



Cite this: *Metallomics*, 2017, 9, 391

## Detection of Zn<sup>2+</sup> release in nitric oxide treated cells and proteome: dependence on fluorescent sensor and proteomic sulfhydryl groups

Mohammad R. Karim and David H. Petering\*

Nitric oxide (NO) is both an important regulatory molecule in biological systems and a toxic xenobiotic. Its oxidation products react with sulfhydryl groups and either nitrosylate or oxidize them. The aerobic reaction of NO supplied by diethylamine NONOate (DEA-NO) with pig kidney LLC-PK<sub>1</sub> cells and Zn-proteins within the isolated proteome was examined with three fluorescent zinc sensors, zinquin (ZQ), TSQ, and FluoZin-3 (FZ-3). Observations of Zn<sup>2+</sup> mobilization from Zn-proteins depended on the specific sensor used. Upon cellular exposure to DEA-NO, ZQ sequestered about 13% of the proteomic Zn<sup>2+</sup> as Zn(ZQ)<sub>2</sub> and additional Zn<sup>2+</sup> as proteome•Zn–ZQ ternary complexes. TSQ, a sensor structurally related to ZQ with lower affinity for Zn<sup>2+</sup>, did not form Zn(TSQ)<sub>2</sub>. Instead, Zn<sup>2+</sup> mobilized by DEA-NO was exclusively bound as proteome•Zn–TSQ adducts. Analogous reactions of proteome with ZQ or TSQ *in vitro* displayed qualitatively similar products. Titration of native proteome with Zn<sup>2+</sup> in the presence of ZQ resulted in the sole formation of proteome•Zn–ZQ species. This result suggested that sulfhydryl groups are involved in non-specific proteomic binding of mobile Zn<sup>2+</sup> and that the appearance of Zn(ZQ)<sub>2</sub> after exposure of cells and proteome to DEA-NO resulted from a reduction in proteomic sulfhydryl ligands, favoring the formation of Zn(ZQ)<sub>2</sub> instead of proteome•Zn–ZQ. With the third sensor, FluoZin-3, neither Zn–FZ-3 nor proteome•Zn–FZ-3 was detected during the reaction of proteome with DEA-NO. Instead, it reacted independently with DEA-NO with a modest enhancement of fluorescence.

Received 30th September 2016,  
Accepted 29th November 2016

DOI: 10.1039/c6mt00220j

www.rsc.org/metallomics

### Significance to metallomics

A significant fraction of Zn<sup>2+</sup> bound within the proteome other than zinc-metlothionein becomes reactive with zinc sensors upon exposure to NO, presumably as a result of sulfhydryl ligand modification. The native cell proteome also contains large numbers of adventitious binding sites for Zn<sup>2+</sup> that hypothetically involve thiol ligands. In the presence of NO, the affinity of these sites is also reduced. As a result, the reactivity of various sensors with proteomic Zn<sup>2+</sup> labilized by NO depends on their metal binding affinity in relation to the reduced affinity of the proteome, both native and adventitious binding sites, for Zn<sup>2+</sup>.

## Introduction

Numerous zinc fluorescent sensors have been designed to provide researchers with tools to observe pools of intracellular Zn<sup>2+</sup> that hypothetically participate in transient cellular events induced by normal or pathological conditions.<sup>1–3</sup> In one of the well described reactions, elevated Zn<sup>2+</sup> activates the MTF-1 transcription factor, leading to the induction of mRNA and the subsequent synthesis of metallothionein (MT) that sequesters excess intracellular Zn<sup>2+</sup> and ZnT1 that transports Zn<sup>2+</sup> from the cytosol into the extracellular medium.<sup>4–6</sup> In addition, it has been proposed that Cd<sup>2+</sup> stimulates MTF-1 binding to its

cognate DNA by displacing Zn<sup>2+</sup> from basal MT, upregulating MTF-1, and eventually leading to the sequestration of Cd<sup>2+</sup> by elevated concentrations of MT.<sup>6</sup>

Nitric oxide (NO) serves both as a regulatory and toxic biomolecule, depending on the situation.<sup>7–10</sup> Because NO and its oxidation products such as NO<sub>2</sub> and N<sub>2</sub>O<sub>3</sub> display strong preference for reacting with sulfhydryl groups, past studies have investigated the reactivity of Zn–MT with these compounds, recognizing that MT contains 20 cysteinyl thiolate groups and uses them to bind up to 7 Zn<sup>2+</sup> ions.<sup>11–13</sup> For example, Pitt and coworkers hypothesized that NO exposure of lung endothelial cells releases Zn<sup>2+</sup> from MT that subsequently causes vasoconstriction.<sup>14</sup> Experiments supporting mobilization of Zn<sup>2+</sup> were based on enhancement of fluorescence of the Zn<sup>2+</sup> sensors zinquin and FluoZin-3 (FZ-3) in the presence of elevated NO production (Fig. 1).<sup>14–16</sup>

Department of Chemistry and Biochemistry, University of Wisconsin–Milwaukee, Milwaukee, WI 53201, USA. E-mail: petering@uwm.edu; Fax: +1-414-229-5530



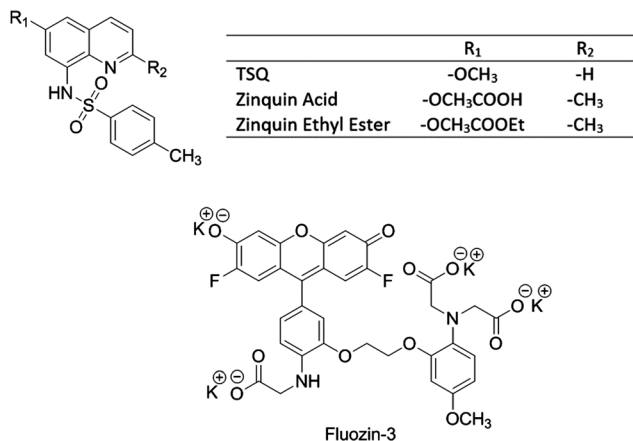
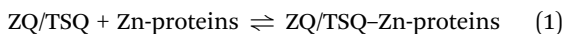


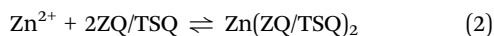
Fig. 1 Structures of Zn<sup>2+</sup> sensors.

Many fluorescent probes for Zn<sup>2+</sup> have been synthesized during the past two decades.<sup>1–3</sup> Commonly, the design, synthesis, and photophysics of new sensors are reported together with a generic test of their ability to detect labile cellular Zn<sup>2+</sup>. For example, some cells have been exposed to NO in order to render Zn<sup>2+</sup>, possibly from the MT pool, available to determine whether the sensor is an effective sensor for Zn<sup>2+</sup>.<sup>14–16</sup> We began to study the reactions of NO with cells because of an interest in the chemical reactivity of Zn–MT with NO and its oxidation products.<sup>17</sup> When it became evident that Zn–MT is not particularly reactive with NO in comparison with sulfhydryl groups throughout the proteome, other questions arose: what is the source of Zn<sup>2+</sup> that Zn<sup>2+</sup> sensors are imaging and what is the underlying bioinorganic chemistry that is responsible for the fluorescence increase?

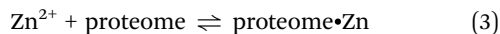
Initial studies showed that ZQ and its close relative, TSQ, image different sets of Zn-proteins within the proteome in the form of ternary adducts between the probe and Zn-proteins:<sup>18–21</sup>



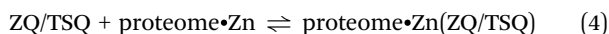
This finding immediately expanded the possibilities for reaction of sensors beyond simply binding Zn<sup>2+</sup>:



For example, studies have shown that Zn<sup>2+</sup> associates with the general proteomic pool,



leading to the possibility that ZQ and TSQ and perhaps other sensors react with proteome•Zn to generate fluorescent ternary species:<sup>19</sup>



In light of the above results, this study was undertaken to assess how ZQ, TSQ, and FZ-3 interact with the cell's complement of Zn-proteins and proteome after mobilization of Zn<sup>2+</sup> from native proteomic binding sites by NO. ZQ and TSQ were compared as closely related sensors that differ primarily at the 2 position of the isoquinoline ring with ZQ bearing a methyl

group instead of a hydrogen that leads to its larger affinity for Zn<sup>2+</sup> (Fig. 1). FZ-3 was chosen as a distinctly different molecular structure that is commonly used as a probe for cellular Zn<sup>2+</sup> including in studies of the interaction of NO with cells.<sup>14,22</sup>

## Materials and methods

### Chemicals and reagents

TSQ was purchased from AnaSpec, Inc., zinquin from Enzo Life Sciences, FluoZin-3 from Molecular Probes, and DEA-NONOate from Cayman Chemical. All sensors were dissolved in DMSO and stored in the dark at –20 °C. DEA-NONOate was dissolved in 0.01 M NaOH and stored in aliquots at –80 °C until use. All the other chemicals and reagents were purchased either from Fisher Scientific or Sigma-Aldrich at the highest grade available.

### Cell culture

The LLC-PK<sub>1</sub> (pig kidney cells) cell line was purchased from the American Tissue Culture Company. Cells were grown in Medium 199 with HEPES modification (Sigma) supplemented with 2.2 g L<sup>–1</sup> NaHCO<sub>3</sub>, 50 mg L<sup>–1</sup> penicillin G, 50 mg L<sup>–1</sup> streptomycin and 4% fetal calf serum (FCS). The cells were incubated at 37 °C in the presence of 5% CO<sub>2</sub>. Media was changed every 2–3 days until confluence. Cells were subdivided by trypsin treatment to free cells from plates.

### Fluorescence spectroscopy of LLC-PK<sub>1</sub> cells

Cultured cells were grown in 100 mm culture plates until confluence was obtained. Culture media was discarded and the plates were washed three times with cold cholate buffer, prepared by dissolving 2.47 g Na<sub>2</sub>HPO<sub>4</sub>, 0.53 g NaH<sub>2</sub>PO<sub>4</sub>, 17.0 g NaCl and 13.33 g choline chloride in 2 L MilliQ water. Cells were then gently removed from the plates using a rubber cell scraper and pooled in Dulbecco's phosphate buffered saline (DPBS) (0.1 g L<sup>–1</sup> MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.2 g L<sup>–1</sup> KCl, 0.2 g L<sup>–1</sup> KH<sub>2</sub>PO<sub>4</sub>, 8.0 g L<sup>–1</sup> NaCl, 1.15 g L<sup>–1</sup> anhydrous Na<sub>2</sub>HPO<sub>4</sub>). Cells were collected by centrifugation at 680 g for 5 min and resuspended in DPBS. An aliquot of 1 mL cell suspension (*ca.* 10<sup>7</sup> cells) was placed in a cuvette and treated with 20 μM zinquin ethyl ester or TSQ and incubated for 30 min at 25 °C followed by 500 μM DEA-NO for another 25 min. Using an excitation wavelength of 370 nm, emission spectra were recorded between 400 nm and 600 nm with a Hitachi F-4500 fluorescence spectrophotometer. Afterwards, 10 μM *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN) was added and the fluorescence spectrum monitored for another 10 min.

### Separation of cell lysate following the reaction of LLC-PK<sub>1</sub> cells and DEA-NO in the presence of zinquin or TSQ

Cells were grown and harvested as above and resuspended in 10 mL DPBS. The cell suspension (*ca.* 10<sup>8</sup> cells) was incubated with 20 μM zinquin ethyl ester or TSQ for 30–45 min followed by 500 μM DEA-NO for another 40 min in the dark. In control experiments, cells were reacted with 20 μM sensor for 70–85 min. Subsequently, cell suspensions were taken through the cycle of



centrifugation and resuspension in fresh DPBS three times to remove extracellular sensor. The last cell pellet was resuspended in 1 mL of cold MilliQ water. Cells were then lysed by sonication and centrifuged at 47 000 g for 20 min at 4 °C to collect the cell lysate or supernatant.

### Isolation of proteome using Sephadex G-75 chromatography

Cell supernatant prepared as described above in the absence of DEA-NO was loaded onto an 80 cm × 0.75 cm gel filtration column of Sephadex G-75 (GE Healthcare) equilibrated with 20 mM Tris buffer (pH 7.4) at room temperature to separate the high and low molecular weight fractions. During gel-filtration chromatography, the column was eluted with degassed 20 mM Tris-Cl (pH 7.4) and fifty fractions were collected. Fluorescence emission spectra of all fractions were obtained as described above. The zinc content in each fraction was also measured by flame atomic absorption spectroscopy (AAS). The high molecular weight fractions were combined and termed the 'proteome'. The fraction of the proteome that binds  $Zn^{2+}$  was termed 'Zn-proteins'. It is noted that metallothionein, migrating as a 10 kDa band of  $Zn^{2+}$  was not detected in these cells nor was any putatively oxidized and polymerized metallothionein protein observed when cell supernatant was preincubated with 1 mM 2-mercaptoethanol to reduce disulfide bonds in polymeric protein and 50  $\mu$ M  $Zn^{2+}$  was added to reconstitute any reduced protein as Zn-MT.

### Reaction of proteome with DEA-NO in the presence of sensor

Proteome in 20 mM degassed Tris buffer (pH 7.4) was incubated with 20  $\mu$ M of the sensor, namely, zinquin acid (45 min), TSQ (40 min) or FluoZin-3 tetrapotassium salt (20 min). Time dependent fluorescence spectra were recorded using an excitation wavelength of 370 nm and emission wavelength scan ranging from 400 nm to 600 nm. Subsequently, the reaction mixture was treated with 500  $\mu$ M DEA-NO for an hour. The final reaction mixture was loaded onto a Sephadex G-75 column, and the fractions were eluted with 20 mM degassed Tris buffer (pH 7.4). All the collected fractions were analyzed for both fluorescence and zinc content.

### Quantification of $Zn^{2+}$ by atomic absorption spectrophotometry

The concentration of  $Zn^{2+}$  in solutions was determined by flame atomic absorption spectrophotometry. A GBC model 904 instrument employed an acetylene torch to atomize samples using an 80:20 mixture of compressed air and acetylene. Zinc measurements were made with deuterium background correction. The instrument was calibrated before each run with standard  $Zn^{2+}$  solutions.

### Quantification of sulfhydryl groups

The concentration of sulfhydryl groups was determined with Ellman's reagent.<sup>23</sup> Specifically, 60  $\mu$ L of proteome sample was diluted with 540  $\mu$ L 20 mM Tris-Cl, pH 7.4 and then incubated with 60  $\mu$ L of 10 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) for 30–60 min before the absorbance at 412 nm was obtained. An extinction coefficient of 13 600  $cm^{-1} M^{-1}$  was used to determine the concentration of reactive thiol groups in the samples.

## Results

### Reactions of LLC-PK<sub>1</sub> cells with DEA-NO in the absence or presence of zinquin

Pig kidney LLC-PK<sub>1</sub> cells were used to investigate the impact of nitric oxide on the availability of intracellular  $Zn^{2+}$  to react with fluorescent zinc sensors. DEA-NO, which releases NO with a half time of about 20 min at room temperature, served as the source of nitric oxide.<sup>24–26</sup> Initially,  $1 \times 10^7$  LLC-PK<sub>1</sub> cells were incubated with 20  $\mu$ M zinquin ethyl ester (ZQ<sub>ee</sub>) for 30 min. During this time the charge neutral probe diffused into the cells and began to undergo ester hydrolysis. A fluorescence emission spectrum centered at 470 nm was observed, indicative of the formation of ternary adduct species, ZQ-Zn-proteins (Fig. 2A and reaction (1)).<sup>19,21</sup> Based on previous estimates, the amount of protein-bound zinc participating in adduct formation represents about 10–15% of the total.<sup>18,20,21</sup> The introduction of 500  $\mu$ M DEA-NO to the cells for another 25 min caused a gradual increase of fluorescence at 470 nm, along with a noticeable shoulder at 490 nm (Fig. 2A). Compared with the reaction of cells with ZQ<sub>ee</sub> alone, a 25% increment in fluorescence intensity was observed (Fig. 2B). Subsequently,

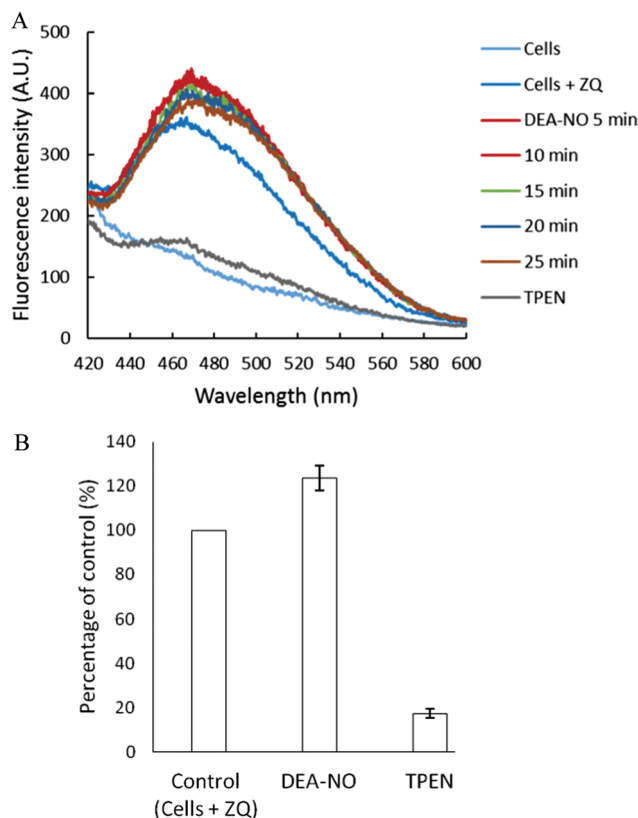
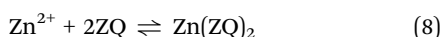
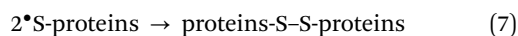
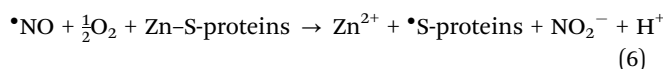
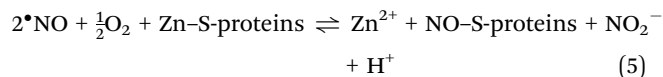


Fig. 2 Reaction of LLC-PK<sub>1</sub> cells with DEA-NO in the presence of zinquin ethyl ester (ZQ<sub>ee</sub>). (A) Fluorescence spectra of the reaction between  $10^7$  LLC-PK<sub>1</sub> cells and 20  $\mu$ M ZQ<sub>ee</sub> for 30 min followed by the treatment with 500  $\mu$ M DEA-NO for another 25 min. (B) Percent enhancement of fluorescence upon addition of 500  $\mu$ M DEA-NO and its reversal by 10  $\mu$ M TPEN. Error bars represent standard errors for at least three measurements and A.U. indicates arbitrary units.



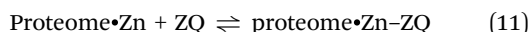
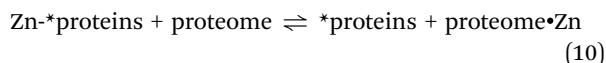
10  $\mu\text{M}$  TPEN, a cell permeable, strong zinc chelator, quenched the fluorescence to 17% of the intensity observed with control cells treated with 20  $\mu\text{M}$  ZQee (Fig. 2B), confirming that enhancement of fluorescence following incubation with ZQ<sub>ee</sub> and DEA-NO involved the participation of  $\text{Zn}^{2+}$ . Moreover, the reaction of cells with DEA-NO reduced its total sulfhydryl concentration from 780 nmol/10<sup>6</sup> cells to 440 nmol/10<sup>6</sup> cells as measured after isolation of supernatant from sonicated cells.

The appearance of the shoulder at 490 nm suggested the mobilization of  $\text{Zn}^{2+}$  from the proteome (Zn-proteins) by NO or one of its oxidation products and, in turn, the production of the  $\text{Zn}(\text{ZQ})_2$  complex (reactions (5)–(8)), which fluoresces with an emission maximum of 490 nm.<sup>19,21</sup> It was hypothesized that oxidation products of NO modified  $\text{Zn}^{2+}$ -bound thiol ligands, thus releasing  $\text{Zn}^{2+}$ .<sup>27</sup>



Another contributor to the fluorescence increase might be  $\text{Zn}^{2+}$  mobilized from Zn-proteins that non-specifically rearranges to adventitious binding sites within the proteome (proteome $\cdot$ Zn) (reaction (3)). In turn, proteome $\cdot$ Zn may react with ZQ to generate proteome $\cdot$ Zn-ZQ that fluoresces at 470 nm (reaction (4)).<sup>19</sup>

To investigate further the basis for the above observations,  $1.25 \times 10^8$  LLC-PK<sub>1</sub> cells in 10 mL were reacted with 20  $\mu\text{M}$  ZQee for 30 min at room temperature before incubation with 500  $\mu\text{M}$  DEA-NO for another 40 min. The cells were then lysed, centrifuged, and the resultant supernatant separated by Sephadex G-75 gel filtration. When the fractions were analyzed for fluorescence and zinc content, proteomic and low molecular weight (LMW) bands of both fluorescence and zinc were found for both control (cells plus ZQee) and DEA-NO exposed (cells and ZQee plus DEA-NO) cells (Fig. 3A and B). Proteome-associated fluorescence that centered at 470 nm increased by 16% in DEA-NO treated cells compared to control, unexposed cells. Evidently, DEA-NO induced the formation of more proteome $\cdot$ Zn-ZQ adducts. Plausibly, the modification of SH groups by DEA-NO either made available altered native Zn-proteomic sites (Zn $\cdot$ proteins) for reaction with ZQ or  $\text{Zn}^{2+}$  at such sites moved to non-specific proteomic sites where ternary adducts were generated (reactions (9)–(11)).



The low molecular weight fluorescence pool of control reaction constituted 19% of the total fluorescence (high molecular weight and low molecular weight), whereas that of DEA-NO treated reaction represented 32% (Fig. 3C). The spectra of low molecular

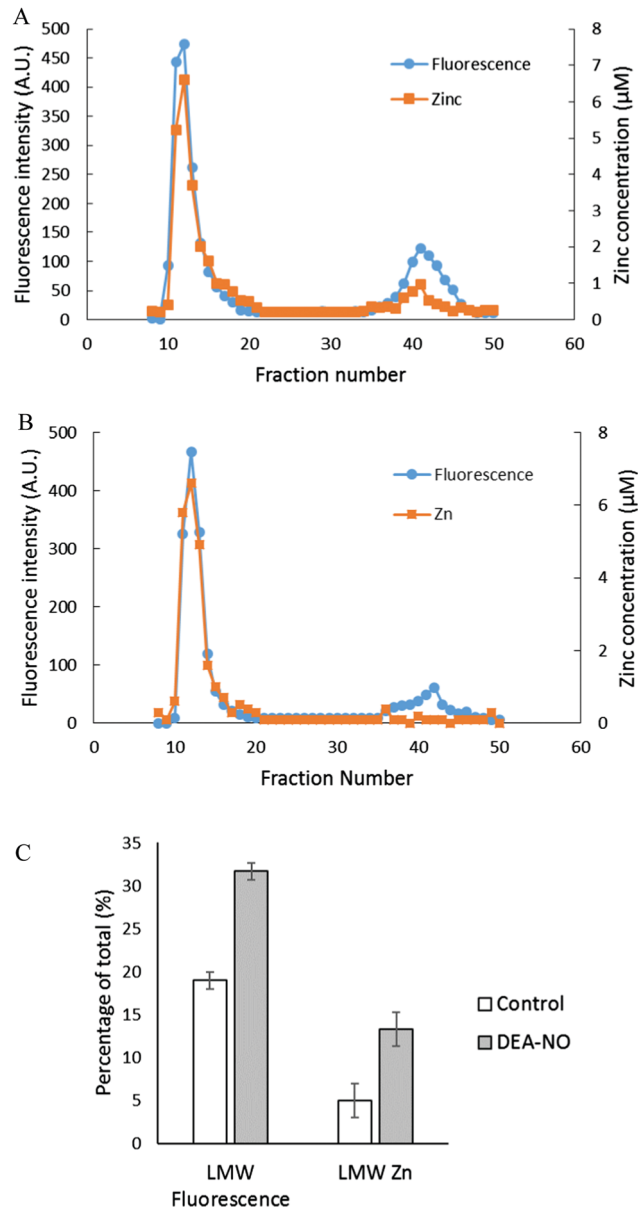
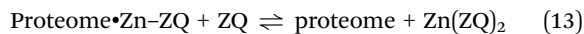
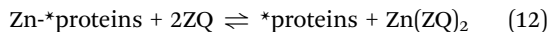


Fig. 3 Sephadex G-75 gel filtration of LLC-PK<sub>1</sub> cell supernatant incubated with 20  $\mu\text{M}$  ZQee followed by 500  $\mu\text{M}$  DEA-NO. (A) 10<sup>8</sup> LLC-PK<sub>1</sub> cells were reacted with 500  $\mu\text{M}$  DEA-NO for 40 min following 20  $\mu\text{M}$  ZQee for 30 min. The cells were lysed, centrifuged, and the supernatant separated using Sephadex G-75 column chromatography. The fractions were analyzed for both fluorescence and zinc. (B) Control: a parallel reaction was run under identical conditions without DEA-NO. (C) Comparison of the low molecular weight (LMW) fluorescence and zinc content between control and DEA-NO exposed cells. Error bars represent standard errors for at least three measurements.

weight fractions centered near 490 nm, the wavelength maximum of  $\text{Zn}(\text{ZQ})_2$ . Consistent with the fluorescence data, after DEA-NO treatment the low molecular weight zinc content was calculated to be 13% of the total zinc, whereas that of control reaction was only 5% (Fig. 3C). The significant increase of both the low molecular weight zinc and its accompanying fluorescence emission centered at 490 nm indicated the formation of  $\text{Zn}(\text{ZQ})_2$  during the reaction of NO with cells. Possibly, ZQ extracted  $\text{Zn}^{2+}$  from native  $\text{Zn}^{2+}$



binding sites that had been modified and weakened (Zn-\*proteins) by nitric oxide to generate Zn(ZQ)<sub>2</sub> (reaction (12)). Alternatively, Zn<sup>2+</sup> labilized at such sites may have shifted to non-specific sites of binding within the proteome (reaction (10)) and then reacted with ZQ to generate Zn(ZQ)<sub>2</sub> (reactions (11) and (13)):



### Reaction of isolated proteome with DEA-NO in the presence of zinquin acid (ZQ<sub>acid</sub>)

The reactions of NO and ZQ with LLC-PK<sub>1</sub> cells were investigated in a simpler system that contained isolated proteome and was not complicated by membrane barriers and plentiful glutathione that are present in cells. Proteome containing 10 μM native protein-bound Zn<sup>2+</sup> was first reacted with 20 μM ZQ<sub>acid</sub> for 45 min at room temperature. As in whole cells, a gradual increase of fluorescence with the emission maximum of 470 nm was observed, indicative of the formation of ZQ-Zn-protein ternary adducts.<sup>21</sup> Subsequently, 500 μM DEA-NO was added for an hour. When the final reaction mixture was separated by Sephadex G-75 chromatography, a noticeable low molecular weight pool of fluorescence and zinc, larger than that of the control sample, was detected (Fig. 4A and B). The LMW fluorescence pool (emission maximum of 490 nm) was measured to be 50% of total fluorescence, which is 1.7 times that of the control (30% of total fluorescence) (Fig. 4C) and displayed an emission maximum at 490 nm. In addition, the LMW zinc pool was determined to be three times larger than that of control proteome (15% of total zinc content *vs.* 5%). The reduction of sulfhydryl content by 41% (from 212 μM to 126 μM) following the treatment with DEA-NO demonstrated the reaction between proteomic sulfhydryl groups and nitric oxide. As in whole cells, the low molecular weight fluorescence and zinc pool in the reaction of isolated proteome indicated the production of Zn(ZQ)<sub>2</sub>. Moreover, proteomic fluorescence intensity increased by 19% in comparison with the control, suggesting the generation of new ternary adduct sites that resulted from the labilization of Zn<sup>2+</sup>. Overall, the *in vitro* results agreed qualitatively with those derived from the exposure of cells to DEA-NO.

### Reaction of LLC-PK<sub>1</sub> cells with DEA-NO in the presence of TSQ

The experiments involving ZQ were repeated substituting TSQ, its closely related analog (Fig. 1). First, TSQ was reacted with LLC-PK<sub>1</sub> cells to form TSQ-Zn-protein ternary adducts (reaction (1)) as was evident from the fluorescence spectrum centered at around 470 nm (Fig. 5A).<sup>18</sup> In this bound form, TSQ like ZQ<sub>acid</sub> is firmly fixed within the cells even though it is a neutral molecule. Subsequent incubation with 500 μM DEA-NO for 40 min caused a two-fold increase of fluorescence, substantially more than obtained with zinquin, and a shift of the emission maximum to 480 nm, which suggested that some Zn(TSQ)<sub>2</sub> had formed. As with ZQ, 10 μM TPEN was able to reduce the fluorescence to 32% of the control value, signaling the participation of Zn<sup>2+</sup> in the enhancement of fluorescence in DEA-NO

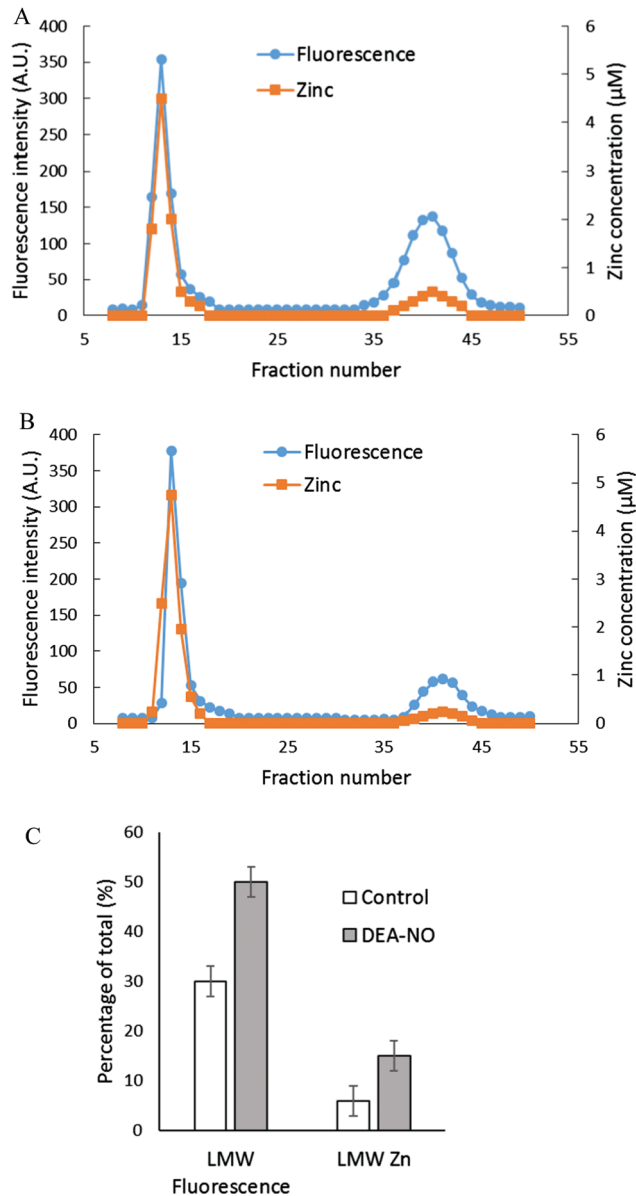


Fig. 4 Sephadex G-75 gel filtration of isolated proteome incubated with 20 μM zinquin acid (ZQ<sub>acid</sub>) followed by 500 μM DEA-NO. (A) Isolated proteome (10 μM Zn<sup>2+</sup>) was reacted with 20 μM ZQ<sub>acid</sub> for 45 min followed by 500 μM DEA-NO for another 1 hour. The final reaction mixture was separated using Sephadex G-75 column chromatography, and the fractions were analyzed for both fluorescence and zinc content. (B) Control: a parallel reaction was run under identical conditions in the absence of DEA-NO. (C) Comparison of the low molecular weight fluorescence and zinc content. Error bars represent standard errors for at least three measurements.

treated as well as control cells (Fig. 5B). Consistent with the fluorescence enhancement, the sulfhydryl concentration declined from 740 nmol/10<sup>6</sup> cells to 450 nmol/10<sup>6</sup> cells as measured after isolation of lysate from sonicated cells.

To characterize the fluorescent species, LLC-PK<sub>1</sub> cells were incubated with 20 μM TSQ for 40 min followed by 500 μM DEA-NO for another 45 min. Cells were then washed, lysed and centrifuged, and the resultant supernatant fractionated using



Sephadex G-75 column chromatography. Compared with the results with zinquin, no detectable pool of low molecular weight fluorescence and  $Zn^{2+}$  as  $Zn(TSQ)_2$  was found in either DEA-NO treated or control cells (Fig. 6A and B). Almost all the fluorescence and zinc were detected in the high molecular weight proteome fractions. The fluorescence intensity of the high molecular weight fractions was 50% greater than that of control (Fig. 6C) and was aligned with the  $Zn^{2+}$  concentrations in the fractions. Moreover, the emission maxima of the proteomic fractions were located at about 470 nm, consistent with the presence of ternary adduct species. In contrast to the results in Fig. 5A, these findings indicate that  $Zn^{2+}$  mobilized by incubation with DEA-NO remained bound completely by the proteome in the presence of TSQ, forming ternary adducts instead of  $Zn(TSQ)_2$ . If some  $Zn(TSQ)_2$  was formed *in vivo* as suggested by Fig. 5A, perhaps proteome•Zn-TSQ species became favored during chromatography as proteome and TSQ were separated and the reaction equilibrium, as in reaction (13), shifted to the left. That is, possibly because of its lower affinity for  $Zn^{2+}$  relative

to ZQ, TSQ cannot compete with the adduct species to retain  $Zn(TSQ)_2$  during gel filtration.<sup>20</sup> However, a rapid one step separation of proteome from low molecular weight molecules using centrifugal filtration (3 kDa cut off Centricon filter) also did not demonstrate any  $Zn(TSQ)_2$ . Thus, it was concluded that mobilized  $Zn^{2+}$  was present only as proteome•Zn-TSQ.

### Reaction of isolated proteome with DEA-NO in the presence of TSQ

The reaction of DEA-NO and TSQ with proteome was also examined (Fig. 6D). Isolated proteome containing 10  $\mu M$   $Zn^{2+}$  was reacted with 20  $\mu M$  TSQ for 40 min before 500  $\mu M$  DEA-NO was added for one hour. The reduction of sulfhydryl concentration by about 41% (from 340  $\mu M$  to 200  $\mu M$ ) indicated a significant reaction between nitric oxide or its oxidation products and proteomic sulfhydryl groups. When the reaction mixture was subjected to Sephadex G-75 gel filtration, all of the fluorescence and  $Zn^{2+}$  resided in proteomic fractions;  $Zn(TSQ)_2$  was not formed in either DEA-NO treated and control reactions.

### Reaction of isolated proteome with added $Zn^{2+}$ in the presence of ZQ or TSQ

The capacity of the proteome to bind adventitious  $Zn^{2+}$  in the absence or presence of DEA-NO and ZQ or TSQ was also examined in order to probe the involvement of reactions (2)–(4). Control proteome (8  $\mu M$   $Zn^{2+}$ ) was titrated with  $Zn^{2+}$  in the presence of excess ZQ or TSQ. The fluorescence emission intensity centered at about 470 nm, due to the formation of proteome•Zn-ZQ/TSQ ternary adducts, increased in parallel with the concentration of added  $Zn^{2+}$  (Fig. 7A and 8A). In both cases, the emission maximum remained unchanged at 470 nm as  $Zn^{2+}$  was added to the proteome. Moreover, when the final reaction mixtures were fractionated by gel filtration with Sephadex G-75, no measurable low molecular weight fluorescence or  $Zn^{2+}$  was detected with either fluorophore (Fig. 7B and 8B). Thus, the proteome contained extensive capacity to bind  $Zn^{2+}$  and formed adducts with  $Zn^{2+}$  and either sensor (reactions (3) and (4)). These results differ from the findings about the reaction of cells or proteome with DEA-NO in the presence of zinquin. In those experiments, a red shifted emission maximum of the DEA-NO treated sample (Fig. 2) and a detectable low molecular weight fluorescence and zinc pool (Fig. 3 and 4) indicated that  $Zn(ZQ)_2$  was produced along with proteome•Zn-ZQ.

The difference in outcome related to the absence or presence of DEA-NO. The results suggested the hypothesis that the behavior of ZQ depends on the existence and extent of modification of sulfhydryl groups that may participate in the adventitious proteomic binding of  $Zn^{2+}$  (reactions (14) and (15)).

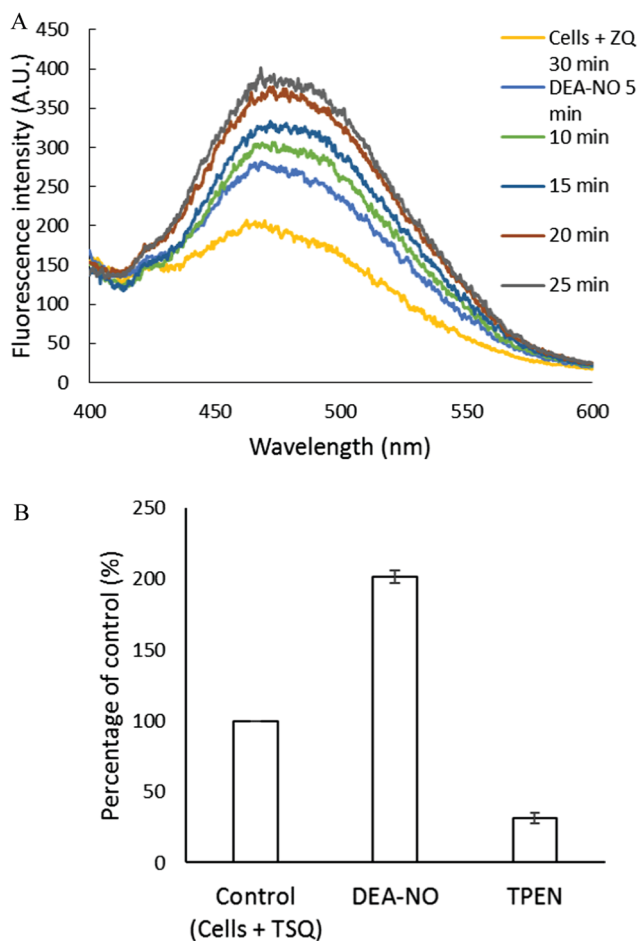
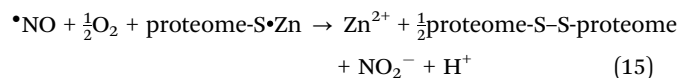
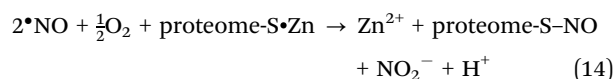
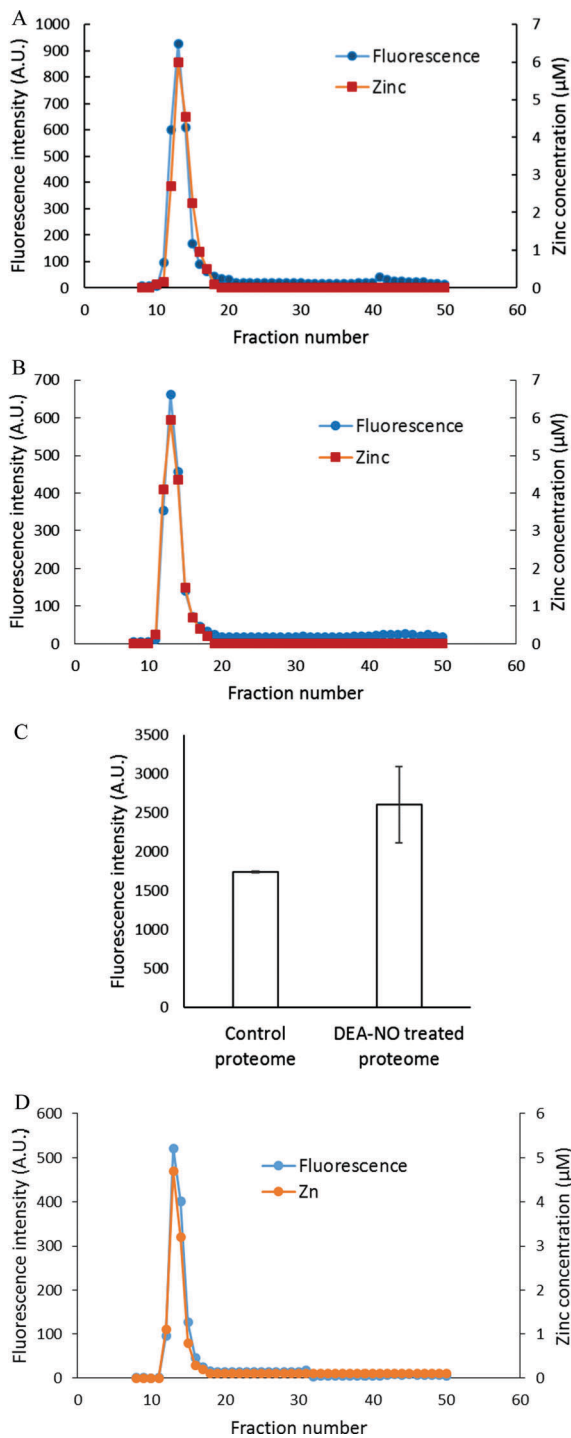
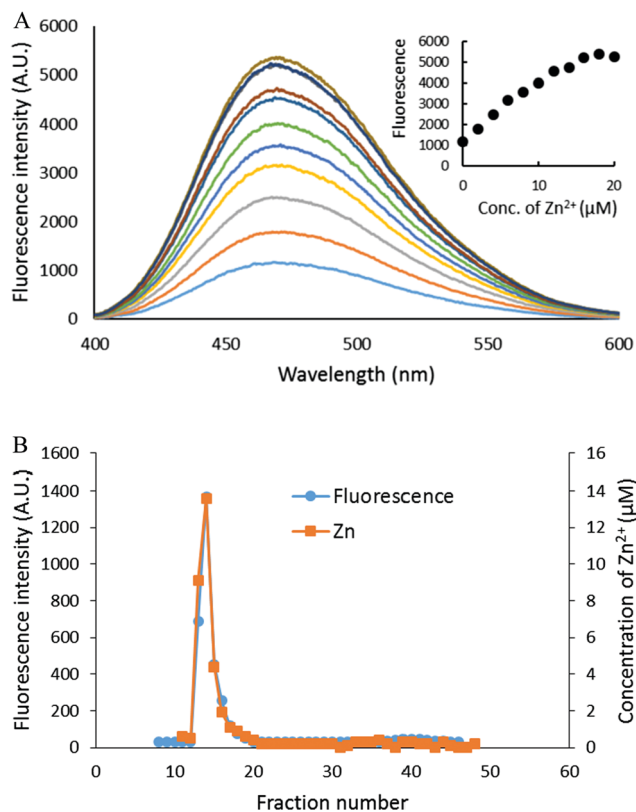


Fig. 5 Reaction of LLC-PK<sub>1</sub> cells with DEA-NO in the presence of TSQ. (A) Fluorescence spectra of the reaction between 10<sup>7</sup> LLC-PK<sub>1</sub> cells and 20  $\mu M$  TSQ for 30 min followed by the treatment with 500  $\mu M$  DEA-NO for another 25 min. (B) Percent enhancement of fluorescence upon addition of 500  $\mu M$  DEA-NO and its reversal by 10  $\mu M$  TPEN. Error bars represent standard errors for at least three measurements.





**Fig. 6** Sephadex G-75 chromatography of LLC-PK<sub>1</sub> cell supernatant or isolated proteome incubated with TSO followed by 500 μM DEA-NO. (A) 10<sup>8</sup> LLC-PK<sub>1</sub> cells were reacted with 500 μM DEA-NO for 45 min following 20 μM TSO for 40 min. The cells were lysed, centrifuged, and the supernatant was separated using a Sephadex G-75 column. The fractions were analyzed for both fluorescence and zinc. (B) Control: a parallel reaction was run at identical condition with no DEA-NO added. (C) Comparison of the integrated fluorescence intensity of proteome. (D) Isolated proteome (10 μM Zn<sup>2+</sup>) was reacted with 20 μM TSO for 40 min followed by 500 μM DEA-NO for another 1 hour. The final reaction mixture was separated using a Sephadex G-75 column, and the fractions were analyzed for both fluorescence and zinc content. Error bars represent standard errors for at least three measurements.



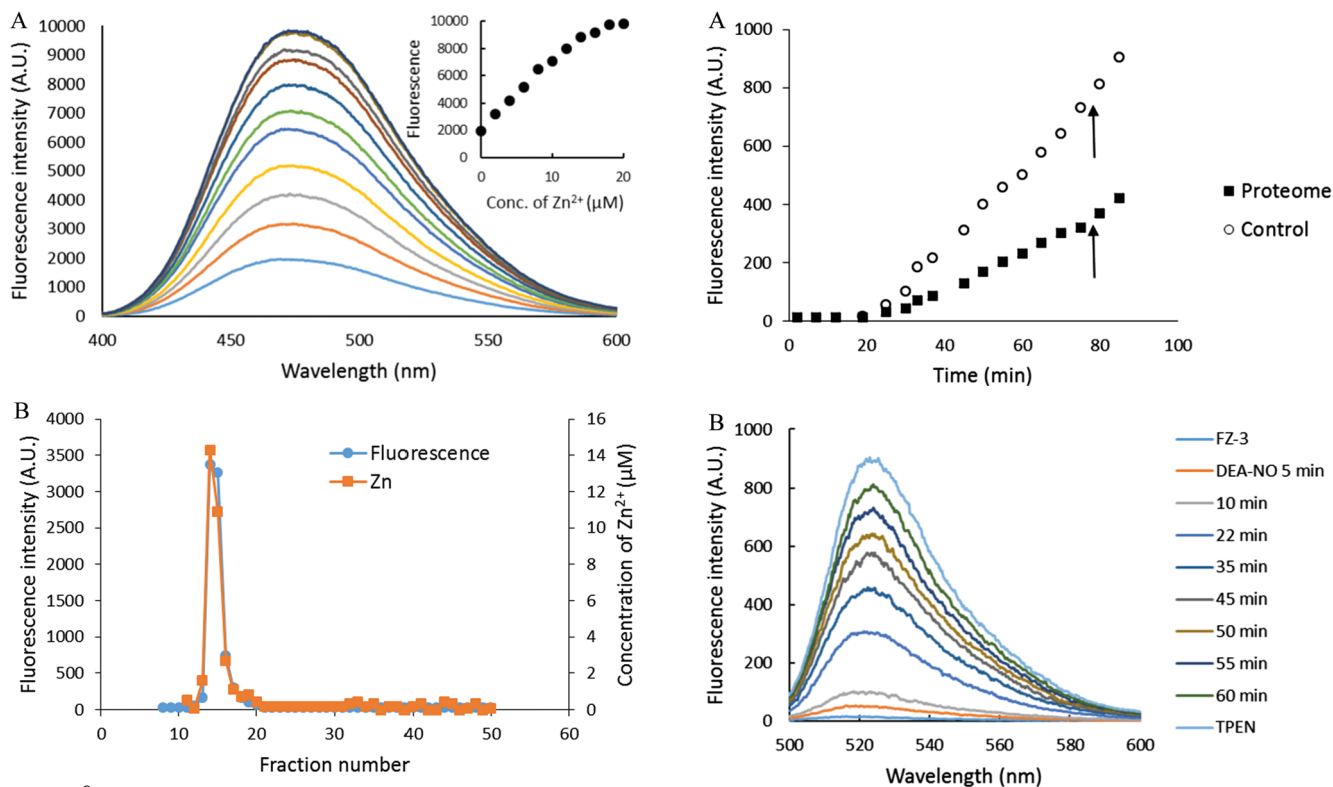
**Fig. 7** Zn<sup>2+</sup> titration of proteome pre-treated with ZQacid. (A) Isolated proteome (8 μM Zn<sup>2+</sup>) was reacted with 20 μM ZQacid for 30 min (the bottom most spectrum). Then, the reaction mixture was titrated with the increasing concentration of ZnCl<sub>2</sub>. (B) The final reaction mixture from (A) was fractionated using Sephadex G-75 gel filtration, and the eluted fractions were analyzed for both fluorescence and zinc content.

As the number of adventitious binding sites for Zn<sup>2+</sup> declines through reaction with NO, the formation of a substantial concentration of Zn(ZQ)<sub>2</sub> becomes more favorable (reaction (8)).

### Reaction of isolated proteome with DEA-NO in the presence of FluoZin-3

The reaction of isolated proteome (10 μM Zn<sup>2+</sup>) with 500 μM DEA-NO was also monitored using another Zn<sup>2+</sup> sensor that does not form ternary complexes with Zn-proteins, FZ-3 (20 μM). The absence of any fluorescence signal during the reaction of proteome and FluoZin-3 for 20 min indicated that Zn-proteome did not react with FZ-3 to form ternary adducts. The introduction of DEA-NO to the reaction mixture of proteome and FZ-3 for 1 hour resulted in the reduction of proteomic sulfhydryl concentration by about 41% (from 342 μM to 202 μM). The presence of DEA-NO caused a gradual increase of fluorescence throughout this period (Fig. 9A and B). Interestingly, addition of 10 μM TPEN, a powerful Zn<sup>2+</sup> chelator, at an intermediate time did not quench any of the enhanced fluorescence. Instead, the fluorescence signal continued increasing. This finding suggested that the increase of fluorescence was not caused by the formation of Zn-FluoZin-3 complex, because its fluorescence should have been quenched upon the addition of TPEN. In a separate experiment, upon fractionation of the final reaction mixture of proteome (10 μM Zn<sup>2+</sup>), 20 μM FZ-3 and 500 μM DEA-NO using a





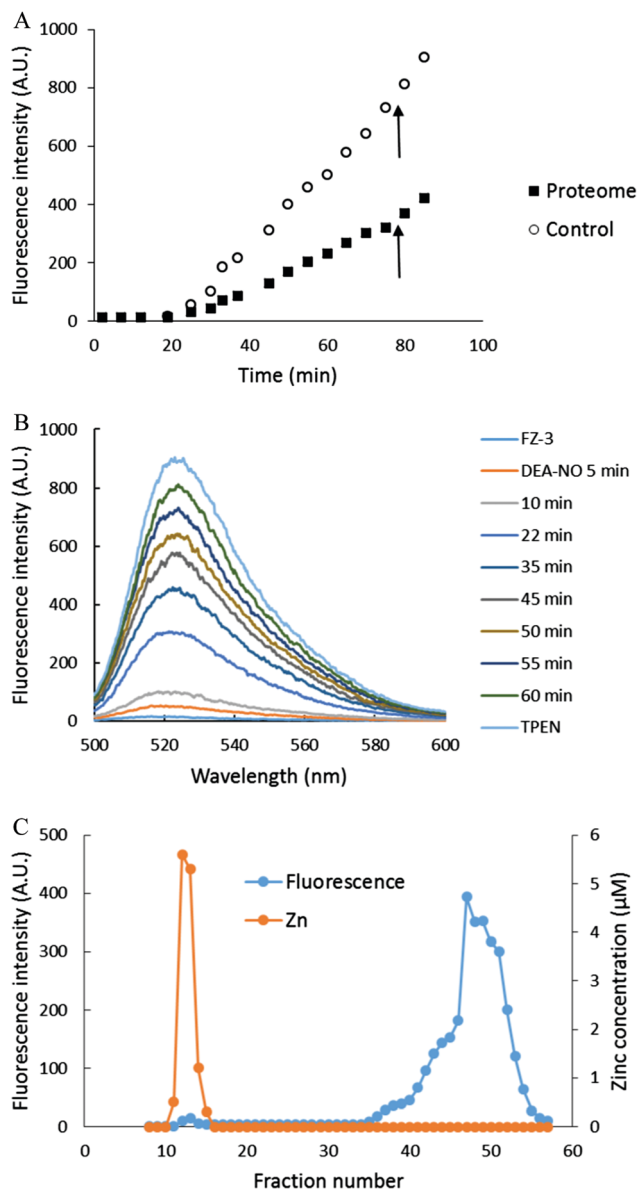
**Fig. 8**  $\text{Zn}^{2+}$  titration of isolated proteome pre-treated with TSQ. (A) Isolated proteome ( $8 \mu\text{M Zn}^{2+}$ ) was reacted with  $20 \mu\text{M TSQ}$  for 30 min (the bottom most spectrum). Following on, the reaction mixture was titrated with increasing concentrations of  $\text{ZnCl}_2$ . The inset summarizes the fluorescence changes as a function of  $\text{Zn}^{2+}$ . (B) The final reaction mixture from (A) was fractionated using Sephadex G-75 gel filtration, and the eluted fractions were analyzed for both fluorescence and zinc content.

Sephadex G-75 column, no LMW Zn-FZ-3 complex was isolated (Fig. 9C) confirming that FluoZin-3 does not chelate proteomic  $\text{Zn}^{2+}$  after reaction of DEA-NO with the proteome. However, virtually all of the fluorescence was observed in the LMW region of the chromatogram, indicating that a FZ-3 related species was responsible for the fluorescence enhancement seen during the reaction.

To investigate the source of this fluorescence enhancement,  $20 \mu\text{M FluoZin-3}$ , itself, was reacted with  $500 \mu\text{M DEA-NO}$  for 1 hour followed by  $10 \mu\text{M TPEN}$  for another 10 min (Fig. 9A). A gradual increase of fluorescence larger than observed in the proteome experiment was recorded. Again,  $10 \mu\text{M TPEN}$  did not reverse the intensification of the fluorescence. It was concluded that NO reacts with FZ-3 with an increase of fluorescence, independent of its reaction with  $\text{Zn}^{2+}$ . FluoZin-3 was also reacted with *S*-nitrosylpenicillamine under similar conditions except that the reactants were kept in the dark to prevent the photochemical cleavage of S-NO bond to release NO. No reaction with FluoZin-3 was observed, indicating that the reaction of NO donors with FZ-3 is specific for nitric oxide or its oxidation products.

## Discussion

Fluorescent sensors of all kinds are in routine use to reveal structures, chemical changes, and ongoing processes in living



**Fig. 9** Reaction of DEA-NO with isolated proteome preincubated with FluoZin-3. (A) Isolated proteome ( $10 \mu\text{M Zn}^{2+}$ ) was reacted with  $20 \mu\text{M FZ-3}$  for 20 min followed by  $500 \mu\text{M DEA-NO}$  for another hour. Finally  $10 \mu\text{M TPEN}$  was added for 10 min. In the control experiment,  $20 \mu\text{M FZ-3}$  in  $20 \text{ mM Tris}$  (pH 7.4) was reacted with  $500 \mu\text{M DEA-NO}$  for an hour followed by  $10 \mu\text{M TPEN}$  for another 10 min. The arrow indicates the time of TPEN addition. (B) Fluorescence spectra of the reaction between  $20 \mu\text{M FZ-3}$  and  $500 \mu\text{M DEA-NO}$ . (C) Sephadex G-75 chromatographic fractionation of the reaction mixture of proteome ( $10 \mu\text{M Zn}^{2+}$ ) and  $500 \mu\text{M DEA-NO}$  in the presence of  $20 \mu\text{M FZ-3}$  for an hour.

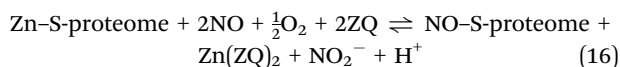
cells.<sup>28–31</sup> Major efforts have been made and are continuing to design probes that selectively detect essential and extraneous metal ions in cells and, thereby, to reveal their participation in normal and pathologic biochemistry.<sup>1,3,30,32,33</sup> In the case of  $\text{Zn}^{2+}$ , for example, the appearance of a fluorescence microscopic signal in the presence of a cell permeable sensor, followed by its diminution in the presence of the powerful membrane permeant chelator, TPEN, has been employed in many studies to conclude





that labile  $\text{Zn}^{2+}$  is present in the cellular process under investigation. Similarly, the absence of such changes signals that mobile  $\text{Zn}^{2+}$  is not involved. In one early study, sheep pulmonary artery endothelial cells (SPAECs) exposed to the NO donor, *S*-nitrosocysteine, and ZQ displayed a 25% increase in fluorescence compared with control cells.<sup>15</sup> This result led to the conclusion that  $\text{Zn}^{2+}$  mobilized by NO plays a key role in NO-dependent vessel constriction.

The results of the present experiments involving cells and isolated proteome are comparable to those of the original study. Furthermore, though not discussed in the original paper, the high background fluorescence of control SPAEC cells incubated with ZQ is consistent with the initial formation of fluorescent ZQ–Zn-protein adducts.<sup>15</sup> Originally, these results were explained by reaction (16):



Oxidation products of NO modify thiol groups at  $\text{Zn}^{2+}$  binding sites, weakening the association of  $\text{Zn}^{2+}$  with the remaining ligands. In this circumstance, ZQ captures  $\text{Zn}^{2+}$  either by direct ligand substitution (reaction (12)) or reacts with  $\text{Zn}^{2+}$  that has dissociated from its original binding sites (reaction (8)).

From a chemical point of view, recent studies and the present experiments reveal that the milieu in which ZQ undergoes reaction is more complex. In cells ZQ binds to a significant fraction of the unmodified Zn-proteome and forms ternary ZQ–Zn-protein adducts (reaction (1) and Fig. 2A, 3B).<sup>21</sup> Depending on the cell type, it competes to sequester a small fraction of  $\text{Zn}^{2+}$  from Zn-proteins as  $\text{Zn(ZQ)}_2$  (Fig. 3B).<sup>21</sup> The introduction of NO increases the fluorescence by *ca.* 20–25%, signaling the formation of additional adduct species at modified sites, ZQ–Zn–NO–S-proteins and/or adducts at adventitious  $\text{Zn}^{2+}$  binding sites within the proteome, proteome-S•Zn–ZQ. More  $\text{Zn(ZQ)}_2$  forms as well (Fig. 2B and 3C). When TSQ, a closely related  $\text{Zn}^{2+}$  probe, replaces ZQ, the results are different:  $\text{Zn(TSQ)}_2$  is not produced (Fig. 5 and 6). Turning to a third sensor, FluoZin-III, yet another response to NO was noted as fluorescence emission increased due to the modification of the fluorophore not because of the binding of  $\text{Zn}^{2+}$  (Fig. 9). No evidence was elicited demonstrating that NO-treated FluoZin-III served as an effective probe for  $\text{Zn}^{2+}$  associated with modified native sites or adventitious proteomic binding sites.

It has been proposed that aerobic NO releases  $\text{Zn}^{2+}$  preferentially from Zn–metallothionein (Zn–MT) because its plethora of sulfhydryl groups bind up to seven  $\text{Zn}^{2+}$  ions within the protein's two domain structure.<sup>11–13</sup> A previous study on the reaction of DEA-NO with cells containing measurable Zn–MT demonstrated that the large pools of thiols in the proteomic and glutathione fractions readily react with oxidation products of NO and that Zn–MT is relatively unreactive with DEA-NO in the presence of other sources of thiol groups.<sup>17</sup>

The present study utilized a cell type that does not contain measurable Zn–MT, which would otherwise be observed midway between high and low molecular weight bands of  $\text{Zn}^{2+}$  in control Sephadex G-75 chromatograms (Fig. 3B). In this setting, it was clear that ZQ mobilized  $\text{Zn}^{2+}$  from Zn-proteins that had reacted

with DEA-NO (Fig. 3 and 4). Thus, Zn-proteins are a major source of  $\text{Zn}^{2+}$  for reaction with fluorescent probes after  $\text{Zn}^{2+}$  was mobilized by the hypothetical reaction of DEA-NO with sulfhydryl, Zn-binding ligands.

The detailed understanding of the origin of the fluorescence enhancement observed after cells or proteome were incubated with ZQ and DEA-NO bears on the meaning of the results. Although ZQ and TSQ can form 2:1 complexes with  $\text{Zn}^{2+}$ , the generation of ternary adduct species with control Zn-proteome is much more favorable (Fig. 3B and 4B). Upon exposure of isolated proteome to DEA-NO, this distinct preference remained for TSQ in the presence of the modified proteome (Fig. 6A and D). In contrast, the differential was lost in the cellular and proteomic reactions with ZQ as both the formation of ternary adducts and  $\text{Zn(ZQ)}_2$  increased in the presence of DEA-NO (Fig. 2A, 3 and 4).

A plausible explanation for the different behavior of ZQ and TSQ relies on the higher affinity of ZQ of  $\text{Zn}^{2+}$  due to the presence in its structure of an electron donating methyl group alpha to the isoquinoline's nitrogen ligand (unpublished information) (Fig. 1).<sup>19,20</sup> The methyl group also sterically favors tetrahedral ligand coordination to coincide with a preferred Zn-ligand geometry. This difference apparently favors the formation of the 2 to 1 ligand to metal complex of ZQ relative to its ternary adducts.  $\text{Zn}^{2+}$  labilized by NO and detected as  $\text{Zn(ZQ)}_2$  might stem from metal ion still bound to sulfhydryl-modified native binding sites or from  $\text{Zn}^{2+}$  that has migrated to adventitious sites (reactions (12) and (13)). In either case it is not free or even loosely bound to cellular ligands within the cell. This conclusion is also consistent with the finding that another sensor with relatively low affinity for  $\text{Zn}^{2+}$ , Newport Green, fails to sequester  $\text{Zn}^{2+}$  from Zn-proteins exposed to NO.<sup>34</sup>

These results qualitatively resemble the observed interactions of ZQ and TSQ with cells and proteome exposed to *N*-ethylmaleimide (NEM).<sup>20</sup> Because NEM is a simple electrophile that is particularly reactive with thiol groups, the similarity of outcomes using DEA-NO and NEM supports the hypothesis that each targets sulfhydryl-containing proteins as well as glutathione. That these should include thiolate metal binding ligands of Zn-proteins as well as free sulfhydryl groups in cells treated with these reagents is intriguing because the thiolate nucleophile when bound to  $\text{Zn}^{2+}$  would seem to be a less reactive nucleophile than the free thiol at pH 7.

The capacity of proteome to bind  $\text{Zn}^{2+}$  in the presence of ZQ or TSQ was explored in titration experiments (Fig. 7 and 8). It sequesters at least 3–5 times more added  $\text{Zn}^{2+}$  than is present in the native Zn-proteome with sufficient stability that the formation of proteome•Zn-(ZQ/TSQ) is preferred exclusively to  $\text{Zn(ZQ/TSQ)}_2$ . The result with ZQ differed from those observed with cells or proteome treated with ZQ and DEA-NO. Native proteome competed effectively with ZQ to form proteome•Zn–ZQ without the appearance of  $\text{Zn(ZQ)}_2$ ; NO-exposed proteome did not (Fig. 3 and 4). These divergent findings suggest that a substantial fraction of adventitious binding sites for  $\text{Zn}^{2+}$  involve sulfhydryl ligands. Once modified by DEA-NO, the proteome has less capability to associate with  $\text{Zn}^{2+}$  and ZQ (reactions (14) and (15)).

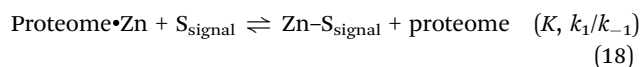


Because TSQ binds  $Zn^{2+}$  with lower affinity than ZQ, the altered proteome still manages to outcompete TSQ for  $Zn^{2+}$  and no  $Zn(TSQ)_2$  forms (Fig. 6). Thus, depending on the fluorescent probe, the  $Zn^{2+}$  binding characteristics of the proteome under different cellular conditions play a significant role in how the sensor functions.

The functional implications of the apparent strong affinity of mobilizable  $Zn^{2+}$  for non-specific binding sites in the proteome have not been sorted out. For example, if the proteome provides a strong buffer for  $Zn^{2+}$ , how does intracellular  $Zn^{2+}$  signaling take place? Signaling has been viewed as a process in which mobilized  $Zn^{2+}$  binds reversibly to specific sites that initiate signal cascades,



such that the chemical basis of signaling are the variable concentration of  $Zn^{2+}$ , a modest stability constant ( $K$ ) for  $Zn-S_{\text{signaling}}$ , and, as a consequence, favorable rates for on and off steps ( $k_1/k_{-1}$ ) of reaction (17). This model will need to be rethought if the signaling reaction is better represented as



in which the stability constant of  $Zn-S_{\text{signal}}$  is large so that  $S_{\text{signal}}$  can compete with proteome for bound  $Zn^{2+}$ .<sup>4</sup>

Lastly, the reaction of FluoZin-3 with DEA-NO treated Zn-proteome was complicated by a side reaction of NO or its oxidation products with the probe that increased its fluorescence emission independent of the presence of  $Zn^{2+}$  (Fig. 9). A previous study of the properties of Newport Green as a sensor of proteomic  $Zn^{2+}$  released by DEA-NO also displayed a similar reaction with DEA-NO.<sup>34</sup> Both sensors utilize a fluorescein-based fluorophore. In fact, NO sensors have been constructed based on the reaction of NO with this fluorophore.<sup>2,35,36</sup> Not surprisingly, therefore, both Newport Green and FluoZin-3 act as weak NO sensors. Thus, when both  $Zn^{2+}$  and NO may be involved in cellular processes that are under study, particular care needs to be taken in analyzing results based on the use of these probes. Lastly, the results of Fig. 9 suggest that NO-modified FluoZin-3 has lost its  $Zn^{2+}$  sensing property. Experiments are ongoing to understand the impact of NO on the chemistry of this probe.

## Acknowledgements

This research was supported by NIH grants ES-024509 and ES-04184 and by a Research Growth Initiative award from the University of Wisconsin–Milwaukee.

## References

- 1 K. P. Carter, A. M. Young and A. E. Palmer, *Chem. Rev.*, 2014, **114**, 4564–4601.
- 2 M. D. Pluth, E. Tomat and S. J. Lippard, *Annu. Rev. Biochem.*, 2011, **80**, 333.

- 3 E. L. Que, D. W. Domaille and C. J. Chang, *Chem. Rev.*, 2008, **108**, 1517–1549.
- 4 A. Nowakowski, M. Karim and D. Petering, *Encyclopedia of Inorganic and Bioinorganic Chemistry*, 2015, pp. 1–10.
- 5 D. H. Petering, *Chemtracts: Inorg. Chem.*, 2004, **17**, 569–580.
- 6 S. J. Langmade, R. Ravindra, P. J. Daniels and G. K. Andrews, *J. Biol. Chem.*, 2000, **275**, 34803–34809.
- 7 J. S. Stamler, D. J. Singel and J. Loscalzo, *Science*, 1992, **258**, 1898.
- 8 S. Habib and A. Ali, *Indian J. Clin. Biochem.*, 2011, **26**, 3–17.
- 9 M. T. Gladwin, J. H. Crawford and R. P. Patel, *Free Radical Biol. Med.*, 2004, **36**, 707–717.
- 10 D. A. Wink and J. B. Mitchell, *Free Radical Biol. Med.*, 1998, **25**, 434–456.
- 11 K.-D. Kroncke, K. Fehsel, T. Schmidt, F. T. Zenke, I. Dasting, J. R. Wesener, H. Bettermann, K. D. Breunig and V. Kolbbaehofen, *Biochem. Biophys. Res. Commun.*, 1994, **200**, 1105–1110.
- 12 K. Katakai, J. Liu, K. Nakajima, L. K. Keefer and M. P. Waalkes, *Toxicol. Lett.*, 2001, **119**, 103–108.
- 13 M. A. Schwarz, J. S. Lazo, J. C. Yalowich, W. P. Allen, M. Whitmore, H. A. Bergonia, E. Tzeng, T. R. Billiar, P. D. Robbins and J. R. Lancaster, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 4452–4456.
- 14 P. J. Bernal, K. Leelavanichkul, E. Bauer, R. Cao, A. Wilson, K. J. Wasserloos, S. C. Watkins, B. R. Pitt and C. M. S. Croix, *Circ. Res.*, 2008, **102**, 1575–1583.
- 15 C. M. S. Croix, K. Wasserloos, K. Dineley, I. Reynolds, E. Levitan and B. Pitt, *Am. J. Physiol.: Lung Cell. Mol. Physiol.*, 2002, **282**, L185–L192.
- 16 A. Gow and H. Ischiropoulos, *Am. J. Physiol.: Lung Cell. Mol. Physiol.*, 2002, **282**, L183–L184.
- 17 J. Zhu, J. Meeusen, S. Krezoski and D. H. Petering, *Chem. Res. Toxicol.*, 2010, **23**, 422–431.
- 18 J. W. Meeusen, H. Tomasiewicz, A. Nowakowski and D. H. Petering, *Inorg. Chem.*, 2011, **50**, 7563–7573.
- 19 A. B. Nowakowski, J. W. Meeusen, H. Menden, H. Tomasiewicz and D. H. Petering, *Inorg. Chem.*, 2015, **54**, 11637–11647.
- 20 A. Nowakowski and D. Petering, *Metalloomics*, 2012, **4**, 448–456.
- 21 A. B. Nowakowski and D. H. Petering, *Inorg. Chem.*, 2011, **50**, 10124–10133.
- 22 W. Lin, B. Mohandas, C. P. Fontaine and R. A. Colvin, *BioMetals*, 2007, **20**, 891–901.
- 23 G. Ellman and H. Lysko, *Anal. Biochem.*, 1979, **93**, 98–102.
- 24 W. Maret and Y. Li, *Chem. Rev.*, 2009, **109**, 4682–4707.
- 25 J. A. Hrabie, J. R. Klose, D. A. Wink and L. K. Keefer, *J. Org. Chem.*, 1993, **58**, 1472–1476.
- 26 K. Schmidt, W. Desch, P. Klatt, W. R. Kukovetz and B. Mayer, *Naunyn Schmiedeberg's Arch. Pharmacol.*, 1997, **355**, 457–462.
- 27 D. Jourdeuil, F. L. Jourdeuil and M. Feelisch, *J. Biol. Chem.*, 2003, **278**, 15720–15726.
- 28 X. Michalet, F. Pinaud, L. Bentolila, J. Tsay, S. Doose, J. Li, G. Sundaresan, A. Wu, S. Gambhir and S. Weiss, *Science*, 2005, **307**, 538–544.
- 29 S. B. VanEngelenburg and A. E. Palmer, *Curr. Opin. Chem. Biol.*, 2008, **12**, 60–65.
- 30 D. W. Domaille, E. L. Que and C. J. Chang, *Nat. Chem. Biol.*, 2008, **4**, 168–175.



- 31 I. Johnson, *Histochem. J.*, 1998, **30**, 123–140.
- 32 X. Peng, J. Du, J. Fan, J. Wang, Y. Wu, J. Zhao, S. Sun and T. Xu, *J. Am. Chem. Soc.*, 2007, **129**, 1500–1501.
- 33 X. Zhang, Y. Xiao and X. Qian, *Angew. Chem., Int. Ed.*, 2008, **47**, 8025–8029.
- 34 M. R. Karim and D. H. Petering, *Metallics*, 2016, **8**, 201–210.
- 35 M. D. Pluth, M. R. Chan, L. E. McQuade and S. J. Lippard, *Inorg. Chem.*, 2011, **50**, 9385–9392.
- 36 M. H. Lim, *Nat. Protoc.*, 2007, **2**, 408–415.

