



## Method

## An assay for the rate of removal of extracellular hydrogen peroxide by cells



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## ABSTRACT

Cells have a wide range of capacities to remove extracellular hydrogen peroxide. At higher concentrations of extracellular  $\text{H}_2\text{O}_2$  (micromolar) the rate of removal can be approximated by a rate equation that is first-order in the concentration of  $\text{H}_2\text{O}_2$  and cell density. Here we present a method to determine the observed rate constant for the removal of extracellular  $\text{H}_2\text{O}_2$  on a per cell basis. In the cells examined, when exposed to  $20 \mu\text{M}$   $\text{H}_2\text{O}_2$ , these rate constants ( $k_{\text{cell}}$ ) range from  $0.46 \times 10^{-12} \text{ s}^{-1} \text{ cell}^{-1} \text{ L}$  for Mia-PaCa-2 cells (human pancreatic carcinoma) to  $10.4 \times 10^{-12} \text{ s}^{-1} \text{ cell}^{-1} \text{ L}$  for U937 cells (human histiocytic lymphoma). For the relatively small red blood cell  $k_{\text{cell}} = 2.9 \times 10^{-12} \text{ s}^{-1} \text{ cell}^{-1} \text{ L}$ . These rate constants,  $k_{\text{cell}}$ , can be used to compare the capacity of cells to remove higher levels of extracellular  $\text{H}_2\text{O}_2$ , as often presented in cell culture experiments. They also provide a means to estimate the rate of removal of extracellular  $\text{H}_2\text{O}_2$ ,  $\text{rate} = -k_{\text{cell}} [\text{H}_2\text{O}_2]$  ( $\text{cells L}^{-1}$ ), and the half-life of a bolus of  $\text{H}_2\text{O}_2$ . This information is essential to optimize experimental design and interpret data from experiments that expose cells to extracellular  $\text{H}_2\text{O}_2$ .

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## Introduction

Considerable research is now focused on the basic biology associated with the cellular production of free radicals, related oxidants, and antioxidants. There is a growing consensus that these species are not just associated with various pathologies and aging, but rather are central to the biology of normal cells and tissues [1–5]. Unfortunately, much of what we know about oxidants and antioxidants in biology is observational in nature due to the high reactivity and low levels of the initial oxidative intermediates [6]. Many popular assays provide relative changes that may not be specific or have a linear response in the readout [7,8]. In addition, once formed these highly reactive species can rapidly react with multiple targets, disappearing into the cellular milieu, resulting in a vanishingly small steady-state level, far below lower-limits-of-detection of most analytical approaches.

Although many kinetic rate constants for the reactions of free radicals, related oxidants and antioxidants, as well as antioxidant enzymes are available, quantitative integration into our understanding of more complex biological systems has been challenging and slow [2,9–14]. Modeling of complex systems with the

integration of physics, chemistry, and biology will allow more thorough analyses, yielding better predictions and understanding of fundamental redox processes and consequences in biology [6,9–17]. Currently, most analyses are presented as qualitative assessments with limited predictive abilities. To establish better mathematical models of biological redox systems we need to develop new approaches to gather quantitative information on fundamental components of the redox circuits that comprise biologic systems.

The integration of free radical and oxidant/antioxidant chemistry and biology are being addressed in the burgeoning field of redox biology, more specifically in the newly developing field of Quantitative Redox Biology (QRB) [17]. To gain the next level of understanding of cellular redox processes, quantitative information on the generation and removal of superoxide and hydrogen peroxide by cells and tissues must be in hand. Here we address the kinetics of the removal of extracellular  $\text{H}_2\text{O}_2$  by intact cells. For example, even though red blood cells produce a low flux of superoxide and  $\text{H}_2\text{O}_2$  intracellularly [18,19], they also efficiently remove extracellular  $\text{H}_2\text{O}_2$  [20–22]. Removal of extracellular  $\text{H}_2\text{O}_2$  of course is not restricted to erythrocytes, many different types of cells are able to remove extracellular  $\text{H}_2\text{O}_2$  [23–32]. Many different enzyme systems are involved in this removal process, and new pathways are still being discovered. For some of the known reactions involved in the removal of  $\text{H}_2\text{O}_2$  the kinetic rate constants have been determined with in vitro experiments using purified enzymes. As a result there is a beginning understanding of their potential contributions to the maintenance of a normal steady-state level of  $\text{H}_2\text{O}_2$  as well as their roles in pathological

Abbreviations: QRB, Quantitative Redox Biology; RBC, Red blood cells

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settings. However, there is no one assay that can experimentally determine the overall rate of removal of extracellular  $\text{H}_2\text{O}_2$  by all systems combined as described here.

Many studies on the mechanisms and consequences of exposure of cells to  $\text{H}_2\text{O}_2$  use a bolus addition of  $\text{H}_2\text{O}_2$  to the culture media over the cells. However, cells can remove this extracellular  $\text{H}_2\text{O}_2$ ; thus, the concentration of  $\text{H}_2\text{O}_2$  will vary with time. The observed consequences of exposure are not only a function of the concentration of  $\text{H}_2\text{O}_2$ , but also how fast it is removed. It is reasonable to assume that the greater the “cell density” the more rapidly the added  $\text{H}_2\text{O}_2$  will be removed [23,24]. Thus, the actual cellular consequences observed may also change with cell density [28,30,33]. The possibility of significant changes in the concentration of  $\text{H}_2\text{O}_2$  with time adds another complicating factor to the interpretation of the data from these types of experiments.

To maintain a constant level of  $\text{H}_2\text{O}_2$  Antunes et al. developed a new approach, the cellular steady-state titration of  $\text{H}_2\text{O}_2$  [30,31,34]. In this approach a stable flux of  $\text{H}_2\text{O}_2$  is produced, e.g. by glucose/glucose oxidase. This production is balanced by the cellular removal of this extracellular  $\text{H}_2\text{O}_2$  to achieve a desired extracellular and intracellular steady-state level of  $\text{H}_2\text{O}_2$  over a long period of time. The cellular consequences can be quite different than experiments employing bolus additions of  $\text{H}_2\text{O}_2$  and may be much more relevant to the *in vivo* biology of cells and tissues [31].

Here we present a method to determine an observed rate constant for the removal of extracellular  $\text{H}_2\text{O}_2$  on a per cell basis. This rate constant provides a quantitative means to compare the capacity of cells to remove extracellular  $\text{H}_2\text{O}_2$ ; results can then be coupled with cell density to determine the rate of removal of  $\text{H}_2\text{O}_2$  in experiments, allowing for better design and interpretation of data. In addition this information is required to mathematically model redox processes in systems to advance the new field of Quantitative Redox Biology.

## Methods

### Cell culture and enumeration

HL-60, U937, MIA-PaCa-2, MDA-MB-231 (ATCC, Manassas, VA) and BAEC (isolated from bovine heart [35]) were maintained in RPMI 1640 media supplemented with 10% FBS and 85 U/mL penicillin and 85  $\mu\text{g}/\text{mL}$  streptomycin (Invitrogen, Grand Island, NY). MCF-7 cells were maintained in MEM with 10% FBS with the same penicillin and streptomycin levels as above. The MCF-7 p51 (GPx-4) stable overexpressing cells [36] with a selectable antibiotic resistance marker were grown in the same media supplemented with Geneticin<sup>®</sup> (G418, 350  $\mu\text{g}/\text{mL}$ ) (Research Products International Corp, Prospect, IL) and 30 nM sodium selenite. All cells were cultured at 37 °C in a humidified incubator with 5%  $\text{CO}_2$ .

Human RBC's obtained from donors with informed consent were isolated after removal of WBC's with Histopaque (Sigma-Aldrich, St. Louis, MO). The isolated RBC's were washed 2 × in PBS pH 7.4, counted and then suspended in HBSS (Invitrogen).

Cells were detached with trypsin (0.25%)–EDTA (0.038%) (Invitrogen) that had been supplemented with 0.1% disodium EDTA. Cells were then washed in media and counted with a Z2<sup>™</sup> Coulter Counter<sup>®</sup> Cell and Particle Counter (Beckman-Coulter, Miami, FL). Typically, cells were seeded with cold media (5 °C) and allowed to attach overnight (< 12 h) in 96-well tissue culture plates incubated at 37 °C with 5%  $\text{CO}_2$ . This experimental approach allowed the cells to attach overnight with little growth. Typically, the number of cells in a well the following day was within 3% of the intended cell-number. Cells that grew as

suspension cultures or cell lines that typically grow as monolayers, but were to be used in a “detached” state, were washed, suspended in HBSS, and then counted with the Z2<sup>™</sup> Coulter Counter<sup>®</sup>. Cells attached as monolayers were rinsed three times with HBSS before being used in experiments: all cells were in wells of clear 96-well microplates (Costar, Corning, NY, or Nunc, Roskilde, Denmark) covered with 50  $\mu\text{L}$  HBSS.

### pHPA extracellular hydrogen peroxide assay

#### Overview of assay

In this assay a known number of cells is introduced into the wells of a 96-well plate. Then extracellular  $\text{H}_2\text{O}_2$  is added to subsets of the wells at different defined times. The cells commence to remove this extracellular  $\text{H}_2\text{O}_2$ . The system is quenched at predetermined times and the concentration of extracellular  $\text{H}_2\text{O}_2$  remaining is determined. The information on number of cells, amount of extracellular  $\text{H}_2\text{O}_2$  remaining, total volume of media, and the different times of exposure of cells to extracellular  $\text{H}_2\text{O}_2$  allows the determination of the capacity of cells to remove extracellular  $\text{H}_2\text{O}_2$ .

Pilot studies with the various cell lines were run to establish the range of optimal experimental parameters, *i.e.* appropriate cell-number per well required and time-frame for removal of extracellular peroxide, which is cell-type and cell density dependent. These experiments established general conditions for each cell line, allowing cell-number and time of exposure to extracellular  $\text{H}_2\text{O}_2$  as variables in our determinations of the rate of removal extracellular  $\text{H}_2\text{O}_2$ . For most adherent cell lines we established the highest number of cells that can attach overnight and cover 90% of the 96-well bottom surface. Cell size influences this experimental parameter. Under conditions in which suspended cells or freshly trypsinized cells were to be used, the cells were counted and placed immediately in HBSS before the assay. The protocol below is for an experiment where the cells are exposed to 20.0  $\mu\text{M}$   $\text{H}_2\text{O}_2$  in the media with a maximum total time of exposure of 10 min. Both of these parameters can be varied as needed. The final concentration of  $\text{H}_2\text{O}_2$  in the analysis is  $\leq 10.0$   $\mu\text{M}$ .

### Solutions

#### Standard solutions of $\text{H}_2\text{O}_2$

Stock solutions of  $\text{H}_2\text{O}_2$  standards are prepared just before use. These stock solutions are added to the designated wells of the 96-well plate while the cells are being exposed to  $\text{H}_2\text{O}_2$ . Standard stock solutions are prepared at 0.0, 2.0, 4.0, 8.0, 12, 16, 20, 24, 32, and 40  $\mu\text{M}$  in HBSS using appropriately diluted 30% v/v ( $\approx 10$  M)  $\text{H}_2\text{O}_2$  (Sigma) stock in HBSS. The actual concentration of the original stock is determined using  $\epsilon_{240} = 39.4 \text{ M}^{-1} \text{ cm}^{-1}$  [37]. The wells to be used for standards are pre-loaded with 50  $\mu\text{L}$  of HBSS; then, the standards are added to wells as 50  $\mu\text{L}$  additions, resulting in  $\text{H}_2\text{O}_2$  standards of 0.0, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12, 14, 16, 18, and 20  $\mu\text{M}$ , (100  $\mu\text{L}$ ): note that at the end of the experiment, 100  $\mu\text{L}$  of the stopping solution is added producing final concentrations in the wells of 0.0, 0.50, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10.0  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . All standards are run in triplicate and corrected to the 0.0  $\mu\text{M}$  (blank) wells. These standard curves allow absolute determination of the concentrations of the remaining  $\text{H}_2\text{O}_2$  in the wells where cells have been exposed to  $\text{H}_2\text{O}_2$ .

Stopping solution: The stopping solution is made as a mixture of: 20 mL HBSS (Invitrogen); 20  $\mu\text{L}$  1 M 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES), pH 7.2–7.5; 10 mg  $\text{NaHCO}_3$  (3 mM), 5 mg *para*-hydroxyphenylacetic acid (pHPA; alias 2-(4-hydroxyphenyl)acetic acid, CAS Nr 156-38-7) (0.8 mM) (Sigma, St Louis, MO); and 2 mg HRP (horseradish peroxidase

Type 1)(Sigma): this solution was prepared fresh immediately before use.

#### Protocol

1. Standard solutions (50  $\mu\text{L}$ ) having ten different concentrations of  $\text{H}_2\text{O}_2$  (0.0–40  $\mu\text{M}$ ; 0.0, 2.0, 4.0, 8.0, 12, 16, 20, 24, 32, 40  $\mu\text{M}$ ) are placed into designated wells of a clear plastic 96-well tissue culture plate, for example wells B2–B11, C2–C11, D2–D11, for triplicate determinations. To these wells HBSS (50  $\mu\text{L}$ ) is also added, a total volume of 100  $\mu\text{L}$  in each well. Final concentrations of  $\text{H}_2\text{O}_2$  in the wells for these standards are 0.0, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 12, 16, 20  $\mu\text{M}$ . Note: to faithfully mimic the exposure of cells to extracellular  $\text{H}_2\text{O}_2$ , the freshly prepared  $\text{H}_2\text{O}_2$  standards are added to the standard wells while the cells are being exposed to  $\text{H}_2\text{O}_2$ , i.e. in parallel with step 3 below.
2. Counted cells in 50  $\mu\text{L}$  of HBSS are placed in other wells of the clear plastic 96-well tissue culture plate; for example into wells E2–E11, F2–F11, G2–G11 to provide triplicate determinations for each time point; or for different cell lines or treatment within a row.
3. Experiments are initiated by adding 50  $\mu\text{L}$  of freshly prepared 40  $\mu\text{M}$   $\text{H}_2\text{O}_2$  stock solution in HBSS to achieve 20  $\mu\text{M}$  final concentration of  $\text{H}_2\text{O}_2$  in each well with cells; 100  $\mu\text{L}$  total volume in each well; additions and progression to new wells is typically done in 1.0-min intervals over a 10-min exposure period. For example,  $\text{H}_2\text{O}_2$  stock solution is added to wells E2, F2, G2, then one minute later to wells E3, F3, G3, etc. for triplicate determinations at each time point. In some circumstances additions may be at 5-min intervals over 45 min, e.g. low number of cells or low  $\text{H}_2\text{O}_2$  removal capacity, or some other appropriate combination of concentration and time.
4. Immediately after the addition of  $\text{H}_2\text{O}_2$  to the final set of three wells (within 10 s), i.e. the end of the experiment and the last time point, 100  $\mu\text{L}$  of a stopping solution is added to each well; this addition is made to both sets of wells, i.e. wells with cells and wells with standards with the aid of a 12-channel multi pipette. The horseradish peroxidase in the stopping solution reacts with the remaining  $\text{H}_2\text{O}_2$  in the wells. The activated HRP then oxidizes pHPA resulting in formation of the fluorescent pHPA dimer, which is used as the readout for the amount of  $\text{H}_2\text{O}_2$  in the wells. Note: because the volume of solution in each well is now doubled, all concentrations of  $\text{H}_2\text{O}_2$  are now halved, here 0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0  $\mu\text{M}$  for the standards with the concentration of the remaining  $\text{H}_2\text{O}_2$  in wells with cells also halved.
5. Inhibition of catalase with 3-AT (optional): Cellular catalase activity was irreversibly inhibited using 3-amino-1,2,4-triazole (Sigma; CAS 61-82-5) before cells were examined in a peroxide-removal assay [38]. Briefly, attached or suspended cells were incubated in full media with 20 mM 3-AT for 1 h at 37  $^\circ\text{C}$ , the media was removed, and cells were washed three times with HBSS before addition of the buffer for the peroxide-removal assay: suspended cells were counted before placing them into the peroxide-removal assay buffer and assigned wells of the assay plate. 3-AT must be removed from the media before initiation of the peroxide-removal assay, as it will react with compound I of HRP, interfering with the quantitation of the remaining  $\text{H}_2\text{O}_2$ .
6. At room temperature the assay color/fluorescence is sufficiently developed and can be read in a fluorescence plate reader after 5 min. We routinely run the assay at room temperature and read the plates using a Tecan SPECTRAfluor PLUS (Tecan US, Research Triangle Park, NC) fluorescence plate reader with excitation at 345 nm while monitoring emission at 425 nm from above the

wells. Typically, the assay plate is read every 5 min over 30 min. This ensures that the chemistry of the assay for the detection of the remaining  $\text{H}_2\text{O}_2$  in the wells has come to completion. Actually, only one reading is needed; the additional readings are to ensure that there are no anomalies. If an anomaly is noted in one of the plate-readings, another can be used to arrive at a best result. Note that both the experimental wells (with cells) and the standards are “stopped” at the same time; reading of all the wells is done essentially simultaneously, i.e. as fast as the plate reader allows; this is essential so that all measurements are internally consistent. This is one of the most important aspects of this assay: All samples are treated the same and analyzed the same way, with no temporal differences in standards and cell samples.

7. For the data analysis it is important to subtract the appropriate “blank” from all other readings, both standards and cell samples. In our assay the blank is the mean (or median) value of the fluorescence from the standards with no  $\text{H}_2\text{O}_2$ . It is important to examine all the readings from the plate to ensure there are no anomalies, especially the blank wells. Note that the blank is prepared so that it matches the media over the cells during the exposure to extracellular  $\text{H}_2\text{O}_2$ .
8. First-order rate constant calculations: The rate of removal of extracellular  $\text{H}_2\text{O}_2$  by cells under the experimental conditions of this assay is approximated well by a first-order rate law,  $d[\text{H}_2\text{O}_2]/dt = -k_{\text{obs}} [\text{H}_2\text{O}_2]$ , where  $k_{\text{obs}}$  is the observed first-order rate constant as provided by the slope of:  $\ln[\text{H}_2\text{O}_2]$  vs. time (in seconds), i.e. time is the abscissa and  $\ln[\text{H}_2\text{O}_2]$  is the ordinate; because the removal of  $\text{H}_2\text{O}_2$  is typically first-order, the readout in arbitrary fluorescent units (corrected for the blank) will allow determination of  $k_{\text{obs}}$ , i.e.  $\ln(\text{fluorescence units})$  vs. time; units for  $k_{\text{obs}}$  are  $\text{s}^{-1}$ . The observed rate constant for each cell will be,  $k_{\text{cell}} = k_{\text{obs}}/(\text{cell L}^{-1})$ , where  $(\text{cell L}^{-1})$  is the number of cells in the well divided by the total volume of media in the well, in liters. This is the volume of media during the time the cells are exposed to the bolus of extracellular  $\text{H}_2\text{O}_2$ , i.e. before the addition of the stopping solution. This rate constant,  $k_{\text{cell}}$ , is not only a measure of the capacity of cells to remove extracellular  $\text{H}_2\text{O}_2$ , but it is also a tool to design and interpret data from experiments in which extracellular  $\text{H}_2\text{O}_2$  is employed, either as bolus or as a flux. The rate of removal of extracellular  $\text{H}_2\text{O}_2$  in an experiment with a known cell density will be:  $\text{rate} = -k_{\text{cell}} [\text{H}_2\text{O}_2]$  (number of cells  $\text{L}^{-1}$ ). The average contribution of one cell to this overall rate will be:  $\text{rate}_{\text{cell}} = -k_{\text{cell}} [\text{H}_2\text{O}_2]$  (1 cell  $\text{L}^{-1}$ ). Very useful for both experimental design and data interpretation is the half-life of a bolus addition of  $\text{H}_2\text{O}_2$  in an experiment with a specific cell density:  $t_{1/2} = 0.693/(k_{\text{cell}} (\text{number of cells L}^{-1}))$ .

#### Statistics

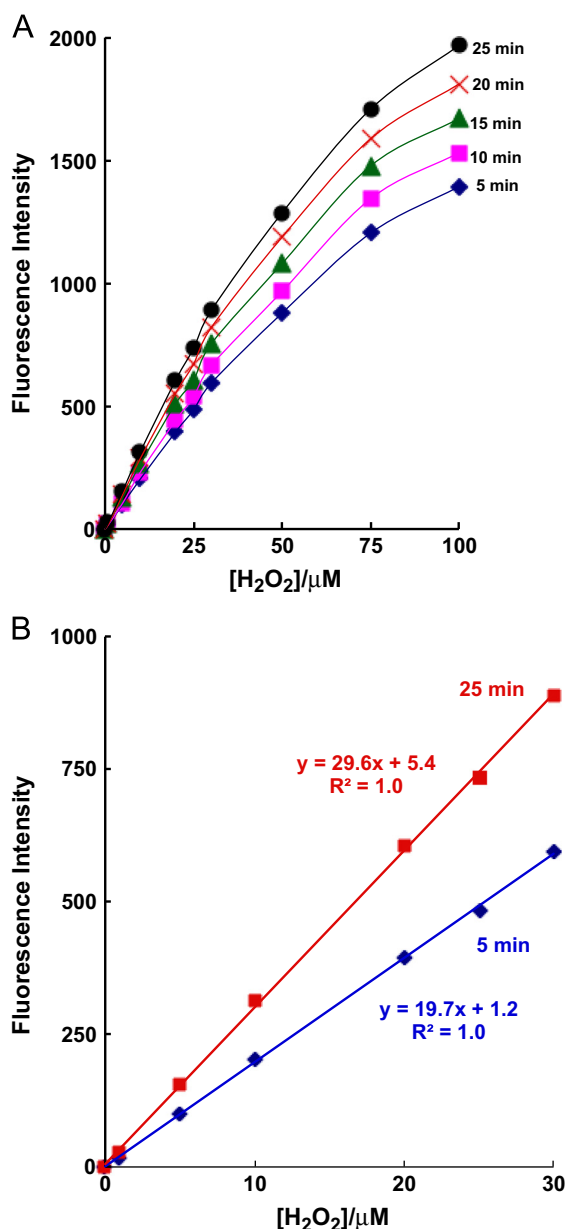
The lower limits of detection (LLD) for standard curves were estimated as described by Anderson [39].

#### Results and discussion

##### Standards for $\text{H}_2\text{O}_2$

Our goal was to develop a simple and flexible microplate reader assay for measuring  $\text{H}_2\text{O}_2$  reliably while being compatible with cells in vitro. We have adapted the flexible  $\text{H}_2\text{O}_2$  assay in which pHPA in the presence of  $\text{H}_2\text{O}_2$  and HRP is oxidized to a dimeric fluorophore  $(\text{pHPA})_2$  [40–42]. This assay is for use in measuring  $\text{H}_2\text{O}_2$  in media and not intended for measuring intracellular generation of  $\text{H}_2\text{O}_2$  or its extracellular accumulation

in media from cells. The assay can measure  $\text{H}_2\text{O}_2$  in a convenient and flexible format utilizing clear tissue culture plates and fluorescence microplate readers with monochromator/grating or commonly used filter sets (Fig. 1A). The fluorescence assay for  $\text{H}_2\text{O}_2$  based on formation of  $p\text{HPA}$  dimer is linear in the range of interest, 0–30  $\mu\text{M}$ . The assay is responsive, but nonlinear at higher levels of  $\text{H}_2\text{O}_2$ , i.e. > 30  $\mu\text{M}$  (This is the final concentration after addition of stopping solution.), Fig. 1A and B. As seen in both Fig. 1A and B the color/fluorescence assay continues to develop



**Fig. 1.** Standard curves for the HRP/ $p\text{HPA}$  assay for  $\text{H}_2\text{O}_2$  change with time and are nonlinear at higher concentrations of  $\text{H}_2\text{O}_2$ . (A) Standards for  $\text{H}_2\text{O}_2$  (5–100  $\mu\text{M}$ , final concentration in the wells) in the HRP/ $p\text{HPA}$ -based assay were read at 5, 10, 15, 20, and 25 min after addition of the stopping solution; see Methods. Assays were run in clear 96-well tissue culture plates (200  $\mu\text{L}$  total volume) and monitored for fluorescence with band-pass filters for  $\lambda_{\text{ex}}=345$  nm,  $\lambda_{\text{em}}=425$  nm; (B) The initial linear portion of the standard curves from 0 to 30  $\mu\text{M}$   $\text{H}_2\text{O}_2$  shown at 5 and 25 min. Each reading of the plate produced a linear response. This demonstrates that for this assay, standards and samples must be run in parallel to ensure the same timeframe. This is a representative assay from one experiment, plotting the average from the triplicate wells of each concentration. It is important in the analysis to subtract the “blank”, i.e. wells with no cells and no  $\text{H}_2\text{O}_2$ , but all other components the same as in the wells that contain cells.

over time and doesn't reach a true end point until at least 30 min. However, the assay is linear from 0 to 30  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$ , whether read at 5 or 25 min after the addition of the stopping solution; valid estimates of  $\text{H}_2\text{O}_2$  concentration are possible at any time between 5 and 30 min. The assay protocol demonstrated the potential for broad applicability: especially in timing with multiple samples in a 96-well plate format.

We further explored the usefulness of the assay with cultured cells by demonstrating that the assay is linear at low  $\text{H}_2\text{O}_2$  concentrations, i.e. at levels that can be reliably measured, while low enough to prevent rapid and overt cellular toxicity. Fig. 2A is a standard curve from a representative experiment with 0.5 to 10  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (final concentration in the wells), using our standard protocol. The assay shows linearity when read at either 5 or 25 min with a lower limit of detection (LLD) of 0.4  $\mu\text{M}$  at 25 min. The  $\text{H}_2\text{O}_2$  concentration range we have used is the range anticipated for most of our cell experiments. As seen in Fig. 1A, standard curves are initially linear with increasing slopes over time; Fig. 2B presents this quantitatively. This demonstrates that the assay does not reach a true end point at the temperatures and times we have employed, and that a low level, systemic oxidation occurs. This low rate of background oxidation highlights and enforces the importance that standard curves and experimental samples must be developed and analyzed simultaneously. Additionally, the assay could be modified and is applicable to kinetic-based assays for estimates of  $\text{H}_2\text{O}_2$  concentration based on rates of  $(p\text{HPA})_2$  fluorescent dimer formation.

#### *pHPA, caveats*

The upper limit of  $\text{H}_2\text{O}_2$  concentration that can be used in the 96-well plate reader assay is 30  $\mu\text{M}$  (final concentration; 60  $\mu\text{M}$  during cell-exposure), which is a limit similar to the 40  $\mu\text{M}$ , using fluorimeters, as reported by Hyslop and Sklar [41]. The loss of linear responses above 30–40  $\mu\text{M}$   $\text{H}_2\text{O}_2$  in standards curves is probably due to the absorbance of  $(p\text{HPA})_2$ , which will affect excitation intensity [41] as well as other photo-induced fluorescence reactions [43]. The lower limit of detection (LLD) can approach 0.4  $\mu\text{M}$ : this of course varies depending on the quality of the standard curve produced when running the assay.

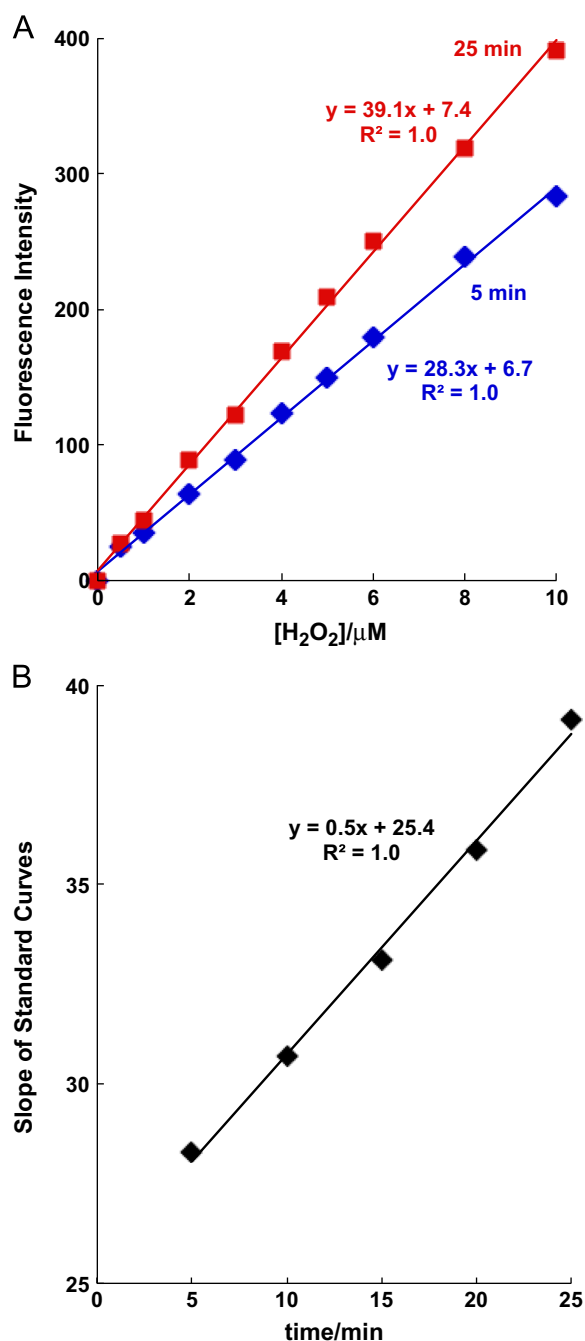
There is a background oxidation of  $p\text{HPA}$  that occurs in standard curves (Fig. 2A and B) as well as cell samples. Using the protocol presented here overcomes this issue in quantitation due to the simultaneous development and analysis of the produced  $p\text{HPA}$  fluorophore. Erroneous results will occur if this background oxidation is not accounted for in experiments where samples and standards are not assayed and run simultaneously. This low level oxidation would be misinterpreted in samples in which cells are being evaluated as the apparent generation or accumulation of  $\text{H}_2\text{O}_2$  from cells.

To simplify the analysis of an experiment the number of cells in each well of the 96-well plate should be the same. The observed kinetics for the rate of removal of extracellular  $\text{H}_2\text{O}_2$  is approximated by a first-order rate equation. Thus, the actual concentration of the  $\text{H}_2\text{O}_2$  remaining in each of the wells with cells, in principle, need not be known. The value of  $k_{\text{obs}}$  can simply be determined by a plot of  $\ln(\text{net fluorescence, in arbitrary units})$  vs. time, where  $\text{net fluorescence} = (\text{fluorescence in well}) - (\text{fluorescence of blank})$ . The standards are run because: they take very little extra time; the cost of reagents is minimal; the actual concentration in the wells is known and can be reported with confidence; but most important, the standards provide quality control as many (but not all) possible abnormalities can be identified.

#### *Removal of extracellular $\text{H}_2\text{O}_2$*

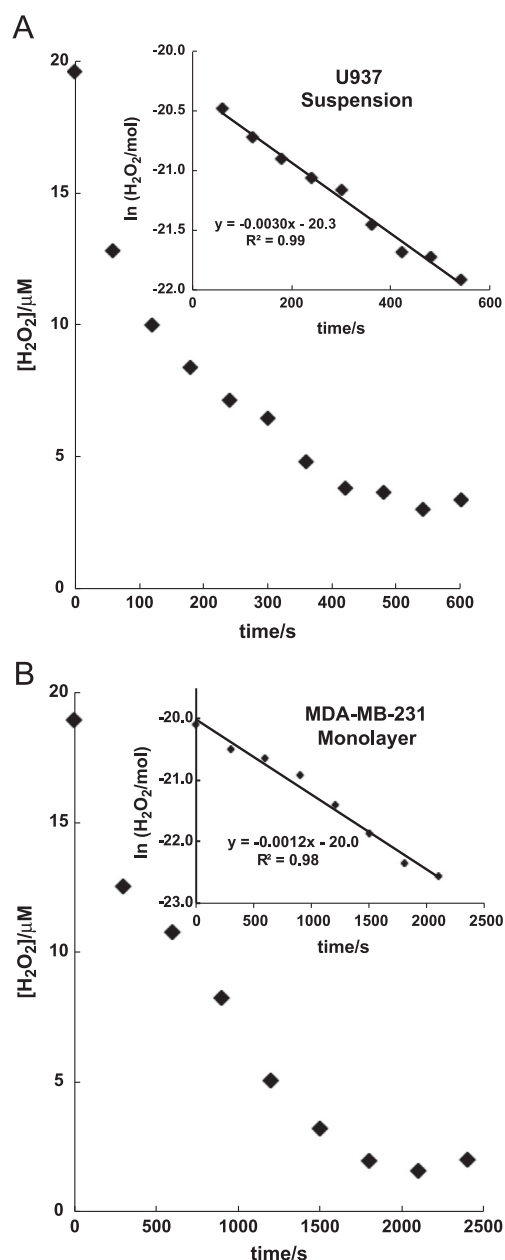
The kinetics of the removal of extracellular  $\text{H}_2\text{O}_2$  is approximated well by a simple first-order rate equation, first-order in  $[\text{H}_2\text{O}_2]$ ,





**Fig. 2.** A typical standard curve performed under the conditions of the assay for removal of extracellular  $H_2O_2$  by cells. (A) Representative standard curve from a HRP/pHPA-based assay for  $H_2O_2$  read with a fluorescence plate reader. The data are the average of triplicate samples on a plate read 5 and 25 min after addition of the stopping solution. The lower limit of detection (LLD) for the 25-min standard curve was  $0.43 \mu M$ . (B) The slope of a standard curve changes linearly with time. Here the plate with the standard samples was read at five different times (5–25 min) after addition of the stopping solution. Again, this demonstrates that standards and samples must be run in parallel with steps in the protocol being congruent as appropriate.

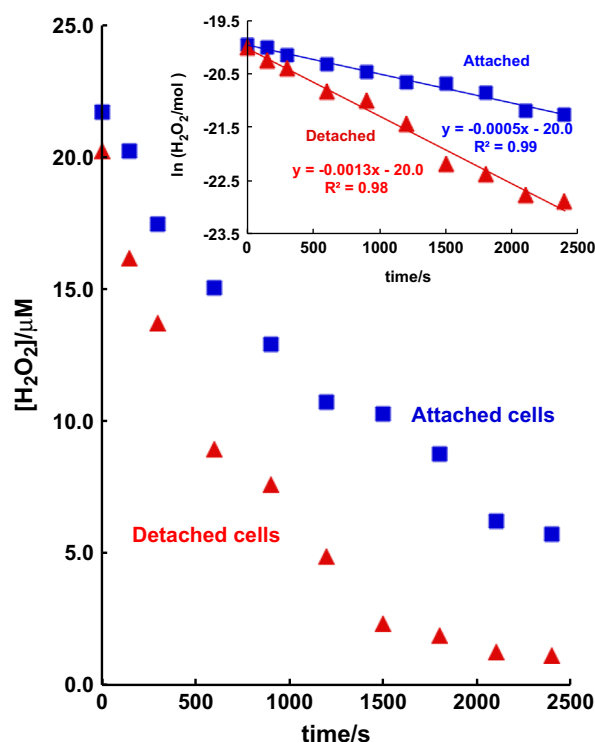
Figs. 3 and 4. However, as seen in Fig. 3, the capacity of cells to remove extracellular  $H_2O_2$  varies drastically between different cell lines; note that the observed first-order rate constant for the removal of extracellular  $H_2O_2$  by the human histiocytic lymphoma suspension cell line U937 is approximately three times larger than the attached monolayer of human mammary adenocarcinoma MDA-MB-231 cells,



**Fig. 3.** Removal of extracellular  $H_2O_2$  by cells grown in suspension culture or as adherent monolayers is a first-order process. (A) U937 cells ( $5.0 \times 10^4$  cells  $well^{-1}$ ) in HBSS were incubated with  $20 \mu M$   $H_2O_2$  for 0–10 min (100  $\mu L$  total volume) at room temperature and then assayed for the remaining extracellular  $H_2O_2$  using the pHPA assay. The observed first-order rate constant for the loss of extracellular  $H_2O_2$  in this experiment was determined to be  $k_{obs} = 3.0 \times 10^{-3} s^{-1}$ . The cell density in this experiment was  $5.0 \times 10^8$  cells  $L^{-1}$ . This yields  $k_{cell} = k_{obs}/(5.0 \times 10^8 \text{ cell } L^{-1}) = 6.0 \times 10^{-12} s^{-1} \text{ cell}^{-1} L$ . (B) MDA-MB-231 human breast cancer cells ( $2.1 \times 10^5$  cells  $well^{-1}$ ) were allowed to attach overnight to the bottom of the tissue culture well, rinsed of old media, and washed with HBSS and covered with 50  $\mu L$  HBSS. The cells were then treated with  $20 \mu M$   $H_2O_2$  (100  $\mu L$  total volume); because of their much lower capacity to remove extracellular  $H_2O_2$  the time of maximum exposure was 40 min. The observed first-order rate constant for the loss of extracellular  $H_2O_2$  in this experiment was determined to be  $k_{obs} = 1.2 \times 10^{-3} s^{-1}$ . The cell density in this experiment was  $2.1 \times 10^9$  cells  $L^{-1}$ . This yields  $k_{cell} = k_{obs}/(2.1 \times 10^9 \text{ cell } L^{-1}) = 0.57 \times 10^{-12} s^{-1} \text{ cell}^{-1} L$ . Both experiments are representatives of typical observations seen in greater than 50 experiments with various cell lines.

despite there being approximately four times more MDA-MB-231 cells in the experiment than U937 cells; also note the difference in the time-scale for each abscissa of Fig. 3A and B. U937 cells grow as a suspension culture and were exposed to extracellular  $H_2O_2$  while in

suspension, whereas the MDA-MB-231 cells are cultured as a monolayer and were exposed to extracellular  $\text{H}_2\text{O}_2$  as attached cells. The difference in this physical aspect of cell culture may contribute to the very different capacities of these cells to remove extracellular  $\text{H}_2\text{O}_2$ .



**Fig. 4. The physical setting of cells affects the rate of extracellular  $\text{H}_2\text{O}_2$  removal.** Bovine aortic endothelial cells were allowed to attach overnight or were freshly trypsinized, counted, and then assayed for their ability to remove extracellular  $\text{H}_2\text{O}_2$ . The same cell densities ( $1.0 \times 10^5$  cells  $\text{well}^{-1}$ ) were used for the assays for both attached and detached cells. The cells were exposed to a bolus addition of  $20 \mu\text{M}$   $\text{H}_2\text{O}_2$  ( $100 \mu\text{L}$  total volume yielding a cell density of  $1.0 \times 10^9$  cells  $\text{L}^{-1}$ ). Because of the low capacity of BAECs to remove extracellular  $\text{H}_2\text{O}_2$  the maximum time of exposure was increased to 40 min, before addition of stopping solution. Insets demonstrate the pseudo-first-order kinetics observed for  $\text{H}_2\text{O}_2$  removal by BAECs. In these sets of experiments  $k_{\text{cell}} = 0.50 \times 10^{-12} \text{ s}^{-1} \text{ cell}^{-1} \text{ L}$  for attached cells and  $k_{\text{cell}} = 1.3 \times 10^{-12} \text{ s}^{-1} \text{ cell}^{-1} \text{ L}$  for suspended cells.

**Table 1**

Rate constant,  $k_{\text{cell}}$ , for removal of extracellular  $\text{H}_2\text{O}_2$  by cells.

Cell (Vol/pL) <sup>b</sup>	State	$k_{\text{cell}}/10^{-12} \text{ s}^{-1} \text{ cell}^{-1} \text{ L}^a$			$k_{\text{cell}}/10^{-12} \text{ s}^{-1} \text{ cell}^{-1} \text{ L}$ with 3-aminotriazole		
		Mean	± Std err	$n^c$	Mean	± Std err	$n^c$
HL-60 Human promyelocytic leukemia (0.64)	Suspended	1.8	0.4	5	0.64		1
U937 Human histiocytic lymphoma (0.93)	Suspended	10.4	1.7	6	2.7		1
MDA-MB-231 Human mammary adenocarcinoma (1.53)	Monolayer	0.47	0.03	8	0.32	0.04	5
MCF-7 Human mammary adenocarcinoma (1.70)	Monolayer	0.64	0.06	5	0.49	0.06	4
MCF-7p51 Human mammary adenocarcinoma (1.84)	Monolayer (GPx-4 Overex pressor)	3.7	1.5	5	1.7	0.5	3
MIA-PaCa-2 Human pancreatic carcinoma (2.03)	Monolayer	0.46	0.04	5	0.38	0.03	5
PC3 human prostate cancer cell line (2.93)	Monolayer	2.0	0.3	9			
BAEC Bovine Aortic Endothelial Cells (0.93)	Monolayer	0.72	0.16	11	0.42	0.09	5
BAEC Bovine Aortic Endothelial Cells (0.93)	Detached	1.9	0.2	13			
RBC's Human red blood cell (0.09)	Suspended	2.9	0.7	10	1.1		1
RBC's mouse red blood cell (0.05)	Suspended	2.4		2			
Jurkat <sup>d</sup> Clone E6–1 (0.78)	Suspended	1.0	0.1	16			

<sup>a</sup> The rate of removal of  $\text{H}_2\text{O}_2$  will be:  $\text{rate} = -k_{\text{cell}} [\text{H}_2\text{O}_2]$  (number of cells  $\text{L}^{-1}$ ). The half-life of  $\text{H}_2\text{O}_2$  in an experiment upon bolus addition of  $\text{H}_2\text{O}_2$  will be approximately  $t_{1/2} = 0.693/(k_{\text{cell}} \text{ (number of cells } \text{L}^{-1}))$ .

<sup>b</sup> Cell volumes are from Ref. [57]; Jurkat volume from [58].

<sup>c</sup>  $n$  is the number of independent experiments. Each experiment had, as minimum, triplicate determinations at each time point and for each standard.

<sup>d</sup> This value of  $k_{\text{cell}}$  was estimated from the data in Ref. [30]. Note that  $k_{\text{cell}}$  of Antunes et al. is  $k_{\text{obs}}$  here. Their cell density was  $1.0 \times 10^9 \text{ cell } \text{L}^{-1}$ .

## Physical constraints on removal of extracellular $\text{H}_2\text{O}_2$

Cells in an adherent culture will have proportionally less surface area available for the diffusion of extracellular  $\text{H}_2\text{O}_2$  into the cell, and thus a possible slower rate of removal of extracellular  $\text{H}_2\text{O}_2$ . To address this possible physical constraint we examined the rate of removal of extracellular  $\text{H}_2\text{O}_2$  by attached and detached bovine aortic endothelial cells (BAEC). BAECs that were attached as a monolayer or detached as a suspension were assayed simultaneously with equal cell numbers per well. The data of Fig. 4 demonstrate that detached BAECs remove bolus additions of extracellular  $\text{H}_2\text{O}_2$  at a rate 2.6 times greater than the same cells when attached as a monolayer at the bottom of tissue culture wells; see also Table 1. In this experiment the cellular first-order rate constants ( $k_{\text{cell}}$ ) for the removal of extracellular peroxide-removal by BAECs were  $0.50 \times 10^{-12} \text{ s}^{-1} \text{ cell}^{-1} \text{ L}$  for attached cells and  $k_{\text{cell}} = 1.3 \times 10^{-12} \text{ s}^{-1} \text{ cell}^{-1} \text{ L}$  for suspended cells (Fig. 4 inset). Thus, the “available” surface area influences the removal of extracellular  $\text{H}_2\text{O}_2$ .

## Inhibition of catalase

In preliminary studies to explore the contribution of different peroxide-removal enzymes responsible for the removal of extracellular  $\text{H}_2\text{O}_2$  in our experimental configuration, we treated cells with the catalase inhibitor 3-aminotriazole. This treatment specifically inhibits catalase irreversibly through a suicide inactivation mechanism dependent on  $\text{H}_2\text{O}_2$  [44]. Because aminotriazole requires  $\text{H}_2\text{O}_2$  to inactivate catalase it can also be used to detect  $\text{H}_2\text{O}_2$  in vitro and in vivo [45–49]. In general we have found that cellular catalase activity can be inhibited to less than 20% of its original activity when incubated for 1 h with 20 mM 3-aminotriazole and that intracellular as well as extracellular  $\text{H}_2\text{O}_2$  can facilitate this inactivation [49].

The inhibition of catalase decreased the rate of peroxide-removal from all the cells tested, but to varying degrees, Table 1. Other peroxide-removal enzymes and mechanisms exist and their contributions are apparent as  $k_{\text{cell}}$  with 3-AT is  $> 20\%$  of  $k_{\text{cell}}$  in the absence of 3-AT. These observations with 3-AT not only demonstrate that extracellular  $\text{H}_2\text{O}_2$  can penetrate into the cytoplasm of cells but additionally into organelles such as peroxisomes, where catalase is located. Changes in  $k_{\text{cell}}$  following inhibition of catalase demonstrate that the assay and methodology reflect real and measurable changes in the ability of cells to remove extracellular  $\text{H}_2\text{O}_2$ .

### Differences in cellular capacity to remove extracellular $\text{H}_2\text{O}_2$

As seen in Table 1,  $k_{\text{cell}}$  for removal of extracellular  $\text{H}_2\text{O}_2$ , as determined using our assay protocol, varies widely in the set of cells examined. The human pancreatic cancer cell line MIA-PaCa-2 and the human mammary adenocarcinoma cell line MDA-MB-231 have the lowest values of  $k_{\text{cell}}$ . In contrast the human histiocytic lymphoma cell line U937 has 20 times greater capacity to remove  $\text{H}_2\text{O}_2$  as estimated by  $k_{\text{cell}}$ . Interestingly, the MCF-7 human breast cancer cell line that overexpresses GPx-4 [50,51] removes  $\text{H}_2\text{O}_2$  at a rate 3.5 times faster than the normal wild-type MCF-7 counterpart. This difference is not due to changes in catalase, GPx1, or attributable to differences in GSH levels, as the GPx-4 over-expressor has similar levels to the parental MCF-7 wild type cell line [50]. As seen with lipid hydroperoxides [52], GPx-4 may be a major contributor to removal of  $\text{H}_2\text{O}_2$ .

Erythrocytes have a remarkable capacity to remove extracellular  $\text{H}_2\text{O}_2$ ,  $k_{\text{cell}} = 2.9 \times 10^{-12} \text{ s}^{-1} \text{ cell}^{-1} \text{ L}$ , despite their relatively small size (0.090 pL) compared to the other cells examined (0.64–2.93 pL). Because RBCs do not have mitochondria, meeting their needs for ATP through glycolysis, the principal intracellular source of oxidants is thought to be the very slow autooxidation of oxyhemoglobin, producing methemoglobin and superoxide. The rate constant of this reaction for human oxyhemoglobin is estimated to be  $\approx 1 \times 10^{-7} \text{ s}^{-1}$  [53,54]; if the concentration of oxyhemoglobin in RBCs is 5 mM, then the rate of superoxide production will be  $5 \times 10^{-10} \text{ M s}^{-1}$  yielding a rate for production of  $\text{H}_2\text{O}_2$  of  $2.5 \times 10^{-10} \text{ M s}^{-1}$ , assuming SOD reacts with the vast majority of superoxide produced. The flux of  $\text{H}_2\text{O}_2$  in each RBC would then be  $0.023 \text{ zmol s}^{-1}$  or 13 molecules of  $\text{H}_2\text{O}_2 \text{ RBC}^{-1} \text{ s}^{-1}$ . In our experiments to determine  $k_{\text{cell}}$  cells were exposed to  $20 \mu\text{M}$   $\text{H}_2\text{O}_2$ ; the initial rate of removal of this extracellular  $\text{H}_2\text{O}_2$  by a cell would be:  $\text{rate} = -(2.9 \times 10^{-12} \text{ s}^{-1} \text{ cell}^{-1} \text{ L}) (20 \times 10^{-6} \text{ M}) (1 \text{ cell L}^{-1}) = -58 \times 10^{-18} \text{ M s}^{-1}$  or for each cell  $58 \text{ amol s}^{-1}$  ( $36 \times 10^6$  molecules  $\text{cell}^{-1} \text{ s}^{-1}$ ). Thus, the capacity to remove  $\text{H}_2\text{O}_2$  by RBCs greatly exceeds the rate of intracellular production. The biconcave shape of RBCs yields a large surface-to-volume ratio that supports rapid exchange of oxygen and carbon dioxide; this also allows rapid diffusion of extracellular  $\text{H}_2\text{O}_2$  into the RBC where the peroxide-removal system has a great capacity to remove this  $\text{H}_2\text{O}_2$ . In whole blood the density of RBCs is about  $5 \times 10^{12} \text{ cells L}^{-1}$ ; this would yield an estimate of  $k_{\text{obs}} = (2.9 \times 10^{-12} \text{ s}^{-1} \text{ cell}^{-1} \text{ L}) (5 \times 10^{12} \text{ cells L}^{-1}) = 15 \text{ s}^{-1}$ , yielding a half-life of  $\text{H}_2\text{O}_2$  in whole blood of less than 50 ms.

This high capacity for removal of extracellular  $\text{H}_2\text{O}_2$  by RBCs is thought to be a means for red blood cells to remove localized pulses of very high fluxes of  $\text{H}_2\text{O}_2$ , such as generated by activated immune cells, thereby protecting other cells and tissues from oxidative damage and yet not suffer extensive damage themselves that would cripple their function [55].

There are several peroxide-removal systems in cells, e.g. peroxiredoxins, glutathione peroxidases, catalase, and others. It must be kept in mind that the fraction of the flux of extracellular  $\text{H}_2\text{O}_2$  removed by any one of these systems depends on the flux of  $\text{H}_2\text{O}_2$  entering the cell and the availability of co-factors required for their individual function [11,12,18,23,24–27,56]. In addition, the biological response observed upon exposure will be a function of the exposure/dose (mole  $\text{cell}^{-1}$ ) or flux (mole  $\text{cell}^{-1} \text{ s}^{-1}$ ) [28,31,32], and references therein. There are literally hundreds of reports where the exposure to a bolus of extracellular  $\text{H}_2\text{O}_2$  is presented as concentration without information on the volume of media or cell density; because in a laboratory most experiments employ the same protocol, i.e. same number of cells and the same volume of media, the results reported are only relative within that protocol. Such results may not be universally interpretable or translatable to other experimental results that used different

protocols. We encourage researchers to provide more quantitative information on the exposure of cells to extracellular  $\text{H}_2\text{O}_2$ , i.e. mole  $\text{cell}^{-1}$  or mole  $\text{cell}^{-1} \text{ s}^{-1}$ . We suggest information be provided on a per cell basis (a biophysical normalization) as well as per mg cell protein (a biochemical normalization). Use of both normalizations will maximize potential information from experiments. Expressing cell density in  $\text{cell L}^{-1}$  and the use of seconds as the unit for time (SI unit) allows direct use when connecting to standard chemical kinetics and mathematical modeling [57]. This also allows direct quantitative comparisons between different cell types, a goal of quantitative redox biology [17,57].

### Declaration of interest

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