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OFFGEL isoelectric focusing and polyacrylamide gel electrophoresis separation of platinum-binding proteins

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ABSTRACT

In this work a 2D electrophoretic separation procedure able to maintain the integrity of platinum–protein bonds has been developed. The method is based on the use of sequential OFFGEL isoelectric focussing (IEF) and PAGE. A systematic study of the reagents used for PAGE, for OFFGEL-IEF separation, and post-separation treatment of gels (such as enzymatic digestion and sample preparation for MS analysis) was tackled regarding their suitability for the identification of platinum binding proteins using standard proteins incubated with cisplatin. The distribution of platinum in high and low molecular weight fractions (separated by cut-off filters) was determined by ICP-MS, which allows evaluating platinum–protein bond stability under the conditions studied. SDS-PAGE in the absence of β-mercaptoethanol or dithiotreitol preserved the platinum–protein bonds. In addition, neither the influence of the electric field during the electrophoretic separation, nor the processes of fixing, staining and destaining of proteins in the gel did result in the loss of platinum from platinum binding proteins. SDS-PAGE under non-reducing conditions provides separation of platinum-binding proteins in very narrow bands with quantitative recoveries. Different amounts of platinum-bound proteins covering the range 0.3–2.0 μg were separated and mineralised for platinum determination, showing good platinum linearity. Limits of detection for a mixture of five standard proteins incubated with cisplatin were between the range of 2.4 and 13.9 pg of platinum, which were satisfactory for their application to biological samples. Regarding OFFGEL-IEF, a denaturing solution without thiourea and without dithiotreitol is recommended. The suitability of the OFFGEL-IEF for the separation of platinum binding proteins of a kidney cytosol was demonstrated.

1. Introduction

Platinum-based antitumor drugs (cisplatin (cis-[$Pt(NH_3)_2Cl_2$]), carboplatin and oxaliplatin) are widely used for the therapy of various types of cancer (e.g. brain, breast, ovarian, prostate, colon) [1]. Severe side effects (nephrotoxicity, ototoxicity, emetogenesis and neurotoxicity) as well as acquired drug resistance, have often been reported during application of these drugs [2,3]. Only little is known about the molecular principles of these effects. A closer look at the specific binding of platinum to proteins in living organisms [4,5] may shed some light on these mechanisms. However, separation methods have to be developed for the preparation, for structural analysis, of very complex biological samples containing hundreds of proteins that may bind to platinum along with other many non-Pt-bound proteins.

At present, two-dimensional gel electrophoresis (2-DE) is a powerful separation technique, which allows separation of thousands of proteins in biological research [6,7]. Based on two distinct procedures, it combines isolectric focusing (IEF), which separates proteins according to their isoelectric point (p*I*), and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which separates them further according to their molecular mass. The separated proteins in 2-DE gels are visualised by, e.g. Coomassie Blue or silver staining, excised, digested with trypsin and identified by matrix-assisted laser desorption/ionisation-time of flight-mass spectrometry (MALDI-TOF-MS) or by high performance liquid chromatography–electrospray ionisation-mass spectrometry/mass spectrometry (HPLC–ESI-MS/MS) resulting in the principal workflow of the proteomic analysis [8]. Other methodologies may be used for the separation of proteins, but if chromatography is employed, it should be multidimensional involving the use of reverse phase, which may be problematic for the quantitative recovery of a mixture of proteins with broad physical–chemical characteristics. On the other hand, higher resolution can be achieved using capillary electrophoresis but it is not useful for preparative purposes.

It has to be remarked that 2-DE was originally designed for the characterisation of proteins without regard for the trace elements which they may be carrying. However, there is no method for a detailed characterisation of metal–protein complexes in liv-

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ing organisms, and the suitability of these methods will depend strongly on the stability of the metal–protein bond, especially using SDS-PAGE. Because of this, the main problem researchers have to face is that it is not always possible to keep these metal-bound protein complexes intact during separation and post-separation processes, and metal losses may occur. Some authors have already discussed these metal losses in different proteins, and in most cases the use of native- or Blue native-PAGE instead of SDS-PAGE, has been proposed to solve the problem [9–13].

Regarding IEF, fractionation under well-established denaturing conditions (urea, thiourea, DTT, ampholytes and glycerol) has also been widely reported for proteomic studies, including the use of immobilised pH gradient gels (IPG IEF) [14] or solution isoelectric focusing (sIEF) [15], which presents the advantage that the sample is recovered in liquid phase. Commercial sIEF systems can be classified according to the way in which the pH gradient is created, namely, through carrier ampholytes, or through the use of isoelectric membranes, and consequently, to how proteins are confined within the gradient. In the case of Agilent's OFFGEL system [16,17], which has been used for the experiments described in the present work, the pH gradient is formed through isoelectric membranes. The OFFGEL system presents the capability of generating stable pH gradients, an effective barrier between successive chambers and superior focusing to those employing carrier ampholytes.

In contrast, there are very few reports on native IEF. Shang et al. [18] successfully performed native IEF on myoglobin and yeast soluble proteins. However, the applicability of this approach to the study of metalloproteome has not been fully investigated. Recently, Pioselli et al. [19] separated mixtures of metalloproteins containing copper, zinc and iron by sIEF with a five-well system under well-established denaturing, and novel non-denaturing separation conditions. Denaturing conditions separate the metalloprotein mixtures with high resolution, although the stability of the complexes was affected. However, non-denaturing conditions showed a lower degree of resolution in the separation, but the stability of the metal–protein complexes was preserved.

The analytical approaches for platinum binding proteins have been recently reviewed by Esteban-Fernández et al. [20]. The use of native 2-DE has been reported by Lustig et al. [21,22] for the separation of human and rabbit serum incubated with $PtCl_4^{2-}$. They concluded that platinum binds very strongly to various serum proteins such as albumin, transferrin and globulin and the binding seems to be strong enough to withstand the electric field, the IEF and the native-PAGE. Moreover, Ma et al. [23] also studied platinum-binding serum proteins after native-PAGE, obtaining representative platinum signals for these proteins. However, for complex samples, SDS-PAGE is preferred due to its higher resolution and reproducibility.

The stability and the strength of the platinum–protein bond in the presence of chemicals, such as urea, 3-[(3-cholamidopropyldimethylammonio]-1-propanesulfonate (CHAPS), dithiotreitol (DTT) and SDS have been evaluated by several authors [22,24,25]. Lustig et al. [22] demonstrated, for rabbit serum incubated with $PtCl_4^{2-}$, the possibility of using urea, even at high concentrations (9 M), and SDS only at concentrations lower than 0.1%, while CHAPS and DTT could not be used at all. However, recently Moreno-Gordaliza et al. [24] demonstrated that the platinum–protein bonds in an insulin-cisplatin model resisted the action of the denaturing and reducing agents (such as urea 8 M and DTT 10 mM) in a Tris buffer, remarking the significant strength of the platinum–protein bonds. Esteban-Fernández et al. [25] also studied the stability and strength of the bond by subjecting the cytosolic fraction of kidney and ear impacted by cisplatin to several treatments common in gel electrophoresis, employing SDS, β-mercaptoethanol (BME), heating and sonication. Analysis by size exclusion chromatography–inductively coupled plasma-mass spectrometry (SEC–ICP-MS) revealed that biomolecule–cisplatin interactions resisted denaturing and reducing conditions, based on the absence of the peak corresponding to free cisplatin in the treated samples and in the non-significant differences in the chromatographic profiles for treated and untreated samples.

Indeed, gel electrophoresis has already been successfully applied for the separation of cisplatin-containing proteins in biological samples. In particular, Allardyce et al. [26] administered cisplatin to *Escherichia coli* cells, and proteins were partially separated by non-reducing SDS-PAGE, followed by a platinum analysis by laser ablation (LA)-ICP-MS. Membrane proteins were identified in a platinum-rich electrophoretic band by reversed phase-electrospray ionisation-time of flight (RP-ESI-Q-TOF), after an in-gel tryptic digestion. In this case, the outer membrane protein A (ompA) was identified and was proposed to be involved in the cellular cisplatin intake.

On the other hand, not only the nature of the electrophoretic (native or SDS) process may alter the metal–protein binding. There are few studies regarding the influence of other extra-electrophoretic conditions (including post-separation process) on possible metal–protein binding break. Using synchrotron radiation X-ray fluorescence as detection system after SDS-PAGE, Gao et al. [27] found that not only the influence of the electric field during electrophoresis separation, but also the processes of fixing, staining and destaining of proteins in the gel may result in loss of metal ions (such as Cu, Fe and Zn) from metalloproteins in human liver cytosol. Recently, Raab et al. [28] found that staining of the gel prior to LA-ICP-MS is not recommended, since most of the protein-bound metal is lost during the staining procedure (except when it is covalently bound), and should be avoided prior to ablation. They also found that native gel electrophoresis is the most suitable technique to separate metal–protein complexes and preparation for metal detection and quantification by LA-ICP-MS.

The aim of this study is to determine the most appropriate separation conditions for 2-DE based on the sequential use of PAGE and OFFGEL-IEF of proteins containing platinum, and to define general parameters for post-separation treatments to make the proteins suitable for structural MS analysis, preserving the platinum–protein bonds along the whole analytical procedure. The location and quantification of platinum in the gel or in the wells after OFFGEL-IEF separation of a mixture of standard proteins incubated with cisplatin, may allow evaluating platinum–protein bond stability under the conditions needed for both separation techniques. To quantify the platinum remaining bound to the protein after 2-DE and to evaluate the origin of possible platinum losses, a model protein, HSA, was incubated with cis-Pt and subjected to each of the procedures implicated in these separations. After the incubation of the HSA–Pt complexes with the different reagents used in PAGE, or in OFFGEL-IEF, or in post-separation treatments, solutions were subjected to ultrafiltration through 3 kDa cut-off filters and platinum was measured by ICP-MS. In addition, the isoelectric fractionation of a kidney tissue extract from a rat treated with cis-Pt was performed to study the recovery of protein-bound Pt under the optimal conditions investigated.

2. Materials and methods

2.1. Chemicals

The platinum-based drug used was cisplatin (Sigma Aldrich Chemie, St. Louis, MO). Human apo-transferrin (TF), human serum albumin (HSA), carbonic anhydrase from bovine erythrocytes (CA), myoglobin from horse heart (MYO) and cytochrome c from horse heart (CYT C) 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.

Table 1
Recoveries for HSA-cisPt expressed as percentage of platinum, after ultrafiltration, mixed 1 + 1 with the different reagents given in the table tested under: A) non-denaturing or denaturing and/or reducing PAGE, B) OFFGEL-IEF or C) during in gel digestion with trypsin. In all the cases, the relative standard deviation is lower than 3% for three determinations.

Reagents	Reaction time (min)	Protein-bound Pt (%)	Non-protein-bound Pt (%)
A. Reagents used under non-denaturing or denaturing PAGE			
(A1) Native buffer[a]	5	94	6
(A2) LSB[b]	5	98	2
(A3) LSB	60	88	12
(A4) LSB (95 °C)	5	98	2
(A5) LSB (95 °C)	60	91	9
(A6) BME (5%)	5	92	8
(A7) BME (5%)	24 h	80	20
(A8) LSB with BME	5	91	9
(A9) LSB with BME	60	65	35
(A10) LSB with BME (95 °C)	1	87	13
(A11) LSB with BME (95 °C)	5	64	36
(A12) LSB with BME (95 °C)	60	41	59
(A13) LSB with DTT	5	96	4
(A14) LSB with DTT (95 °C)	1	95	5
(A15) Running buffer[c]	60	100	0
(A16) Bio-Safe™ Coomassie stain	60	92	8
(A17) Fixing solution	60	98	2
B. Reagents used under OFFGEL-IEF			
(B1) Thiourea (2.5 M)	5	57	43
(B2) Thiourea (2.5 M)	24 h	61	39
(B3) DTT (5%)	5	87	13
(B4) DTT (5%)	24 h	85	15
(B5) Denaturing solution (DS)[d]	16 h	57	43
(B6) DS without DTT	24 h	68	34
(B7) DS without thiourea	24 h	79	23
(B8) DS without thiourea and DTT	24 h	98	2
C. Reagents used during in gel digestion with trypsin			
(C1) NH_4HCO_3 (50 mM, 22 °C)	60	99	1
(C2) NH_4HCO_3 (25 mM, 22 °C)	60	99	1
(C3) NH_4HCO_3 (25 mM, 37 °C)	16 h	93	7
(C4) Tris–HCl pH 7.8 (25 mM, 37 °C)	16 h	94	6
(C5) Formic acid 5%	60	98	2

[a] Composition of Native buffer (Tris–HCl 62.5 mM pH 6.8, glycerol 40%, bromophenol blue 0.01%).
[b] Composition of Laemmli sample buffer (Tris–HCl 62.5 mM pH 6.8, glycerol 25%, SDS 2%, bromophenol blue 0.01%).
[c] Composition of running buffer (Tris–HCl 25 mM pH 8.3, glycine 192 mM, SDS 0.1%).
[d] Composition of denaturing solution (DS): urea 8 M, thiourea 2.5 M, DTT 0.08 M, ampholytes and glycerol.

Louis, MO) were used for the preparation of the incubation solution under physiological conditions.

High-purity HNO_3, used both for the pH adjustment of the previously mentioned incubation media and for the mineralisation of the electrophoretic gels, and HCl were obtained by distillation of the analytical-grade reagents (Merck, Darmstadt, Germany) in an acid distiller (Berghof B BSB-939IR, Eningen, Germany). Hydrogen peroxide (30%, w/v, Panreac Química SA, Barcelona, Spain) was used for sample digestion. 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. All solutions were prepared with deionised water (Milli-Q Ultra pure water systems, Millipore, USA).

2.2. In vitro incubations and protein quantification

To reproduce the physiological intracellular saline and pH conditions, TF, HSA, CA, MYO and CYT C (62 μM) were incubated, separately, with cisplatin (620 μM) at a molar ratio 1:10 in a buffer containing NaCl (4.64 mM) and Tris–NO_3 (10 mM, pH 7.4) at 37 °C in a thermostatic bath (Neslab RTE-111). Control samples were also prepared by incubating the same proteins under the same conditions already described but in the absence of cisplatin. To remove free cisplatin, samples were filtered through a Amicon Ultra-0.5 mL Ultracel-3 (3 kDa cut-off filter, Millipore) by centrifugation at 14,000 × *g* during 30 min, reversing the filter and recovering the retained fraction containing cisplatin-bound proteins by centrifugation at 1000 × *g* for 2 min. Both fractions (retained and non-retained) were analysed by ICP-MS.

Total protein concentration in the solutions was determined by the Quick Start Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The kit supplied Bradford reagent in a solution containing methanol and phosphoric acid and the standard proteins in NaCl (0.9%) and NaN_3 (0.05%). Bovine serum albumin (BSA) at seven different concentrations: 2, 1.5, 1.0, 0.75, 0.5, 0.25 and 0.125 mg mL^{-1} were used as standards for quantification. The 1× dye reagent was added to each standard and to the sample solutions, vortexed and incubated at room temperature for at least 5 min. The assay was performed in 2.0 mL disposable plastic cuvettes and the absorbance measured at 595 nm in an HP8453 UV-visible spectrophotometer (Agilent Technologies Inc., Waldbronn, Germany). The linear range obtained for BSA was 125–1000 μg mL^{-1}.

2.3. Stability of the platinum–protein adducts

Aliquots of 250 μL of the retained fraction on the cut-off filter for HSA incubated with cisplatin, which was prepared as described in Section 2.2, were mixed with 250 μL of the different reagents used under non-denaturing or denaturing PAGE, OFFGEL-IEF or digestion with trypsin, as shown in Table 1. After these treatments, samples were ultrafiltrated again and the retained and filtrated solutions were analysed for total Pt content determination by ICP-MS.

Firstly, it was studied if platinum–protein bonds are stable under incubation with the different chemical compounds used in PAGE,

either, in non-denaturing (Native buffer, A1 in Table 1) or in reducing and denaturing conditions (LSB containing 2% SDS together with 5% BME heated at 95 °C for 5 min, A11). As a comparison, LSB was also tested in milder conditions: non-reducing, without BME, at 25 or 95 °C (A2 and A4, respectively), reducing (with BME) at low temperatures, 25 °C (A8) or even replacing BME by 0.08 M DTT at 25 or 95 °C (A13 and A14, respectively). Incubations were made trying to emulate the time needed for the separation of proteins by SDS-PAGE (between 5 and 60 min for samples A3, A5, A9 and A12). Moreover, the influence of BME alone was also studied for longer reaction times: 5 min or 24 h (A6 and A7). Other reagents and incubation times studied were: running buffer, for 1 h (A15), Bio-Safe™ colloidal Coomassie Blue G-250 stain, for 1 h (A16) or H_2O/methanol/acetic acid (72.5/20/7.5), which is used for protein fixation to the gel prior to staining, for 1 h (A17).

On the other hand, it was also studied if the Pt bound to HSA was preserved after treatment with the reagents typically involved in OFFGEL-IEF separations. Either 2.5 M thiourea or 80 mM DTT alone was tested separately at different reaction times: 5 min and 24 h (B1, B2, B3 and B4, respectively in Table 1), as well as a denaturing solution containing 2.5 M thiourea, 80 mM DTT, 8 M urea, ampholytes and glycerol for 16 h (DS) (B5). Moreover, the effect of thiourea (B6), DTT (B7) or both DTT and thiourea (B8) in the denaturing solution was also evaluated.

Finally, in order to study the influence of the reagents used in the post-separation treatments of gels (such as enzymatic digestion and sample preparation for MS analysis), the protein bound platinum fraction (i.e. >3 kDa) was also diluted 1 + 1 with NH_4HCO_3 at different concentrations, temperatures and for different reaction times: 50 mM at 22 °C, shaking for 60 min (C1 in Table 1), 25 mM at 22 °C, shaking for 60 min (C2), 25 mM at 37 °C, shaking for 16 h (C3), Tris–HCl pH 7.8 (25 mM, 37 °C, shaking at 300 rpm for 16 h) (C4) or 5% formic acid (C5).

Total protein concentration in the solutions was also determined by the Quick Start Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), as described above in Section 2.2. In all the cases studied, approximately 95% of the total protein content was recovered in the fraction higher than 3 kDa, finding no protein traces in the fraction below 3 kDa.

2.4. PAGE separation

PAGE was carried out with a Mini Protean Tetra Cell Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, CA) using 3 and 12.5% of polyacrylamide for the stacking and the resolving gels, respectively.

For denaturing and reducing conditions, 5% (710 mM) of the reducing agent (BME) was added to the Bio-Rad's Laemmli sample buffer (LSB), containing Tris–HCl (62.5 mM, pH 6.8), glycerol (25%), SDS (2%) and bromophenol blue (0.01%) (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples were diluted 1 + 1 with the above mixture, and heated for 5 min at 95 °C. For SDS-PAGE separations under non-reducing conditions, samples were diluted with LSB containing the same reagents, in the absence of BME, instead. The gels were run at constant current (12 mA for 20 min and 20 mA for 3 h).

Precision Plus Protein standards, unstained (Bio-Rad Laboratories, Inc., Hercules, CA), were used to provide a ladder of convenient and consistent molecular sizes. It contained 10 recombinant protein bands of 10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa. They were provided in the loading buffer (which contained glycerol (30% w/v), SDS (2%), Tris (62.5 mM, pH 6.8), DTT (50 mM), EDTA (5 mM), sodium azide (NaN_3, 0.02%) and bromophenol blue (0.01%), ready to load, with no dilution required for its application. The running buffer contained Tris–HCl (25 mM, pH 8.3), glycine (192 mM) and SDS (0.1%).

After separation, gels were washed in deionised water for 20 min and the proteins fixed on the gel for 1 h in a solution containing H_2O/methanol/acetic acid (72.5/20/7.5). Gels were visualized by staining with (a) Bio-Safe™ colloidal Coomassie Blue G-250 (Bio-Rad Laboratories, Inc., Hercules, CA) for 1 h or (b) Dodeca Silver Stain kit (Bio-Rad Laboratories, Inc., Hercules, CA).

2.5. Gel mineralisation for platinum analysis by ICP-MS

Gels were cut into pieces, which were digested in mini-Teflon vessels with 1.0 mL of HNO_3 and 0.5 mL of H_2O_2 and evaporated to dryness. Two more evaporation steps were performed, adding 1.0 mL of aqua regia and then 1.0 mL of HCl. Finally, the samples were diluted to 2.0 mL with HCl 0.24 M for ICP-MS analysis.

A Quadrupole ICP-MS Thermo X-series (Thermo Electron, Windford, Cheshire, UK) equipped with a Meinhard nebuliser, 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: forward power, 1250 W; plasma gas, 15 L min^{-1}; auxiliary gas, 0.73 L min^{-1}; nebuliser gas, 0.85 L min^{-1}; channels per AMU, 10; and integration time, 0.6 ms. Platinum content measurements were acquired in continuous mode, monitoring m/z 194 (Pt), 195 (Pt), and 191 (Ir). Non-spectral interferences (matrix effects) were not observed; thus, quantification of platinum was carried out by external calibration over the working range (0.5–100 μg L^{-1}) with 20 μg L^{-1} iridium as internal standard (IS).

2.6. OFFGEL-IEF

2.6.1. Sample preparation

About 0.250 g of kidney tissue from a rat which was treated with a monodose of 16 mg of cisplatin per m^2 of corporal surface and sacrificed three days after the treatment, as already described by Esteban-Fernández et al. [25], was dissected and homogenised in a Potter with 3 mL of a buffer containing Tris–HCl (10 mM), NaCl (25 mM) and 12.5 μL of a protease inhibitor cocktail. The homogenates were centrifuged at 15,000 × g for 40 min. All the preparative steps were performed at 4 °C to minimise the risk of species degradation or transformation. Then, protein precipitation was performed by adding six volumes of acetone (80% at −20 °C) 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 pellet was then washed with a small amount of acetone, centrifuged and the supernatant was again discarded.

2.6.2. OFFGEL electrophoresis

For the pI-based protein separation, the 3100 OFFGEL Kit pH 3–10 (Agilent Technologies Inc., Waldbron, Germany) with a 12 or 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 8 M, thiourea 2.5 M, DTT 0.08 M, ampholyte and glycerol, or urea 8 M, DTT 0.08 M, ampholyte and glycerol) per well. The pellet from the cytosolic fraction (4.5 mg total protein content) or the mixture of standard proteins (625 μg total protein content) was diluted with focusing buffer to a final volume of 3.6 mL, and 150 μL of sample was loaded in each well. The sample was then focused with a maximum current of 50 μA, and typical voltages ranging from 500 to 4000 V until 50 kV h was reached after 24 h. The recovered fractions (volumes between 100 and 150 μL) were diluted to 1.5 mL with distilled water for ICP-MS analysis.

2.7. In-gel tryptic digestion of proteins separated by SDS-PAGE and analysis by MALDI-TOF-MS

When protein identification was needed, *in situ* digestion with trypsin was performed after gel separation, the produced peptides were extracted and identification was obtained by peptide mass fingerprinting (PMF) following the protocols described elsewhere [8]. In brief, gel slices were washed for at least 1 h in 500 μL of 50 mM ammonium bicarbonate (NH_4HCO_3) at 22 °C, with shaking (1000 rpm) in a Thermomixer (Eppendorf AG, 22331 Hamburg, Germany). Then, the solvent was discarded and the gel slices were washed in 500 μL of acetonitrile/NH_4HCO_3 (50% / 50 mM) with shaking (1000 rpm) for 1 h. Again, the wash off was discarded; each slice was cut into 2–3 pieces and transferred to a 200 μL Eppendorf-like PCR tube. The gel slices were kept wet with the wash solution to facilitate cutting and transfer. Next, 50 μL acetonitrile was added to shrink the gel pieces. After 10–15 min, the solvent was removed and the gel slices were dried in the Concentrator Plus (Eppendorf AG, 22331 Hamburg, Germany). Finally, the gel pieces were reswelled with 40 μL of 50 mM NH_4HCO_3 containing 12.5 ng μL^{-1} modified porcine trypsin (Promega, Madison, WI, USA). Once the gels had been completely reswollen, 10–20 μL of 50 mM NH_4HCO_3 was added to cover the gel pieces and incubated overnight at 37 °C.

A MALDI-TOF mass spectrometer (Voyager DE-PRO, Applied Biosystems) was used to analyse protein digests. The instrumental parameters were set as follows: detector, reflectron mode; accelerating voltage, 20 kV; grid, 75%; guide wire, 0.002; delay time, 100 ns. Acquisition was made in the *m*/*z* range 800–4000. A total of 100 shots were performed per spectrum, and six spectra were accumulated per sample in order to increase the S/N ratio. Spectra were acquired in the positive ion mode.

Samples for MALDI-TOF-MS analysis were prepared following the dried droplet method. A volume of 0.5 μL of the prepared digest was mixed with 0.5 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid as matrix. The mixture was deposited into a stainless steel MALDI sample target and air-dried. Calibration was performed externally using the monoisotopic masses of the singly charged ions produced by the peptide standards bradkinin 1–7 (757.3997), angiotensin II (1046.5423), P14R (1533.8582) and ACTH 18–39 (2465.1989). The peaks from porcine trypsin at 841.50 and 2210.10 were used for internal mass calibration. Peptide mass fingerprinting was performed searching protein databases with either Mascot (Matrix Science, London, UK), or ProFound (Proteometrics, New York, USA) search engines. Peptide mass tolerance was set to 100 ppm, allowing two missed cleavages per peptide. Methionine oxidation was considered as variable modification. All the searches were performed without constraining proteins molecular weight (Mr) and p*I*, and without any taxonomic specifications. Positive identifications were considered according to the statistics of the program used, being the assignments manually checked as well.

3. Results and discussion

A systematic study on the reagents used for PAGE, for OFFGEL-IEF separation, or for post-separation treatments of gels, such as enzymatic digestion and sample preparation for MS analysis, was tackled regarding their suitability for the identification of platinum binding proteins. As a pre-screening step, incubated HSA–cisPt, chosen as a model protein, was subjected to treatment with those reagents involved in the mentioned methodologies, in order to check the degree of remaining protein-bound Pt. The preservation of Pt–protein bonds was also checked after the separation of a mixture of Pt-binding proteins by either PAGE or OFFGEL-IEF. The latter was also applied for the separation of a rat kidney extract containing Pt–proteins.

3.1. PAGE separation

3.1.1. Study of the stability of platinum–protein bonds

The stability of Pt–protein bonds after treatment with the reagents used for PAGE in non-denaturing, non-reducing, or in reducing and denaturing conditions, was studied using a HSA–Pt model, as described in Section 2.3.

Table 1A shows the percentage of Pt found in the retained and filtered fractions, calculated over the total Pt content measured in both fractions, being the protein losses, due to unspecific adsorption to the filters, around 5% for all the samples. As can be seen, sample preparation for native-PAGE, provides a high platinum content in the fraction higher than 3 kDa (94%), indicating that the platinum-bound protein was stable after the incubation in these mild conditions, avoiding buffer systems with platinophile N- or S-donor groups. However, traditional sample preparation for SDS-PAGE (A11) produced a lower recovery (64%) of platinum bound to protein, which decreased (41%) when heating for a longer period of time (60 min). Nevertheless, the recovery after heating for only 1 min was acceptable (87%). To evaluate which of the reagents used in these conditions was the origin of the platinum losses, BME alone was tested for different reaction times, 5 min and 24 h, without heating (A6 and A7, respectively). It can be seen that the recovery after 5 min of incubation was acceptable, but relatively high platinum losses were obtained for a longer reaction time (80% recovery). On the other hand, recoveries obtained under sample preparation conditions for SDS-PAGE using DTT instead of BME were very similar, either at room temperature, for 5 min: 96 (A13) vs. 91% (A8) or at 95 °C for 1 min: 87 (A10) vs. 95% (A14). Consequently, it may be concluded that the presence of a reducing agent with thiol groups (BME or DTT) at high temperatures or during long incubation times (60 min, A9 and A12) produces deleterious effects in the binding between platinum and proteins. Results also indicate that the presence of SDS at concentrations up to 1% does not affect platinum–protein bonds, even after incubation at high temperatures (A2 and A4), being the platinum recoveries in the low molecular weight fraction the lowest as compared to all the conditions studied: 2%. Moreover, Table 1A also shows that the exposure of the HSA-platinum complex for 1 h to a fixing solution and to Bio-Safe™ colloidal Coomassie Blue G-250 stain (containing less than 5% v/v phosphoric acid) resulted in a platinum loss of approximately 2% and 8%, respectively. These results demonstrate that methanol and phosphoric acid contained in the fixing solution did not significantly disrupt platinum-HSA bonds, and it is possible to stain the gel prior to MS analysis. Unsuccessful results were elsewhere reported for Fe-, Cu- and Zn-protein complexes that suffer a loss of up to 90% protein-bound metal during the staining procedure [28], indicating that the study of metalloproteins by SDS-PAGE when the metal is not covalently bound is more problematic and technically demanding.

As a result of that, it is possible to conclude that native- or SDS-PAGE in the absence of BME or DTT (A2 conditions given in Table 1A) may be appropriate to separate platinum-containing proteins. Moreover, after PAGE separation, the use of methanol/acetic acid for fixing and Colloidal Coomassie blue for staining is recommended, because these reagents do not alter the native platinum species by stripping the metal off the protein.

3.1.2. Quantification of platinum in protein bands after PAGE separation

A method was developed to follow up the separation of the Pt–protein bands after PAGE separation and for the quantification through the gel of the platinum which remained bound to the proteins. This methodology is needed for a further evaluation of the stability of platinum–protein bonds in a mixture of proteins separated by SDS-PAGE. 10 μg of either HSA or HSA–cisPt (fraction

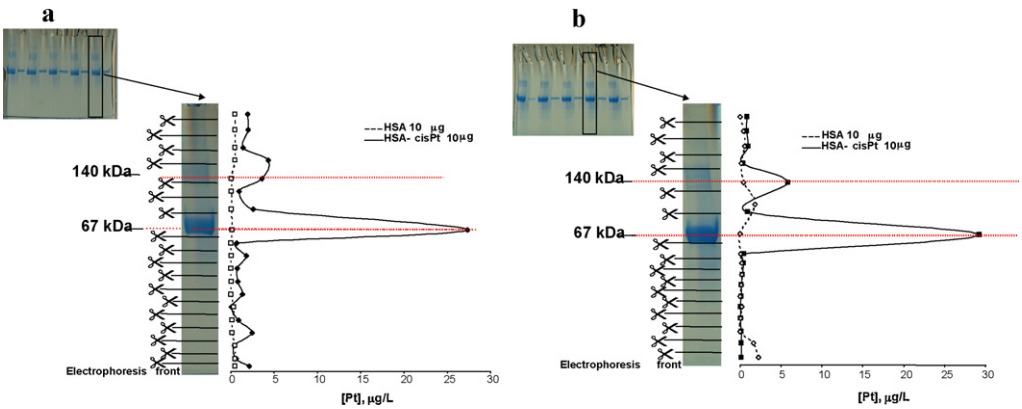

Fig. 1. Platinum profile obtained for a stained gel containing HSA or HSA–cisPt after treatment with: (a) LSB with 5% BME, or (b) LSB with 5% BME heated at 95 °C for 5 min. The stained gel was cut in 19 pieces. Left side: photo of the gel, molecular weight of standard proteins and cut areas.

higher than 3 kDa) were subjected to SDS-PAGE, using LSB with 5% BME, without heating the sample. After fixing the gel, HSA or HSA–cisPt detection was achieved using Bio-Safe™ colloidal Coomassie Blue G-250 stain. Then, several gel lanes were selected to study the distribution of platinum across their total length. Selected lanes were cut into 19 pieces, which were mineralised (see Section 2.5 for full details) and solutions obtained were diluted to 2.0 mL with HCl 0.24 M. Finally, the platinum contained in each of the gel pieces was determined by ICP-MS.

Fig. 1a shows the distribution of platinum through the 19 pieces of the gel showing the highest ICP-MS signal for platinum located on a small area corresponding to the stained area containing the protein (indicated by a red dotted line). This profile proves, first, that platinum is found in the expected band, bound to HSA, and second, that no platinum is lost during the separation since no platinum is detected at the front, where low molecular mass products are expected. Fig. 1a also shows a band containing only HSA (black dotted line), which is used as a control lane. It can be seen the total absence of platinum on the stained area as well as in the rest of the gel lane. Results confirm a reduced platinum background due to the platinum electrodes because in vertical gel electrophoresis the gel comes into very little contact with the electrodes, usually thin platinum wire. It is also important to remark that no contamination was observed due to the several steps involved in the mineralisation of the gel pieces.

Experiments were conducted to reduce the time needed for mineralisation of the gel lanes; thus, only two evaporation steps were performed, excluding the evaporation with aqua regia. Results show that although platinum distribution is the same as that with aqua regia, the response found was lower (data not shown). Therefore, for the rest of the work, mineralisation was carried out following the three evaporation steps described in Section 2.5.

The platinum data for HSA–cisPt was expressed as a percentage of the total platinum introduced in the gel lane, which is measured directly by ICP-MS, showing a recovery of 78%. This recovery is lower than the value given in Table 1 (91%), probably due to the influence of the reaction time for platinum–protein samples containing BME. However, it is important to remark the presence of most of the Pt bound to the protein even in denaturing and reducing separation conditions. This fact is very important for the analysis of platinum in biological samples where the use of these conditions may be needed.

The distribution of platinum through the gel lanes when the loaded samples, containing either 10 μg of HSA or HSA–cisPt, were previously incubated at 95 °C for 5 min with LSB containing BME was also studied. Results shown in Fig. 1b confirm the previous platinum distribution, even in the conditions in which part of the platinum had been removed from the protein. Platinum was always absent in the lanes loaded only with HSA, as there were no traces over the blank level either in the HSA band, or in the rest of the lane. The platinum data for HSA–cisPt expressed as a percentage of the total platinum loaded in the gel lane, which was also measured directly by ICP-MS, showed a recovery of 71%. This recovery is in accordance with the values given in Table 1A (64%). Moreover, in all the lanes loaded with HSA–cisPt, a less intense upper platinum-containing band is observed. This band is due to HSA dimerisation. Results are in agreement with those reported by Ivanov et al. [29], who described, using SEC-UV, that cisplatin leads to HSA dimerisation, detecting platinum-species of around 140 kDa.

3.1.3. *Evaluation of the Pt–protein bonds stability in a mixture of proteins under denaturing or denaturing and reducing PAGE*

Mixtures of proteins (TF, HSA, CA, MYO and CYT C) which had been incubated separately in the absence or presence of cisPt were employed to evaluate the metal–protein bond stability under SDS-PAGE separations, using 0.3, 0.5, 1.0 and 2.0 μg of each protein. Coomassie-stained gels corresponding to the separation of the proteins by either non-reducing SDS-PAGE or reducing SDS-PAGE (using LSB with BME heated at 95 °C for 5 min) are shown in Fig. 2a and b, respectively. Moreover, Fig. 2c and d shows the platinum distribution of the selected area in Fig. 2a and b (black dotted line), respectively. As can be seen, stained proteins showed a similar protein distribution in both cases, independently on the conditions used. However, better resolution was achieved by using reducing and denaturing conditions (Fig. 2b). Estimated molecular weights of the proteins were ~76, ~67, ~29, ~17 and ~12 kDa, which were in agreement with the theoretical Mr values of TF, HSA, CA, MYO and CYT C. Moreover, it was observed that in all the lanes less intense bands appeared, corresponding to protein dimerisation (as was previously reported for HSA).

To determine the amount of platinum remaining bound to proteins under the different PAGE separation conditions, the lanes corresponding to protein bands (2 μg), which are indicated by a dotted black line, were cut in 19 pieces. The different pieces were mineralised and the platinum content was determined by ICP-MS. The platinum profiles are shown in Fig. 2c and d. As can be seen in Fig. 2a and b, the electrophoretic resolution under the described conditions allows differentiating sharp Coomassie-stained protein bands. The platinum profiles, shown in Fig. 2c and d, for these gels, seem to have less resolution, but this is an artifact as for these measurements, lanes were not cut in tiny bans; the goal was only to show that the platinum was still bound to protein bands and only to these bands. In all the cases, a big peak can be discerned, at a mass around 150 kDa, which corresponds to the TF and HSA

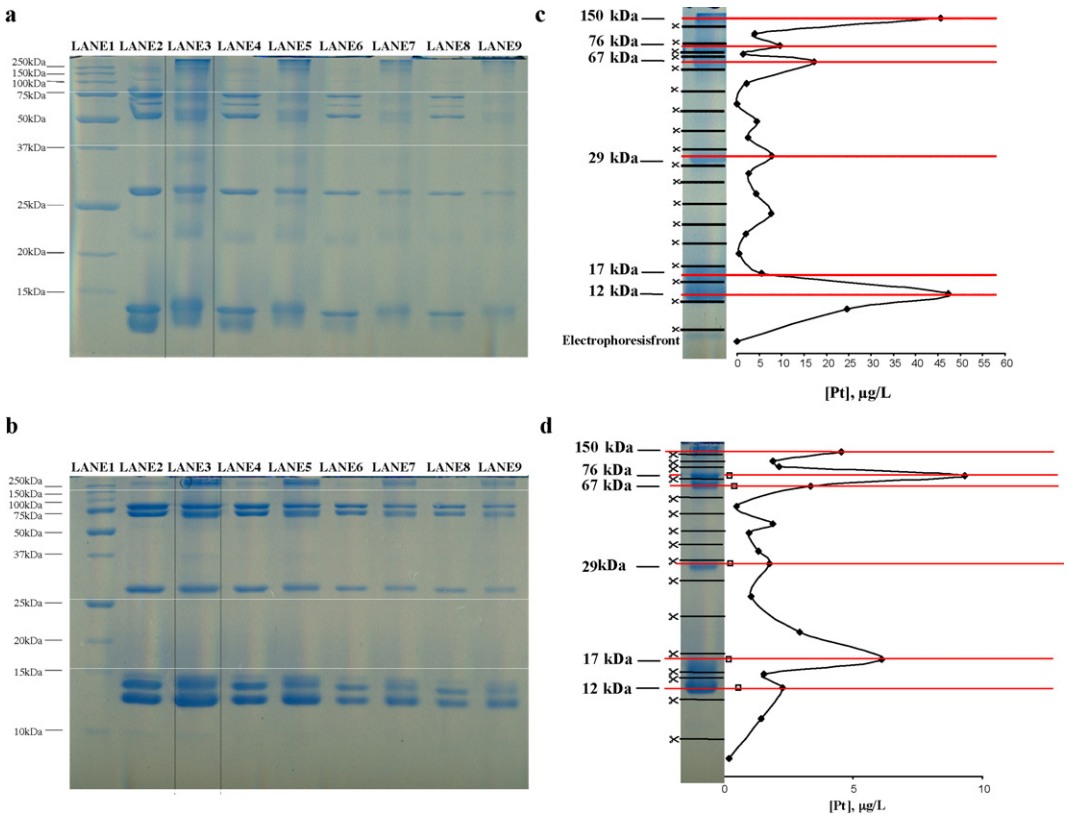

Fig. 2. Separation of a mixture of standard proteins (TF, HSA, CA, MYO and CYT C) under different PAGE conditions: (a) denaturing and (b) denaturing and reducing. (c and d) The platinum distribution of the selected area in (a) and (b), respectively. (d) White squares, which correspond to the platinum content found in the gel loaded with the same mixture of proteins, but without cisplatin. Left side of (a), (b): molecular weight of standard proteins. In (a) and (b), Lane 1: Precision Plus Protein standards; Lanes 2, 4, 6 and 8: differing amounts (2, 1, 0.5 and 0.3 µg, respectively) of mixed standard proteins (TF, HSA, CA, MYO and CYT C); Lanes 3, 5, 7 and 9: differing amounts (2, 1, 0.5 and 0.3 µg, respectively) of mixed standard platinum–proteins (TF, HSA, CA, MYO and CYT C).

dimerisation. Two other high peaks are observed at around 76 and 67 kDa, corresponding to TF and HSA, respectively. Furthermore, proteins below 37 kDa (CA), around 20 kDa (MYO) and higher than 15 kDa (CYT C) also contain a fairly high amount of platinum. It is also important to remark the high amount of platinum in the stained bands of the gel. Recovery value for platinum was of 110% for non-reducing SDS-PAGE (Fig. 2c), which confirms the preservation of the platinum–protein bond. However, a lower platinum recovery, 25% (Fig. 2d), was found for traditional reducing SDS-PAGE conditions, due to the high influence of BME and temperature in platinum–protein bonds.

Therefore, it is concluded that SDS-PAGE should be performed under non-reducing conditions in order to preserve Pt–protein bonds during the separation of mixtures of Pt–proteins.

3.1.4. Detection limits

Different amounts of platinum–proteins covering the range 0.3–2.0 µg (for single proteins) were also mineralised, showing the platinum quantification, by ICP-MS, a good linearity ($r = 0.951$ for TF, $r = 0.992$ for HSA, $r = 0.996$ for CA, $r = 0.998$ for MIO and $r = 0.998$ for CYT C) for all the platinum–proteins studied. Furthermore, the background recorded for all proteins is quite similar, indicating that the gel analysed could be considered as platinum-free. The limits of detection (based on 3 times the standard deviation of 6 blanks measured from one gel for the denaturing PAGE using LSB separation) were 10.1 pg for TF–cisPt, 10.3 pg for HSA–cisPt, 13.9 pg for CA–cisPt, 2.4 pg for MYO–cisPt and 4.7 pg for CYT C–cisPt. These limits of detection are satisfactory for their application to biological samples.

3.2. Separation by OFFGEL-IEF

3.2.1. Study of the effect of the reagents used for OFFGEL-IEF separations on the stability of platinum–protein bonds

In the pre-screening step it was also studied if the complex HSA–cisPt retained the platinum after treatment with the reagents typically used during OFFGEL-IEF, most often involving denaturing and reducing solutions containing urea, thiourea and DTT, as described in Section 2.3. Results are shown in Table 1B, which are also given as the percentage of platinum found in each fraction with respect to the total content in the sample. It can be seen that thiourea (Table 1, B1, B2) or denaturing solutions containing thiourea (Table 1, B5) led to the loss of a high percentage of the platinum bound to the protein (losses of about 40%) independently on the reaction time employed (5 min or 24 h). This is due to the strong reactivity of platinum compounds toward S-donor molecules and the formation of very stable Pt^{II}–S bonds, which results in a competition between thiourea and the protein for platinum. A similar competition was reported between intracellular thiols and DNA, resulting in a resistance to antitumor platinum drugs [2,3].

However, the platinum protein bond resisted the action of 8 M urea, buffer ampholytes and glycerol (Table 1, B8, 98% Pt recovery), and also tolerated the DTT concentrations used for OFFGEL-IEF (B4, 85% Pt recovery). Even the treatment with a combination of the previously mentioned reagents in denaturing solutions containing DTT provided acceptable recoveries (B7, 79% Pt recovery), as long as they did not contain thiourea. The significant strength of the platinum–protein bonds was already demonstrated by Moreno-Gordaliza et al. [24].

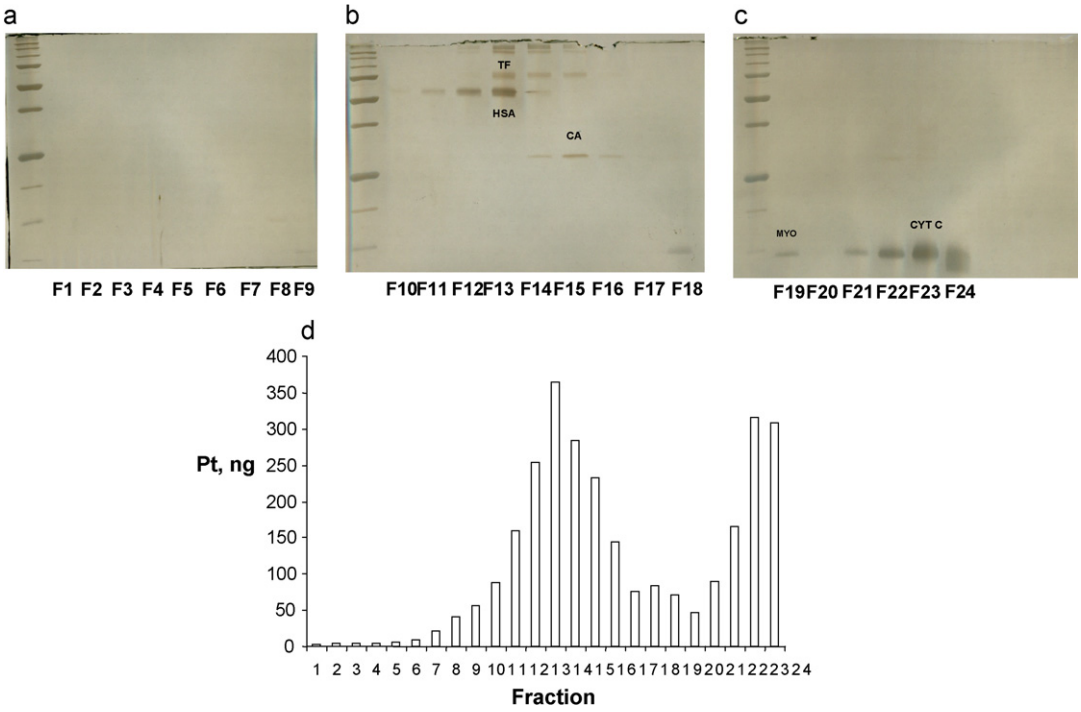

Fig. 3. Non-reducing SDS-PAGE of OFFGEL-IEF liquid fractions (24 fractions using strips with a pH range between 3 and 10) obtained under denaturing and non-reducing conditions in the absence of thiourea for the separation of a mixture of standard proteins (TF, HSA, CA, MYO and CYT C) incubated with cisplatin: (a) fractions 1–9, (b) fractions 10–18 and (c) fractions 19–24. In all figures lane 1 corresponds to Precision Plus Protein standards; (d) platinum profile obtained in the OFFGEL-IEF fractions described above with ICP-MS analysis. Gels were visualised by staining with Dodeca Silver Stain kit.

From the above results, it can be concluded that the use of thiourea has to be avoided for sample solubilisation of proteins containing platinum. Although it has been reported that the use of thiourea in addition to urea improves solubilisation, particularly of membrane proteins [30], for the separation of platinum–proteins by IEF, a denaturing solution without thiourea is recommended, being the presence or absence of DTT not so important.

3.2.2. Evaluation of the Pt–protein bonds stability in a mixture of proteins under OFFGEL-IEF separation conditions

A mixture of standard proteins (TF, HSA, CA, MYO and CYT C) incubated with cisplatin was also used to investigate the OFFGEL-IEF separation under denaturing and non-reducing conditions (without DTT) in the absence of thiourea. Protein separation takes place in a two-phase system with an upper liquid phase that is divided in compartments and a lower phase that is a conventional rehydrated IPG strip. Typically, the sample is diluted in the focusing buffer and loaded into all wells. Because there is no fluidic connection between the wells, proteins are forced to migrate through the IPG gel where the actual separation takes place. After IEF, the 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 3.00 and 10.00. Then, proteins were identified in their respective IEF fractions visualising the proteins after a consecutive separation of the different fractions by non-reducing SDS PAGE, which is shown in Fig. 3a–c.

As can be seen, the procedure separated the proteins mainly in the following fractions (the data in brackets correspond to the expected pH ranges per fraction given by the supplier): TF (~76 kDa) in fraction F13 (6.50–6.76), F14 (6.76–7.03) and F15 (7.03–7.29); HSA (~67 kDa) in fraction F11 (5.98–6.24), 12 (6.24–6.50), F13 (6.50–6.76) and F14 (6.76–7.03); CA (~29 kDa) in fraction F14 (6.76–7.03), F15 (7.03–7.29) and F16 (7.29–7.55); MYO (~17 kDa) in fraction F18 (7.81–8.08) and F19 (8.08–8.34); and CYT C (~12 kDa) in fraction F21 (8.60–8.86), F22 (8.86–9.13), F23 (9.13–9.29) and F24 (9.39–9.65). It can be concluded that these results are consistent with their theoretical p*I* values (6.8, 5.9, 6.4, 7.2, and 9.6 for TF, HSA, CA, MYO and CYT C, respectively). It should be noted that experimental p*I* values for Pt–proteins are displaced with respect to p*I* values obtained in a parallel experiment for proteins without cisplatin (data not shown). Moreover, Fig. 3b also shows the presence of protein dimers in the different fractions mentioned above. In addition, to evaluate the applicability of the separation protocol for platinum–protein analysis, platinum determination is needed to correlate with the presence of proteins in the fractions produced following separation by IEF. This determination was performed by ICP-MS. Fig. 3d showed the migration patterns for the mixed platinum–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 platinum–protein bond and the absence of free platinum. A recovery value of 60% was obtained for platinum.

As has been observed, proteins treated with cisplatin show important changes in their electrophoretical behavior, as they may, either dimerise or react with diverse amounts of Pt atoms with different possible ligands remaining coordinated to them, which can affect the separation by SDS-PAGE and IEF, respectively. So, separation methodologies for these samples present added difficulties. Nevertheless, in real samples, Pt-bound proteins present a lower degree of heterogeneity, due to lower Pt concentrations present in biological samples after cisplatin treatment. The OFFGEL separation method described in this work is usable when coupled to gel electrophoresis and in gel digestion of the separated bands for further peptide identification by LC–MS/MS. But most importantly, along all these procedures, it is imperative to keep platinum attached to the proteins, as was achieved and demonstrated by the previous experiments.

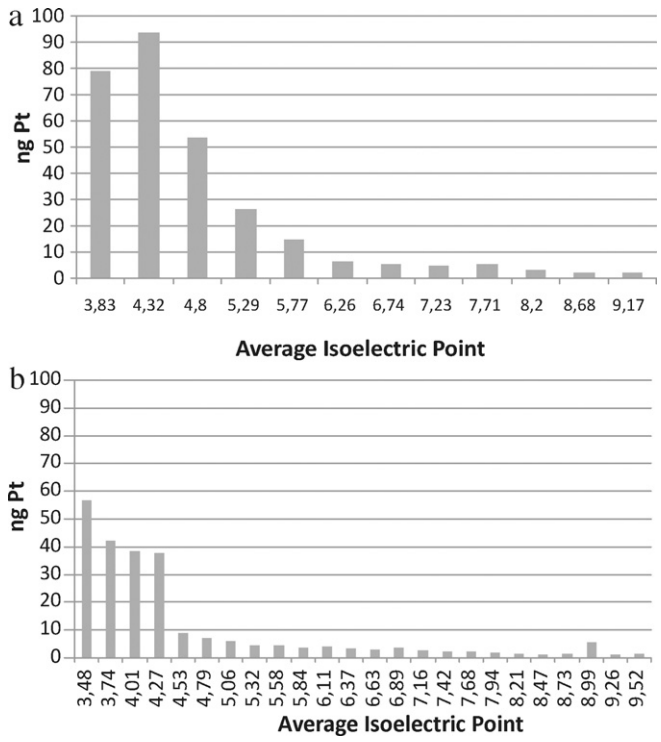

Fig. 4. Platinum profile obtained by ICP-MS analysis for the OFFGEL-IEF liquid fractions under: (a) well-established denaturing conditions (urea, thiourea, ampholytes, DTT and glycerol) (12 fractions using strips with a pH range between 3 and 10), or (b) with denaturing conditions without using thiourea or DTT (24 fractions using strips with a pH range between 3 and 10) for the separation of kidney tissue extracts containing platinum.

3.2.3. Evaluation of the stability of Pt–protein bonds in a rat kidney tissue extract under OFFGEL-IEF separation conditions

Kidney cytosols from rats treated with cis-Pt were isoelectrically fractionated in the conditions previously described, as an alternative to the classical IEF, which is performed in the presence of thiourea, for the separation of platinum binding proteins. Prior to the separation it was necessary to precipitate the proteins present in the cytosolic fraction (following the procedure described in Section 2.6.1) due to the incompatibility of the cytosolic extraction solution with the OFFGEL-IEF system. The cytosolic proteins were separated by OFFGEL-IEF under denaturing conditions (B5, in Table 1), which showed a complete solubilisation of the proteins. Proteins were separated in 12 fractions, using strips with a pH range between 3 and 10. Moreover, for comparison purposes the same tissue extracts were separated under denaturing conditions without thiourea and DTT (Table 1, B8), which showed a solubilisation of approximately 60% of the proteins. Proteins 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 OFFGEL-IEF, are shown in Fig. 4. A similar platinum distribution under both conditions can be seen, and a high amount of platinum was obtained mainly for the first four fractions. However, each fraction was subjected to ultrafiltration in order to measure the platinum remaining bound to the proteins. Recoveries of 23% (Fig. 4a) and 85% (Fig. 4b) for platinum bound to the proteins, were obtained after separation under denaturing and reducing conditions or denaturing conditions without DTT and thiourea, respectively. These results indicate the suitability of the OFFGEL-IEF in the absence of thiourea and DTT to separate and maintain the integrity of platinum–protein complexes.

3.3. Sample treatments for the analysis of platinum containing proteins by mass spectrometry

Structural identification of platinum binding proteins in the separated electrophoretic bands requires performing an enzymatic digestion followed by MALDI-TOF-MS or ESI-MS analysis. Thus, the stability of platinum containing proteins to some of the reagents used along an in-gel tryptic digestion and extraction procedures was also evaluated.

The influence of NH_4HCO_3 was investigated because this reagent is employed during the in-gel tryptic digestion of proteins. Overnight digestion in a Tris–HCl buffer was also included as control due to the stability under these conditions of platinum–proteins complexes already reported by Moreno-Gordaliza et al. [24]. The Pt-bound HSA fraction (i.e. >3 kDa) was also diluted 1 + 1 with NH_4HCO_3 at different concentrations, temperatures and for different reaction times, as described in Section 2.3.

As shown in Table 1C the recovery of protein bound platinum was high for these samples, between 93 and 99%, showing the suitability of all the conditions studied for tryptic digestion of platinum–proteins. Moreover, a high recovery value (98%, Table 1, C5) was also obtained in the presence of formic acid for 1 h, indicating the suitability of this reagent, widely used for ESI-MS analysis. These results may indicate that platinum-containing proteins may be able to resist the effect of the reagents tested, which are used along a classical proteomic in-gel digestion with trypsin.

3.3.1. Identification of proteins by MALDI-TOF-MS

Studies have been conducted to confirm that the Pt–protein bands, appearing at higher molecular weights in SDS-PAGE were, positively, the dimers of the treated proteins. Identification of those intense proteins bands in the gel following the SDS-PAGE separation under non-reducing conditions of the solution containing the five cisplatin-proteins complexes, running at approximately 160, 140, 60, 35 and 24 kDa, was carried out by MALDI-TOF-MS, following spot excision and tryptic *in-gel* digestion. Peptide mass fingerprinting was performed searching protein databases with either Mascot or Profound search engines. It can be concluded that TF, HSA, CA, MYO and CYT C are clearly present in the selected bands by peptide matching, which indicates protein dimerisation. Moreover, as expected, TF, HSA, CA, MYO and CYT C are also present in their corresponding bands (76, 67, 29, 17 and 12 kDa, respectively) by peptide matching. The presence of heterogeneity in proteins treated with cisplatin has been previously observed by our group (unpublished results), and are due to both, dimerisation and platinum-catalyzed specific protein fragmentation. However, only no metal-containing peptides could be found in the peptide maps and future studies will focus on the identification of the platinum binding sites and platinum-containing peptides using ESI coupled to tandem mass spectrometry.

4. Conclusions

The suitability of a 2D electrophoretic separation procedure able to preserve platinum–protein bonds has been demonstrated. This method is based on the use of sequential OFFGEL-IEF and PAGE. Furthermore, general parameters for post-separation treatments to make the proteins suitable for further structural MS analysis, preserving the platinum–protein bonds along the whole analytical procedure, have been settled. The main reason for the loss of protein-bound platinum during the separation lies on the use of some reagents, such as BME, DTT or thiourea. Therefore, the use of SDS-PAGE in the absence of BME or DTT is recommended. However, neither the influence of the electric field during electrophoresis

separation, nor the processes of fixing, staining and destaining proteins in the gel seemed to result in the loss of platinum from platinum binding proteins. The suitability of the separation of platinum binding proteins from a kidney tissue extract by OFFGEL-IEF was demonstrated.

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