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

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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

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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*