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$_2^•^-$, Eq. 1.1), the primary reactive oxygen species (ROS) [14]. Superoxide can also be produced enzymatically by NADPH oxidase [14].

$$O_2 + e^- \rightarrow O_2^{•^-} \quad (1.1)$$

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$_2$O$_2$), the hydroxyl radical ($•$OH), hypochlorite ion (OCl$^-$) and singlet oxygen ($^1$O$_2$), whereas RNS include the nitric oxide radical (NO$^•$) and the peroxynitrite anion (ONOO$^-$) [3].

Superoxide rapidly undergoes dismutation, catalysed by a family of enzymes called superoxide dismutases (SODs), to form H$_2$O$_2$ (Eq. 1.2, Fig. 1.1), which is relatively more stable (half-life $\simeq 1$ ms) [16, 17]. Unlike other ROS, H$_2$O$_2$ 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 \( \text{H}_2\text{O}_2 \) to act as a signalling molecule, but at high concentrations it can cause oxidative damage over large distances within the cell [16].

\[
2\text{O}_2^* + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2 \tag{1.2}
\]

Low levels of \( \text{H}_2\text{O}_2 \) are maintained by the action of a family of enzymes—glutathione peroxidases (GPXS), which transfer electrons from two molecules of glutathione (GSH) to \( \text{H}_2\text{O}_2 \) to form water (Eq. 1.3, Fig. 1.1) [16]. The enzyme catalase can convert six million \( \text{H}_2\text{O}_2 \) molecules per minute to water and oxygen (Eq. 1.4) [16].

\[
2\text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{GPX}} \text{GSSG} + \text{H}_2\text{O} \tag{1.3}
\]

\[
\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} \text{H}_2\text{O} + \text{O}_2 \tag{1.4}
\]

The hydroxyl radical (\( \bullet\text{OH} \)) 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 \( \bullet\text{OH} \)-mediated oxidative damage occurs in close proximity to these species [18]. Furthermore, because the cells lack specific mechanisms to eliminate \( \bullet\text{OH} \), its excess production can have severe pathological consequences [19].

\[
\text{O}_2^* + \text{H}_2\text{O}_2 \xrightarrow{\text{Fe}^{3+}} \text{\bullet OH} + \text{OH} \tag{1.5}
\]

The hypochlorite ion (\( \text{OCl}^- \)), is formed from the reaction of \( \text{H}_2\text{O}_2 \) with chloride ions (\( \text{Cl}^- \)), catalysed by the enzyme myeloperoxidase (MPO, Eq. 1.6, Fig. 1.1) [20]. The myeloperoxidase-\( \text{H}_2\text{O}_2-\text{Cl}^- \) system is crucial for the proper functioning of phagocytes, giving these cells the ability to kill a wide range of pathogens. Although \( \text{OCl}^- \) is essential for a proper immune response, uncontrolled production of HOCl within the phagocytes is detrimental to the host tissue [20].

\[
\text{H}_2\text{O}_2 + \text{Cl}^- \xrightarrow{\text{MPO}} \text{OCl}^- + \text{\bullet OH} + \text{Fe}^{3+} \tag{1.6}
\]

Singlet oxygen (\( ^1\text{O}_2 \)), unlike other ROS, results from the UV illumination of molecular oxygen (Fig. 1.1), and is moderately stable (half-life \( \simeq 3\mu\text{s} \)). At high concentrations, it diffuses across the nuclear membrane and oxidatively damages the DNA through selective oxidation of guanine to 8-hydroxyguanine. However, \( ^1\text{O}_2 \) can be quenched by vitamins A (\( \beta \)-carotene) and E (\( \alpha \)-tocopherol).

The primary source of RNS is the nitric oxide radical (\( \text{NO}^* \)), 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• 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• with O_2^- results in the production of peroxynitrite (ONOO^−, Eq. 1.7, Fig. 1.1) [25]. With a short half-life (≃1 µs), ONOO^− is highly reactive, oxidising tyrosine residues of proteins to form stable 3-nitrotyrosine thereby impacting the structure and functions of proteins [25, 26].

\[
O_2^{-} + NO^{•} \rightarrow ONOO^{-}
\]  

(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).
1.1 The Toxicity of Oxygen

- **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 hydroxytoluene 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 H₂O₂, 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 (-SO₂H) and sulfonic (-SO₃H) 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-hydroxyguanine, 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•), 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,
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
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.
1.3 Evaluating Cellular Redox State

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 such 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, F₂-isoprostanes, NO₂-Tyr, AGE                      |
| Atherosclerosis        | MDA, HNE, Acrolein, F₂-isoprostanes, NO₂-Tyr                                  |
| Cancer                 | MDA, GSH/GSSH ratio NO₂-Tyr, 8-OH-dG                                          |
| Cardiovascular disease | HNE, GSH/GSSH ratio, Acrolein, F₂-isoprostanes, NO₂-Tyr                      |
| Diabetes mellitus      | MDA, GSH/GSSH ratio, S-glutathionylated proteins, F₂-isoprostanes, NO₂-Tyr, AGE |
| Ischemia               | GSH/GSSH ratio, F₂-isoprostanes                                              |
| Parkinsons disease     | HNE, GSH/GSSH ratio, Carbonylated proteins, Iron level                        |
| Rheumatoid arthritis   | GSH/GSSH ratio, F₂-isoprostanes                                              |
| Obesity                | MDA, GSH/GSSH ratio, HNE, F₂-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 nitro 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-β-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 •OH and O₂•⁻ respectively [61, 62, 64, 65]. More advanced versions of these spin traps, namely 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) andmito-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).
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$_2$O$_2$ 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$_2$O$_2$.

The majority of H$_2$O$_2$-selective sensors reported to date are based on boronate groups, harnessing the fact that boronic esters are selectively hydrolysed by H$_2$O$_2$ [83, 84]. One or more boronic esters are incorporated onto a fluorophore to give a pro-fluorescent probe, which upon hydrolysis by H$_2$O$_2$ 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...
resulting in low fluorescence. H$_2$O$_2$ 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$_2$O$_2$ [98].
In addition to the boronic esters, another responsive group exploited for its selectivity towards H₂O₂-mediated oxidation is the sulfonyl moiety, as in fluorescein-pentafluorobenzenesulfonyl ester (FPBS, Fig. 1.7a) [99]. Hydrolysis of the sulfonyl ester by H₂O₂ results in lactone ring opening. In conjunction with the enzyme-driven loss of acetate, this results in enhanced fluorescence intensity.

A similar approach to H₂O₂ sensing was demonstrated in fluorescein-benzil (NBzF) (Fig. 1.7b) [100]. A Baeyer-Villiger type reaction of the nitro-benzil group with H₂O₂ 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 H₂O₂ over other ROS, but a small fluorescence increase with tBuOOH or ONOO⁻ was observed [100]. NBzF was employed to monitor H₂O₂ 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 (I509/I439) upon treatment with 30 equivalents of HOCl.

![Fig. 1.8](https://example.com/fig1_8.png)

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.
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, \( \text{NO}^\bullet \) undergoes rapid oxidation to yield \( \text{NO}^+ \), 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 \( \text{NO}^\bullet \) bearing diacetyl groups for better cell permeability (Fig. 1.9a), with a more than a 100-fold increase in fluorescence upon reaction with \( \text{NO}^\bullet \) [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 \( \text{NO}^\bullet \) with \( o \)-phenylenediamine, with example probe DAF-2 DA [104]; \( b \) Cu(FL\(_n\)), whichdetects \( \text{NO}^\bullet \) 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
1.5 Fluorescent Redox Probes

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-

![HKGreen-1](image1.png)

**HKGreen-1**

![HKGreen-2](image2.png)

**HKGreen-2**

![PET](image3.png)

**PET donor**

![NiPSY](image4.png)

**NiPSY-1**

**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
Intelligence for ONOO\textsuperscript{−} sensing utilises its potent nitrating ability. Nitration of NiSPYs upon reaction with ONOO\textsuperscript{−} removes the PET-quenching, unmasking the probe’s fluorescence (Fig. 1.10b) [116].

1.5.1.5 Probes for Superoxide

The superoxide radical anion (\textit{\textit{\textsuperscript{1}O\textsubscript{2}\textsuperscript{−}}}) is the primary ROS, a consequence of one-electron reduction of molecular oxygen. Sensing of this ROS particularly focuses 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\textsubscript{2}O\textsubscript{2} has also been applied for the detection

![Fig. 1.11](image-url) 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]
1.5 Fluorescent Redox Probes

**Fig. 1.12** Mechanism of •OH-mediated production of •CH₃ which brings about PET-quenching alleviation upon radical oxidation of Fluorescamine-nitroxide [124]

![Mechanism of •OH-mediated production of •CH₃](image)

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 •OH in biological systems [124]. These sensors consist of a PET quenching TEMPO moiety attached to a fluorophore. Introduction of •OH in the presence of DMSO resulted in the generation of methyl radicals (•CH₃) 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 •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 ¹O₂, 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 ¹O₂ [126]. ¹O₂-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].
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 $\text{H}_2\text{O}_2$ 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

![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.](image-url)
could then be reversed by air re-oxidation \cite{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 \cite{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 \cite{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 $[\text{Ru(bpy)}_2(\text{bpy-Q})]^2^+ \text{ (Fig. 1.15b)}$ gave rise to a 4-fold increase in luminescence upon electrochemical reduction in acetonitrile ($E_{\text{red}} = -0.2\text{ V}$) \cite{139}.

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

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**Fig. 1.15** a reversible redox reactivity of quinone/hydroquinone pair; b example of ruthenium-based redox-controlled luminescent switch \cite{139}; c mechanism of intracellular response of Da-Cy probe to $\text{H}_2\text{O}_2$/thiol redox pair \cite{140}; d reduced non-fluorescent form of TCA probe \cite{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$_2$O$_2$ 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$_2$O$_2$/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.
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 ([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 dinucleotide (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-
1.5 Fluorescent Redox Probes

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 NaBH₃CN reversibly for up to 3 cycles, but the process is not selective for these oxidising and reducing agents. Extensive electrochemical and spectroelectrochemical 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 Zn²⁺-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 Na₂S₂O₄ 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-
The Current State of Redox Sensing

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 homeostasis.

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.

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