins, Alzheimer's disease, multiple sclerosis, hypertensive cerebrovascular injury, aluminium overload, allergic encephalomyelitis, potentiation of traumatic injury
Eye	Photic retinopathy, ocular haemorrhage, cataractogenesis, retrolental fibroplasia, degenerative retinal damage, retinopathy of prematurity
Heart and cardiovascular system	Atherosclerosis, adriamycin cardiotoxicity, keshan disease, alcohol cardiomyopathy
Kidney	Metal ion mediated nephrotoxicity, aminoglycoside nephrotoxicity, Autoimmune nephrotic syndromes
Gastrointestinal tract	NSAID-induced GI tract lesions, oral iron poisoning, endotoxin liver injury, diabetogenic actions of alloxan, halogenated hydrocarbon liver necrosis, free fatty acid induced pancreatitis
Inflammatory immune injury	Rheumatoid arthritis, glomerulonephritis, autoimmune diseases, vasculitis (hepatitis B virus)
Iron overload	Nutritional deficiencies (kwashiorkar), thalassemia and other chronic anaemias, idiopathic haemochromatosis, Dietary iron overload
Red blood cells	Fanconi's anaemia, sickle cell anaemia, haemolytic anaemia, favism, Malaria, protoporphyrin photo-oxidation
Lungs	Bronchopulmonary dysplasia, mineral dust pneumoconiosis, bleomycin and paraquat toxicity, hypoxia, cigarette smoke effect (necrosis), emphysema, adult respiratory distress syndrome (ARDS)
Ischaemia reperfusion	Myocardial infarction/stroke, organ transplantation, frostbite
Other	Cancer, aging, alcoholism, radiation injury, porphyria, contact dermatitis

resulting in a heightened baseline oxidation state, a condition termed oxidative stress (Fig. 1.2). High local and global concentrations of ROS/RNS cause the damage of vital biomolecules like nucleic acids, lipids and proteins, and result in a myriad of pathological conditions.

1.2.3 Cellular Redox State

It is therefore evident that in order to maintain a healthy environment, cells are required to tune their redox balance, or oxidative capacity. Too low an oxidative
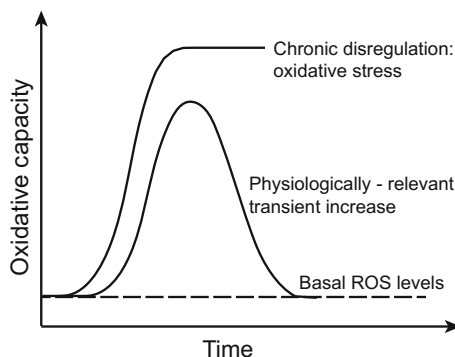
Fig. 1.2 Variations in cellular ROS levels. Transient increases are vital for physiological functions such as signalling, whereas chronic elevations are damaging and cause diseases. Reprinted with permission from *Angewandte Chemie*, Volume 55, Issue 5, published by John Wiley & sons, Inc.

capacity would mean sub-optimal signal transduction and other physiological functions, while too great an oxidative capacity is indicative of potentially-damaging ROS [47]. The oxidative damage caused by ROS depends on the intracellular concentrations of these reactive species as well as the equilibrium between ROS and AO. The redox balance of a cell, i.e. the pro-oxidant/anti-oxidant equilibrium, at any given time is representative of the cellular redox state, which is defined as the ability of a cell to act as a reducing agent or oxidising agent (Fig. 1.3). Lower concentrations of ROS or higher AO concentrations indicate a reducing cell environment. However, when the redox balance is perturbed in favour of ROS, beyond the cells ability to scavenge or repair the damage caused by ROS, oxidative stress develops, leading to oxidising cell environment [47].

Despite the established synergism between oxidative stress and disease, little is known about the extent of oxidative changes and the mechanisms involved. This clearly substantiates the need to develop tools that can report on the real time redox state of a cell and aid in elucidating the mechanisms involved.

1.3 Evaluating Cellular Redox State

To date, various methods have been developed and introduced to measure redox state in cells. However, many of these methods are not robust and suffer from drawbacks like simplicity, non-invasiveness, efficiency, and lack of real time monitoring of redox state. Traditionally, two different approaches have been exploited:

1. Measuring the consequences of ROS/RNS reactivity (biomarkers) in cellular fluids.
2. Directly quantifying ROS/RNS in cells.

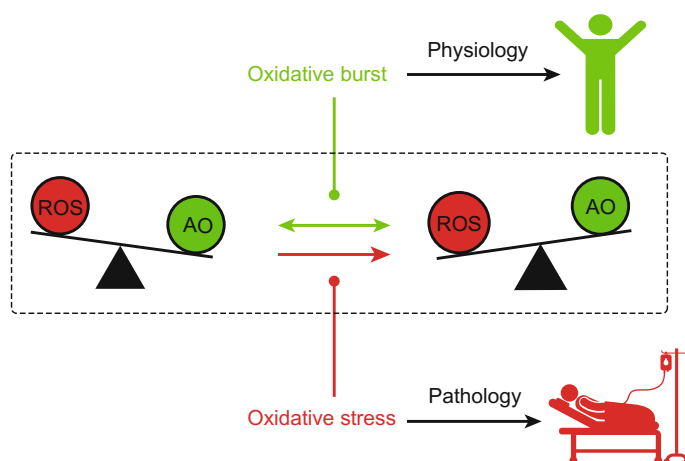Each will be addressed briefly below.

Fig. 1.3 The redox balance. Reversible increases in the concentrations of ROS are necessary for adequate cell physiology, whereas irreversible depletion of antioxidants (AO) or over production of ROS results in oxidative stress and related pathological consequences

1.3.1   Measuring Consequences of Oxidative Stress

Damaging effects of ROS/RNS on key biomolecules are well established (Sect. 1.2.2). This strategy focusses on quantifying the species produced as a result of the attack by ROS/RNS on various biomolecules. Several biomarkers of oxidative stress have been reported [40, 41, 48–56] as means of quantifying the damaging effects of ROS and establishing their role in inflicting a myriad of clinical conditions, these include:

1. Total antioxidant capacity of plasma.
2. Consumption of antioxidants.
3. Local activity of antioxidant enzymes.
4. In-vivo concentration of free radicals.
5. Markers of downstream consequences of oxidation.

Of all these, markers of oxidation have been extensively investigated, and are recognised hallmarks of various pathological conditions [55, 57–59]. As a result of these investigations, several key biomarkers associated with each disease have been identified (Table 1.3).

The above-mentioned biomarkers accumulate to detectable concentrations in cell, tissue or body fluids auch as plasma, lymph and urine and can be quantified using techniques such as HPLC, mass spectrometry and ELISA. Although these biomarkers are quite stable and eliminate the shortcomings of the very short half-life of

Table 1.3 Key biomarkers of oxidative stress associated with human diseases [55, 57–59]

Disease	Key biomarkers
Alzheimers disease	MDA, HNE, GSH/GSSH ratio, F_2-isoprostanes, NO_2-Tyr, AGE
Atherosclerosis	MDA, HNE, Acrolein, F_2-isoprostanes, NO_2-Tyr
Cancer	MDA, GSH/GSSH ratio NO2-Tyr, 8-OH-dG
Cardiovascular disease	HNE, GSH/GSSH ratio, Acrolein, F_2-isoprostanes, NO_2-Tyr
Diabetes mellitus	MDA, GSH/GSSH ratio, S-glutathionylated proteins, F_2-isoprostanes, NO_2-Tyr, AGE
Ischemia	GSH/GSSH ratio, F_2-isoprostanes
Parkinsons disease	HNE, GSH/GSSH ratio, Carbonylated proteins, Iron level
Rheumatoid arthritis	GSH/GSSH ratio, F_2-isoprostanes
Obesity	MDA, GSH/GSSH ratio, HNE, F_2-isoprostanes

ROS/RNS, the key issue with these biomarkers is that they are either upstream effectors of oxidative stress or more commonly downstream effects [55, 56], and hence cannot be considered an exact reflection of the in vivo redox state.

1.3.2 Approaches to Measure ROS/RNS in Cells

Due to their high reactivity and the cells numerous scavenging mechanisms, in vivo concentrations of ROS/RNS lie in the picomolar to nanomolar range. The detection and quantification of these species in biological systems must therefore be direct, efficient and non-invasive, whilst reporting the redox state in a biological cell. Given the sub-micromolar concentrations of ROS/RNS, another important requirement is sensitivity. The last two decades have witnessed immense focus in the literature on exploring multitude of strategies that would enable real time monitoring of ROS and RNS in biological systems, and the two most extensively used approaches are electron spin resonance (ESR) spectroscopy and fluorescence imaging [60, 61].

One of the earliest analytical approaches developed for the direct detection of ROS/RNS was ESR spectroscopy, which reports on the magnetic properties of unpaired electrons and their molecular environment [62]. The drawbacks associated with the concentration and half-lives of biological free radicals have been efficiently overcome by the use of spin traps. These molecules, mostly nitrone and nitroxide derivatives, bring about the conversion of short lived primary radical species into longer lived radical adducts with characteristic ESR responses [63].

Extensive use of spin traps such as phenyl-t-butylnitrone (**PBN**) and 5,5-dimethylpyrroline-N-oxide (**DMPO**) is reported in the literature, as an aid for the detection of organic radical products of lipid peroxidation and $^\bullet$OH and $O_2^{\bullet-}$ respectively [61, 62, 64, 65]. More advanced versions of these spin traps, namely 5-diethoxyphosphory-5-methyl-1-pyrroline-N-oxide (DEPMPO) and mito-DEPMPO,

have also been developed to eliminate the problems associated with the kinetics and selectivity of PBN and DMPO [63, 66–68]. One of the serious problems associated with these spin traps is that, in presence of cellular reductants like glutathione and ascorbate, the ESR active nitrone-ROS adducts can be rapidly reduced, rendering the adduct ESR inactive and causing underestimation of cellular ROS production. Spin traps such as cyclic hydroxylamines which are less vulnerable to reduction by cellular reductants have been developed and successfully employed in in vivo quantification of ROS [69, 70]. Nevertheless, this strategy has not escaped limitations including instability of spin traps, unwanted reactions with cellular metabolites and lack of spin trap specificity.

1.4 Fluorescence Imaging—an Alternative Approach

Fluorescence imaging is a robust and versatile tool that is applied to visualise structural (such as sub-cellular organelles, protein fibres and membrane-components) and biochemical features (such as pH, redox state, metal ion concentration and temperature) within a biological system [71]. This requires the addition of an exogenous chemical probe that displays the desired fluorescence properties upon binding to the structural component or in response to the analyte of interest [72, 73]. More recently, scientific studies have exploited fluorescence imaging techniques as a strategy to study cellular oxidative capacity [74]. This can be attributed to the specific advantages of fluorescence imaging over other techniques, which include greater sensitivity, high spatial resolution, high specificity and efficiency, precise information on cellular and sub-cellular location, possibility to observe in vivo dynamics, and the synthetic ease of modifying the fluorophore and its photophysics [71].

Owing to the extensive benefits of this technique, the last decade has seen an enormous increase in the development of novel fluorophores particularly suitable for sensing and probing applications. Particular attention has been on the use of these molecules as potential sensors for a variety of cellular analytes including ROS/RNS [74], thiols [75], as well as metals [76].

1.5 Fluorescent Redox Probes

In recent decades, a wide range of fluorescent responsive probes to study redox processes have been developed. Based on their sensing mechanism, these tools can be broadly classified into reaction-based probes and reversible probes. While selective probes usually respond to specific ROS/RNS via a specific irreversible reaction (Fig. 1.4a), reversible probes on the contrary can respond to multiple oxidation-reduction cycles (Fig. 1.4b).
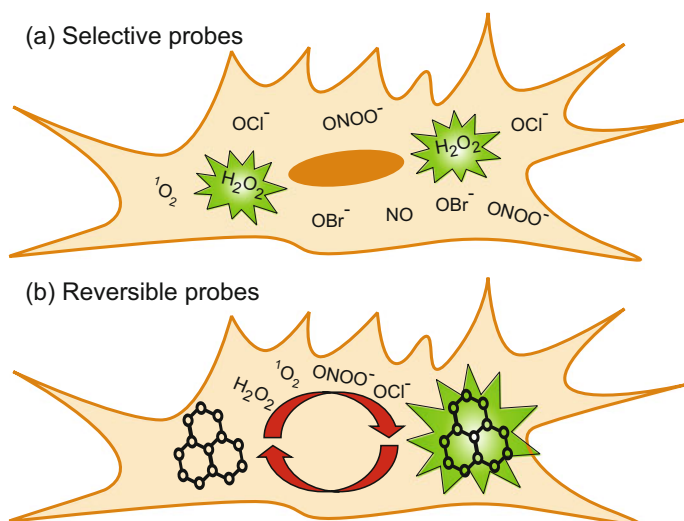
Fig. 1.4 Two important criteria for the development of redox probes: **a** selective response to a specific ROS/RNS, and **b** reversible response to oxidation-reduction cycles. Reprinted with permission from *Antioxidants and Redox Signalling*, Volume 24, Issue 13, published by Mary Ann Leibert, Inc., New Rochelle, NY

1.5.1 Selective Probes for Detection of ROS/RNS

1.5.1.1 Probes for Hydrogen Peroxide

One of the most extensively utilised fluorescent probes to measure cellular H_2O_2 levels is 2,7 -dichlorodihydrofluorescein (**DCFH**) [77], a non-fluorescent molecule that undergoes a two electron oxidation to the fluorescent 2,7-dichlorofluorescein (Fig. 1.5) [78]. In addition to being oxidised by other ROS/RNS, as well as by enzymatic reactions, recent studies suggest that partial oxidation of **DCFH** can result in a free radical intermediate, which reacts with molecular oxygen, resulting in the formation of superoxide. **DCFH** has further shortcomings such as reduced cellular retention of the oxidised form, auto-oxidation and photosensitivity [79–82]. These problems spurred a quest to identify functional groups or scaffolds that would specifically react with H_2O_2.

The majority of H_2O_2-selective sensors reported to date are based on boronate groups, harnessing the fact that boronic esters are selectively hydrolysed by H_2O_2 [83, 84]. One or more boronic esters are incorporated onto a fluorophore to give a pro-fluorescent probe, which upon hydrolysis by H_2O_2 releases a fluorescent molecule (Fig. 1.6) [85]. Initial attempts in this direction resulted in the preparation of aminocoumarin boronate ester (**AMC**) [84] and Peroxyfluor-1 (**PF1**, Fig. 1.6b) [85], both of which possess a pinacol boronic ester that masks the phenolic oxygen
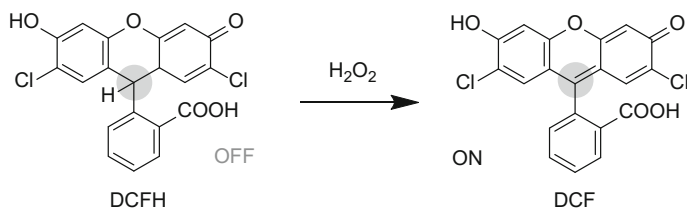
Fig. 1.5 Oxidation reaction of DCFH. Circles indicate the site of redox response. Reprinted with permission from *Antioxidants and Redox Signalling*, Volume 24, Issue 13, published by Mary Ann Leibert, Inc., New Rochelle, NY

Fig. 1.6 a Scheme of boronate cleavage-based detection of H_2O_2 with example probes: **b PF1** [85], **c** ratiometric FRET-based **RPF1** [86]. In all figures, the fluorescence quenching motif is highlighted in a dashed circle, and the moiety enabling fluorescence is shown in a grey circle. Reprinted with permission from *Antioxidants and Redox Signalling*, Volume 24, Issue 13, published by Mary Ann Leibert, Inc., New Rochelle, NY

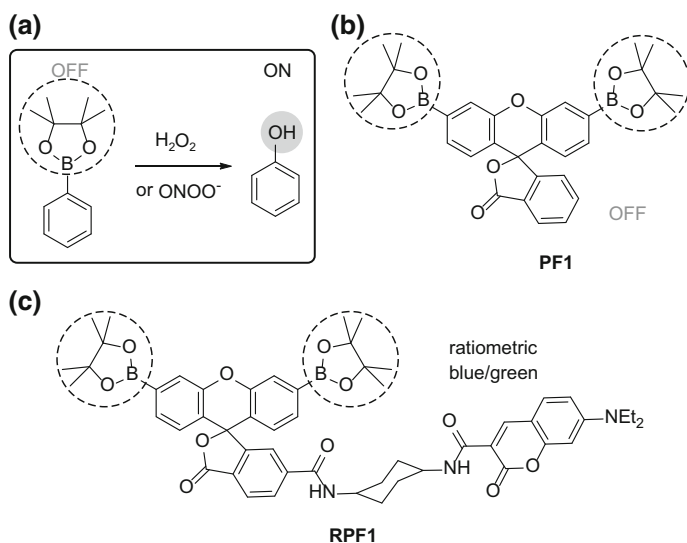resulting in low fluorescence. H_2O_2 chemoselectively oxidises the boronic ester to a hydroxyl group, restoring the fluorescence.

Building off this strategy, a myriad of fluorescent probes have been developed, with excitation-emission profiles that span the entire visible spectrum [83, 85, 87–94], including the ratiometric Ratio-Peroxyfluor-1 (**RPF1**, Fig. 1.6c) [86], mitochondrially-localising **MitoPY1** [95], nuclear **NucPE1** [96] and lysosomal **LNB** (Fig. 1.6d) [97]. However, recent work has indicated boronic esters can also be hydrolysed by peroxynitrite ($ONOO^-$), a reaction that proceeds approximately 100-fold faster than with H_2O_2 [98].
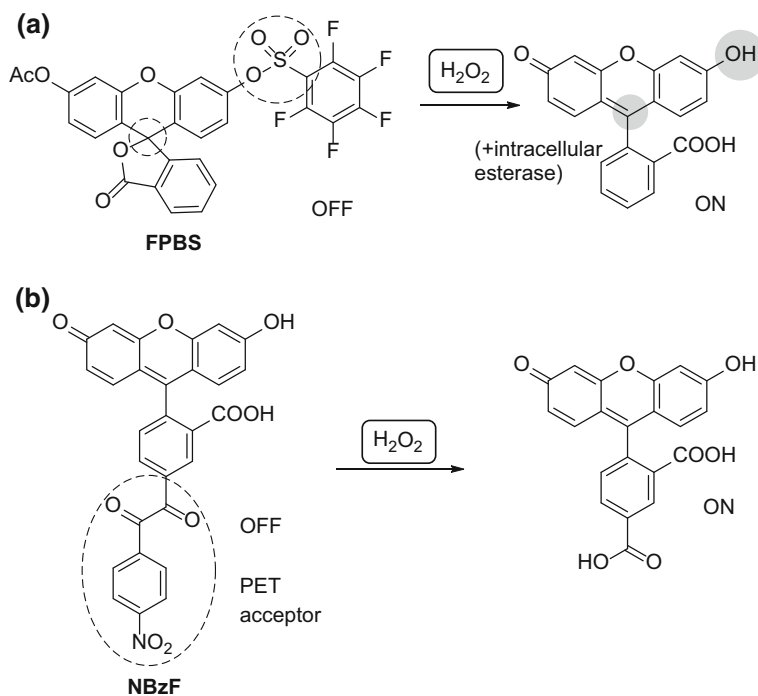
Fig. 1.7 Other irreversible transformations applied for the design of reaction-based H_2O_2-selective probes: **a** hydrolysis of the sulfonyl ester of **FPBS** [99], **b** H_2O_2-induced nitrobenzil cleavage in **NBzF** [100]

In addition to the boronic esters, another responsive group exploited for its selectivity towards H_2O_2-mediated oxidation is the sulfonyl moiety, as in fluorescein-pentafluorobenzenesulfonyl ester (**FPBS**, Fig. 1.7a) [99]. Hydrolysis of the sulfonylester by H_2O_2 results in lactone ring opening. In conjuction with the enzyme-driven loss of acetate, this results in enhanced fluorescence intensity.

A similar approach to H_2O_2 sensing was demonstrated in fluorescein-benzil (**NBzF**) (Fig. 1.7b) [100]. A Baeyer-Villiger type reaction of the nitro-benzil group with H_2O_2 yields a benzoic anhydride, which on further hydrolysis releases the fluorescent 5-carboxy fluorescein (Fig. 1.7b). The probe was shown to be selective towards H_2O_2 over other ROS, but a small fluorescence increase with tBuOOH or $ONOO^-$ was observed [100]. **NBzF** was employed to monitor H_2O_2 production in phorbol-12-myristate-13-acetate (PMA)-stimulated RAW 264.7 murine macrophages and in A431 epidermoid carcinoma cells stimulated with an epidermal growth factor [100].

1.5.1.2 **Probes for Hypochlorous Acid**

Most HOCl-selective fluorescent probes developed thus far are based on the HOCl-facilitated rhodamine ring opening mechanism (Fig. 1.8a). This was first demonstrated in **HySOx**, a fluorescent probe selective for HOCl detection [101]. Reaction of HOCl with the thioether moiety in the rhodamine scaffold resulted in ring opening to give the corresponding sulfonate, and a simultaneous increase in fluorescence. **HySOx** was reported to have a good selectivity towards HOCl and was also employed for the detection of HOCl in porcine neutrophils during phagocytosis. Subsequent work yielded thiolactone (**R19S** and **R101S**) and selenolactone (**R19Se**) containing rhodamine probes for HOCl [102].

The first instance of employing HOCl-mediated oxidative hydrolysis of oximes to develop a selective probe was a ratiometric fluorescent probe based on a phenanthroimidazole moiety (**PAI**) bearing oxime functionality (Fig. 1.8b) [103]. Reaction with HOCl resulted in the cleavage of the oxime group to generate the electron withdrawing aldehyde group leading to an intra-molecular charge transfer (ICT) based red-shift in fluorescence. This ratiometric probe was reported to be highly selective for HOCl, and exhibited a 10-fold increase in the ratio (I_{509}/I_{439}) upon treatment with 30 equivalents of HOCl.
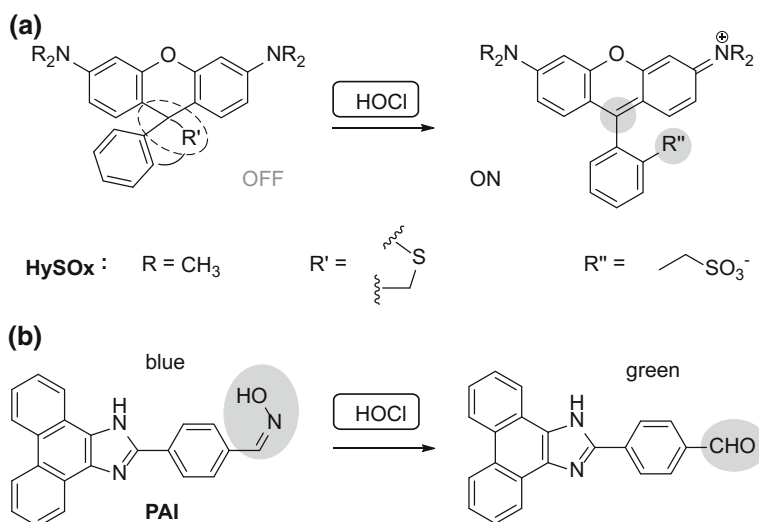

Fig. 1.8 a Examples of HOCl-induced reactions of ring opening in rhodamine-based probes, exemplified by **HySOx** [101]; **b** response of ratiometric probe, **PAI**, to HOCl [103]. Reprinted with permission from *Antioxidants and Redox Signalling*, Volume 24, Issue 13, published by Mary Ann Leibert, Inc., New Rochelle, NY.eps

1.5.1.3 Probes for Nitric Oxide

The development of fluorescent probes for nitric oxide has taken place in two major domains: organic fluorophores and transition metal-based probes. The first reported organic fluorophore-based nitric oxide probe utilised the PET donor ability of the *o*-phenylenediamine scaffold, resulting in the fluorescence quenching of a tethered dye. Under aerobic conditions, NO$^\bullet$ undergoes rapid oxidation to yield NO$^+$, which reacts with *o*-phenylenediamine via an *N*-nitrosation reaction to generate a more fluorescent triazole-containing fluorophore (Fig. 1.9a).

This strategy was employed in the development of diaminofluorescein-2-diacetate (**DAF-2 DA**), a selective probe for NO$^\bullet$ bearing diacetyl groups for better cell permeability (Fig. 1.9a), with a more than a 100-fold increase in fluorescence upon reaction with NO$^\bullet$ [104]. Although **DAF-2 DA** demonstrated the utility of *o*-phenylenediamine as an NO responsive scaffold, subsequent probe development that sought to overcome issues related to pH and retention, gave rise to probes with a broad palette of emission colours [107–112].
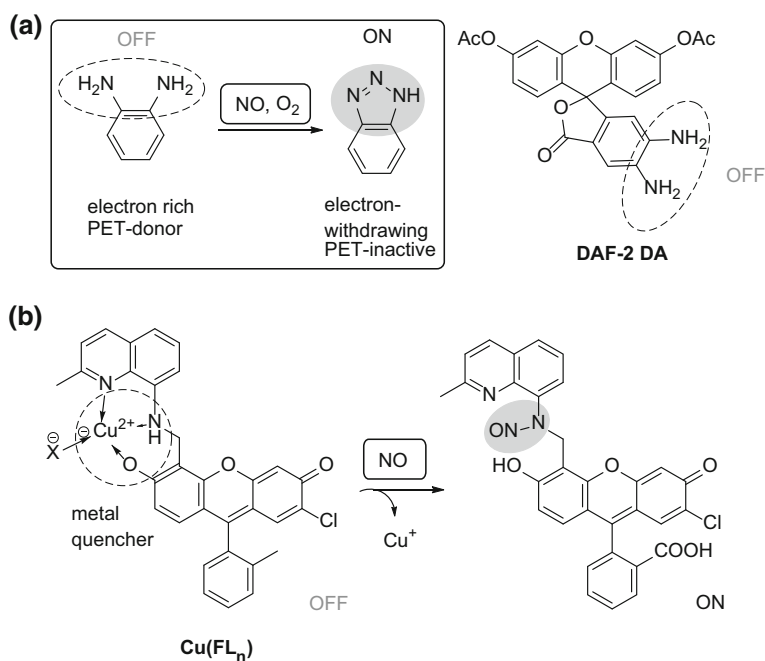

Fig. 1.9 Reaction-based probes for the selective detection of nitric oxide: **a** reaction of NO$^\bullet$ with *o*-phenylenediamine, with example probe **DAF-2 DA** [104]; **b Cu(FL$_n$)**, which detects NO$^\bullet$ via redox-induced release of the fluorophore [105, 106]. Reprinted with permission from *Antioxidants and Redox Signalling*, Volume 24, Issue 13, published by Mary Ann Leibert, Inc., New Rochelle, NY

Metal complex probes for NO• typically contain a fluorophore coordinated to a ligand bearing a paramagnetic metal ion, which quenches the fluorescence of the fluorophore. Biocompatible Cu(II)-containing probes **Cu(FL$_n$)** have been developed, with ligands bearing quinoline-fluorescein conjugates (Fig. 1.9b) [105, 106]. NO• mediated chemo-selective reduction of copper(II) to copper(I) with simultaneous *N*-nitrosation of fluorescein results in the release of the metal ion from the chelate and in a 16-fold increase in fluorescence.

1.5.1.4 Probes for Peroxynitrite

In addition to the boronate-based probes that have been shown to respond to both ONOO$^-$ and H$_2$O$_2$, probes based on the oxidation of an ethyltrifluoromethylketone group to a dioxirane intermediate have also been developed. **HKGreen-1**, which contains a fluorescein scaffold linked to an ethyltrifluoromethylketone by an aryl-ether bond, exploits this strategy (Fig. 1.10a) [113]. The ketone is oxidised to a dioxirane intermediate that brings about the ether bond cleavage and release of the fluorophore. **HKGreen-2** and **HKGreen-3** probes also respond to ONOO$^-$ via formation of a dioxirane intermediate, unmasking probes fluorescence [114, 115]. Another strat-
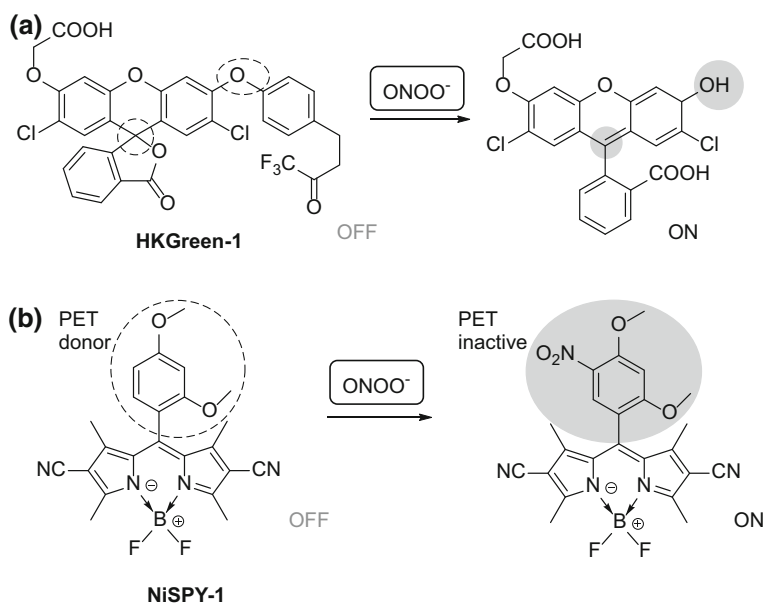

Fig. 1.10 ONOO$^-$ detection by irreversible dioxirane formation leading to: **a** liberation of a fluorophore (**HKGreen-1**) [113], **b** Example of ONOO$^-$ detection via PET-quenching alleviation upon peroxynitrite-induced nitration (**NiPSY1**) [116]. Reprinted with permission from *Antioxidants and Redox Signalling*, Volume 24, Issue 13, published by Mary Ann Leibert, Inc., New Rochelle, NY

egy for ONOO$^-$ sensing utilises its potent nitrating ability. Nitration of **NiSPYs** upon reaction with ONOO$^-$ removes the PET-quenching, unmasking the probe's fluorescence (Fig. 1.10b) [116].

1.5.1.5 Probes for Superoxide

The superoxide radical anion ($^1O_2^{\bullet-}$) is the primary ROS, a consequence of one-electron reduction of molecular oxygen. Sensing of this ROS particularly focusses on the use of dihydroethidine (**DHE**, Fig. 1.11a) through a mechanism that has been shown by Kalyanaraman et al. to involve the formation of 2-hydroxyethidium (**2-HE**) following a reaction between superoxide and hydroethidine [117, 118]. A different sensing mechanism is concerned with the oxidation of non-fluorescent 2,3-dihydrobenzothiazoles (Fig. 1.11b), for example in **H.Py.Bzt** (2-(2-pyridil)-benzothiazoline) to corresponding strongly fluorescent benzothiazole derivatives [119].

Another strategy involving the deprotection of benzenesulfonate derivatives. for example in **BESSo** (Fig. 1.11c) to sense H$_2$O$_2$ has also been applied for the detection
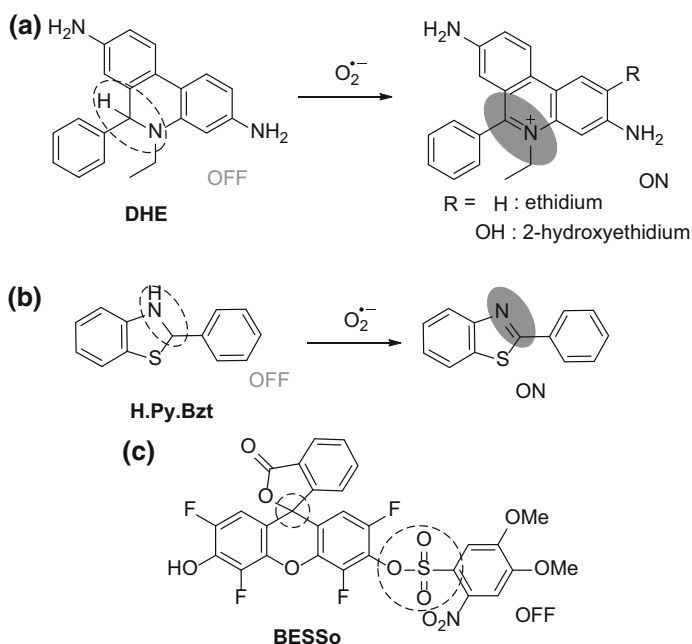

Fig. 1.11 Superoxide detection by oxidation of **a DHE** to ethidine and 2-hydroxyethidium [117, 118] **b** benzothiazole moiety in **H.Py.Bzt** [119], and **d** deprotection of benzenesulfonate group in **BESSo** [120, 121]

Fig. 1.12 Mechanism of
$^\bullet$OH-mediated production of
$^\bullet$CH$_3$ which brings about
PET-quenching alleviation
upon radical oxidation of
Fluorescamine-nitroxide
[124]

Fluorescamine-nitroxide
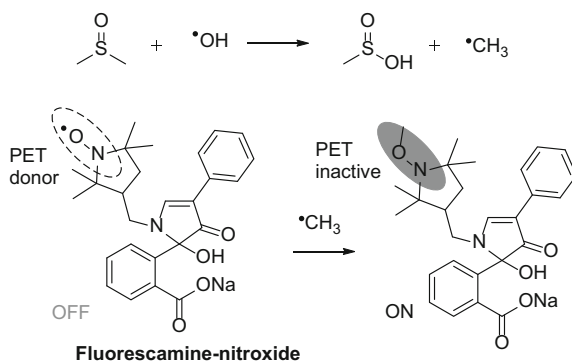
of superoxide, this indicates unclear preference of such groups in their reactivity towards ROS [120, 121].

1.5.1.6 Probes for Hydroxyl Radical

Following the work of Blough and colleagues who first initiated the use of nitroxide-conjugated fluorescent sensor [122, 123], Pou and co-workers developed **Fluorescamine-nitroxide** (Fig. 1.12) for sensing $^\bullet$OH in biological systems [124]. These sensors consist of a PET quenching TEMPO moiety attached to a fluorophore. Introduction of $^\bullet$OH in the presence of DMSO resulted in the generation of methyl radicals ($^\bullet$CH$_3$) which then react with the nitroxide and convert it into *o*-methylhydroxylamine derivative with enhanced fluorescence emission.

Several other nitroxide based probes have been reported, such as **TEMPO-BDP** [125], but significant background fluorescence and other side reactions caused by the presence of DMSO along with high reactivity of nitroxide itself have limited the use of these probes for $^\bullet$OH sensing in biological systems.

1.5.1.7 Probes for Singlet Oxygen

Probes for singlet oxygen have been designed based on the reactivity of the anthracene moiety towards $^1O_2$, to form an endoperoxide (Fig. 1.13a). The Nagano group developed 9,10-diphenylanthracene (DPA) conjugated xanthene probes (**DPAXs**, Fig. 1.13b) for fluorescent detection of $^1O_2$ [126]. $^1O_2$-mediated endoperoxide formation results in significant reduction of the PET quenching by the DPA scaffold and therefore efficient emission from the xanthene fluorophore. Faster singlet oxygen-responsive xanthene-based probes have been developed (**DMAX**) that employ a 9,10-dimethylanthracene trap [127].

Fig. 1.13 a Detection
mechanism of singlet oxygen
based on endoperoxide
formation within aromatic
rings [126]. **b** example of
fluorescein-based probes
(**DPAXs**) based on this
strategy [127]
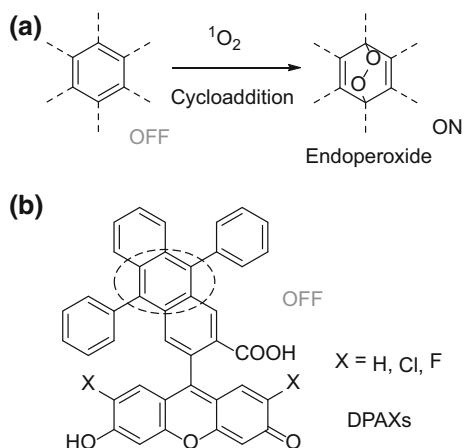
1.5.2 Reversible Probes for Redox Sensing

Although the scientific community has turned greatest attention towards the development of selective reaction-based probes, such probes are not able to distinguish between transient bursts in ROS production typical of physiological events and chronically-elevated ROS levels, characteristic of pathological oxidative stress. This is because both situations can result in high ROS measurement at a single timepoint. In order to make this distinction, monitoring time-resolved changes in the redox state of the cells is essential. This ability solely depends on the use of reversible probes, which can cycle back and forth with successive oxidation and reduction events. Using such a probe, chronic oxidative stress will be distinguished by high ROS-levels over time, while transient oxidation events will be imaged as high ROS-levels which subsequently decrease.

Reversible redox sensing abilities have been successfully developed in a set of redox-responsive fluorescent proteins which possess vital properties such as excellent photostability, bio-compatibility, and ease of intracellular targeting. For example, **HyPer** selectively and reversibly responds to H_2O_2 with a ratiometric emission change [128]. This and other fluorescent protein-based redox probes have been successfully used in a variety of in vivo models, and continue to deliver information that was previously beyond the access of scientific community [129]. However, such probes often require an invasive and usually laborious genetic modification of the system, and so cannot be applied to a variety of samples, nor will they have potential clinical applications. In this context, small molecule probes are very promising in overcoming the intrinsic limitations of genetically-encoded probes. The reversible redox probes discussed in this section have been classified according to the redox responsive group.

1.5.2.1 Nitroxide-Based Probes

To enable fluorescent detection, the nitroxide moiety can be covalently tethered to a fluorophore of choice via a suitable linker. The nitroxyl free radical will quench the fluorophores fluorescence [130], which can be restored upon reversible reduction to the diamagnetic hydroxylamine, or irreversible formation of alkoxyamine derivatives (Fig. 1.14a). Since fluorescence is activated upon reduction, these probes are often referred to as profluorescent nitroxides, by analogy to prodrugs. The first proof-of-concept nitroxyl-based fluorophore, **NO-naphthalene** (Fig. 1.14b) [123], exhibits a 10-fold increase in fluorescence quantum yields upon reduction. This strategy has also been applied to other fluorophores in the development of **NO-dansyl** and **NO-perylene** probes [131].

While the nitroxyl radical hydroxylamine redox couple is reversible, the reversibility of nitroxide-based probes is generally not reported. Reversibility of response has been demonstrated for **fluorescein-TEMPO** (Fig. 1.14b), for which one-electron reduction by excess hydrazine hydrate gave rise to an increase in fluorescence, which
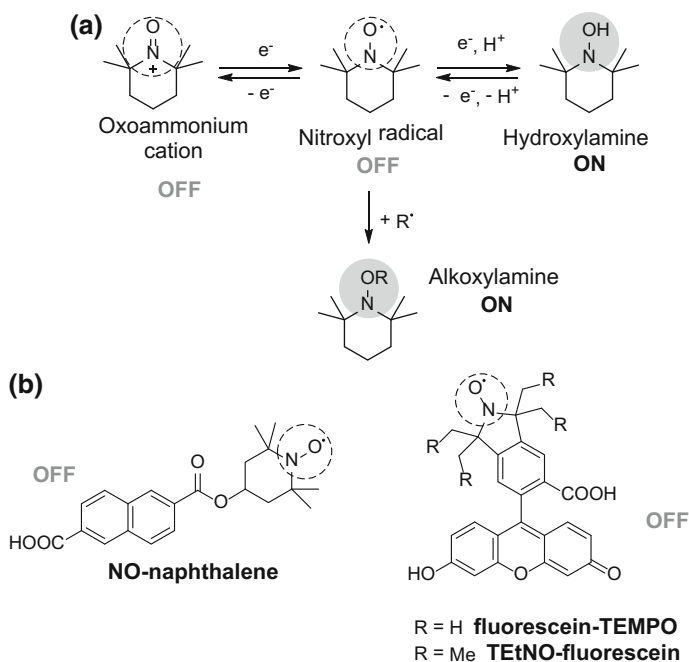
Fig. 1.14 a Possible redox reactions of nitroxyl radicals in biological media. Circles indicate sites of redox response, with fluorescence quenching motifs highlighted in dashed circles and moieties enabling fluorescence shown in grey circles; **b** selected examples of nitroxide-based redox-responsive fluorescent probes, with the fluorescence quenching nitroxyl radical shown in the dashed circle [123, 130, 132–134]. Reprinted with permission from *Angewandte Chemie*, Volume 55, Issue 5, published by John Wiley & sons, Inc.

could then be reversed by air re-oxidation [132, 135]. While this re-oxidation persisted over three redox cycles, with each cycle there was a slight increase in the basal fluorescence intensity, indicative of irreversible destruction of a fraction of the radical.

1.5.2.2 Quinones

The quinone/hydroquinone reversible redox couple (Fig. 1.15a) has been widely studied for almost hundred years [136] and is therefore an obvious choice in the design of redox-responsive optical probes. Quinone-based probes employ a strategy of luminescence quenching by PET from the luminophore in the excited state to the electron-poor quinone motif, as widely reported for porphyrin-based systems [137, 138]. By this strategy, a number of reversible quinone-containing redox sensors based on ruthenium complexes have been reported. For example, the red-luminescent probe **[Ru(bpy)**$_2$(bpy-Q]$^{2+}$ (Fig. 1.15b) gave rise to a 4-fold increase in luminescence upon electrochemical reduction in acetonitrile ($E_{red} = -0.2$ V) [139].

Other ruthenium-quinone complexes have been developed [142, 143], and despite electrochemical reversibility in near biologically-relevant potentials (-50 to -300 mV) and significant luminescence response, none of these complexes was examined in more biologically-relevant aqueous conditions, nor was their chemical reversibil-

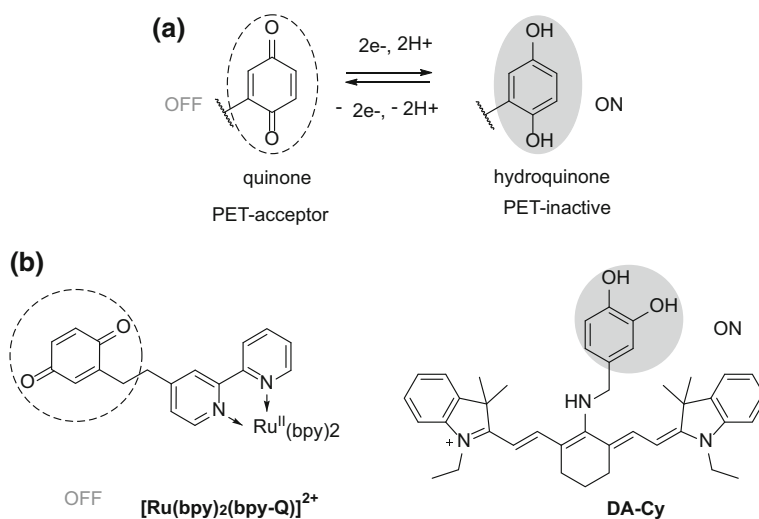**Fig. 1.15** **a** reversible redox reactivity of quinone/hydroquinone pair; **b** example of ruthenium-based redox-controlled luminescent switch [139]; **c** mechanism of intracellular response of **Da-Cy** probe to H_2O_2/thiol redox pair [140]; **d** reduced non-fluorescent form of **TCA** probe [141]. Reprinted with permission from *Angewandte Chemie*, Volume 55, Issue 5, published by John Wiley & sons, Inc.

ity demonstrated. To ensure a redox-dependent fluorescence response in a biological setting, an intracellular redox-active dopamine was directly attached to a cyanine dye (**DA-Cy**, Fig. 1.15c) yielding an on-off-on probe [140]. H_2O_2 oxidation of the 1,2-hydroquinone moiety of dopamine gave a 20-fold fluorescence quenching, and this effect could be counteracted by addition of thiols, in an irreversible process. Disappearance of **DA-Cy** fluorescence upon oxidative stress and subsequent thiol-dependent recovery in HL-7702, HepG2 and RAW 264.7 cells, as well as in rat hippocampal tissue slices, demonstrates the biological utility of **DA-Cy** to study H_2O_2/thiol redox pair. However, the irreversible reduction upon thiol addition excludes the possibility of imaging more than one oxidation/reduction cycle.

1.5.2.3 Chalcogen-Based Fluorescent Redox Probes

A broad class of reversible redox probes involve sulfur, selenium and tellurium in their sensing groups. These can be further divided into those that sense oxidation through formation of a disulfide, diselenide or ditelluride bridge (dichalcogenides, Fig. 1.16a), and those that involve oxidation of the chalcogen itself to the oxide form (Fig. 1.17a).

Dichalcogenides
The sulfide-disulfide oxidation (exemplified in the cysteine to cystine oxidation) is central to countless biological processes and structures. Ratios of thiol-disulfide (whether **GSH/GSSG** or cysteine/cystine) within cells are therefore widely accepted to be good indicators of cellular oxidative stress [144], ensuring that disulfide is therefore a suitable redox sensing moiety on which the development of fluorescent redox sensors can be based. Likewise, the selenide-diselenide oxidation plays an important role in biology, such as in the catalytic site of glutathione peroxidase [145]. These redox switches have been employed in the development of a limited number of probes discussed below. One of the first reported reversible redox probes based on disulfides, **carbostyril-Tb**, incorporated a carbostyril chromophore separated from a terbium complex by a hexapeptide linker (Fig. 1.16b) [146]. Upon oxidation, the two cysteine residues in the linker form a disulfide bridge that brings the carbostyril and Tb close enough to enable sensitised luminescence, with constant emission intensity at 400 nm enabling ratiometric readout. The reduction potential of this probe has been reported to be −0.243 mV, which lies well within the biological range. While this probe was not tested in biological systems, the authors identify the ease with which the reduction potential can be tuned by modification of the linker. The probe **FSeSeF**, which consists of two fluorescein molecules linked by a diselenide bridge (Fig. 1.16b), utilises an approach similar to the sulfide-disulfide oxidation [75].

Chalcogenoxides
Another strategy for developing fluorescent redox sensors has been to employ chalcogens (S, Se and Te), which can be readily and reversibly oxidised to the respective chalcogenoxides (sulfoxides, selenoxides and telluroxides; Fig. 1.17a). The strategy of employing chalcogen-chalcogenoxide oxidation was pioneered by the Han group
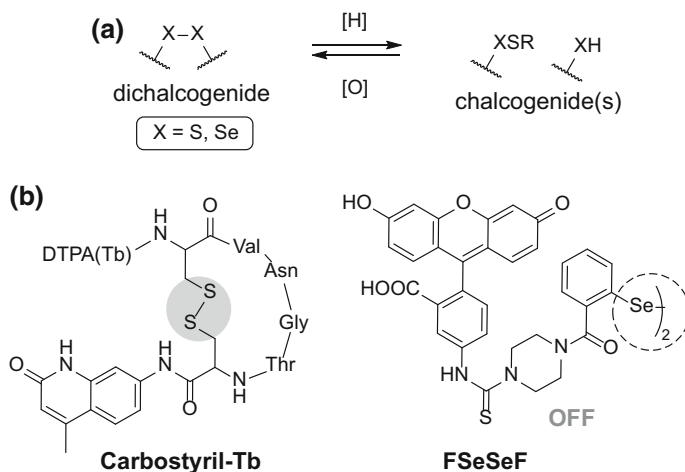
Fig. 1.16 Reversible redox transformations of **a** dichalcogenide and **b** examples of dichalcogenide-type probes [75, 146, 147]. Reprinted with permission from *Angewandte Chemie*, Volume 55, Issue 5, published by John Wiley & sons, Inc.

following the decoding of the catalytic sites of GPx, which showed that selenium in the catalytic pockets reacts with ROS to form selenoxides [145]. The first such probe, **Cy-PSe** (Fig. 1.17b), a near IR emitter, was based on photoinduced electron transfer (PET) between a cyanine signal transducer and a phenyl selenium modulator [148]. Upon oxidation to Se = O, PET quenching is alleviated, resulting in a turn-on in fluorescence. **Cy-PSe** was reported to be selectively oxidised by peroxynitrite and reduced by glutathione and cysteine. The probe was used to measure peroxynitrite in activated mouse macrophages, and its reversibility in biological systems was demonstrated.

A number of further probes have been developed based on this strategy such as **MPhSe-BOD** [151], **diMPhSe-BOD** [151], **HCSe** [152]. and **NI-Se** [153]. This strategy has also been extended towards the development of probes based on other chalcogenides—tellurium (**2Me TeR** [149], Fig. 1.17c) and sulfur ($[\mathbf{Ru(bpy)}]_3^{2+}$]-**PTZ** [150], Fig. 1.17d).

1.5.2.4 Nicotinamides and Flavins

Biological systems exhibit complex mechanisms of redox regulation, but the majority of regulatory systems utilise members of the vitamin B group nicotinamides (B3) and flavins (B2). These vitamins, and particularly their nucleotide derivatives, nicotinamide adenine dinucleotide (NAD), flavin adenine dinucelotide (FAD), and flavin mononucleotide (FMN), act as redox active cofactors and coenzymes in cellular redox reactions (Fig. 1.18a, b). The first reported use of such vitamins in redox sen-
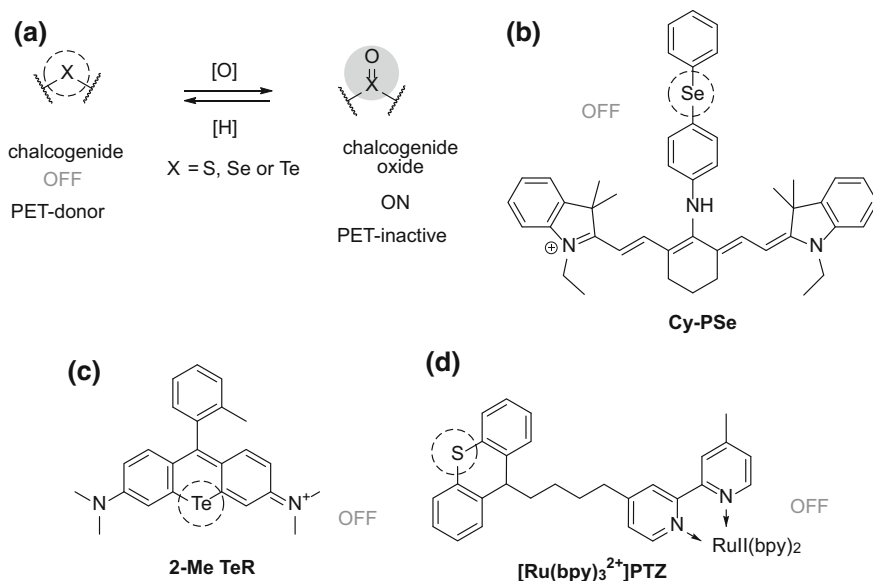
Fig. 1.17 **a** Reactivity of reversible chalcogenide /chalcogenoxide redox couple and **b, c** selected examples of this type of probes based on **b** Se [148], **c** Te [149] and **d** S [150]. Reprinted with permission from *Angewandte Chemie*, Volume 55, Issue 5, published by John Wiley & sons, Inc.

sors was a PET-based redox molecular switch consisting of a perylene scaffold linked to nicotinamide, **Perylene-NAD** (Fig. 1.18c), which undergoes a 10-fold increase in emission upon oxidation [154]. This molecular switch can be oxidised by *p*-chloranil and reduced by NaBH$_3$CN reversibly for up to 3 cycles, but the process is not selective for these oxidising and reducing agents. Extensive electrochemical and spectro-electrochemical studies give an insight into the reduction potential of this sensor and the plausible electron transfer mechanisms, and highlight the value of these studies for all redox sensors. There are no other reports of nicotinamide-based fluorescent redox probes and it appears that the scientific community has underestimated the potential of the reversible redox properties of nicotinamide.

One of the first reports utilising the reversible redox properties of flavins came from the Aoki group, which developed Zn^{2+}-tetraazacyclododecane complexes bearing lumiflavin and tryptophan [155]. Although these complexes were designed for use as DNA photolyase mimics, the studies emphasised the photochemistry and redox properties of the flavin scaffold. Later, the same group reported **CMFL-BODIPY**, containing carboxymethylflavin (Fig. 1.18d). This probe could be reduced by Na$_2$S$_2$O$_4$ with a 9-fold decrease in fluorescence emission [156]. The reduction potential of this probe was reported to be −240 mV, similar to that of cellular flavins. Although the probes reversibility and selectivity were not reported, studies in HeLa cells demonstrated the potential of the sensing strategy. Again, the potential of this sensing group has been underutilised.
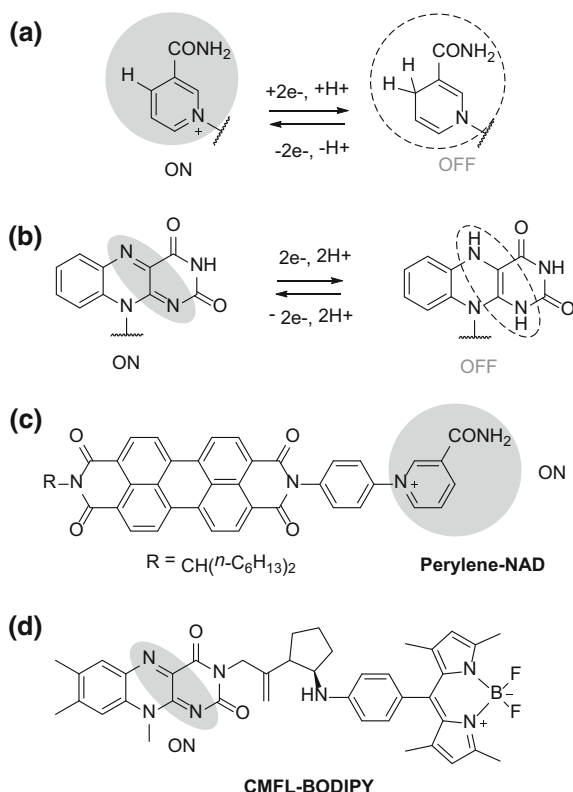
1.6 The Current State of Redox Sensing

This survey of currently available probes reveals the vast potential of the field. In comparison to the field of irreversible probes, many of which have been reported, the development of reversible probes has been relatively slow, and certainly warrants further study.

This literature survey has helped to identify the most promising features of current probes and determine areas that warrant further attention. The nature of the fluorescence change upon oxidation/reduction is crucial in determining the biological utility of a probe. For turn-on (intensity-based) probes, it is important to consider whether the signal is enhanced upon reduction (as for nitroxide-based probes) or upon oxidation (as for most other classes of probes). For the former, probes are likely to be best able to probe questions of hypoxia, or AO efficacy, while the latter will have utility in uncovering new roles of ROS/RNS. However, more promising still are ratiometric probes, for which both oxidised and reduced form can be imaged. In addition to minimising interference from background effects such as probe concentration, ratiometric probes bear the possibility to enable quantification of relative or abso-

lute reduction potential, although this remains to be realised. Despite the promise of probes developed to date, there has been very little work on the rational targeting of probes to specific cell types, or to sub-cellular organelles. Since the redox environment is highly compartmentalised within cells, there is much to be gained from tools that are able to report on location-specific changes in redox state. Another key perspective that is often ignored when designing redox probes is tuning the redox potential of the probes. It is crucial that the probes have a redox potential that falls within the biologically relevant range (-50 to -300 mV). This is essential to ensure that the probe which is introduced within the cells to measure its redox state, doesn't alter the redox potential of the cell itself.

Development of probes utilising the cells' own redox regulators such as nicotinamides and flavins remains underexplored till date. Redox responsive scaffolds such as nicotinamides and flavins not only offer reversible redox properties, but also have reduction potentials well-tuned to the cellular redox events. This suggests that redox probes based on flavins and nicotinamides would not alter the redox potential of the cell. In addition, the reduction potential of flavin and nicotinamide scaffolds can be desirably altered by making suitable structural modifications [157–159]. This ability to tune the reduction potential of a probe will enable development of sets of probes that enable accurate determination of reduction potentials within the cell (analogous to the use of a range of pH indicators to determine acidity). Furthermore, the use of naturally existing molecules eliminates concerns related to cytotoxicity, biological compatibility and cell permeability. Therefore, there is great promise in the development of fluorescent redox probes based on the naturally existing redox molecules.

While fluorescence response is routinely screened in a analysis of a new probe, many studies do not assess the reversibility of the response, the biological sensitivity, or the biological compatibility, but such information is essential: a reversible probe that does not enter cells is not likely to be useful in probing intracellular redox state, nor will a probe with a reduction potential outside the physiologically-relevant range. Key data to gather includes: reversibility over repeated cycles of oxidation-reduction; response to a range of oxidants and reductants (to verify selectivity or global response); stability of signal in the presence of possible interferents such as metal ions, proteins, or pH changes; effect on cell viability; sensitivity to biologically-relevant redox changes as well as the stability, retention and photostability of the probe in time in cellular studies.

Furthermore, the more widespread adoption of fluorescent redox probes will be facilitated by verification that probes can work by more modalities than just confocal microscopy, such as flow cytometry and in plate reader assays. While there is still much work to be done, the promising strategies identified thus far are likely to yield reversible fluorescent sensors of redox state that can be used to distinguish chronic oxidative stress from physiological oxidative bursts not only in cultured cells, but also in vivo studies. The challenge remains to ensure that such probes are put to best use, and that they are employed beyond the laboratory in which they were developed, instead becoming invaluable tools for the redox biology community.

1.7 Objectives

The survey of the literature indicated that there exists a wide array of reaction-based fluorescent redox probes. The aims of the project described in this thesis were to design and synthesise a new class of fluorescent redox-responsive probes. The primary requirement for such tools is reversibility of response to repeated cycles of oxidation and reduction. In order to have utility in biological studies, however, a number of other important aspects must be satisfied, which include:

1. The probe must respond globally to cellular oxidative changes. While reaction-based (irreversible) probes selective for a particular ROS or redox-active pair provide valuable details about the role of redox signalling in a particular biochemical pathway, reversible probes that respond to global oxidative changes are able to report on the overall oxidative capacity of a cell and its recovery from stress.
2. Clear fluorescence response, in which the probe has a high quantum yield and sufficient Stoke's shifts, as well as a large difference in the signal between oxidised and reduced forms. Furthermore, a ratiometric response, in which emission wavelength changes are measured rather than emission intensity, ensures an internal reference that nullifies any concentration, background and instrument-based effects.
3. The fluorescence properties of the probe should be amenable for use with existing imaging technologies, such as confocal microscopy and flow cytometry.
4. Fast reaction kinetics are essential for instantaneous equilibration with the steady state of specific ROS/RNS in the local cellular environment and for maximum spatio-temporal resolution of the signal.
5. Sensitivity of response requires that the fluorescence change is triggered by biologically-relevant redox potentials, or by biologically-meaningful concentrations of ROS/RNS and/or AO.
6. Tunability of redox potential will ensure that probes can be developed that cover the whole range of biologically-relevant redox potentials. Such potentials can differ significantly depending not only on the stage of cellular development, cell type and intracellular localization, but also on the considered biologically-relevant redox pair.
7. Biological compatibility requires the optimization of parameters such as sufficient cellular permeability, and specific sub-cellular localization to enable monitoring of oxidative stress at the organelle level. Furthermore, particularly for reversible probes that offer the potential to monitor cells over time, probes must be non-toxic, and have minimal effect on cellular homoeostasis.
8. The developed probe should be investigated for responsive behaviour in a variety of biological systems in order to understand the extent and limitations of its applicability.

This thesis details the work performed towards the design, synthesis and biological application of reversible redox probes. Chapters 2–4 describe the development of

flavin-based redox probes, for imaging both cytoplasmic and mitochondrial oxidative capacity. Chapter 5 presents the strategies investigated for the development of ratiometric redox probes based on nicotinamides. Finally, Chaps. 6–8 discuss the biological applications of the developed probes.

References

1. F.I.F. Benzie, Evolution of antioxidant defence mechanisms. Eur. J. Nutr. **39**, 53–61 (2000)
2. B. Halliwell, Antioxidant defence mechanisms: from the beginning to the end (of the beginning). Free Radic. Res. **31**, 261–272 (1999)
3. S.I. Liochev, Reactive oxygen species and the free radical theory of aging. Free Radic. Biol. Med. **60**, 1–4 (2013)
4. O.I. Aruoma, H. Kaur, B. Halliwell, Oxygen free radicals and human diseases. J. R. Soc. Health **111**, 172–177 (1991)
5. M. Valko, D. Leibfritz, J. Moncol, M.T. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. **39**, 44–84 (2007)
6. R. Gerschman, D.L. Gilbert, S.W. Nye, P. Dwyer, W.O. Fenn, Oxygen poisoning and X-irradiation: a mechanism in common. Science **119**, 623–626 (1954)
7. N.S. Dhalla, R.M. Temsah, T. Netticadan, Role of oxidative stress in cardiovascular diseases. J. Hypertens. **18**, 655–673 (2000)
8. E.H. Sarsour, M.G. Kumar, L. Chaudhuri, A.L. Kalen, P.C. Goswami, Redox control of the cell cycle in health and disease. Antioxid. Redox Signal. **11**, 2985–3011 (2009a)
9. T.C. Jorgenson, W. Zhong, T.D. Oberley, Redox imbalance and biochemical changes in cancer. Cancer Res. **73**, 6118–6123 (2013)
10. H. Kamata, H. Hirata, Redox regulation of cellular signalling. Cell. Signal. **11**, 1–14 (1999)
11. B. D'Autreaux, M.B. Toledano, ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat. Rev. Mol. Cell Biol. **8**, 813–824 (2007)
12. C.C. Winterbourn, The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells. Biochimica et Biophysica Acta (BBA)—General Subjects **1840**, 730–738 (2014)
13. B. Kalyanaraman, V. Darley-Usmar, K.J.A. Davies, P.A. Dennery, H.J. Forman, M.B. Grisham, G.E. Mann, K. Moore, L.J. Roberts, H. Ischiropoulos, Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. Free Radic. Biol. Med. **52**, 1–6 (2012)
14. M.D. Brand, C. Affourtit, T.C. Esteves, K. Green, A.J. Lambert, S. Miwa, J.L. Pakay, N. Parker, Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. Free Radic. Biol. Med. **37**, 755–767 (2004)
15. T. Finkel, N.J. Holbrook, Oxidants, oxidative stress and the biology of ageing. Nature **408**, 239–247 (2000)
16. B. Halliwell, Generation of hydrogen peroxide, superoxide and hydroxyl radicals during the oxidation of dihydroxyfumaric acid by peroxidase. Biochem. J. **163**, 441–448 (1977)
17. M. Valko, H. Morris, M.T.D. Cronin, *Metals, Toxicity and Oxidative Stress* (2005)
18. C.H. Foyer, H. Lopez-Delgado, J.F. Dat, I.M. Scott, Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. Physiol. Plant. **100**, 241–254 (1997)
19. E. Pinto, T.C.S. Sigaud-kutner, M.A.S. Leitão, O.K. Okamoto, D. Morse, P. Colepicolo, Heavy metal-induced oxidative stress in. J. Phycol. **39**, 1008–1018 (2003)
20. J.M. Pullar, M.C.M. Vissers, C.C. Winterbourn, Living with a killer: the effects of hypochlorous acid on mammalian cells. IUBMB Life **50**, 259–266 (2000)

21. L.J. Ignarro, Nitric oxide: a unique endogenous signaling molecule in vascular biology (Nobel Lecture). Angew. Chem. Int. Ed. **38**, 1882–1892 (1999)
22. R.F. Furchgott, Endothelium-derived relaxing factor: discovery, early studies, and identifcation as nitric oxide (nobel lecture). Angew. Chem. Int. Ed. **38**, 1870–1880 (1999)
23. D. Hirst, T. Robson, Nitric oxide physiology and pathology, in *Nitric Oxide*, ed. by H.O. McCarthy, J.A. Coulter), vol. 704, Chap. 1 (Humana Press, 2011), pp. 1–13
24. S. Moncada, R.M. Palmer, E.A. Higgs, Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. **43**, 109–142 (1991)
25. M.P. Murphy, M.A. Packer, J.L. Scarlett, S.W. Martin, Peroxynitrite: a biologically significant oxidant. Gen. Pharmacol.: Vasc. Syst. **31**, 179–186 (1998)
26. P.Á.L. Pacher, J.S. Beckman, L. Liaudet, Nitric oxide and peroxynitrite in health and disease. Physiol. Rev. **87**, 315–424 (2007)
27. V. Sosa, T. Moline, R. Somoza, R. Paciucci, H. Kondoh, L.L. Me, Oxidative stress and cancer: an overview. Ageing Res. Rev. **12**, 376–390 (2013)
28. P.H.G.M. Willems, R. Rossignol, C.E.J. Dieteren, M.P. Murphy, W.J.H. Koopman, Redox homeostasis and mitochondrial dynamics. Cell Metab. **22**, 207–18 (2015)
29. M. Schieber, N.S. Chandel, ROS function in redox signaling and oxidative stress. Curr. Biol. **24**, R453–R462 (2014)
30. K. Sinha, J. Das, P.B. Pal, P.C. Sil, Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. Arch. Toxicol. **87**, 1157–1180 (2013)
31. A.A. Starkov, The role of mitochondria in reactive oxygen species metabolism and signaling. Ann. N. Y. Acad. Sci. **1147**, 37–52 (2008)
32. M. Rojkind, J.A. Dominguez-Rosales, N. Nieto, P. Greenwel, Role of hydrogen peroxide and oxidative stress in healing responses. Cell. Mol. Life Sci. **59**, 1872–1891 (2002)
33. M. Noble, J. Smith, J. Power, M. Mayer-Proschel, Redox state as a central modulator of precursor cell function. Ann. N. Y. Acad. Sci. **991**, 251–271 (2003)
34. T. Finkel, Signal transduction by reactive oxygen species. J. Cell Biol. **194**, 7–15 (2011)
35. C.C. Winterbourn, M.B. Hampton, Thiol chemistry and specificity in redox signaling. Free Radic. Biol. Med. **45**, 549–561 (2008)
36. T. Finkel, From sulfenylation to sulfhydration: what a thiolate needs to tolerate. Sci. Signal. **5**, pe10(2012)
37. E.H. Sarsour, M.G. Kumar, L. Chaudhuri, A.L. Kalen, P.C. Goswami, Redox control of the cell cycle in health and disease. Antioxid. Redox Signal. **11**, 2985–3011 (2009)
38. E.R. Stadtman, B.S. Berlett, Reactive oxygen-mediated protein oxidation in aging and disease. Drug Metab. Rev. **30**, 225–243 (1998)
39. J.S. Dawane, V.A. Pandit, Understanding redox homeostasis and its role in cancer. J. Clin. Diagn. Res. **6**, 1796–1802 (2012)
40. R. Zhu, Y. Wang, L. Zhang, Q. Guo, Oxidative stress and liver disease. Hepatol. Res. **42**, 741–749 (2012)
41. H.K. Vincent, A.G. Taylor, Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. Int. J. Obes. (Lond) **30**, 400–418 (2006)
42. D. Athanasiou, M. Aguila, D. Bevilacqua, S.S. Novoselov, D.A. Parfitt, M.E. Cheetham, The cell stress machinery and retinal degeneration. FEBS Lett. **587**, 2008–2017 (2013)
43. Y.J.H.J. Taverne, A.J.J.C. Bogers, D.J. Duncker, D. Merkus, Reactive oxygen species and the cardiovascular system. Oxid. Med. Cell. Longev. **2013**, 15 (2013)
44. M. Mohsenzadegan, A. Mirshafiey, The immunopathogenic role of reactive oxygen species in Alzheimer disease. Iran J. Allergy Asthma Immunol. **11**, 203–216 (2012)
45. C. Garcia-Ruiz, J.C. Fernandez-Checa, Redox regulation of hepatocyte apoptosis. J. Gastroenterol. Hepatol. **22**(Suppl 1), S38–42 (2007)
46. M. Ristow, K. Zarse, A. Oberbach, N. Klöting, M. Birringer, M. Kiehntopf, M. Stumvoll, C.R. Kahn, M. Blüher, *Antioxidants prevent health-promoting effects of physical exercise in humans* (Proc. Natl. Acad, Sci, 2009)
47. H. Sies, Oxidative stress: oxidants and antioxidants. Exp. Physiol. **82**, 291–295 (1997)

48. A. Skoumalova, J. Hort, Blood markers of oxidative stress in Alzheimer's disease. J. Cell Mol. Med. **16**, 2291–2300 (2012)
49. D. Montero, G. Walther, A. Perez-Martin, E. Roche, A. Vinet, Endothelial dysfunction, inflammation, and oxidative stress in obese children and adolescents: markers and effect of lifestyle intervention. Obes. Rev. **13**, 441–455 (2012)
50. J. Skrha, Effect of caloric restriction on oxidative markers. Adv. Clin. Chem. **47**, 223–247 (2009)
51. H. Yin, New techniques to detect oxidative stress markers: mass spectrometry-based methods to detect isoprostanes as the gold standard for oxidative stress in vivo. BioFactors **34**, 109–124 (2008)
52. H.K. Kim, A.C. Andreazza, The relationship between oxidative stress and post-translational modification of the dopamine transporter in bipolar disorder. Expert Rev. Neurother. **12**, 849–859 (2012)
53. A.M. Pickering, L. Vojtovich, J. Tower, A.D. KJ, Oxidative stress adaptation with acute, chronic, and repeated stress. Free Radic. Biol. Med. **55**, 109–118 (2013)
54. W.A. Pryor, S.S. Godber, Noninvasive measures of oxidative stress status in humans. Free Radic. Biol. Med. **10**, 177–184 (1991)
55. M.A. Smith, C.A. Rottkamp, A. Nunomura, A.K. Raina, G. Perry, Oxidative stress in Alzheimer's disease. Biochimica et Biophysica Acta (BBA)—Mol. Basis Disease **1502**, 139–144 (2000)
56. I. Dalle-Donne, R. Rossi, R. Colombo, D. Giustarini, A. Milzani, Biomarkers of oxidative damage in human disease. Clin. Chem. **52**, 601–623 (2006)
57. H.N. Xu, J. Tchou, L.Z. Li, Redox imaging of human breast cancer core biopsies: a preliminary investigation. Acad. Radiol. **20**, 764–768 (2013)
58. T. Kullisaar, S. Turk, K. Kilk, K. Ausmees, M. Punab, R. Mandar, Increased levels of hydrogen peroxide and nitric oxide in male partners of infertile couples. Andrology **1**, 850–858 (2013)
59. R. Stocker, J.F. Keaney Jr., Role of oxidative modifications in atherosclerosis. Physiol. Rev. **84**, 1381–1478 (2004)
60. D. Ortiz de Orue, Lucana, Redox sensing: novel avenues and paradigms. Antioxid. Redox Signal. **16**, 636–638 (2012)
61. S.I. Dikalov, D.G. Harrison, Methods for detection of mitochondrial and cellular reactive oxygen species. Antioxid. Redox Signal. **20**, 372–382 (2014)
62. E. Finkelstein, G.M. Rosen, E.J. Rauckman, Spin trapping of superoxide and hydroxyl radical: practical aspects. Arch. Biochem. Biophys. **200**, 1–16 (1980)
63. M.M. Tarpey, D.A. Wink, M.B. Grisham, Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. Am. J. Physiol. Regul. Integr. Comp. Physiol. **286**, R431–44 (2004)
64. A. Keszler, B. Kalyanaraman, N. Hogg, Comparative investigation of superoxide trapping by cyclic nitrone spin traps: the use of singular value decomposition and multiple linear regression analysis. Free Radic. Biol. Med. **35**, 1149–1157 (2003)
65. N. Khan, C.M. Wilmot, G.M. Rosen, E. Demidenko, J. Sun, J. Joseph, J. O'Hara, B. Kalyanaraman, H.M. Swartz, Spin traps: in vitro toxicity and stability of radical adducts. Free Radic. Biol. Med. **34**, 1473–1481 (2003)
66. C. Frejaville, H. Karoui, B. Tuccio, F. Le Moigne, M. Culcasi, S. Pietri, R. Lauricella, P. Tordo, 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide: a new efficient phosphorylated nitrone for the in vitro and in vivo spin trapping of oxygen-centered radicals. J. Med. Chem. **38**, 258–265 (1995)
67. M. Hardy, A. Rockenbauer, J. Vasquez-Vivar, C. Felix, M. Lopez, S. Srinivasan, N. Avadhani, P. Tordo, B. Kalyanaraman, Detection, characterization, and decay kinetics of ROS and thiyl adducts of mito-DEPMPO spin trap. Chem. Res. Toxicol. **20**, 1053–1060 (2007)
68. V. Roubaud, S. Sankarapandi, P. Kuppusamy, P. Tordo, J.L. Zweier, Quantitative measurement of superoxide generation using the spin trap 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide. Anal. Biochem. **247**, 404–411 (1997)

69. S. Dikalov, M. Skatchkov, E. Bassenge, Quantification of peroxynitrite, superoxide, and peroxyl radicals by a new spin trap hydroxylamine 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine. Biochem. Biophys. Res. Commun. **230**, 54–57 (1997)

70. S. Dikalov, M. Skatchkov, E. Bassenge, Spin trapping of superoxide radicals and peroxynitrite by 1-Hydroxy-3-carboxy-pyrrolidine and 1-Hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine and the Stability of Corresponding Nitroxyl Radicals Towards Biological Reductants. Biochem. Biophys. Res. Commun. **231**, 701–704 (1997)

71. J. Lakowicz, *Principles of Fluorescence Spectroscopy* (Kluwer Academic/Plenum Publishers, New York, Boston, Dordrecht, London, Moscow, 1999)

72. M.Y. Berezin, S. Achilefu, Fluorescence lifetime measurements and biological imaging. Chem. Rev. **110**, 2641–2684 (2010)

73. N.J. Emptage, Fluorescent imaging in living systems. Curr. Opin. Pharmacol. **1**, 521–525 (2001)

74. A. Gomes, E. Fernandes, J.L. Lima, Fluorescence probes used for detection of reactive oxygen species. J. Biochem. Biophys. Methods **65**, 45–80 (2005)

75. Z. Lou, P. Li, X. Sun, S. Yang, B. Wang, K. Han, A fluorescent probe for rapid detection of thiols and imaging of thiols reducing repair and H2O2 oxidative stress cycles in living cells. Chem. Commun. **49**, 391–393 (2013)

76. G. He, X. Zhao, X. Zhang, H. Fan, S. Wu, H. Li, C. He, C. Duan, A turn-on PET fluorescence sensor for imaging Cu2+ in living cells. New J. Chem. **34**, 1055–1058 (2010)

77. A.S. Keston, R. Brandt, The fluorometric analysis of ultramicro quantities of hydrogen peroxide. Anal. Biochem. **11**, 1–5 (1965)

78. S. Watanabe, In vivo fluorometric measurement of cerebral oxidative stress using 2'-7'-dichlorofluorescein (DCF). Keio J. Med. **47**, 92–98 (1998)

79. N.W. Kooy, J.A. Royall, H. Ischiropoulos, Oxidation of 2',7'-dichlorofluorescin by peroxynitrite. Free Radic. Res. **27**, 245–254 (1997)

80. C. Rota, C.F. Chignell, R.P. Mason, Evidence for free radical formation during the oxidation of 2-7-dichlorofluorescin to the fluorescent dye 2-7-dichlorofluorescein by horseradish peroxidase: Possible implications for oxidative stress measurements. Free Radic. Biol. Med. **27**, 873–881 (1999)

81. P. Wardman, Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. Free Radic. Biol. Med. **43**, 995–1022 (2007)

82. M. Wrona, K. Patel, P. Wardman, Reactivity of 2,7-dichlorodihydrofluorescein and dihydrorhodamine 123 and their oxidized forms toward carbonate, nitrogen dioxide, and hydroxyl radicals. Free Radic. Biol. Med. **38**, 262–270 (2005)

83. J.C. Sanchez, W.C. Trogler, Polymerization of a boronate-functionalized fluorophore by double transesterification: applications to fluorescence detection of hydrogen peroxide vapor. J. Mater. Chem. **18**, 5134–5141 (2008)

84. L.-C. Lo, C.-Y. Chu, Development of highly selective and sensitive probes for hydrogen peroxide. Chem. Commun., 2728–2729 (2003)

85. E.W. Miller, A.E. Albers, A. Pralle, E.Y. Isacoff, C.J. Chang, Boronate-based fluorescent probes for imaging cellular hydrogen peroxide. J. Am. Chem. Soc. **127**, 16652–16659 (2005)

86. A.E. Albers, V.S. Okreglak, C.J. Chang, A FRET-based approach to ratiometric fluorescence detection of hydrogen peroxide. J. Am. Chem. Soc. **128**, 9640–9641 (2006)

87. A.E. Albers, B.C. Dickinson, E.W. Miller, C.J. Chang, A red-emitting naphthofluorescein-based fluorescent probe for selective detection of hydrogen peroxide in living cells. Bioorgan. Med. Chem. Lett. **18**, 5948–5950 (2008)

88. B.C. Dickinson, D. Srikun, C.J. Chang, Mitochondrial-targeted fluorescent probes for reactive oxygen species. Curr. Opin. Chem. Biol. **14**, 50–56 (2010)

89. B.C. Dickinson, C.J. Chang, Chemistry and biology of reactive oxygen species in signaling or stress responses. Nat. Chem. Biol. **7**, 504–511 (2011)

90. L. Du, M. Li, S. Zheng, B. Wang, Rational design of a fluorescent hydrogen peroxide probe based on the umbelliferone fluorophore. Tetrahedron. Lett. **49**, 3045–3048 (2008)

91. F. He, Y. Tang, M. Yu, S. Wang, Y. Li, D. Zhu, Fluorescence-amplifying detection of hydrogen peroxide with cationic conjugated polymers, and its application to glucose sensing. Adv. Funct. Mater. **16**, 91–94 (2006)

92. F. He, F. Feng, S. Wang, Y. Li, D. Zhu, Fluorescence ratiometric assays of hydrogen peroxide and glucose in serum using conjugated polyelectrolytes. J. Mater. Chem. **17**, 3702–3707 (2007)

93. E.W. Miller, C.J. Chang, Fluorescent probes for nitric oxide and hydrogen peroxide in cell signaling. Curr. Opin. Chem. Biol. **11**, 620–625 (2007)

94. D. Srikun, A.E. Albers, C.I. Nam, A.T. Iavarone, C.J. Chang, Organelle-targetable fluorescent probes for imaging hydrogen peroxide in living cells via SNAP-tag protein labeling. J. Am. Chem. Soc. **132**, 4455–4465 (2010)

95. B.C. Dickinson, C.J. Chang, A targetable fluorescent probe for imaging hydrogen peroxide in the mitochondria of living cells. J. Am. Chem. Soc. **130**, 9638–9639 (2008)

96. B. Dickinson, Y. Tang, Z. Chang, C. Chang, A nuclear-localized fluorescent hydrogen peroxide probe for monitoring sirtuin-mediated oxidative stress responses invivo. Chem. Biol. **18**, 943–948 (2011)

97. D. Kim, G. Kim, S.-J. Nam, J. Yin, J. Yoon, Visualization of endogenous and exogenous hydrogen peroxide using a lysosome-targetable fluorescent probe. Sci. Rep. **5** (2015)

98. A. Sikora, J. Zielonka, M. Lopez, J. Joseph, B. Kalyanaraman, Direct oxidation of boronates by peroxynitrite: mechanism and implications in fluorescence imaging of peroxynitrite. Free Radic. Biol. Med. **47**, 1401–1407 (2009)

99. H. Maeda, Y. Fukuyasu, S. Yoshida, M. Fukuda, K. Saeki, H. Matsuno, Y. Yamauchi, K. Yoshida, K. Hirata, K. Miyamoto, Fluorescent probes for hydrogen peroxide based on a non-oxidative mechanism. Angew. Chem. Int. Ed. **43**, 2389–2391 (2004)

100. M. Abo, Y. Urano, K. Hanaoka, T. Terai, T. Komatsu, T. Nagano, Development of a highly sensitive fluorescence probe for hydrogen peroxide. J. Am. Chem. Soc. **133**, 10629–10637 (2011)

101. S. Kenmoku, Y. Urano, H. Kojima, T. Nagano, Development of a highly specific rhodamine-based fluorescence probe for hypochlorous acid and its application to real-time imaging of phagocytosis. J. Am. Chem. Soc. **129**, 7313–7318 (2007)

102. X. Chen, K.-A. Lee, E.-M. Ha, K.M. Lee, Y.Y. Seo, H.K. Choi, H.N. Kim, M.J. Kim, C.-S. Cho, S.Y. Lee, W.-J. Lee, J. Yoon, A specific and sensitive method for detection of hypochlorous acid for the imaging of microbe-induced HOCl production. Chem. Commun. **47**, 4373–4375 (2011)

103. W. Lin, L. Long, B. Chen, W. Tan, A ratiometric fluorescent probe for hypochlorite based on a deoximation reaction. Chemistry: Eur. J. **15**, 2305–2309 (2009)

104. H. Kojima, N. Nakatsubo, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata, T. Nagano, Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. Anal. Chem. **70**, 2446–2453 (1998)

105. M.H. Lim, B.A. Wong, W.H. Pitcock, D. Mokshagundam, M.-H. Baik, S.J. Lippard, Direct nitric oxide detection in aqueous solution by copper(II) fluorescein complexes. J. Am. Chem. Soc. **128**, 14364–14373 (2006)

106. M.H. Lim, D. Xu, S.J. Lippard, Visualization of nitric oxide in living cells by a copper-based fluorescent probe. Nat. Chem. Biol. **2**, 375–380 (2006)

107. Y. Gabe, Y. Urano, K. Kikuchi, H. Kojima, T. Nagano, Highly sensitive fluorescence probes for nitric oxide based on boron dipyrromethene chromophorerational design of potentially useful bioimaging fluorescence probe. J. Am. Chem. Soc. **126**, 3357–3367 (2004)

108. S. Izumi, Y. Urano, K. Hanaoka, T. Terai, T. Nagano, A simple and effective strategy to increase the sensitivity of fluorescence probes in living cells. J. Am. Chem. Soc. **131**, 10189–10200 (2009)

109. H. Kojima, M. Hirotani, N. Nakatsubo, K. Kikuchi, Y. Urano, T. Higuchi, Y. Hirata, T. Nagano, Bioimaging of nitric oxide with fluorescent indicators based on the rhodamine chromophore. Anal. Chem. **73**, 1967–1973 (2001)

110. H. Kojima, Y. Urano, K. Kikuchi, T. Higuchi, Y. Hirata, T. Nagano, Fluorescent indicators for imaging nitric oxide production. Angew. Chem. Int. Ed. Engl. **38**, 3209–3212 (1999)

111. M.J. Plater, I. Greig, M.H. Helfrich, S.H. Ralston, The synthesis and evaluation of o-phenylenediamine derivatives as fluorescent probes for nitric oxide detection. J. Chem. Soc. Perkin Trans. **1**, 2553–2559 (2001)

112. E. Sasaki, H. Kojima, H. Nishimatsu, Y. Urano, K. Kikuchi, Y. Hirata, T. Nagano, Highly sensitive near-infrared fluorescent probes for nitric oxide and their application to isolated organs. J. Am. Chem. Soc. **127**, 3684–3685 (2005)

113. D. Yang, H.-L. Wang, Z.-N. Sun, N.-W. Chung, J.-G. Shen, A highly selective fluorescent probe for the detection and imaging of peroxynitrite in living cells. J. Am. Chem. Soc. **128**, 6004–6005 (2006)

114. T. Peng, D. Yang, HKGreen-3: a rhodol-based fluorescent probe for peroxynitrite. Org. Lett. **12**, 4932–4935 (2010)

115. Z.-N. Sun, H.-L. Wang, F.-Q. Liu, Y. Chen, P.K.H. Tam, D. Yang, BODIPY-based fluorescent probe for peroxynitrite detection and imaging in living cells. Org. Lett. **11**, 1887–1890 (2009)

116. T. Ueno, Y. Urano, H. Kojima, T. Nagano, Mechanism-based molecular design of highly selective fluorescence probes for nitrative stress. J. Am. Chem. Soc. **128**, 10640–10641 (2006)

117. H. Zhao, S. Kalivendi, H. Zhang, J. Joseph, K. Nithipatikom, J. Vasquez-Vivar, B. Kalyanaraman, Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. Free Radic. Biol. Med. **34**, 1359–1368 (2003)

118. C.D. Georgiou, I. Papapostolou, N. Patsoukis, T. Tsegenidis, T. Sideris, An ultrasensitive fluorescent assay for the in vivo quantification of superoxide radical in organisms. Anal. Biochem. **347**, 144–151 (2005)

119. B. Tang, L. Zhang, L.-L. Zhang, Study and application of flow injection spectrofluorimetry with a fluorescent probe of 2-(2-pyridil)-benzothiazoline for superoxide anion radicals. Anal. Biochem. **326**, 176–82 (2004)

120. H. Maeda, K. Yamamoto, Y. Nomura, I. Kohno, L. Hafsi, N. Ueda, S. Yoshida, M. Fukuda, Y. Fukuyasu, Y. Yamauchi, N. Itoh, A design of fluorescent probes for superoxide based on a nonredox mechanism. J. Am. Chem. Soc. **127**, 68–69 (2005)

121. H. Maeda, K. Yamamoto, I. Kohno, L. Hafsi, N. Itoh, S. Nakagawa, N. Kanagawa, K. Suzuki, T. Uno, Design of a practical fluorescent probe for superoxide based on protection-deprotection chemistry of fluoresceins with benzenesulfonyl protecting groups. Chemistry (Weinheim an der Bergstrasse, Germany) **13**, 1946–1954 (2007)

122. D.J. Kieber, N.V. Blough, Determination of carbon-centered radicals in aqueous solution by liquid chromatography with fluorescence detection. Anal. Chem. **62**, 2275–2283 (1990)

123. N.V. Blough, D.J. Simpson, Chemically mediated fluorescence yield switching in nitroxide-fluorophore adducts: optical sensors of radical/redox reactions. J. Am. Chem. Soc. **110**, 1915–1917 (1988)

124. S. Pou, Y.I. Huang, A. Bhan, V.S. Bhadti, R.S. Hosmane, S.Y. Wu, G.L. Cao, G.M. Rosen, A fluorophore-containing nitroxide as a probe to detect superoxide and hydroxyl radical generated by stimulated neutrophils. Anal. Biochem. **212**, 85–90 (1993)

125. P. Li, T. Xie, X. Duan, F. Yu, X. Wang, B. Tang, A new highly selective and sensitive assay for fluorescence imaging of *OH in living cells: effectively avoiding the interference of peroxynitrite. Chemistry (Weinheim an der Bergstrasse, Germany) **16**, 1834–1840 (2010)

126. K. Tanaka, N. Umezawa, K. Kikuchi, Y. Urano, T. Higuchi, Novel fluorescent probes for singlet oxygen. Angew. Chem. Int. Ed. Engl. **38**, 2899–2901 (1999)

127. K. Tanaka, T. Miura, N. Umezawa, Y. Urano, K. Kikuchi, T. Higuchi, T. Nagano, Rational design of fluorescein-based fluorescence probes mechanism-based design of a maximum fluorescence probe for singlet oxygen. J. Am. Chem. Soc. **123**, 2530–2536 (2001)

128. V.V. Belousov, A.F. Fradkov, K.A. Lukyanov, D.B. Staroverov, K.S. Shakhbazov, A.V. Terskikh, S. Lukyanov, Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. Nat. Meth. **3**, 281–286 (2006)

129. A.J. Meyer, T.P. Dick, Fluorescent protein-based redox probes. Antioxid. Redox Signal. **13**, 621–650 (2010)
130. S.A. Green, D.J. Simpson, G. Zhou, P.S. Ho, N.V. Blough, Intramolecular quenching of excited singlet states by stable nitroxyl radicals. J. Am. Chem. Soc. **112**, 7337–7346 (1990)
131. E. Lozinsky, V.V. Martin, T.A. Berezina, A.I. Shames, A.L. Weis, G.I. Likhtenshtein, Dual fluorophore-nitroxide probes for analysis of vitamin C in biological liquids. J. Biochem. Biophys. Methods **38**, 29–42 (1999)
132. B.J. Morrow, D.J. Keddie, N. Gueven, M.F. Lavin, S.E. Bottle, A novel profluorescent nitroxide as a sensitive probe for the cellular redox environment. Free Radic. Biol. Med. **49**, 67–76 (2010)
133. F. Yu, P. Song, P. Li, B. Wang, K. Han, Development of reversible fluorescence probes based on redox oxoammonium cation for hypobromous acid detection in living cells. Chem. Commun. **48**, 7735–7737 (2012)
134. Y. Liu, S. Liu, Y. Wang, TEMPO-based redox-sensitive fluorescent probes and their applications to evaluating intracellular redox status in living cells. Chem. Lett. **38**, 588–589 (2009)
135. H.-Y. Ahn, K.E. Fairfull-Smith, B.J. Morrow, V. Lussini, B. Kim, M.V. Bondar, S.E. Bottle, K.D. Belfield, Two-photon fluorescence microscopy imaging of cellular oxidative stress using profluorescent nitroxides. J. Am. Chem. Soc. **134**, 4721–4730 (2012)
136. L.F. Fieser, The tautomerism of hydroxy quinones. J. Am. Chem. Soc. **50**, 439–465 (1928)
137. D. Gust, T. Moore, Photosynthetic model systems, in *Photoinduced Electron Transfer III*, ed. by J. Mattay, vol. 159, Chap. 3, (Springer, Berlin, 1991), pp. 103–151
138. M.R. Wasielewski, Photoinduced electron transfer in supramolecular systems for artificial photosynthesis. Chem. Rev. **92**, 435–461 (1992)
139. V. Goulle, A. Harriman, J.-M. Lehn, An electro-photoswitch: redox switching of the luminescence of a bipyridine metal complex. J. Chem. Soc. Chem. Commun., 1034–1036 (1993)
140. F. Yu, P. Li, P. Song, B. Wang, J. Zhao, K. Han, Facilitative functionalization of cyanine dye by an on-off-on fluorescent switch for imaging of H2O2 oxidative stress and thiols reducing repair in cells and tissues. Chem. Commun. **48**, 4980–4982 (2012)
141. W. Zhang, X. Wang, P. Li, F. Huang, H. Wang, W. Zhang, B. Tang, Elucidating the relationship between superoxide anion levels and lifespan using an enhanced two-photon fluorescence imaging probe. Chem. Commun. **51**, 9710–9713 (2015)
142. A.C. Benniston, G.M. Chapman, A. Harriman, S.A. Rostron, Reversible luminescence switching in a Ruthenium(II) Bis(2,2:6,2-terpyridine)-Benzoquinone Dyad. Inorg. Chem. **44**, 4029–4036 (2005)
143. Y.-X. Yuan, Y. Chen, Y.-C. Wang, C.-Y. Su, S.-M. Liang, H. Chao, L.-N. Ji, Redox responsive luminescent switch based on a ruthenium(II) complex Ru(bpy)(2)(PAIDH) (2+). Inorg. Chem. Commun. **11**, 1048–1050 (2008)
144. B. Palmieri, V. Sblendorio, Current status of measuring oxidative stress, in *Advanced Protocols in Oxidative Stress II*, ed. by D. Armstrong, vol. 594, Chap. 1 (Humana Press, 2010), pp. 3–17
145. J.T. Rotruck, A.L. Pope, H.E. Ganther, A.B. Swanson, D.G. Hafeman, W.G. Hoekstra, Selenium: biochemical role as a component of glutathione peroxidase. Nutr. Rev. **38**, 280–283 (1980)
146. K. Lee, V. Dzubeck, L. Latshaw, J.P. Schneider, De Novo designed peptidic redox potential probe: linking sensitized emission to disulfide bond formation. J. Am. Chem. Soc. **126**, 13616–13617 (2004)
147. K. Xu, M. Qiang, W. Gao, R. Su, N. Li, Y. Gao, Y. Xie, F. Kong, B. Tang, A near-infrared reversible fluorescent probe for real-time imaging of redox status changes in vivo. Chem. Sci. **4**, 1079–1086 (2013)
148. F. Yu, P. Li, G. Li, G. Zhao, T. Chu, K. Han, A near-IR reversible fluorescent probe modulated by selenium for monitoring peroxynitrite and imaging in living cells. J. Am. Chem. Soc. **133**, 11030–11033 (2011)
149. Y. Koide, M. Kawaguchi, Y. Urano, K. Hanaoka, T. Komatsu, M. Abo, T. Terai, T. Nagano, A reversible near-infrared fluorescence probe for reactive oxygen species based on Te-rhodamine. Chem. Commun. **48**, 3091–3093 (2012)

150. F. Liu, Y. Gao, J. Wang, S. Sun, Reversible and selective luminescent determination of ClO-/H2S redox cycle in vitro and in vivo based on a ruthenium trisbipyridyl probe. Analyst **139**, 3324–3329 (2014)
151. B. Wang, P. Li, F. Yu, J. Chen, Z. Qu, K. Han, A near-infrared reversible and ratiometric fluorescent probe based on Se-BODIPY for the redox cycle mediated by hypobromous acid and hydrogen sulfide in living cells. Chem. Commun. **49**, 5790–5792 (2013)
152. S.-R. Liu, S.-P. Wu, Hypochlorous acid turn-on fluorescent probe based on oxidation of diphenyl selenide. Org. Lett. **15**, 878–881 (2013)
153. Z. Lou, P. Li, Q. Pan, K. Han, A reversible fluorescent probe for detecting hypochloric acid in living cells and animals: utilizing a novel strategy for effectively modulating the fluorescence of selenide and selenoxide. Chem. Commun. **49**, 2445–2447 (2013)
154. P. Yan, M.W. Holman, P. Robustelli, A. Chowdhury, F.I. Ishak, D.M. Adams, Molecular switch based on a biologically important redox reaction. J. Phys. Chem. B **109**, 130–137 (2004)
155. Y. Yamada, S. Aoki, Efficient cycloreversion of cis, syn-thymine photodimer by a Zn2+1,4,7,10-tetraazacyclododecane complex bearing a lumiflavin and tryptophan by chemical reduction and photoreduction of a lumiflavin unit. J. Biol. Inorg. Chem. **11**, 1007–1023 (2006)
156. Y. Yamada, Y. Tomiyama, A. Morita, M. Ikekita, S. Aoki, BODIPY-based fluorescent redox potential sensors that utilize reversible redox properties of flavin. ChemBioChem **9**, 853–856 (2008)
157. J.D. Walsh, A.F. Miller, Flavin reduction potential tuning by substitution and bending. J. Mol. Struct. (Thoechem) **623**, 185–195 (2003)
158. C.O. Schmakel, K.S.V. Santhanam, P.J. Elving, Nicotinamide adenine dinucleotide (NAD+) and related compounds. Electrochemical redox pattern and allied chemical behavior. J. Am. Chem. Soc. **97**, 5083–5092 (1975)
159. P. Bourbon, Q. Peng, G. Ferraudi, C. Stauffacher, O. Wiest, P. Helquist, Synthesis, photophysical, photochemical, and computational studies of coumarin-labeled nicotinamide derivatives. J. Organ. Chem. **77**, 2756–2762 (2012)