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Review Article

Redox-sensitive probes for the measurement of redox chemistries within phagosomes of macrophages and dendritic cells

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ABSTRACT

There is currently much interest in factors that affect redox chemistries within phagosomes of macrophages and dendritic cells. In addition to the antimicrobial role of reactive oxygen species generation within phagosomes, accumulating evidence suggests that phagosomal redox chemistries influence other phagosomal functions such as macromolecular degradation and antigen processing. Whilst the redox chemistries within many sub-cellular compartments are being heavily scrutinized with the increasing use of fluorescent probe technologies, there is a paucity of tools to assess redox conditions within phagosomes. Hence the systems that control redox homeostasis in these unique environments remain poorly defined. This review highlights current redox-sensitive probes that can measure oxidative or reductive activity in phagosomes and discusses their suitability and limitations of use. Probes that are easily targeted to the phagosome by using established approaches are emphasized.

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Introduction: redox systems in phagosomes

Phagosomes are vacuolar organelles resulting from the engulfment of extracellular material by specialized immune cells called phagocytes, such as macrophages and dendritic cells. The resultant organelle goes through a series of fusion and fission events with endosomes and subsequently lysosomes to produce a hybrid organelle – the phagolysosome. Throughout the phagosome's maturation, it accumulates antimicrobial and degradative components that serve to kill and digest phagocytosed material such as microbes [1]. The production of reactive oxygen species (ROS) within phagosomes by NADPH oxidase (NOX2) has been shown to be a major contributor to the antimicrobial function of this organelle, and has been studied extensively [2]. This contribution is highlighted in patients with chronic granulomatous disease in which the absence of functional NOX2 leads to recurrent infections [3]. Whilst much of the field has focused on the oxidative characteristics of the phagosome, the reductive capacity of the phagosome has received less attention. Nonetheless, increasing evidence indicates that reductive chemistries within the phagolysosome play

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critical roles in phagosomal function and homeostasis [4,5]. For instance, enzymes capable of reducing disulfide bonds are required for the efficient processing of disulfide-bond-containing protein antigens within the acidic phagosomal lumen [6]. Furthermore, oxidation-sensitive proteases, such as the lysosomal cysteine cathepsins, require a reducing environment for activity [7,8]. Rybicka et al. have recently shown that ROS production by NOX2 negatively regulates both disulfide reducing mechanisms and thiol-dependent proteases in the phagosome [4,9]. As factors that affect antigen processing and subsequent presentation to the adaptive immune system have major implications in a variety of pathological conditions, phagosomal redox chemistries may be of great significance to health and disease. Surprisingly, very little is known about the mechanisms that control and maintain the redox microenvironment within these compartments. A major impediment to this line of research has been the lack of convenient technologies that can accurately measure redox chemistries within the phagosome. In this review current techniques and probes that are used to measure oxidative and reductive events in the phagosomes of macrophages and dendritic cells are highlighted.

Approaches: measurement of phagosome-specific redox chemistries

In order to accurately assess redox events in the phagosome, ideal probes must be highly sensitive and compartment-specific, and should allow for both temporal and spatial resolution. In addition they must be specific to the type of redox event being measured, and resistant to interference by other nearby chemistries which are present in the phagosome (such as pH and proteolysis). In the case of oxidative processes, the probes used should ideally be specific for particular ROS. The detection of reductive processes should be similarly specific, as it must be able to distinguish between small molecule activity (*e.g.* cysteine, glutathione) and enzymatic processes. This review focuses on fluorescence-based probes due to their sensitivity and relative ease of detection with conventional laboratory instrumentation. As will be discussed in this review, many reagents can measure redox changes with high sensitivity, however not all are suitable for use within the phagosomal lumen.

A common approach utilizes redox-sensitive fluorescent probes covalently- or non-covalently coupled to experimental particles, which are subsequently given to phagocytes [10–12]. Since each probe is directly conjugated to an engulfed particle, this approach allows for the reliable measurement of phagosome-specific activity. By synchronizing the phagocytosis of the particles (*e.g.* by a short pulse followed by removal of extracellular particles), phagosomal parameters can be measured throughout their maturation in a time-resolved manner. Typically, the experimental particles used are 1–3 μm in diameter and are composed of polystyrene latex or silica, to which probes are conjugated through functionalized surface chemistries (typically $-NH_2$ or –COOH). 3 μm silica particles are preferred in the Yates lab as they are dense (easy to synchronize as they quickly fall onto cellular monolayers) and generally have a higher availability of surface chemistries that can be used for conjugation. In addition to the redox-sensitive fluorophore or substrate, a second fluorophore which is insensitive to the chemistries within the phagosome is usually conjugated to the particles for calibration purposes. It is also possible to manipulate the mode of uptake by conjugating various phagocytic receptor ligands to the experimental particles [9]. Following phagocytosis, fluorescence can be monitored in a population-based format using a spectrofluorometer or fluorometric plate reader, or in a single cell-based format using confocal microscopy or flow cytometry (Fig. 1) [11].

Fluorometers are well suited for determining population-based differences in phagosomal redox activity [11]. The latest generation of fluorescence plate readers allows rapid read times for high temporal resolution, and can be equipped with a plethora of optical options/combinations to allow measurement of fluorescence lifetime and fluorescence polarization in addition to standard fluorescence intensity-based analysis. Multi-well plate readers are also easily adaptable for high-throughput analysis [4]. Whilst plate and cuvette-based fluorometers offer highly sensitive and robust readouts from populations of cells, phagosomal or cell heterogeneity is not able to be assessed. To assess these attributes, quantitative fluorescence microscopy can be employed [9,11,13]. The ability to visualize samples also reduces background signal, as well as potential artifacts such as extracellular experimental particles that can occur in fluorometry- and cytometry-based assays. Although the sample throughput, dynamic range and temporal resolution of this modality are typically modest, the ability to determine differences between phagosomes within single cells or those of different cells makes this approach popular in many studies [14]. Another approach that allows phagosomal heterogeneity between cells (but not within cells) is flow cytometry. Cytometry-based analyses can also quickly determine population-based differences in phagosomal redox activity [15,16]. However since sample preparation for these assays are laborious (often require fixing, counter-staining and numerous wash steps), temporal resolution is low and there is a potential for artifacts being introduced between the biological redox event and the time of measurement. Another major drawback of cytometry-based approaches is the low sensitivity of fluorescent detection, which makes the resolution of subtle differences between samples difficult.

A drawback to the approaches outlined above, is that since the majority of commonly available redox-sensitive probes are not specific, they cannot precisely identify the particular redox products being produced (as discussed in more detail elsewhere [17,18]). A complementary approach would be to utilize HPLC coupled with mass spectral analysis of the probes, which has been shown to identify specific redox modifications in combination with the real time assays outlined here [19,20].

Measuring phagosomal ROS production

NOX2 is the prototypical phagocyte oxidase and the first identified enzyme that creates ROS as an intentional product [21]. NOX2 consists of the cytosolic components, p67, p47, p40 and the small GTPase RAC1/2, which upon activation form a complex with the membrane bound proteins, p22 and gp91 [22]. The assembly of NOX2 on the phagosome can be induced by a variety of stimuli such as Fc receptor activation during the phagocytosis of IgG-opsonized particles [23,24]. Expression of NOX2 in professional phagocytes is induced during differentiation and can also be increased following exposure to activating stimuli such as interferon gamma [25]. The production of ROS in phagosomes begins with the generation of superoxide. Superoxide can dismutate spontaneously or through the enzyme superoxide dismutase, resulting in hydrogen peroxide. Further transformation of these oxidants either enzymatically or through the interaction with metals or reactive nitrogen species lead to downstream reactive species including hydroxyl radicals, hypochlorous acid (in neutrophils) and peroxynitrite [21,26].

Chemiluminescence-based techniques were some of the first assays used to study ROS production in cells [27]. Lucigenin and luminol are commonly used reagents for ROS detection; however the lack of specificity to individual oxidants and susceptibility to interfering chemistries, as well as difficulties in determining spatial resolution, limit their use in studying phagosomal ROS