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NOTE

Development of non-denaturing off-gel isoelectric focusing for the separation of uranium–protein complexes in fish

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Abstract An off-gel non-denaturing isoelectric focusing (IEF) method was developed to separate uranium–biomolecule complexes from biological samples as a first step in a multidimensional metalloproteomic approach. Analysis of a synthetic uranium–bovine serum albumin complex demonstrated the focusing ability of the liquid-phase IEF method and the preservation of most of the uranium–protein interactions. The developed method was applied to gill cytosol prepared from zebrafish (*Danio rerio*) exposed to depleted uranium. The results were compared in terms of resolution, recovery, and protein identities with those obtained by in-gel IEF using an immobilized pH gradient gel strip.

Keywords Off-gel isoelectric focusing · Uranium · Protein · Non-denaturing · Zebrafish gill

Introduction

The chronic toxicity of uranium (U) is largely dependent on interactions of this element with proteins [1]. The description of these interactions in vivo is hampered by typically low levels of U in the environment and their non-covalent character [2].

Xu et al. [3] recently developed a non-denaturing isoelectric focusing (IEF) method using immobilized pH gradient (IPG) gel strips and laser ablation inductively coupled plasma-mass spectrometry (LA ICP-MS) detection for in vivo quantitative analysis of U–protein complexes. The principal drawbacks of IPG strip gel-based techniques for the study of non-covalent metal–biomolecule complexes include the limited sample load and the virtual impossibility to recover the intact metal–protein complex and even intact protein from the gel for further characterization [4]. These drawbacks can be alleviated by liquid-phase IEF (i.e., off-gel IEF).

The aim of this work was to develop an off-gel non-denaturing isoelectric focusing method to fractionate zebrafish (*Danio rerio*) gill cytosol after exposure to waterborne depleted U in view of the identification of U-binding proteins. The results were compared in terms of resolution, recovery, and protein identities with those obtained using IPG strip IEF–LA ICP-MS discussed elsewhere [3].

Materials and methods

Details about chemicals, reagents, biological samples, instrument settings, and procedures are available in the Electronic Supplementary Material.

Off-gel IEF experiments were carried out at 4 °C (in a cold room) with a MicroRotofor® (Bio-Rad, Hercules, CA) device. The ion exchange membranes were equilibrated in the appropriate electrolyte overnight (12 h) and thoroughly rinsed with water before use. Freshly prepared zebrafish gill cytosol, 1.5 mL, was mixed with a commercial carrier ampholyte (CA) solution and ultrapure water to reach a total volume of 3 mL and CA percentage (all in *w*/*v*) of 2 % Brd (broad: pH 3–10), 0.5 % Brd, or 0.5 % Nrw (narrow: pH 4–7). The resulting mixture was loaded in the focusing chamber of the MicroRotofor® (~2.5 mL). The catholyte solution was

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0.1 M NaOH and anolyte solutions were 0.1 M H_3PO_4 (for Brd-type CA) and 0.5 M CH_3COOH (for Nrw-type CA), respectively. The voltage applied for the focusing was controlled by setting 1 W constant power, and the focusing was stopped after 30 min of voltage/current stabilization. The liquid fractions (10×250 μL) were immediately and simultaneously harvested. The pH of each fraction was measured using a micro-electrode calibrated daily in the 4–10 pH range (precision 0.02 pH units). After each experiment, the focusing chamber, needle array, and harvesting tray were washed three times with 200 mM NH_4HCO_3 solution to remove traces of U and then thoroughly rinsed with water.

Protein, U, and Fe recoveries were assessed by comparing the quantities loaded in the off-gel IEF system with those recovered in the 10 liquid fractions. The strip IEF–LA ICP-MS, tryptic digestion, and μRPC–electrospray ionization (ESI)–MS/MS protocols optimized elsewhere [3] were adapted to the analysis of zebrafish gill cytosol using the same instruments. Synthetic U–bovine serum albumin (BSA) complexes (nU/nBSA ratio=4.7×10^{-4}, representative of the nU/nprotein ratio in studied cytosol of gill samples) were prepared as described elsewhere [3] and subsequently focused by off-gel IEF (0.5 % Nrw). The resulting U–BSA-rich fraction was subsequently analyzed by reversed-phase liquid chromatography (RPLC) (C_4) coupled to both ICP sector field mass spectrometry (SFMS) and ESI–MS.

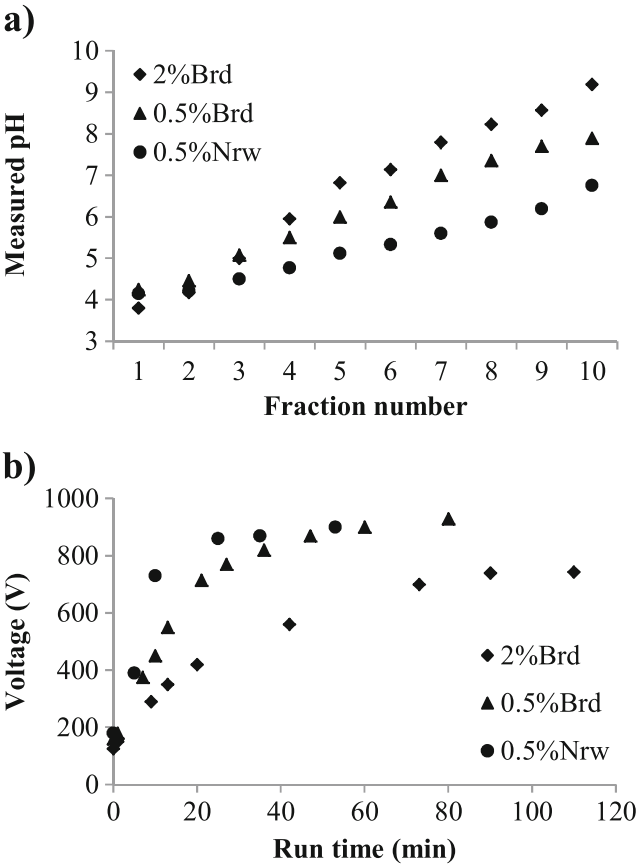

Fig. 1 pH gradient (**a**) and voltage profile/runtime (**b**) obtained after off-gel IEF of different cytosol samples with different types (broad *Brd* and narrow *Nrw*) and percentages (0.5 and 2 %) of carrier ampholytes

Results and discussion

Selection of CA

Commercial CA is a mixture of low molecular weight (200–1,200 Da) synthetic oligopeptides. The reduction of CA percentage was therefore motivated by (i) the minimization of potential CA–protein/U interactions and (ii) the prevention of ionization efficiency alteration and background noise elevation for species with $m/z<1{,}000$ in further ESI–MS/MS analysis due to the presence of CA. Figure 1 shows the influence of the CA percentage and type on the measured pH gradient (a) and on the voltage profile and runtime (b) for cytosol sample analysis. The reduction of the CA percentage from 2 % Brd (condition recommended by the manufacturer) to 0.5 % Brd restricted the pH interval from 3.8–9.2 to 4.1–7.9, respectively. Neither of these intervals covered satisfactorily the expected 3–10 pH range, but both pH gradients were quite linear (R^2=0.9815 and 0.9916, respectively) with a minimum 0.5 pH unit resolution (pH difference between two adjacent compartments). However, cytosol analysis using the manufacturer-recommended condition (Electronic Supplementary Material, Fig. S1) showed that most of the U and proteins were focused in the 4–7 pH interval. A linear 4–7 pH gradient (R^2=0.9836) was then obtained with 0.5 % Nrw, and the resolution was 0.30±0.05 pH unit. The runtime was twice shorter compared to the recommended condition, but the stabilization voltage increased from 750 to 900 V (Fig. 1b) which is still one third of the voltage applied in the in-gel IEF procedure. Limiting runtime and voltage could favor the decrease of dissociation of weak U–protein complexes.

As previous studies indicated a specific affinity of U for some metal-containing proteins [5], monitoring other metals (Fe, Cu, or Zn) is of interest. The main obstacle was the metal content of CA as it could result in the false-positive detection of metals in some cytosolic fractions and biased metal distribution patterns. Only the Fe level in narrow range CA was low enough for unbiased monitoring. The U contamination of both CA types was low (~100 pg U) and comparable to the amount of U present in cytosol prepared from unexposed (control) zebrafish whereas amounts in exposed groups were at least 10 times higher.

U–BSA complex preservation

Figure S2 (Electronic Supplementary Material) shows U and BSA distributions after off-gel IEF with 0.5 % Nrw of a synthetic mixture of U and BSA with an initial U/BSA molar

ratio of 4.7×10^{-4}. The average (n=3) BSA and U recoveries were 75±10 and 41±6 %, respectively. Most (84 %) of the recovered BSA was focused in agreement with the BSA pI interval given in the product information datasheet (pI 4.9–5.3, Sigma-Aldrich A2058). The recovered U was distributed along the entire 4–7 pH gradient with a maximum (20 %) focused with BSA at pH 5.3 where the U/BSA molar ratio decreased by ~45 % compared to the initial ratio. This loss of U was explained by a moderate affinity of U for BSA (log K= 4.8 [6]). The complexation constant of this weak synthetic complex could be representative of an average association constant found in vivo.

An aliquot of the pH 5.3 fraction was further analyzed by RPLC (C_4) coupled to ICP SFMS and ESI–MS. The co-elution of U (Electronic Supplementary Material, Fig. S3) and BSA (Electronic Supplementary Material, Fig. S4) at 17 min confirmed the preservation of the U–BSA complex after off-gel IEF. However, only free BSA (66,421±12 Da) was identified in the ESI–MS spectra, and the absence of signal from an intact U–BSA complex (66,700 Da for uranyl–BSA) was attributed to the low and environmentally relevant U/protein molar ratio (~10^{-4} range).

Application to zebrafish gill cytosol and comparison with in-gel IEF

Histograms in Fig. 2 show the average U, Fe, and protein distributions for a pool of zebrafish gill cytosol fractionated by off-gel IEF with 0.5 % Nrw. For comparison, U and Fe electrophoregrams obtained by IPG IEF–LA ICP-MS for the corresponding sample are presented in Fig. 2 (red line). The resolution was overall better for in-gel IEF (sharp U and Fe peaks) than for off-gel IEF. This was attributed to the design of the focusing chamber (only 10 pH fractions) whereas the pH gradient was continuous on the IPG strip.

From the separation point of view, a good agreement was found for both U- and Fe-rich fractions between off-gel and in-gel techniques. Nevertheless, two differences could be pointed out regarding the U distribution (Fig. 2a). The U background was elevated (>100 cps) below pH 5 in the in-gel electrophoregram while there was little (<2.5 %) U in this off-gel pH region. On the contrary, the U background was very low (<20 cps) in-gel above pH 6.5, while about 10 % of U was found in the corresponding off-gel pH fraction. These differences could be attributed to (i) different U–CA and U–immobiline interactions, (ii) differences in terms of focusing time and voltage potentially affecting the stability of the U–protein complexes, or (iii) differences in the prefocusing steps. Indeed, the pH gradient was preexistent on the IPG strip, and the sample faced a wide pH range during the 12-h rehydration step. This could potentially change U speciation before focusing. The off-gel IEF pH gradient was only formed after the focusing process began, and the sample mixture with 0.5 %

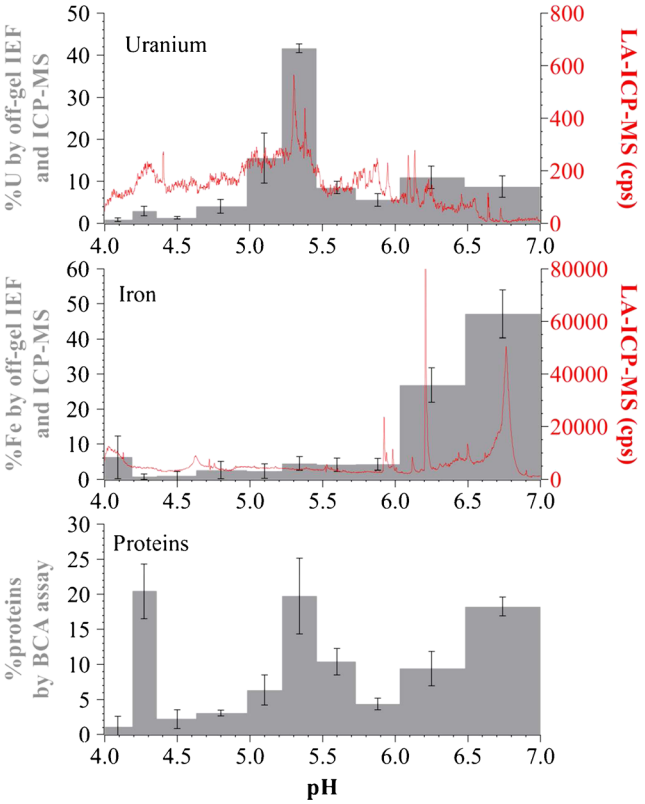

Fig. 2 Distribution of U, Fe, and proteins in zebrafish gill cytosol by off-gel IEF (*histograms*) (0.5 % Nrw, pH 4–7 gradient, n=3, pool of four individuals, mean±SD) and by in-gel IEF (*red line*, U and Fe only) (IPG strip 13 cm, pH 4–7 linear gradient)

CA had an average pH of 7.3. This pH value is typical for cytosolic compartment and therefore close to the native sample pH.

The average (n=3) U, Fe, and protein recoveries for off-gel IEF (0.5 % Nrw) were 25±8, 52±25, and 53±5 %, respectively. They were significantly lower than for the synthetic U–BSA sample. The U, Fe, and protein losses were most probably caused by adsorption in the focusing cell, ion exchange membranes, and harvesting device. The average (n=3) U recovery for strip IEF (pH 4–7) was measured at 48±20 % according to the method described elsewhere [3]. The average U recoveries of both off-gel and in-gel techniques were not significantly different, but the reproducibility was better for off-gel IEF as shown by a smaller standard deviation.

Protein identification in two off-gel IEF fractions (pH 5.3 and 6.2) and the corresponding in-gel IEF bands (Electronic Supplementary Material, Table S1) showed a satisfactory overlap of the identified proteins (~60 %) and similar sequence coverage between both methods. Shifts observed between measured and theoretical pI are common to both techniques and mainly attributed to posttranslational modifications. The ability to detect lower-abundance proteins with the off-gel method was essentially attributed to the absence

of the protein extraction step in the case of liquid-phase IEF fractions compared to IPG strip gel bands.

Conclusion

The developed off-gel IEF method offered comparable and complementary results to in-gel IEF for the separation of in vivo U–protein complexes in terms of elemental distribution and protein identification. The focusing time was 12 times shorter, and the maximum voltage applied, three times lower for off-gel IEF compared to in-gel IEF. These reduced time and voltage could contribute to the preservation of weak U–protein interactions as demonstrated with the analysis of a synthetic U–BSA complex. The handling and further analysis of liquid IEF fractions are simplified. Metal content could be assessed directly by ICP-MS without the need of laser ablation. No extraction of the proteins from the gel is required to characterize the complexes. The protein concentration in off-gel fractions could be determined without staining, and the actual pH could be measured. The drawbacks of the off-gel IEF include the presence of CA, a limited resolution, and a risk of cross contamination (vs. single-use IPG strips).

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References

1. Bresson C, Ansoborlo E, Vidaud C (2011) Radionuclide speciation: a key point in the field of nuclear toxicology studies. J Anal At Spectrom 26(3):593–601
2. Frelon S, Guipaud O, Mounicou S, Lobinski R, Delissen O, Paquet F (2009) In vivo screening of proteins likely to bind uranium in exposed rat kidney. Radiochim Acta 97(7):367–373. doi:10.1524/ract.2009.1619
3. Xu M, Frelon S, Simon O, Lobinski R, Mounicou S (2013) Non-denaturating isoelectric focusing gel electrophoresis for uranium–protein complexes quantitative analysis with LA-ICP MS. Anal Bioanal Chem:1–10. doi:10.1007/s00216-013-7033-8
4. Mounicou S, Szpunar J, Lobinski R (2009) Metallomics: the concept and methodology. Chem Soc Rev 38(4):1119–1138
5. Michon J, Frelon S, Garnier C, Coppin F (2010) Determinations of uranium(VI) binding properties with some metalloproteins (transferrin, albumin, metallothionein and ferritin) by fluorescence quenching. J Fluoresc 20(2):581–590
6. Yang Y, Feng Y, Wang Y, Wang L, Shi W (2013) Interactions between U(VI) and bovine serum albumin. J Radioanal Nucl Chem:1–6. doi:10.1007/s10967-013-2487-x