Methods for measuring myeloperoxidase activity toward assessing inhibitor efficacy in living systems

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ABSTRACT
Myeloperoxidase aids in clearance of microbes by generation of peroxidase-mediated oxidants that kill leukocyte-engulfed pathogens. In this review, we will examine 1) strategies for in vitro evaluation of myeloperoxidase function and its inhibition, 2) ways to monitor generation of certain oxidant species during inflammation, and 3) how these methods can be used to approximate the total polymorphonuclear neutrophil chemotaxis following insult. Several optical imaging probes are designed to target reactive oxygen and nitrogen species during polymorphonuclear neutrophil inflammatory burst following injury. Here, we review the following 1) the broad effect of myeloperoxidase on normal physiology, 2) the difference between myeloperoxidase and other peroxidases, 3) the current optical probes available for use as surrogates for direct measures of myeloperoxidase-derived oxidants, and 4) the range of preclinical options for imaging myeloperoxidase accumulation at sites of inflammation in mice. We also stress the advantages and drawbacks of each of these methods, the pharmacokinetic considerations that may limit probe use to strictly cell cultures for some reactive oxygen and nitrogen species, rather than in vivo utility as indicators of myeloperoxidase function. Taken together, our review should shed light on the fundamental rational behind these techniques for measuring myeloperoxidase activity and polymorphonuclear neutrophil response after injury toward developing safe myeloperoxidase inhibitors as potential therapy for chronic obstructive pulmonary disease and rheumatoid arthritis. J. Leukoc. Biol. 99: 541–548; 2016.

PHYSIOLOGIC IMPORTANCE OF MPO FUNCTION
MPO, a heme-containing peroxidase in myeloid cells (PMNs and monocytes), has a central role in the development of the nascent inflammatory response and the perpetuation of chronic inflammation in diseases such as RA. From the initial trigger (i.e., damage or infection), patrolling monocytes [1], mast cells [2], and dendritic cells [3] respond to the acute injury almost immediately by increasing vascular permeability through histamine release and simultaneous establishment of chemokine and chemotactant gradients required to arrest circulating leukocytes (see Fig. 1). A hallmark PMN burst occurs when responding leukocytes crawl to a slow roll through interaction of leukocyte-derived L-selectin and endothelial E-selectin and P-selectin [4]. In the mouse, PMN and other leukocytes essentially stop as the burden of the drag exerted on the cell overcomes blood flow pressures through, in part, binding of LFA-1 and Mac1 (CD11b) with newly exposed ICAM on the activated EC surface (ICAM-1). This progress occurs near the damage and just before diapedesis [5]. Once at the inflammatory site, PMN, and later PMN-signal monocytes, begin the task of clearing foreign material (i.e., microbes) or cell debris [4]. To combat pathogens in the area, PMNs ensnare microbes through exocytosis of chromosomal material called neutrophil extracellular traps enabling phagocytosis of ensnared pathogens by neighboring PMNs [6, 7]. At the same time, there is probably an increased extracellular concentration of MPO in the local microenvironment as a direct result of MPO contributed by apoptotic PMNs [8]. PMNs also experience an influx of K+ [9] and an increased pH [10], which awakens dormant proteases [11]. Given this, the engulfed pathogens ultimately face harsh pH conditions in the

Abbreviations: 5-HT = 5-hydroxytryptamide, ADHP = 10-acetyl-3,7-dihydroxyphenoxazine, APF = 2-[6-(4′-aminophenoxy)-3H-xanthen-3-onyl]benzoic acid, BODIPY = boron-dipyrromethene, CPH = 1-hydroxy-3-carboxy-2,2,5,5-tetramethyl-pyrrolidine hydrochloride, DHR123 = dihydrorhodamine 123, DMPO = 5,5-dimethyl-1-pyrroline N-oxide, EC = endothelial cell, HOCl = hypochlorous acid, ICAM = intercellular adhesion molecule, LFA-1 = lymphocyte function-associated antigen-1, MPO = myeloperoxidase, PMNs = polymorphonuclear neutrophils, RA = rheumatoid arthritis.

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MPO levels in chronic obstructive pulmonary disease [15], RA [16], production during oxidative stress has been linked to heightened DNA and protein adducts [14]. For example, ROS and RNS

the powerful HOCl oxidant generated by the prolific MPO-H2O2 system [13].

In humans, MPO has become a biomarker for heightened inflammation because of its role in the production of oxidized DNA and protein adducts [14]. For example, ROS and RNS production during oxidative stress has been linked to heightened MPO levels in chronic obstructive pulmonary disease [15], RA [16], atherosclerosis [17–21], and even some cancers [22, 23]. Therefore, MPO is considered a druggable target with small-molecule inhibitors already identified and more expected for limiting ROS/RNS production. In preclinical study, several MPO inhibitors have limited inflammation in diseases, including thioxanthine-2 in chronic obstructive pulmonary disease studies [15], PF-1355 in vasculitis and glomerulonephritis [24], and INV315 in atherosclerosis [25]. In this review, we will mainly discuss the recent, emerging tools for in vivo assessment of MPO as a target with the goal of monitoring disease progression, tracking PMN migration, and detecting inflammatory sites for preclinical or clinical applications.

WHAT SETS MPO APART FROM OTHER PEROXIDASES?

It is generally accepted that there are 2 distinct peroxidase superfamilies. One superfamily comprises all plant, fungal, and bacterial peroxidases, and the other comprises the mammalian peroxidases [26]. One primary difference between mammalian peroxidases (e.g., MPO, LPO, EPO, and thyroid peroxidase) and nonmammalian peroxidases (e.g., horseradish peroxidase, bacterial catalase-peroxidase, lignin peroxidase, and ascorbate peroxidase) is the presence of specific linkages between the respective heme prosthetic group and the main peroxidase protein scaffold [26]. There are generally 2 ester bond linkages, but in the case of MPO, the presence of an additional unique sulfonium linkage distorts the heme from its normal planar state and allows MPO to produce HOCl.

Generally, all peroxidases have an active-site heme group that contains a central Fe atom that maintains its oxidation state (i.e., reactivity) through coordination with a distal His residue.

MPO has a ferric heme, MPO-Fe(III), which is oxidized to a short-lived intermediate, termed Compound I (half life ~100 ms [27]), by reacting with a 100–10,000-fold lower relative concentration of H2O2, leading to generation of a ferryl porphyrin π cation radical, MPO-Fe(IV) = O" + + (Equation 1) [28, 29]. MPO can regenerate its ferric state in 2 consecutive 1-electron steps with a classic peroxide electron donor (AH2) within a typical peroxidase catalytic cycle (Equations 2–3). The porphyrin radical is reduced to a ferryl heme, known as Compound II, in the first step (Equation 2). Compound II then is reduced back to ferric enzyme A by AH2 in the following step (Equation 3). At the same time, AH2 is oxidized to the free radical (AH*) [30, 31].

\[
\begin{align*}
\text{MPO} - \text{Fe(III)} + \text{H}_2\text{O}_2 & \rightarrow \text{MPO} - \text{Fe(IV)} = \text{O}^+ + \text{H}_2\text{O} \\
\text{Compound I} & \\
\text{Compound I} + \text{AH}_2 & \rightarrow \text{MPO} - \text{Fe(IV)} = \text{O} + \text{AH}^+ \\
\text{Compound II} & \\
\text{Compound II} + \text{AH}_2 & \rightarrow \text{MPO} - \text{Fe(III)} + \text{AH}^+ + \text{H}_2\text{O} \\
\text{Compound II} & \\
\text{Compound I} + \text{Cl}^- & \rightarrow \text{MPO} - \text{Fe(III)} + \text{HOCI} \\
\text{Compound I} &
\end{align*}
\]

In the chlorination cycle of MPO, Cl- ions are uniquely oxidized to HOCI by Compound I, and in the process, the MPO ferric state is regenerated (Equation 4).

EPO = eosinophil peroxidase, EPR = electron paramagnetic resonance, Fe = iron, Gd = gadolinium, H2DCFDA = 2,7'-dichlorodihydrofluorescein diacetate, H2O2 = hydrogen peroxide, HC = heavy chain of myeloperoxidase, HoBr = hypobromous acid, HoCl = hypochlorous acid, HPF = 2-(6-(4-hydroxy)phenoxy-3H-xanthen-3-on-9-yl)benzoic acid, LPO = lactoperoxidase, MPO = myeloperoxidase, NO = nitric oxide radical, O2 = superoxide radical, ONOO- = peroxynitrite, PDB = Protein Data Bank, PMN = polymorphonuclear neutrophil, RA = rheumatoid arthritis, ROS = reactive oxygen species, RNS = reactive nitrogen species.
Several MPO crystal structures have been solved that demonstrate complex formations between the MPO enzyme and a variety of ligands and inhibitors, which has provided a wealth of insight into MPO catalysis and inhibitor binding. Substrates gain access to the proximal surface of the cavern, wherein the heme prosthetic group acts as the floor of the MPO active site. A narrower substrate-binding chase leads from the enzyme and can be seen in the MPO isoform C structure (see PDB accession number 1LXP), MPO cocrystallized with cyanide and thioxanthine (1DNW), the complex of MPO with cyanide (1DNU), the thioxanthine-inhibited MPO (3ZSI), the ceruloplasmin-bound MPO (4EJX), and the MPO with aromatic hydroxamates (4C1M) [32–38]. As mentioned, the protoporphyrin IX macrocycle of MPO has 3 covalent bonds with the parent enzyme as shown in Fig. 2, and the planar distortion of the heme leads to the distant spectral signature of MPO [26]. These linkages correspond to the following: 1) an ester bond between the light chain of the myeloperoxidase Asp24 residue of the MPO light chain and the heavy chain (highlighted as yellow). The catalytically essential residues Glu140, His242, and Arg239 (highlighted as red) compose the distal heme pocket; in the proximal site, the heme iron is coordinated through the His246 imidazole residue (highlighted as turquoise).

To study MPO in any complex mixture, there must be an effective means to differentiate between MPO-dependent ROS/RNS products and other reactive products. As such, a number of sensors have been developed to meet this need, such as ‘OH, singlet oxygen, O$_2^-$, ONOO$^-$, and $\cdot$OCl. A summary of these sensors is shown in Table 1. This section will focus on introducing most of these sensors, attributing unique properties that drive their utility, and imparting their relative limitations.

Generally, the cellular redox homeostasis is maintained by a series of checks and balances between the production of ROS/RNS and the antioxidant system, wherein superoxide dismutase produces H$_2$O$_2$ from O$_2^-$, and this is counteracted by the activity of catalase that degrades H$_2$O$_2$ into H$_2$O. Other antioxidants act as general scavengers, such as NADPH, glutathione, thioredoxin, and peroxiredoxin [44], which further modulate the levels of certain oxidants. Up-regulated NADPH oxidases increase the relative O$_2^-$ levels, contributing to certain pathologies, such as cardiac hypertrophy, fibrosis, ischemic stroke, and neurodegenerative diseases [45]. Central to this and important to the discussion here is that MPO functions to generate the potent oxidants HOCl from Cl$^-$ and H$_2$O$_2$, while generating other ROSs (e.g., ‘OH, singlet oxygen, and ONOO$^-$) [46].

Given the relative importance of the redox state of the catalytic Fe in MPO, the use of EPR or spin resonance spectroscopy has become a sensitive method for in vitro assessment of the inhibitor- or substrate-altered redox state [47]. Some probes for this application include DMPO for trapping O$_2^-$ [48], DEPMPO for MPO and MPO-derived oxidants [49], 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine for the spin trapping of ONOO$^-$ and O$_2^-$ [48], CPH for stable trapping O$_2^-$ [47], 1-hydroxy-4(2-triphenylphosphonio)-acetamidio-2,2,6,6-tetramethylpiperidine for detection of mitochondrial O$_2^-$ [50], and 3,5-dibromo-4-nitrosobenzensulfonic acid for trapping NO$^\cdot$, ONOO$^-$, and O$_2^-$ in biologic systems [51]. The distinct advantage of the CPH probe is that it generates a more-stable NO$^\cdot$ with a longer half-life, which provides for a better detection window, whereas the use of nitrore spin traps, such as DMPO and DEPMPO, produce the more-unstable intermediate O$_2^-$ [47]. In addition, CPH probes are membrane permeable and can provide insights into the generation of ROS across both extracellular and intracellular membranes, allowing cytostal concentrations to be compared with those in the mitochondria [52].

There are also cell-permeable, fluorogenic ROS/RNS sensors, such as H$_2$DCFDA and DHR123, which are oxidized by ROO$^\cdot$, ONOO$^-$, and $\cdot$OCl [53]. H$_2$DCFDA is converted into 2',7'-dichlorofluorescein by intracellular esterases within the cell [54]. Apart from the relative nonselectivity to ROS species and oxidants, both cytochrome c and heme peroxidases catalyze the oxidation of 2',7'-dichlorofluorescein and DHR123 [55]. NO$^-$ is able to oxidize another fluorescein-based probe 4-amino-5-methylamino-2',7'-dihydrofluorescein to the fluorescent dye benzotriazole via oxidative cyclooxygenation reactions [56, 57]. The hydroethidium-based probe mito-SOX can detect O$_2^-$ in the mitochondria, whereas dihydroethidium can detect O$_2^-$ generally within cells [58]. These
probes are not directly used for the detection of MPO-derived products but can be used to confirm the oxidant generated or present in a complex mixture.

Other ROS, cell-permeable, fluorogenic probes include APF and HPF, which undergo O-dearylization oxidation to form highly fluorescent products in the presence of ‘OH, ONOO\(^-\), and \(\mathrm{O}_2^-\) [57, 59]. HPF can selectively detect the presence of ‘OH and ONOO\(^-\), whereas \(\mathrm{O}_2^-\) can also oxidize APF. MPO-derived HOCl and EPO-derived HOBr were specifically detected in PMNs and eosinophils by the combination of APF and HPF, respectively.

### TABLE 1. A summary of the sensors for detection of MPO-derived oxidants and other ROSs/RNSs

<table>
<thead>
<tr>
<th>Modality/ Agent</th>
<th>MW (Da)</th>
<th>Excitation (\lambda) (nm)</th>
<th>Emission (\lambda) (nm)</th>
<th>Target</th>
<th>Cell culture (in vitro)</th>
<th>Preclinical (in vivo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMPO</td>
<td>113.2</td>
<td>—</td>
<td>—</td>
<td>‘OH, (\mathrm{O}_2^-)</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>DEPMPO</td>
<td>235.2</td>
<td>—</td>
<td>—</td>
<td>‘OH, (\mathrm{O}_2^-)</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>CMH</td>
<td>201.3</td>
<td>—</td>
<td>—</td>
<td>ONOO(^-), (\mathrm{O}_2^-)</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>CPH</td>
<td>223.7</td>
<td>—</td>
<td>—</td>
<td>ONOO(^-), (\mathrm{O}_2^-)</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>mito-TEMPO-H</td>
<td>511.1</td>
<td>—</td>
<td>—</td>
<td>(\mathrm{O}_2^-)</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>DBNBS</td>
<td>367.0</td>
<td>—</td>
<td>—</td>
<td>NO(^-), ONOO(^-), (\mathrm{O}_2^-), MPO</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>Fluorescence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APF</td>
<td>423.4</td>
<td>490</td>
<td>515</td>
<td>‘OH, ONOO(^-), and HOCl/Br</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>HPF</td>
<td>424.4</td>
<td>490</td>
<td>515</td>
<td>‘OH, ONOO(^-)</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>DAF-FM</td>
<td>412.4</td>
<td>495</td>
<td>515</td>
<td>NO(^-)</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>H2 DCFDA</td>
<td>487.3</td>
<td>495</td>
<td>527</td>
<td>‘OH, ROO, ONOO(^-), HOCl</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>HKGreen-3</td>
<td>613.1</td>
<td>520</td>
<td>535</td>
<td>ONOO(^-)</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>mito-SOX</td>
<td>759.7</td>
<td>510</td>
<td>580</td>
<td>(\mathrm{O}_2^-), heme peroxidases</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>DHR123</td>
<td>364.6</td>
<td>500</td>
<td>536</td>
<td>ONOO(^-), (\mathrm{NO}_2^-), HOCl</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>HKOCl-I</td>
<td>512.2</td>
<td>520</td>
<td>541</td>
<td>HOCl</td>
<td>×</td>
<td>✓</td>
</tr>
<tr>
<td>DHE</td>
<td>315.4</td>
<td>518</td>
<td>605</td>
<td>(\mathrm{O}_2^-), heme peroxidases</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>ADHP</td>
<td>257.2</td>
<td>571</td>
<td>585</td>
<td>Heme peroxidases</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>SNAPF</td>
<td>640.0</td>
<td>614</td>
<td>676</td>
<td>HOCl</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Quenched nanoparticle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxazine nanoparticle</td>
<td>423.3 (oxazine)</td>
<td>620</td>
<td>672</td>
<td>ONOO(^-), HOCl</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>LS601R-PEG40</td>
<td>41,000</td>
<td>785</td>
<td>810</td>
<td>‘OH, (\mathrm{O}_2^-)</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Lucigenin</td>
<td>510.5</td>
<td>—</td>
<td>470</td>
<td>(\mathrm{O}_2^-) and (\mathrm{H}_2\mathrm{O}_2)</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>BLI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminol</td>
<td>177.2</td>
<td>—</td>
<td>425</td>
<td>‘OH, ONOO(^-), (\mathrm{O}_2^-), HOCl and HOBr</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>L-012</td>
<td>288.7</td>
<td>—</td>
<td>425</td>
<td>‘OH, ONOO(^-), HOCl and HOBr</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>MCLA</td>
<td>291.7</td>
<td>—</td>
<td>465</td>
<td>(\mathrm{O}_2^-), MPO-derived oxidants, ONOO(^-)</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Pholasin</td>
<td>34,600</td>
<td>—</td>
<td>Blue light</td>
<td>‘OH, ONOO(^-), (\mathrm{O}_2^-) and MPO</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>CRET</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Luminol-QD800</td>
<td>N/A</td>
<td>—</td>
<td>800</td>
<td>MPO-derived HOCl</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>MCLA- BP-AF594</td>
<td>N/A</td>
<td>—</td>
<td>620</td>
<td>(\mathrm{O}_2^-), and MPO-derived oxidants</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>FRET</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HyPer sensor</td>
<td>52,000</td>
<td>420/500</td>
<td>535</td>
<td>(\mathrm{H}_2\mathrm{O}_2)</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Photo-acoustic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIR light-absorbing RSPN1</td>
<td>553.7 (IR775S)</td>
<td>680</td>
<td>838</td>
<td>ONOO(^-), HOCl</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>MRI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gd(<em>{2})5-HT-DTPA /Gd(</em>{2})5-HT-DOTA</td>
<td>707.2, 788.7</td>
<td>—</td>
<td>—</td>
<td>MPO</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Abbreviations: BLI, bioluminescence imaging; CMH, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine; CRET, chemiluminescence resonance energy transfer; DAF-FM, 4-amino-5-methylamino-2',7'-dihalofluorescein; DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid; DHE, dihydroethidium; DTPA, diethylentriaminepentaacetic acid; SPN, semiconducting polymer nanoparticle; mito-TEMPO-H, 1-hydroxy-4-{2-triphenylphosphonio)acetamido}-2,2,6,6-tetramethylpiperidine; N/A, not applicable.
[59, 60]. Other oxidation-mediated probes include HKGreen-3, which detects ONOO⁻ in vitro or in RAW 264.7 macrophages, generating fluorescent Nmethylrhodol by an N-dearylation reaction [61]. Similarly, a BODIPY-based fluorescent probe, HKOCl-1, detects HOCl from the formation of benzoquinone from p-methoxyphenol [61, 62]. A red-shifted fluorogenic sensor of peroxidase activity is ADHP, also known as Amplex Red, which has a strict H₂O₂ dependency, allowing H₂O₂ to be used as a trigger for inhibitor studies, whereby the enzyme and the compound can be allowed to reach equilibrium before initiation of the chemical reaction (Fig. 3A). Previously, we used stopped flow spectroscopy and the ADHP substrate to study MPO inhibition by benzoic acid hydrazide and its analogs [41, 42]. ADHP has also been used in tissue-based assays for determining the presence of MPO activity, as a surrogate for assessing inflammation [63]. There is evidence that ADHP can undergo autoraual formation under prolonged exposure to excitation energy [58]. The BODIPY-based fluorescent probe HKOCl-1, specific for the detection of MPO-derived HOCl, has never, to our knowledge, been used apart from the original reported study. Significant effort exists toward adapting these probes for use in intravital PMN recruitment and transmigration studies [64–66].

### STRATEGIES FOR IMAGING MPO ACTIVITY IN PRECLINICAL MOUSE MODELS

Biodistribution studies indicate that there are static spatial and temporal differences in expression of mammalian peroxidases throughout host cells and tissues. Mobile immune cells are also an excellent source of stored peroxidases because these enzymes are sequestered in granules, as is the case for MPO and EPO [67]. In response to stimuli, PMNs flood injured tissue with MPO to catalyze the production of the disinfectant HOCl. Quantification of MPO levels can indicate overall inflammatory burden. Chronic inflammation leads to prolonged signal production and constant cellular recruitment, and, as such, both plasma and synovial fluids taken from patients with RA have increased MPO levels compared with healthy controls [16]. In addition, genetic production of MPO deficient-mice attenuated RA severity in mouse models without altering circulating cytokine levels [68], indicating that MPO is a critical mediator of joint inflammation and damage in experimental RA. Cytokine levels are not altered in these mice, which may show the lack of MPO-dependent feedback signaling, but that is conjecture at this stage.

Although use of the aforementioned ROS/RNS probes seems compelling, their hydrodynamic and pharmacokinetic properties limit their utility in animal studies. Their narrow imaging window is driven by their enhanced washout kinetics and reduced circulation half-life. For example, solid tumors and regions of inflammation are known to be hyperpermeable to microparticles and nanoparticles due to enhanced permeability and retention properties [69]. Currently, there are already several in vivo imaging methods available, such as MRI of MPO-induced oligomerization of chelated Gd containing serotonin analogs [70], fluorescence imaging of MPO-induced release of oxazine probes from a nanoparticle scaffold [71], and MPO-induced chemiluminescence using luminol (Sigma-Aldrich, St. Louis, MO, USA) [72, 73]. Previously, bioluminescence imaging of MPO activity was successfully conducted in spontaneous, large, granular lymphocytic tumors in Gzmb mice with intraperitoneal administration of luminol [72]. On the surface, these results are not surprising because PMNs infiltrated these tumors. This “pseudo” in vivo Western blot is an interesting tool for studies of MPO activity but is limited to superficial sites because of photon scattering and the absorptive properties of blood and tissue [74]. This methodology is employed in Fig. 4. In this example, intraperitoneal injection of

**Figure 3.** Fluorescence reaction for measuring MPO activity. (A) General mechanism of ADHP oxidation in a proposed 2-step reaction, whereby the MPO–H₂O₂ complex generates 2 ADHP radicals that undergo a subsequent enzyme-independent dismutation reaction to complete formation of 1 resorufin and 1 ADHP molecule. (B) Classic biochemical assays are possible using absorbance changes caused by the MPO–H₂O₂ system and the use of new fluorogenic probes, such as ADHP. Pictures are cuvettes containing increasing concentrations of the MPO–H₂O₂ system, as indicated, at a static ADHP level (40 μM). (C) Stopped-flow progress curves of resorufin generation by MPO (25 nM) initiated by the addition of H₂O₂ (22 μM) for a series of given ADHP concentrations (adapted from Huang et al. [41]).
luminol was used to detect MPO activity in inflammation sites associated with an acute cutaneous infection (Fig. 4B). This method has some limitations in that luminol has an extremely short half-life (20 min) in mice [72] and is 90% cleared by the kidney and excreted through the urine [75]. Another limitation is that this imaging method can only be used as a paninflammation-type sensor because it does not discriminate between sterile inflammation and active infection [76]. This process is highly H$_2$O$_2$-dependent, and without an optimal H$_2$O$_2$ concentration available to MPO, the light production is severely affected (Fig. 4C).

To overcome the depth limitation of the luminol-based and normal fluorescent reflectance methods, hybrid techniques were developed that combines a near-infrared–emitting quantum dot, tethered to a light-production engine, by way of the MPO-mediated oxidation of the luminol payload [73]. This probe, in an inflammatory environment, allows for bioluminescence-resonance energy transfer to occur between the oxidized luminol and the probe. Another option available for in vivo probe sensors is to push to longer wavelengths to abrogate tissue interference, which was the case for the APF-derived, near-infrared dye sulfonaphthoamino-phenyl fluorescein, used to assess HOCl production from human whole blood and advanced atherosclerotic plaques [77]. Previously, we were also able to modulate the poor pharmacokinetics of these small-molecule ROS/RNS probes by attaching an activatable, nearinfrared oxazine probe to the surface of an iron-oxide nanoparticle (increasing the half-life to ~9 h) [71]. In this case, the probe was oxidized and released from the particle only in the presence of HOCI or peroxynitrite [71]. Another report of similar conceptual design is the quenched nanoparticle LS601R-PEG40, a nonfluorescent hydrocyanine probe conjugated with PEG40, which can be oxidized to fluorescent LS601 in the presence of various ROSs [78]. Shifts are also being seen in the in vivo methods used to collect this ROS/RNS information, namely, the advent of the photoacoustic or optoacoustic imaging modalities. One such probe used in this way was IR775S, a cyanine dye derivative sensitive to ONOO⁻ and HOCl oxidation and used in a variety of inflammatory states [79].

Another option used in the prclinical setting is MRI, which allows for the capture of noninvasive, anatomic information, while gradient modulation can lead to differential relaxation profiles and probe visualization. An example of this is the MPO-dependent polymerization of 5-HT chelated to Gd, namely Gd-bis-5-HT-dithylenetriaminepentaacetic acid [70, 80]. As a polymer, the sensor has an enhanced magnetic resonance signal, which is prolonged up to 120 mins with the MPO-Gd probe in matrigel experiments, mice brains, and the infarct zone in mice [81–85]. This probe has been used extensively for in vivo inflammatory models that have been established in such a way as to prove the MPO-dependent nature of the reporter using MPO-deficient mice [81–83].

**WHAT DOES THE FUTURE HOLD FOR MONITORING MPO ACTIVITY AND PMN RECRUITMENT NONINVASIVELY?**

New, emerging technology will allow for the development of better optical ROS/RNS sensors that take advantage of the spectrum of near-infrared wavelengths to limit potential interference and scatter from blood and tissue during in vivo animal imaging studies. To complement this, there are ongoing attempts to develop the next generation of probes for in vivo imaging of MPO, which, hopefully, will have better pharmacodynamic properties for in vivo work to improve the detectability of MPO by increasing the signal-to-noise ratio. Besides new probe chemistry, improved imaging hardware has already begun to provide better resolution for researchers using optical imaging, especially with the advent of photoacoustic/optoacoustic techniques [86–88]. Distinct from fluorescence, strong absorbing dyes are desired because the thermal expansion of the probe causes a resultant and proportional ultrasound signal [89, 90]. The benefit of these technologies over the standard reflectance-fluorescence imaging is that they reduce the signal scattering as the light passes through tissue because sound waves are used instead of light for the later portion of the imaging strategy [91].

**CONCLUDING REMARKS**

Imaging MPO activity and assessing the imaging of this important peroxidase remains an active area of research because of its central role in inflammation. In designing new MPO inhibitors, the physiologic role of the enzyme, the elements that lead to HOCl generation, the gamut and specificity of secondary ROS/RNS reporter probes, and the lead compounds that ultimately cause toxicity in vivo must be understood. Often the best inhibitor for MPO is not the safest (e.g., sodium azide is a potent MPO inhibitor but toxic when applied to an animal). Taken together, a more-holistic approach can be implemented, which would provide a streamlined approach for screening positive traits to accelerate new anti-inflammatory drug designs.
AUTHORSHIP


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DISCLOSURES

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