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# Real-Time Amperometric Analysis of Reactive Oxygen and Nitrogen Species Released by Single Immunostimulated Macrophages

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*Macrophages are key cells of the immune system. Immunologically activated macrophages are known to release a cocktail of reactive oxygen and nitrogen species. In this work, RAW 264.7 macrophages were activated by interferon-γ and lipopolysaccharide, and the reactive mixture released by single cells was analyzed, in real time, by amperometry at platinized carbon microelectrodes. In comparison with untreated macrophages, significant increases in amperometric responses were observed for activated macrophages. Nitric oxide ($NO^{\bullet}$), nitrite ($NO_2^-$), and peroxynitrite ($ONOO^-$) were the main reactive species detected. The amounts of these reactive species were quantified, and their average fluxes released by a single, activated macrophage were evaluated. The detection of $ONOO^-$ is of particular interest, as its role and implications in various physiological conditions have been widely debated. Herein, direct evidence for the formation of $ONOO^-$ in stimulated macrophages is presented. Finally, the presence of 1400W, a selective inducible nitric oxide synthase (iNOS) inhibitor, led to an almost complete attenuation of the amperometric response of activated RAW 264.7 cells. The majority of the reactive species released by a macrophage are thus likely to be derived from $NO^{\bullet}$ and superoxide ($O_2^{\bullet-}$) co-produced by iNOS.*

## Introduction

Macrophages are key immune cells responsible for the phagocytosis of pathogens and cellular debris, as well as for the activation of other immune elements. It is now generally accepted that the antimicrobial activities of immunostimulated macrophages involve the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS).[1–9] However, severe differences of opinion remain over the exact identities of the reactive species involved, and in particular, about the occurrence of peroxynitrite ($ONOO^-$/ONOOH).[10–13]

Peroxynitrite is a potent oxidizing and nitrating agent formed via the near diffusion-limited reaction ($k \approx 10^{10}\,M^{-1}\,s^{-1}$) of nitric oxide ($NO^{\bullet}$) and superoxide ($O_2^{\bullet-}$).[14] This coupling occurs faster than the superoxide dismutase (SOD) catalyzed disproportionation of $O_2^{\bullet-}$ to $O_2$ and $H_2O_2$ ($k \approx 10^9\,M^{-1}\,s^{-1}$).[12] In other words, $ONOO^-$ is expected to be formed in situations where both $NO^{\bullet}$ and $O_2^{\bullet-}$ are produced at similar rates. Indeed, several reports in the literature suggest that the majority of $NO^{\bullet}$ produced by activated macrophages is converted to $ONOO^-$, which is presumed to be a major actor in the antimicrobial activity of macrophages.[1–4] It is also well established that the bolus addition of $ONOO^-$ to cells at physiological pH can lead to oxidation, nitrosylation, and nitration of a vast array of biomolecules, as well as to cell death.[11,15–19] However, in spite of the afore-mentioned observations, it is still debated whether $ONOO^-$ is actually formed in vivo, and if so, whether it plays any pathophysiological role.

This controversy, we believe, is largely due to the lack of reliable detection methods for evidencing minute release of peroxynitrite in real time directly after cell activation. In many studies, the presence of 3-nitrotyrosine (3-NT) is considered to be evidence of prior $ONOO^-$ formation in vivo. This is based on observations that the addition of $ONOO^-$ to tyrosine, or to proteins containing tyrosine residues, lead to the formation of 3-NT under physiological conditions.[12,15] However, alternative mechanisms of tyrosine nitration, such as via the nitrite/$H_2O_2$/myeloperoxidase pathway,[20–24] have since been proposed, leading to the specificity of 3-NT as a "footprint" for in vivo $ONOO^-$ formation to be called into question.[12,24,25]

Most of the other techniques (such as fluorescence, chemiluminescence) used to measure $ONOO^-$ are also indirect methods that rely on the measurement of secondary species.[26–30] In order for these techniques to be accurate, the molecular probes must be completely specific and highly sensitive for $ONOO^-$ over any other ROS/RNS that might be present. This is, unfortunately, difficult to ascertain in practice. For example, dihydrorhodamine 123 (DHR) and 2,7-dichlorodihydrofluorescein (DCFH) are two of the more commonly used fluorescent probes for $ONOO^-$.[31] However, it has been shown that DHR is oxidizable by species such as cytochrome *c*, HOCl, or $H_2O_2$ in the presence of peroxidases, whereas DCFH can also be oxidized by species such as $HO^{\bullet}$, $ROO^{\bullet}$, $NO^{\bullet}$, $H_2O_2$, cytochrome *c*,

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and xanthine oxidase.[27–29] In other studies, $ONOO^-$ is detected based on its reaction with luminol to yield chemiluminescence. Like DHR and DCFH, luminol is not specific for $ONOO^-$, and is, as a matter of fact, also used as a probe for $O_2^{\bullet-}$.[26,27] To further complicate matters, probes may sometimes interfere actively in the analysis by generating ROS/RNS themselves: luminol radicals, for example, are known to be able to reduce $O_2$ to $O_2^{\bullet-}$.[26,27,29,30] It is thus clear that great care must then be exercised with the use of indirect analytical techniques so as to minimize erroneous interpretations and inadvertent measurements of artifacts.

Electrochemistry, on the other hand, offers the possibility of direct, on-line and short-time measurements of important biological species, such as neurotransmitters or neuromodulators, released by cells.[32–34] Microelectrodes, which can be positioned in close proximity to single living cells with submicrometric accuracy, are able to detect the release of biological species with attomole and subsecond resolutions.[35,36] We have previously reported the use of amperometry at platinized carbon microelectrodes to detect and quantify ROS/RNS released by human lymphocytes[37] or skin cells,[38] as well as the application of this technique to biomedical studies concerning initial oxidative mechanisms of skin carcinogenesis.[39] We have also measured the oxidative bursts produced by single macrophages stimulated by their mechanically induced membrane depolarization.[40] Similarly, the release of ROS/RNS by macrophages cultured in a microfluidic chamber and stimulated by the microinjection of a calcium ionophore was measured with platinized band electrodes.[36]

As a consecutive effort, we present herein amperometric studies of ROS/RNS that are released by single immunostimulated macrophages. In this work, macrophages were stimulated with interferon-γ (IFN-γ) and lipopolysaccharide (LPS) in order to induce expression of the inducible isoform of nitric oxide synthase (iNOS, NOS2), which is known to produce large amounts of $NO^\bullet$ over a prolonged period.[8] The ensuing release of ROS/RNS by a single macrophage was then followed in real time by amperometry. The various ROS/RNS were quantified, and in particular, $ONOO^-$ was shown to be present at high levels. This is, to the best of our knowledge, the first direct evidence that $ONOO^-$ is released by immunologically activated macrophages. The biological implications of these findings will be discussed.

## Results

### Nitrite determination by fluorimetry

Nitrite ($NO_2^-$) is one of the stable end products of $NO^\bullet$ and $ONOO^-$. The production of $NO^\bullet$-derived species by immunostimulated macrophages over time can thus be estimated by measuring the accumulation of $NO_2^-$ in the culture medium. This was performed by fluorimetry, because of the low cell density (see the Experimental Section), for both IFN-γ/LPS treated and untreated macrophages, as presented in Figure 1.

The variation of the culture supernatant's $NO_2^-$ concentration, accumulated over a period of 8 to 48 h, was insignificant

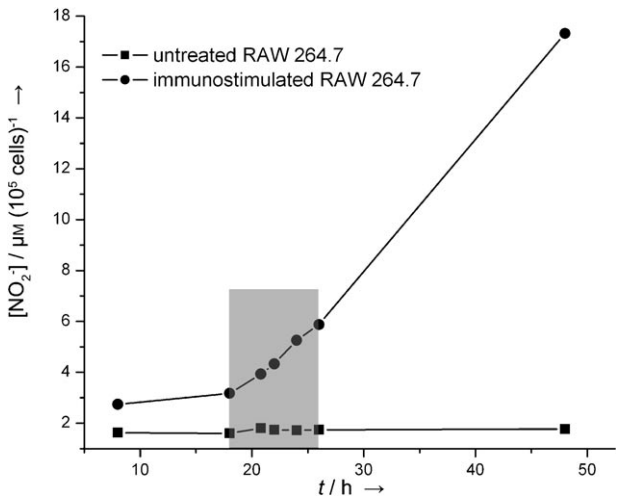


**Figure 1.** Nitrite production by $10^5$ macrophages treated (●) and untreated (■) with IFN-γ (20 units $mL^{-1}$) and LPS (50 ng $mL^{-1}$). The shaded area indicates the time window over which the amperometric measurements (and hence, the calculations of charges) represented in Figures 3 and 4 were made.

for untreated RAW 264.7 macrophages. This was expected, as iNOS should not have been expressed in these untreated cells. With stimulated RAW 264.7 macrophages, the release rate reveals two distinct parts: between 8–18 h, the increase in $NO_2^-$ concentration was slight, featuring an induction period; a strong and linear increase was then observed for the period between 18–48 h. The slow increase in $NO_2^-$ accumulation observed at the start is consistent with the fact that the iNOS gene must first be transcribed (lag time of approximately 5–6 h)[8] and after which, additional time was required for the accumulation of $NO_2^-$ to sufficiently high levels for detection by fluorimetry. Linear regression of the data over 18–48 h indicated that $NO_2^-$ was accumulated in the cell culture medium (2 mL) at a rate of 0.486 μM per $10^5$ cells per hour ($R = 0.997$) in fully activated macrophages. This corresponds to the release of several attomoles of $NO^\bullet$ derivatives by a single activated macrophage every second. This should thus be detectable at our platinized carbon microelectrodes (see Introduction), yielding a more precise view of ROS/RNS release. Unlike the fluorimetric method, which only allows for the selective detection of relatively high quantities of $NO_2^-$, amperometry at the platinized carbon microelectrodes would allow for the detection of minute release of four different species - $H_2O_2$, $ONOO^-$, $NO^\bullet$, and $NO_2^-$ (vide infra).

### Single-cell amperometric measurements

Amperometric analysis of the release of ROS/RNS was performed by positioning a platinized carbon disk microelectrode in close proximity with the cell membrane of an isolated macrophage (see Figure 2). This method was similar to that, termed "artificial synapse" configuration, which was previously described for investigating vesicular exocytosis in bovine chromaffin cells.[41] The only difference was that the carbon microelectrodes used in the current work were platinized to increase

  

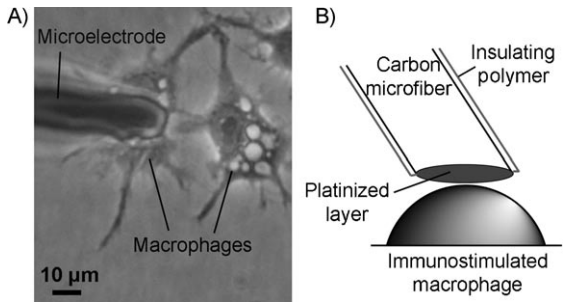


**Figure 2.** A) Photomicrograph and B) schematic representation of the experimental setup. The microelectrode in (A) appears slightly out of focus as it is above the plane of the cell.

the sensitivity and selectivity towards ROS/RNS. There were no visually observable differences between the overall morphology of cells that were in, or not in, close proximity to a microelectrode over the course of the experiments.

The release of ROS/RNS was detected in real time by amperometry at a constant potential, *E*, versus a sodium-saturated calomel reference electrode (SSCE). Amperometric measurements were made at *E* of +300 mV, +450 mV, +650 mV, and +850 mV versus SSCE to allow for detection and quantification of each released species. These potentials were selected based on previous in vitro voltammetric studies of the oxidation of independent $H_2O_2$, $ONOO^-$, $NO^\bullet$, and $NO_2^-$ phosphate-buffered saline (PBS) solutions.[34,40] Linear combinations of the measured currents obtained at each value of this set of potentials were found to specifically characterize and quantify each of the four species [see Eqs. (1)–(4)].

A typical response of the amperometric measurements of single immunostimulated macrophages is presented in Figure 3. Two distinct features are observed in the responses that were obtained. Firstly, broad and relatively weak peaks, persisting over several tens of minutes, were observed. Examples of such peaks can be seen in Figure 3, ranging approximately over the interval of 300–2000 s. Secondly, sharp "bursts" superimposed upon the afore-mentioned broad peaks. These amperometric spikes usually have half-widths of several tens of milliseconds, and less frequently, up to several tens of seconds (see Figure 3 insets).

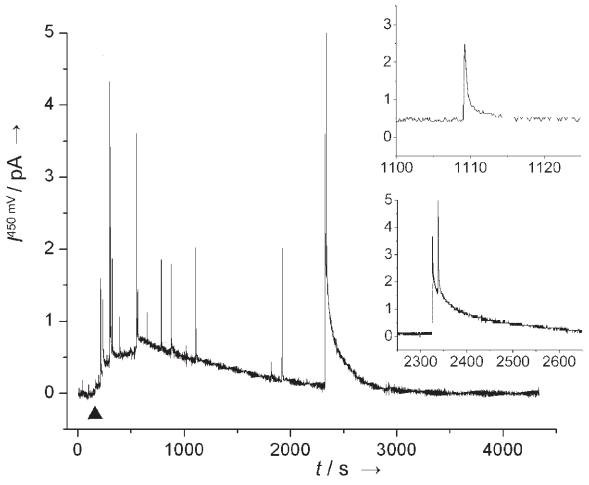


**Figure 3.** A typical amperometric response from a single IFN-γ/LPS-stimulated macrophage, measured at +450 mV versus SSCE after 19.5 h stimulation in this case. The black triangle indicates the time at which the platinized carbon microelectrode was placed in contact with the cell. Insets show representative zooms of two amperometric spikes.

In order to quantify the various species released, the baseline current drifts were subtracted from the individual amperometric responses (see the Experimental Section). Each resultant curve was then integrated over time (1 h duration) to obtain the total charge, *Q*, that corresponds to the overall oxidation process(es) occurring at each specific measurement potential.

We have established previously[34] that the detected current at each potential, $I^{potential}$, can be written as a linear combination of the responses of each of the four species,[40] with different weights. The weights, dependent only on the detection potential and the type of electrode used, are experimentally determined from in vitro steady state voltammograms of each respective species. $NO^\bullet$ and $NO_2^-$ oxidation waves are well separated from the other two species,[40] and are close to their plateau potentials at +650 mV and +850 mV versus SSCE, respectively. The difference between $I^{450\ mV}$ and $I^{650\ mV}$, and that between $I^{650\ mV}$ and $I^{850\ mV}$, thus corresponds to currents originating from the electrooxidation of $NO^\bullet$ and $NO_2^-$, respectively. The voltammograms of $H_2O_2$ and $ONOO^-$, however, overlap. At +450 mV versus SSCE, the oxidation waves of $H_2O_2$ and $ONOO^-$ are both near their plateau potentials, whereas at +300 mV versus SSCE, the $H_2O_2$ wave is close to its plateau potential while that of $ONOO^-$ is close to its half-wave potential (see Figure 3 of ref. [40]). The amperometric responses measured at +300 mV and +450 mV are thus cumulative responses of both $H_2O_2$ and $ONOO^-$, albeit with different weights. In summary, we have:

$$I^{850\ mV} = I_{H_2O_2} + I_{ONOO^-} + I_{NO} + I_{NO_2^-} \quad (1)$$

$$I^{650\ mV} = I_{H_2O_2} + I_{ONOO^-} + I_{NO} \quad (2)$$

$$I^{450\ mV} = 0.99 \times I_{H_2O_2} + 0.90 \times I_{ONOO^-} \quad (3)$$

$$I^{300\ mV} = 0.85 \times I_{H_2O_2} + 0.29 \times I_{ONOO^-} \quad (4)$$

The system of linear equations is readily solved to deconvolute the currents due to each of the four individual species. The emission fluxes ($\Phi_{species}$) of each species can then be calculated from their respective current intensities obtained above, by using Faraday's Equation (5):

$$\Phi_{species} = I_{species}/(n_{species} \times F) \quad (5)$$

where $n_{species}$ is the number of electrons per molecule exchanged for the oxidation of one species ($n_{species}=2$ for $H_2O_2$ and $NO_2^-$; $n_{species}=1$ for $ONOO^-$ and NO)[40] and *F* is the Faraday constant. Therefore, amperometric measurements of statistically significant numbers of single macrophages at four different potentials (+300, +450, +650, and +850 mV versus SSCE)

  

enable a direct quantification with excellent precision of the release of $H_2O_2$, $ONOO^-$, $NO^\bullet$, and $NO_2^-$.

Finally, at each potential, the current may be time integrated to provide the overall detected charge over any time interval ($t_0$, $t_{0+\theta}$) [Eq. (6)]:

$$Q_{species} = \int_{t0}^{t0+\theta} I_{species}\, dt \qquad (6)$$

where $I_{species}$ is given by Equations (1)–(4). Owing to the linearity of Equations (1)–(4), the same equations apply to the charges detected at each potential.

### Detection and quantification of the reactive species released by immunostimulated RAW 264.7 macrophages

The results of the above amperometric measurements of ROS/RNS release by immunostimulated RAW 264.7 macrophages are presented in Figure 4 in terms of the charges detected

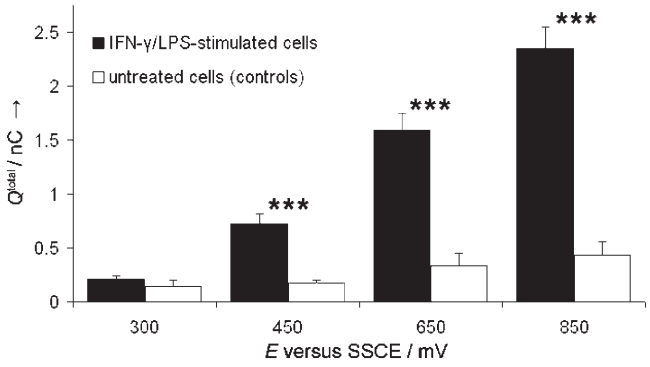


**Figure 4.** Total charges ($Q^{total}$) involved in oxidation processes, over a period of 1 h, at four different potentials ($E$). Single cell measurements were performed for macrophages that had been activated by IFN-γ/LPS over a period of 18–24 h (corresponding to the shaded area in Figure 1). The black and white bars represent the average charge detected over single immunostimulated RAW 264.7 cells ($n > 30$ at each potential), and untreated RAW 264.7 cells ($n \geq 8$ at each potential), respectively. Error bars represent SEM. Note that the charge detected at each potential is obtained through time-integration of the current monitored at the measurement potential [see Eq. (6)].

over a 1 h period. At all potentials, except that of +300 mV, the total charges transferred were significantly larger for IFN-γ/LPS-stimulated macrophages than untreated, control macrophages. This is direct evidence that the induction of iNOS expression leads to an increased release of oxidizable ROS/RNS species from a macrophage.

The fluxes of emission of each species were calculated as described above. The effective quantities of each species were then obtained by taking the integral of the fluxes over the period of measure (1 h). Overall, each immunostimulated macrophage released, on average, approximately 8.9 ± 1.9 fmol of $NO^\bullet$, 7.5 ± 0.9 fmol of $ONOO^-$, and 4.0 ± 1.3 fmol of $NO_2^-$ per hour. Interestingly, no significant amount of $H_2O_2$ was detected over IFN-γ/LPS stimulated RAW 264.7 macrophages, though it should be mentioned that the release of $H_2O_2$ is evidenced over phorbol 12-myristate 13-acetate (PMA) activated macrophages of this same cell line.[42]

In a further series of experiments, the highly selective iNOS inhibitor, 1400W, was added to the culture medium during the IFN-γ/LPS-activation of macrophages. Measurements were performed at potentials of +450, +650, and +850 mV versus SSCE. In all measurements ($n = 15$) of macrophages co-treated with 1400W, only very weak, basal levels of amperometric responses were obtained (Figure 5). Taken together, our results

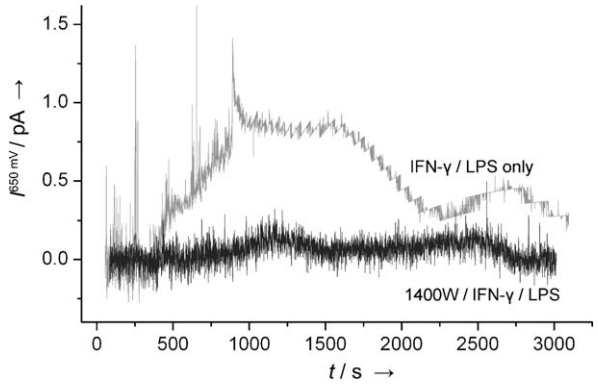


**Figure 5.** A typical amperometric response (black) from a single immunostimulated macrophage that was co-treated with 1400W. This particular response was measured at +650 mV versus SSCE, which would allow for the detection of any $H_2O_2$, $ONOO^-$, and $NO^\bullet$ released. In comparison with the corresponding response obtained at the same potential, but in the absence of 1400W (gray), it is evidenced that the majority of ROS/RNS released by IFN-γ/LPS-stimulated RAW 264.7 macrophages were derived from iNOS.

strongly suggest that the induction of iNOS expression is crucial to the production of several ROS/RNS in RAW 264.7 macrophages.

### Effects of peroxynitrite scavenging

We have previously reported a detailed electrochemical characterization of $ONOO^-$ at our platinized carbon microelectrodes, demonstrating that this elusive species can be detected quantitatively by the method used herein.[35] The results of the amperometric measurements reported above establish that $ONOO^-$ is one of the major reactive species released by activated macrophages after expression of iNOS. In this case, one would expect observing partial decreases in amperometric responses when the measurements were carried out in the presence of $ONOO^-$ scavengers, provided these scavengers may act efficiently before $ONOO^-$ is captured and oxidized by the electrode surface. Measurements were performed at +450 mV versus SSCE because, as evidenced above, this potential allows for the quantification of $ONOO^-$ as no $H_2O_2$ is detected [viz., $I_{H_2O_2} = 0$ in Eq. (3)].

Uric acid[43] (UA; 1 μM) or (−)-epicatechin[44] (EC; 0.1 mM), both of which are considered to be efficient scavengers of $ONOO^-$, were used for this series of experiments. In addition,

  

$ONOO^-$ is known to react rapidly with $CO_2$ ($k = 3 \times 10^4\ M^{-1}s^{-1}$),[45] and "$CO_2$"—added as $NaHCO_3$ (5 mM)—was also used to "scavenge" $ONOO^-$. Under our experimental conditions, PBS solutions of UA, EC, and $NaHCO_3$ were checked to have no effects on the microelectrodes' sensitivity or stability at the concentrations used (data not shown). Indeed, as one can see from Figure 6, amperometric responses at +450 mV versus SSCE were observed to decrease significantly in the presence of UA, EC, or $NaHCO_3$, though the control level was not reached.

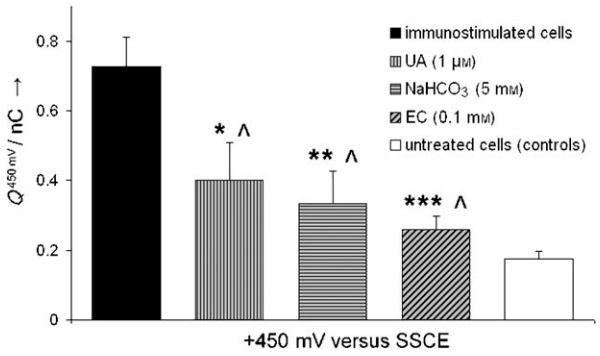


**Figure 6.** Effects of peroxynitrite scavenging by uric acid (UA; gray bar with vertical stripes), $NaHCO_3$ (gray bar with horizontal stripes), or (−)-epicatechin (EC; gray bar with diagonal stripes) on amperometric responses measured at +450 mV versus SSCE. Significant decreases (*) were observed when compared with immunostimulated cells without $ONOO^-$ scavenging (black bar). The responses in the presence of $ONOO^-$ scavengers were, nonetheless, still more significant (Λ) than that detected for unactivated RAW 264.7 macrophages (white bar), thus evidencing that scavenging was not totally due to the competition with the electrode oxidation.

## Discussion

The preliminary fluorimetric measurements of nitrite accumulation (Figure 1) in the culture medium strongly suggested that NO•-derived species are released by stimulated macrophages. However, the actual identities of the released species could not be determined by using this method. Conversely, in the present study, the reactive cocktail released by immunostimulated macrophages was easily detected, characterized, and quantified in real time by amperometry at platinized carbon microelectrodes. More specifically, stimulation of RAW 264.7 macrophages was achieved by incubation in the presence of IFN-γ and LPS, which is known to induce high levels of iNOS expression. As would be expected, both the above-mentioned amperometric and fluorimetric methods yielded similar estimates for the release rates of NO• derivatives. The excellent temporal resolution offered by the electrochemical method vis-à-vis the fluorimetric method was, however, evident. For the latter method, estimates were obtained based on the accumulation of $NO_2^-$ over prolonged periods. In other words, with the fluorimetric method, one could obtain information about the total amount of NO• derivatives produced over a certain period, but not information about any temporal variations in the rates at which these were released. Amperometry, on the other hand, offered the possibility of real-time detection with much better temporal resolutions. Indeed, the amperometric responses that were obtained feature the superimposition of two distinct types of release: weak broad peaks, and sharp amperometric spikes. These features would not have been detected by using solely the comparative fluorimetric method.

The weak broad peaks are likely to be due to the quasi-continuous detection of one or more types of ROS/RNS. Certain ROS/RNS, such as NO•[18,46] and $ONOO^-$/ONOOH,[47] are known to be able to freely diffuse across cellular membranes. A steady production of such species would then lead to a continuous flux being detected at the microelectrode, which would account for the weak broad peaks observed. On the other hand, the sharp amperometric spikes were similar, albeit with slower kinetics, to what we and others had previously observed during vesicular exocytosis of catecholamines at chromaffin cells.[41] This suggests that the observed sharp spikes in this work might be due to cellular events such as exocytosis of late endosomes and recycling endosomes, or perhaps secretion during membrane trafficking and fusion.[48] For example, Nelson and co-workers[49] have reported a correlation of amperometric spikes and changes in membrane electrical capacitance during the exocytosis of phagosomes by J774 macrophages. As we have shown in our previous works on chromaffin exocytosis,[41] much useful information can be obtained by studying the kinetics of such spikes. More detailed studies of the spikes that were observed thus appear to be of interest in our measurements of stimulated macrophages; this is, however, beyond the scope of the current paper and will be disclosed in a forthcoming work.

Quantification of ROS/RNS released by immunostimulated macrophages was achieved by treatment of the amperometric responses based on Equations (1)–(6). The average amounts of ROS/RNS that were detected from single immunostimulated macrophages are summarized in Table 1. For comparison, the amounts of ROS/RNS that were detected upon mechanically induced membrane depolarization of RAW 264.7 cells are also shown, though these corresponded to intense bursts limited to a few minutes of activity.[40]

**Table 1.** Reactive oxygen and nitrogen species released by a single stimulated RAW 264.7 macrophage.

| ROS/RNS Stimuli | $H_2O_2$ [fmol] | $ONOO^-$ [fmol] | NO• [fmol] | $NO_2^-$ [fmol] |
|---|---|---|---|---|
| IFN-γ/LPS | 0[a] | 7.5 ± 0.9[a] | 8.9 ± 1.9[a] | 4.0 ± 1.3[a] |
| Mechanically induced membrane depolarization[40] | 5 ± 1[b] | 9 ± 1[b] | 14 ± 2[b] | 6 ± 1[b] |

[a] Total quantity released sampled over 1 h. [b] Total quantity released during the few minutes of intense activity following mechanical membrane depolarization.

Indeed, we have previously demonstrated that macrophages, stimulated by depolarization of their membranes, release significant amounts of all four ROS and RNS species ($H_2O_2$,

  

$ONOO^-$, $NO^\bullet$, $NO_2^-$).[40] In the present work, under conditions mimicking an in vivo inflammatory situation, we again detected the presence of $ONOO^-$ amongst the complex cocktail of reactive nitrogen species produced, yet no $H_2O_2$ was detected. Immunostimulated macrophages were found to release $ONOO^-$ at an average rate of $(2.1 \pm 0.3)$ amol per cell per s. Other groups[1–4,17,50]—albeit with the use of indirect detection methods—have also reported significant levels of $ONOO^-$ production by macrophages. In particular, Ischiropoulos and coworkers[1] estimated the rate of $ONOO^-$ formation in activated macrophages to be $(1.8 \pm 0.3)$ amol per cell per s, which is in excellent agreement with our direct measurements. Evidence for the presence of $ONOO^-$ was further strengthened by the observation that the amperometric responses of IFN-γ/LPS-stimulated macrophages were significantly decreased (40–60%) in the presence of $ONOO^-$ "scavengers" such as UA, EC, or $CO_2$. The responses were, nonetheless, still higher than those due to unactivated cells. This is most likely due to incomplete scavenging of $ONOO^-$, as the close microelectrode–cell distance limits the available scavenging time.

At this point, we wish to highlight the usefulness of the direct amperometric measurements by "artificial synapses" for the detection of ROS/RNS such as $ONOO^-$. It has been reported that $ONOO^-$-dependent oxidations or nitrations might be affected in an excess of either $NO^\bullet$ or $O_2^{\bullet-}$.[51–53] This is clearly the case for IFN-γ/LPS-stimulated macrophages, which, as shown herein, release significant amounts of both $NO^\bullet$ and $ONOO^-$. It is thus likely that the rates and efficiencies of protein tyrosine nitration or DHR oxidation by macrophage-produced $ONOO^-$ are affected. This being the case, it follows that reliance on these indirect methods of $ONOO^-$ detection (such as 3-NT detection or fluorescence) necessarily leads to systematic errors in $ONOO^-$ estimates. On the other hand, as $ONOO^-$ is oxidized and directly detected at platinized carbon electrodes, the modulation of $ONOO^-$ reactivities by excess $NO^\bullet$ does not have any effect on its detection and quantification by amperometry. In fact, this would also hold true for all the three other detectable ROS/RNS species as they are also directly oxidized at the electrodes.

It is noteworthy that macrophages stimulated by physical depolarization of their membranes release $H_2O_2$, whereas immunologically activated RAW 264.7 macrophages do not (see Table 1). Based on our previous work,[34,35,40] it is suggested that membrane depolarization simultaneously activates constitutive NO synthases and NADPH oxidases, leading to co-production of $NO^\bullet$ and $O_2^{\bullet-}$ at different cell locations. $H_2O_2$ may then be produced by the (SOD-catalyzed) disproportionation reaction of the superoxide ion whereas this latter species diffuses before it may meet the diffusive $NO^\bullet$ front. In this work, a different set of enzymes was activated. Whereas the stimulation of macrophages with IFN-γ and LPS induces iNOS expression, it is not known to lead to NADPH oxidase activation.[3] It is thus expected that the stimulated macrophages produce higher fluxes of $NO^\bullet$ as compared to $O_2^{\bullet-}$. Most of the $O_2^{\bullet-}$, if produced, would thus be coupled with $NO^\bullet$ to form $ONOO^-$ before having any chance to disproportionate into $H_2O_2$ at significant amounts. Consequently, only minute amounts of $H_2O_2$, if any, could be formed by the disproportionation of the remaining $O_2^{\bullet-}$ (see Introduction). Very low fluxes of $H_2O_2$ would be immeasurable if the slight variations in amperometric currents due to these fluxes are of similar, or smaller, magnitude compared to the background noise. The other reactive species were, however, produced in significant quantities and could be detected with excellent precision at the microelectrodes. We were thus able to quantify both excess $NO^\bullet$, as well as $ONOO^-$ that was formed. In addition, $NO_2^-$, presumably derived from the spontaneous decomposition of $ONOO^-$,[54] was also quantified. Indeed, while we cannot totally exclude the formation of $NO_2^-$ from the decomposition of $NO^\bullet$ in the presence of $O_2$, this pathway appears less likely because of the relatively slow reaction rate.

In a further series of experiments, macrophages were activated in the presence of 1400W. 1400W is known to be one of the most effective iNOS inhibitors, and is reported to be at least 5000 and 200 times more selective for iNOS than eNOS or nNOS, respectively.[55] As only basal levels of responses were measured from macrophages co-incubated with 1400W, we conclude that the ROS/RNS released from IFN-γ/LPS-stimulated macrophages are formed mainly, if not exclusively, by iNOS. In fact, it has been previously reported that iNOS produces $O_2^{\bullet-}$ following cytosolic depletion of L-arginine.[50] The ROS/RNS detected in this work may therefore be likely to have stemmed from the co-production of $NO^\bullet$ and $O_2^{\bullet-}$ by iNOS. As $NO_2^-$ could only result from the decomposition of $ONOO^-$, which in turn was formed from equimolar fluxes of $NO^\bullet$ and $O_2^{\bullet-}$, then each $NO_2^-$ or $ONOO^-$ molecule detected would have been derived from one $NO^\bullet$ and one $O_2^{\bullet-}$ released primarily. Residual $NO^\bullet$ was detected directly. One can thus estimate that immunostimulated macrophages release at least $(5.7 \pm 0.7)$ amol per cell per s of $NO^\bullet$ and $(3.2 \pm 0.5)$ amol per cell per s of $O_2^{\bullet-}$, of which the majority are likely to have been produced by iNOS.

At this point, it should be mentioned that the values for $NO^\bullet$ and $O_2^{\bullet-}$ release rates calculated above are likely to be underestimates. Certain ROS/RNS, such as $^\bullet OH$ and $NO_2^\bullet$, are too reactive—and consequently too short lived—to be detected, though their release cannot be excluded.[40] Also, $NO_3^-$, a stable end product of RNS decompositions, is not electroactive at our electrodes, and is thus undetectable.[34] Similarly, hypochlorous acid ($HOCl/OCl^-$) cannot be oxidized at our electrodes at the imposed potentials (data not shown), and as such, is also undetectable. There is, however, no substantial evidence of $HOCl/OCl^-$ production by IFN-γ/LPS-stimulated macrophages, though it has been reported for myeloperoxidase-expressing macrophages under particular circumstances, such as in human atherosclerotic lesions and Alzheimer's disease.[56] Nonetheless, as we have demonstrated herein and elsewhere,[34,37–40] amperometry at platinized carbon microelectrodes remains an effective means of monitoring the cellular release of ROS/RNS in real time. Indeed, our estimate of the rate of $NO^\bullet$ production by macrophages was found to be in excellent agreement with previous literature values reported by Lewis et al.[57] ($(6.0 \pm 0.4)$ amol per cell per s), as well as by Nalwaya and Deen[58] ($(4.9 \pm 0.6)$ amol per cell per s). In addition, consistent with what we report herein, Porterfield et al. previ-

  

ously reported the direct detection of $NO^{\bullet}$ fluxes from IFN-γ-stimulated RAW 264.7 macrophages with the use of $NO^{\bullet}$ microelectrodes.[46]

Literature estimates of $O_2^{\bullet-}$ production by RAW 264.7 macrophages were, however, more variable: ranging from (0.32 ± 0.07) amol per cell per s,[58] to (1.3 ± 0.4) amol per cell per s,[59] as well as to 2.9 amol per cell per s.[57] It is not clear why the values differ so much, but it is plausible that different intracellular L-arginine levels led to different rates of $O_2^{\bullet-}$ generation by iNOS. It should also be pointed out that different indirect methods of $O_2^{\bullet-}$ quantification were used in each case. For example, Nalwaya and Deen[58] determined the rate of $O_2^{\bullet-}$ synthesis by monitoring the reduction of ferricytochrome *c* to ferrocytochrome *c*. In their studies, incubation of macrophages was performed in the presence of a NOS inhibitor, $N^G$-monomethyl-L-arginine (L-NMMA), to prevent $NO^{\bullet}$ from competing for ferricytochrome *c*. Unfortunately, by doing so, any production of $O_2^{\bullet-}$ from iNOS would have been underestimated, which might possibly account for their relatively low $O_2^{\bullet-}$ estimate. In addition, it is also possible that the rate of $O_2^{\bullet-}$ production varies with time. In this work, measurements were made after 18–24 h of stimulation, a period corresponding to the beginning of the intense and steady production of ROS/RNS, as determined from fluorimetry (see Figure 1). Measurements were made by Nalwaya and Deen,[58] Brune et al.,[59] and Lewis et al.,[57] at 0–10, 6–8, and 16 h, after stimulation, respectively, that is, at different stages of the incubation period shown in Figure 1.

Finally, we would like to caution that, tempting as it might be, the presence of $ONOO^-$ in activated macrophages, as demonstrated in this paper, does not necessarily prove its implication in all in vivo oxidative/nitrative reactions. For example, some of us have previously reported that endogenous tyrosine nitration of iron regulatory protein-1 (IRP-1) in stimulated macrophages was more likely due to the nitrite/$H_2O_2$/peroxidase pathway, rather than the $ONOO^-$ pathway.[20] Work reported by other groups[21–23] similarly favored the former pathway. Taking those results together with this work, it seems likely that, whereas $ONOO^-$ is produced by stimulated macrophages, the reactivity of $ONOO^-$ towards tyrosine nitration is modulated, possibly because of the presence of excess $NO^{\bullet}$ as discussed above. The relatively high in vivo concentrations of $CO_2$ might also lead to further modulations of $ONOO^-$ reactivity.[45] Indeed, the compartmentalized co-production of $NO^{\bullet}$ and $O_2^{\bullet-}$ by iNOS might possibly be a means of facilitating $ONOO^-$ formation during phagocytosis. However, the role played by $ONOO^-$ is versatile: it might be that of an antimicrobial agent,[1] or as a messenger as demonstrated in human leukocytes,[60] or to protect the macrophage against IFN-γ/LPS-induced cell death.[61] In all likelihood, multiple roles and in vivo reactions are possible, depending on various factors such as the exact physiological and redox conditions.

## Conclusions

This work has demonstrated that the cell release of reactive oxygen and nitrogen species can be directly detected and quantified by amperometry at platinized carbon microelectrodes. RAW 264.7 macrophages, stimulated by IFN-γ and LPS, release a cocktail comprising mainly of $NO^{\bullet}$, $NO_2^-$, and, of particular interest, $ONOO^-$. This offers, to the best of our knowledge, the first direct evidence that $ONOO^-$ is released by immunostimulated macrophages. Finally, our results also strongly suggest that the majority of ROS/RNS released derived from the co-production of $NO^{\bullet}$ and $O_2^{\bullet-}$ by iNOS.

## Experimental Section

**Chemicals**: Murine recombinant interferon-γ (IFN-γ; specific activity, $2\times10^7$ units $mg^{-1}$) was provided by R&D Systems. *Escherichia coli* lipopolysaccharide (LPS), uric acid (UA), (−)-epicatechin (EC), $NaHCO_3$, and *N*-(3-(aminomethyl)benzyl)acetamidine (1400W) dihydrochloride were from Sigma. Unless otherwise stated, phosphate-buffered saline (PBS) was used in all experiments. PBS (pH 7.4; 0.137 M NaCl, 0.01 M $Na_2HPO_4$, and 0.003 M KCl) was prepared by dissolving tablets (Sigma) in water. Purified water from a Milli-Q purification system (resistivity = 18 MΩ $cm^{-1}$; Millipore) was used in the preparation of all solutions.

**Cell culture and treatment**: The murine macrophage RAW 264.7 (American Type Culture Collection) cell line was cultured at 37 °C under a 5.5% $CO_2$ atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 1.0 g $L^{-1}$ D-glucose and sodium pyruvate (Invitrogen). The medium was supplemented with 5% fetal bovine serum (Invitrogen) and 20 g $mL^{-1}$ gentamycin (Sigma). 18 to 24 h prior to electrochemical studies, confluent monolayers of RAW 264.7 cells were harvested mechanically, and resuspended in Petri dishes (35 mm diameter; Nunc), with or without the addition of IFN-γ (20 units $mL^{-1}$) and LPS (50 ng $mL^{-1}$) to induce the production of endogenous reactive species. In some experiments, cells were co-treated with 1400W (400 nM), a highly selective iNOS inhibitor, in the presence of L-arginine (400 μM).

**Nitrite measurements by fluorimetry**: RAW 264.7 macrophages are cultured to a density of $10^5$ cells per well in phenol red-free DMEM (2 mL), with or without IFN-γ (20 units $mL^{-1}$) and LPS (50 ng $mL^{-1}$) treatment. At the times indicated in the figure and text, the cell culture supernatant was filtered (Centricon 5 K, Millipore), and the filtered medium (200 μL) was allowed to stand for 30 min in the presence of 2,3-diaminonapthalene (DAN; 40 mM, 10 μL), water (400 μL), and HCl (1 N, 100 μL). NaOH (1 N, 150 μL) was then added, and fluorescence measurement was immediately done (excitation wavelength 375 nm, emission wavelength 405 nm). Nitrite concentration was calculated from a sodium nitrite standard curve.

**Microelectrodes fabrication**: The preparation of microelectrodes has been previously described in detail.[40,62] Briefly, individual carbon fibers (10 μm diameter; Thornel P-55S, Cytec Engineered Materials) were sealed into pulled-glass capillaries (1 mm diameter; GC120F-10, Clark Electromedical Instruments), and the protruding carbon fibers were insulated by electrochemical deposition of poly(oxyphenylene) following literature procedure.[63] The tip of the insulated carbon fiber was then polished at an angle of 45° on a diamond particle whetstone microgrinder (Model EG-4, Narishige) to expose a clean, elliptical carbon surface. The polished carbon surface was then platinized by reducing hydrogen hexachloroplatinate (Sigma) in the presence of lead acetate (Sigma) at −60 mV versus SSCE. The platinization process, followed on a computer, was interrupted when the electrical charge of the signal reached

  

5 μC, which corresponds to the optimal activity of the electrode surface in this series of experiments.

**Single-cell measurements**: Experiments were performed at controlled room temperature (22±1 °C) on the stage of an inverted microscope (Axiovert 135, Zeiss) placed in a Faraday cage. Immediately prior to measurements, the medium was emptied from a Petri dish containing adherent macrophages, which was then rinsed three times and filled with PBS. In some experiments, the buffer used contained UA (1 μM), EC (0.1 mM), or $NaHCO_3$ (5 mM). The microelectrode was positioned about 30 μm above the cell of interest and polarized for 3 min at the start of each measurement. The microelectrode tip was then lowered slowly with a micromanipulator (MHW-103, Narishige) till it was just in contact with the cell. The release of ROS/RNS was detected in real time by amperometry (AMU130 amperometer, Radiometer Analytical) at a constant potential, *E*, versus a sodium-saturated calomel reference electrode. Values of *E* corresponding to +300, +450, +650, and +850 mV were chosen based on previous in vitro voltammetric studies of the oxidation of independent $H_2O_2$, $ONOO^-$, $NO^\bullet$, and $NO_2^-$ solutions.[34] The time course of the amperometric current was monitored and stored on a computer (Latitude D600, Dell) through a D/A converter (Powerlab 4SP, ADInstruments) and its software interface (Chart version 4.2 for Windows, ADInstruments). The exponential decrease of the baseline current due to microelectrode polarization over the course of the measurement was fitted and subtracted by using commercial software (Origin version 7.0, OriginLab Corporation).

**Data analysis:** Graphs were plotted from data expressed as mean ± standard error of the mean. Data was statistically analyzed by using Student's *t*-test (two populations). Results were considered significantly different at: * $p \leq 0.1$; ** $p \leq 0.05$; *** $p \leq 0.01$.

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