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Boronate Probes for the Detection of Hydrogen Peroxide Release from Human Spermatozoa

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Abstract

Human spermatozoa are compromised by production of reactive oxygen species (ROS) and detection of ROS in spermatozoa is important for the diagnosis of male infertility. Probes 2',7'-dichlorohydrofluorescein diacetate (DCFH), dihydroethidium (DHE) and MitoSOX red (MSR) are commonly used for detecting ROS by flow cytometry, however these probes lack sensitivity to hydrogen peroxide (H_2O_2), which is particularly damaging to mammalian sperm cells. This study reports the synthesis and use of three aryl boronate probes, peroxyfluor-1 (PF1), carboxy peroxyfluor-1 (CPF1) and a novel probe 2(2-ethoxyethoxy)ethoxy peroxyfluor-1 (EEPF1) in human spermatozoa. PF1 and EEPF1 were found to be effective in detecting H_2O_2 and peroxynitrite ($ONOO^-$) produced by spermatozoa when stimulated with menadione or 4-hydroxynonenal. EEPF1 was more effective at detection of ROS in spermatozoa than DCFH, DHE and MSR; furthermore it distinguished poorly motile sperm as shown by greater ROS production. EEPF1 should therefore have a significant role in diagnosis of oxidative stress in male infertility, cryopreservation, age, lifestyle and exposure to environmental toxicants.

Introduction

Reactive oxygen species (ROS) produced by human spermatozoa compromise sperm function [1-5] and as such their detection is important for the diagnosis of male infertility [6]. ROS are typically detected in human spermatozoa using fluorescent probes such as dihydroethidium (DHE), MitoSOX Red (MSR) and 2',7'-dichlorohydrofluorescein diacetate (DCFH) (Figure 1) [7]. DHE is an intracellular ROS probe that fluoresces within both the head and the mitochondrial midsection of the spermatozoa upon oxidation. It is most commonly used for detection of superoxide ($O_2^{\bullet -}$), although it also reacts with hydrogen peroxide (H_2O_2) in the

presence of peroxidases, and with oxidases and cytochromes [8]. MSR is a charged variant of DHE that localises in the mitochondrial matrix to predominantly respond to and measure the generation of $O_2^{\bullet-}$. DCFH is a fluorescein-based nonspecific probe that reportedly reacts with H_2O_2 [9] and other ROS, particularly hydroxyl radicals ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$) [10]. This probe has some disadvantages, since it requires the concomitant presence of peroxidases to react with H_2O_2 [11], can undergo autoxidation and is known to catalyse $O_2^{\bullet-}$ production [9]. An aryl boronate probe reported by Chang et al. [12], peroxyfluor-1 (PF1), reacts with both H_2O_2 and $ONOO^-$; but not $^{\bullet}OH$, $O_2^{\bullet-}$, nitrous oxide (NO) or hypochlorite (^-OCl) [12, 13]. This class of probe has found wide use for the *in vivo* detection of H_2O_2 [14], including research into ROS production in cryopreserved mouse spermatozoa [15]. The ability of aryl boronates to detect the low levels of ROS generated by mammalian spermatozoa suggests this class of probe as a potential diagnostic tool for the selective detection of ROS, particularly H_2O_2 in sperm cells. This would be of clinical significant as several independent studies have indicated that H_2O_2 is particularly damaging to mammalian sperm function [16-18].

A number of aryl boronates have been developed for use in a range biological applications [14]. We chose to use PF1, carboxyPF1 (CPF1) and a new probe 2(2-ethoxyethoxy)ethoxy-PF1 (EEPF1) for the study as they are structurally similar to allow for direct and meaningful comparison, while being relatively easy to prepare on both small and larger scale. This is an important consideration for future work in this area. CPF1 is a variant of PF1 originally synthesised for attachment to other functional groups [19, 20]. EEPF1 contains a truncated polyethylene glycol (PEG) group with increased hydrogen bond acceptors to enhance the aqueous solubility relative to PF1. A series of comparative studies were performed to define the relative ability of all three probes to detect ROS generation by human spermatozoa in a sensitive and selective manner. This study examines the relative capacities of these probes to detect H_2O_2 and $ONOO^-$ spontaneously generated by human spermatozoa exhibiting impaired motility. The results have important diagnostic implications for the facilitated detection of oxidative stress in mammalian spermatozoa exhibiting signs of impaired functionality.

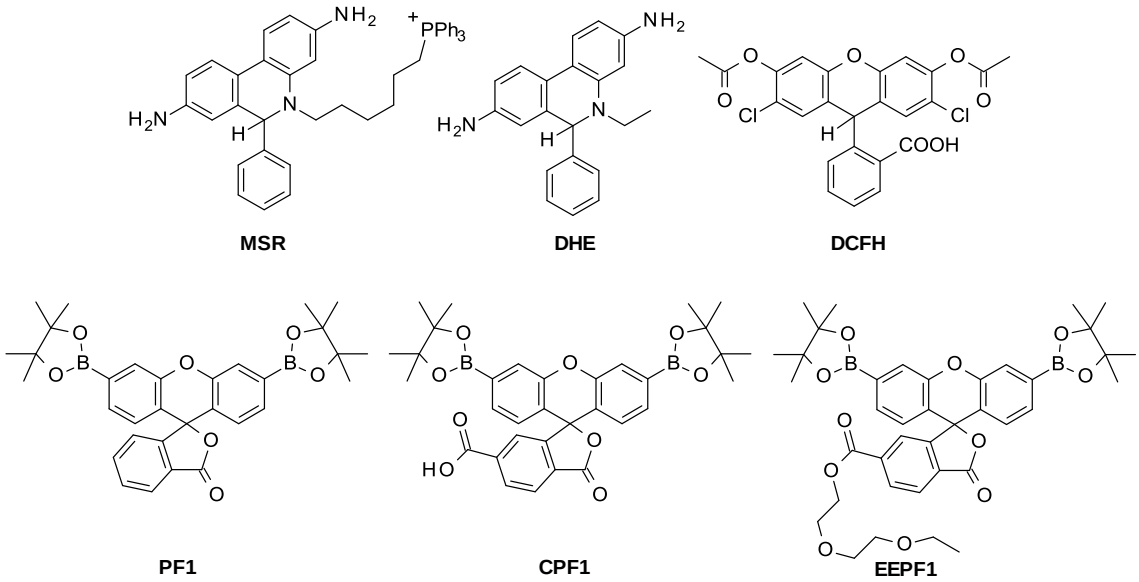


Figure 1. Chemical structures of the ROS sensors used in this study. MSR, DHE and DCFH are oxidised by removal of the indicated hydrogens to produce a fluorescent aromatic structure. PF1, CPF1 and EEPF1 are oxidised by the deprotection of the pinacolatoboron groups to produce highly fluorescent structures.

Materials and Methods

Materials:

Unless otherwise stated all chemicals were purchased from Sigma Aldrich. N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC-HCl) was obtained from GL Biochem (Shanghai). 4-Hydroxynonenal (4HNE) was from Sapphire Biosciences; MitoSox Red, dihydroethidium and Live/dead fixable FAR red stain were from Life Technologies. 2',7'-Dichlorofluorescein diacetate was from molecular probes. Freshly prepared Biggers, Whitten and Whittingham (BWW) medium was used for all experiments, supplemented with 1 mg/ml polyvinyl alcohol, 5 units/ml penicillin and 5 mg/ml streptomycin, and the osmolarity was kept between 290 and 310 mOsm/kg [21].

Semen Samples:

The University of Newcastle human ethics committee and the NSW state Minister for Health approved the use of semen samples for research. A cohort of unselected, normozoospermic donors, mainly university students of unknown fertility status, supplied semen samples for this study. Semen samples were produced into a sterile container and delivered to the laboratory within 1 hour of ejaculation.

Sample Preparation:

Spermatozoa were isolated by discontinuous Percoll gradient centrifugation using a simple 2-step design incorporating 44% and 88% Percoll as described previously [22]. Purified spermatozoa were recovered and washed with HEPES-

buffered BWW supplemented with 1 mg/ml PVA [21], centrifuged at 500 ***g*** for 5 minutes, and resuspended at a concentration of 2×10^7 cells/ml.

Leukocyte Removal:

Where indicated, all residual traces of leukocyte contamination in the sperm suspensions were removed using magnetic beads (Dynabeads, Dynal, Oslo, Norway) coated with a monoclonal antibody against the common leukocyte antigen, CD45 (Invitrogen, Carlsbad, C). Following Percoll isolation, 5×10^6 cells in 100 µl BWW were added to pre-washed antibody-bound Dynabeads and then placed on a rotor for 30 min. Following incubation, each sample was placed in a magnetic holder to separate leukocyte-bound Dynabeads from purified sperm cells in BWW. Luminol-peroxidase mediated chemiluminescence was then used in order to confirm the removal of leukocytes from each sperm suspension; for this purpose 20 µl of zymosan opsonized with autologous serum was added to each 400 µl sample, 5 min from the beginning of the luminometry run [7].

Treatments:

Spermatozoa were treated with menadione (0-50 µM), arachidonic acid (AA; 0-50 µM) and H_2O_2 (0-4 mM) for 15 min at 37 °C. Treatments with 4HNE (0-400 µM) were for 30 min at 37 °C. Stock solutions of menadione were made up fresh daily in dimethyl sulfoxide (DMSO), with a minimum dilution of 1/100 in BWW before being added to spermatozoa.

Staining:

After spermatozoa were treated they were incubated with PF1, CPF1 and EEPF1 for 30 mins @ 37°C at a final concentration of 10 µM. Stock solutions were made up using (DMSO) at a concentration of 10 mM.

Flow Cytometry:

A FACS-Canto Flow Cytometer (Becton Dickinson) was employed using a 488 nm argon laser coupled with emission measurements using the 530/30 band pass (green) FITC channel. Ten thousand sperm events were recorded after non-sperm events were gated out. Data were analysed using BD Diva Software (Becton Dickinson).

Statistical Analysis:

All graphed results are expressed as the mean ± standard error of the mean (SEM). Experiments were replicated at least three times with independent samples. Data was then analysed by one-way analysis of variance (ANOVA) using Graphpad Prism 6, followed by post-hoc comparison by Fisher's LSD (Least Significant Difference).

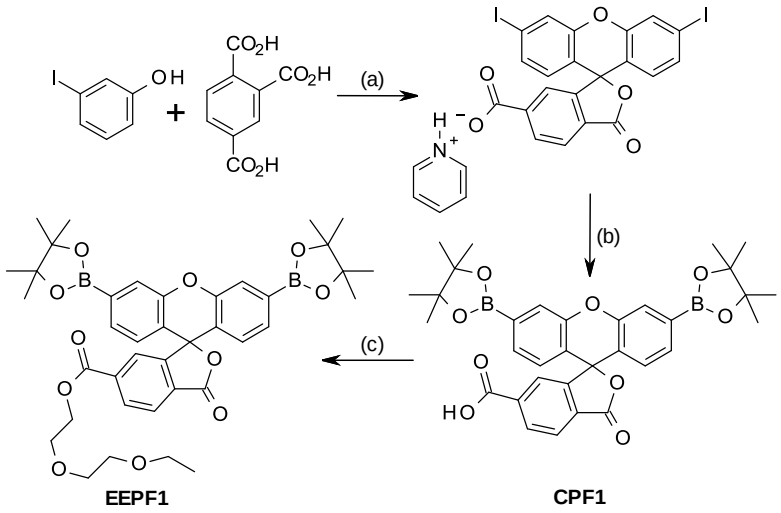
ROS Selectivity Study:

Solutions of PF1, CPF1, EEPF1 and DCFH in 20 mM HEPES buffer at pH 7.4 were treated with 100 µM of ROS: H_2O_2, $ONOO^-$, ^-OCl, $^{\bullet}OH$, $O_2^{\bullet-}$, NO, and *tert*-butyl hydroperoxide (TBHP). A stock solution of approximately 100 mM H_2O_2 in Milli-Q

water was prepared from a 30% H_2O_2 solution in water and the exact concentration was determined by UV absorption at 240 nm (ε_{240} = 43.6 $M^{-1}cm^{-1}$) using a Cary UV-Vis-NIR 5000 Spectrophotometer. A stock solution of NaOCl was similarly prepared and the ^{-}OCl concentration determined by UV absorption at 292 nm (ε_{292} = 350 $M^{-1}cm^{-1}$). A solution of $ONOO^{-}$ was prepared by a known method [23], and its concentration determined using UV absorption at 302 nm (ε_{302} = 1670 $M^{-1}cm^{-1}$). $^{\bullet}OH$ was produced by the Fenton reaction of 100 µM H_2O_2 with 1 mM $FeClO_4$. $O_2^{\bullet-}$ was also produced by a known method [24], using a xanthine/xanthine oxidase system for production of $O_2^{\bullet-}$ and catalase as a scavenger for any H_2O_2 produced. NO was generated from S-nitrosoglutathione, and TBHP was diluted from a stock solution. The ROS were added to each probe and the fluorescence was monitored using a Biotek Synergy H4 fluorescence plate reader (excitation 450 nm, emission 520 nm) over 40 min.

Fluorescence Controls for Menadione, AA and 4HNE

Solutions of PF1, CPF1, EEPF1 in BWW were treated with menadione (0-50 µM), arachidonic acid (0-50 µM) or 4-hydroxynonenal (0-400 µM) to give a final probe concentration of 10 µM. Samples were incubated for 15 min (30 min for 4HNE) at 37°C, then fluorescence emission quantified using a Fluostar Optima (BMG Labtech),with settings of excitation filter 485 ± 10 nm, and emission filter at 520 nm.



Scheme 1: Synthesis of CPF1 and EEPF1. (a) 1. $MeSO_3H$, 140 °C, 72 h 2. Recrystallisation 2:1 acetic anhydride: pyridine [25] (b) Bis(pinacolato)diboron, Pd(dppf)Cl_2, KOAc, DMF, mic. synth., 100 °C, 3 h (c) 1. N-Hydroxysuccinimide, EDC-HCl, DMF, 1 h. 2. 2-(2-ethoxyethoxy)ethanol, 2 h

Synthesis:

Peroxyfluor-1 (**PF1**) was prepared as described [12] using microwave irradiation in place of conventional heating: 3',6'-diiodofluoran[12] (89 mg, 0.16 mmol), bis(pinacolato)diboron (160 mg, 0.63 mmol), potassium acetate (141 mg, 0.63 mmol) and Pd(dppf)Cl_2 (14 mg, 0.02 mmol) pre-dried *in vacuo,* were dissolved in DMF (4 mL) under N_2 atmosphere in a sealed microwave vial fitted with a Teflon cap. The light brown mixture was reacted in a CEM Discover microwave synthesiser

(Matthews, NC) at 80 °C for 2 h. The solvent was removed under reduced pressure to give a dark brown powder which was purified by column chromatography eluting with 4:1 hexane:ethyl acetate to give PF1 as a white solid. (40 mg, 45%) The sample was characterised by 300 MHz proton nuclear magnetic resonance spectroscopy (^{1}HNMR) in deuterated chloroform. ^{1}HNMR data: δ 8.03 (1H, m), 7.74 (2H, s), 7.60 (2H, m), 7.43 (2H, dd, J_1=7.8Hz, J_2=1.1Hz), 7.06 (1H, m), 6.86 (2H, d, J=7.8Hz), 1.35 (24H, s).

CarboxyPeroxyfluor-1 (**CPF1**) [19] was similarly prepared: 3',6'-Diiodo-6-carboxyfluoran pyridinium salt [25] (109 mg, 0.16 mmol), bis(pinacolato)diboron (160 mg, 0.63 mmol), potassium acetate (142 mg, 0.63 mmol) and Pd(dppf)Cl_2 (13.9 mg, 0.017 mmol) were dissolved in dry DMF (4 mL) in an anhydrous N_2 atmosphere. The resultant solution was reacted in a sealed microwave vial sealed with a Teflon cap at 100 °C for 3 h in a CEM Discover microwave synthesiser (Matthews, NC). The solution was evaporated under reduced pressure to give a dark brown powder, which was purified by flash column chromatography eluting with neat ethyl acetate to give CPF1 as a light brown solid. (55 mg, 58%) The sample was similarly characterised: ^{1}HNMR ($CDCl_3$, 300MHz): δ 8.29 (dd, 1H, J_1=7.8Hz, J_2=1.4Hz), 8.11 (d, 1H, J=7.8Hz), 7.79-7.73 (m, 3H), 7.43 (dd, 2H, J_1=7.8Hz, J_2=1.1Hz), 6.81 (d, 2H, J=7.8Hz), 1.35 (s, 24H).

2-(2-Ethoxyethoxy)ethoxy Peroxyfluor-1 (**EEPF1**): CPF1 (50 mg, 0.08 mmol), N-hydroxysuccinimide (11 mg, 0.08 mmol) and EDC-HCl (26 mg, 0.13 mmol) were added to DMF (1 mL) in a dry N_2 glovebox and stirred for 1 h. 2-(2-Ethoxyethoxy)ethanol (23 µL, 0.17 mmol) in dry DMF (0.5mL) was added and the solution stirred for a further 2 h. The solvent was removed under reduced pressure, and the resultant solid was purified by column chromatography eluting with ethyl acetate to give EEPF1 as a white powder. (28 mg, 47%) The sample was similarly characterised by ^{1}HNMR ($CDCl_3$, 500MHz): δ 8.28 (1H, dd, J_1=8.0Hz, J_2=1.0Hz), 8.09 (1H, d, J_1=8.0Hz), 7.76 (2H, s), 7.71 (1H, s), 7.44 (2H, d, J_1=8.0Hz), 6.82 (2H, d, J=7.5Hz), 4.42 (2H, t, J=4.5Hz), 3.76 (2H, t, J=4Hz), 3.61 (2H, t, J=2.75), 3.52 (2H, t, J=4.5Hz), 3.45 (2H, q, J=7Hz), 1.35 (24H, s), 1.14 (3H, t, J=7Hz). This new boronate probe was also characterised by 500MHz carbon 13 nuclear magnetic resonance spectroscopy (^{13}CNMR) and high resolution mass spectrometry (HRMS).^{13}CNMR ($CDCl_3$, 125MHz): δ 168.6, 164.9, 154.0, 150.5, 136.5, 131.2, 130.0, 129.7, 129.4, 129.0, 128.8, 126.9, 125.3, 125.1, 123.8, 120.5, 84.2, 70.6, 69.8, 68.9, 64.8, 24.9, 15.1. HRMS: calculated 712.3226, found 712.3237.

Results and Discussion

ROS Characterisation of Probes

The sensitivity of EEPF1 to H_2O_2 was defined by incubating separate samples with 0-100 µM H_2O_2 and monitoring the resultant fluorescence, see Figure 2A. A clear, dose dependant response is evident, with an emission maximum at 525 nm. EEPF1 was also incubated with 100 µM of each individual ROS to determine the selectivity of EEPF1 for various ROS and the reactivity profiles are summarised in Figure 2B. For comparison, the reaction of DCFH with each ROS was also characterised and

the results are shown in Figure 2C. Similar reactivity profiles for PF1 and CPF1 are reported in the supplementary data. EEPF1 showed good reactivity with H_2O_2, and although there is a significant initial fluorescence response from $ONOO^-$, a greater overall fluorescence was observed for H_2O_2 over 40 min. This is likely due to the more transient nature of $ONOO^-$ compared with H_2O_2. Limited fluorescence was observed for the reaction of EEPF1 with all other ROS studied, with a similar result for the other aryl boronates (Figure S1). By contrast, DCFH reacts best with both $ONOO^-$ and $^{\bullet}OH$ (Figure 2C) and to a lesser extent with ^-OCl and $O_2^{\bullet-}$, but not H_2O_2, as shown in Figure 2C. This lack of reactivity to H_2O_2 is consistent with some literature [10], but contrasts other reports that infer the detection of H_2O_2 using this probe [9]. However, DCFH is able to detect other ROS such as $^{\bullet}OH$ produced from H_2O_2 *in* vivo, and also H_2O_2 in the presence of peroxidases [11]. Thus, the aryl boronates have a clear advantage over DCFH for the detection of H_2O_2 in the absence of any external catalyst.

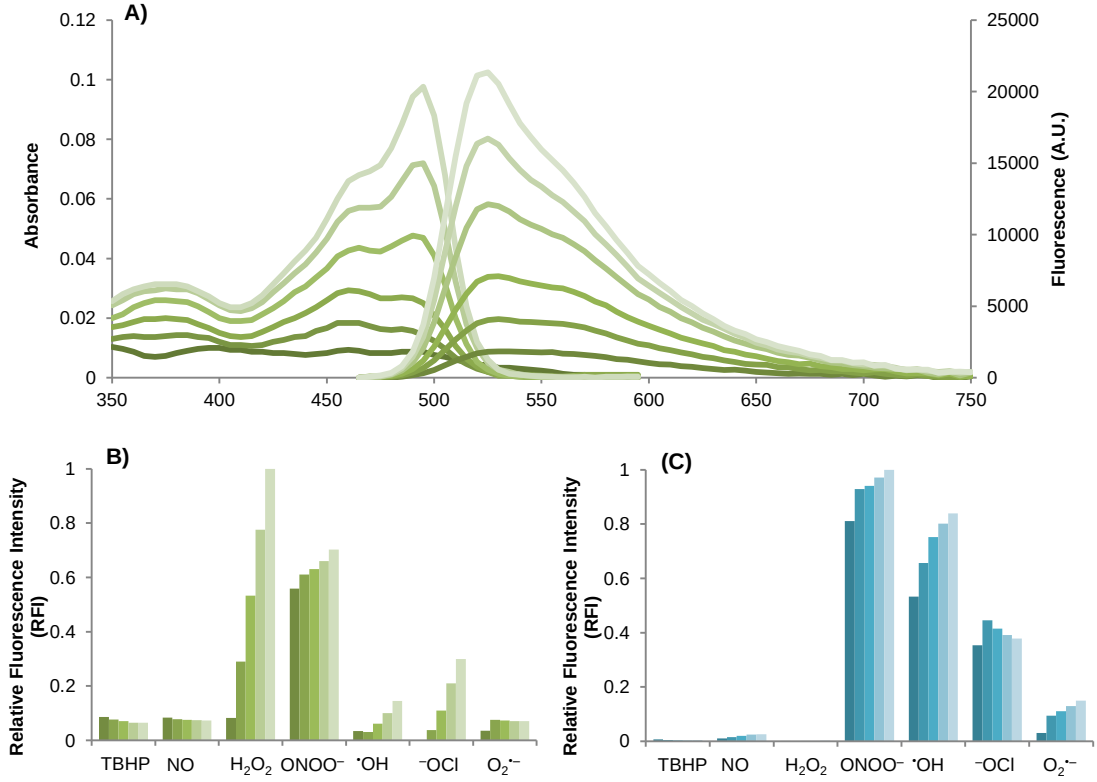


Figure 2. Fluorescence characterisation of EEPF1 and comparison with DCFH for selectivity to ROS. A) Absorption and emission spectra of EEPF1 when treated with 0, 10, 25, 50, 75 and 100 µM H_2O_2 and incubated at 37 °C for 40 min in 20 mM HEPES buffer. (Excitation at 450 nm). B) EEPF1 and C) DCFH selectivity data, each incubated at 37 °C with 100 µM ROS and measured at 0, 10, 20, 30 and 40 min. (Excitation 450 nm, emission 520 nm).

Comparative Study on ROS Production in Human Spermatozoa

The ability of the aryl boronates PF1, CPF1, EEPF1 and other probes described in the literature (the dihydrofluorescein DCFH and the hydroethidiums DHE and MSR)

to detect ROS in spermatozoa was then studied in populations of human spermatozoa, with a view to defining their relative abilities to detect cellular ROS generation. ROS production in human spermatozoa was induced by treating samples with three different compounds, menadione, AA and 4HNE [7]. Menadione is a redox cycling quinone known to produce significant oxidative stress due to quinone oxidoreductase activity [26, 27]. AA is a cis-unsaturated fatty acid associated with the production of $O_2^{\bullet-}$ by sperm mitochondria [28, 29], while 4HNE is a lipid-derived aldehyde responsible for the induction of mitochondrial ROS generation in senescent spermatozoa [30]. Further samples were also treated with 4mM H_2O_2 as a positive control for probes sensitive to H_2O_2 (DCFH, PF1, CPF1, EEPF1). The ROS-producing compounds were incubated separately with each probe (DCFH, DHE, MSR, PF1, CPF1 and EEPF1) and the resulting fluorescence response was measured by flow cytometry.

Figure 3 shows the percentage of fluorescent spermatozoa, indicating the percentage of spermatozoa that generated ROS as measured by each probe. MSR showed the largest background fluorescence in the negative control samples (Figure 3A), with significantly higher fluorescent populations ($P < 0.05$) than aryl boronates PF1 and CPF1, presumably reflecting the active generation of mitochondrial ROS by populations of human spermatozoa, as previously described [31]. For the treated sperm samples, those incubated with DHE exhibited the smallest fluorescent populations for all stimuli (Figures 3B-D). Conversely, those stained with EEPF1 consistently showed the largest fluorescent populations, with over 90% responding positively following treatment with the ROS-generating reagents (Figures 3B-D). PF1 also showed readily measurable fluorescent responses to the stimuli, with comparable or larger fluorescent populations than DCFH, DHE and MSR (Figures 3B-D). Staining spermatozoa with CPF1 gave the lowest fluorescent populations of all three aryl boronates. Nevertheless, treated sperm were more fluorescent with CPF1 than with DHE and DCFH within the sperm treated with AA (Figure 3C) and 4HNE (Figure 3D) treatment groups.

Thus the aryl boronates PF1 and EEPF1 were the most broadly sensitive of the probes to ROS produced by spermatozoa on stimulation with menadione, AA and 4HNE (Figures 3B-D) with the latter clearly being the most sensitive. The third aryl boronate (CPF1), while sensitive, gave less consistent results. Of the other probes, MSR gave the greatest response to each stimuli (Figure 3B-D), while DHE was the least sensitive.

The fluorescent populations shown in Figure 3 also provide some insights into which ROS are produced by spermatozoa on treatment with menadione and 4HNE (results for AA are discussed separately, see Figure 4). Thus for samples stained with MSR, a smaller proportion of fluorescent cells was apparent for 4HNE treatments (Figure 3D) compared to menadione (Figure 3B), suggesting that the latter is the more efficient stimulator of $O_2^{\bullet-}$ production. In contrast, fluorescence of the aryl boronates PF1, CPF1 and EEPF1 were similar between menadione and 4HNE treatments (Figures 3B & 3D), indicating similar levels of H_2O_2/$ONOO^-$ production. This suggests an efficient conversion of $O_2^{\bullet-}$ (detected by MSR) to H_2O_2 or $ONOO^-$ (detected by the aryl boronates) in those samples treated with 4HNE. However, similar to MSR, DCFH showed a lack of sensitivity for the ROS produced in response

to 4HNE (Figure 3D) compared with the menadione (Figure 3B) treatments. Yet DCFH is also known to react with H_2O_2 and $ONOO^-$, as do the aryl boronates, suggesting that DCFH should have similar sensitivity to the 4HNE treatment as to menadione. This incongruity could be due to the production of $^{\bullet}OH$ in samples treated with menadione and not those treated with 4HNE. The $^{\bullet}OH$ radical reacts with DCFH but not with the aryl boronates, which would result in the greater positive populations observed for DCFH in samples treated with menadione than 4HNE (Figures 3B & 3D). Another likely explanation is the need for oxidation of DCFH to be facilitated by peroxidase which may not be trivial due to the highly compartmentalised nature of the spermatozoon, limiting the distribution and hence catalytic availability of the peroxidase. This again highlights the significant advantage of the aryl boronates over DCFH in this regard, as no external catalysis is required to produce a fluorescent response.

We next investigated the fluorescence of PF1, CPF1 and EEPF1 on incubation with menadione, AA and 4HNE in absence of spermatozoa, to directly assess the reactivity of the probes to these compounds. Menadione (12.5, 25, 50µM), AA (12.5, 25, 50µM) and 4HNE (100, 200, 400µM) were separately incubated with PF1, CPF1 and EEPF1 and the resulting fluorescence measured. A fluorescent response was not observed when PF1, CPF1 or EEPF1 were incubated with menadione or 4HNE (see Figure 4). However, AA did cause a dose-dependent response with PF1 ($r = 0.9999$), CPF1 ($r = 0.9898$) and EEPF1 (0.9994) as shown in Figure 4. This is possibly due to auto-oxidation of AA, generating a hydroperoxide [32] capable of deprotecting the aryl boronates of the probes, thus leading to a fluorescent response. This may explain the observed increase in fluorescent population for PF1, CPF1 and EEPF1 in the presence of AA compared to those populations stained with DCFH, DHE and MSR. AA was not used to induce ROS production in further experiments using spermatozoa with PF1, CPF1 and EEPF1.

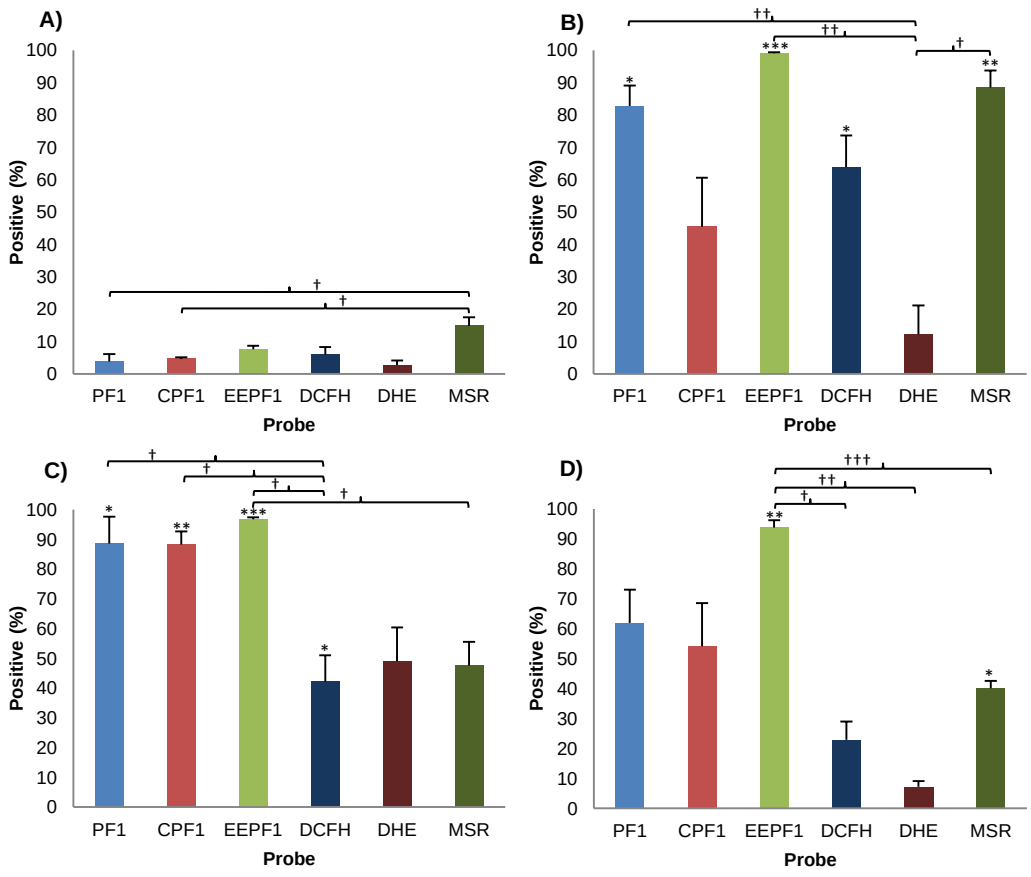


Figure 3. Analysis of flow cytometry results, showing the percentage of human sperm populations which indicated a fluorescent response. A) Negative control sample with no external stimulus. Samples exposed to stimuli: B) 50 µM menadione for 15 min, C) 50 µM AA for 15 min or D) 200 µM 4HNE for 30 min. Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001 compared with untreated sample. †P < 0.05, ††P < 0.01, †††P < 0.001 as compared on graphs.

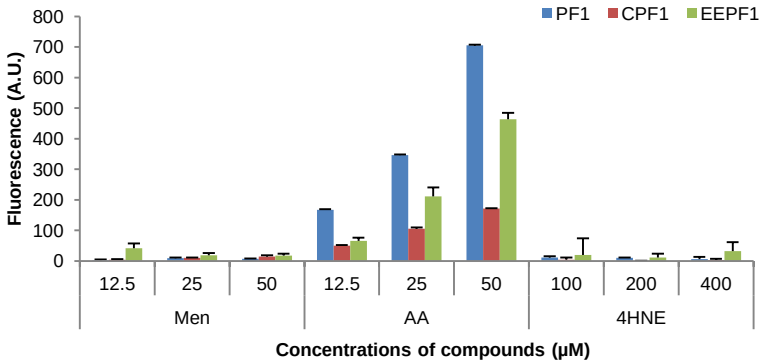


Figure 4. Fluorescence response of PF1, CPF1 and EEPF1 to menadione, AA and 4HNE in the absence of spermatozoa. Fluorescence readings were recorded in BWW solution after 30 min of incubation at 37 °C by a microplate reader. Excitation wavelength was 485 nm, emission recorded at 520 nm. Each probe gave a dose-dependent response upon exposure to AA, however no significant response was recorded when incubated with either menadione or 4HNE.

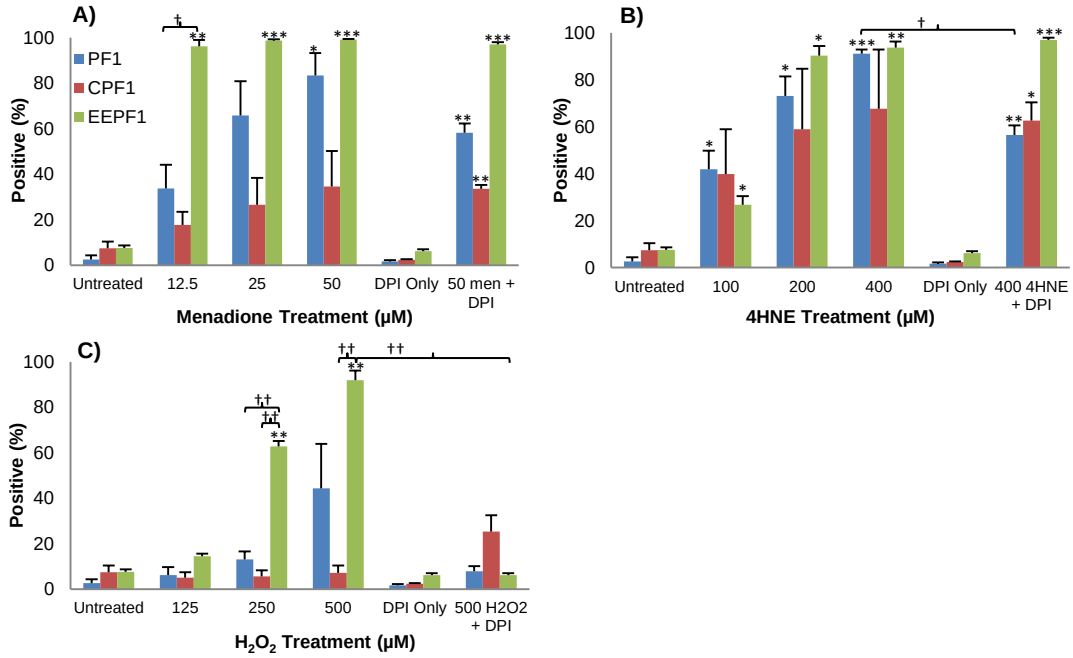


Figure 5. Analysis of flow cytometry results for menadione, 4HNE and H_2O_2 treated spermatozoa. A) Menadione treated spermatozoa exhibit dose-response correlation with PF1 and CPF1; however EEPF1 show large positive populations to 12.5, 25 and 50 μM treatments. B) PF1, CPF1 and EEPF1 all exhibit dose-response correlations to sperm stimulated with 4HNE. C) EEPF1 show a dose-response correlating to spermatozoa treated with H_2O_2 concentrations 0-500 μM. Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001 compared with untreated sample. †P < 0.05, ††P < 0.01 as compared on graph.

Sensitivity of Aryl Boronates to ROS Production in Human Spermatozoa

Dose-dependent studies were subsequently carried out to further define the sensitivity of the three new aryl boronates PF1, CPF1 and EEPF1 in spermatozoa stimulated with menadione, 4HNE and H_2O_2. Diphenylene iodonium (DPI), an inhibitor of NO and $O_2^{\bullet-}$ production by flavoproteins, was used to further test the sensitivity of the probes for ROS generation.

Menadione

Populations of spermatozoa treated with menadione and incubated with EEPF1 show the largest fluorescent populations, see Figure 5A. This result is consistent with the earlier study comparing the aryl boronates to previously studied probes, where EEPF1 gave the greatest fluorescent response of the six probes as shown in Figure 3. However, the populations stained with EEPF1 (Figure 5A) were over 90 % positive at even the lowest concentration of menadione (12.5 μM), significantly larger than PF1 ($P < 0.05$). Populations stained with PF1 also revealed a dose-dependent increase in activity when treated with 12.5-50 μM menadione (Figure 5A, $r = 0.937$). In contrast, the fluorescent responses of populations stained with CPF1 were not statistically significant even at 50 μM menadione (Figure 5A). Thus it appears that the new probe EEPF1 is the most effective of the probes for ROS production in spermatozoa on stimulation with menadione. The existing aryl boronate PF1 is

significantly less effective, while CPF1 is the least effective of all three. Microscope images of spermatozoa stained with CPF1 and EEPF1 were obtained (see supplementary data). ROS produced by both the head and the mitochondria-rich midpiece can be seen, indicating that neither CPF1 nor EEPF1 stained specifically for a location inside the cell.

Co-incubation of DPI with 50 µM menadione did not significantly decrease the positive population for EEPF1 and although suggested, no statistically significant reduction in signal was recorded in the presence of PF1 and CPF1 (Figure 5A) when treated with DPI. Overall, incubation with menadione indicated greater sensitivity of EEPF1 over PF1 and CPF1, suggesting that it may be a particularly useful probe for the detection of intracellular ROS.

4HNE

Figure 5B shows the fluorescent responses for spermatozoa treated with 4HNE. Samples stained with PF1 or EEPF1 revealed significant fluorescent populations at 100, 200 and 400 µM treatments of 4HNE. However those samples incubated with CPF1 did not produce a significant fluorescent response as a consequence of the large variation associated with these measurements. The signal generated in the presence of PF1 was significantly reduced ($P < 0.05$) in the presence of DPI. However, incubating with DPI did not reduce the fluorescent response for samples treated with EEPF1, again suggesting an increased efficacy over PF1. From these results it is clear that both PF1 and EEPF1 were capable of detecting significant H_2O_2 or $ONOO^-$ production by 4HNE-stimulated spermatozoa.

H_2O_2

EEPF1 gave the greatest fluorescent response to ROS in populations of spermatozoa treated with H_2O_2, see Figure 5C. These fluorescent populations correlated with the concentration of H_2O_2 added ($r = 0.946$, $P < 0.01$). Figure 5C also shows that in contrast, samples stained with PF1 only gave a limited increase in positivity at 250 µM and 500 µM of H_2O_2 while CPF1 generated a negligible fluorescence response, even for samples treated with up to 500 µM of H_2O_2. These results again indicate greater efficacy of EEPF1 over PF1 and CPF1 for the detection of ROS produced by human spermatozoa stimulated with H_2O_2.

The mechanism by which H_2O_2 stimulates enhanced ROS generation by human spermatozoa is thought to involve the induction of lipid peroxidation followed by the covalent binding of lipid aldehydes such as 4HNE to proteins in the mitochondrial electron transport chain (ETC), particularly, succinic acid dehydrogenase [23]. The adduction of proteins within the ETC is, in turn, thought to lead to electron leakage and sustained ROS generation. The fact that DPI, an inhibitor of flavoproteins involved in mitochondrial electron transport such as succinic acid dehydrogenase, could significantly impair the ROS response to H_2O_2 as detected by EEPF1 is in good agreement with this model.

In light of these results, EEPF1 is clearly able to sense ROS produced by human spermatozoa more effectively than PF1, CPF1, DCFH, DHE or MSR. Hence, we suggest that EEPF1 should be used in preference to DCFH, DHE or PF1, particularly

for detection of low concentrations (0-100 μM) of H_2O_2 or $ONOO^-$ in human spermatozoa.

The different fluorescent responses of PF1, CPF1 and EEPF1 to ROS produced by spermatozoa (Figures 3 and 5) also provide some preliminary insights into structure-activity relationships for the aryl boronate class of probe. CPF1 consistently detected lower numbers of reactive spermatozoa than PF1 and EEPF1. As fluorescein-based probes are susceptible to photobleaching, 10 μM solutions of CPF1 and PF1 were irradiated with a 100 mW 488 nm argon laser to ensure the cause for differing fluorescent responses was not a photobleaching effect. CPF1 and PF1 showed comparable rates of photobleaching, as such, the reduced CPF1 positive populations observed in Figure 5 are not the results of differences in rates of photobleaching. However, for spermatozoa to be analysed by flow cytometry, the probe must be able to cross the plasma membrane in order to react with intracellular ROS and generate a fluorescent signal. It is therefore likely that the impaired cellular uptake of CPF1 is responsible for its lack of activity. Any localised fluctuation in pH would affect the ionisation of the carboxyl group of CPF1, which would, in turn, be expected to influence cell permeability and hence the intracellular concentration of this probe. By contrast, EEPF1 is esterified with a truncated PEG (see Scheme 1) so would be expected to possess a higher capacity for intracellular penetration. Furthermore, hydrolysis of the PEG ester by intracellular esterases may enhance cellular retention of the active species, as reported for a similar aryl boronate probe, PF6-AM [33].

Spontaneous ROS Generation by Human Spermatozoa

Finally, the use of PF1, CPF1 and EEPF1 to detect spontaneous ROS generation by human spermatozoa was investigated to validate the use of these probes for detecting the increased ROS production associated with poorly motile sperm. To this end, the spermatozoa were separated on discontinuous Percoll gradients into subpopulations exhibiting high and low levels of motility respectively [7] (see methods section for detail). These sperm populations were then separately incubated with either PF1, CPF1 or EEPF1. Figure 6 clearly demonstrates the increased generation of ROS by poorly motile spermatozoa compared with their more motile counterparts. A relative increase in the proportion of ROS–generating cells was detected in the poorly motile cells with PF1, CPF1 and EEPF1. The largest increase was seen with EEPF1, which detected ROS generation in around 40% of the poorly motile cells compared with < 15% with PF1 and CPF1. These results confirm EEPF1 as the most effective of these probes for the detection of released ROS in human spermatozoa. As such, EEPF1 is recommended for use as an intracellular probe for detection of ROS in human spermatozoa.

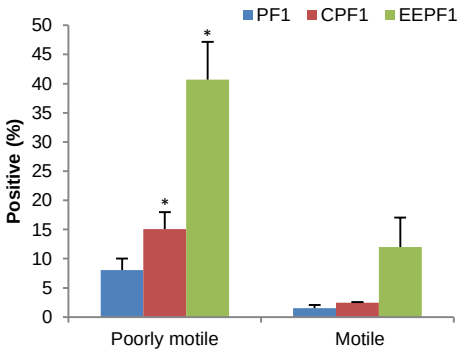


Figure 6. Analysis of flow cytometry results showing the percentage of poorly motile and motile samples of human spermatozoa populations indicating a fluorescent response. EEPF1 provides a greater fluorescent response to the ROS produced in poorly motile sperm. Significance level relative to motile sample: *P < 0.05

Conclusion

The aryl boronate probes discussed here react directly with H_2O_2 and therefore present a distinct advantage over DCFH, which we show to be insensitive to H_2O_2. PF1 and EEPF1 were also shown to be effective fluorescent probes for the detection of both H_2O_2 and $ONOO^-$ in human spermatozoa. Both PF1 and EEPF1 were significantly more effective at detection of ROS by flow cytometry compared to DCFH and DHE when stimulated using menadione and 4HNE. In particular, EEPF1 was the most effective of the studied probes for externally stimulated and spontaneously generated ROS produced by human spermatozoa. This particular probe should therefore have a significant role to play in the diagnosis of oxidative stress in spermatozoa in the context of a variety of circumstances including spontaneous male infertility, cryopreservation, age, lifestyle and exposure to environmental toxicants.

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