Talanta 120 (2014) 433–442

Contents lists available at ScienceDirect

Talanta

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

Combining TBP-based rOFFGEL-IEF with FASP and nLC–ESI-LTQ-MS/MS for the analysis of cisplatin-binding proteins in rat kidney

I. Moraleja, E. Moreno-Gordaliza, M.L. Mena, M.M. Gómez-Gómez *

Analytical Chemistry Department, Universidad Complutense de Madrid, Avda. Complutense s/n, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 3 August 2013
Received in revised form
22 November 2013
Accepted 28 November 2013
Available online 22 December 2013

Keywords:
Platinum-binding proteins
Kidney tissues
OFFGEL-IEF
FASP
ICP-MS
nLC–ESI-LTQ-MS/MS

ABSTRACT

In this work, a methodology based on a reducing IEF separation in combination with a FASP tryptic digestion able to maintain the integrity of cisplatin–protein complexes has been developed. The method is based on OFFGEL-IEF under conditions provided by the thiol-free reducing agent TBP, which allowed the separation of cisplatin-binding proteins in liquid fractions. The FASP procedure is applied as an intermediate stage between the IEF separation and MS analysis where the proteins are retained and concentrated in a commercially available ultrafiltration device. The filter unit acts as a proteomic reactor for detergent removal, buffer exchange, chemical modification (reduction and alkylation) and protein digestion. Finally, purified peptides are recovered by centrifugation. This procedure provides efficiencies comparable to standard in-solution digestion and the risk of platinum-complexes loss is minimized due to the fact that reagents employed along the process are subsequently eliminated before the following step. The stability of platinum–protein complexes under the FASP tryptic digestion, either using TBP or DTT as reducing agents, was maintained, allowing the identification of several platinum-containing peptides from cisplatin–HSA. This methodology was applied to the separation of platinum-enriched protein fractions obtained by SEC–ICP-MS in a kidney tissue extract from a rat treated with cisplatin, followed by further identification by nLC–ESI-LTQ-MS/MS after FASP tryptic digestion of selected platinum-containing liquid fractions.

1. Introduction

Cis-diamminedichloroplatinum (II) (cisplatin) is by far the most widely employed anticancer agent in the treatment of solid tumors [1,2]. However, it presents several side effects such as nephrotoxicity, neurotoxicity, ototoxicity and emetogenesis, which limit the dose that can be administered. Among all these effects, nephrotoxicity is the major dose-limiter in cisplatin therapy [3], which may result in acute renal failure [4–6]. Recent bioimaging studies performed by LA-ICP-MS [7] revealed the distribution and accumulation of platinum in rat kidney after cisplatin treatment, showing the highest content in the cortex and corticomedullary junction. The damage exerted by the drug mainly occurs in the RPTECs of the cortex [8]. This demonstrates the connection between platinum accumulation and renal damage. The origin of these toxic effects is thought to be related to the ability of platinum to form complexes with proteins [9], mainly coordinating to S- or N-containing amino acid residues. Therefore, in the last years, efforts have been directed toward the characterization of platinum–protein complexes in biological samples under cisplatin treatment [10].

At present, the coupling of liquid chromatography to tandem mass spectrometry (LC–MS/MS) is the preferred strategy for protein identification in biological samples. But to achieve the greatest amount of identified proteins, samples must be previously separated in multiple dimensions due to their complexity [11,12]. A widely used method for fractionating complex protein samples is 2-DE [13,14], which provides separations with high resolution, but is technically demanding and difficult to automate. Furthermore, large scale analysis of proteomes is challenging because protein spots have to be visualized by staining, individually excised, digested and the peptides generated need to be extracted from the gel prior to analysis by LC-MS/MS, which is a tedious and time-consuming process. To avoid these limitations, fractionation

Abbreviations: IEF, isoelectric focusing; FASP, filter-aided sample preparation; TBP, tributylphosphine; DTT, dithiothreitol; LA-ICP-MS, laser ablation-inductively coupled plasma mass spectrometry; RPTECs, renal proximal tubule epithelial cells; 2-DE, two-dimensional gel electrophoresis; IPG, immobilized pH gradient; IAA, iodoacetamide; nLC–ESI-LTQ-MS/MS, nanoliquid chromatography coupled to electrospray linear ion trap tandem mass spectrometry; rOFFGEL-IEF, reducing OFFGEL-IEF; nrOFFGEL-IEF, non-reducing OFFGEL-IEF; SEC–ICP-MS, size exclusion chromatography–inductively coupled plasma-mass spectrometry; TF, human apotransferrin; HSA, human serum albumin; CA, carbonic anhydrase; MYO, myoglobin; CYT C, cytochrome c

* Corresponding author. Tel.: +34 394 5146.
E-mail addresses: irenemoraleja@ucm.es (I. Moraleja), estefania.moreno@quim.ucm.es (E. Moreno-Gordaliza), mariluz@ucm.es (M.L. Mena), mmgomez@ucm.es (M.M. Gómez-Gómez).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.11.084

devices based on solution IEF have been developed and combined successfully with MS for proteomic applications [15–17]. Recently, several studies have demonstrated the feasibility of the OFFGEL-IEF fractionators for protein separation [18–20]. With this approach, the protein separation takes place in a two-phase system with an upper liquid phase that is divided into wells and a lower phase that is a rehydrated IPG gel strip. Typically, the protein mixture is diluted with a buffer solution, which contains urea, thiourea, DTT, carrier ampholytes and glycerol and loaded into all the wells. Because there is no liquid connection between the wells, proteins are forced to migrate through the IPG gel, where the real separation takes place. Then, focused proteins diffuse into the liquid phase and thus can be easily recovered at the end of the IEF separation, which is its main feature.

It has to be remarked that OFFGEL-IEF was originally designed for proteins without regard for the trace element which they may be carrying, and the suitability of this method for metalloproteomic separations will depend strongly on the stability of the metal–protein complexes. In fact, platinum losses may occur due to the strong reactivity of platinum compounds toward S-donor molecules, such as thiourea or DTT. We recently demonstrated that the presence of a reducing agent with thiol groups at high temperatures or during long incubation time produces deleterious effects in the binding between platinum and proteins [21]. The same was reported to occur with thiourea. As a result, it was suggested that traditional denaturing and reducing OFFGEL-IEF does not seem to maintain the whole integrity of platinum–protein complexes and separations should be performed under non-reducing conditions and in absence of thiourea in order to preserve platinum–protein complexes.

Considering these facts and the lower resolution and solubility offered under non-reducing conditions, the use of TBP is considered in this work as a possible alternative to DTT for the separation of platinum-containing proteins. The use of TBP was reported by Herbert et al. [22] as the reducing agent in both the sample solution for the first-dimensional isoelectric focusing and during the IPG equilibration procedure, and its use has increasingly spread in biochemical applications over the past years [23–27]. This is due to the fact that TBP improves protein solubility during IEF, which results in shorter run times and increased resolution. One explanation for the improved resolution is that TBP, an uncharged reducing agent, maintains reducing conditions for the entire IEF process (unlike DTT), thereby minimizing aggregation that could occur through disulfide bonding. Furthermore, TBP operates in a stoichiometric reaction, allowing the use of lower concentrations of the reagent (2 mM instead of 64 mM used for DTT). Moreover, the fact that TBP lacks sulfur groups and presents phosphorous instead, which may be less reactive towards Pt (II), makes this type of reducing agents very appealing candidates for platinum–protein complexes analysis.

After OFFGEL-IEF fractionation, proteolytic digestion prior to the analysis by LC-MS/MS needs to be carried out, to facilitate protein identification [28]. Digestions are typically done in-solution [29] and reduction and alkylation prior to digestion lead to improved peptide recoveries. Although the use of DTT and IAA within in-solution digestions still allowed detecting cisplatin–insulin peptides by nESI-MS [30], these conditions impaired the detection of certain platinum–peptides during in-gel digestion of cisplatin-incubated proteins [31], due to the aggressiveness of this kind of digestion. Therefore, the effect of reagents on the stability of platinum–protein complexes might be correlated with different factors, such as their concentration, exposure time and conditions, protein amount, platination degree and type of modified amino acid residues. Thereby, although the employment of sulfur-containing reagents is generally not recommended for platinum–protein complexes, their use may still be possible under certain experimental conditions.

Recently, a filter-aided sample preparation (FASP) procedure has been reported [32–34] as an alternative to in-solution digestion. In the FASP procedure, proteins are retained and concentrated in a commercially available ultra filtration device (spin filter). The filter unit then acts as a proteomic reactor for detergent removal, buffer exchange, chemical modification (reduction and alkylation) and protein digestion in the upper chamber. Finally, purified peptides are recovered by centrifugation through the membrane. It is remarkable that this procedure provides digestion efficiencies comparable to standard in-solution digestion, eliminates the reagents used in the reduction and alkylation steps prior to digestion and also allows the removal of urea and glycerol that would interfere with the MS analysis. The fact that this procedure eliminates the reagents employed along the process before the following step could minimize the risk of platinum loss for platinum–protein complexes.

The aim of this work is to evaluate the suitability of TBP for the rOFFGEL-IEF separation of the complexes formed between cisplatin and proteins. With this purpose, it was first studied on a model protein scale; and finally on high platinum to protein ratio fractions from a kidney tissue extract from a rat treated with cisplatin, where renal proteins were identified by nLC–ESI-LTQ-MS/MS. Moreover, the stability of cisplatin–protein complexes was also evaluated in the sequential reduction, alkylation and tryptic digestion under conditions provided by the FASP procedure.

2. Materials and methods

2.1. Chemicals

The platinum-based drug used was cisplatin (Sigma Aldrich Chemie, St. Louis, MO, USA). TF, HSA, CA from bovine erythrocytes, MYO from horse heart and CYT C from horse heart were also purchased from Sigma Aldrich. Sodium chloride (Panreac Química, SA, Barcelona, Spain) and 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris, Sigma Aldrich Chemie, St. Louis, MO, USA) were used for the preparation of the incubation solution under physiological conditions.

High-purity HNO_3, used for pH adjustment of the incubation media, and HCl were obtained by distillation of the analytical-grade reagents (Merck, Darmstadt, Germany) in an acid distiller (Berghof B BSB-939IR, Eningen, Germany). Stock solutions of platinum and iridium (1000 mg L^{-1}, Merck, Darmstadt, Germany) were diluted with HCl (0.24 mol L^{-1}) to prepare ICP-MS standard solutions. Working solutions were prepared daily and diluted with HCl (0.24 mol L^{-1}) to final concentration.

For tryptic digestions, Porcine Trypsin Gold mass spectrometry grade (Promega (Madison, WI, USA)) was used. All solutions were prepared in de-ionized water (Milli-Q Ultrapure water systems, Millipore, USA), excluding those solutions which were used for tryptic digestions and nLC–ESI-LTQ-MS/MS analysis, where mass spectrometry grade water from Scharlab (Barcelona, Spain) was used.

2.2. Standard proteins–cisplatin incubations

To reproduce the physiological intracellular saline and pH conditions, TF, HSA, CA, MYO and CYT C (62 μM) were incubated separately with cisplatin at a protein: cisplatin molar ratio 1:10 in a buffer containing Tris-NO_3 (10 mM, pH 7.4) and NaCl (4.64 mM), at 37 °C in a thermostatic bath (Neslab RTE-111, MedWOW, New Hampshire, USA) for 96 h. To remove unreacted cisplatin, samples were filtered through an Amicon Ultra-0.5 mL Ultracel-3 (3.0 kDa cut-off filter, Millipore, USA) by centrifugation at 14,000*g* during 30 min. The retained fraction containing cisplatin-bound proteins was recovered by reversing the filter and centrifugation at 1000*g*

for 2 min. A further washing step of the retained protein fraction with the incubation buffer was also carried out.

Total protein concentration in the solutions was determined by the Quick Start Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The platinum content in both fractions (retentate and filtrate) were analyzed by ICP-MS, as described in Section 2.6.

2.3. Rat kidney cytosolic extracts

2.3.1. Sample preparation

About 0.250 g of a kidney tissue from a rat, which was treated with a monodose of 16 mg cisplatin/m^2 of corporal surface and sacrificed three days after the treatment, as previously described [35], were dissected and homogenized in a Potter with 3.0 mL of a buffer containing Tris–HCl (10 mM), NaCl (25 mM), pH 7.40 and 12.5 μL of a protease inhibitor cocktail (Sigma Aldrich Chemie, St. Louis, MO, USA). The homogenates were centrifuged at 15,000*g* for 40 min. All the preparative steps were performed at 4 °C to minimize the risk of species degradation or transformation.

For subsequent OFFGEL-IEF separations (as described in Section 2.5), a clean-up step was performed by protein precipitation. First, six volumes of acetone (80% at −20 °C) were added to one volume of the cytosolic fraction (4.5 mg total protein content) to a final volume of 2.0 mL, incubating the mixture overnight at −20 °C. Next, the mixture was centrifuged at 13,000*g* for 10 min and the supernatant was carefully removed and discarded. The protein pellet was then washed with 500 μL of acetone, centrifuged as stated before, and the supernatant was again discarded.

2.3.2. SEC–ICP-MS

In a parallel experiment, pre-fractionation of platinum–protein complexes in the cytosolic renal extract was performed by size exclusion chromatography (SEC) using a Superdex™ 75 10/300 GL column (GE Healthcare Bio-Sciences AB, Sweden, separation range between 3 and 70 kDa). For the chromatographic separation, a high-pressure quaternary gradient pump (Jasco PU-2089), equipped with an injection valve (Rheodyne, USA), was used as the delivery system. The mobile phase employed was Tris–HCl 10 mM and NaCl 25 mM and pH 7.4, with a flow rate of 0.8 mL min^{-1} and 200 μL as injection volume. For elemental monitoring, the chromatographic system was coupled to an ICP-MS. Transient mode was set to chromatographic acquisitions (channels per AMU: 10 and integration time: 0.6 ms), monitoring *m/z* 194 (Pt) and 195 (Pt). The SEC column was calibrated with protein standards. Proteins used for SEC calibration were blue dextran (>2000 kDa), bovine albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa) and aprotinine (6.5 kDa) (Sigma-Aldrich Chemie).

The kidney cytosolic fraction was filtered through a 0.22 μm filter and injected into the SEC column and fractions were collected every minute (0.8 mL each). Both the platinum and protein content were determined in every fraction by ICP-MS and the Bradford assay, respectively. This allowed the location of fractions with high platinum content: e.g. fraction F17. The pool of ten identical fractions selected was subjected to the following procedure: 8.0 mL of the cytosolic extract was ultrafiltrated through an Amicon Ultra-0.5 mL Ultracel-3 (3.0 kDa cut-off filter, Millipore, USA), as already described in Section 2.2. The retentate was made up to 3.6 mL (150 μg total proteins content) with OFFGEL-IEF buffer containing TBP as reductant for further OFFGEL-IEF separations, as described in Section 2.5. Selected fractions were FASP tryptic digested as described in Section 2.4.1.

2.4. FASP procedure

2.4.1. Tryptic digestion

HSA (45 μg) or TF (45 μg), which had been incubated with cisplatin, were diluted with focusing buffer (urea 6.6 M, TBP 2 mM, ampholytes and glycerol 9.6%) to a final volume of 150 μL and added to the upper chamber of a Vivacon spin filter (Sartorius Stedim Biotech, Goettingen, Germany) with a nominal cutoff of 10 kDa, with slight modifications to the procedure previously described [33,36].

The sample was mixed with UA solution (0.2 mL of 8 M urea in NH_4HCO_3 50 mM), and centrifuged at 14,000*g* for 15 min. The retentates were further diluted in the devices with 0.2 mL of UA solution and centrifuged again. Next, a reduction step was performed, where two reducing agents were studied (TBP 5.0 mM or DTT 5.0 mM), followed by incubation during 30 min at 37 °C. Then, IAA 20 mM in NH_4HCO_3 50 mM was added, and incubated for 30 min at room temperature in the darkness (final volume in the reducing and alkylation stages between 150 and 200 μL). After centrifugation, the concentrate was diluted with UB solution (0.1 mL of 1 M urea in NH_4HCO_3 50 mM) as a clean-up step, and concentrated again. This step was repeated twice. Next, trypsin was added at 1:50 (w:w) enzyme: substrate ratio in 100 μL of UB solution, and the samples were incubated at 37 °C overnight. Peptides were collected by centrifugation, followed by three additional 30 μL washes with UB solution. Moreover, by adding 0.1 mL of SDS 1% and reversing the filter, the retained fraction containing non-digested platinum–proteins was collected by centrifugation at 1000*g* for 2 min. This step was performed twice.

The FASP procedure was also applied for the reduction, alkylation and tryptic digestion of cisplatin-containing standard proteins or cytosolic extracts. For the mixture of proteins or the protein pellet from the renal cytosolic fraction, a total protein content of 250 μg was loaded and digested in the spin filter, as described above.

Total protein concentration in the solutions was determined by the Quick Start Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The platinum content in the retentate and filtrate fractions was analyzed by ICP-MS, as described in Section 2.6.

2.4.2. Separation of platinum-binding peptides by SEC–ICP-MS analysis

In a parallel experiment, the detection of platinum-containing peptides in the filtrate fraction obtained after FASP tryptic digestion was performed by SEC using a Superdex™ Peptide 10/300 GL column (GE Healthcare Bio-Sciences AB, Sweden, separation range between 0.1 and 7 kDa). For the chromatographic separation, a high-pressure quaternary gradient pump (Jasco PU-2089), equipped with an injection valve (Rheodyne, USA), was used as the delivery system. The mobile phase employed was NH_4HCO_3 50 mM as mobile phase, with a flow rate of 0.8 mL min^{-1} and 200 μL as injection volume. For elemental monitoring, the chromatographic system was coupled to an ICP-MS. Transient mode was set to chromatographic acquisitions (channels per AMU: 10 and integration time: 0.6 ms), monitoring *m/z* 194 (Pt) and 195 (Pt).

2.4.3. Analysis of platinum-binding peptides from cisplatin–HSA by nLC–ESI-LTQ-MS/MS analysis

Selected platinum-contained peptides from cisplatin–HSA tryptic digestion samples (around 150 μL) were desalted by micro-solid phase extraction using OMIX C_{18} (100 μL, Millipore), eluted in 70% acetonitrile, 0.1% trifluoroacetic acid and evaporated in a vacuum centrifuge. Finally, digests were dissolved in 15 μL of a solution containing 2.0% acetonitrile and 0.1% formic acid and analyzed by nLC–ESI-LTQ-MS/MS as described in Section 2.7

2.5. OFFGEL-IEF separations

For the pI-based protein separation, the 3100 OFFGEL Kit pH 3–10 (Agilent Technologies Inc., Waldbron, Germany) with a 24 well setup was used according to the protocol of the supplier. Ten minutes prior to sample loading, 24 cm long IPG gel strips, with a linear pH gradient ranging from 3 to 10, were rehydrated in the assembled device with 40 μL of focusing buffer (either urea 6.6 M, DTT 64 mM, ampholytes and 9.6% glycerol; or urea 6.6 M, TBP 2 mM, ampholytes and 9.6% glycerol) per well.

The mixture of standard proteins (625 μg total protein content) or the pellet from the cytosolic fraction (4.5 mg total protein content) was diluted with the focusing buffer, which contains urea 6.6 M, TBP 2.0 mM, ampholytes and 9.6% glycerol, to a final volume of 3.6 mL, and 150 μL of sample were loaded in each well. The sample was then focused at 20 °C with a maximum current of 50 μA, and typical voltages ranging from 500 to 4000 V until 50 kVh^{-1} was reached after 24 h approximately. The recovered fractions (volumes between 100 and 150 μL) were diluted to 1.5 mL with de-ionized water for platinum determination by ICP-MS, as described in Section 2.6.

For comparison purposes, the same above mentioned amount of cisplatin-bound proteins were also separated by rOFFGEL-IEF under DTT reducing conditions. Samples were diluted with the focusing buffer, which contains urea 6.6 M, DTT 64 mM, ampholytes and 9.6% glycerol, to a final volume of 3.6 mL, and 150 μL of sample were loaded in each well.

2.6. Platinum analysis by ICP-MS

A Quadrupole ICP-MS Thermo X-series (Thermo Electron, Windford, Cheshire, UK) equipped with a Meinhard nebulizer, a Fassel torch, and an Impact Bead Quartz spray chamber cooled by a Peltier system was employed for total platinum determination. ICP-MS operating conditions were as follows: forward power, 1250 W; plasma gas, 15 L min^{-1}; auxiliary gas, 0.73 L min^{-1}; nebulizer gas, 0.85 L min^{-1}; channels per AMU, 10; and integration time, 0.6 ms. Signals were acquired in continuous mode, monitoring *m/z* 194 (Pt), 195 (Pt), and 191 (Ir). Non-spectral interferences (matrix effects) were not observed; therefore, quantification of platinum was carried out by external calibration over the working range (0.5–10.0 μg L^{-1}) with 10 μg L^{-1} iridium as internal standard (IS).

2.7. nLC–ESI-LTQ-MS/MS analyses

For peptide analysis by nLC–ESI-LTQ-MS/MS, a dual gradient system nanoLC pump (nanoLC ultra 1D Plus, Eksigent) with a Thermo Electron Micro AS autosampler was used. Aliquots of samples (5 μL) were injected, using a 20 μL loop and a pick-up method, and loaded on a trap-column (Reprosil pur C18, 3 μm particle size, 0.3 mm × 10 mm, 120 Å pore size, SGE) at a 3 μL min^{-1} flow rate using 2% acetonitrile, 0.1% HCOOH as mobile phase. The preconcentrated peptides were eluted and delivered in reversed flow direction at 200 nL min^{-1} to a reverse phase micro capillary analytical column (Acclaim PepMap 100, C18, 3-μm particle size, 75 μm × 15 cm, 100 Å pore size, Dionex, LC Packings). Peptide elution was performed applying a three-step gradient: 5–15% B linear for 5 min, 15–40% B linear for 40 min and 40–80% B linear for another 15 min, holding the system at 80% B for 10 min. Mobile phase B used was 99.9% ACN, 0.1% HCOOH. The column was connected to a stainless steel nano-bore emitter (O.D. 150 μm, I.D. 30 μm, Proxeon, Odense, Denmark) for spraying. The nHPLC system was coupled with either with a linear ion trap LTQ XL or with a linear ion trap LTQ-Orbitrap (Thermo Scientific, San Jose, CA, USA) equipped with a nano-electrospray (nESI) source from Proxeon (Odense, Denmark). Peptides were scanned and fragmented using a triple play scan method, consisting on acquisition of full enhanced MS scans in the positive ion mode, over the *m/z* range 400–1600, followed by zoom scans and further full enhanced MS/MS, acquired in profile mode, of the three most intense peaks in the full MS scan. CID activation of ions was applied in MS/MS experiments, with 35% relative collision energy and 30 ms activation time, being isolation width of the precursor ions set to 4. Dynamic exclusion was enabled with a repeat count of 1, using a 180 s exclusion duration window. During the analysis, the parameters were typically set to capillary temperature, 200 °C; spray voltage, 1.7 kV.

For data analysis, spectra were assessed with the Xcalibur Qual Browser software (Thermo Scientific). MS/MS spectra search on NCBI protein databases using SEQUEST and MASCOT allowed the identification of proteins. The search was performed against a rat (*Rattus norvegicus*) NCBI database, assuming monoisotopic masses and fully enzymatic digestion by trypsin. Oxidation of methionines and carbamidomethylation of cysteine residues were set as variable modifications. Two missed cleavages were allowed and tolerances of 2.0 Da and 0.5 Da were selected for peptide and fragment masses. Proteins identified both with a MASCOT significant protein score using $p < 0.01$ as a significance threshold, with at least a peptide with a score above the identity threshold, with $p < 0.05$; and a Sequest P (protein) $< 10^{-3}$ were taken as valid.

Platinum peptides were recognized and identified taking into account the isotopic patterns both in the zoom scans of the precursor ions and in the MS/MS spectra. In the case of model proteins, such as HSA, search on human Uniprot KB protein databases using SEQUEST allowed the confirmation of the identity of proteins and the coverage obtained.

3. Results and discussion

3.1. Evaluation of the stability of cisplatin–protein complexes under the FASP-assisted tryptic digestion procedure

In this work, the FASP procedure will be applied as an intermediate stage between OFFGEL-IEF separation and the nLC–ESI-LTQ-MS/MS analysis. Therefore, the suitability of such tryptic digestion for cisplatin-binding proteins has been tested. First, it was studied at a model protein scale, followed by its application to a kidney tissue extract from a rat treated with cisplatin.

HSA, previously incubated with cisplatin, was employed to evaluate the cisplatin–protein complex stability under the FASP procedure. Thus, 45 μg of cisplatin–HSA was diluted with the OFFGEL-IEF focusing buffer (to simulate the OFFGEL-IEF recovered fractions), added to the upper chamber of a commercial spin filter (10 kDa) and then, washed twice with urea 8.0 M in NH_4HCO_3 50 mM. Next, the cisplatin-coordinated protein was reduced with TBP 5 mM, alkylated with IAA 20 mM and finally, washed again twice with urea 1.0 M in NH_4HCO_3 50 mM. For comparison purposes, the stability of the cisplatin–HSA using DTT 5 mM as reductant was also studied. Following, cisplatin–HSA retained in the upper chamber of the spin filter were digested overnight with trypsin in a solution containing urea 1.0 M and NH_4HCO_3 50 mM (trypsin: protein ratio 1:50, w-w), and peptides were collected by centrifugation, followed by two additional 30 μL washes with urea 1.0 M and NH_4HCO_3 50 mM. The recovery of the tryptic peptides obtained was about 50% of the loaded protein as measured by the Bradford protein assay in the retentate. This recovery is in agreement with those reported by Wisniewski et al. [36] for tryptic peptides on classical proteomic studies for the same spin-filters.

To evaluate the possible losses of platinum along the procedure, the platinum content in the filtrate fractions after the washing, reducing and alkylating steps was determined by ICP-MS. For both reducing agents (TBP and DTT), the percentage of platinum found in all the three filtrate fractions was lower than 15%, calculated over the total platinum content measured in the retentate and filtrate fractions, being the protein losses, due to unspecific adsorption to the filters, around 5% for all the samples. Moreover, the percentage of platinum found in the filtrate fraction after the tryptic digestion, which contains the peptides was about 40% and 39% for TBP and DTT, respectively. These results pointed out that the platinum-bound protein was stable after the reducing step using both TBP and DTT reagents. The fact that cisplatin–protein bonds even remain stable during the reducing step with DTT could be due to the short exposure time during the FASP process. Moreover, for comparing purposes, the FASP procedure was also performed under non-reducing and non-alkylating conditions, which led to a recovery of only 16% of platinum in the peptide fraction. This result clearly shows that reducing and alkylating conditions are required to obtain appropriate peptide recoveries, which is especially critical for cystine-rich proteins such as HSA.

In a further attempt to improve peptide recovery, either a higher amount of trypsin (trypsin:protein ratio 1:25, w-w) or a second tryptic digestion step were studied. A higher trypsin:protein ratio did not significantly increase the platinum–peptide recovery (around 45%). Considering the almost negligible improvement and the fact that the presence of a high concentration of trypsin can hinder the subsequent identification of the peptides by ESI, the 1:50 trypsin:protein ratio was selected. On the other hand, after a second digestion step with fresh trypsin, the additional platinum–peptide recovery obtained was 20%. By combining both filtrates, a significant high platinum–peptide global recovery (around 60%) was obtained. Therefore this latter strategy will be employed in subsequent experiments.

In order to demonstrate the applicability of the FASP method for platinum–protein complexes analysis, it was necessary to check if platinum present in the peptide fraction still remained bound to peptides. For this purpose, the cisplatin–peptide complexes obtained after the FASP procedure from cisplatin–HSA were separated by SEC–ICP-MS. The platinum SEC–ICP-MS profile for the cisplatin–peptide complexes is shown in Fig. 1a. Moreover, free cisplatin incubated for 8 h in NH_4HCO_3 50 mM (that is the medium in which peptides were present) was also separated by SEC–ICP-MS for comparing purposes, and the platinum profile is shown in Fig. 1b. As can be seen in Fig. 1a, several platinum species were obtained between 12 and 28 min, which indicates the absence of free cisplatin (which presents several platinum species with retention times approximately between 25 and 45 min, as shown in Fig. 1b). These results point out that the platinum–protein bonds in cisplatin–HSA complexes are strong enough to resist the overnight tryptic digestion, as already was reported for in-solution tryptic digestion of insulin incubated with cisplatin [9].

On the other hand, cisplatin–peptide complexes obtained after the FASP procedure on cisplatin–HSA using DTT as reductant were also analyzed by nLC–ESI-LTQ-MS/MS, leading to the protein identification with 77.3% coverage of the amino acid sequence after Sequest search. Several ions were detected with an isotopic pattern expected from platinum-containing peptides at *m/z* 455.44, 628.06 and 765.80, as can be seen in Figs. 2a, S1 and S2a, respectively. The CID fragmentation spectrum of the ion at *m/z* 455.44 (3+), which is shown in Fig. 2a allows the identification of the peptide as $[^{65}SLHTLFGDK^{73}+Pt(NH_3)_2+Tris]^{3+}$. With respect to the CID-MS/MS spectrum, non-platinated *y-series* ions were found up to y_4^+. On the other hand, a platinated y_7^{2+} ion was observed at *m/z* 574.28 and 565.68, corresponding to $[HTLFGDK+Pt+NH_3+Tris]^{2+}$ and $[HTLFGDK+Pt+Tris]^{2+}$, respectively. Moreover, platinated b_3^+, b_4^+, and b_5^+ ions

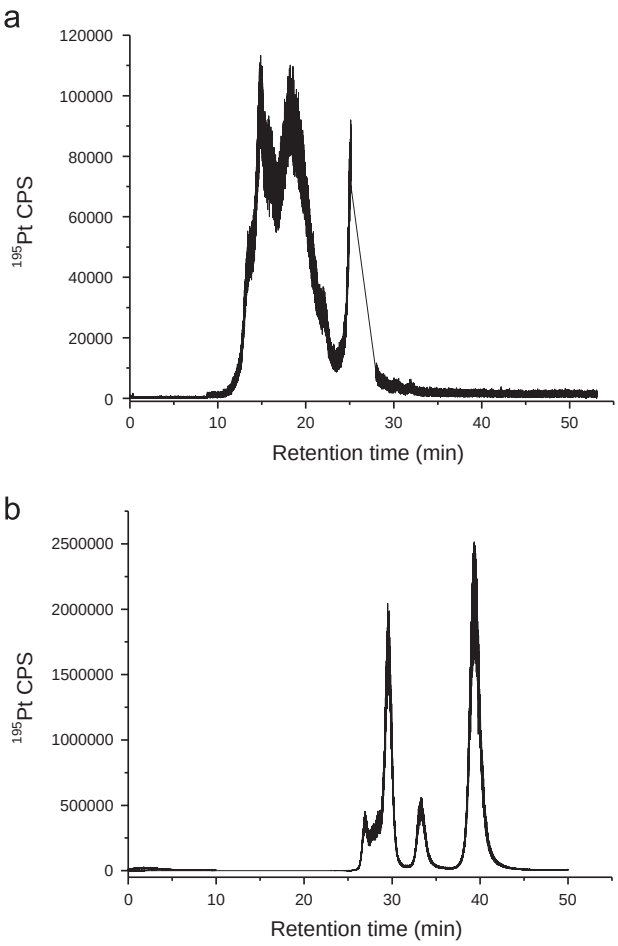

Fig. 1. SEC–ICP-MS chromatogram monitoring ^{195}Pt for (a) cisplatin–peptide complexes obtained after the FASP procedure for cisplatin–HSA, (b) cisplatin (10 μM) in NH_4HCO_3 50 mM for 8 h.

were found at *m/z* 668.16, 769.08 and 882.28, corresponding to $[SLH+Pt+NH_3+Tris]^+$, $[SLHT+Pt+NH_3+Tris]^+$, and $[SLHTL+Pt+NH_3+Tris]^+$, respectively. The $[SLHTLF+Pt+NH_3+Tris]^{2+}$ and $[SLHTLF+Pt+Tris]^{2+}$ fragment ions at *m/z* 515.20 and 506.64, respectively, were also detected and the unplatinated b_2^+ ion was found at *m/z* 201.04. These results point out that platinum is bound to H67. Furthermore, the peptide at *m/z* 628.06 (3+) was identified as $[^{360}C^*C^*AAADPHEC^*YAK^{372}+Pt(NH_3)+Tris]^{3+}$ as can be seen in Fig. S1. According to the CID-MS/MS spectrum, unplatinated-b ions were observed up to b_6^+. Nevertheless, the first platinated b ion found was also the same ion b_6^+, identified as $(C^*C^*AAAD+Pt+Tris)^+$, besides the ions b_8^+ and b_{11}^+. Regarding to y series, two unplatinated ions were observed (y_5^+ and y_7^{2+}), but as in the case of b ions, the first platinum-containing ion detected was y_7^{2+} (which is $y_7^{2+}+Pt$, $y_7^{2+}+Pt+Tris$, and $y_7^{2+}+Pt+NH_3+Tris$ at *m/z* 548.7, 609.02, and 617.7, respectively). These facts indicate that Pt may be coordinated to several binding sites within the sequence, probably to C361 and H367. Finally, the MS/MS spectrum of the peptide ion at *m/z* 765.80 (3+), allowed its identification as $[^{241}VHTEC^*C^*HGDLLEC^*ADDR^{257}+Pt(NH_3)]^{3+}$, being C* carbamidomethylcysteine. Unplatinated y^+ ions were detected up to y_5^+, and ions y_7^+ and y_{10}^+ were also identified, but the sequence could not be followed further. On the other hand, platinated b ions were found from b_7^{2+} to b_{15}^{2+}, as can be seen in Fig. S2a. No lower-mass platinated b-series ions could be found. These results point out H247 as the most probable binding site, although the exact platinum binding site could not be accurately located. Moreover, since the unplatinated *b* series is not observed, platinum may be

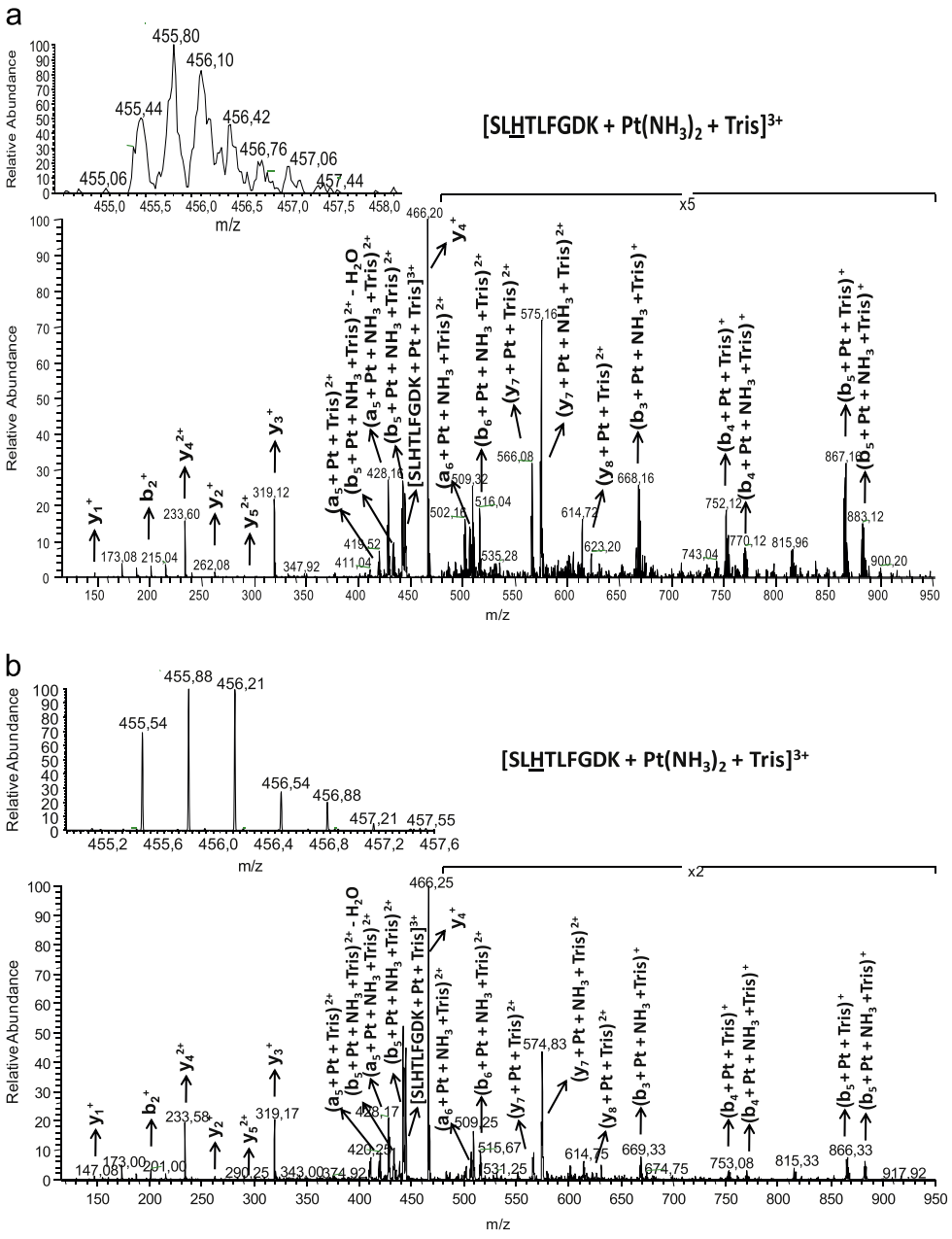

Fig. 2. CID-MS/MS spectrum of the platinum-containing peptide ion at (a) *m/z* 455.4 (3+), corresponding to [SLHTLFGDK+Pt$(NH_3)_2$+Tris]$^{3+}$ (DTT reducing conditions), and (b) *m/z* 455.54 (3+) corresponding to [SLHTLFGDK+Pt$(NH_3)_2$+Tris]$^{3+}$(TBP reducing conditions) for the nLC–ESI-MS/MS analysis of the HSA tryptic digests obtained after the FASP procedure. Inset: zoom scan MS of the platinated precursor ion. **H** represents the platinum bonding site.

coordinated to several binding sites within the sequence, probably to the cysteines and histidines, as is reported in previous studies [32].

For comparison purposes, cisplatin–peptide complexes obtained after the FASP procedure from cisplatin–HSA using TBP as reductant were also analyzed by nLC–ESI-LTQ-MS/MS, leading to the protein identification with 74.8% coverage of the amino acid sequence after Sequest search. Several ions were also detected with an isotopic pattern expected from platinum-containing peptides at *m/z* 455.54, 703.54 and 1061.78, as can be seen in Figs. 2b, S2b and S3, respectively. The CID fragmentation spectrum of the ion at *m/z* 455.54 (3+), which is shown in Fig. 2b allows the identification of the peptide as [^{65}SLHTLFGDK^{73}+Pt $(NH_3)_2$+Tris]$^{3+}$. These results point out that platinum is bound to H67. Furthermore, the peptide at *m/z* 703.54 (4+) was identified as [^{241}VHTEC*C*HGDLLEC*ADDRADLAK^{262}+Pt$(NH_3)_2$]$^{4+}$ as can be seen in Fig. S2b. Several unplatinated y^{2+} ions were detected, as y_6^{2+} and y_7^{2+}, and y^{2+} ions from y_{11}^{2+} to y_{15}^{2+}. Moreover, platinated b_7^{2+} and b_9^{2+} ions were found at *m/z* 558.65, 549.65 and 644.67, corresponding to [VHTEC*C*H+Pt]$^{2+}$, [VHTEC*C*H+Pt−H_2O]$^{2+}$, and [VHTEC*C*HGD+Pt]$^{2+}$, respectively. The platinated a_7^{2+} ion was also found at *m/z* 544.65. No lower-mass platinated b-series ions could be found. These results indicate that H247 is the most probable binding site, although the exact platinum binding site could not be accurately located. Finally, the CID fragmentation spectrum of the ion at *m/z* 1061.78 allows its identification as [^{287}SHC*IAEVENDEMPADLPSLAADFVESK^{313}+ Pt (NH_3)]$^{3+}$, which is shown in Fig. S3. These results point out that platinum is bound to M298.

According to the protein structure, all residues of the identified sites for TBP or DTT are located at the surface, as can also be shown in Fig. S4, being accessible to cisplatin during the incubation step.

As a result of that, it can be concluded that the FASP tryptic digestion using TBP as reductant proved to be appropriate for the

identification of several platinum binding sites on the HSA, which are all located at the protein surface. Several histidines (H67, H247) and methionine (M298) were identified from the CID-MS/MS, due to the considerable affinity of platinum (II) to the imidazole nitrogens of histidine or the thioether sulfur in methionine as was earlier reported. When DTT is used as reducing agent, a number of platinum binding sites, which are also located at the surface of HSA, were also identified, corresponding to the same previous histidines (H67, H247) and histidine (H367) or cysteine (C361). These results demonstrated that platinum bound to histidine or methionine withstands the action of 5 mM TBP or 5 mM DTT during the short incubation time (1 h) used for the FASP tryptic digestion. However, when DTT is used at high concentrations (10 mM) and longer incubation time (16 h) during in-gel digestion, as was earlier reported by Moreno-Gordaliza et al. [31], only one platinum–peptide was detected, which corresponds to the major binding site M298, evidencing once more the deleterious effect of thiol-containing reagents on the platinum–peptide bonds after long reaction time with a reducing agent with thiol groups (such as DTT).

Experiments were then conducted to investigate the FASP procedure for cisplatin–TF, a mixture of standard proteins (TF, HSA, CA, MYO and CYT C) and kidney cytosols from rats treated with cisplatin. Recovery values for total platinum loaded in the spin filter was 69%, 55% and 55%, for TF, the mixture of standard proteins and the kidney cytosol, respectively. Moreover, in all cases the peptide fractions obtained were also separated by SEC–ICP-MS, demonstrating that cisplatin still bound to peptides (data not shown).

As a result of that, it is possible to conclude that the FASP procedure both using TBP or DTT as reductants is presented as a very appealing alternative for the reduction, alkylation and tryptic digestion of cisplatin–protein complexes in biological samples.

3.2. *Evaluation of the cisplatin–protein complexes stability under TBP-based rOFFGEL-IEF separation*

With the aim to improve the OFFGEL-IEF separation resolution during the analysis of cisplatin–protein complexes in biological samples, a rOFFGEL-IEF methodology compatible with the preservation of the cisplatin–protein bonds was investigated.

First, the metal–protein complex stability was evaluated under rOFFGEL-IEF separation using TBP as reductant, in absence of thiourea. With this purpose, a mixture of proteins (TF, HSA, CA, MYO and CYT C), which had been individually incubated in the presence of cisplatin, were used, loading 125 μg of each protein in the OFFGEL-IEF. Then, the sample was diluted in the focusing buffer (containing urea, TBP, ampholytes and glycerol) and loaded into wells. After IEF, proteins are present in the liquid phase and can be recovered conveniently from the wells for further processing. Proteins were separated in 24 fractions, using strips with a pH range between 3 and 10 (data not shown). It can be concluded that experimental pI values are consistent with the theoretical values (6.8, 5.9, 6.4, 7.2, and 9.6 for TF, HSA, CA, MYO and CYT C, respectively). In addition, to evaluate the applicability of the separation protocol for cisplatin–protein analysis, platinum determination is needed to establish its correlation with the presence of proteins in the fractions produced following separation by IEF. This determination was performed by ICP-MS. Fig. 3a showed the platinum content in each well, which reflects the migration patterns for the mixed cisplatin–proteins. It can be seen that high amounts of platinum were obtained for the different fractions containing the different proteins, which indicates the preservation of the cisplatin–protein complexes. A recovery value of 50% was obtained for platinum, calculated over the total platinum amount loaded into the rOFFGEL-IEF. Furthermore, each fraction was also subjected to ultra-filtration through 3.0 kDa cut-off filters in order to measure the platinum remaining bound to proteins. Recoveries around 95% were obtained for the platinum bound to proteins in each well.

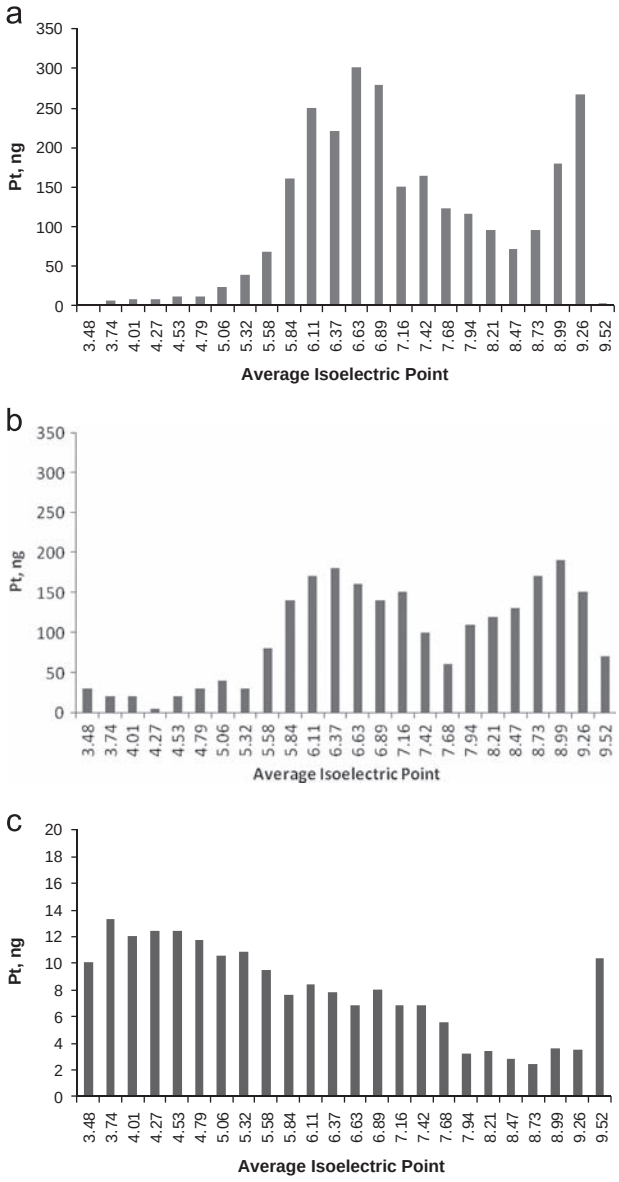

Fig. 3. Platinum profile obtained in the rOFFGEL-IEF fractions with ICP-MS analysis for: the separation of a mixture of standard proteins (TF, HSA, CA, MYO and CYT C) using (a) TBP or (b) DTT as reducing agent, or (c) the separation of kidney tissue extracts containing cisplatin using TBP as reducing agent. In all cases, the separation was performed in 24 fractions using strips with a pH range between 3 and 10.

For comparison purposes, a mixture of proteins (TF, HSA, CA, MYO and CYT C) was also separated by DTT-based rOFFGEL-IEF (Fig. 3b), providing a platinum recovery value of 34%. These results indicate that denaturing solutions containing DTT led to the release of a significant amount of the platinum bound to proteins. The significant platinum losses obtained for DTT-based rOFFGEL-IEF could be due to the high concentration of DTT (64 mM) used and the long exposure time involved during the separation (24–36 h). However, the lower concentration (5 mM) and shorter incubation time with DTT used in the FASP-assisted tryptic digestion do not produce deleterious effects in the binding between platinum and proteins, as was earlier reported in Section 3.1. These results are in agreement with early work, in which was stated that the effect of reagents on the stability of

platinum–protein complexes is correlated with different factors, such as their concentration, exposure time and conditions [21].

Next, kidney cytosols from rats treated with cisplatin were isoelectrically fractionated by TBP-based rOFFGEL-IEF. Prior to the separation, it was necessary to precipitate the proteins present in the cytosolic fraction (following the procedure described in Section 2.3.1) due to the incompatibility of the cytosolic extraction solution with the OFFGEL-IEF system. The pellet obtained showed a complete solubilization of the proteins in the focusing buffer solution (containing urea 6.6 M, TBP 2.0 mM, ampholytes and 9.6% glycerol) for the OFFGEL-IEF separation. The results on the platinum determination in the different fractions produced following separation by rOFFGEL-IEF are shown in Fig. 3c. Again, quite a good recovery value (52%) was obtained for platinum in the fractions, calculated over the total platinum amount loaded into the OFFGEL-IEF. Interestingly, this value is very close to the reference protein recovery range $(65 \pm 10)\%$ assessed by the manufacturer for different standard protein mixtures using the same type of OFFGEL strips. Furthermore, recoveries around 95% were also obtained for the platinum bound to proteins in each well. This clearly indicates that TBP seems to preserve the original Pt–protein complexes during rOFFGEL-IEF.

For comparison purposes, the kidney cytosolic proteins were also separated by DTT-based rOFFGEL-IEF (data not shown), providing a platinum recovery value of 28%. These results confirm that separations with denaturing solutions containing DTT, in contrast to TBP, lead to the release of a significant amount of the platinum bound to proteins.

Therefore, it can be concluded that platinum–proteins separations by OFFGEL-IEF under reducing conditions provided by TBP are an improved alternative to non-reducing conditions, with no significant protein-bound platinum loss involved. For this reason, this reducing agent was selected for further Pt–protein separations in complex biological samples.

3.3. *Analysis of cisplatin–protein complexes in a rat kidney tissue extract*

The high complexity of tissue-derived protein extracts and the relatively low abundance of platinum-coordinated proteins content found along the focusing fractions result in the need to design analytical methodologies which include strategies for sample enrichment in platinum species. This way, the difficult task of identifying platinum–protein complexes could be more feasible. With this regard, two main approaches were included: (a) protein extraction of cortex (isolated from cisplatin-treated rat kidneys), which presents the highest platinum content in the organ (3-fold with respect to the medulla [37]), and where the renal damage is mainly located; (b) a pre-fractionation by SEC–ICP-MS of kidney cytosols, performed to identify the protein fractions with the highest platinum content, which were selected for further TBP-based rOFFGEL-IEF separation, followed by FASP tryptic digestion using TBP as reducing agent and nLC–ESI-MS/MS analysis.

Proteins present in the renal cortex cytosolic fraction from rats treated with cisplatin (obtained according to the procedure described in Section 2.3.1) were first separated by SEC. Fig. 4a shows the corresponding SEC–ICP-MS chromatogram for ^{195}Pt monitoring. As can be seen in Fig. 4a, platinum species were found all over the protein separation range (from 6.5 to 70 kDa, based on the molecular weight of the calibration curve for the SEC column), mainly corresponding to platinum-bound proteins. Prominent platinum peaks were found above 70 kDa (top of the calibration range, corresponding to retention times between 8 and 11 min) and below 29 kDa, at retention times between 15 and 22 min. Next, the cytosol was injected again into the SEC column and fractions were collected every minute (0.8 mL each). The protein (Fig. 4b) and platinum

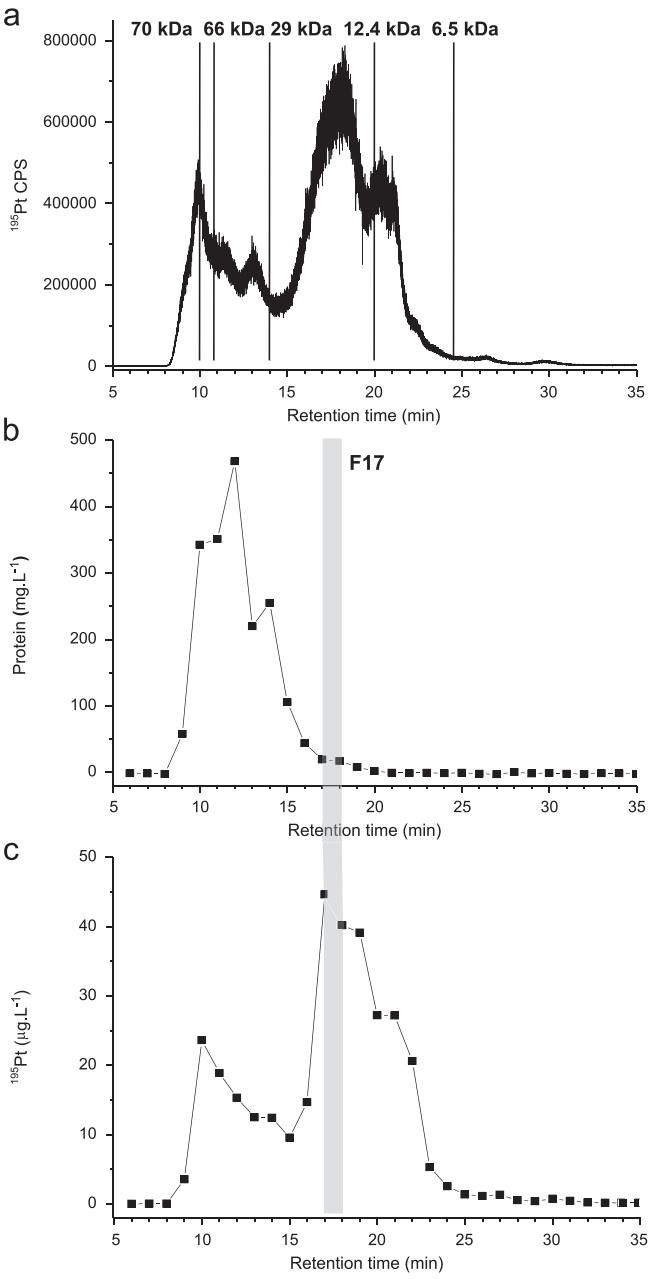

Fig. 4. (a) SEC–ICP-MS chromatogram monitoring ^{195}Pt for a kidney cytosol from a rat treated with cisplatin. Numbers correspond to molecular weight calibration markets: 70 kDa, 66 kDa, 29 kDa, 12.4 kDa and 6.5 kDa; (b) and (c) show the protein and platinum contents found in each collected 1-min interval fractions, respectively.

(Fig. 4c) content were measured (by Bradford assay and ICP-MS, respectively) in each fraction in order to identify those with a high platinum to protein ratio. As can be seen, the fractions collected at retention times between 16 and 22 min showed the highest platinum to protein ratios.

In order to avoid protein losses during the centrifugation stages of the FASP procedure (where 10 kDa cut-off filters are used), it is necessary to estimate the approximate molecular weight of proteins from the selected fractions. For this purpose, fractions of interest were filtrated through 3 and 10 kDa cut-off filters, and the platinum content was determined by ICP-MS. Results showed that around 75% protein-bound platinum contained in the fractions which elute between 16 and 18 min have a MW higher than 10 kDa. As a consequence, platinum-enriched protein fraction F17, corresponding

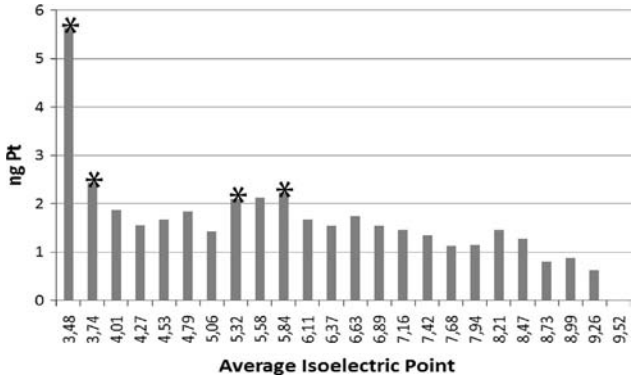

Fig. 5. Platinum profile obtained in the TBP-based OFFGEL-IEF fractions with ICP-MS analysis for the separation of the fraction F17 from a renal cortex kidney tissue extracts containing cisplatin. (*) Denotes selected wells to perform the FASP procedure and the nLC–ESI-LTQ-MS/MS analysis. The separation was performed in 24 fractions using strips with a pH range between 3 and 10.

to retention times of 17–18 min was selected for a further TBP-based rOFFGEL-IEF separation. Then, selected SEC fraction F17 with a high platinum to protein ratio content was collected after ten injections of a renal cortex cytosol from a rat treated with cisplatin, and each fraction was pooled (8.0 mL total volume). Pooled fraction was subjected to a preconcentration step (employing cut-off filters of 3 kDa) as reported in Section 2.3.2 and separated by TBP-based rOFFGEL-IEF. Proteins (150 μg) were separated in 24 fractions, using strips with a pH range between 3 and 10. The results on the platinum determination, which was performed by ICP-MS analysis, in the different fractions produced following separation by TBP-based rOFFGEL-IEF, are shown in Fig. 5. As can be seen, platinum is distributed uniformly along the 24 fractions, showing a particularly higher amount of platinum in the first fraction. Further nLC–ESI-LTQ-MS/MS analysis of selected FASP tryptic digested platinum protein fractions indicated in Fig. 5 (with the average isoelectric points 3.48, 3.74, 5.32 and 5.84) followed by SEQUEST and MASCOT search in the *Rattus norvegiccus* NCBInr database, allowed the identification of a number of proteins.

Complete details on the proteins identified in the platinum-enriched protein fraction F17 from the rat kidney sample are summarized in Tables S1, S2, S3 and S4. Some of the proteins identified have been previously reported to be able to react with cisplatin. These include albumin and cytochrome c [31]; or thioredoxin and chaperonin 60, which were also found for *Escherichia coli* cells treated with the anticancer drug [38]. Interestingly, the copper transporter ATX 1 was also found, being copper transporters related to the cisplatin uptake and efflux mechanisms through the cell [39].

It has to be noted that no peptides containing platinum could be found in the identified proteins, based on a parallel SEQUEST search introducing the different possible platinum moieties masses as variable modifications. This problem can be partially due to a significantly lower abundance of platinum species compared to unmodified peptides and their difficult ionization in ESI. Nevertheless, platinum was detected in low abundance proteins in the TBP-based rOFFGEL-IEF fractions due to the higher sensitivity of the ICP-MS. To date it is still a remaining challenge to detect low abundance metal-containing peptides in biological samples, as reported by several authors [7,40].

4. Conclusions

Cisplatin-binding proteins have been successfully separated by TBP-based rOFFGEL-IEF appearing as an attractive alternative to OFFGEL-IEF separations under non-reducing conditions. The negative effect that thiol-containing reducing agents, such as DTT, have on the integrity of the platinum–protein complexes on the OFFGEL-IEF separation has been confirmed.

Furthermore, the problem to perform in-solution digestions of platinum-containing proteins under reducing conditions has been clearly overcome. The FASP procedure allows performing tryptic digestion of platinum–protein complexes, including previous reduction and alkylation steps, even when thiol-containing reducing agents are employed. The short time that the proteins remain in contact with the reducing agent (around 1 h) could be the key factor for preserving the platinum–protein complexes. Moreover, the adequate peptide recoveries obtained through this kind of digestion allowed the identification of several platinum–containing peptides from HSA.

Overall, the suitability of the thiol-free rOFFGEL-IEF for the separation of cisplatin-enriched protein fractions obtained by SEC–ICP-MS in a kidney tissue extract from a rat treated with cisplatin followed by a further identification by nLC–ESI-LTQ-MS/MS after FASP tryptic digestion was demonstrated.

In addition, the use of TBP could be clearly an advance in metalloproteomics electrophoretic separations in general, which have been claimed for many years to be necessarily performed under non-reducing conditions for metalloprotein preservation. FASP digestion is also envisioned as an advantageous and attractive option for other metalloproteins studies.

Acknowledgment

This work was financially supported by the Spanish CICYT project CTQ-2011–24585. I. Moraleja acknowledges Universidad Complutense de Madrid for a predoctoral fellowship.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.11.084.

References

[1] S.E. Sherman, S.J. Lippard, Chem. Rev. 87 (1987) 1153–1181.
[2] S. Culine, Oncologie 10 (2008) 446–450.
[3] I. Arany, R.L. Safirstein, Semin. Nephrol. 23 (2003) 460–464.
[4] V. Sahni, D. Choudhury, Z. Ahmed, Nat. Rev. Nephrol. 8 (2009) 450–462.
[5] N.A. Santos, C.S. Catao, N.M. Martins, C. Curti, M.L. Bianchi, A.C. Santos, Arch. Toxicol. 7 (2007) 495–504.
[6] R.W. Schrier, J. Clin. Invest. 110 (6) (2002) 743–745.
[7] E. Moreno-Gordaliza, C. Giesen, A. Lázaro, D. Esteban-Fernández, K. Lehmann, B. Humanes, B. Cañas, U. Panne, A. Tejedor, N. Jakubowski, M.M. Gómez-Gómez, Anal. Chem. 83 (2013) 7933–7940.
[8] N. Pabla, Z. Dong, Kidney Int. 73 (2008) 994–1007.
[9] E. Moreno-Gordaliza, B. Cañas, M.A. Palacios, M.M. Gómez, Anal. Chem. 81 (2009) 3507–3516.
[10] D. Esteban-Fernández, E. Moreno-Gordaliza, B. Cañas, M.A. Palacios, M.M. Gómez-Gómez, Metallomics 2 (2010) 19–38.
[11] P.G. Righetti, A. Castagna, B. Herbert, F. Reymond, J.S. Rossier, Proteomics 3 (2003) 1397–1407.
[12] J. Bailey, P. Mrozinski, T. Preckel, C. Miller, J. Martosella, R. Kincaid, Curr. Trends Mass Spectrom. 11 (2006) 16–21.
[13] O. Carrette, P.R. Burkhard, J.C. Sanchez, D. Hochstrasser, Nat. Protoc. 2 (2006) 812–823.
[14] P.H. O'Farrel, J. Biol. Chem. 250 (1975) 4007–4021.
[15] M. Han, M. Herlyn, A.B. Fisher, D.W. Speicher, Electrophoresis 29 (2008) 695–705.
[16] J.K. Myung, G. Lubec, J. Proteome Res. 5 (2008) 1267–1275.
[17] P.E. Michel, D. Crettaz, P. Morier, M. Heller, D. Gallot, J.D. Tissot, F. Reymond, J.S. Rosier, Electrophoresis 27 (2006) 1169–1181.
[18] P. Hörth, C.A. Miller, T. Preckel, C. Wenz, Mol. Cell. Proteomics 5 (2006) 1968–1974.
[19] P.E. Michel, F. Reymond, I.L. Arnaud, J. Josserand, H.H. Girault, J.S. Rossier, Electrophoresis 24 (2003) 3–11.

[20] M. Heller, M. Ye, P.R. Michel, P. Morier, D. Stadler, M.A. Junger, R. Aebersold, F. Reymond, J.S. Rossier, J. Proteome Res. 4 (6) (2005) 2273–2282.
[21] M.L. Mena, E. Moreno-Gordaliza, I. Moraleja, B. Cañas, M.M. Gómez-Gómez, J. Chrom. A 1218 (2011) 1281–1290.
[22] B.R. Herbert, M.P. Molloy, A.A. Gooley, B.J. Walsh, W.G. Bryson, K.L. Willians, Electrophoresis 19 (1998) 845–851.
[23] G.B. Smejkal, C. Li, M.H. Robinson, A.V. Lazarev, N.P. Lawrence, E. Chernokalskaya, J. Proteome Res. 5 (2006) 983–987.
[24] K. Melchior, A. Tholey, S. Heisel, A. Keller, H.P. Lenhof, E. Meese, C.G. Huber, J. Chrom. A 1217 (2010) 6159–6168.
[25] V.A. Petyuk, W.J. Qian, R.D. Smith, D.J. Smith, Methods 50 (2) (2010) 77–84.
[26] G.M. Campo, A. Avenoso, A. D'Ascola, M. Scuruchi, V. Prestipino, A. Calatroni, S. Campo, Gene 494 (1) (2012) 24–35.
[27] J. Serrano, L.A. Higgins, B.A. Witthuhn, L.B. Anderson, T. Markowski, G.W. Holcombe, P.A. Kosian, J.J. Korte, J.E. Tietge, S.J. Degitz, Comp. Biochem. Physiol. Part D: Genomics Proteomics 5 (2) (2010) 138–150.
[28] R. Aebersold, M. Mann, Nature 422 (2003) 198–207.
[29] K.R. Williams, K.L. Stone, Mol. Biotechnol. 8 (1997) 155–159.
[30] E. Moreno-Gordaliza, B. Cañas, M.A. Palacios, M.M. Gómez, Analyst 135 (2010) 1288–1298.
[31] E. Moreno-Gordaliza, B. Cañas, M.A. Palacios, M.M. Gómez, Talanta 88 (2012) 599–608.
[32] L.L. Manza, S.L. Stamer, A.J. Ham, S.G. Codreanu, D.C. Liebler, Proteomics 5 (2005) 1742–1745.
[33] J.R. Wisniewski, A. Zougman, N. Nagaraj, M. Mann, Nat. Methods. 6 (2009) 359–362.
[34] D.C. Liebler, A.L. Ham, Nat. Methods 6 (2009) 785–786.
[35] D. Esteban-Fernández, J.M. Verdaguer, R. Ramírez-Camacho, M.A. Palacios, M.M. Gómez, J. Anal. Toxicol. 32 (2008) 140–146.
[36] J.R. Wisniewski, D.F. Zielinska, M. Mann, Anal. Biochem. 410 (2011) 307–309.
[37] B. Humanes, A. Lázaro, S. Camano, E. Moreno-Gordaliza, J.A. Lázaro, M. Blanco-Codesido, J.M. Lara, A. Ortiz, M.M. Gómez-Gómez, P. Martín-Vasallo, A. Tejedor, Kidney Int. 82 (2012) 652–663.
[38] J. Will, W.S. Sheldrick, D. Wolters, J. Biol. Inorg. Chem. 13 (2008) 421–434.
[39] R. Safaei, M.H. Maktabi, B.G. Blair, C.A. Larson, S.B. Howell, J. Inorg. Biochem. 103 (2009) 333–341.
[40] J.S. Becker, S. Mounicou, M.V. Zoriy, J.S. Becker, R. Lobinski, Talanta 76 (2008) 1183–1188.