Biosensors and Bioelectronics 51 (2014) 274–279

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Short communication

Whole-cell *Escherichia coli*-based bio-sensor assay for dual zinc oxide nanoparticle toxicity mechanisms

Jonathan S. Mcquillan, Andrew M. Shaw*

Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter EX4 4QD, UK

ARTICLE INFO

Article history:
Received 8 April 2013
Received in revised form
13 June 2013
Accepted 12 July 2013
Available online 22 July 2013

Keywords:
Zinc oxide nanoparticle
Escherichia coli
Stress response
Whole cell biosensor
Real time PCR

ABSTRACT

A whole-cell biosensor assay for dual ZnO nanoparticle toxicity mechanisms has been developed based on the transcriptional response of *Escherichia coli* to: (1) Zn^{2+} from ZnO nanoparticle dissolution with genes *zntA* (Zn^{2+} efflux) and *znuABC* (Zn^{2+} uptake); and (2) redox stress from ZnO nanoparticle photo-electron production under ultraviolet light with genes *soxS* and *katG*. Both processes occur in a dispersion of ZnO nanoparticles leading to toxicity. ZnO nanoparticle dissolution was measured independently by ICP-MS and photo-radical generation was confirmed by the stochiometric reduction of the redox dye, 2, 6-dichloroindolphenol (DCPIP). The whole-cell biosensor can detect both toxicity mechanisms and is a species-specific assay capable of discriminating between ZnO nanoparticles and the Zn^{2+} dissolution product.

1. Introduction

Zinc oxide nanoparticles are produced in high tonnage industrially for incorporation into a number of products including sunscreen and cosmetics, ultimately ending up in the environment with potential toxicological effects (Klingshirn, 2007). The direct mechanism for ZnO nanoparticle toxicity is dissolution and release of zinc ions (Gulson et al., 2010; Miao et al., 2010). A second toxicity mechanism is the generation of photo-radicals by ZnO nanoparticles in ultraviolet light, promoting electrons across the material band gap (Comparelli et al., 2005; Hoffmann et al., 1995; Mills and Le Hunte, 1997). The two toxicity mechanisms together offer a species-specific assay for ZnO nanoparticles in complex media. Both mechanisms can be ameliorated by the presence of surface ligands (Mitchnick et al., 1999; Waalewijn-Kool et al., 2013) but the efficacy of the ligand-induced reduction in toxicity requires a reliable, quality assured assay. However, both toxicity mechanisms are difficult to measure physically in the environment or complex media where zinc ions are quickly dispersed and free radicals are rapidly neutralised with half-lives in the order of nanoseconds.

Whole cell biosensors offer an alternative approach to physical methods for detecting toxic substances (Bousse, 1996), specifically where transcriptional biomarkers for a toxic stress response are measured minutes-hours following exposure. These typically feature promoter-reporter gene fusion constructs whereby positive regulation of the gene corresponds to an increase in a detectable product (Mitchell and Gu, 2004; Riether et al., 2001). Specifically, recombinant reporter strains of *E. coli* have been used to probe Zn^{2+} toxicity (Ivask et al., 2010; Ivask et al., 2002, 2009). However, an alternative approach is to detect changes in gene regulation directly in non-recombinant strains by measuring levels of the corresponding gene transcript by real-time PCR. This allows simultaneous measurement of two toxicity mechanisms using multiple transcriptional biomarkers for the corresponding stress responses as it does not require individual promoter-reporter constructs for each gene.

E. coli bacteria are particularly suited as whole-cell biosensors as the role of individual genes and proteins has been intensively studied (Keseler et al., 2011). Specifically, the organism responds to excess Zn^{2+} by regulating the expression of genes encoding divalent cation-specific transporters that control the flux of Zn^{2+} across the plasma membrane (Yamamoto and Ishihama, 2005). Specifically, ZnuABC is a Zn^{2+} import protein (Patzer and Hantke, 1998), which is regulated through the activity of the Zinc Uptake Regulator (Zur) (Patzer and Hantke, 2000), and ZntA is a Zn^{2+} efflux protein (Beard et al., 1997; Binet and Poole, 2000; Rensing et al., 1997) which is regulated through the activity of ZntR (Brocklehurst et al., 1999; Outten et al., 1999). The genes are inversely regulated in response to femtomolar changes in the free Zn^{2+} concentration in the cytosol (Outten and O'Halloran, 2001). Similarly, the *E. coli* SoxR and OxyR proteins are redox-dependant switches that regulate the expression of genes to counteract oxidative stress (Demple, 1999). SoxR is activated by oxidation of redox-sensitive Fe–S clusters and promotes transcription of a

* Corresponding author. Tel.: +44 1392263495; fax: +44 1392263434.
E-mail address: andrew.m.shaw@exeter.ac.uk (A.M. Shaw).

0956-5663/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2013.07.024

second transcription factor, SoxS which regulates multiple anti-oxidant functions including expression of superoxide dismutase genes *sodA* and *sodC*. OxyR is activated by oxidation of cysteine residues and di-sulfide bond formation leading to gene regulatory activity including the positive regulation of *katG*, a gene encoding Catalase; a potent antioxidant.

In this study, we use the *E. coli* bacterium as a whole cell biosensor for simultaneous detection of the ZnO nanoparticle toxicity mechanisms. We show physically the dissolution of ZnO nanoparticles in a complex medium supporting bacterial growth (Neidhardt's medium (Neidhardt et al., 1974)) using ultra-centrifugation and total Zn determination by ICP-MS, and also the rate of photo-electron production leading to a stoichiometric colour change in the redox dye DCPIP. Both dissolution and photo-radical generation are toxic to the bacterium and activate changes in gene transcription which can be utilised as biomarkers for both toxicity mechanisms in a single assay.

2. Experimental

2.1. Nanoparticle characterisation and handling

Zinc oxide nanoparticles (Z-COTE®) were purchased from BASF UK (Cheadle, UK). The specific material batch has been characterised by the National Physics Laboratory of the UK (NPL) as part of the Nanotechnology Industries Association (NIA) PROSPECT project (Ecotoxicology Test Protocols for Representative Nanomaterials in Support of the OECD Sponsorship Programme). The dry nanoparticles had a surface area determined by BET isotherm of 12–14 $m^2\ g^{-1}$ and a mean diameter of 150 ± 60 nm, measured by high resolution Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

Experiments were carried out using dispersions of the nanoparticles in water or Neidhardt's medium (Neidhardt et al., 1974). The composition of the medium and details of the dispersion procedure are given in the Supporting information. For TEM analysis the particles were first dispersed in Neidhardt's medium and then the suspension was centrifuged in a vessel containing a polyethylene plug in the bottom supporting copper–palladium 200 mesh grids with carbon-formvar supports (TAAB, Aldermaston, UK). We found that this process coats the grids evenly and reduces drying artefacts. The grids were dried at room temperature before imaging. The nanoparticles were also imaged after suspension with *E. coli* bacteria (MG1655) wherein the samples were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) before drying and imaging.

The stability of nanoparticle suspensions was determined by recording absorption spectra using a scanning UV–vis spectrophotometer (Bibby Scientific, Stone, UK) over a time course, wherein a characteristic λ_{max} at 375 nm is indicative of a colloidal suspension.

2.2. Dissolution rate measurements

Zinc oxide nanoparticles were suspended Neidhardt's medium and samples were removed over the time course 0, 1, 2, 5, 11, 23 and 47 h. The nanoparticles were separated from the medium by ultracentrifugation (50,000*g*, 30 min, Beckman) and then the medium was acidified (10% nitric acid) and microwave digested at 200 °C for 10 min (Milestone Inc., Connecticut, USE). Digests were measured with a 7500 ICP-MS (Agilent, UK) in standard analysis mode and total Zn concentration in the samples was determined by measuring the isotopes ^{64}Zn, ^{66}Zn, ^{67}Zn and ^{68}Zn. Quantification was performed by external calibration using Zn standard solutions and ^{103}Rh as an internal standard, correcting each sample for a procedural blank.

2.3. Zinc ion toxicity tests

E. coli K12 (MG1655) were cultured aerobically at 37 °C in Neidhardt's medium with 0.1% (w/v) glucose. Log phase cultures were mixed 1:1 with sterile, pre-warmed minimal salts medium containing zinc oxide nanoparticles or zinc chloride. Viable cell numbers were determined over a time course of 6 h by the plate counting method.

2.4. DCPIP assay for photo-electron production

A suspension of ZnO nanoparticles in deionised water was mixed with an aqueous solution of DCPIP (Sigma, Gillingham, UK) to a final concentration of 1 mM. The mixture was either kept in the dark or irradiated with 375 ± 5 nm light from a lid-mounted LED (NICHIA, Japan) producing a power of 2 mW. Photo-electron production from the ZnO nanoparticles was measured by monitoring the absorbance change at 595 nm using a spectrophotometer. A further $ZnCl_2$ photo-control was also performed: no photo-radicals were detected by the DCPIP assay.

2.5. Photo-electron toxicity tests

Exponentially replicating *E. coli* were harvested by centrifugation (5000*g*, 5 min) and re-suspended with a suspension of the ZnO nanoparticles in deionised water at a density of 10^6 CFU mL^{-1}. The suspensions were irradiated with 375 nm light from the lid-mounted LED used in the DCPIP assay or kept in the dark. Bacterial survival was measured by CFUs compared to the appropriate control; either *E. coli* in water in the dark or *E. coli* in water with 375 nm light.

2.6. Real-time PCR

E. coli cultures were exposed to Zn as described for the zinc ion toxicity tests and exposed to the nanoparticles either in the dark or irradiated with UV radiation at 375 nm. An additional control was exposure to UV without the nanoparticles. The exposure time was 10 min. The abundance of specific mRNA sequences in the total RNA extracted from the suspensions was compared to untreated controls, which were *E. coli* in unmodified minimal salts medium in the dark. Real time PCR was carried out using the SYBR green DNA detection chemistry and the method of Pfaffl (Pfaffl, 2001), and as described in other work (McQuillan et al., 2012). Primer sequences are listed in the Supporting information, Table S1. The internal reference gene was *rrsB* as used by others in metal stress measurements for *E. coli* and whose expression does not change in response to excess Zn^{2+} (Yamamoto and Ishihama, 2005).

3. Results and discussion

3.1. Nanoparticle characterisation

Commercially available, uncoated Z-Cote® zinc oxide nanoparticles were used in our experiments to demonstrate an *E. coli* 'whole cell biosensor assay' for ZnO dissolution and photo-radical generation in a complex medium. The ZnO nanoparticles have an irregular morphology and a mean diameter of 150 ± 60 nm in the Neidhardt's medium consistent with the size of the dry powder, Fig. 1A. This medium was chosen as it supports the growth of *E. coli* and suspensions of the nanoparticles were metastable over the time course of approximately 8 h, sufficient for microbiological experiments to be conducted. The stability of the ZnO nanoparticle suspensions was determined by measuring characteristic UV–vis absorption spectra of colloidal ZnO nanoparticles over time (data not shown). The size and shape of the nanoparticles, as well as