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# The development and *in vitro* characterisation of an intracellular nanosensor responsive to reactive oxygen species

James R. Henderson[a,*], David A. Fulton[b], Calum J. McNeil[a], Philip Manning[a]

[a] *Diagnostic & Therapeutic Technologies, Institute of Cellular Medicine, The Medical School, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK*
[b] *School of Chemistry, Newcastle University, NE1 7RU, UK*

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ABSTRACT

Advances in sensor technologies have enhanced our understanding of the roles played by reactive oxygen species (ROS) in a number of physiological and pathological processes. However, high inter-reactivity and short life spans has made real-time monitoring of ROS in cellular systems challenging. Fluorescent dyes capable of intracellular ROS measurements have been reported. However, these dyes are known to be intrinsically cytotoxic and thus can potentially significantly alter cellular metabolism and adversely influence *in vitro* data. Reported here is the development and *in vitro* application of a novel ROS responsive nanosensor, based on PEBBLE (Probes Encapsulated By Biologically Localised Embedding) technology. The ROS sensitive fluorescent probe dihydrorhodamine 123 (DHR 123) was employed as the sensing element of the PEBBLE through entrapment within a porous, bio-inert polyacrylamide nanostructure enabling passive monitoring of free radical flux within the intracellular environment. Successful delivery of the nanosensors into NR8383 rat alveolar macrophage cells via phagocytosis was achieved. Stimulation of PEBBLE loaded NR8383 cells with phorbol-12-myristate-13-acetate (PMA) enabled real time monitoring of ROS generation within the cell without affecting cellular viability. These data suggest that PEBBLE nanosensors could offer significant advantages over existing technologies used in monitoring the intracellular environment.



## 1. Introduction

Reactive oxygen species (ROS) are known to play important roles in cellular systems and therefore enhancing our understanding of the contributions made by ROS to both pathological and physiological processes has been the focus of much interest (Aitken et al., 2007; Babior, 1984; Babior et al., 1973; Brechard and Tschirhart, 2008; Drose and Brandt, 2008; Goud et al., 2008). Reactive oxygen species are important in the clearance of invading microorganisms by phagocytic cells of the innate immune system. Individuals whose cell-mediated immune system cannot produce adequate amounts of ROS can develop chronic granulomatous disease (CGD), highlighting the clinical importance of ROS in the immune system (De Ravin et al., 2008; Feld et al., 2008; Kang and Malech, 2009). However, during periods of oxidative stress, when ROS production exceeds the tissue's ability to counteract the negative effects of increased ROS levels, pathological processes such as migraine, ischaemic injury and cardiovascular disease can develop (Bindokas et al., 1996; Cross et al., 1987; Heurtaux et al., 2004; Khalil and Khodr, 2001; Khodr and Khalil, 2001; Zulueta et al., 1997). Increasing our awareness of the exact role that individual species, and ROS in general, play during periods of oxidative stress could enhance our ability to treat and counteract such pathologies. The high inter-reactivity and short lifespan of ROS makes *in vitro* monitoring of these species especially challenging. However, the development of advanced analytical systems has, in part, allowed *in vitro* ROS levels to be monitored directly and in real-time using a diverse range of techniques (Chang et al., 2005a,b; Manning et al., 1998, 2001; McNeil et al., 1989, 1992; McNeil and Manning, 2002; Scheller et al., 1999).

Clark and co-workers (1998) reported the development of an optical nanosensor technology, known as PEBBLE (Probes Encapsulated By Biologically Localised Embedding), capable of reporting changes that occurred in the intracellular environment of cultured cells. Since then PEBBLE nanosensors responsive to a number of different analytes, including pH, calcium and glucose have been described (Clark et al., 1999a; Clark et al., 1999b; Hammond et al., 2008; Sumner et al., 2002; Xu et al., 2002). Reported here is the development and application of a ROS responsive nanosensor based on the commercially available fluorescent probe, dihydrorhodamine 123 (DHR 123). DHR 123 has been

*Abbreviations:* Ham's F12K, Kaign's Modification of Ham's F12 medium; L-NAME, *N*-nitro-L-arginine methyl ester; PMA, phorbol-12-myristate 13-acetate; XOD, xanthine oxidase.

* Corresponding author. Tel.: +44191 222 7991; fax: +44191 222 7991.
*E-mail address:* J.R.Henderson@newcastle.ac.uk (J.R. Henderson).

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shown to respond to a number of species including superoxide ($O_2^{-}\cdot$), hydrogen peroxide ($H_2O_2$) and peroxynitrite ($ONOO\cdot$) (Chang et al., 2005a; Crow, 1997; Goud et al., 2008; Henderson and Chappell, 1993; Qin et al., 2008; Roesler et al., 1991), which oxidise DHR 123 to yield the fluorescent product rhodamine 123 (R 123). The fluorescence observed is therefore directly proportional to intracellular concentrations of ROS making DHR 123 an ideal candidate for the sensing element of a generic ROS responsive PEBBLE nanosensor. It is important to note that this sensor technology was developed not to assess the effect of one specific species on the cell but to gauge temporal fluxes in the general level of oxidative stress within the intracellular environment.

Membrane permeable 'free' dyes and particle-conjugated probes have been extensively reported in the literature (Chang et al., 2005a; Henderson and Chappell, 1993; Jiang et al., 1997; Palazzolo-Ballance et al., 2007). However, the use of fluorescent dyes encapsulated in the polyacrylamide matrix of PEBBLE nanosensors offers a number of advantages over previously reported probes (Clark et al., 1998; Webster et al., 2005). First, the bio-inert matrix of the PEBBLEs protects the intracellular environment from any potentially cytotoxic effects that long-term exposure to fluorescent probes may illicit. Simultaneously, through size exclusion, the matrix enables minimal interaction between non-specific interferents such as cellular proteins and the encapsulated probes. This minimises the occurrence of false positives as highlighted by Sumner et al. (2002). Such properties also allow the sensors to remain within cells for extended periods of time without compromising sensitivity. A further advantage is that PEBBLE technology allows ratiometric sensors to be fabricated through the co-entrapment of an analyte sensitive probe with a reference dye within the polymer matrix. In this study the reference dye used was Alexa Fluor$^{568}$ conjugated to 10,000 mw Dextran, co-immobilised with DHR 123. The ratiometric nature of the PEBBLEs helps to eliminate sensor variations caused by any environmental changes, e.g. temperature or photobleaching of the probes, therefore reducing the chance of erroneous data. Further to this, the small size of the sensors (20–200 nm in diameter) allows delivery into cells with minimum perturbation or membrane damage.

Prior to delivering the nanosensors into the intracellular environment the PEBBLEs required physical characterisation to ensure dye entrapment, consistency of size and responsiveness to ROS. These three criteria were addressed using $^1$H NMR spectroscopy, dynamic light scattering measurements and enzymatically generated ROS, respectively.

A number of methods have been investigated for delivering PEBBLEs into the intracellular domain of mammalian cells, including cell penetrating peptides (CPP), liposomal transfection, Gene Gun® and cell directed delivery (CDD) e.g. phagocytosis (Webster et al., 2007). In this study ROS sensitive PEBBLEs were delivered into macrophage cells through phagocytosis. Successful introduction of the nanosensors into the cells would enable real-time fluxes in ROS generation within the intracellular environment to be measured. The impact of these sensors on normal cellular physiology would be assessed using the MTT cytotoxicity assay. Further evidence of cellular perturbation could be gained from the use of highly selective chronoamperometric electrodes capable of monitoring real-time changes in key ROS in the extracellular environment. Specifically nitric oxide (NO) and $H_2O_2$ detection would give a partial but none the less significant indication of the ability of macrophages to release these species into the extracellular environment following loading with nanosensors. Such observations would give valuable insight into what impact the presence of the nanosensors may have on macrophage cell function.

## 2. Materials and methods

### 2.1. Cell culture

The rat alveolar macrophage cell line, NR8383 (ATCC via LGC Promochem, UK) was cultured in Kaign's Modification of Ham's F12 medium (Ham's F12K) (LGC Promochem, UK) modified with 15% foetal calf serum (Sigma, UK), at 37 °C in a humidified, 5% $CO_2$ atmosphere.

### 2.2. Nanosensor synthesis and characterisation

The polyacrylamide PEBBLE nanosensors were synthesised using a modified version of the methods reported previously (Clark et al., 1999b; Coupland et al., 2008; Webster et al., 2005). In brief, a solution consisting of acrylamide and *N*,*N*′-methylenebisacrylamide (Sigma–Aldrich, UK) at a ratio of 1:3.37, respectively, was produced by dissolving in distilled water and sonicating for 5 min. To this 20 μl of a 10 mg/ml solution of DHR 123 (Sigma–Aldrich, UK) and 20 μl of a 10 mg/ml solution of Alex Fluor$^{568}$-10,000 mw Dextran conjugate (Invitrogen, UK) were added and gently mixed. Surfactants Brij® 30 (Fluka, UK) and Dioctyl sulphosuccinate sodium salt (98%) (Sigma–Aldrich, UK) at a ratio of 1:1.94 were added to 42 ml of deoxygenated hexane to produce a microemulsion. The microemulsion was stirred continuously in an oxygen-purged environment for 15 min before adding 2 ml of the aqueous phase acrylamide-fluorescent probe solution. Polymerisation was initiated through the addition of 30 μl of ammonium persulphate (10%, w/v) (Sigma–Aldrich, UK) followed by 15 μl of *N*,*N*,*N*′,*N*′-tetramethylethylethylenediamine (Sigma–Aldrich, UK). The reaction was allowed to proceed for a minimum of 2 h under oxygen-purged conditions at room temperature, stirring constantly. All hexane was then removed through rotary evaporation yielding a nanosensor slurry which was rinsed repeatedly in absolute ethanol until all residual surfactant was removed. A final filtration step to separate the nanosensors from the ethanol was carried out and the sensors dried and stored in air tight containers at 4 °C prior to use.

### 2.3. PEBBLE characterisation by $^1$H NMR spectroscopy

$^1$H NMR spectroscopy of the PEBBLEs, performed on a JEOL Lamda500 spectrometer operating at 500.16 MHz, further confirmed the presence of the fluorescent dyes within the sample. The samples were dissolved in $D_2O$ and the water signal was suppressed during acquisition.

### 2.4. PEBBLE sizing by dynamic light scattering measurements

The average PEBBLE size was obtained prior to use. Dynamic light scattering was performed with a Wyatt DAWN HELEOS II instrument fitted with a QELS detector operating in batch mode at 30 °C with a 635 nm laser. Measurements were made at a detection angle of 99° and Wyatt ASTRA V software was utilized to analyse the data. The hydrodynamic radius was calculated using a non-linear least squares fitting algorithm contained within the ASTRA V software to fit the measured correlation function. The particle size distribution analysis was performed with the DYNALS regularization algorithm contained within the ASTRA V software. All determinations were made in triplicate. Samples were prepared by dissolving PEBBLEs in deionised water (concentration 1 mg/ml) then sonicating for 30 min. The samples were analysed both neat and after filtration through a 0.2 μm cellulose acetate syringe filter (Sartorius, UK), giving identical results. Particle size distributions were estimated using the regularization analysis within the ASTRA V software.