Bio-inspired small molecule tools for the imaging of redox biology

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CONSPECTUS

The availability of electrons to biological systems underpins the mitochondrial electron transport chain (ETC) that powers living cells. It is little wonder, therefore, that the sufficiency of electron supply is critical to cellular health. Considering mitochondrial redox activity alone, a lack of oxygen (hypoxia) leads to impaired production of adenosine triphosphate (ATP), the major energy currency of the cell; whereas excess oxygen (hyperoxia) is associated with elevated production of reactive oxygen species (ROS) from the interaction of oxygen with electrons that have leaked from the ETC. Furthermore, the redox proteome, which describes the reversible and irreversible redox modifications of proteins, controls many aspects of biological structure and function. Indeed, many major diseases, including cancer and diabetes, are now termed “redox diseases”, spurring much interest in the measurement and monitoring of redox states and redox-active species within biological systems.

In this Account, we describe recent efforts in the development of magnetic resonance (MR) and fluorescence imaging probes for studying redox biology. These two classes of molecular imaging tools have proved invaluable in supplementing the structural information that is traditionally provided by MRI and fluorescence microscopy, respectively, with highly-sensitive chemical information. Importantly, the study of biological redox processes requires sensors that operate at biologically-relevant reduction potentials, which can be achieved by use of bio-inspired redox-sensitive groups. Since oxidation-reduction reactions are so crucial to modulating cellular function, and yet also have the potential to damage cellular structures, biological systems have developed highly sophisticated ways to regulate and sense redox changes. There is therefore a plethora of diverse chemical structures in cells with biologically-relevant reduction potentials, from transition metals to organic molecules to proteins. These chemical groups can be harnessed in the development of exogenous molecular imaging agents that are well-tuned to biological redox events.

To date, small molecule redox sensitive tools for oxidative stress and hypoxia have been inspired from four classes of cellular regulators. The redox-sensitive groups found in redox cofactors, such as flavins and nicotinamides, can be used as reversible switches in both fluorescent and MR probes. Enzyme substrates that undergo redox processing within the cell can be modified to provide fluorescence or MR readout, while maintaining their selectivity. Redox-active first row transition metals are central to biological homeostasis, and their marked electronic and magnetic changes upon oxidation/reduction have been used to develop MR sensors. Finally, redox-sensitive amino acids, particularly cysteine, can be utilized in both fluorescent and MR sensors.

REDOX STRESS

Central to contemporary studies of redox biology is a focus on reactive oxygen and nitrogen species (ROS/RNS), which are electron-deficient molecules produced through a series of reactions stemming from the primary ROS, superoxide, a by-product of the ETC. It was traditionally believed that ROS/RNS played only damaging roles through their reactions with proteins, nucleic acids and fatty acids, and the term "oxidative stress" has long been used to describe the build-up of oxidants in the cell with harmful consequences.[1] It is now appreciated that at lower levels, ROS/RNS play essential roles in healthy cells, including in signaling and immune response.[2] The terms "eustress", describing physiological, positive effects of ROS/RNS, and "distress", referring to pathological, deleterious effects, have therefore been recently adopted.[3]

In contrast, "hypoxia" describes conditions in which there is a lack of oxygen. Tissue may be hypoxic under pathological conditions, where blood flow is disrupted, such as heart attack or stroke, but hypoxia is also essential for normal development of the mammalian embryo.[4] At present, there is also considerable attention on the hypoxic environments in poorly-vascularized tumors, to better understand the progression of cancers,[5] and for the development of hypoxia-selective cancer therapies.[6]

While hypoxia and oxidative stress may appear to be antithetical conditions, the two are closely linked. Not only does oxidative stress from reperfusing blood rapidly follow hypoxia in stroke and heart attack, but ROS generation has been reported to occur simultaneously to hypoxia in various conditions, including cerebral infarction,[7] acute respiratory distress syndrome,[8] and even increased physical activity.[9]

Cellular redox changes, particularly those involved in physiological eustress responses, are inherently transient, and are therefore difficult to capture for monitoring purposes. Furthermore, the post-processing protocols commonly adopted to study cells, involving fixation and lysis, will alter the redox environment, precluding studies of the native state. Indeed, all cellular manipulations, including sorting cells, alters redox biology.[10] A key criterion in sensing cellular redox state is therefore minimal perturbation to the native system, and molecular imaging can go some way to achieve this.

MOLECULAR IMAGING TOOLS

Molecular imaging agents are generally exogenous small molecules that provide molecular information about the system in conjunction with conventional imaging techniques. There is much activity in the development of molecular imaging tools for all modes of imaging, with traditional positron emission tomography (PET) agents, MRI contrast agents and fluorescent probes now joined by ultrasound[11] and near-infrared imaging agents.[12]

Considerations in the design of molecular imaging tools for biology include:

1. Ratiometricity. A longstanding challenge in the development of molecular imaging tools is overcoming the concentration dependence of signal. When probe response is monitored using a single parameter, it is difficult to distinguish accumulation of the probe from the stimulus-triggered response. This can be overcome by the development of ratiometric probes, in which the sensing event is reported through a change in two different outputs. The ratio of these two changes is independent of the probe concentration.[13]

2. Probe localization. Molecular imaging tools will only be able to report on regions in which they accumulate, and the design of new tools must therefore identify the requirement to localize to a specific cell type, be retained in the extracellular space, where redox buffering systems are vastly different, or accumulate in a sub-cellular organelle.
3. Selectivity *vs* generality of response. While significant effort in molecular imaging of redox biology focusses on the development of selective, irreversible sensors that are capable of distinguishing individual ROS/RNS, here we focus on sensors that report on overall redox balance, as these enable real-time monitoring of cellular populations.[14]
4. Reduction potential. A crucial design feature for a biologically-relevant redox sensor is the identification of systems with appropriate reduction potentials. A probe that tends to be oxidized in the relevant biological environment will be most sensitive to hypoxia, while a probe that is naturally in the reduced form will be able to report on oxidative stress. Although there have been several successful chemical probes including boronates, nitroxides, quinones, genetically-encoded and metal-based probes for redox sensing that have been extensively reviewed in literature,[14-16] an effective method for ensuring a biologically-relevant reduction potential (Table 1) is to employ redox-responsive groups that are already utilized in biological systems. In addition to considering intracellular redox regulation, there is also some value in extracellular sensing of redox changes, such as in hypoxic regions, which tend to be less reducing than the intracellular environment. This review focusses on redox-responsive sensors bearing a bio-inspired redox-sensitive group.

Table 1: Redox potentials and cytoplasmic concentrations of key cellular redox couples.

Cellular redox reactions	Standard reduction potential / mV	Cellular concentrations
$NADH \overset{2e^-/H^+}{\rightleftharpoons} NADH_2$	-316	Up to 10 μM
$TrxSS \overset{2e^-/H^+}{\rightleftharpoons} TrxSH_2$	-280	2-10 μM
$2GSH \overset{e^-/H^+}{\rightleftharpoons} GSSG$	-280 to -320	Up to 15 mM
$FMN \overset{2e^-/H^+}{\rightleftharpoons} FMNH_2$	-219	6-45 nM
$FAD \overset{2e^-/H^+}{\rightleftharpoons} FADH_2$	-219	70 – 480 nM
$Fe^{3+} \overset{e^-}{\rightleftharpoons} Fe^{2+}$	-700 to +400	18-50 μM

There is little work in the development of non-invasive position emission tomography (PET)-based redox probes for *in vivo* imaging.[17] A notable example is the superoxide-responsive dihydroethidium-based PET sensor **[^{18}F]ROStrace** that has been demonstrated to report on elevated superoxide levels in neuroinflammation.[18] PET-based sensors will not be discussed further here. Below we briefly summarize typical approaches to developing fluorescent and MR molecular imaging tools.

Fluorescent probes

Fluorescent probes have become a mainstay of the biological research laboratory, enabling the study of numerous cellular chemical processes.[19] Emission intensity or wavelength can be modulated by a number of electronic processes, which can be harnessed in the design of redox-responsive systems. Ratiometric fluorescent probes typically involve changes in emission

intensity, with a commonly-used method being Förster resonance energy transfer (FRET), a distance-dependent energy transfer mechanism involving non-radiative transfer of energy from a donor fluorophore in its excited state to an acceptor fluorophore. A less commonly-employed promising strategy for achieving ratiometricity is to measure changes in fluorescence lifetime.[20]

MRI contrast agents

MRI contrast agents, typically based on paramagnetic metal ions, are commonly used in clinical and research settings to increase water relaxation rates and hence improve MRI sensitivity.[21] Responsive MRI contrast agents are those that can report on chemical changes in their environment.[22] The required administered dose of MRI contrast agents is quite high (mM), making them insensitive to analytes that exist in the cell at far lower concentrations, but ideal for measuring chemical conditions, such as oxidative stress or hypoxia. The design of redox-responsive MRI contrast agents can utilize either a redox-active metal center, or a redox-active ligand.[23] Responsive MRI contrast agents reported to date are predominated by relaxivity or chemical exchange agents.

Relaxivity agents are those that induce changes in the longitudinal (T_1) or transverse (T_2) relaxation times of water protons. The most common agents of this type are based on Gd(III) (primarily T_1) and iron oxide nanoparticles (primarily T_2). Relaxivity can be modulated by altering the interaction of water molecules with the metal center, such as by varying the hydration state of the complex or controlling the molecular motion of the complex.[24] The concentration dependence problem is a particular challenge for this class of agents, as Gd(III) complexes and iron oxide nanoparticles will exhibit considerable contrast in both "off" and "on" forms, as the prevailing paramagnetism of the metal center predominates the response. Strategies to overcome this have been comprehensively

reviewed elsewhere,[25] and include measurement of the ratio of transverse to longitudinal contributions to relaxation rates, combination of MRI with other imaging modalities, and use of magnetogenic metals that switch between diamagnetic and paramagnetic states.[26]

The chemical exchange saturation transfer (CEST) protocol involves selective irradiation to saturate the resonance of exchangeable solute protons. Exchange of these protons with water protons results in saturation transfer to bulk water, and attenuation of the water signal. The CEST agent may be an endogenous molecule such as glycogen, or an exogenous agent such as ^{129}Xe.[27] Paramagnetic metal complexes can also be employed for CEST imaging (paraCEST).[28] Systems with two exchange sites will give two CEST signals, and therefore a ratiometric response.

BIOINSPIRED MOLECULAR IMAGING TOOLS

Redox cofactors

The main redox cofactors in the cell are flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD^+). The redox-sensing portions of these molecules, flavin and nicotinamide, are also utilized in biology as vitamins riboflavin and niacin, and as cofactors in redox-active proteins.

The first reported use of nicotinamide as a sensing group was **Perylene-NAD** (Figure 1(a)), comprising a perylene fluorophore linked to nicotinamide.[29] Photoinduced electron transfer (PET) quenching of the perylene emission by the reduced nicotinamide is alleviated upon oxidation. Inspired by the excellent reversibility of the nicotinamide redox switch, we recently reported **GdNR1** (Figure 1(b)), a Gd complex bearing a nicotinimidium-conjugated DO3A ligand.[30] Reduction of the complex results in a 2.5-fold increase in relaxivity due to a change in hydration

state. This is a rare example of a redox-responsive MRI contrast agent bearing a biologically-inspired redox active ligand.

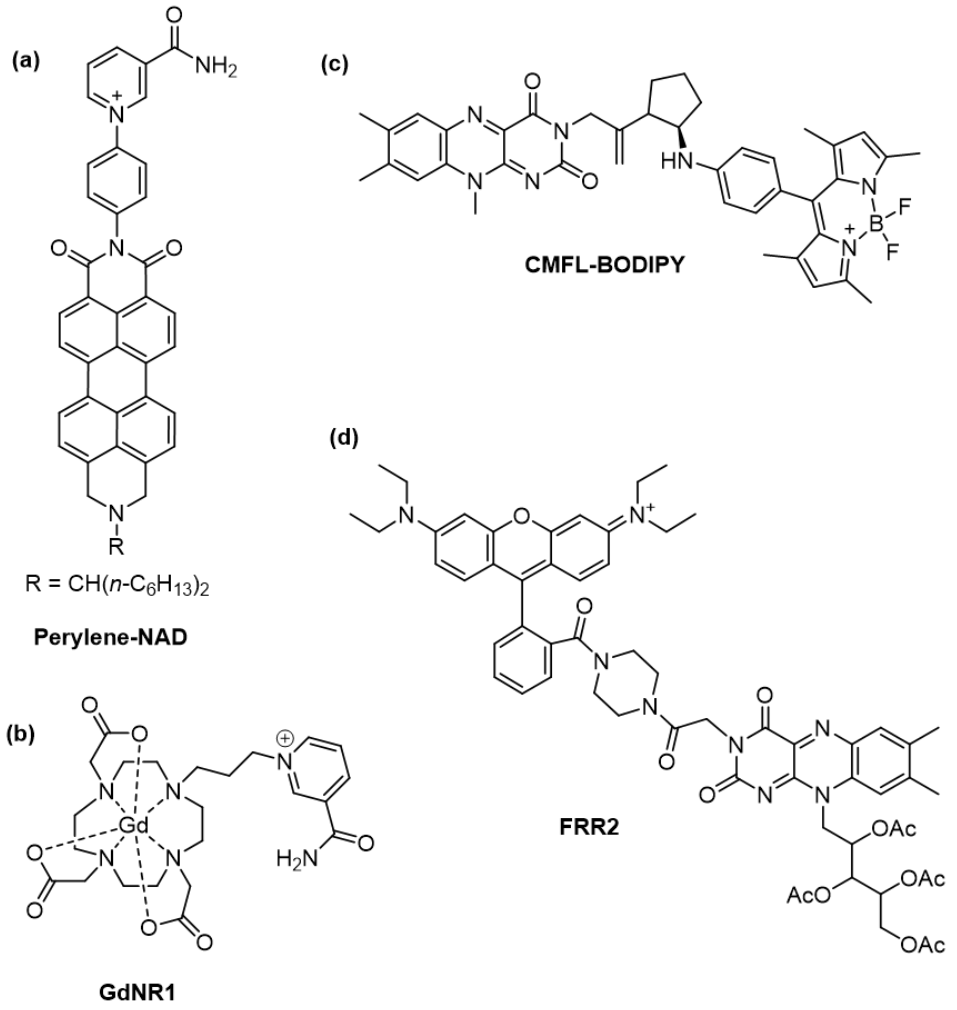

Figure 1: (a) Chemical structures of key fluorescent redox sensors incorporating bio-inspired sensing motifs - nicotinamide in (a) **Perylene NAD** and (b) **GdNR1** and flavin in (c) **CMFL-BODIPY** and (d) **FRR2.**

Aoki and co-workers first utilized flavins as redox-sensing groups, incorporating the moiety into Zn^{2+}/tetraazacyclododecane complexes.[31] They subsequently reported **CMFL-BODIPY** (Figure 1 (c)), in which carboxymethylflavin was conjugated to a BODIPY fluorophore.[32] Flavin reduction resulted in a nine-fold decrease in fluorescence emission. We subsequently reported naphthalimide-flavin redox sensor 1 (**NpFR1**), which showed slightly red-shifted fluorescence

excitation and emission compared to native flavin groups, and a reduction potential (-336 mV *vs* SHE) within the range of cellular flavins.[33] **NpFR1** exhibits a greater than 100-fold increase in fluorescence upon oxidation, and can undergo multiple cycles of oxidation and reduction without losing signal (Figure 2, Table 2).

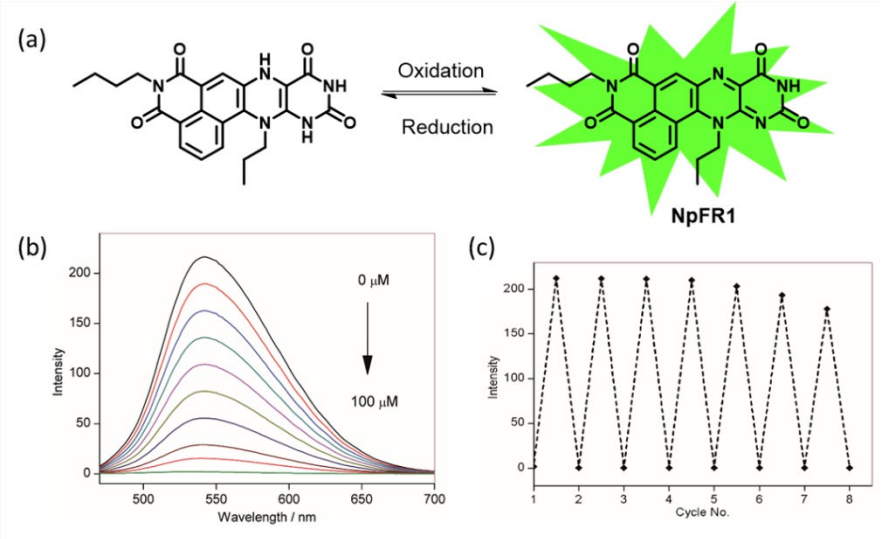

Figure 2: (a) Chemical structures of **NpFR1** in reduced and oxidized forms. Fluorescence response of NpFR1 to (b) incremental addition of $Na_2S_2O_4$ and (c) cycles of reduction (100 μM $Na_2S_2O_4$) and oxidation (250 μM H_2O_2). Adapted from Ref. [33] Copyright (2014) Royal Society of Chemistry.

Given the considerable interest in sensing mitochondrial redox biology,[34] we prepared mitochondrially-targeted analogue **NpFR2** (Figure 3(a)), by incorporating the triphenyl phosphonium group commonly employed for mitochondrial delivery.[35] **NpFR2** showed similar fluorescence switching and reversibility to its cytoplasmic counterpart, with colocalization experiments confirming the mitochondrial accumulation of the probe (Figure 3(b)). We subsequently demonstrated its use in flow cytometry studies of hematopoietic cells (Figure 3(c)).

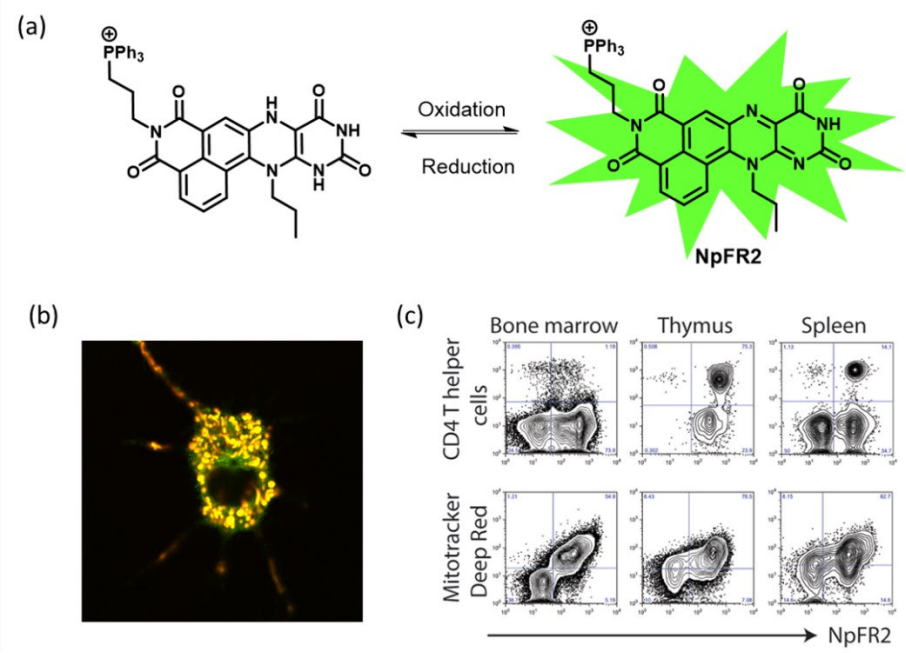

Figure 3: (a) Chemical structures of **NpFR2** in reduced and oxidized forms. (b) Co-localization image of macrophages (RAW 264.7) treated with **NpFR2** (20 μM, λ_{ex} = 488 nm, λ_{em} = 495-600 nm) and Mitotracker Deep Red (100 nM, λ_{ex} = 633 nm, λ_{em} = 650-750 nm) for 15 min. Yellow regions indicate co-localization. (c) Flow cytometry imaging of **NpFR2** fluorescence (mitochondrial redox state) in comparison with mitochondrial number (Mitotracker Deep Red) in CD4-expressing T helper lymphocytes from hematopoietic organs. Adapted from Ref. [35] Copyright (2015) Royal Society of Chemistry.

Using this same flavin redox switch, we reported **FCR1**, a ratiometric redox sensor consisting of a redox-insensitive coumarin donor and a redox-responsive flavin acceptor.[36] The ratio of flavin:coumarin emission intensities serves as an indicator of the redox state (Figure 4), undergoing a 6-fold decrease upon reduction by chemical or electrochemical reduction. We demonstrated that **FCR1** could be used to report on redox changes using two-photon and fluorescence lifetime imaging microscopy (FLIM) as well as flow cytometry.

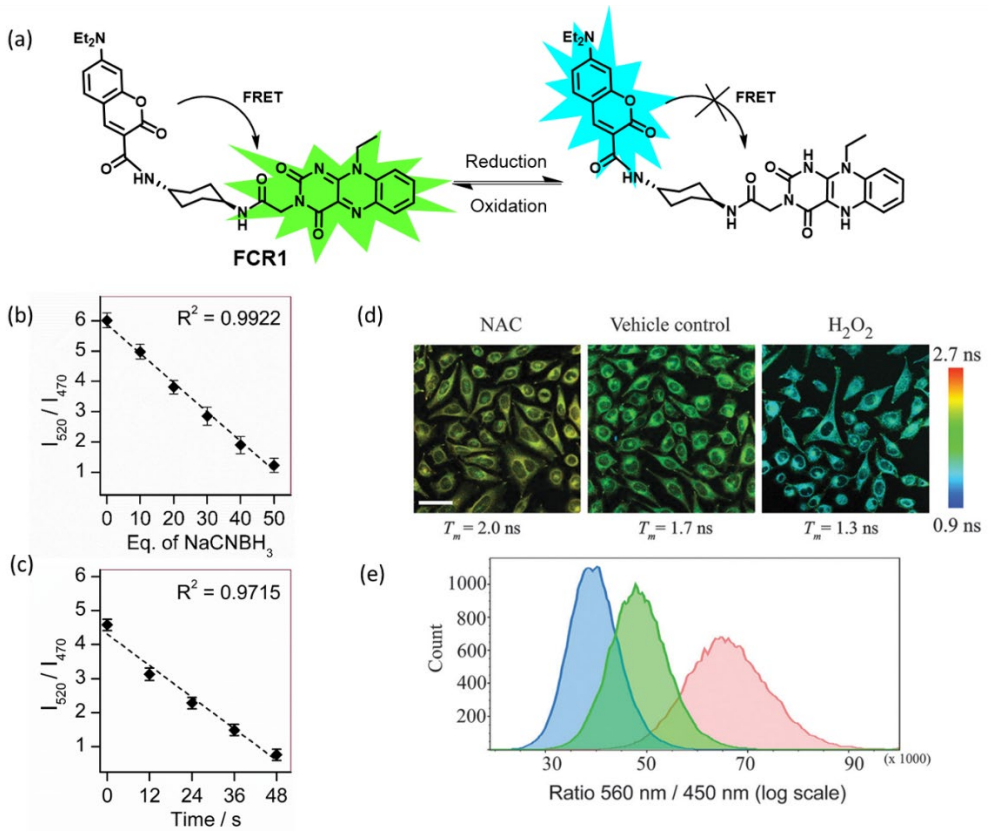

Figure 4: (a) Chemical structures of FCR1 in oxidized and reduced forms. Ratio of flavin/coumarin (I_{520}/I_{475}) emission of FCR1 (10 μM) (b) with incremental reduction with $NaBH_3CN$ and (c) over time after the application of a potential of -1.3 V to FCR1 (100 μM) in MeCN. (d) Fluorescence lifetimes of the donor (420–470 nm) in HeLa cells treated with FCR1 (10 μM, λ_{ex} = 820 nm) and N-acetyl cysteine, vehicle control and H_2O_2. (e) Flow cytometry imaging of FCR1 (10 μM, λ_{ex} = 405 nm) in HeLa cells treated with N-acetyl cysteine (blue), vehicle control (green) and H_2O_2 (red). Reproduced from Ref. [36] Copyright (2015) Royal Society of Chemistry.

Using similar principles, we then developed a pair of flavin-rhodamine redox sensors (**FRR1** and **FRR2**) (Figure 1(d)), in which a flavin group (*N*-ethylflavin or acetylated riboflavin) was used as the FRET donor, and rhodamine as the FRET acceptor. Since reduced flavins do not absorb visible light, the reduced probe must be excited directly at rhodamine (530 nm) to elicit a measurable signal. Due to the lipophilic cationic nature of the rhodamine group, **FRR1** and **2** show excellent

mitochondrial localization, and respond to changes in mitochondrial ROS levels. We used **FRR2** to image the mitochondrial oxidative burst that accompanies stimulation of macrophages.

While flavins and nicotinamides are the most common redox cofactors, in fact this strategy can also be applied to other biologically-relevant small molecule redox regulators, such as antioxidants. This has been elegantly demonstrated by Cosa and co-workers, who have developed a series of fluorogenic analogues of α-tocopherol, an antioxidant of the vitamin E family,[37,38] as well as the essential cofactor ubiquinone (coenzyme Q10).[39]

Table 2: Fluorescence and redox properties of flavin-based probes.

Probe	Redox potential (mV vs SHE)	Ex/Em wavelength (nm)	Ratiometric	Cellular localization
NpFR1	-336	405/545	No	Cytoplasmic
NpFR2	As for NpFR1	488/545	No	Mitochondrial
FCR1	-310	405/470 and 530	Emission ratiometric	Cytoplasmic
FRR2	-290	488 and 560/590	Excitation ratiometric	Mitochondrial

Enzyme substrates

Fluorogenic substrates are commonly used to study enzyme activity and localization.[40] Modified substrates are also widely used for drug delivery, activation and targeting.[41] This strategy has been used for the development of pro-drugs that are selectively activated in hypoxia: active drugs are masked by a group that is reduced and released by one of a number of reductase enzymes.[42] The process is hypoxia-selective, as such reductions are reversible in the presence of oxygen. Various bioreductive groups include quinones, aromatic azides and azobenzene derivatives.

The bio-reductive nitrobenzene group is commonly used in pro-drug design: the nitroaromatic group undergoes one electron reduction in the presence of the ubiquitous nitroreductase enzyme, and subsequent fragmentation and reduction under hypoxic conditions to an aniline (Figure 5(a)). This hypoxia-selective reduction has also been harnessed in the development of fluorescent sensors, as nitro- and amino- substituents tend to exert markedly different effects on fluorophores.[43] Sensors using this strategy include those with cyanine-based fluorophores,[44] as well as aggregation-induced fluorogens.[45]

Having demonstrated distinct emission spectral changes induced by altering the substitution pattern of 4-amino-1,8-naphthalimides,[46] we were interested in investigating the potential for nitro-substituted naphthalimides to report on tissue hypoxia. We therefore investigated the chemical properties and biological behavior of four naphthalimide hypoxia (**NpHy**) sensors (Figure 5(b)).[47] **NpHy2** showed the most promising ratiometric behavior upon reduction (Figure 5(c)), but its relatively high reduction potential meant that it was reduced too rapidly to differentiate normoxic and hypoxic cells. **NpHy1** was reduced even more rapidly, being reduced in normoxic cells within minutes, but **NpHy3** had a sufficiently low reduction potential to enable study of hypoxia sensitivity in mesenchymal stem cells. In contrast, the rapid reduction of **NpHy1** was well-tuned to extracellular hypoxia, so **NpHy4** was designed to be retained in the extracellular space, successfully reporting on hypoxia within tumor spheroids (Figure 5(d)). This study demonstrates the importance of tuning the reduction potential to the biological location and question being investigated.

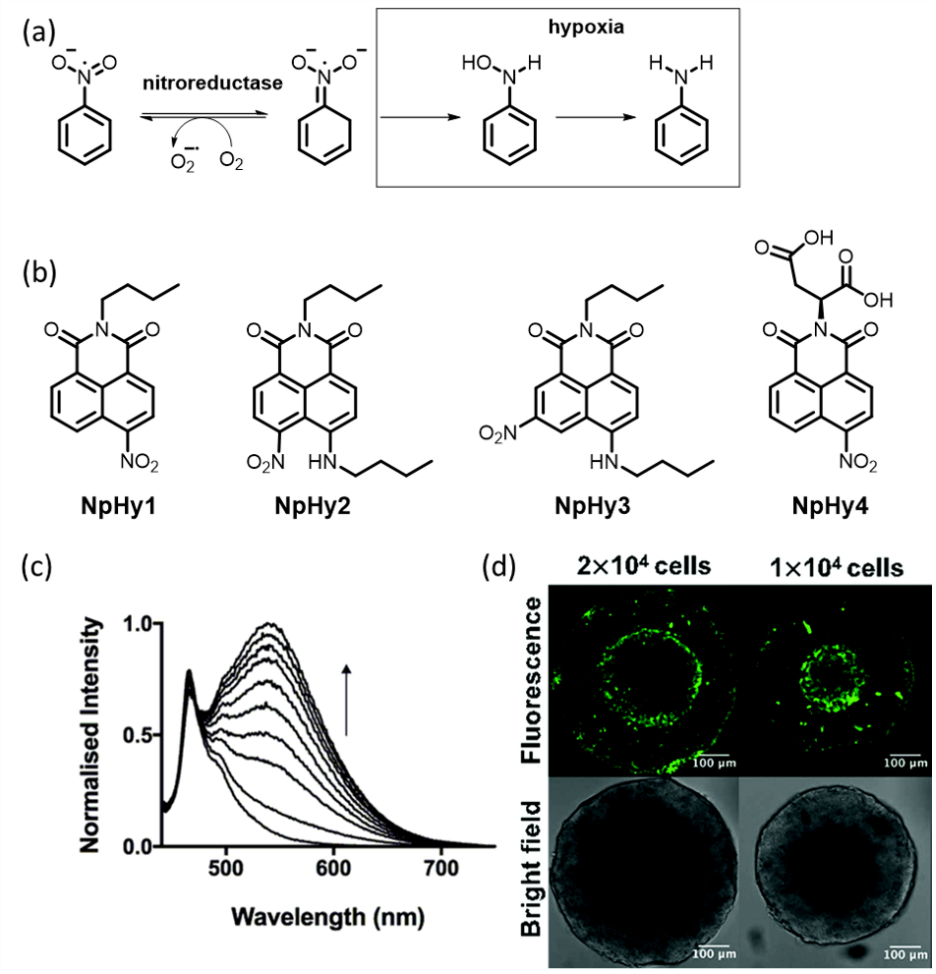

Figure 5: (a) Mechanism of aromatic nitro reduction in cells, (b) NpHy probes for hypoxia sensing, (c) fluorescence titration of NpHy2 (10 µM) with increasing sodium dithionite reduction (PBS, pH 7.4, λ_{ex} = 420 nm), (d) Two-photon microscopy of NpHy4 (5 µM, 20 h) in DLD-1 spheroids of different sizes. Top: Brightfield images (acquired with 515 nm laser), bottom: fluorescence images (810 nm two-photon excitation, 515–600 nm emission). Adapted from ref. [47] Copyright (2018) Royal Society of Chemistry.

The nitrobenzene to aniline reduction has also been used for the development of hypoxia-responsive MRI contrast agents. Nagano and co-workers prepared a set of gadolinium complexes bearing a nitrobenzenesulfonamide substituent.[48] In hypoxic conditions, reduction to the aminobenzenesulfonamide greatly altered the pK_a of this group, leading to decoordination of the sulfonamide from the gadolinium, and allowing coordination of an additional water molecule. This

increased hydration state resulted in increased relaxivity. More recently, Liu *et al.* reported a gadolinium complex bearing a nitrobenzene group, which upon reduction to aniline undergoes a self-immolative process to reveal Gd-DOTA (Dotarem), a widely-used MRI contrast agent.[49] This reduction resulted in a 15% increase in relaxivity (r_1=3.51 to 4.02 mM^{-1} s^{-1}).

Redox-active metals

Redox-active first row transition metals are essential for a multitude of redox processes in the cell, from the transport of oxygen by iron in hemoglobin to the disproportionation of superoxide by copper in superoxide dismutase. Indeed, the abundance and speciation of redox-active metals, particularly iron, but also copper, manganese and cobalt, significantly influences cellular redox status. Sensing these metal ions themselves can therefore serve to provide proxy information about redox events within the cell.[50] More powerfully, the biologically-relevant redox switching of metal ions can be used as the basis of sensors of hypoxia and oxidative stress. Much activity in this area has been around MRI contrast agents, rather than fluorescent sensors, as metal ions tend to quench fluorescence due to electron and energy transfer between metal and fluorophore,[51] and paramagnetic interactions.[52]

A promising strategy for the design of redox-responsive MRI contrast agents is the biologically-relevant and magnetogenic Co(III)-Co(II) redox switch: Co(III) is typically diamagnetic low spin while Co(II) bears three unpaired electrons in high spin form. This sensing strategy was first demonstrated by Morrow *et al.*, who reported an octahedral, MRI silent Co(III) complex (Figure 6(a)) that can be reduced to the MRI active Co(II).[53]

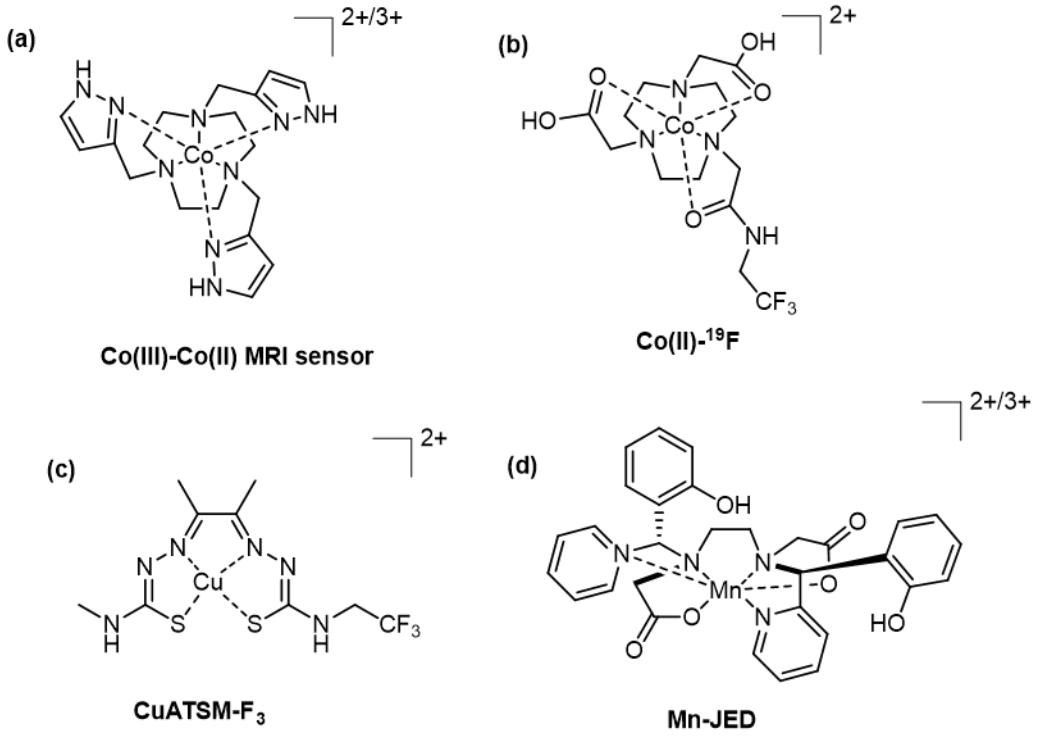

Figure 6: Chemical structures of MRI-active redox sensors based on redox-sensitive metals (a,b) cobalt, (c) copper and (d) manganese.

Simultaneously, we were developing Co(III) complexes containing a tris(2-pyridylmethyl)amine (TPA) ligand, inspired by hypoxia-activated prodrugs of the same ligand prepared by our colleagues at the University of Sydney.[54] **CoTPA0** could be cycled back and forth multiple times between MRI-silent Co(III) and MRI-active Co(II) forms.[55] We also showed that varying the charge on the ligand by incorporating carboxylate groups could modulate the penetration of complexes into tumor spheroids, and used NMR imaging experiments to confirm that the complexes were indeed reduced within these spheroids (Figure 7).[56]

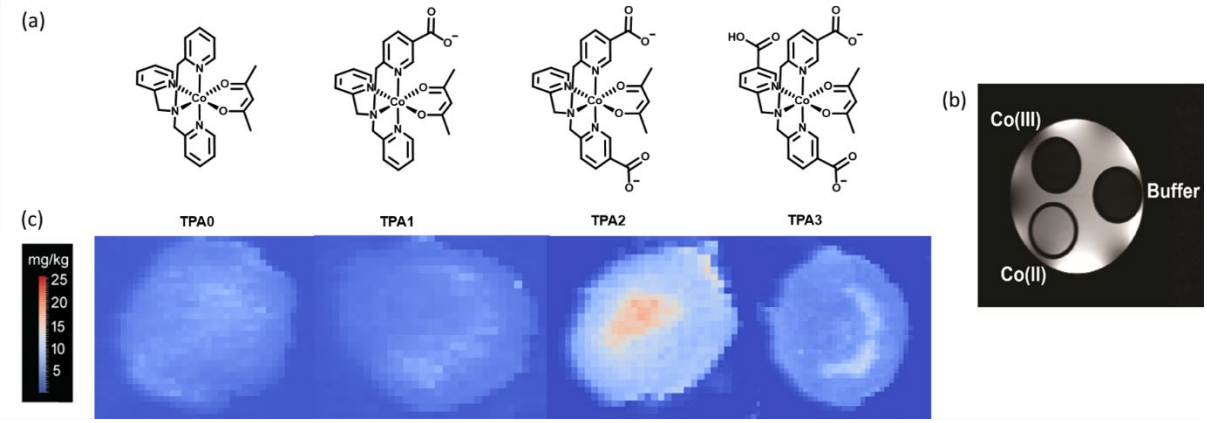

Figure 7: (a) Reduction-activated Co(III) MR contrast agents, (b) phantom images (9.4 T) of **Co(III)TPA0** (8 mM), **Co(II)TPA0** (8 mM) and buffer alone in deoxygenated 10% D_2O, 0.15 M NaCl, 0.1 M Tris buffer, pH 7.4, (c) representative cobalt LA-ICP-MS images showing cyrosectioned slices of DLD-1 spheroids (15,000 cells) incubated with **TPAx** (2 mM) for 24 h; scale bar 500 μm. Adapted from Ref.[55] and [56] Copyright (2016) Royal Society of Chemistry and Copyright (2017) American Chemical Society.

Que and co-workers recently reported a set of Co complexes, again utilizing the hypoxia-activated Co(III) to Co(II) transition, but harnessing the Co(II)-induced paramagnetic relaxation enhancement of the ^{19}F nuclei on the appended trifluoromethyl group (Figure 6(b)), which decreases the ^{19}F NMR signal compared to the diamagnetic Co(II) system.[57] Que's group also utilized another bioinspired redox-active metal, copper, in the development of a similar set of ^{19}F-MR probes for hypoxia (Figure 6(c)).[58]

Mn(II), with its 5 unpaired electrons in the high spin form, has a much greater effect on water relaxivity than Cu(II) or Co(II). The biologically-relevant Mn(III)/Mn(II) redox couple has been utilized by Caravan and co-workers in the design of redox-responsive systems. For example, by harnessing the less effective relaxation caused by Mn(III) compared to Mn(II) (Figure 6(d)), and

designing a ligand that could bind stably to both ions, they could observe a 9-fold change in relaxivity upon reduction.[59]

The development of paraCEST probes based on redox-active metals was pioneered by Morrow and co-workers, utilizing the Co(III)-Co(II) switch.[53,60] More recently, Harris and colleagues achieved ratiometric quantitation of redox status by utilizing the Fe(III)-Fe(II) switch in an iron etidronate complex.[61]

While fluorescent complexes of these redox-active metals have not been reported to date for redox sensing, the reported emission properties of complexes of iron, manganese, cobalt, and copper, and the marked electronic changes upon oxidation/reduction of these metals suggest that this is likely to be a valuable strategy in the future.

Redox-sensitive amino acids

Another bio-inspired approach to sensing redox changes in biological systems is to utilize redox-responsive amino acids. The reversible oxidation of cysteine and selenocysteine to disulfide and diselenide bridges, respectively, is a key process in living systems, modulating protein structure and function, and mediating redox buffering, principally through the glutathione (GSH:GSSG) system. This transformation has been adopted in a small number of fluorescent and MRI probes, and there is certainly much more that can be achieved in this domain.

One of the first reversible redox sensors, **RedoxFluor-1 (RF-1)** reported by Chang and co-workers is based on a disulfide integrated into the fluorescein scaffold (Figure 8(a)).[62] The authors suggest

that reduction of **RedoxFluor-1** results in the formation of dihydrofluorescein species from the disulfide and involves internal charge transfer, and protonation. Schneider and co-workers reported **carbostyril-Tb** (Figure 8 (b)), bearing a terbium complex tethered through a peptide linker to carbostyril, which can act as a sensitizer of terbium emission.[63] Formation of a disulfide bond between the two cysteine residues in the linker brings the two groups into sufficiently close proximity for sensitization, therefore inducing a ratiometric fluorescence change. A similar mechanism has been utilized for a diselenide probe, FSeSeF, in which fluorescence of fluorescein is quenched when two fluorescein molecules are tethered by a diselenide linker: reduction of the diselenide results in separation of the fluoresceins, restoring their fluorescence.[64]

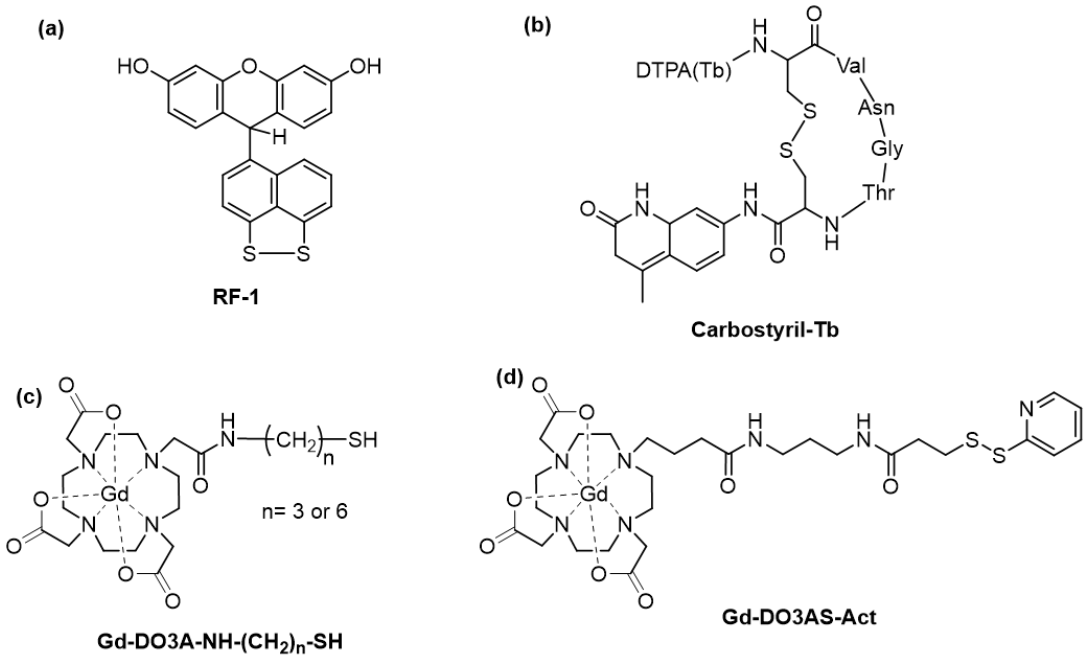

Figure 8: Chemical structures of redox sensors based on redox-active disulfide bridges.

This same intramolecular formation of disulfide/diselenide bridges should also be applicable to the development of redox-responsive MRI contrast agents, as slowed molecular tumbling increases relaxivity, but no such systems have been reported to date. Raghunand *et al.* utilized intermolecular

disulfide bond formation in their gadolinium complexes bearing a thiol group that can bind to the redox-sensitive thiol group in human serum albumin (HSA) (Figure 8(c)), slowing the tumbling and increasing the relaxivity.[65] Botta and co-workers encapsulated gadolinium complexes inside perthiolated β-cyclodextrin-based nanocapsules: degradation of the nanocapsules in reducing conditions released the gadolinium complexes, resulting in a significant decrease in relaxivity.[66]

The modulation of the relaxivity of MRI contrast agents by changing hydration state was employed in the design of a thiol-containing Gd(III) complex, which undergoes a decrease in hydration state from q=2 to q=1, and a corresponding two-fold decrease in relaxivity, upon interaction with cellular thiols (Figure 8(d)).[67]

SUMMARY AND OUTLOOK

Here we have summarized recent advances in the development of bioinspired MR and fluorescent sensors of redox biology. The advantage of utilizing diverse redox switches is that a range of reduction potentials can be accessed, enabling selection of the most appropriate probe for each biological question.

Much remains to be achieved in this field: there are relatively fewer MR probes than fluorescent sensors, and currently MR probes are seldom applied to advanced research studies, let alone in clinical applications. While some approaches have been reported to overcome the concentration dependence problem, broader adoption of these methods for redox-responsive agents will greatly move the field forward. Mitochondrially-localized redox sensors are now widely-reported, but

there is also interest in understanding redox changes in other locales, so future research efforts in our group, as well as those of many others, is towards other sub-cellular targets, and cell types.

Given the current interest in understanding redox biology in biological and medical research, the continued development of molecular imaging tools will be of great importance to studies across a huge range of investigations in physiology and pathology.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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BIOGRAPHICAL INFORMATION

Amandeep Kaur undertook her Masters studies at VIT University, India in 2012 including a research internship at ENSCCF, France. In 2016 she completed her PhD at the University of

Sydney, where she worked with Elizabeth New on the development of reversible fluorescent redox sensors for understanding the role of oxidative stress in physiology and pathology. From 2016-2018 she was a Postdoctoral Research Fellow at the University of New South Wales in the EMBL Node for Single Molecule Science. She is currently a University of Sydney Fellow working towards the development of fluorescent probes for super-resolution imaging.

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, where she is now Associate Professor and Westpac Research Fellow. Her research is focused on the development of small-molecule fluorescent and magnetic resonance probes for the study of biological systems.

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