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# Determination of the affinity of biomimetic peptides for uranium through the simultaneous coupling of HILIC to ESI-MS and ICP-MS

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## 1 Abstract

2 Several proteins have been identified in the past decades as targets of uranyl ( $\text{UO}_2^{2+}$ ) *in vivo*. However,  
3 the molecular interactions responsible for this affinity are still poorly known which requires the  
4 identification of the  $\text{UO}_2^{2+}$  coordination sites in these proteins. Biomimetic peptides are efficient  
5 chemical tools to characterize these sites. In this work, we developed a dedicated analytical method to  
6 determine the affinity of biomimetic, synthetic, multi-phosphorylated peptides for  $\text{UO}_2^{2+}$  and evaluate  
7 the effect of several structural parameters of these peptides on this affinity at physiological pH. The  
8 analytical strategy was based on the implementation of the simultaneous coupling of hydrophilic  
9 interaction chromatography (HILIC) with electrospray ionization mass spectrometry (ESI-MS) and  
10 inductively coupled plasma mass spectrometry (ICP-MS). An essential step had been devoted to the  
11 definition of the best separation conditions of  $\text{UO}_2^{2+}$  complexes formed with di-phosphorylated peptide  
12 isomers and also with peptides of different structure and degrees of phosphorylation. We performed the  
13 first separations of several sets of  $\text{UO}_2^{2+}$  complexes by HILIC ever reported in the literature. A dedicated  
14 method had then been developed for identifying the separated peptide complexes online by ESI-MS and  
15 simultaneously quantifying them by ICP-MS, based on uranium quantification using external  
16 calibration. Thus, the affinity of the peptides for  $\text{UO}_2^{2+}$  was determined and made it possible to  
17 demonstrate that (i) the increasing number of phosphorylated residues (pSer) promotes the affinity of  
18 the peptides for  $\text{UO}_2^{2+}$ , (ii) the position of the pSer in the peptide backbone has very low impact on this  
19 affinity (iii) and finally the cyclic structure of the peptide favors the  $\text{UO}_2^{2+}$  complexation in comparison  
20 with the linear structure. These results are in agreement with those previously obtained by spectroscopic  
21 techniques, which allowed to validate the method. Through this approach, we obtained essential  
22 information to better understand the mechanisms of toxicity of  $\text{UO}_2^{2+}$  at the molecular level and to  
23 further develop selective decorporating agents by chelation.

24

25

## 26 Introduction

27 The occurrence of uranium (U) in the environment is due to natural and anthropogenic sources [1].  
28 Being part of the actinide family, U has no biological function and exhibits chemical and radiological  
29 toxicity, depending on its isotopic composition. Natural U ( $U_{\text{nat}}$ ) displays mainly chemical toxicity  
30 driven by the interactions of the uranyl cation ( $UO_2^{2+}$ ) with target biomolecules *in vivo* [2]. However,  
31 the mechanisms of toxicity of  $UO_2^{2+}$  at the cellular and molecular level are still poorly understood. The  
32 full identification of biomolecules binding specifically  $UO_2^{2+}$  *in vivo* and *in vitro* and the characterization  
33 of their interactions are essential to better describe these mechanisms and to further develop selective  
34 decorporating agents. Although few target proteins of  $UO_2^{2+}$  have been identified *in vivo* [3,4], the  
35 coordination sites responsible for their strong affinity for  $UO_2^{2+}$  are still not known. In this context  
36 biomimetic approaches, based on the synthesis of model peptides designed specifically to mimic the  
37 coordination sites of  $UO_2^{2+}$  in proteins, are very useful to determine the key parameters governing this  
38 affinity [5]. Recently, multi-phosphorylated cyclopeptides have been synthesized as models of  $UO_2^{2+}$   
39 coordination sites in osteopontin (OPN) (Fig.1), a hyperphosphorylated target protein of  $UO_2^{2+}$  [4,6].  
40 Such biomimetic peptides need to be validated to be representative of the suggested  $UO_2^{2+}$  coordination  
41 sites. In this purpose, studies have been devoted to the characterization of  $UO_2^{2+}$  interactions with these  
42 peptides and the determination of the associated stability constants using spectroscopic techniques such  
43 as fluorescence spectroscopy, Extended X-ray absorption fine structure (EXAFS), circular dichroism  
44 (CD) and also electrospray ionization mass spectrometry (ESI-MS) [6–10]. Through these studies, the  
45 affinity of these peptides for  $UO_2^{2+}$  was shown to increase with the number of phosphoserine residues  
46 (pSer) in their scaffold, to reach the highest value for tetra-phosphorylated peptide (pS1368), being close  
47 to the affinity constant of  $UO_2(\text{OPN})$  [6].  
48 The aim of this work was to develop a dedicated analytical method to determine in a single step an  
49 affinity scale of multi-phosphorylated biomimetic peptides for  $UO_2^{2+}$ . The strategy was based on the  
50 setting up of the simultaneous coupling of hydrophilic interaction liquid chromatography (HILIC) to  
51 electrospray ionization mass spectrometry (ESI-MS) and inductively coupled plasma mass spectrometry  
52 (ICP-MS) [11,12]. The  $UO_2(\text{peptide})$  complexes could then be separated, online identified by ESI-MS

53 and quantified by ICP-MS in a simultaneous manner. Hence, the quantitative distribution of  $\text{UO}_2^{2+}$   
54 within the different complexes could be determined in one single step, leading to the further  
55 determination of an affinity scale. Through this approach, complementary data to those obtained by  
56 spectroscopic techniques could be acquired in combination with the reduction of analysis time and  
57 sample consumption, which is a major advantage when only small amount of the peptides can be  
58 synthesized. A major challenge was to achieve the chromatographic separation of the  $\text{UO}_2^{2+}$  complexes  
59 while preserving the integrity of their structure, knowing that their labile character and their electrostatic  
60 interactions can lead to their dissociation during the separation processes [13]. The separation mode of  
61 HILIC seemed promising to meet this challenge since it is dedicated to the separation of polar,  
62 hydrophilic, neutral or charged compounds and is described as being suitable for the separation of metal  
63 complexes, even the labile ones [14]. To our knowledge, the separation of  $\text{UO}_2^{2+}$  complexes by HILIC  
64 has never been reported in the literature.

65 The first part of this work was devoted to the definition of HILIC separation conditions of several model  
66 systems containing (i)  $\text{UO}_2^{2+}$  with di- and tetra-phosphorylated peptides, pS16 and pS1368, in order to  
67 determine the effect of the number of pSer residues on  $\text{UO}_2^{2+}$  affinity (ii)  $\text{UO}_2^{2+}$  and di-phosphorylated  
68 peptide isomers, pS16 and pS18, in order to evaluate the impact of the position of pSer residues on  
69  $\text{UO}_2^{2+}$  affinity, (iii)  $\text{UO}_2^{2+}$  in presence of cyclic pS1368 and linear linS1368 tetra-phosphorylated  
70 peptides, in order to evaluate the impact of the peptide structure on its affinity for  $\text{UO}_2^{2+}$ . Then a  
71 quantification method of  $\text{UO}_2^{2+}$  based on external calibration was developed to determine online the  
72 distribution of  $\text{UO}_2^{2+}$  within the separated complexes. Such a dedicated method allowed to measure in  
73 a single step the affinity of biomimetic peptides towards  $\text{UO}_2^{2+}$  when they are in competing  
74 complexation reaction and to determine the effect of the structural parameters of the peptides on their  
75 affinity.

# 1. Experimental part

## 1.1. Chemicals

Acetonitrile (ACN, CH<sub>3</sub>CN, LC-MS grade) and ammonia NH<sub>3</sub> (20-22%) were purchased from VWR prolabo (Briare le canal, France). Ammonium acetate (NH<sub>4</sub>CH<sub>3</sub>CO<sub>2</sub>) and toluene (C<sub>6</sub>H<sub>5</sub>CH<sub>3</sub>, purity > 99.7 %) were supplied by Sigma Aldrich (Saint Quentin Fallavier, France). Ultrapure water (18.2 MΩ cm at 25°C) was obtained from Milli-Q purification system (Merck millipore, Guyancourt, France). Uranium (U) and bismuth (Bi) standard solutions (1000 µg mL<sup>-1</sup>) in HNO<sub>3</sub> 2% w/w, were provided by the SPEX Certiprep Group (Longjumeau, France). L-Tryptophan (Trp) (99% purity) was purchased from Acros Organics. Nitric acid solutions (HNO<sub>3</sub> 2%) were prepared by diluting in ultrapure water, HNO<sub>3</sub> 65% (Merck, France) which was distilled with evapoclean from Analab (France).

## 1.2. Preparation of stock solutions and samples

### 1.2.1. Uranium and peptide stock solutions

The uranium stock solution was prepared by diluting in ultrapure water an in-house U<sub>nat</sub> solution [15] prepared in 0.5 mol L<sup>-1</sup> ultrapure HNO<sub>3</sub> (SCP Science), to achieve a U<sub>nat</sub> concentration of 10,000 µg mL<sup>-1</sup> (5 x 10<sup>-2</sup> mol L<sup>-1</sup>). The U<sub>nat</sub> concentration of the stock solution was determined by ICP-MS based on external calibration using U<sub>nat</sub> standard solutions in HNO<sub>3</sub> 2% and was 9,447.2 µg mL<sup>-1</sup> (3.97 x 10<sup>-2</sup> mol L<sup>-1</sup>).

Di-phosphorylated peptide isomers pS16 and pS18 were synthesized, characterized and supplied by the CIBEST team at SyMMES (Univ. Grenoble Alpes, CEA, CNRS, IRIG, 38 000 Grenoble - France) following the procedures described elsewhere [8]. Linear and cyclic tetra-phosphorylated peptides pS1368 and linS1368, were supplied by Cambridge peptides (Cambridge, UK) following the procedure developed by the CIBEST team [6]. The peptide stock solutions were prepared by dissolving the adequate amount of the targeted peptide powder in 20 mmol L<sup>-1</sup> NH<sub>4</sub>CH<sub>3</sub>CO<sub>2</sub> (pH ~ 7.4) to reach a concentration between 2 and 4 mmol L<sup>-1</sup>. The concentration of the peptides was determined by external calibration, using HILIC coupled to UV/VIS in series with ESI-MS, by quantifying the Tryptophan (Trp) contained in the sequence of all peptides (UV absorption at λ = 280 nm). Trp standard solutions were obtained by diluting a Trp solution prepared at 10<sup>-2</sup> mol L<sup>-1</sup> in 20 mmol L<sup>-1</sup> NH<sub>4</sub>CH<sub>3</sub>CO<sub>2</sub>, in 70/30

103 ACN/H<sub>2</sub>O containing 20 mmol L<sup>-1</sup> NH<sub>4</sub>CH<sub>3</sub>CO<sub>2</sub> to reach concentrations that ranged between 5 x10<sup>-6</sup>  
104 mol L<sup>-1</sup> and 2 x 10<sup>-4</sup> mol L<sup>-1</sup>.

### 105 *1.2.2. Preparation of the samples*

106 In a first step, UO<sub>2</sub>(peptide) contact solutions were prepared by adding a very small volume (around 1.5  
107 μL) of U<sub>nat</sub> stock solution in 125-250 μL of peptide stock solution, to obtain UO<sub>2</sub><sup>2+</sup> concentration ranging  
108 between 2 and 5 x 10<sup>-4</sup> mol L<sup>-1</sup> and the desired UO<sub>2</sub><sup>2+</sup>/peptide ratio. The pH was adjusted to 7.4 using  
109 ammonia (20-22%). All solutions were systematically prepared the day before the analysis. Finally, the  
110 working samples were freshly prepared before analysis by diluting the UO<sub>2</sub>(peptide) contact solutions  
111 by a factor of 2 or 5 in the adequate mobile phase, to reach a final UO<sub>2</sub><sup>2+</sup> concentration of 10<sup>-4</sup> mol L<sup>-1</sup>,  
112 which was quantified by ICP-MS as described in section 1.4.1. The concentration of the peptides in the  
113 contact solution was determined by weight while taking into account the concentration of the peptide  
114 stock solution.

## 115 **1.3. Instrumentation**

### 116 *1.3.1. Hydrophilic interaction liquid chromatography*

117 An ultimate 3000 UHPLC<sup>+</sup> Dionex/ThermoFisher scientific (Courtaboeuf, France), made of a degasser,  
118 a dual RS pump, an RS autosampler, a column compartment and an RS diode array detector, was used.  
119 The separation of the different sets of UO<sub>2</sub>(peptide) complexes was carried out using Acquity BEH  
120 Amide (100 x 2.1; 1,7 μm, Waters), YMC Triart Diol (100 x 2; 1.9 μm, YMC) and Acquity BEH HILIC  
121 (100 x 2.1; 1,7 μm, Waters) columns. The composition of the desired mobile phases was obtained by  
122 online mixing in the adequate proportions, solvent A (60/40 ACN/H<sub>2</sub>O v/v containing 20 mmol L<sup>-1</sup>  
123 NH<sub>4</sub>CH<sub>3</sub>CO<sub>2</sub>) and solvent B (80/20 ACN/H<sub>2</sub>O v/v containing 20 mmol L<sup>-1</sup> NH<sub>4</sub>CH<sub>3</sub>CO<sub>2</sub>).

124 For the quantification of pS16, pS18 and pS1368 stock solutions, an YMC Triart diol (100 x 2 mm; 1,9  
125 μm) column was used with a mobile phase composed of 70/30 ACN/H<sub>2</sub>O v/v and 20 mmol L<sup>-1</sup>  
126 NH<sub>4</sub>CH<sub>3</sub>CO<sub>2</sub>. For the quantification of linS1368, an Acquity BEH HILIC (100 x 2,1; 1,7μm) column  
127 was used and the mobile phase was made of 68/32 ACN/H<sub>2</sub>O v/v and 20 mmol L<sup>-1</sup> NH<sub>4</sub>CH<sub>3</sub>CO<sub>2</sub>. In all  
128 the cases, the separation was run in isocratic mode at a flow rate of 300 μL min<sup>-1</sup> and the injection  
129 volume was 3 μL.

130 The retention factor  $k$  of the analytes were calculated following the equation (1):

$$131 \quad k = \frac{(t_R - t_0)}{t_0} \text{ (Equation 1)}$$

132 Where  $t_R$  is the retention time (min) of the analyte, determined by HILIC-ESI-MS,  $t_0$  is the void time of  
133 unretained marker, toluene ( $10^{-4}$  mol L $^{-1}$ ,  $V_{inj} = 1$   $\mu$ L), determined by HILIC-UV/VIS at  $\lambda = 254$  nm.

134 The selectivity and resolution factors of the separations,  $\alpha$  and  $R_s$  respectively, were calculated based on  
135 the equations 2 and 3:

$$136 \quad \alpha = \frac{k_2}{k_1} \text{ (Equation 2)}$$

$$137 \quad R_s = 1.18 \times \frac{t_{R2} - t_{R1}}{W_{0.5h1} + W_{0.5h2}} \text{ (Equation 3)}$$

138 With analyte 2 more retained than analyte 1.  $W_{0.5}$  corresponds to full width half-maximum of each peak.

### 139 *1.3.2. Mass spectrometers*

140 The ESI mass spectrometer was a triple quadrupole TSQ Quantum Ultra<sup>TM</sup> (Thermo Fisher scientific,  
141 San Diego CA, USA) equipped with an H-ESI II ionization probe. All mass spectra were recorded in  
142 negative ionization mode with the following parameters: spray voltage -3.5 kV, temperature of the probe  
143 120°C and temperature of the capillary transfer 360°C. In all cases and in agreement with previous  
144 studies [6,8], double charged  $[\text{UO}_2(\text{peptide})]^{2-}$  complexes with 1:1 stoichiometry were observed. For  
145 the sake of clarity, the  $\text{UO}_2^{2+}$  complexes were denoted along the manuscript  $\text{UO}_2(\text{peptide})$ , by omitting  
146 the charge. Mass spectra were acquired in full scan mode ( $m/z$  400-1500) and in single ion monitoring  
147 (SIM) mode, by selecting the  $m/z$  ratio associated to the targeted free peptides and  $\text{UO}_2(\text{peptide})$   
148 complexes (spectral width:  $\pm 0.5$   $m/z$ ).

149 The ICP-MS instrument was a single quadrupole XSeriesII (Thermo Fisher Scientific). The sample  
150 introduction system consisted of a perfluoroalkoxy PFA-ST nebulizer operating at 200  $\mu$ L min $^{-1}$   
151 followed by a quartz cyclonic spray chamber thermostated at 3 °C (PC3 system, ESI). The simultaneous  
152 coupling of HILIC to ESI-MS and ICP-MS was performed according to the setting up described in our  
153 previous work [11] and presented in Fig.2. In order to prevent any carbon deposition due to the use of  
154 organic solvents, additional 8 mL min $^{-1}$  oxygen flow rate was introduced in the plasma, through an  
155 “additional gas port” located in the spray chamber [16,17]. A platinum skimmer, a sampler cone and a



156 1 mm inner diameter injector were additionally used for this purpose. The parameters were checked  
157 daily using a 25 ng mL<sup>-1</sup> U<sub>nat</sub> standard solution introduced in the ICP-MS at 6.7 μL min<sup>-1</sup> along with a  
158 10 ng mL<sup>-1</sup> Bi in HNO<sub>3</sub> 2% at 140 μL min<sup>-1</sup> (Fig.2). The chromatograms were recorded based on the  
159 signal of <sup>238</sup>U and <sup>209</sup>Bi with an integration time of 90 ms for each isotope.

#### 160 **1.4.Online quantification of the UO<sub>2</sub>(peptide) complexes**

161 Samples were analyzed using the simultaneous coupling of HILIC to ESI-MS and ICP-MS. The  
162 separated UO<sub>2</sub>(peptide) complexes were online identified by ESI-MS and simultaneously quantified by  
163 ICP-MS. For this purpose, external calibration was selected as quantification method for the  
164 determination of total UO<sub>2</sub><sup>2+</sup> concentration in the samples, denoted [UO<sub>2</sub><sup>2+</sup>]<sub>total</sub>, since the uncertainty  
165 range (5-15%) provided by this method is adequate for our study.

##### 166 *1.4.1. Determination of the total UO<sub>2</sub><sup>2+</sup> concentration in the working samples*

167 Calibration was performed by introducing U<sub>nat</sub> standard solutions into the ICP-MS under flow injection  
168 analysis mode (FIA), employing the same mobile phase composition and flow rate as used when  
169 coupling to the separation. In non-complexing medium, hydrolysis dominates the speciation of UO<sub>2</sub><sup>2+</sup>,  
170 potentially leading to its precipitation depending on its concentration [18]. Therefore, the tetra-  
171 phosphorylated peptide pS1368 was added to the U<sub>nat</sub> standard solutions in an equimolar ratio to prevent  
172 this phenomenon, but also to yield specific standard solutions of UO<sub>2</sub>(peptide) complexes. Five levels  
173 of calibration were considered with U<sub>nat</sub> concentration ranging from 8 to 40 μg mL<sup>-1</sup>. The calibration  
174 curve was established daily by plotting the mean value of the peak areas as a function of U<sub>nat</sub>  
175 concentrations; each standard being injected in triplicate. The repeatability of the measurements,  
176 determined from the RSD of the peak areas for each concentration, was between 1% and 4% (< 15%).

##### 177 *1.4.2. Quantification of UO<sub>2</sub><sup>2+</sup> in the separated UO<sub>2</sub>(peptide) complexes*

178 This step was performed by integrating the total area of the chromatographic peak of each UO<sub>2</sub>(peptide)  
179 complex and by further determining the corresponding concentration using the calibration curve. During  
180 the separation, adsorption of free or weakly complexed UO<sub>2</sub><sup>2+</sup>, on the stationary phase can occur. To  
181 recover UO<sub>2</sub><sup>2+</sup> adsorbed on the column, successive injections of the tetra-phosphorylated peptide pS1368  
182 were carried out after each chromatographic run until the background of <sup>238</sup>U signal was less than 10<sup>3</sup>

183 cps. The concentration of this fraction of  $\text{UO}_2^{2+}$ , denoted  $[\text{UO}_2^{2+}]_{\text{free}}$ , was then determined based on  
184 external calibration, by summing the area of the peaks resulting from the injections of pS1368. A  
185 cleaning process was further carried out to eliminate any adsorbed peptide residues, using 5/95  
186 ACN/ $\text{H}_2\text{O}$  v/v containing 20 mmol  $\text{L}^{-1}$   $\text{CH}_3\text{COOH}$ , followed by the column regeneration with the  
187 working mobile phase working.  
188 The quantitative distribution of  $\text{UO}_2^{2+}$  among the eluted species, expressed in percent (%), was  
189 determined according to the Equation 4.

$$190 \quad \% \text{UO}_2^{2+}_X = \frac{[\text{UO}_2^{2+}]_X}{[\text{UO}_2^{2+}]_{\text{total}}} \text{ (Equation 4)}$$

191 With X being free  $\text{UO}_2^{2+}$  or  $\text{UO}_2(\text{peptide})$  complexes.

192 The mass balance was expressed as the ratio of the sum of the concentrations of  $\text{UO}_2^{2+}$  in its free and  
193 complexed forms, to the total concentration of  $\text{UO}_2^{2+}$  in the sample, according to Equation 5.

$$194 \quad \text{Mass balance (\%)} = \frac{\sum [\text{UO}_2^{2+}]_{\text{UO}_2(\text{peptide}1)} + [\text{UO}_2^{2+}]_{\text{UO}_2(\text{peptide}2)} + [\text{UO}_2^{2+}]_{\text{Free}}}{[\text{UO}_2^{2+}]_{\text{total}}} \text{ (Equation 5)}$$

195 During the HILIC-ICP-MS coupling and the FIA mode, the stability of the  $^{238}\text{U}$  signal was monitored  
196 using bismuth ( $^{209}\text{Bi}$ ) as internal standard, added at 10 ng  $\text{mL}^{-1}$  in the nitric acid makeup solution (Fig.).  
197 The stability of the  $^{209}\text{Bi}$  signal during an acquisition cycle was checked by calculating the relative  
198 standard deviation RSD (%) of measurements acquired continuously. The RSD was 1.9%, which reflects  
199 good stability of the signal during an acquisition cycle (< 15%). The stability of the ICP-MS response  
200 was also daily checked by calculating the standard deviation of the averaged  $^{209}\text{Bi}$  measurements for all  
201 acquisitions cycles. As for example, for a maximum of 11 acquisition cycles, an RSD between 1 and  
202 5.9% was obtained, which is acceptable for our applications.

203

## 204        **2. Results and discussion**

### 205        **2.1. Definition of the HILIC conditions for separating UO<sub>2</sub>(peptide) complexes**

206        The achievement of the HILIC separation of the UO<sub>2</sub>(peptide) complexes is a crucial step to be able to  
207        develop our method. Due to the particularity of UO<sub>2</sub><sup>2+</sup> complexes, few chromatographic techniques are  
208        suitable to perform such challenging separations [13]. The ones encountered in the literature are mainly  
209        size exclusion chromatography (SEC) [3,19–23] and immobilized metal affinity chromatography  
210        (IMAC) [24], to investigate the interactions of UO<sub>2</sub><sup>2+</sup> with proteins. Furthermore, the limitation of these  
211        separation techniques lies in their low separation resolution and in some cases their incompatibility to  
212        ESI-MS coupling. The preservation of UO<sub>2</sub><sup>2+</sup> complexes during elution is of great concern since  
213        dissociation is often observed for such labile species during the chromatographic process. Even though  
214        the UO<sub>2</sub>(peptide) complexes are known to be fully formed in the samples as indicated by their stability  
215        constants measured at pH 7.4 [6,10], they may fully or partially dissociate on the column. One way to  
216        limit this dissociation is to use an excess of the peptides in the samples. However, full dissociation was  
217        observed for complexes containing cyclic peptides with no or only one pSer in their sequence, whatever  
218        the column and the UO<sub>2</sub><sup>2+</sup>:peptide proportion (data not shown). By contrast, UO<sub>2</sub><sup>2+</sup> complexes with  
219        multi-phosphorylated peptides and having stability constants higher or equal to 10<sup>10</sup>, could be detected  
220        using an excess of di- and tetra-phosphorylated peptides, from 2 to 10 equivalents. It must be mentioned  
221        that several UO<sub>2</sub><sup>2+</sup>:peptide proportions were tested, but only the chromatograms obtained with the  
222        proportions that allowed to detect all the separated complexes, at least by ICP-MS, are presented in this  
223        part.

224        In our previous work, the Acquity BEH amide column with a mobile phase made of 70/30 ACN/H<sub>2</sub>O  
225        v/v and 20 mmol L<sup>-1</sup> NH<sub>4</sub>CH<sub>3</sub>CO<sub>2</sub> allowed a successful separation of the free multi-phosphorylated  
226        peptides [25]. Therefore, we first evaluated these conditions to separate the UO<sub>2</sub><sup>2+</sup> complexes containing  
227        these peptides, but successful results were obtained only for the complexes formed with the ps16 and  
228        ps18 isomers (Fig.1). As shown in Fig.3-a, UO<sub>2</sub>(ps16) and UO<sub>2</sub>(ps18) complexes were separated with  
229        a good selectivity ( $\alpha=1.3$ ) and baseline resolution ( $R_s= 2.2$ ). The availability of the pSer group in  
230        different positions in the peptide scaffold (Fig.1) could explain the achievement of the separation

231 through differential interactions of the pSer with the amide function of the stationary phase, as  
232 previously observed for the free peptides [25].

233 As stated above, these chromatographic conditions did not allow the separation of the  $\text{UO}_2(\text{pS16})/$   
234  $\text{UO}_2(\text{pS1368})$  and  $\text{UO}_2(\text{pS1368})/ \text{UO}_2(\text{linS1368})$  sets of complexes, even by increasing the acetonitrile  
235 content of the mobile phase while keeping the salt concentration constant. A less polar stationary phase,  
236 grafted by diol functional group (YMC Triart Diol) led to the separation of  $\text{UO}_2(\text{pS16})$  and  $\text{UO}_2(\text{pS1368})$   
237 with an improved selectivity ( $\alpha = 2.3$ ) and resolution ( $R_s = 3.9$ ), using 72% of ACN in the mobile phase  
238 (Fig.3-b). As seen in (Fig.3-b), the  $\text{UO}_2(\text{pS16})$  complex could not be observed by ESI-MS despite the  
239 high excess of pS16 added to pS1368 and  $\text{UO}_2^{2+}$ . In the chromatogram recorded by ICP-MS, we could  
240 observe a peak of low intensity eluting at the same retention time as free pS16, which may be reasonably  
241 assigned to  $\text{UO}_2(\text{pS16})$ . The peak eluting at 4 min was attributed to  $\text{UO}_2(\text{pS1368})$ , thanks to its  
242 identification by ESI-MS. Furthermore, free pS1368 was not detected by ESI-MS, allowing to suggest  
243 that this peptide, which exhibits a high affinity for  $\text{UO}_2^{2+}$ , is fully coordinated.

244 Acquity BEH HILIC hybrid silica column was selected to separate  $\text{UO}_2(\text{pS1368})$  and  $\text{UO}_2(\text{linS1368})$   
245 containing tetra-phosphorylated peptides having the same peptide sequence but different cyclic/linear  
246 structure. A mobile phase composed of 68/32 ACN/ $\text{H}_2\text{O}$  v/v and 20 mmol  $\text{L}^{-1}$   $\text{NH}_4\text{CH}_3\text{CO}_2$  yielded the  
247 best separation of the two complexes with good selectivity ( $\alpha=8.1$ ) and resolution ( $R_s=5.6$ ) (Fig.3-c).  
248 The  $\text{UO}_2^{2+}$  complex formed with the cyclic peptide eluted earlier than the complex containing the linear  
249 one. This allows to suggest that the accessibility of the polar groups of the stationary phase to interact  
250 with the complexes is linked to the structuration of the peptide upon its complexation to  $\text{UO}_2^{2+}$ . To our  
251 knowledge, the use of hybrid silica column has never been described in the literature to separate metal  
252 complexes. Only one study reports the use of a bare silica column to separate gadolinium-based contrast  
253 agents [26], while most of the separations of transition metal complexes such as Fe, Cu and Ni, were  
254 carried out with stationary phases grafted by zwitterionic, diol and amide functions [14,27]. In our study,  
255 dedicated HILIC conditions were set up to successfully separate a set of  $\text{UO}_2(\text{peptide})$  complexes with  
256 good selectivity and baseline resolution. These results highlight the potential of HILIC to separate  $\text{UO}_2^{2+}$   
257 complexes, known to be challenging. Thus, we reported the first separations of  $\text{UO}_2^{2+}$  complexes ever  
258 described in the literature, using HILIC.

## 2.2 Evaluation of the effect of the structure of the peptides on $\text{UO}_2^{2+}$ affinity

### 2.2.1. Effect of the number of phosphorylated residues in the peptide backbone

When designing biomimetic peptides, the selection of the amino acid to build the peptide backbone is of prime importance since the functional complexing groups of these amino acids will be involved in the  $\text{UO}_2^{2+}$  coordination. Phosphorylated amino acids, mainly pSer are of major concern, knowing that  $\text{UO}_2^{2+}$  exhibits high affinity for phosphate groups [28]. Furthermore, some of  $\text{UO}_2^{2+}$  target proteins such as OPN, are highly phosphorylated and are suspected to interact with  $\text{UO}_2^{2+}$  through their pSer sites [6,29]. Therefore, the effect of phosphorylation on  $\text{UO}_2^{2+}$  affinity has gained a lot of interest and was studied by using a peptide representative of site 1 of calmodulin [30,31] and phosphorylated model peptides of a specific sequence of  $\beta$ -casein [32]. The phosphorylation of these model peptides induced an increase of their affinity towards  $\text{UO}_2^{2+}$  in comparison to their non-phosphorylated counterparts.

In our work, the effect of phosphorylation on  $\text{UO}_2^{2+}$  affinity was evaluated in a single step by applying the analytical approach that we developed through the simultaneous coupling of HILIC to ESI-MS and ICP-MS. Several ratios of di- and tetra-phosphorylated peptides pS16 and pS1368 were added to  $\text{UO}_2^{2+}$  at pH 7.4. Knowing that the conditional stability constant of  $\text{UO}_2(\text{pS16})$  is lower than that of  $\text{UO}_2(\text{pS1368})$  (Fig.1), a higher excess of pS16 was added to  $\text{UO}_2^{2+}$  in comparison to pS1368. The quantitative distribution of  $\text{UO}_2^{2+}$  among the separated peptide complexes was determined through the quantification method described in the experimental part, which allowed to assess the differential affinity of the peptides towards  $\text{UO}_2^{2+}$ . The total  $\text{UO}_2^{2+}$  concentration in each sample was firstly measured in duplicate by ICP-MS before the separation and the values are listed in Table 1, as well as targeted and experimental  $x\text{UO}_2^{2+}:y\text{pS1368}:z\text{pS16}$  proportions.

Table 1: Total concentration of  $\text{UO}_2^{2+}$  in each sample  $[\text{UO}_2^{2+}]_{\text{total}}$  measured in duplicate, relative deviation\* of the values, experimental concentration of pS1368 and pS16 in the samples and experimental  $x\text{UO}_2^{2+}:y\text{pS1368}:z\text{pS16}$  proportions. For simplification, the samples containing different x:y:z proportions were assigned by a letter (A-E).

Targeted $x\text{UO}_2^{2+}:y\text{pS1368}:z\text{pS16}$	$[\text{UO}_2^{2+}]_{\text{total}}$ $\mu\text{g mL}^{-1}$ ( $\text{mol L}^{-1}$ )		Relative deviation* (%)	$[\text{pS1368}]$ $\text{mol L}^{-1}$	$[\text{pS16}]$ $\text{mol L}^{-1}$	Experimental $x\text{UO}_2^{2+}:y\text{pS1368}$ $:z\text{pS16}$ (sample N)
	Value 1	Value 2				
<b>2:0:20</b>	20.5 ( $8.6 \times 10^{-5}$ )	20.3 ( $8.5 \times 10^{-5}$ )	1.2	0	$9.2 \times 10^{-4}$	2:0:21.2 (A)
<b>2:0.5:20</b>	20.8 ( $8.7 \times 10^{-5}$ )	20.9 ( $8.8 \times 10^{-5}$ )	0.7	$2.3 \times 10^{-5}$	$9.1 \times 10^{-4}$	2:0.5:20.9 (B)
<b>2:2:2</b>	30.5 ( $1.3 \times 10^{-4}$ )	31.4 ( $1.3 \times 10^{-4}$ )	2.9	$1.2 \times 10^{-4}$	$1.2 \times 10^{-4}$	2:1.9:1.9 (C)
<b>2:3:1</b>	39.8 ( $1.7 \times 10^{-4}$ )	39.7 ( $1.7 \times 10^{-4}$ )	0.3	$1.7 \times 10^{-4}$	$6.0 \times 10^{-5}$	2:2.1:0.7 (D)

<b>2:4:0</b>	33.9 (1.4 x10 <sup>-4</sup> )	35.5 (1.5 x10 <sup>-4</sup> )	4.6	4.6 x10 <sup>-4</sup>	0.0	2:6.4:0 (E)
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\*Relative deviation (value1-value 2)/(value 1)

284 As can be seen in Table 1, a relative deviation ranging between 0.3 and 4.6 % shows good repeatability  
 285 of the measurements.

286 The chromatograms simultaneously recorded by ESI-MS and ICP-MS, using the YMC Triart Diol  
 287 column to separate the complexes, are presented in Fig.4. The peak of free tetra-phosphorylated peptide  
 288 pS1368 was observed by ESI-MS only when it was in excess relatively to UO<sub>2</sub><sup>2+</sup>, whereas the one of  
 289 pS16 was detected for all proportions. The peak corresponding to UO<sub>2</sub>(pS1368) was detected by ESI-  
 290 MS and ICP-MS for all proportions whilst the one of UO<sub>2</sub>(pS16) was observed exclusively by ICP-MS  
 291 and only when pS16 was in large excess compared to UO<sub>2</sub><sup>2+</sup> and pS1368, that is for samples A and B.  
 292 This indicates that pS1368 seems to fully complex UO<sub>2</sub><sup>2+</sup> when they are in equimolar proportions or  
 293 less, while pS16 is weakly complexed even when it is present in large excess with respect to UO<sub>2</sub><sup>2+</sup>. The  
 294 quantitative distribution of total UO<sub>2</sub><sup>2+</sup> among the di- and tetra-phosphorylated peptide complexes and  
 295 the mass balance, expressed in percent (%) could be calculated using Equation 4 and 5, respectively and  
 296 are presented in the diagram of Fig.5. In the presence of three equivalents of pS1368 (Sample E), 93%  
 297 of total UO<sub>2</sub><sup>2+</sup> was involved in UO<sub>2</sub>(pS1368) and 5% was under free form. When the proportion of  
 298 pS1368 decreased compared to that of pS16, only the fraction of free UO<sub>2</sub><sup>2+</sup> increased to reach 42% for  
 299 equimolar conditions 2UO<sub>2</sub><sup>2+</sup>:2pS1368:2pS16 (sample C), while 56% of total UO<sub>2</sub><sup>2+</sup> remained involved  
 300 in UO<sub>2</sub>(pS1368). Even when pS16 was in large excess compared to pS1368 and UO<sub>2</sub><sup>2+</sup> (Sample B), up  
 301 to 91% of total UO<sub>2</sub><sup>2+</sup> was under free form and the remaining fraction was involved in UO<sub>2</sub>(pS1368).  
 302 Since UO<sub>2</sub>(pS16) could not be quantified, its fraction was estimated to be 0.2% by calculating the ratio  
 303 of the peak areas of this complex to that of total UO<sub>2</sub><sup>2+</sup>.

304 Thus, it can be concluded that the addition of three equivalents of pS1368 (Sample E) to UO<sub>2</sub><sup>2+</sup> led to  
 305 the complexation of 93% of the latter, while 10 equivalents of pS16 (Sample A) were not enough to  
 306 complex less than 1% of UO<sub>2</sub><sup>2+</sup>. When these two peptides were present in equimolar proportion with  
 307 respect to UO<sub>2</sub><sup>2+</sup>, half of the total UO<sub>2</sub><sup>2+</sup> was complexed by pS1368 while the remaining half was under  
 308 free form. This results confirms that the tetra-phosphorylated peptide exhibits a higher affinity for UO<sub>2</sub><sup>2+</sup>

309 compared to the di-phosphorylated one, in agreement with the values of the conditional stability  
310 constants previously reported, which allows to validate the method [6].

### 311 *2.2.2. Effect of the position of phosphorylated residues in the peptide backbone*

312 In addition to the major role played by the number of pSer residues to complex  $\text{UO}_2^{2+}$ , the coordination  
313 ability of these residues might be affected by their position in the peptide backbone and their neighboring  
314 amino acids through steric effect [8]. In this part, the impact of the position of pSer residues on  $\text{UO}_2^{2+}$   
315 affinity was evaluated by adding di-phosphorylated peptide isomers pS16 and pS18 in equimolar  
316 proportions to  $\text{UO}_2^{2+}$ , knowing that these two peptides exhibit the same sequence but the pSer are in  
317 different positions in the cyclic peptide scaffold (Fig.1). The formed complexes were separated using  
318 the Acquity BEH amide column, and the chromatograms obtained simultaneously by ESI-MS and ICP-  
319 MS are presented in Fig.3-a.

320 Since these complexes are identical in terms of structure and charge, the amount of free  $\text{UO}_2^{2+}$  potentially  
321 adsorbed on the column was considered to be also identical. Therefore, the quantitative distribution of  
322  $\text{UO}_2^{2+}$  among the two isomeric complexes was determined by calculating the ratio of the peak areas  
323  $A(^{238}\text{U}_{\text{UO}_2(\text{pS18})})/A(^{238}\text{U}_{\text{UO}_2(\text{pS16})})$  from the chromatograms recorded by ICP-MS, for measurements carried  
324 out in triplicate. The average ratio was 1.30 and the RSD was 6.2%, reflecting good repeatability of the  
325 measurements. The ratio close to unity shows that  $\text{UO}_2^{2+}$  did not preferentially coordinate one of the two  
326 isomers, allowing to deduce that the position of the pSer group in the peptide backbone has very weak  
327 influence on the affinity of these peptides towards  $\text{UO}_2^{2+}$ . This result is in agreement with the conditional  
328 stability constants of the two complexes, being  $\log K^{\text{pH}=7.4}(\text{UO}_2(\text{pS18})) = 10.1$  and  $\log K^{\text{pH}=7.4}$   
329  $(\text{UO}_2(\text{pS16})) = 10.3$  [8].

### 330 *2.2.3. Effect of the cyclic/linear structure of the peptide backbone*

331 The structure of the biomimetic peptides plays a key role in the coordination of  $\text{UO}_2^{2+}$ , which is directly  
332 related to the degree of flexibility of the peptide backbone [5]. The pre-organized cyclic peptides  
333 considered in this work were designed specifically to complex  $\text{UO}_2^{2+}$  in its equatorial plane (Fig.1).  
334 Linear peptides of equivalent sequence are more flexible and must therefore adapt their conformation  
335 around  $\text{UO}_2^{2+}$  for an efficient coordination. The structure of the peptide has therefore a direct effect on

336 its affinity for  $\text{UO}_2^{2+}$  [7]. In this part, the method developed by HILIC-ESI-MS/ICP-MS was applied to  
 337 measure online and in a single step the affinity towards  $\text{UO}_2^{2+}$ , of tetra-phosphorylated peptides with the  
 338 same sequence but cyclic (pS1368) and linear (linS1368) structure. Variable proportions of the pS1368  
 339 and linS1368 were added to  $\text{UO}_2^{2+}$ , in a competitive complexation reaction.  
 340 Measured total  $\text{UO}_2^{2+}$  concentration in each sample before separation, targeted and experimental  
 341  $x\text{UO}_2^{2+}:\text{ypS1368}:\text{zlinS1368}$  proportions are summarized in Table 2.

342 Table 2 : Total concentration of  $\text{UO}_2^{2+}$  in each sample  $[\text{UO}_2^{2+}]_{\text{total}}$  measured in duplicate, relative deviation (%)  
 343 of the values, experimental concentration of pS1368 and linS1368 in the samples and experimental  
 344  $x\text{UO}_2^{2+}:\text{ypS1368}:\text{zlinS1368}$  proportions. For simplification, the samples containing different x:y:z proportions  
 345 were assigned by a letter (A-E).

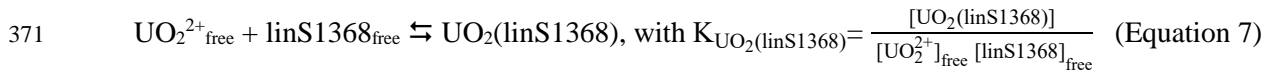
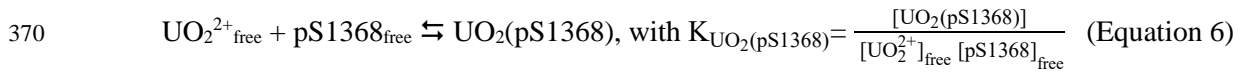
Targeted $x\text{UO}_2^{2+}:\text{ypS1368}:\text{zlinS1368}$	$[\text{UO}_2^{2+}]_{\text{total}}$ $\mu\text{g mL}^{-1}$ ( $\text{mol L}^{-1}$ )		Relative deviation (%)	$[\text{pS1368}]$ $\text{mol L}^{-1}$	$[\text{linS1368}]$ $\text{mol L}^{-1}$	Experimental $x\text{UO}_2^{2+}:\text{ypS1368}:\text{zlinS1368}$ (sample N)
	Value 1	Value 2				
<b>2:4:0</b>	28.2 ( $1.2 \times 10^{-4}$ )	28.4 ( $1.2 \times 10^{-4}$ )	0.7	$4.5 \times 10^{-4}$	0	2:7.5:0 ( <b>A</b> )
<b>2:3:1</b>	27.5 ( $1.2 \times 10^{-4}$ )	27.6 ( $1.2 \times 10^{-4}$ )	0.3	$1.9 \times 10^{-4}$	$6.1 \times 10^{-5}$	2:3.2:1 ( <b>B</b> )
<b>2:2:2</b>	33.9 ( $1.4 \times 10^{-4}$ )	34.8 ( $1.5 \times 10^{-4}$ )	2.7	$1.4 \times 10^{-4}$	$1.4 \times 10^{-4}$	2:2:1.9 ( <b>C</b> )
<b>2:0.5:3.5</b>	24.0 ( $1.0 \times 10^{-4}$ )	23.9 ( $1.0 \times 10^{-4}$ )	0.6	$3.7 \times 10^{-5}$	$2.4 \times 10^{-4}$	2:0.7:4.6 ( <b>D</b> )
<b>2:0:4</b>	28.7 ( $1.2 \times 10^{-4}$ )	28.1 ( $1.2 \times 10^{-4}$ )	2.1	0	$5.6 \times 10^{-4}$	2:0:9 ( <b>E</b> )

346 As reported in Table 2, the relative deviation between the values obtained for each replicate was between  
 347 0.3 and 2.7%, showing good repeatability of the measurements. The chromatograms simultaneously  
 348 recorded by ESI-MS and ICP-MS, using the Aquity BEH HILIC column to separate the complexes, are  
 349 shown in Fig.6.

350 As shown in Fig.6, the peak of free pS1368 in the chromatograms recorded by ESI-MS was observed  
 351 only when it was in excess relatively to  $\text{UO}_2^{2+}$  (samples A and B), whilst the one of linS1368 was  
 352 detected for all ratios. Furthermore, the peak corresponding to  $\text{UO}_2(\text{pS1368})$  was observed by ESI-MS  
 353 and ICP-MS for all proportions whilst the one of  $\text{UO}_2(\text{linS1368})$  was detected exclusively when  
 354 linS1368 was in large excess compared to  $\text{UO}_2^{2+}$ . All these observations indicate that pS1368 is fully  
 355 bound to  $\text{UO}_2^{2+}$  in equimolar proportion or less, while linS1368 is weakly engaged in the complexation  
 356 even when it was added in large excess. The quantitative distribution of total  $\text{UO}_2^{2+}$  among the different  
 357 complexes and the mass balance, expressed in percent (%) could be calculated using Equation 4 and 5,  
 358 respectively and are presented in the diagram of Fig.7. When  $\text{UO}_2^{2+}$  was in the presence of an excess of  
 359 pS1368, 97% of total  $\text{UO}_2^{2+}$  were involved in the complexation while the remaining fraction was under  
 360 free form (Sample A). When the proportion of pS1368 decreased in favor of that of its linear equivalent,



361 the fraction of free  $\text{UO}_2^{2+}$  increased significantly, while the fraction of  $\text{UO}_2^{2+}$  engaged in  $\text{UO}_2(\text{linS1368})$   
 362 slightly increased (Sample B-D). Finally, excess of linS1368 peptide led to the involvement of only 12%  
 363 of the total  $\text{UO}_2^{2+}$  in the corresponding complex, while 77% was in the free form (Sample E).  
 364 All of these results show that  $\text{UO}_2^{2+}$  coordinates preferentially the cyclic peptide in comparison with the  
 365 linear one. The cyclic structure of the peptide confers a higher stability to the  $\text{UO}_2^{2+}$  complexes than the  
 366 linear structure, through the more efficient coordination conferred by the preorientation of the four  
 367 phosphate groups in the equatorial plane of  $\text{UO}_2^{2+}$  [33]. Owing to our approach, we were able to  
 368 determine the affinity constant of  $\text{UO}_2(\text{linS1368})$ . The conditional stability constants of  $\text{UO}_2(\text{pS1368})$   
 369 and  $\text{UO}_2(\text{linS1368})$  are expressed according to the equilibria shown in equations 6 and 7.



372 From equations 6 and 7, the logarithmic expression of  $\frac{K_{\text{UO}_2(\text{pS1368})}}{K_{\text{UO}_2(\text{linS1368})}}$  is:

373 
$$\log \frac{K_{\text{UO}_2(\text{pS1368})}}{K_{\text{UO}_2(\text{linS1368})}} = \log K_{\text{UO}_2(\text{pS1368})} - \log K_{\text{UO}_2(\text{linS1368})} = \log \frac{[\text{linS1368}]_{\text{free}}}{[\text{pS1368}]_{\text{free}}} \quad (\text{Equation 8})$$

374 
$$\log K^{\text{pH}7.4}(\text{UO}_2(\text{linS1368})) = \log K^{\text{pH}7.4}(\text{UO}_2(\text{pS1368})) - \log \frac{[\text{linS1368}]_{\text{free}}}{[\text{pS1368}]_{\text{free}}} \quad (\text{Equation 9})$$

375 The intersection point of the distribution curves of  $\text{UO}_2(\text{pS1368})$  and  $\text{UO}_2(\text{linS1368})$  fractions (Fig.7)  
 376 corresponds to an equal percentage of both complexes. allowing to deduce their concentration:  
 377  $[\text{UO}_2(\text{pS1368})] = [\text{UO}_2(\text{linS1368})] = 7\%$  of  $[\text{UO}_2^{2+}]_{\text{total}} = 7.11 \pm 0.12 \times 10^{-6} \text{ mol L}^{-1}$ , knowing that  
 378  $[\text{UO}_2^{2+}]_{\text{total}} = 10^{-4} \text{ mol L}^{-1}$ .

379 The concentration of linS1368 was deduced from the intersection point Fig.7 and was  $3.59 \pm 0.06 \times 10^{-4}$   
 380  $\text{mol L}^{-1}$ , while that of pS1368 was estimated at  $2.34 \pm 0.08 \times 10^{-5} \text{ mol L}^{-1}$  by plotting the same curves,  
 381 but as a function of the pS1368 concentrations. Therefore, a concentration of the linear peptide 15 times  
 382 higher than that of the cyclic peptide was needed to obtain an equal distribution of  $\text{UO}_2^{2+}$  between the

383 linear and the cyclic peptide complexes. The total concentrations of the peptides are expressed according  
384 to equations 10 and 11:

385 
$$[\text{linS1368}]_{\text{Total}} = [\text{UO}_2(\text{linS1368})] + [\text{linS1368}]_{\text{free}} = 3.59 \pm 0.06 \times 10^{-4} \text{ mol L}^{-1} \text{ (Equation 10)}$$

386 
$$[\text{pS1368}]_{\text{Total}} = [\text{UO}_2(\text{pS1368})] + [\text{pS1368}]_{\text{free}} = 2.34 \pm 0.08 \times 10^{-5} \text{ (Equation 11)}$$

387 Taking into account the concentrations of the different species calculated above, as well as the value of  
388  $\log K^{\text{pH}7.4}(\text{UO}_2(\text{pS1368})) = 11.3$  [6], the equation 8 becomes:

389 
$$\log K^{\text{pH}7.4}(\text{UO}_2(\text{linS1368})) = 11.3 - \log(21.64 \pm 1.63) = 9.97 \pm 0.03 \text{ (Equation 12)}$$

390 The constant determined through our methodology allows to deduce that the affinity of the linear tetra-  
391 phosphorylated peptide for  $\text{UO}_2^{2+}$  is more than one order of magnitude lower than its cyclic counterpart  
392 at pH 7.4. This result confirms the impact of the peptide structure on the affinity for  $\text{UO}_2^{2+}$ , that is in  
393 agreement with the behavior also observed for proteins. For example, the structure of OPN is not defined  
394 and upon complexation with  $\text{UO}_2^{2+}$ , its secondary structure undergoes modifications to effectively  
395 position 4 pSer residues in the  $\text{UO}_2^{2+}$  equatorial plane [6]. Thus, a structure fitting the coordination of  
396  $\text{UO}_2^{2+}$  is necessary at the scale of an entire protein as well as at the scale of a small peptide to form  
397  $\text{UO}_2^{2+}$  complexes of high stability.

## 398 **Conclusion**

399 In this work, a dedicated analytical approach was developed to separate  $\text{UO}_2^{2+}$  complexes formed with  
400 multi-phosphorylated biomimetic peptides, to characterize, and quantify them online and in a single  
401 step. To reach this aim, we implemented the simultaneous coupling of HILIC to ESI-MS and ICP-MS.  
402 Separations of the complexes containing positional isomers, peptides with two and four pSer residues  
403 as well as cyclic and linear tetra-phosphorylated peptides of the same sequence, were successfully  
404 achieved using columns with amide, diol and hybrid silica stationary phases. We developed the first  
405 separations of  $\text{UO}_2^{2+}$  complexes by HILIC ever described in the literature for complexes with conditional  
406 stability constants of  $10^{10}$  or higher at pH 7.4. Owing to the dedicated method that we developed,  
407 quantify we were able to determine on line the quantitative distribution of  $\text{UO}_2^{2+}$  among the separated  
408 complexes. Thus, the effect of the structure of the peptides on their affinity towards  $\text{UO}_2^{2+}$  was  
409 determined and we showed that (i) the cyclic tetra-phosphorylated peptide pS1368 has the highest

410 affinity for  $\text{UO}_2^{2+}$  compared to peptides with a lower degree of phosphorylation, highlighting the high  
411 affinity of  $\text{UO}_2^{2+}$  for phosphate-rich binding sites in proteins. (ii) the pSer position in the peptide  
412 sequence had no significant impact on the affinity for  $\text{UO}_2^{2+}$ , in agreement with the literature results  
413 obtained by multiple spectroscopic techniques independently for each sample (iii) the cyclic structure  
414 of the tetra-phosphorylated peptide favors the  $\text{UO}_2^{2+}$  complexation compared to its linear analogue. In  
415 addition, we determined the affinity constant of the linear tetra-phosphorylated peptide, being  
416  $(\log K^{\text{pH}7.4}(\text{UO}_2(\text{linS1368})) = 9.97 \pm 0.03$ .

417 Overall, this work highlights the powerful simultaneous coupling of HILIC to ESIMS and ICPMS to  
418 determine in a single step the effect of several structural parameters of biomimetic peptides on their  
419 affinity towards  $\text{UO}_2^{2+}$  when they are in a competing complexation reaction. A quick and reliable  
420 determination of stability of the  $\text{UO}_2(\text{peptides})$  complexes is of prime importance to access deeper  
421 fundamental information on  $\text{UO}_2^{2+}$  toxicity but also to be able to develop *in vivo* decorporating agents  
422 based on chelation. This approach can be extended to the evaluation of the affinity of these biomimetic  
423 peptides for other actinides (Pu, Am, Cm...). Moreover, it can be implemented for the screening of the  
424 complexation properties of various classes of chelating molecules towards elements of interest in the  
425 fields of energy, toxicology and the environment

426

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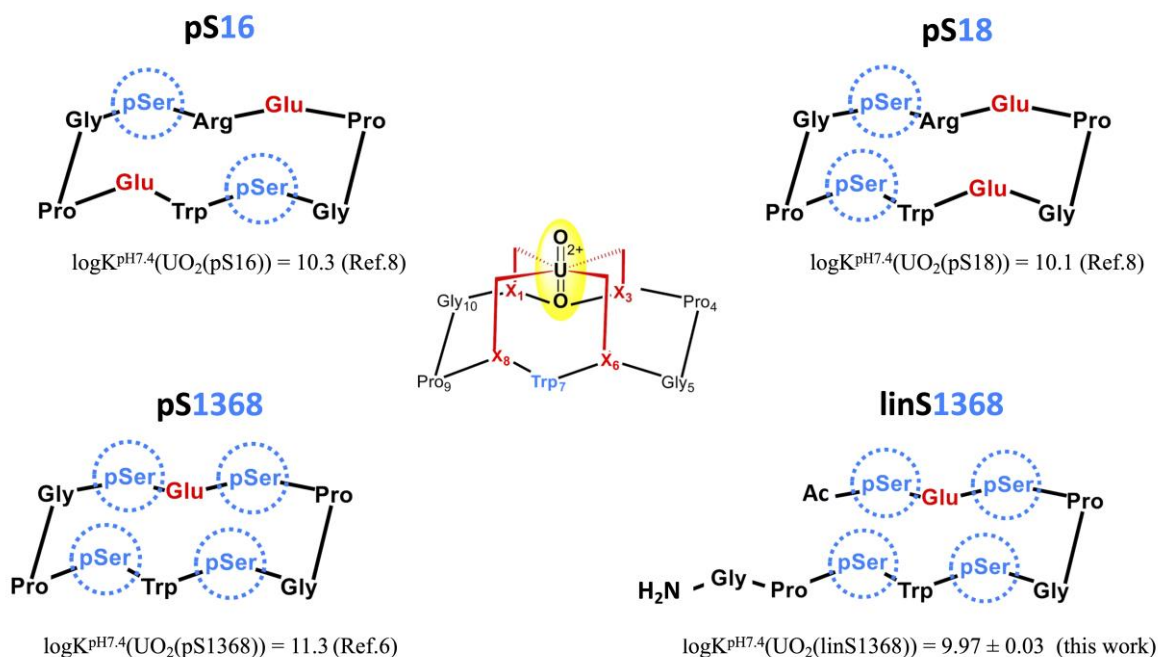
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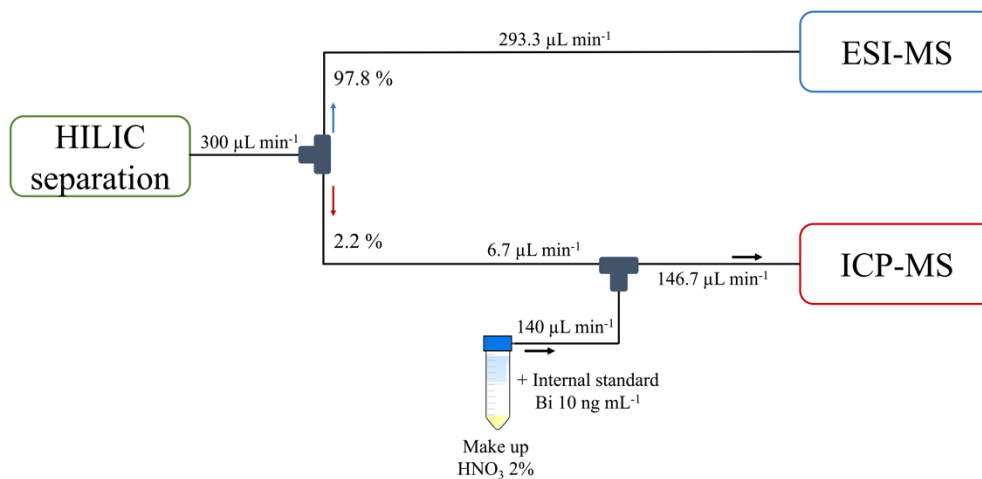
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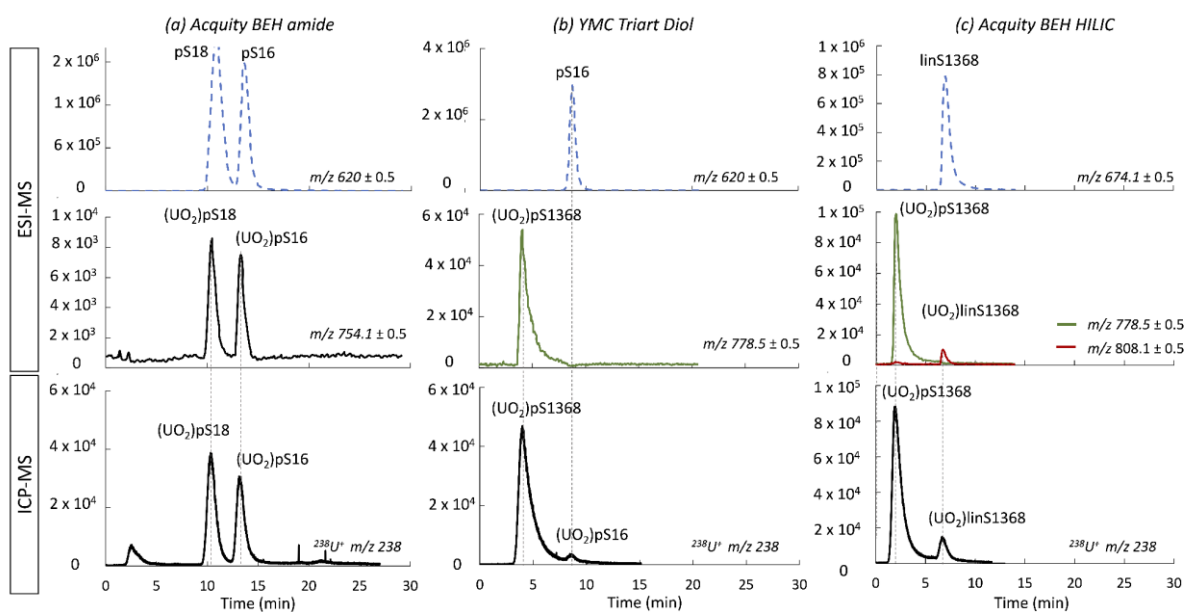
438  
 439 Fig.1: Multi-phosphorylated peptides considered in this work. Peptides were named according to their cyclic/linear structure  
 440 and to the number and position of the pSer residues. Di-phosphorylated peptides pS18 and pS16, contain two pSer residues in  
 441 trans 1,6 and cis 1,8 positions respectively. Tetra-phosphorylated pS1368 and linS1368 peptides contain four pSer residues in  
 442 1,3,6 and 8 position but have cyclic and linear structure.

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 445 Fig.2: Schematic representation of the simultaneous coupling of HILIC to ESI-MS and ICP-MS according to [11].

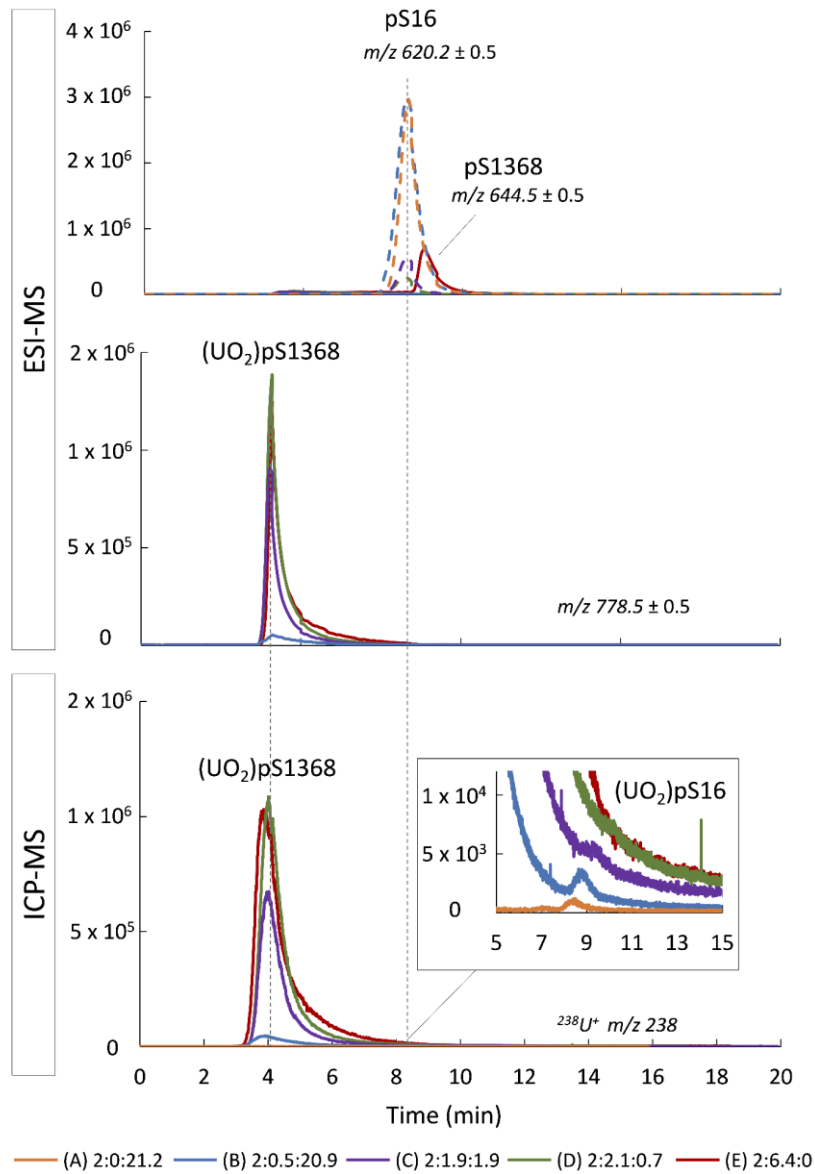
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448 Fig.3: Chromatograms simultaneously acquired by ESI-MS using SIM mode for targeted monitoring of  $m/z$  ratios of free  
 449 peptides and  $\text{UO}_2$ (peptide) complexes and by ICP-MS recording the  $^{238}\text{U}$  signal with an integration time of 90 ms. (a)  
 450 Sample:  $2\text{UO}_2^{2+}$ :10pS16:10pS18. Column: Acquity BEH amide 100 x 2.1 mm; 1.7 $\mu\text{m}$ . Mobile phase: 70/30 ACN/ $\text{H}_2\text{O}$  v/v  
 451 with 20 mmol  $\text{L}^{-1}$   $\text{NH}_4\text{CH}_3\text{CO}_2$ . (b) Sample:  $2\text{UO}_2^{2+}$ :0.5pS1368:20pS16. Column: YMC Triart Diol 100 x 2,1 mm; 1,9  $\mu\text{m}$ .  
 452 Mobile phase: 72/28 ACN/ $\text{H}_2\text{O}$  v/v with 20 mmol  $\text{L}^{-1}$   $\text{NH}_4\text{CH}_3\text{CO}_2$ . (c) Sample:  $2\text{UO}_2^{2+}$ :0.5pS1368:3.5linS1368. Column:  
 453 Acquity BEH HILIC 100 x 2,1; 1,7  $\mu\text{m}$ . Mobile phase: 68/32 ACN/ $\text{H}_2\text{O}$  v/v with 20 mmol  $\text{L}^{-1}$   $\text{NH}_4\text{CH}_3\text{CO}_2$ . In all cases, the  
 454 flow rate was 300  $\mu\text{L min}^{-1}$ , the elution mode isocratic and  $V_{\text{inj}} = 3 \mu\text{L}$ .

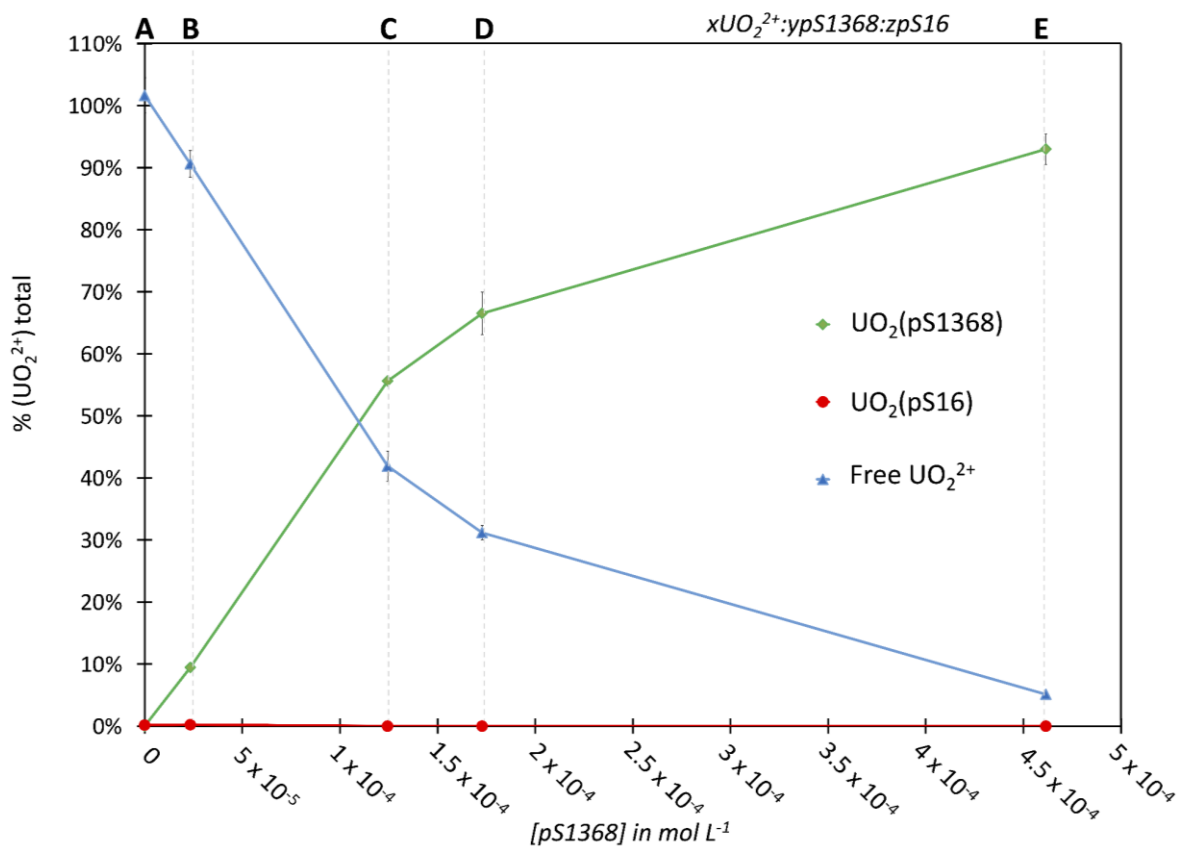
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458 Fig.4: Chromatograms simultaneously acquired by ESI-MS using SIM mode for targeted monitoring of  $m/z$  ratios of free  
 459 peptides and  $\text{UO}_2(\text{peptide})$  complexes and by ICP-MS recording the  $^{238}\text{U}$  signal with an integration time of 90 ms. Column:  
 460 YMC Triart Diol 100 x 2.1 mm; 1.9  $\mu\text{m}$ . Mobile phase: 72/28 ACN/ $\text{H}_2\text{O}$  v/v with 20  $\text{mmol L}^{-1}$   $\text{NH}_4\text{CH}_3\text{CO}_2$ . Flow rate was  
 461 300  $\mu\text{L min}^{-1}$ , the elution mode isocratic and  $V_{\text{inj}} = 3 \mu\text{L}$ .

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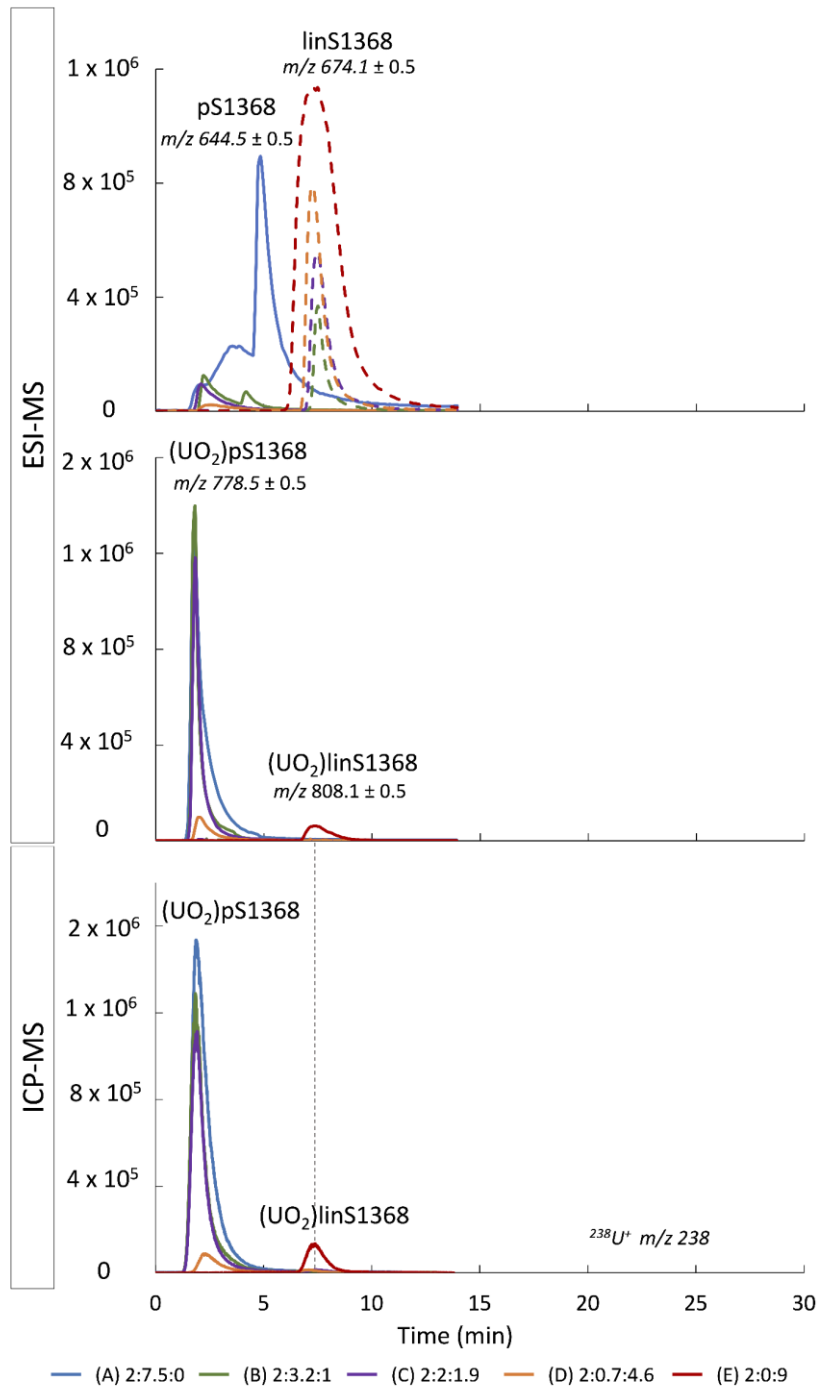
465 Fig.5 : Quantitative distribution of total  $\text{UO}_2^{2+}$  expressed in percentage (%) among the different complexes as a function of

466 pS1368 concentration and  $x\text{UO}_2^{2+}:\text{ypS1368}:\text{zpS16}$  proportions in the samples (A) 2:0:21.2, (B) 2:0.5:20.9, (C) 2:1.9:1.9, (D)

467 2:2.1:0.7 and (E) 2:6.4:0 with average mass balance of  $101.8 \pm 3\%$ ,  $100.3 \pm 2\%$ ,  $97.5 \pm 2\%$ ,  $97.7 \pm 5\%$ ,  $98.1 \pm 3\%$ , respectively. The

468 dots correspond to the average of the values of two replicates and the error bars represent their standard deviation.

469



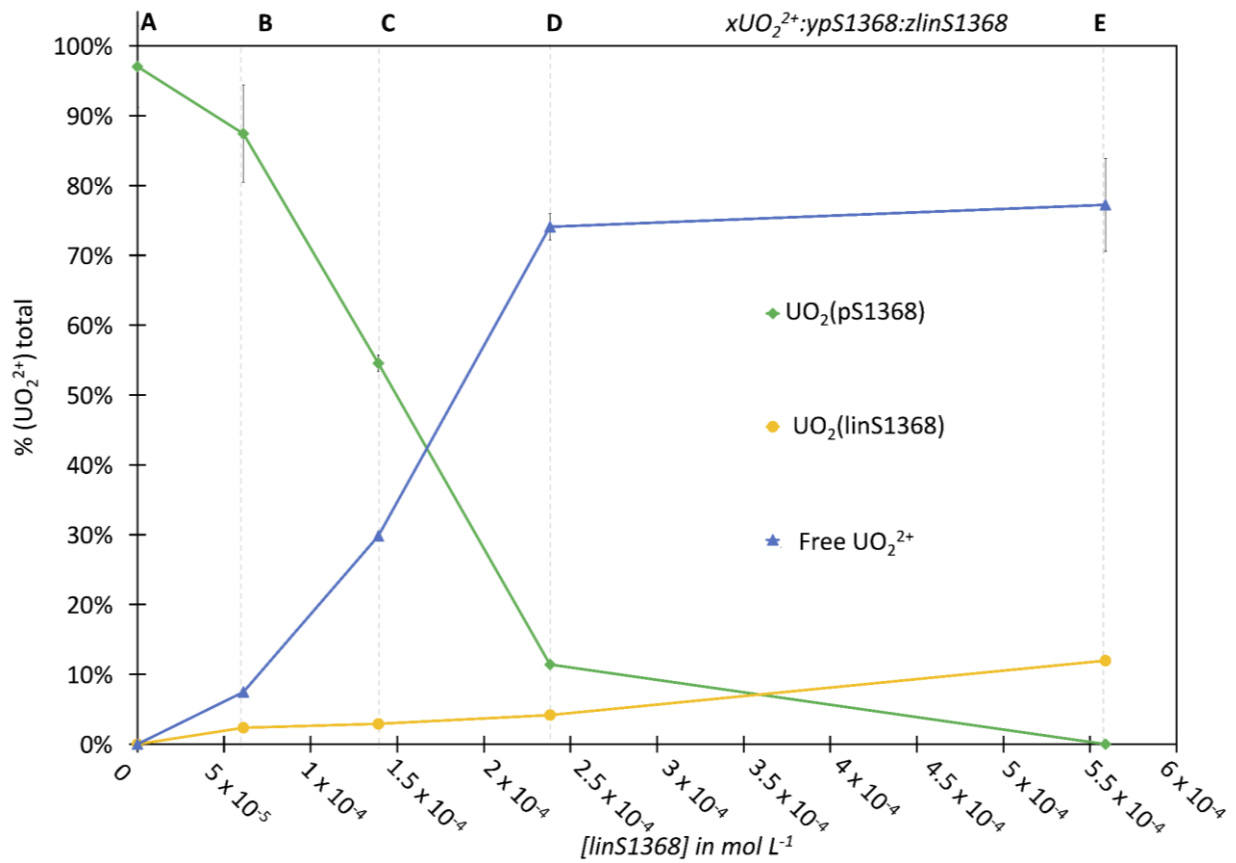
471

472 Fig.6: Chromatograms simultaneously acquired by ESI-MS using SIM mode for targeted monitoring of  $m/z$  ratios of free  
 473 peptides and  $UO_2$  complexes and by ICP-MS recording the  $^{238}U$  signal with an integration time of 90 ms. Column: Acquity  
 474 BEH HILIC 100 x 2,1 mm; 1,7  $\mu$ m. Mobile phase: 68/32 ACN/ $H_2O$  v/v with 20 mmol  $L^{-1}$   $NH_4CH_3CO_2$ . Flow rate was 300  
 475  $\mu$ L  $min^{-1}$ , the elution mode isocratic and  $V_{inj} = 3 \mu$ L.

476



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479 Fig.7 : Quantitative distribution of total UO<sub>2</sub><sup>2+</sup> expressed in percentage (%) among the different complexes as a function of  
 480 linS1368 concentration and the xUO<sub>2</sub><sup>2+</sup>:ypS1368:zlinS1368 proportions in the samples (A) 2-7,5-0, (B) 2-3,2-1, (C) 2-2-1,9,  
 481 (D) 2-0,7-4,6 and (E) 2-0-9 with average mass balance of 97.0±6%, 97.3±7%, 87.3±1%, 89.7±1% and 89.2±6%, respectively.

482 The dots correspond to the average of the values of two replicates and the error bars represent their standard deviation.

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486

## 487 **Figure captions**

488 Fig.1: Multi-phosphorylated peptides considered in this work. Peptides were named according to their  
489 cyclic/linear structure and to the number and position of the pSer residues. Di-phosphorylated peptides  
490 pS18 and pS16, contain two pSer residues in trans 1,6 and cis 1,8 positions respectively. Tetra-  
491 phosphorylated pS1368 and linS1368 peptides contain four pSer residues in 1,3,6 and 8 position but  
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499 an integration time of 90 ms. (a) Sample:  $2\text{UO}_2^{2+}:10\text{pS16}:10\text{pS18}$ . Column: Acquity BEH amide 100 x  
500 2.1 mm; 1.7 $\mu\text{m}$ . Mobile phase: 70/30 ACN/ $\text{H}_2\text{O}$  v/v with 20 mmol  $\text{L}^{-1}$   $\text{NH}_4\text{CH}_3\text{CO}_2$ . (b) Sample:  
501  $2\text{UO}_2^{2+}:0.5\text{pS1368}:20\text{pS16}$ . Column: YMC Triart Diol 100 x 2,1 mm; 1,9  $\mu\text{m}$ . Mobile phase: 72/28  
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503 Acquity BEH HILIC 100 x 2,1; 1,7  $\mu\text{m}$ . Mobile phase: 68/32 ACN/ $\text{H}_2\text{O}$  v/v with 20 mmol  $\text{L}^{-1}$   
504  $\text{NH}_4\text{CH}_3\text{CO}_2$ . In all cases, the flow rate was 300  $\mu\text{L min}^{-1}$ , the elution mode isocratic and  $V_{\text{inj}} = 3 \mu\text{L}$ .

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509 ACN/ $\text{H}_2\text{O}$  v/v with 20 mmol  $\text{L}^{-1}$   $\text{NH}_4\text{CH}_3\text{CO}_2$ . Flow rate was 300  $\mu\text{L min}^{-1}$ , the elution mode isocratic  
510 and  $V_{\text{inj}} = 3 \mu\text{L}$ .

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512 Fig.5 : Quantitative distribution of total  $\text{UO}_2^{2+}$  expressed in percentage (%) among the different  
513 complexes as a function of pS1368 concentration and  $x\text{UO}_2^{2+}:y\text{pS1368}:z\text{pS16}$  proportions in the  
514 samples (A) 2:0:21.2, (B) 2:0.5:20.9, (C) 2:1.9:1.9, (D) 2:2.1:0.7 and (E) 2:6.4:0 with average mass

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518 Fig.6: Chromatograms simultaneously acquired by ESI-MS using SIM mode for targeted monitoring of  
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520 integration time of 90 ms. Column: Acquity BEH HILIC 100 x 2,1 mm; 1,7  $\mu\text{m}$ . Mobile phase: 68/32  
521 ACN/ $\text{H}_2\text{O}$  v/v with 20 mmol  $\text{L}^{-1}$   $\text{NH}_4\text{CH}_3\text{CO}_2$ . Flow rate was 300  $\mu\text{L min}^{-1}$ , the elution mode isocratic  
522 and  $V_{\text{inj}} = 3 \mu\text{L}$ .

523  
524 Fig.7 : Quantitative distribution of total  $\text{UO}_2^{2+}$  expressed in percentage (%) among the different  
525 complexes as a function of linS1368 concentration and the  $x\text{UO}_2^{2+}:\text{ypS1368}:\text{zlinS1368}$  proportions in  
526 the samples (A) 2-7,5-0, (B) 2-3,2-1, (C) 2-2-1,9, (D) 2-0,7-4,6 and (E) 2-0-9 with average mass balance  
527 of  $97.0\pm 6\%$ ,  $97.3\pm 7\%$ ,  $87.3\pm 1\%$ ,  $89.7\pm 1\%$  and  $89.2\pm 6\%$ , respectively. The dots correspond to the  
528 average of the values of two replicates and the error bars represent their standard deviation.

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