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Reversible Fluorescent Probes for Biological Redox States

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The redox chemistry of the cell is key to its function and health, and the development of chemical tools to study redox biology is important. While fluxes in oxidative state are essential for healthy cell function, a chronically elevated oxidative capacity is linked to disease. It is therefore essential that probes of biological redox states distinguish between these two conditions by the reversible sensing of changes over time. In this review, we discuss the current progress towards such probes, and identify key directions for future research in this nascent field of vital biological interest.

1. Introduction

Redox reactions are vital to the existence of living organisms. A healthy cell naturally undergoes transient increases in reactive oxygen and nitrogen species (ROS and RNS), which play an important role in signaling processes and in protection against pathogens.[1] However, if the balance between oxidants and reductants is chronically disrupted, the oxidizing species can cause irreparable damage to the structure and/or function of proteins, DNA, and lipids. Oxidative damage is linked to the development of various pathologies including diabetes,[2] neurodegenerative[3] and cardiovascular[4] diseases, and cancer.[5] Therefore, the ability to distinguish between transient and chronic changes in oxidative capacity in living organisms, in real time, is of crucial importance.

In recent decades, a wide range of fluorescent responsive probes to study redox processes have been developed. Most of these tools take advantage of irreversible reactions with a specific ROS or RNS, thus giving rise to an irreversible interaction between the probe and analyte. Probes of this type are helpfully summarized in multiple reviews.[6] However, such probes are not able to distinguish between transient bursts in ROS production typical of physiological events and chronically elevated ROS levels, which are characteristic of pathological oxidative stress (Figure 1). 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. 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. In this minireview, we aim to summarize the approaches that have been taken to date, and identify promising strategies for future work.

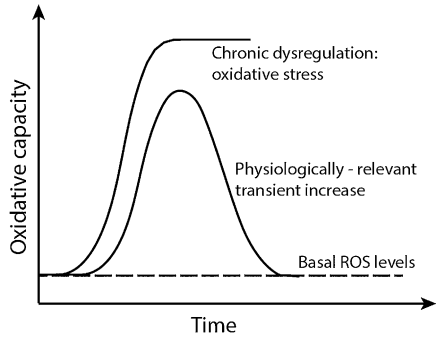

Figure 1. Variations in cellular ROS levels. Transient increases are vital for physiological functions such as signalling, whereas chronic elevations are damaging and cause diseases.

2. Requirements for Reversible Probes of Cellular Redox States

The primary requirement for such tools is reversibility of response to repeated cycles of oxidation and reduction. To have utility in biological studies, however, a number of other important aspects must be considered, and include:

1) Selectivity versus global response: While reaction-based (irreversible) probes selective for a particular ROS or

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redox-active pair provide valuable details about the role of redox signaling in a particular biochemical pathway, reversible probes which respond to global oxidative changes are able to report on the overall oxidative capacity of a cell and its recovery from stress (Figure 1). A combination of both classes of probes would be an ideal tool box for complete evaluation of the physiological and pathological roles of cellular redox state.

2) Clear fluorescent response, in which the probe has a high quantum yield and sufficient Stokes shifts, as well as a large difference in the signal between oxidized and reduced forms. Furthermore, a ratiometric response, in which emission wavelength changes are measured rather than emission intensity, ensures an internal reference which nullifies any concentration, background, and instrument-based effects.
3) 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.
4) Sensitivity of response requires that the fluorescence change is triggered by biologically relevant redox potentials, or by biologically meaningful concentrations of ROS/RNS and antioxidants.
5) Tunability of redox potential will ensure that probes can be developed, which 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.
6) Biological compatibility requires the optimization of parameters such as sufficient cellular permeability and specific subcellular localization to enable monitoring of oxidative stress at the organelle level. Furthermore, particularly for reversible probes which offer the potential to monitor cells over time, probes must be nontoxic and have minimal effect on cellular homeostasis.

A class of probes, which fulfill many of these requirements of optimal reversible probes, is the set of redox-responsive fluorescent proteins, and they have been summarized in detail elsewhere.[7] Aside from the general advantages of fluorescent proteins, such as photostability, biocompatibility, and ease of intracellular targeting, it is possible to design ratiometric fluorescent proteins, thus ensuring an unambiguity of the detected signal. For example, HyPer selectively and reversibly responds to H_2O_2 with a ratiometric emission change.[8] Probes of this type 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. 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. In comparison to the fluorescent protein literature, relatively little work has been reported regarding small-molecule redox probes, and there is a particular lack of detailed biological studies using such tools. This review focuses on the still very limited examples of reversible fluorescent small-molecule probes for redox state in biology (Table 1), with particular attention to promising design features. Our discussion is arranged according to the redox-responsive group, and aims to inspire further development in the field by outlining the primary advantages and limitations of the designs reported to date.

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Elizabeth New undertook her undergraduate and masters studies at the University of Sydney, and completed her PhD in 2009 with Prof. David Parker at Durham University. From 2010–2011 she was a postdoctoral fellow with Prof. Chris Chang at the University of California (Berkeley). In 2012, she returned to the University of Sydney as a Lecturer in Inorganic Chemistry. Her research is focused on the development of small-molecule fluorescent and magnetic resonance probes for the study of biological systems.

3. Nitroxide-Based Probes

Nitroxyl radicals are unusually long-lived free radicals, which have long been used as spin labels for electron spin (paramagnetic) resonance (ESR/EPR) because of their paramagnetism.[9] In biological systems, nitroxyl radicals undergo redox switching between diamagnetic (closed shell) and paramagnetic (radical) states (Figure 2a), and have been explored as responsive probes for EPR[10] and MRI.[10,11] To enable fluorescent detection, the nitroxide moiety can be covalently tethered to a fluorophore of choice through a suitable linker. The nitroxyl free radical will quench the fluorophore's fluorescence,[12] which can be restored either

Table 1: Fluorescence and redox properties of reversible redox probes which have been employed in cellular or animal model studies. Probes are categorized according to the redox responsive moiety and arranged in the order of their appearance in text.

Probe	Selectivity	Ex/Em [nm]	Reported change in fluorescence	Biological system – treatment or stimulus
Nitroxides				
TMB[19]	n.a.	491/507	10-fold increase	HepG2 cells - H_2O_2
R2c[26]	n.a.	683/ca. 700	2.5- to 5-fold increase	HeLa cells- Ascorbate
TEtNO-Anthracene and TEtNO-Fluorescein[21]	n.a.	465/480 490 (970)^{TP}/511	70-fold increase 7-fold increase	CHO cells - H_2O_2, CellROX deep red
Fluorescein-TEMPO[22]	$O_2^{\bullet-}$ and NO$^{\bullet}$	492/514	2.2- to 2.7-fold increase	hTERT-immortalized fibroblasts - 2DG and rotenone
MitoRP[23]	n.a.	460/495	3-fold increase	HeLa cells, isolated mitochondria - NADH, rotenone, succinate and malonate
ME-TRN[27]	$O_2^{\bullet-}$	556/590	N/A	Albino Spraguee Dawley rats - acute retinal ischaemia-reperfusion - lutein
ORCAFluor[28]	Ascorbate and GSH	640/730	2- to 3.5-fold increase	Mice - endogenous levels of ascorbate
mCy-TemOH and Cy-TemOH[24]	HOBr	445 and 610/550 and 632 702/755	632/550 2.5-fold decrease 6-fold decrease	RAW 264.7 cells- H_2O_2, EPO, KBr and GST
Quinones				
RF-1[29]	n.a.	490/503	$>$50-fold increase	HEK 29 - H_2O_2
Rh-Q, Rh-QOMe[30]	n.a.	566/592	6-fold, 4-fold decrease	HeLa cells - no response from $[Cu(phen)2]^{2+}$ or H_2O_2
DA-Cy[31]	H_2O_2 and $^{\bullet}OH$/GSH and cellular thiols	630/755	$\approx$20-fold decrease	HL-7702 cells - H_2O_2, NEM; HepG2 cells - H_2O_2 RAW264.7 cells - PMA, GST, α-lipoic acid Rat hippocampus tissue - H_2O_2, NEM, α-lipoic acid
TCA[32]	$O_2^{\bullet-}$	491 (800)^{TP}/515	$>$1000-fold increase	HL 7702 cells - PMA, tiron HepG2 cells - PMA, GSH, BSO Zebra fish, mouse liver - Ischemia-reperfusion injury
PY-CA[33]	$O_2^{\bullet-}$	405 (800)^{TP}/520	$>$1000-fold increase	4T1 cells - 2ME, ascorbic acid, tiron, Mouse melanoma tumor tissue *C.elegans* - paraquat, tiron
Chalcogenides				
RSe-N[34]	GSH	525/530-640	$\approx$6-fold increase	HepG2, HL7702 - none
Cy-O-Eb[35]	H_2O_2	768/794	$\approx$8-fold increase	HepG2 cells - GSH/H_2O_2 redox cycles HepG2, HL7702 - BSO induced apoptosis Zebra fish larvae - tail fin wounding
FSeSeF[36]	GSH	488/514	4-fold decrease	HeLa - H_2O_2, α-lipoic acid and NEM
Chalcogenoxides				
Cy-PSe[37]	$ONOO^-$	758/775	23.3-fold increase	RAW 264.7 - SIN-1, GST, LPS, IFN-γ, PMA, NOC-5 and TEMPO
MPh-Se-BOD[38]	-OCl	460/475-650	5 fold increase	RAW 264.7 - PMA, Taurine, Xanth./Xanth. oxidase
HCSe[39]	-OCl	510/526	138-fold increase	RAW 264.7 - NaOCl and GSH
NI-Se[40]	-OCl	420/523	12-fold increase	RAW 264.7 - LPS, PMA and SHA Mice - LPS
diMPhSe-BOD[41]	HOBr	610/635, 700	230-fold increase	RAW 264.7 - H_2O_2, EPO, KBr and H_2S
Bis(BODIPY)diselenide[42]	$O_2^{\bullet-}$	504/514	30-fold increase	MCF-7/ADR cells - PMA
2Me TeR[43]	-OCl ($^{\bullet}OH$), ($ONOO^-$)	600/669	200-fold increase	HL-60 cells - H_2O_2 and ABAH
Cy-NTe[44]	$ONOO^-$	793/820	$\approx$80-fold increase	RAW 264.7 - LPS, IFN-γ, PMA, AG, paraquat, NOC-5 and NaOCl BALB/c mice - L-cysteine, LPS, IFN-γ, PMA, AG, GST
$[Ru(bpy)_3]^{2+}$-PTZ[45]	HOCl	450/605	$\approx$7-fold increase	Mice - NaOCl, H_2S and PABA
FO-PSe[46]	HOCl	415 (800)^{TP}/520	$\approx$40-fold increase	RAW 264.7 - PMA, ABAH Zebrafish - Zymosan and Mice abdomen
Flavins				
CMFL-BODIPY[47]	n.a.	450/512	$\approx$9-fold increase	HeLa - $Na_2S_2O_4$ and H_2O_2
NpFR1[48]	Global	405/545	125-fold increase	3T3-L1 preadipocytes and differentiated adipoctyes - Glucose and H_2O_2
NpFR2[49]	Global	488/545	115-fold increase	Mice bone marrow, thymus and spleen - H_2O_2 and Mitotracker deep red.
FCR1[50]	Global	820^{TP}/470, 520	520/470 6-fold increase	HeLa - NAC and H_2O_2

Table 1: (Continued)

Probe	Selectivity	Ex/Em [nm]	Reported change in fluorescence	Biological system – treatment or stimulus
Miscellaneous				
RHyCy5[51]	n.a. (O_2 levels)	596/641	7- to 8-fold decrease	A549 cells - on air and under cover glass
Ox-PS-NP[52]	O_2 levels	635/670, 800	800/670 4-fold decrease	MH-S macrophages - O_2 in atm.; NMRI-Fox1 mice with AsPC-1 cells tumour, Na_2SO_3
Ru-Py[53]	O_2 levels	450/595	≈10-fold decrease	A549 cells - O_2 in atm.; BALB/cSlc mice - ligation in leg
Resorufin-TFB[54]	n.a.	571/583	11-fold increase	Macrophages - NaSH then glucose + GOx or NaOH

ABAH = aminobenzoic acid hydrazide, AG = aminoguanidine, BSO = buthionine sulfoximine, DETA/NO = diethylenetriamine/nitric oxide adduct, 2DG = 2-deoxy-D-glucose, EPO = eosinophil peroxidase, GSH = glutatione (reduced), GST = glutathione-S-transferase, HRP = horse radish peroxidase, IFN-γ = interferon gamma, LPS = lipopolysaccharide, 2-ME = 2-methoxyestradiol, NAC = *N*-acetyl cysteine, NEM = *N*-ethylmaleimide, NOC-5 = 3-aminopropyl-1-hydroxy-3-isopropyl-2-oxotriazene, PABA = *para*-aminobenzoic acid, PMA = phorbol 12-mirystate-13-acetate, SHA = salicyl hydroxamic acid, SIN-1 = 3-Morpholinosydnonimine TP = two photon, Xanth. = xanthine.

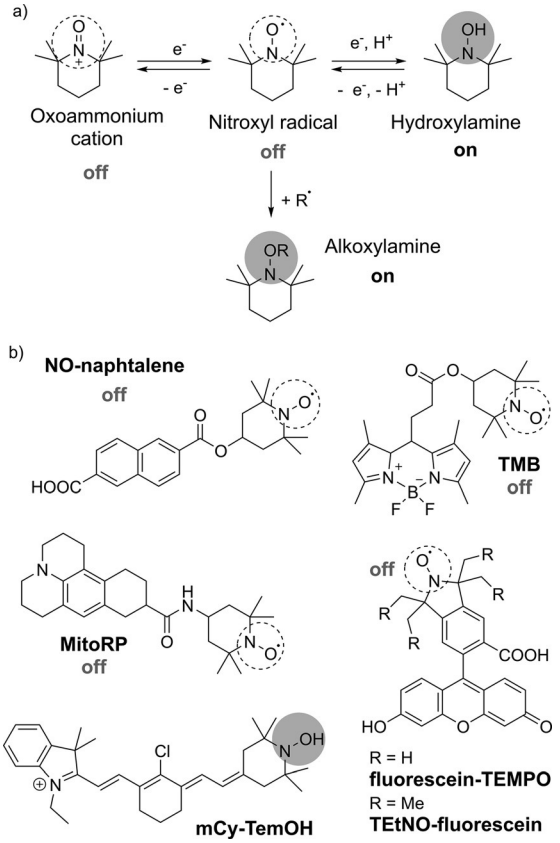

Figure 2. a) Possible redox reactions of nitroxyl radicals in biological media. Circles indicate sites of redox response, with fluorescence-quenching motifs highlighted in dashed circles and moieties enabling fluorescence shown in gray circles. b) Selected examples of nitroxide-based redox-responsive fluorescent probes, with the fluorescence-quenching nitroxyl radical shown in the dashed circle.[13,19,21–24]

upon reversible reduction to the diamagnetic hydroxylamine or irreversible formation of alkoxyamine derivatives (Figure 2a). Since fluorescence is activated upon reduction, these probes are often referred to as profluorescent nitroxides, in analogy to prodrugs.

The first proof of concept nitroxyl-based fluorophore, NO-naphthalene (Figure 2b),[13] exhibits a tenfold increase in fluorescence quantum yields upon reduction. An early development involved collection of both fluorescence and paramagnetism (ESR) readouts from NO-dansyl and NO-perylene probes.[14] Since then, profluorescent nitroxide probes (PFNs) have been used to detect radicals in a wide range of biological and nonbiological applications,[15] whether for the study of particulate samples[16] or for assessing the stability of polymers,[17] and they have been summarized elsewhere.[18] Therefore in this review we will focus only on a few subjectively selected examples aimed at the detection of the redox state in biological settings. One of the probes that successfully addressed the limited applicability of PFNs with short wavelength excitations in biological studies is TMB (Figure 2b), a green-emitting BODIPY fluorophore decorated with a nitroxide moiety, with a sixfold increase in emission upon reduction.[19] This probe could detect H_2O_2-induced oxidative stress in Hep2G hepatocellular carcinoma cells, and was stable to the hydrolytic activity of trypsin, for a time period of 60 minutes, in vitro.[20]

While further red-shifting of excitation and emission wavelengths was achieved by conjugating the TEMPO radical to either a styryl BODIPY, Nile Red, or a ruthenium polypyridyl complex, biological application of these probes was precluded by low quantum yields (0.002 to 0.050) and relatively small dynamic ranges (two- to eightfold changes).[25] In another study, a water-insoluble silicato-phtalocyanine-based profluorescent nitroxide (R2c), with emission at $\lambda =$ 700 nm, was encapsulated in liposomes and used to quantify ascorbic acid levels in HeLa cells.[26]

Near-IR excitation can also be achieved by two-photon excitation, and to this end, a series of profluorescent radicals based on a range of fluorophores was prepared, and fluorescent responses measured.[21] Along with fluorescence increase of up to 70-fold upon reduction, fluorescence lifetimes also increased by up to two orders of magnitude. Two radicals, TEtNO-anthracene and TEtNO-fluorescein (Figure 2b), were selected for biological experiments on the basis of their significant two-photon absorption cross-sections. Linear and two-photon absorption fluorescence microscopy experiments in Chinese hamster ovarian (CHO) cells revealed that these probes were nontoxic and could report on exogenously added H_2O_2.

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 (Figure 2b), for which one-electron reduction by excess hydrazine hydrate gave rise to an increase in fluorescence, and could then be reversed by air reoxidation.[22] While this reoxidation persisted over three redox cycles, with each cycle there was a slight increase in the basal fluorescence intensity, which is indicative of irreversible destruction of a fraction of the radical. Flow cytometry and molecular-imaging experiments in hTERT immortalized human fibroblasts demonstrated the potential of the probe for real-time monitoring of global redox changes in the cellular environment. More recently, a related rhodamine–nitroxide conjugate, ME-TRN, enabled real-time monitoring of fluxes of oxidative stress in the retinas of live rats,[27] thus opening up a new avenue to the wider in vivo application of PFN redox probes. While there have not been examples of targeted nitroxide probes to date, the probe MitoRP (Figure 2b), based on coumarin 343, demonstrated preferential localization to the mitochondria.[23]

Only recently, dual modality MRI-fluorescence bottle-brush polymeric probes containing nitroxyl radicals (ORCA-Fluors), have been reported.[28] Incorporation of a cyanine dye in spatial proximity to the radicals induced a partial fluorescence quenching. In the presence of either ascorbate or ascorbate/GSH, a reaction with nitroxyl radicals led to 2- and 3.5-fold increases, respectively, in the NIR-fluorescence. The probes were successfully used in vivo to report on tissue-dependent inhomogeneities in ascorbate concentrations in mice.

An interesting alternative nitroxide-based sensing mechanism is demonstrated by mCy-TemOH (Figure 2b) and Cy-TemOH, which utilize an oxoammonium cation rather than paramagnetic nitroxyl radical (Figure 2a). These probes undergo a two-electron transformation which is selectively and reversibly achieved by the hypobromous acid/ascorbic acid redox pair, rather than by global redox environment.[24] In particular, mCy-TemOH exhibits ratiometric behavior, with a redox-sensitive peak at $\lambda = 632$ nm and a redox-insensitive peak at $\lambda = 550$ nm, and was successfully applied to image cycles of ascorbate and hypobromous acid treatment in RAW 264.7 macrophages.[24]

4. Quinones

The quinone/hydroquinone reversible redox couple has been widely studied for almost hundred years[55] and is therefore an obvious choice in the design of redox-responsive optical probes.

One of the most commonly used tools to study redox changes in biological systems is dichlorofluorescein (DCF; Figure 3a). Although originally developed to detect H_2O_2,[56] its response to ROS is nonselective, and it suffers from a variety of limitations (reviewed elsewhere).[57,6b,g] While oxidation of the weakly fluorescent DCFH gives rise to the fluorescent semiquinoidal DCF structure with a restored push-pull mechanism of fluorescence, the process is irreversible and therefore will not be covered in this review. The reversibility of the redox response of a fluorescein-like fluorophore has been achieved by incorporating a naphthyl-disulfide motif in RF1 (Redoxfluor 1; Figure 3b). This was also the first example of a small-molecule fluorescent redox probe which demonstrated reversibility in cells, but no reduction potential was reported.[29]

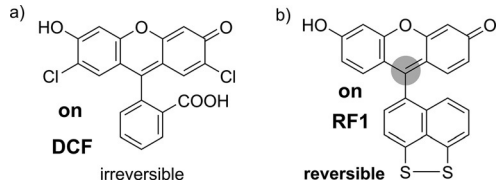

Figure 3. a) Oxidized form of irreversible DCF probe. b) Fluorescein-type reversible redox probe RF1.[29]

Subsequent quinone-based probes employed a strategy of luminescence quenching upon photoinduced electron transfer from the luminophore in the excited state to the electron-poor quinone motif (Figure 4a), as widely reported for porphyrin-based systems.[58] By this strategy, a number of reversible quinone-containing redox sensors based on ruthenium complexes have been reported. For example, the red-luminescent

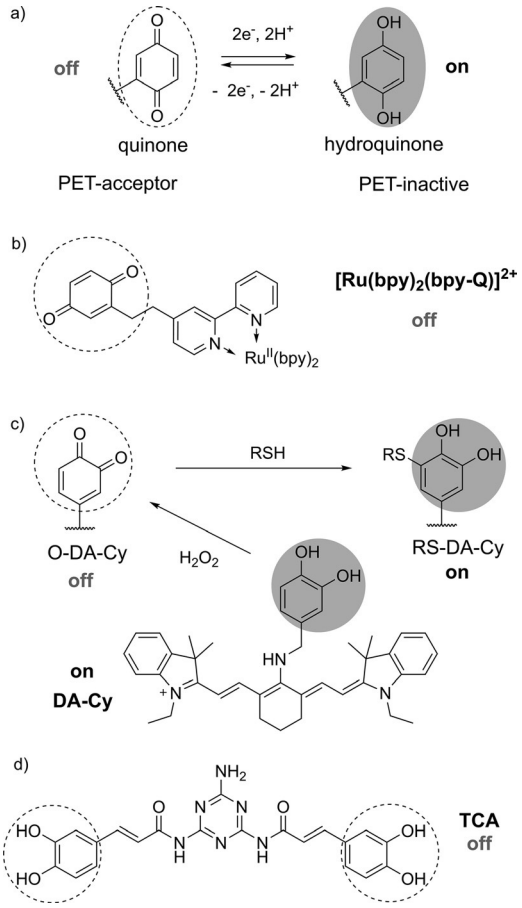

Figure 4. a) Reversible redox reactivity of quinone/hydroquinone pair. b) Example of ruthenium-based redox-controlled luminescent switch.[59] c) Mechanism of intracellular response of Da-Cy probe to H_2O_2/thiol redox pair.[31] d) Reduced nonfluorescent form of TCA probe[32].

probe $[Ru(bpy)_2(bpy\text{-}Q)]^{2+}$ (Figure 4b; bpy = 2,2′-bipyridine) gave rise to a fourfold increase in luminescence upon electrochemical reduction in acetonitrile ($E_{red} = -0.2$ V).[59] While electrochemical reduction of the $[Ru(terpy)]^{2+}$-type (terpy = 2,2′;6′,2″-terpyridine) complex RQ, containing a rigidly linked quinone, led to an increase of luminescence only at low temperatures,[60] $[Ru(bpy)_2(PAIDH)]^{2+}$ (PAIDH = 2-pyridyl-1*H*-anthra[1,2-*d*]imidazole-6,11-dione) showed an electrochemically induced 600-fold increase in luminescence (QY = 0.02) at room temperature ($E_{red} = -0.5$ V).[61] Despite electrochemical reversibility in near-biologically-relevant potentials and significant luminescence response, none of these complexes was examined in more-biologically-relevant aqueous conditions, nor was chemical reversibility demonstrated.

To design redox-responsive probes for biology, the quinone moiety has been covalently attached to a variety of organic fluorophores. Robust electrochemical reversibility of a BODIPY/quinone dyad with a rigid phenyl linker (BD-PQ) was accompanied by a 2.5-fold increase in fluorescence upon reduction ($E_{red} = -0.46$ V).[62] The chemical reversibility was demonstrated by sequential treatment with H_2O_2 and ascorbate, with a fraction of the observed fluorescence change (10–30%) attributed to irreversible redox reactions on the BODIPY dye.

In another design, rhodamine was linked to two different hydroquinones, thus demonstrating the possibility of tuning the redox potential.[30] 1,4-Benzoquinone/rhodamine (Rh-Q) and 2-methoxy-1,4-benzoquinone/rhodamine (Rh-QOMe) probes undergo, respectively, sixfold and fourfold decreases in fluorescence upon oxidation by an excess of $[Cu(phen)_2]^{2+}$ (phen = 1,10-phenanthroline), and these changes were rapidly reversed by addition of cysteine. While they were efficiently internalized by HeLa cells, no oxidation could be observed in cellulo even upon oxidative stimulation, probably because of the reduction of the probe by intracellular GSH. However, the authors speculate that this can be addressed by lowering the probe's redox potential by varying the hydroquinone moiety.

To ensure a redox-dependent fluorescent response in a biological setting, an intracellular redox-active dopamine was directly attached to a cyanine dye (DA-Cy, Figure 4c), thus yielding an on-off-on probe.[31] 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.

The group of Tang reported a quinone-triazine-based one- and two-photon excitable probe, TCA (Figure 4d), which can be reversibly and selectively oxidized by superoxide ($O_2^{\bullet-}$) to generate TCAO. TCAO was shown to have a 1000- and 3-fold greater fluorescence upon one- and two-photon excitation, respectively.[32] TCAO could be reduced by glutathione and the probe was shown to respond to more than three redox cycles. The authors tested the reversibility of TCA fluorescence by sequential treatment of HepG2 cells with the ROS-inducer phorbol-12-myristate-13-acetate (PMA) and the reductant glutathione. In addition, the authors have employed TCA to image redox changes in zebra fish and mice. The group recently published another probe with a similar strategy, and PY-CA can be oxidized by $O_2^{\bullet-}$ selectively to give fluorescent PY-CAO.[33] Like TCA, this probe was also shown to be respond to more than three $O_2^{\bullet-}$/GSH redox cycles and was utilized for in vivo imaging in cells and mouse tumours.

Recently, incorporation of the redox-responsive resorcinol (1,3-dihydroxyphenol) moiety into the hemiporphyrazine backbone yielded a redox-switching, near-IR probe. Its oxidized NIR fluorescent aza-quinoidal form could be reversibly switched to a nonfluorescent resorcinol structure up to seven times by a $NaBH_4$/2,3-Dichloro-5,6-dicyano-1,4-benzoquinone(DDQ) couple.[63] Nevertheless, this switch, developed for optoelectronic applications, was demonstrated only in chloroform, which precludes the evaluation of its utility in biological systems.

5. Chalcogen-Based Fluorescent Redox Probes

A broad class of reversible redox probes involve sulfur, selenium, and tellurium in their sensing groups. These classes can be further divided into those that sense oxidation through formation of a disulfide, diselenide, or ditelluride bridge (dichalcogenides: Section 5.1, Figure 5a), and those that involve oxidation of the chalcogen itself to the oxide form (Section 5.2, Figure 6a).

5.1. Dichalcogenides

The sulfide–disulfide oxidation (exemplified in the cysteine to cystine oxidation) is central to countless biological processes and structures (Figure 5a). Ratios of thiol/disulfide (whether GSH/GSSG or cysteine/cystine) within cells are therefore widely accepted to be good indicators of cellular oxidative stress,[64] and means that disulfide is therefore a suitable redox sensing moiety on which to base the development of fluorescent redox sensors. Likewise, the selenide–diselenide oxidation plays an important role in biology, such as in the catalytic site of glutathione peroxidase (GPx).[65] 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 (Figure 5c).[66] Upon oxidation, the two cysteine residues in the linker form a disulfide bridge which brings the carbostyril and Tb close enough together to enable sensitized luminescence, with constant emission intensity at $\lambda = 400$ nm, thus enabling a 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

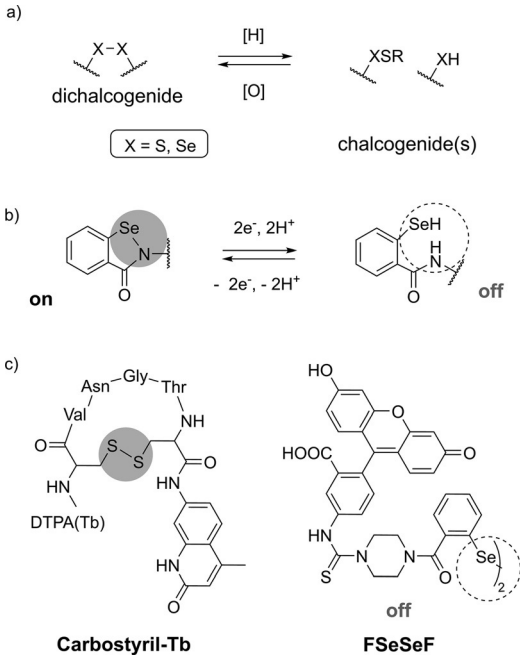

Figure 5. Reversible redox transformations of dichalcogenide (a) and an ebselen-type motif (b). c) Selected examples of dichalcogenide-type probes; DTPA = Diethylenetriaminepentaacetic acid.[29,36,66]

identify the ease with which the reduction potential can be tuned by modification of the linker.

Despite extensive research towards the development of ebselen-based selenium-containing antioxidants (Figure 5b), the use of selenium in fluorescent probes is only relatively recent. The first such probe was based on rhodamine, with a Se–N redox-active bond (RSe-N), and was reported as a thiol sensor without further testing of its reversibility.[34] More recently, Tang and co-workers reported Cy-O-Eb, a redox probe containing an ebselen moiety tethered to a cyanine dye.[35] Extensive studies demonstrating the reversibility of Cy-O-Eb to the GSH/H_2O_2 redox couple were performed, and the probe was applied to image peroxide levels in wounded zebra fish. The selectivity for peroxide over all other ROS is inconclusive, as reports suggest that ebselen can also react with peroxynitrite.[67]

In contrast, the probe FSeSeF, which consists of two fluorescein molecules linked by a diselenide bridge (Figure 5c), utilizes an approach similar to the sulfide–disulfide oxidation.[36] The diselenide bond in the weakly fluorescent FSeSeF can be reduced by cellular thiols such as glutathione and cysteine, thus resulting in the cleavage of the diselenide bridge to give two separate, and fluorescent, molecules—one containing a selenyl sulfide group (FSeSG) and the other, a selenol (FSeH). The response of FSeSeF to glutathione was fourfold faster than the previously reported probes. The authors demonstrate that the products of the reduced probe (FSeSG and FSeH) can be reversibly oxidized by H_2O_2, both in vitro and in vivo, but the reversibility in cellular systems will be highly limited by random diffusion of these molecules as they are unlikely to be in close enough proximity in the event of oxidation.

5.2. Chalcogenoxides

Another strategy for developing fluorescent redox sensors has been to employ chalcogens (S, Se and Te), which can be readily and reversibly oxidized to the respective chalcogenoxides (sulfoxides, selenoxides ,and telluroxides; Figure 6a).

5.2.1. Selenides

The strategy of employing chalcogen–chalcogenoxide oxidation was pioneered by the group of Han after the decoding of the catalytic sites of GPx, and they showed that selenium in the catalytic pockets reacts with ROS to form selenoxides.[65] The first such probe, Cy-PSe, a near-IR emitter, was based on photoinduced electron transfer (PET) between a cyanine signal transducer and a phenyl selenium modulator.[37] Upon oxidation to Se=O, PET quenching is alleviated, thus resulting in a turn-on in fluorescence. Cy-PSe was reported to be selectively oxidized by peroxynitrite and

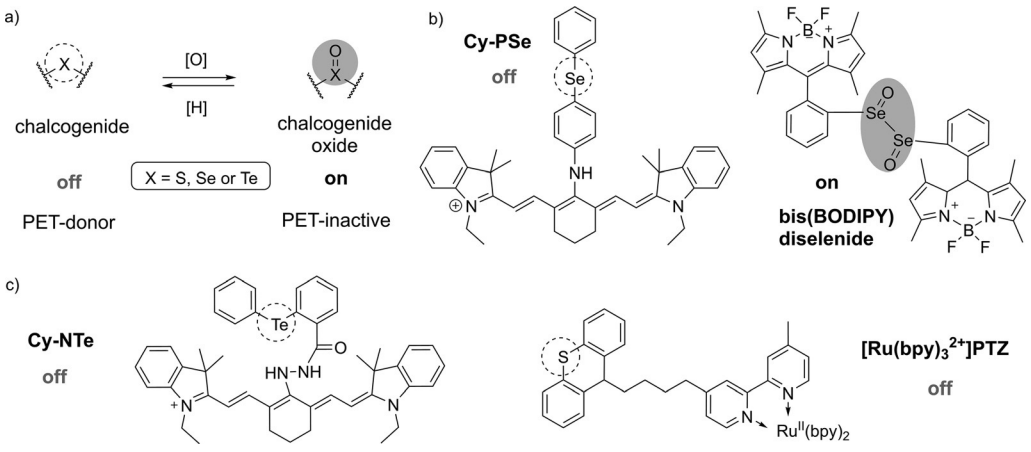

Figure 6. a) Reactivity of reversible chalcogenide/chalcogenoxide redox couple. Selected examples of this type of probe based on Se[37,42] (b) and either Te[44] or S[45] (c) are shown.

reduced by glutathione and cysteine. The probe was used to measure peroxynitrite in activated mouse macrophages, and its reversibility in biological systems demonstrated. The group subsequently developed MPhSe-BOD, containing the BODIPY fluorophore, which instead shows selectivity for oxidation by OCl^-.[38]

A number of additional probes have been developed based on this strategy. HCSe contains a BODIPY scaffold linked to a diphenyl selenide, and undergoes reversible oxidation with OCl^- to the green fluorescent HCSeO, but no information on the selectivity for reducing agents is available.[39] NI-Se contains a 1,8-naphthalimide fluorophore, and is sensitive to the OCl^-/H_2S redox couple.[40] This probe operates by the twisted intramolecular charge-transfer (TICT) mechanism, in which the selenoxide formed upon oxidation restricts the internal rotation of the molecule, thus leading to enhanced fluorescence emission.

Han and co-workers also used the selenium redox chemistry to develop diMPhSe-BOD, a probe for hypobromous acid (HOBr).[41] Unlike previous, intensity-based selenoxide-based sensors, this was the first ratiometric probe of this type, with a blue-shift, upon oxidation, resulting from the electron-withdrawing effect of selenoxides. In contrast to other selenium-based probes, which were nonselectively reduced by thiols, diMPhSeO-BOD was shown to be reduced selectively by H_2S. The biological applicability of diMPhSe-BOD was demonstrated in RAW 264.7 cells

A further contribution to the pool of selenium-based sensors came from the group of Churchill, with the development of bis(BODIPY)diselenide (Figure 6b), in which two BODIPY scaffolds are tethered by a diselenide bridge.[42] The selenides in the probe were reported to undergo selective oxidation in the presence of superoxide ($O_2^{\bullet-}$) over other ROS, thus resulting in the mono-oxidation of both selenium centers, and a turn-on in the fluorescent response, which could be reversed by bio-thiols.

Another recent probe employing the selenium strategy was reported by the group of Tang who developed FO-PSe, the selenium atoms of which can be oxidized to Se=O by HOCl selectively to give a green fluorescent FO-PSeO. The oxidized FO-PSeO could be reduced to FO-PSe by GSH, and the $O_2^{\bullet-}$/GSH redox cycle could be monitored for three or more cycles. FO-PSe was tested in extensive biological studies to investigate HOCl levels in PMA-stimulated macrophages and zymosan-loaded zebrafish, as well as mice abdomen.[46]

5.2.2. Tellurides

The use of tellurium in fluorescent redox probes was inspired by results indicating diaryl tellurides undergo selective and rapid oxidation with singlet oxygen in the presence of a sensitizer.[68] The first reported tellurium-containing redox sensor was 2Me TeR, in which the xanthene O10 of rhodamine was replaced with tellurium, thus giving a nonfluorescent molecule as a result of the heavy-atom effect.[43] The organotellurium center was shown to be oxidized by the hydroxyl radical ($^{\bullet}OH$), peroxynitrite ($ONOO^-$), and hypochlorite (OCl^-) to give the corresponding telluroxide, which emits in the near-IR. Furthermore, this oxidation was shown to be reversible upon treatment with GSH. Interesting biological studies were performed using 2Me TeR in HL-60 cells, which expressed different levels of myeloperoxidase, an antioxidant enzyme.

Two years later, novel probes based on an annulated BODIPY tethered to either selenide or telluride were reported.[69] Both analogues could be oxidized by hypochlorite to the respective oxides, thus resulting in a blocked PET process and enhanced fluorescence properties. Of these probes, the oxidation of the organotelluride by hypochlorite was reported to be extremely sensitive and rapid, and could be reversed by various bio-thiols.

A recent tellurium-based redox probe, Cy-NTe (Figure 6c), comprises a diaryl tellurium integrated into a cyanine platform.[44] Interestingly, oxidation occurs selectively upon reaction with peroxynitrite ($ONOO^-$) to give a near-IR fluorescent product which can be re-reduced by cellular thiols. The authors neatly demonstrate the redox responsive properties and reversibility of Cy-NTe in mice models.

5.2.3. Sulfides

While the past few years have seen development of a large number of chalcogen–chalogenoxide probes based on selenium and tellurium, the more-biologically-relevant sulfur–sulfoxide oxidation has been relatively underutilized in fluorescent redox sensors. To date, the only example is the reversible luminescent redox probe $[Ru(bpy)_3^{2+}]$PTZ, which contains phenothiazine, a sulfur-containing moiety shown to have a reversible redox properties, linked to a luminogenic ruthenium trisbipyridyl complex (Figure 6c).[45] The sulfur in the phenothiazine scaffold is selectively oxidized by hypochlorite (OCl^-), thus resulting in a significant luminescence increase. This oxidation could be reversed in the presence of H_2S. The reversible redox response could be repeated for at least 12 cycles, and $[Ru(bpy)_3^{2+}]$PTZ was used to image redox cycles in live mice.

The marked variations in selectivity of oxidation and/or reduction of the selenium-, tellurium-, and sulfur-based probes is believed to arise from variations in structural characteristics,[70] but determination of structure–activity relationships would be valuable in the future design of probes with desired selectivity. Nevertheless, this set of probes is an interesting demonstration of the tunability of redox probes to different biological redox couples.

6. Nicotinamides and Flavins

Biological systems exhibit complex mechanisms of redox regulation, but the majority of regulatory systems utilize derivatives of members of the vitamin B group, that is, nicotinamides and flavins. These vitamins, and particularly their nucleotide derivatives, NAD and FAD/FMN, respectively, act as redox-active cofactors and coenzymes in cellular redox reactions. Such groups are inherently biocompatible, and their reduction potentials are tuned to cellular redox reactions, thus making them suitable candidates for redox sensing (Figure 7a,b). The first reported use of such vitamins

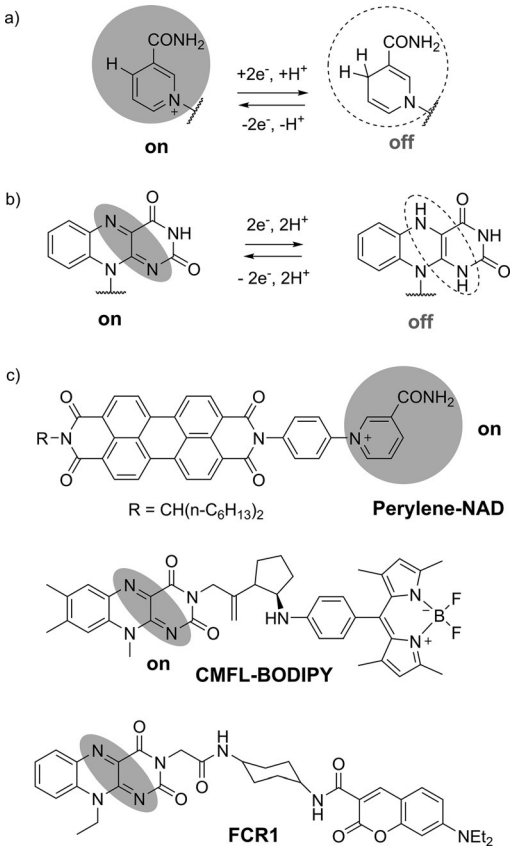

Figure 7. Reversible redox transformations of nicotinamide (a) and the flavin moiety (b). c) Selected examples of probes developed on the basis of these redox-responsive groups.[47,50,71]

in redox sensors was a PET-based redox molecular switch consisting of a perylene scaffold linked to nicotinamide, Perylene-NAD (Figure 7c), which undergoes a tenfold increase in emission upon oxidation.[71] This molecular switch can be oxidized by *p*-chloranil and reduced by $NaBH_3CN$ reversibly for up to three cycles, but the process is not selective for these oxidizing and reducing agents. Extensive electrochemical and spectroelectrochemical studies give 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. Unfortunately, there are no other reports of nicotinamide-based fluorescent redox probes as it appears that the scientific community has underestimated the potential of the reversible redox properties of nicotinamide.

One of the first reports utilizing the reversible redox properties of flavins came from the group of Aoki, who developed Zn^{2+}/tetraazacyclododecane complexes bearing lumiflavin and tryptophan.[72] Although these complexes were designed for use as DNA photolyase mimics, the studies emphasized the photochemistry and redox properties of the flavin scaffold. Later, the same group reported CMFL-BODIPY, containing carboxymethylflavin (Figure 7c). This probe could be reduced by $Na_2S_2O_4$ with a ninefold decrease in fluorescence emission.[47] The reduction potential of this probe was reported to be −240 mV, which is similar to that of cellular flavins. Although the probe's reversibility and selectivity were not reported, studies in HeLa cells demonstrated the potential of the sensing strategy.

Subsequent studies employing flavin-based redox sensors yielded NpFR1, a naphthalimide/flavin conjugate which showed slightly red-shifted fluorescence properties compared to those of intracellular flavins, thus eliminating the issues associated with cellular auto-fluorescence,[48] and its mitochondrially targeted analogue NpFR2.[49] Both probes demonstrate greater than 100-fold decreases in fluorescence upon reduction, with excellent reversibility behavior with a range of oxidizing agents. NpFR1 was used to reveal the impact of glucose on oxidative stress in 3T3-L1 adipocytes, while NpFR2 was employed in flow cytometry studies to distinguish between cell populations with different mitochondrial oxidative capacities within the bone marrow, thymus, and spleen of mice. A second-generation ratiometric redox probe, FCR1 (Figure 7c), was also reported by our group, and consisted of a coumarin/flavin, donor–acceptor FRET pair.[50] In the oxidized form, a FRET process results in green emission from the flavin acceptor, while upon reduction, energy transfer is inhibited, thus resulting in blue donor emission. In addition to fluorescence microscopy and flow cytometry, FCR1 was also shown to report on cellular oxidative capacity by fluorescence lifetime imaging microscopy (FLIM).

7. Other Approaches to Redox Sensing

One of the most widely utilized probes of cellular redox state is dihydroethidine (DHE), a blue fluorescent product formed from the two-electron reduction of the red fluorescent DNA stain ethidium bromide.[73] DHE and its targeted analogues, Mito-HE and MitoSOX, have been used for the detection of superoxide produced in a variety of biological systems, ranging from single cells to whole animal imaging. Nevertheless, its specificity to superoxide, reversibility, stability, and disproportionation products have been widely debated.[74]

Another original design involved a redox-active copper/thiacyclam complex tethered covalently to an anthracene moiety (Cu-Anthracene),[75] thus enabling electrochemically reversible switching between the fluorescent Cu^{I} and fluorescence-quenching Cu^{II} form in MeCN. However, unverified stability of both forms of the probe in biological media and a reduction potential (> 1 V) lying significantly outside the biologically relevant range, precludes the applicability of metal-redox-couple-based strategy to biological systems.

A PET-based redox switch bearing a tetrathiafulvalene (TTF) unit linked to anthracene has been reported, thus exploiting the electron-donating and electron-accepting abilities of the TTF scaffold.[76] Oxidation of the molecule by $Fe(ClO_4)_3$ results in increased blue fluorescence, but while electrochemical studies indicate a reversible two-step oxidation process, the $E_{1/2}$ values of −660 mV and −890 mV lie outside the biologically relevant range. Similar potential probes using a ferrocene redox switch tethered to pyrene[77] and perylene diimide[78] also have limited application, but if the redox properties of these highly reversible switches could be tuned, they could yield promising biological probes.

RHyCy5 is a reversible near-IR probe for hypoxia imaging, and employs the cyanine Cy5 as a FRET donor and the rhodamine derivative, QSY-21, as a FRET acceptor (Figure 8).[51] RHyCy5 harnesses the strong absorbance of

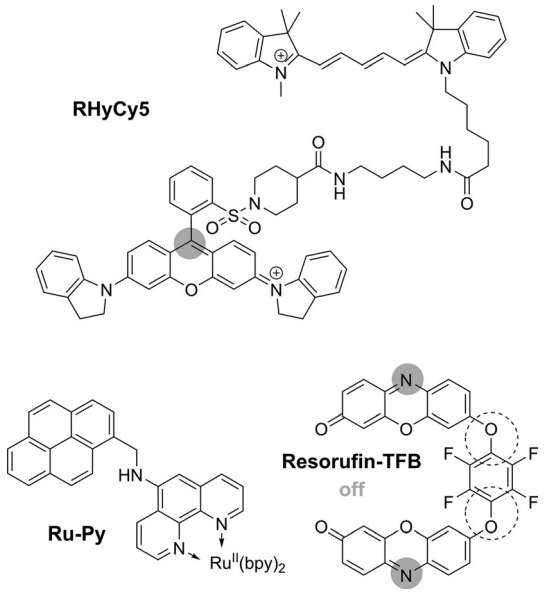

Figure 8. Structures of other probes based on different sensing strategies.[51,53–54]

QSY-21 in the far-red and near-IR regions, absorbance which decreases upon one-electron enzymatic reduction under hypoxia. The authors confirm the formation of a stable radical upon enzymatic reduction, and the reversibility of the process to normoxia/hypoxia cycles in A549 human breast cancer cells. Other hypoxia imaging probes based on metal chelates of hydrophobic ligands such as porphyrins (Ox-PS-NP)[52] and phenanthrolines (Ru-Py; Figure 8)[53] harness the oxygen-mediated quenching of luminescent properties of the complexes. Exciting results from single cells and mice highlight the possibility of using such complexes for imaging oxygen levels in cells.

A recent study has reported a dual-functional probe capable of sequential thiol detection and redox homeostasis based on two *para*-substituted resorufin units on tetrafluorobenzene (Resorufin-TFB; Figure 8).[54] Thiol-mediated cleavage of the tetrafluorophenyl group results in the release of fluorescent resorufin units, which can then be reversibly reduced to the nonfluorescent dihydroresorufin. This report is a unique example of a single probe that can be used to image both intracellular thiols and redox homeostasis, with demonstrated utility in macrophages, but its response varies with different thiols.

8. Summary and Outlook

This survey of reversible fluorescent redox sensors reveals the promise of this burgeoning field. Recent years have seen the identification of a number of promising strategies, and the development of sophisticated probes that are able to sense biological redox state, even in animal studies. Each general strategy that we have outlined, from nitroxyl radicals and quinones to thiol switches to vitamin-based sensors, has proved to be a valuable approach to the sensing of biological redox states. Crucially, there is value in simultaneously using more than one class of probe, as each shows different selectivity, sensitivity, and electrochemistry, and will therefore be able to answer distinct questions. Interestingly, the majority of the sensing mechanisms discussed herein take inspiration from nature, not only harnessing the sophisticated redox sensing processes already used in biology, but also ensuring the biological compatibility and relevance of the probes developed by these strategies.

From this survey we can identify the most promising features of current probes and determine areas that warrant further attention. More recent development of near-IR emitters has enabled application of probes to in vivo studies. 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 antioxidant efficacy, while the latter will have utility in uncovering new roles of ROS/RNS. However, still more promising are ratiometric probes, for which both oxidized and reduced forms can be imaged. In addition to minimizing interference from background effects such as probe concentration, ratiometric probes bear the possibility to enable quantification of relative or absolute reduction potential, although this remains to be realized.

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 subcellular organelles. Since the redox environment is highly compartmentalized within cells, there is much to be gained from tools which are able to report on location-specific changes in redox state. Furthermore, an ability to tune the reduction potential of a probe will enable development of sets of probes which enable accurate determination of reduction potentials within the cell (analogous to the use of a range of pH indicators to determine acidity). While this goal has not been achieved to date, each of the redox-sensing moieties described herein should be amenable to redox tuning through structure variation.

Furthermore, this review has highlighted the inconsistencies between probe validation protocols. While fluorescent response is routinely screened, 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. A comprehensive approach to probe testing is essential, not only to enable meaningful comparisons between them, but also to ensure that researchers who may be interested in utilizing published probes are able to identify the most suitable ones for their purposes. 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 over time in cellular studies. Furthermore, the more widespread adoption of fluorescent redox probes will be facilitated by verification that probes can work by modalities other 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 a redox state and can be used to distinguish chronic oxidative stress from physiological oxidative bursts not only in cultured cells, but also in in vivo studies. The challenge remains to ensure that such probes are put to the best use, and that they are employed beyond the laboratory in which they were developed, thus becoming invaluable tools for the redox biology community.

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