Making light of stress

Jefferson Chan & Christopher J Chang

A nanoparticle that senses reactive oxygen and nitrogen species in the liver should improve *in vivo* detection of cellular stress.

When drugs are withdrawn from the market, the most common reason is liver toxicity. Detecting the likelihood of hepatotoxicity in advance of regulatory approval is often difficult because available safety assays rely on biomarkers with low predictive power. In this issue, Rao and colleagues[1] report an approach that could lead to improved screening for hepatotoxicity. They describe a semiconducting polymer nanoparticle (SPN) capable of sensing and distinguishing reactive oxygen species and reactive nitrogen species *in vivo*. This technological breakthrough could facilitate detection of cellular stress in a range of contexts, including ranking of drug candidates in preclinical safety studies.

Production of reactive oxygen and nitrogen species is an early event in hepatotoxicity and a mechanistic indicator of subsequent cellular and tissue damage, which occurs mainly through the destruction of protein and nucleic acid function. More generally, there has been a growing appreciation that these transient small molecules can be signal or stress agents, depending on the context of their production, trafficking and consumption[2–4]. During the hepatotoxic cascade, the main reactive oxygen species is hydrogen peroxide and the main reactive nitrogen species is peroxynitrite. Hydrogen peroxide is generated either as a by-product of enzyme-mediated oxidative metabolism of drugs or from the reaction of radical drug metabolites with oxygen. In contrast, peroxynitrite production is linked to drug-induced mitochondrial dysfunction.

Because of the importance of specific reactive oxygen and nitrogen species in biology, methods for detecting them have been the subject of intensive research. Bioimaging with fluorescent small-molecule dyes that respond chemoselectively to hydrogen peroxide and peroxynitrite is an attractive, noninvasive approach. However, many of the existing fluorescent probes are useful only for cellular assays and are not amenable to *in vivo* applications. Previous work from the Rao laboratory described a different SPN that can be tracked in mice by photoacoustic imaging[5]. This particle can also detect hypochlorite, a reactive oxygen species, and peroxynitrite, a reactive nitrogen species, although not differentially. Detection of these species relies on IR775S, a cyanine dye that rapidly loses its fluorescence signature after oxidative cleavage of key

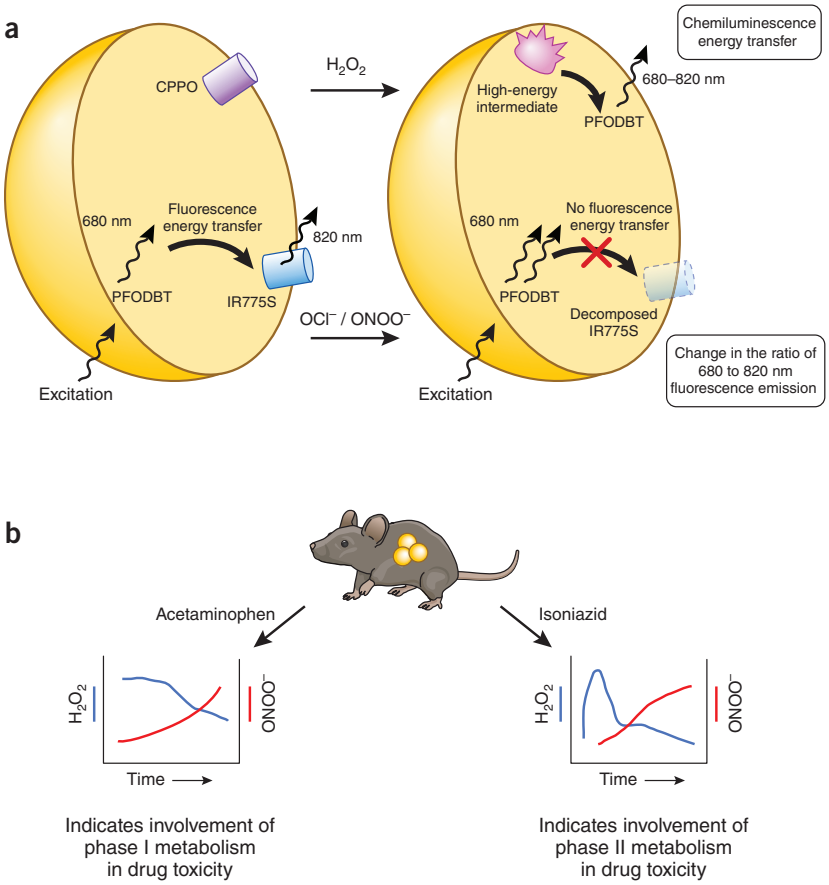

Figure 1 Detection of reactive oxygen and nitrogen species *in vivo* by CF-SPNs. (**a**) CF-SPNs have sensors that detect hydrogen peroxide and peroxynitrite differentially by means of chemiluminescence and fluorescence readouts. CPPO decomposes in the presence of hydrogen peroxide, forming a high-energy intermediate that excites PFODBT, a near-infrared semiconducting polymer, which acts as the chemiluminescence resonance energy transfer acceptor. The fluorescence-based sensor is IR775S, which irreversibly decomposes in the presence of peroxynitrite or hypochlorite. In the absence of these reactive species, fluorescence resonance energy transfer occurs from PFODBT to IR775S. Changes in fluorescence emission reflect the generation of peroxynitrite because CF-SPNs are targeted to hepatocytes, which do not produce hypochlorite. (**b**) The kinetics of hydrogen peroxide and peroxynitrite generation in the liver of drug-treated mice detected by CF-SPNs can act as indicators of the pathways involved. High doses of acetaminophen generate hydrogen peroxide before peroxynitrite, reflecting involvement of phase I metabolism. In contrast, the antituberculosis drug isoniazid elicits a rapid and short-lived burst of hydrogen peroxide and sustained production of peroxynitrite, indicating involvement of phase II metabolism.

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polymethine linkers mediated by hypochlorite or peroxynitrite.

Unlike other probes of reactive oxygen and nitrogen species, the new probe developed by Rao and colleagues[1] combines two sensors—a chemiluminescence resonance energy transfer sensor and a fluorescence resonance energy transfer sensor—in a single particle called a CF-SPN. The sensors interact in different ways with a near-infrared matrix made of PFODBT (poly(2,7-(9,9-dioctylfluorene)-*alt*-4,7-bis(thiophen-2-yl)benzo-2,1,3-thiadiazole)). PFODBT, used here for the first time in a nanoparticle, acts as both a chemiluminescence resonance energy transfer acceptor and a fluorescence resonance energy transfer donor (**Fig. 1a**).

The fluorescence sensor in CF-SPNs is IR775S, as in the authors' previous work[5]. Because hepatocytes do not produce hypochlorite, IR775S in the liver detects only the reactive nitrogen species peroxynitrite. The reaction abolishes the fluorescence resonance energy transfer from PFODBT to IR775S that would predominate in the absence of peroxynitrite. Thus, the ratio of PFODBT and IR775S fluorescence emission signals can be used as an accurate readout to probe *in vivo* nitrosative stress conditions.

The chemiluminescence sensor, which detects the reactive oxygen species hydrogen peroxide, is bis-(2,4,5-trichloro-6-(pentyloxycarbonyl) phenyl) oxalate (CPPO), a hydrophobic peroxyoxalate molecule. CPPO reacts with hydrogen peroxide to form the high-energy intermediate 1,2-dioxetanedione, which generates light by sensitizing fluorophores in close proximity, such as PFODBT and IR775S, triggering chemiluminescence. Indeed, *in vitro* calibrations show that hydrogen peroxide can selectively mediate chemiluminescence resonance energy transfer from CPPO to both near-infrared acceptors.

The authors target their probe to the mouse liver using poly(ethylene glycol) and galactose moieties that associate with asialoglycoprotein receptors expressed on the sinusoidal membrane of hepatocytes. They show that production of hydrogen peroxide and peroxynitrite can be readily detected in the liver after a challenge with high doses of acetaminophen or isoniazid, two commonly used drugs with known toxicity.

Notably, the timing and extent of hydrogen peroxide and peroxynitrite generation varies between the two drugs, providing insights into the different pathways and mechanisms of toxicity that are induced by each (**Fig. 1b**). In acetaminophen overdose, hydrogen peroxide generation precedes that of peroxynitrite. In isoniazid overdose, there is a short-lived oxidative burst and a more rapid and sustained induction of nitrosative stress. These subtle yet important differences would undoubtedly be missed using insensitive assays that rely on the detection of electrophilic reactive metabolites through covalent modification with endogenous nucleophiles such as glutathione.

In the context of drug design, the reactive oxygen and nitrogen species profiles detected with CF-SPNs can act as indicators of drug metabolism pathways, reporting on whether phase I or phase II metabolism is involved (**Fig. 1b**). In both, drug potency is reduced, but in phase I metabolism this occurs through the introduction of polar functionalities by enzymes such as cytochrome P450 (CYP450), whereas in phase II metabolism the drug is conjugated to reactive species such as glutathione, increasing its molecular weight.

Rao and colleagues[1] also show clearly that hydrogen peroxide and peroxynitrite production in the liver can be detected before the onset of histological changes that occur in hepatocellular degeneration, presumably through CYP450 activation reactions, which are the major, broad-spectrum scavenging pathways for therapeutics taken into the body.

CF-SPNs offer opportunities for a diverse array of studies in both clinical and basic research applications involving redox biology. They are versatile, biocompatible materials, and because they are engineered entirely from organic components and lack heavy metal ions, they should have minimal toxicity. Beyond drug toxicity testing, CF-SPNs may be helpful in unraveling the etiology and pathophysiology of many health conditions that involve alterations of oxidative and nitrosative stress, including chronic inflammatory disease, neurodegenerative disease and cancer. Extension to other imaging modalities, such as MRI, PET or photoacoustic imaging, which are more amenable to deep-tissue imaging, can also be envisioned. As the work of Rao and colleagues[1] suggests, combining new chemical detection methods and translational research is a promising avenue for advancing the diagnosis of human disease.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Cas9 in close-up

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Structural and biochemical studies elucidate DNA targeting by the Cas9 endonuclease.

In the past year, a prokaryotic adaptive immune response—the clustered, regularly interspaced, short palindromic repeat (CRISPR) system—has been harnessed to effect genome targeting and engineering in many cell types and organisms[1–3]. These studies involved RNA-guided targeting of an endonuclease, Cas9, to the desired genomic site. Ensuring the specificity of the Cas9-DNA interaction is critical to many future applications of the CRISPR system, particularly clinical applications, but improving the specificity of the native endonuclease will require a deeper structural and functional understanding of its activity. Three recent papers[4–6] in *Cell*, *Science* and *Nature* now provide an important advance in this regard through high-resolution crystal structures of Cas9, with or without bound RNA or DNA, and biochemical analyses.

In many bacteria and almost all Archaea, the CRISPR gene-silencing pathway[7] incorporates short DNA sequences derived from a foreign source, such as phage, into the genome. Processing of primary transcripts containing these sequences produces effector CRISPR RNAs (crRNAs), which form endonuclease-RNA complexes that cleave the foreign nucleic acid, thus conferring resistance to the invader. Type II CRISPR systems use a single, large endonuclease, Cas9, which generates double-strand breaks in target DNA. In eukaryotic cells, double-strand DNA breaks are repaired by nonhomologous end joining or homologous recombination; these pathways can be exploited to achieve gene knockout or gene editing.

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