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# Accepted Manuscript

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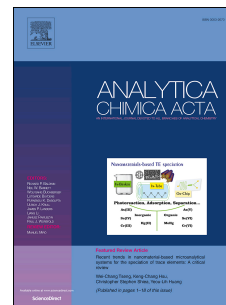
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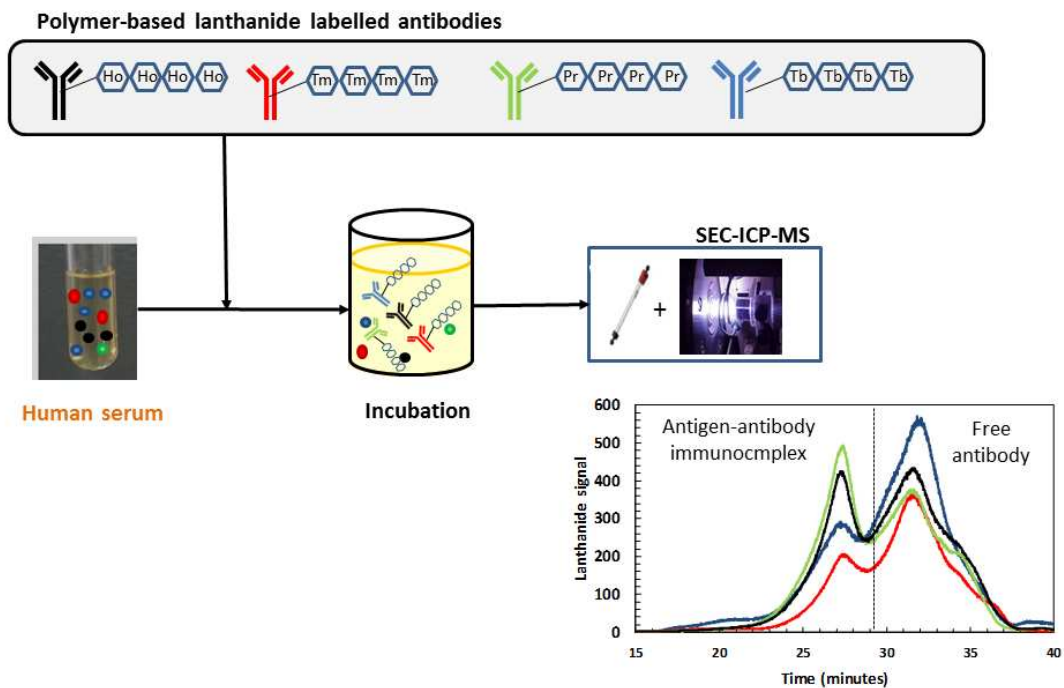
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ACCEPTED MANUSCRIPT

1 **Lanthanide polymer labels for multiplexed determination of biomarkers in**  
2 **human serum samples by means of size exclusion chromatography-**  
3 **inductively coupled plasma mass spectrometry**

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12

13 **Abstract**

14 Lanthanide polymer-labelled antibodies were investigated to improve the  
15 analytical figures of merit of homogeneous immunoassays with inductively  
16 coupled plasma mass spectrometry (ICP-MS) detection for multiplexed  
17 biomarker analysis in human serum samples. Specific monoclonal antibodies  
18 against four cancer biomarkers (CEA, sErbB2, CA 15.3 and CA 125) were  
19 labelled with different polymer-based lanthanide group to increase the number  
20 of metal labels per binding site. After the immunoreaction of the biomarkers with  
21 the specific antibodies, antigen-antibody complexes were separated by size-  
22 exclusion chromatography followed by ICP-MS detection. The polymer label  
23 could be loaded with 30-times more atoms of the lanthanide than the lanthanide-  
24 DOTA complex traditionally used for this purpose elsewhere [1] which resulted  
25 in a 10-fold improvement in both sensitivity and detection limits. Analytical

26 figures of merit obtained with the lanthanide polymer labelling strategy make the  
27 detection of the biomarkers feasible below the threshold reference values used  
28 in clinical analysis. This labelling method was successfully validated by  
29 analyzing a control human serum spiked with the four biomarkers at three  
30 different concentration levels. For all the biomarkers studied, the recovery  
31 values ranged from 95% to 110% whereas inter-assay and intra-assay precision  
32 were lower than 8%. Results obtained with this approach were equivalent to  
33 those obtained by heterogenous-based immunoassays based on the detection  
34 by electro-chemiluminescence or ELISA. However, the method developed  
35 offers better analytical figures of merit using a smaller amount of sample.

36

37 **Keywords:** polymer-based lanthanide-labelled antibody, multiplexed  
38 homogeneous-based immunoassay, biomarker, size exclusion chromatography,  
39 inductively coupled plasma mass spectrometry.

40

## 41 1. Introduction

42 Recently, a number of immunoassays based on the use of metal-labelled  
43 antibodies and the determination of antibody-antigen complexes by inductively  
44 coupled plasma mass spectrometry (ICP-MS) have been proposed for the  
45 determination of biomolecules, and, in particular, proteins [2,3]. The ICP-MS  
46 quantification offers several advantages over the conventional detection  
47 techniques employed in immunoassays (colorimetry, fluorimetry, etc.), such as,  
48 e.g., (i) specificity to heteroatom detection; (ii) compound-independent detection  
49 sensitivity; (iii) high elemental sensitivity and dynamic range; (iv) limited sample  
50 treatment; (v) stability of the reagents against time, temperature and light (the

51 isotopic masses do not change, bleach or degrade); (vi) reduction of non-  
52 specific background; (vii) independence of analytical response from incubation  
53 or storage times and (viii) multiplexed detection [3,4].

54 In general, the immunoassay procedures employed with ICP-MS  
55 detection has been carried out in heterogenous phase in different type of  
56 formats (e.g. sandwich-based, Western blot, etc.). Antibodies are usually  
57 labelled by either metal nanoparticles [5,6] or lanthanides [7,8]. The advantage  
58 of elemental nanoparticles is the possibility of the introduction of a significant  
59 number of atoms per conjugate which allows the amplification of the analytical  
60 response. This advantage is set off by the high affinity of nanoparticles to  
61 surface of labware and/or ICP-MS sample introduction system, increasing  
62 wash-in and wash-out times, and by the difficulty to synthesize nanoparticles of  
63 uniform size. Lanthanides are introduced as DOTA or DTPA chelates due to its  
64 extraordinary thermodynamic stability [7]. The similar chemical properties make  
65 lanthanides well suited for multiplex assays: different antibodies can be easily  
66 and specifically labelled with different lanthanides in the same experimental  
67 workflow.

68 Despite higher simplicity, homogeneous immunoassays with ICP-MS  
69 detection have been scarcely investigated in the literature in comparison with  
70 their heterogenous counterparts. Terenghi et al. [9] showed that a mixture of  
71 antibodies, each labelled with a different lanthanide, could react with different  
72 biomarkers in liquid samples and the antigen-antibody (Ag-Ab) complexes  
73 formed could be isolated by size-exclusion chromatography (SEC) and  
74 specifically determined by ICP-MS. The main benefits of this approach were: (i)  
75 multiplexed capability; (ii) small sample amount consumption; and (iii) virtually

76 no sample preparation. However, signal amplification was limited since only a  
77 single lanthanide atom was introduced per binding site of the antibody. Several  
78 authors demonstrated that the number of lanthanide atoms per antibody can be  
79 increased by using metal-loaded polymers [10-14], which leads to an increase  
80 in sensitivity. This labelling strategy was successfully employed for single-cell  
81 ICP-MS analysis [11-13]. Waentig et al. [14] compared polymer-based  
82 lanthanide labelling with other lanthanide-based labelling strategies for protein  
83 quantification in solid phase immunoassays (Western Blot, SDS-PAGE, etc.).  
84 These authors noted that this labelling strategy improves significantly sensitivity  
85 which result in limits of detection in the low fmol range. However, there has  
86 been no attempt so far to investigate the potential of antibodies conjugated with  
87 metal-labelled polymers in homogenous assays.

88 The aim of this work was to evaluate lanthanide polymer labels for  
89 multiplexed biomarker analysis by the use of homogeneous immunoassay in  
90 which Ag-Ab complex, free metal-labelled antibody and free metal are  
91 separated by SEC and quantified with ICP-MS. To this end, monoclonal  
92 antibodies against four biomarkers (CEA, sErbB2, CA 15.3 and CA 125) usually  
93 present in human serum samples were labelled with a different polymer-based  
94 lanthanide moiety. Size-exclusion chromatography was used to isolate the Ag-  
95 Ab complexes whereas ICP-MS on-line detection was used for quantification.  
96 The method was benchmarked against those at using the labelling with DOTA-  
97 chelates [9].

98

## 99 **2. Experimental**

### 100 *2.1. Regents and materials*

101 Carcinoembryonic antigen (CEA) was obtained from Sigma-Aldrich (St.  
102 Quentin-Fallavier, France). The soluble form of human epidermal growth factor  
103 receptor 2 (sErbB2) was purchased from antibodies-online (Aachen, Germany).  
104 Cancer antigen 15.3 (CA 15.3) was obtained from MyBioSource (San Diego,  
105 CA) and CA 125 was from Fitzgerald (MA). Goat polyclonal antimouse  
106 immunoglobulin (IgG) antibody (H&L) was purchased from Abcam (Cambridge,  
107 UK).

108 Mouse IgG subclass 1 (IgG<sub>1</sub>) antihuman monoclonal antibody (mAb) for  
109  $\alpha$ -CEA (clone 1C11) and mouse IgG<sub>1</sub> antihuman mAb for  $\alpha$ -CA 125 (clone  
110 X325) were purchased from Gene Tex (Irvine, CA). Mouse IgG<sub>1</sub> antihuman  
111 mAb for  $\alpha$ -sErbB2 (clone 5J297) was obtained from antibodies-online (Aachen,  
112 Germany) and mouse IgG<sub>1</sub> antihuman mAb for  $\alpha$ -CA 15.3 (clone M002204) was  
113 from LifeSpan BioSciences (Seattle, WA). The antibody solutions should not  
114 contain additives, such as bovine serum albumin (BSA) or gelatin, because the  
115 latter could be labelled as well and cause interferences. Upon reception, mAb  
116 were divided into single working aliquots and stored at -20°C.

117 MAXPAR<sup>TM</sup>- polymer -Ab labelling kits were obtained from Fluidigm (Les  
118 Ulis, France). Human Albumin Albutein<sup>TM</sup> 20% was purchased from Grifols  
119 Biologicals Inc. (Los Angeles, CA). 1,4,7,10 – Tetraazacyclododecane - 1,4,7 -  
120 tris(aceticacid) - 10-maleimido - ethylacetamide (DOTA) was obtained from  
121 Macrocyclics (Dallas, TX). Tris (2-carboxyethyl) - phosphine hydrochloride  
122 (TCEP), Trizma<sup>TM</sup> base, lanthanide chlorides (HoCl<sub>3</sub>, TbCl<sub>3</sub>, TmCl<sub>3</sub>, PrCl<sub>3</sub>) with  
123 natural isotopic abundance, ammonium acetate ( $\geq$  98%, for molecular biology),  
124 monosodium phosphate, disodium phosphate, ethylenediaminetetraacetic acid  
125 disodium salt (EDTA), dimethyl sulfoxide (DMSO), sodium chloride and



126 polyethylene glycol sorbitan monolaurate (Tween 20) were from Aldrich  
127 (Schelldorf, Germany). Acetic acid glacial and 69% w w<sup>-1</sup> nitric acid were  
128 purchased from Panreac (Barcelona, Spain). Rare earth 100 µg mL<sup>-1</sup> Complete  
129 Standard was provided by Inorganic Ventures (Lakewood, Colorado) and DC<sup>TM</sup>  
130 *Protein Assay Kit* was from Bio-Rad (Marnes-la-Coquette, France).

131       Ultrapure water 18 MΩ cm from a Milli-Q water purification system  
132 (Millipore, Paris, France) was used throughout the work.

133       Amicon<sup>TM</sup> Ultra-0.5 mL centrifugal filters for DNA and protein purification  
134 and concentration (Merck Millipore, Cork, Ireland) with different cutoff limits (3,  
135 30 and 50 kDa) were used throughout the work for washing steps and buffers  
136 exchange during labelling procedure of Abs using a Eppendorf<sup>TM</sup> microcentrifuge  
137 5415R (Eppendorf AG, Hamburg, Germany).

138

## 139 2.2. *Buffers*

140       The buffers used were: (a) ammonium acetate buffer (100 mM, pH 6.8 as  
141 elution buffer and 20 mM, pH 6.0 for metal complexation), (b) phosphate buffer  
142 (100 mM, pH 7.2, 2.5 mM EDTA) for the partial reduction of the antibody with  
143 TCEP and (c) Tris buffer saline (20 mM Tris-HCl, 0,45% NaCl, pH 7.0 for  
144 antibody storage medium and 20 mM Tris-HCl, 0,45% NaCl, 10 mM EDTA, pH  
145 7.0 for removing TCEP).

146

## 147 2.3. *Serum samples*

148       Serum samples were provided from Hospital General Universitario of  
149 Alicante (Alicante, Spain).

150

## 151 2.4. Instrumentation

## 152 2.4.1. Size Exclusion Chromatography

153 The chromatographic analyses were performed on an Agilent 1200  
154 series (Agilent Technologies, Santa Clara, CA) equipped with an autosampler.  
155 Separations were carried out isocratically at 0.5 mL min<sup>-1</sup> using 100 mM  
156 ammonium acetate (pH 6.8) as mobile phase and sample injection volume of  
157 100 µL. Two size exclusion columns of different separation range (GE  
158 Healthcare, Buckinghamshire, UK) were tested: a Superose 6 Increase 10/300  
159 GL (cross-linked agarose composite stationary phase; 10 mm x 300 mm x 8.6  
160 µm average beads size) with the approximate bed volume of 24 mL and an  
161 optimum separation range of 5-5000 kDa for globular proteins and a Superdex  
162 200 HR 10/300 (cross-linked agarose and dextran composite stationary phase;  
163 10 mm x 300 mm x 8.6 µm average beads size) with the approximate bed  
164 volume of 24 mL and an optimum separation range of 10-600 kDa for globular  
165 proteins. The performance of the size exclusion column Superose 6 Increase  
166 10/300 GL was verified with a mixture of blue dextran (Mr 2000 kDa),  
167 thyroglobulin (Mr 669 kDa), ferritin (Mr 440 kDa), aldolase (Mr 158 kDa),  
168 ovalbumin (Mr 44 kDa), ribonuclease A (Mr 13.7 kDa) and ubiquitin from bovine  
169 (Mr 8.6 kDa) using UV-VIS detection at 280 nm with baseline evaluation at 800  
170 nm. Retention times (in minutes) plotted versus the logarithm of molecular mass  
171 (in kDa) did not give a straight line (two straight lines were obtained:  $y = -0.049x$   
172  $+4.104$ ;  $r^2 = 1$  for proteins which  $Mr \geq 440$  kDa and  $y = -0.170x + 7.754$ ;  $r^2 =$   
173  $0.998$  for proteins which  $Mr \leq 440$  kDa). Concerning the size exclusion column  
174 Superdex 200 HR 10/300, it was calibrated similarly to the Superose 6 Increase  
175 10/300 GL but without using blue dextran. Retention times (in minutes) plotted

176 versus the logarithm of molecular mass (in kDa) gave a straight line ( $y = -0.103x$   
177  $+4.881$ ;  $r^2 = 0.991$ ).

178

#### 179 *2.4.2. Inductively Coupled Plasma Mass Spectrometry*

180 Detection was carried out by means of a model 7700x quadrupole – ICP-  
181 MS system (Agilent) equipped with a pneumatic concentric nebulizer and a  
182 double-pass spray chamber. The connection between the exit of the column  
183 and the nebulizer was performed directly by means of polyether ether ketone  
184 (PEEK) tubing. The operating conditions and the nuclides measured are listed  
185 in Table 1. Instrumental conditions for ICP-MS were daily optimized according  
186 to the protocol described in the user's manual. In order to evaluate the plasma  
187 ionization conditions and the matrix load of the plasma, the  $^{138}\text{Ba}^{2+}/^{138}\text{Ba}^+$  and  
188  $^{156}\text{CeO}^+/^{140}\text{Ce}^+$  signal ratios were also registered. Quantification was based on  
189 peak areas using the Agilent ChemStation software.

190

#### 191 *2.5. Antibody labelling procedure*

192 Antibodies (Ab) have been labelled with either a lanthanide-labelled  
193 polymer or a DOTA-lanthanide chelate complex. Antibody labelling procedure  
194 was based on a chemical reaction between a maleimide residue employed as a  
195 linker of the different metal labels and free sulfhydryl groups obtained after a  
196 partial reduction of the Ab's cysteine-based disulfide bridges with TCEP. This  
197 procedure was preferred over other approaches due to its lower complexity [7].

198

##### 199 *2.5.1. Partial reduction of the antibody*

200 The labelling method started with a pre-rinse of the ultrafiltration  
201 membranes with phosphate buffer. Thereupon, a monoclonal antibody (mAb)  
202 washing by centrifugation (1 x 500  $\mu$ L, 10000 x g, 15 min, 4°C) with phosphate  
203 buffer and a partial reduction of the mAb using an excess of TCEP for 30 min at  
204 37°C were carried out. According to the polymer Ab labelling kit protocol, a  
205 molar excess of 60 of TCEP relative to Ab molarity has to be used which has  
206 been optimized for a multitude of IgG isotypes. However, it was observed that  
207 the Ab did not show antigen selectivity in the immunereaction and, hence,  
208 TCEP concentration was optimized. The reduction step for DOTA labelling was  
209 carried out using a 6-fold molar excess [9]. It has to be noted that TCEP is not  
210 particularly stable in phosphate buffers, especially at neutral pH; so the working  
211 solutions have to be prepared immediately before use.  
212 Ethylenediaminetetraacetic acid disodium salt was added to prevent oxidation  
213 of the generated sulfhydryl groups by trace metals [Error! Bookmark not  
214 defined.]. The mAb was quickly washed (1 x 500  $\mu$ L) with Tris buffer saline to  
215 remove the TCEP in solution by centrifugation and resuspended in the same  
216 buffer at 1 mg mL<sup>-1</sup>. Then, the mAb was labelled following different procedures,  
217 namely: (i) DOTA-chelate complexes or (ii) polymer labelling kit.

218

### 219 2.5.2. Antibody labelling via the polymer labelling kit

220 The mAb was labelled following the protocol of the reagent supplied.  
221 Briefly, the polymer was pre-loaded with a lanthanide for 30 - 40 min at 37°C.  
222 Then, the mAb was conjugated with the lanthanide - loaded polymer for 1 h at  
223 37°C. The excess of the ligand was removed from the mAb solution by  
224 ultracentrifugation.

225

226 *2.5.3. Antibody labelling via DOTA-chelate complexes*

227 This labelling procedure was based on that described by Terenghi et al  
228 [Error! Bookmark not defined.]. Briefly, the mAb was reacted with a 50-fold  
229 molar excess of DOTA for 1 h at 37°C. Then, the lanthanide (III) ion was made  
230 react with DOTA for 30 min at 37°C. The excess of the ligand was removed  
231 from the mAb solution by ultracentrifugation.

232

233 In both labelling strategies, it is important to avoid moisture  
234 condensation; otherwise the maleimide moiety will hydrolyze and become non-  
235 reactive. The four mAbs towards the four protein molecules chosen: CEA,  
236 sErbB2, CA 15.3, CA 125, were labelled with the lanthanide ions:  $^{165}\text{Ho}$ ,  $^{159}\text{Tb}$ ,  
237  $^{169}\text{Tm}$  and  $^{141}\text{Pr}$ , respectively, following both labelling methods described above.  
238 Element-labelled mAbs were stored at  $-20^{\circ}\text{C}$  in Tris buffer saline until use.

239

240 *2.6. Determination of the antibody labelling degree*241 *2.6.1. Protein quantification*

242 The concentrations of labelled mAbs were measured by a microplate  
243 spectrophotometer (SPECTROstar Nano, BMG LabTech, Champigny s/Marne,  
244 France) at 750nm using a *DC<sup>TM</sup> Protein Assay Kit*. The *DC<sup>TM</sup>* (detergent  
245 compatible) protein assay is a colorimetric assay, similar to the well-  
246 documented Lowry assay [15], for protein concentration following detergent  
247 solubilization. Bovine serum albumin was used as calibration standard.

248

249 *2.6.2 ICP-MS analysis of metal content*

250 A 0.15  $\mu\text{L}$  volume of all mAbs conjugated with the labelled polymer and  
251 3 $\mu\text{L}$  of mAbs labelled with the DOTA-chelate complexes were diluted up to 5 mL  
252 with 3.5% V V<sup>-1</sup> nitric acid for the determination of the labelling degree of the  
253 mAbs. An external calibration series from 1 ng mL<sup>-1</sup> to 1  $\mu\text{g mL}^{-1}$  was prepared  
254 using a rare earth multielemental standard solution. Samples were analyzed by  
255 ICP-MS using the operating conditions listed in Table 1.

256

### 257 *2.7. Immunoassay procedure*

258 A human serum aliquot (120  $\mu\text{L}$ ) was incubated overnight at 4°C with a  
259 mixture of labelled mAbs 2  $\mu\text{g mL}^{-1}$  or 10  $\mu\text{g mL}^{-1}$ , for the polymer and DOTA-  
260 chelate labels, respectively, and subsequently, analyzed by SEC-ICP-MS.

261 The incubation was performed at 4°C in order to avoid protein  
262 degradation.

263

## 264 **3. Results and discussion**

### 265 *3.1. Preliminary studies with lanthanide-labelled polymer in SEC-ICP-MS*

266 Given that polymer-based lanthanide labels have not been tested for the  
267 analysis of biomolecules in homogeneous-based immunoassays so far, a proof  
268 of concept test was initially carried out to evaluate the potential benefits and  
269 drawbacks of this labelling approach. First, following the procedure described in  
270 the experimental section, a goat polyclonal antimouse IgG antibody (pAb) was  
271 labelled with the <sup>165</sup>Ho polymer reagents and analyzed by SEC-ICP-MS.  
272 Likewise, for the purpose of evaluating the results obtained, this assay was also  
273 carried out using <sup>165</sup>Ho DOTA chelate complexes.

274 Fig. 1 shows the chromatograms obtained for a solution containing a  
275 nominal concentration of  $10 \mu\text{g mL}^{-1}$  pAb labelled with  $^{165}\text{Ho}$  polymer or  $^{165}\text{Ho}$   
276 DOTA using the Supereose 6 Increase 10/300 GL column. Irrespective of the  
277 labelling approach selected, two  $^{165}\text{Ho}$ -related peaks were approximately  
278 obtained at 34 and 40 min. In agreement with the theoretical values expected  
279 from the column calibration curve and UV-Vis measurements at 280 nm, the  
280 first peak corresponds to the  $^{165}\text{Ho}$ -labelled pAb; whereas the second one was  
281 identified as metal impurities from the Ab labelling procedure. In fact, the  
282 retention time of the second peak was similar to that obtained from a solution  
283 containing either free  $^{165}\text{Ho}$  polymer or free  $^{165}\text{Ho}$  DOTA chelate complexes. As  
284 can be seen in Fig. 1, in the case of using  $^{165}\text{Ho}$  polymer, the signal of the  
285 labelled pAb (measured as peak height) was approximately two orders of  
286 magnitude higher than that obtained for the  $^{165}\text{Ho}$  DOTA chelate complexes.  
287 These results are totally expected taking into account that there is an average  
288 of 30 chelators per polymer label [1]. Nevertheless, given the signal difference  
289 between both labelling approaches, it could be concluded that the Ab labelling  
290 efficiency achieved with the polymer reagents was at least three times higher  
291 than that afforded by the DOTA-chelate complexes.

292 Next, with the aim to verify the pAb activity and the immunocomplex  
293 formation, a  $10 \mu\text{g mL}^{-1}$  mouse IgG<sub>1</sub> Ab (antigen) solution in ammonium acetate  
294 was incubated overnight at 4°C with pAb labelled with either  $^{165}\text{Ho}$  polymer or  
295  $^{165}\text{Ho}$  DOTA chelate complex at nominal concentration of  $10 \mu\text{g mL}^{-1}$  and, then,  
296 the mixture obtained was analyzed by SEC-ICP-MS (Fig. 2). In the case of  
297 using the polymer reagents (Fig. 2.A), the elution profile shows, in addition to  
298 those shown in Fig. 1, two new peaks at 17 and 31 min, respectively. Given that

299 the separation in SEC is based on the size of the molecules as they pass  
300 through the column, these results suggest that two different immunocomplexes  
301 have been formed: the first peak corresponds to a high molecular weight  
302 (HMW) immunocomplex whereas the second one to a low molecular (LMW)  
303 immunocomplex. According to the retention time observed for blue dextran  
304 (16.4 min) and thyroglobulin (26.2 min) during column mass calibration, the size  
305 of the HMW immunocomplex might be ranged between 2000 and 700 kDa. On  
306 the other hand, the LMW immunocomplex peak might be related to small  
307 antigen-pAb complex given its proximity to the unreacted pAb peak. The peak  
308 corresponding to the unreacted pAb was still observed either because of the  
309 excess of the pAb used or because of its partial deactivation during the labelling  
310 procedure. Interestingly, the chromatographic profile registered for the mixture  
311 of the antigen with the  $^{165}\text{Ho}$  DOTA labelled pAb (Fig. 2.B) was different to that  
312 obtained using the  $^{165}\text{Ho}$  polymer-labelled pAb. The elution profile just showed  
313 one new peak at 17 min that, in agreement with the literature [9] and previous  
314 observations with the  $^{165}\text{Ho}$  polymer-labelled pAb, should be related to a HMW  
315 immunocomplex. No peak corresponding to other type of immunocomplexes  
316 was registered. Therefore, it could be concluded that the HMW immunocomplex  
317 formation was favored by the use of  $^{165}\text{Ho}$  DOTA labelled pAb over the use of  
318  $^{165}\text{Ho}$  polymer-labelled one. The origin of this behavior could be related to steric  
319 effects caused by the polymer chains linked to the Ab which make difficult the  
320 formation of big antigen-antibody (Ag-Ab) aggregates. In fact, both HMW and  
321 LMW immunocomplex signals were observed to be strongly dependent on the  
322 Ag:Ab ratio tested. Thus, for a given antigen amount, a reduction of the Ab  
323 concentration favored the HMW immunocomplex formation at the expense of



324 the LMW immunocomplex. Conversely, the LMW immunocomplex formation  
325 was improved increasing the Ab concentration. So, when the  $^{165}\text{Ho}$  polymer-  
326 label was used, either HMW or LMW immunocomplex signals could be  
327 theoretically employed for protein quantitation purposes. Nevertheless, given  
328 the interdependence among both immunocomplexes, the analytical figures of  
329 merit were expected to be strongly dependent on the Ab concentration  
330 employed in the immunoassay. The above mentioned phenomenon was not  
331 observed for the  $^{165}\text{Ho}$  DOTA labelled pAb and, hence, protein quantification  
332 could only be performed using the signal of the HMW immunocomplex [9].

333 Previous works in SEC showed that unwanted interactions between the  
334 sample components and the chromatographic stationary phase could occur,  
335 thus negatively affecting quantitative analysis [9,16,17]. For this reason,  
336 lanthanide content emerging from the Superose 6 Increase 10/300 GL column  
337 for both labelling strategies was compared to that initially present in the sample  
338 before the chromatographic run. Holmium recovery using the polymer Ab  
339 labelling kit was quantitative ( $106 \pm 3\%$ ) but not for the DOTA-chelate  
340 complexes ( $70 \pm 5\%$ ). The origin of the low recoveries obtained with the latter  
341 approach was unclear. The chromatographic recovery was therefore  
342 determined using an alternative SEC column (Superdex 200 HR 10/300) to that  
343 initially employed in this work (Superose 6 Increase 10/300 GL). While the  
344 lanthanide recovery for DOTA labelling with the alternative column was  
345 quantitative and acceptable ( $113 \pm 13\%$ ), the peak resolution between the LMW  
346 immunocomplex and the unreacted Ab for lanthanide-labelled polymer was  
347 compromised. No further differences were observed in the chromatograms  
348 between both columns. Therefore, further studies for the mentioned labelling

349 strategies were carried out using different SEC columns: the Superose 6  
350 Increase 10/300 GL column for lanthanide-labelled polymer and the Superdex  
351 200 HR 10/300 column for DOTA-chelate complexes.

352

353 *3.2. Analysis of cancer biomarkers in human serum by means of SEC-ICP-*  
354 *MS and polymer-labelled antibodies*

355 Once the feasibility of using the lanthanide-labelled polymer for protein  
356 analysis in homogeneous-based immunoassays was successfully proved, this  
357 labelling approach was applied for the multiplex determination of cancer  
358 biomarkers in human serum samples; namely: CEA, sErbB2, CA 15.3 and CA  
359 125. To this end, mAbs against the above-mentioned biomarkers have been  
360 labelled with  $^{165}\text{Ho}$ ,  $^{159}\text{Tb}$ ,  $^{169}\text{Tm}$  and  $^{141}\text{Pr}$ , respectively.

361

362 *3.2.1. Optimization of polymer-labelled antibodies synthesis*

363 The labelling degree of the polymer-labelled mAbs depends on the  
364 number of sulfhydryl groups obtained after reducing the Ab's cysteine-based  
365 disulfide bridges with TCEP. To achieve the highest labelling efficiency, it is  
366 necessary to reduce as many disulfide bridges of the mAb as possible.  
367 However, the experimental conditions should not be too harsh so the labelled  
368 Ab still shows antigen selectivity in the immunoreaction. In other words, the  
369 conditions have to be as mild as possible, so that the Ab is not separated into  
370 its heavy and its light chain by the breaking of too many disulfide bridges.  
371 Preferably, the disulfide bridges of the hinge region can be cleaved resulting in  
372 two identical and still binding Ab fragments. To evaluate both labelling efficiency  
373 and mAb's activity, it was proceeded as follows. First, aliquots of the different

374 mAbs were reduced with a given molar excess of TCEP, respectively. Next,  
375 after labelling the different mAb with the polymer reagent, a solution containing  
376 a nominal concentration of  $1 \mu\text{g mL}^{-1}$  of labelled mAb was made to react with  
377 different amounts of their corresponding antigen ( $0\text{-}50 \text{ ng mL}^{-1}$  for CEA,  $0\text{-}100$   
378  $\text{ng mL}^{-1}$  for sErbB2,  $0\text{-}100 \text{ IU mL}^{-1}$  for CA 15.3 and  $0\text{-}100 \text{ IU mL}^{-1}$  CA 125) in  
379 human serum. Finally, the mixture was analyzed by SEC-ICP-MS.

380         Initially, a molar excess rate of 60 of TCEP (concentration recommended  
381 by the reagent supplier) relative to mAb molarity was tested but no  
382 immunocomplexes were registered for all the mAb tested. Similar findings were  
383 observed for 20-fold molar excess, suggesting that the reduction of the disulfide  
384 bridges was too harsh leading to a denaturation of the mAbs. These results  
385 were totally unexpected taking into account polymer manufacturer  
386 recommendations and previous data reported by Waentig et al. [14]. As in the  
387 IgG<sub>1</sub> subclass the 2 heavy chains are connected in the hinge region by 2  
388 disulfide bonds [16] and each disulfide bridge needs at least to be reduced by 2  
389 protons from the TCEP, the molar excess of TCEP was further reduced ranging  
390 from 2 to 8-fold. For a molar excess of TCEP lower than or equal to 8-fold, the  
391 chromatographs obtained showed the four peaks previously mentioned (free  
392 lanthanide-labelled polymer, unreacted labelled mAb, LMW and HMW  
393 immunocomplexes) thus showing that the mAbs conserved their binding  
394 properties. The elution time for all peaks was similar to that previously pointed  
395 out. In general, with a decreasing amount of TCEP, the intensities of the  
396 different immunocomplexes also decreased. Because of signal differences  
397 between 4-fold and 8-fold molar excess of TCEP were lower than 5% and the

398 reduction step is critical in keeping mAb binding properties, the former was  
399 finally chosen for further studies.

400 Finally, the labelling degree of the mAbs (the number of lanthanide atoms  
401 labelled to the mAb) was evaluated as described before (section 2.6.). To this  
402 end, the metal content of the labelled mAbs was measured by means of ICP-  
403 MS. In advance, the total amount of the mAb after labelling was measured with  
404 the DC™ Protein Assay Kit in a microtiter plate because during sample  
405 preparation and in particular during the purification step losses can occur. On  
406 average, in the case of using polymer reagents, there were 29 lanthanides per  
407 mAb and, considering that the lanthanide-labelled polymer contains an average  
408 of 30 chelators per label [1], it points out that almost one polymer label is  
409 attached to each Ab (Table S1). These values are about 6 times lower than  
410 those reported elsewhere [14] but it should be taking into account that the molar  
411 excess of TCEP employed for the partial reduction of the mAb in this work was  
412 15 times lower. For the sake of comparison, the mAbs were also labelled with  
413 DOTA-chelate complexes. The experimental conditions selected were those  
414 previously described by Terenghi et al. [9] where a 6-fold molar excess of TCEP  
415 (with regard to the Ab) was used for the partial reduction of the mAbs. It was  
416 observed that the use of DOTA-chelate complexes was a less efficient  
417 approach for mAb labelling (Table S1). In agreement with previous works [14],  
418 approximately every thirtieth Ab was modified with SCN-DOTA which covalently  
419 bound to amino groups. From these experiments, and considering the  
420 differences in the lanthanide content, better analytical figures of merit should be  
421 expected for the lanthanide-labelled polymer.

422

423 3.2.2. *Influence of the incubation medium on immunocomplex formation*

424 Thereupon different solution media were evaluated for incubating the  
425 polymer-labelled mAbs with the biomarkers. Previous works [19] have shown  
426 that nonspecific proteins may assist the formation and stabilization of Ag-Ab  
427 complexes maintaining the correct conformation of the Ab and antigen for  
428 optimum binding. For this purpose, a solution containing a nominal  
429 concentration of  $1 \mu\text{g mL}^{-1}$  of the polymer-labelled mAbs was incubated  
430 overnight at  $4^{\circ}\text{C}$  with the maximum concentration of wished-to be determined  
431 antigens (namely:  $50 \text{ ng mL}^{-1}$  CEA,  $100 \text{ ng mL}^{-1}$  sErbB2,  $100 \text{ IU mL}^{-1}$  CA 15.3  
432 and  $100 \text{ IU mL}^{-1}$  CA 125) in the pertinent incubation medium. The resulting  
433 mixture was subsequently analyzed by SEC-ICPMS. The incubation media  
434 tested were: (i)  $100 \text{ mM}$  ammonium acetate (SEC carrier); (ii)  $0.1 \% \text{ w w}^{-1}$   
435 Tween 20; (iii)  $6\% \text{ w w}^{-1}$  human serum albumin; and (iv) human serum. In this  
436 experiment, the antigen and the mAb concentration was modified regarding  
437 previous sections. The antigen concentration was selected according to the  
438 concentration range of interest in clinical sample analyses whereas the mAb  
439 nominal concentration was decreased 10-fold due to the high signals afforded  
440 by the polymer-labelled Abs and the low biomarker concentration tested.

441 As expected, regardless of the biomarker, HMW and LMW  
442 immunocomplexes were observed using  $0.1 \% \text{ w w}^{-1}$  Tween 20,  $6\% \text{ w w}^{-1}$   
443 human serum albumin or human serum as incubation medium. No detectable  
444 immunocomplex signal was obtained for ammonium acetate despite this  
445 medium was successfully employed in the preliminary studies (Table S2). From  
446 these data, it was concluded that, given the low levels of the biomarkers  
447 expected in human serum samples, the incubation medium should contain

448 surfactants and/or proteins to favor immunocomplex formation [9,19]. In fact,  
449 the absence of both HMW and LMW immunocomplexes signals with ammonium  
450 acetate could be probably attributed to the low levels of the biomarkers tested  
451 and the incubation medium inefficiency to stabilize the Ab and the Ag-Ab  
452 complexes.

453 Human serum from a healthy person contains significant levels of all the  
454 biomarkers studied (CEA, sErbB2, CA 15.3 and CA 125) and, hence, the  
455 concentration values obtained for unknown human serum samples will be  
456 relative to their content in the control human serum employed in the incubation  
457 step. While this situation is not the ideal from an analytical point of view, it  
458 should not be especially troublesome for clinical sample analyses since its main  
459 interest is focused on status changes from reference range concentrations.  
460 Obviously, this makes imperative to use a control human serum with a known  
461 concentration of all the biomarkers. In this work, a pooled serum, prepared from  
462 15 healthy patients with a declared amount of tumor biomarkers determined  
463 with the conventional heterogeneous immunoassays usually employed in the  
464 clinical analytical laboratories, was used. The concentration levels for all the  
465 biomarkers studied in the control human serum were:  $1.7 \text{ ng mL}^{-1}$  CEA,  $7 \text{ ng}$   
466  $\text{mL}^{-1}$  sErbB2,  $15 \text{ IU mL}^{-1}$  CA 15.3 and  $13 \text{ IU mL}^{-1}$  CA 125.

467

### 468 3.2.3. *Optimization of the concentration of the polymer-labelled antibody*

469 As it has been pointed out (section 3.1. and elsewhere [20]), the Ag:Ab  
470 ratio employed in the immunoreaction determines which types of  
471 immunocomplexes are formed. To investigate this effect in detail, two types of  
472 experiments were carried out. First, a human serum sample containing a fixed

473 amount of each biomarker was incubated with variable amounts of the  
474 corresponding polymer-labelled mAb. Alternatively, the concentration of the  
475 polymer-labelled mAb was fixed and the biomarker concentration was modified.

476 Fig. 3 shows the chromatograms obtained after incubation overnight at  
477 4°C of a human serum sample spiked with 50 ng mL<sup>-1</sup> CEA and with the  
478 corresponding <sup>165</sup>Ho polymer-labelled mAb at a nominal concentration of 6 ng  
479 mL<sup>-1</sup> or 2 µg mL<sup>-1</sup>. As expected, the Ag:Ab ratio employed was critical on  
480 immunocomplex formation. Thus, incubating the antigen with the polymer-  
481 labelled mAb at a nominal concentration of 6 ng mL<sup>-1</sup>, just the HMW  
482 immunocomplex was formed and no LMW immunocomplex signal was  
483 detectable. The opposite behavior was observed for the polymer-labelled mAb  
484 at a nominal concentration of 2 µg mL<sup>-1</sup>.

485 Alternatively, human serum samples containing concentrations from 5 to  
486 50 ng mL<sup>-1</sup> of CEA were incubated with the corresponding <sup>165</sup>Ho polymer-  
487 labelled mAb at the nominal concentrations of 6 ng mL<sup>-1</sup> or 2 µg mL<sup>-1</sup> (Table 2).  
488 Interestingly, the HMW immunocomplex signal did not increase at increasing  
489 antigen concentration when the polymer-labelled mAb nominal concentration  
490 was 6 ng mL<sup>-1</sup>. Nevertheless, the LMW immunocomplex signal did show a  
491 linearly increased response for a polymer-labelled mAb nominal concentration  
492 of 2 µg mL<sup>-1</sup>. The fact that, in the former case, the assay dose response had a  
493 maximum is related to the Hook effect [21] and it is caused by excessively high  
494 concentrations of antigen saturating all of the available binding sites of the Ab  
495 without forming complexes. Consequently, the immunocomplex formation is not  
496 favored and the SEC-ICP-MS signal decreases instead of increasing. This  
497 phenomenon is common in one-step immunometric assays, as the one

498 developed in this work, affecting negatively the dynamic linear range. The Hook  
499 effect can be mitigated by either decreasing the amount of antigen or increasing  
500 the concentration of the Ab. From a practical point of view, the only feasible  
501 approach to deal with this problem is to modify the concentration of the  
502 polymer-labelled mAb. However, as indicated above, when the concentration of  
503 the polymer-labelled mAb was increased, the LMW immunocomplex was clearly  
504 favored over the HMW one. As a result, the use of the HMW immunocomplex  
505 signal for quantitative purposes must be discarded in favor of the LMW  
506 immunocomplex signal. No Hook effect was observed when the LMW  
507 immunocomplex signal was used for quantification since the Ag-Ab reaction did  
508 not go into antigen excess. These findings were similar for all the biomarkers  
509 studied (Fig. S1) and, hence, the mAb nominal concentration was set at 2  $\mu\text{g}$   
510  $\text{mL}^{-1}$  for further studies.

511 At this point, it is interesting to compare the above-mentioned findings  
512 with experimental data obtained for DOTA labelled mAbs. In agreement with  
513 Terenghi et al. [Error! Bookmark not defined.] observations, no Hook effect  
514 was observed for biomarker quantification using the HMW immunocomplex  
515 signal. This behavior is explained considering that optimum mAb nominal  
516 concentration used ( $10 \mu\text{g mL}^{-1}$ ) was 5-fold higher than that using reagents due  
517 to the lower signal amplification afforded by DOTA-chelate complexes.

518

519

#### 520 3.2.4. Method validation

521 The analytical method developed for biomarker analysis was validated  
522 according to ICH guidelines for analytical procedures [22]. The linearity and



523 figures of merit were determined by establishing the calibration graphs for the  
524 four biomarker proteins tested, each at its concentration range of interest in  
525 clinical sample analyses. The limit of detection (LoD), limit of quantification  
526 (LoQ), dynamic range, linearity, sensitivity (defined as the slope of the  
527 calibration curve) and the correlation coefficient for each biomarker using the  
528 polymer reagents are given in Table 3. It must be taken into account that control  
529 human serum employed as incubation medium (blank solution) is not antigen-  
530 free. Therefore, theoretical LoD and LoQ were roughly estimated by dividing the  
531 standard deviation of the instrument response by the slope of the calibration  
532 curve, 3 and 10 times, respectively. The estimation of the instrument response  
533 was based on the standard deviation of the blank. In all cases, analytical figures  
534 of merit were able to detect the biomarkers at concentration below the threshold  
535 reference values used in clinical analysis (namely: [CEA] < 5 ng mL<sup>-1</sup>, [sErbB2]  
536 < 15 ng mL<sup>-1</sup>, [CA 15.3] < 30 IU mL<sup>-1</sup>, [CA 125] < 35 IU mL<sup>-1</sup>) to differentiate  
537 healthy and disease states.

538 Due to the lack of a certified biomarker reference material for CEA,  
539 sErbB2, CA 15.3 and CA 125 antigen, the method accuracy was evaluated  
540 comparing the results of the proposed analytical procedure with those obtained  
541 using an accurate well-established procedure (i.e., the routine immunoassay  
542 procedure employed in the hospital where the serum samples were obtained).  
543 Thus, the reference method for CEA, CA 15.3 and CA 125 was based on an  
544 heterogenous sandwich-type immunoassay with electrochemiluminescence  
545 detection [23-25] whereas the sErbB2 analytical procedure was based on  
546 ELISA sandwich-type immunoassay kit [26]. To perform this comparison, a  
547 control human serum was spiked with the four tumor biomarkers at three

548 different known concentration levels. This assay was performed using the  
549 optimum operating conditions described in previous sections. Data in Table 4  
550 indicate that the results obtained for the biomarkers investigated with SEC-  
551 ICPMS were equivalent to those afforded by the heterogeneous immunoassays  
552 usually employed in clinical analysis laboratories. For all the biomarkers tested,  
553 the recovery values using SEC-ICPMS were quantitative ranging from 95 to  
554 110%. The repeatability was assessed using nine determinations covering the  
555 specific range for the procedure (i.e. three concentrations/three replicates each)  
556 on the same day. The relative standard deviation (RSD) of the four biomarkers  
557 at the different concentration levels was below 5%. The intermediate precision  
558 was also verified by analyzing the spiked human serum samples in four different  
559 days with RSD ranging from 4 to 8%. These results demonstrate the robustness  
560 of the method proposed for biomarker analysis in human serum samples.

561

### 562 3.2.5. Comparison with other methodologies

563 Analytical figures of merit of the CEA, sErbB2, CA 15.3 and CA 125  
564 analysis using polymer labelled mAbs and SEC-ICPMS detection have been  
565 compared with those obtained with DOTA labelling (Table S3). Experimental  
566 immunoassay conditions for DOTA labelling were those employed in the  
567 preliminary studies (Section 3.1) using human serum as the incubation medium.  
568 In this case, since no LMW immunocomplex signal was observed, the  
569 calibration was carried out using the signal of the HMW immunocomplex. In the  
570 case of CEA, analytical figures of merit were similar to those reported by  
571 Terenghi et al. [9]. In general, the sensitivity and the LoD obtained using  
572 polymer reagents were improved 10-fold (on average) regarding the DOTA

573 labelling. These results were poorer than theoretically anticipated according to  
574 the differences in the labelling degree between both approaches. It should be  
575 considered that both unreacted labelled mAb and LMW immunocomplex were  
576 not baseline-resolved in the chromatogram and, hence, the signal  
577 reproducibility for low biomarker concentrations was partially compromised. It is  
578 interesting to note that linear dynamic range was also improved by 10-fold using  
579 polymer reagents. From these data, and despite the low chromatographic  
580 resolution, there is no doubt that polymer–labelling significantly improves the  
581 analytical figures of merit of DOTA labelling approach employed in ICP-MS  
582 homogeneous-based immunoassays for biomolecules analysis. Moreover, it is  
583 worth to mention that the concentration of the polymer-labelled mAb required in  
584 the immunoreaction is decreased 5-fold to that required using DOTA-labelled  
585 mAbs..

586 Table 5 shows the analytical figures of merit of the different biomarkers  
587 determined with previously methods described in the literature. Except of the  
588 work by Terenghi et al. [9] previously mentioned, no further comparison is  
589 feasible with other homogenous immunoassays using SEC-ICPMS detection.  
590 Analytical figures of merit for the method developed were similar to those  
591 afforded by heterogeneous-based immunoassays (with or without ICP-MS  
592 detection). Nevertheless, comparing to commercial immunoassay procedures  
593 (i.e. sandwich ELISA spectrophotometric kits and electrochemiluminescence),  
594 LoDs are usually improved. Thus, LoD for CEA was improved 3-fold whereas  
595 for CA15.3 and CA 125 was improved up to 10-fold. For sErbB2, however, LoD  
596 was significantly deteriorated (50-fold) regarding commercial heterogeneous  
597 immunoassay. The main advantage of the proposed method is its possibility to

598 determine simultaneously several biomarkers thus reducing analysis cost and  
599 sample consumption.

600

#### 601 **4. Conclusions**

602 This work shows that lanthanide-labelled polymers conjugated with  
603 antibodies can be successfully employed for multiplexed biomarkers analysis  
604 using a homogeneous-based immunoassay and SEC-ICPMS detection. This  
605 new approach improves both sensitivity and detection limits 10-fold regarding  
606 the lanthanide-DOTA complex traditionally employed for antibody conjugation  
607 using this type of immunoassay procedure. Results in this work show that  
608 analytical figures of merit are not limited by the detection step but they are  
609 limited by the resolution between the antigen-antibody immunocomplex and the  
610 free metal labelled antibody signal peaks. Therefore, even better analytical  
611 figures of merit could be expected improving the chromatographic separation.  
612 The method was validated by the demonstration that it produced similar results  
613 to those obtained by heterogenous-based immunoassays based on the  
614 detection by electrochemiluminescence or ELISA.

615

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623

624

625 **Appendix A. Supplementary data**

626 Supplementary data to this article can be found online at doi:...'.

ACCEPTED MANUSCRIPT

627 **Figure captions.**

628 **Fig. 1.** SEC-ICP-MS chromatograms of a goat polyclonal antimouse IgG  
629 antibody (pAb) labelled with  $^{165}\text{Ho}$  polymer reagents (black line) and  $^{165}\text{Ho}$   
630 DOTA chelate complex (red line). Polyclonal antibody nominal concentration: 10  
631  $\mu\text{g mL}^{-1}$ , column: Superose 6 Increase 10/300 GL.

632

633 **Fig. 2.** SEC-ICP-MS chromatograms obtained after incubation of a mouse IgG<sub>1</sub>  
634 antibody solution with a goat polyclonal antimouse IgG antibody (pAb) labelled  
635 with (A)  $^{165}\text{Ho}$  polymer reagents and (B)  $^{165}\text{Ho}$  DOTA chelate complex. (1) High  
636 molecular weight immunocomplex; (2) low molecular weight immunocomplex;  
637 (3) unreacted labelled pAb; (4) free lanthanide label. pAb nominal  
638 concentration: 10  $\mu\text{g mL}^{-1}$ ; antigen concentration: 10  $\mu\text{g mL}^{-1}$ ; incubation  
639 medium: 100 mM ammonium acetate; column: Superose 6 Increase 10/300 GL.

640

641 **Fig. 3.** SEC-ICPMS chromatograms obtained after incubation of a human  
642 serum sample spiked with 50  $\text{ng mL}^{-1}$  CEA and with its corresponding  $^{165}\text{Ho}$   
643 polymer labelled mAb at a nominal concentration of: (A) 6  $\text{ng mL}^{-1}$  or (B) 2  $\mu\text{g}$   
644  $\text{mL}^{-1}$ . Column: Superose 6 Increase 10/300 GL.

645

646

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767

1 **Table 1.** Operating conditions of SEC-ICPMS.

|   |  |
|---|--|
| <b>Parameters</b>                       |  |
| RF Power (W)                            | 1500   |
| Argon flow rate (L min <sup>-1</sup> ): |  |
| Plasma gas                              | 15   |
| Auxiliary gas                           | 0.9  |
| Carrier gas                             | 1.15   |
| Carrier                                 |  |
| Type                                    | 100 mM Ammonium acetate (pH 6.8)   |
| Flow rate (mL min <sup>-1</sup> )       | 0.5  |
| Sample introduction system              |  |
| Injection volume (μL)                   | 100  |
| Nebulizer                               | Pneumatic concentric   |
| Spray chamber                           | Double-pass Scott  |
| Nuclides                                | <sup>141</sup> Pr, <sup>159</sup> Tb, <sup>165</sup> Ho, <sup>169</sup> Tm |

2

3

- 4 **Table 2.** Influence of the CEA concentration on the immunocomplexes integrated signals after incubation with  $^{165}\text{Ho}$  polymer-
- 5 labelled mAb at nominal concentrations of  $6 \text{ ng mL}^{-1}$  or  $2 \mu\text{g mL}^{-1}$ . Incubation medium: human serum.

|               | CEA concentration ( $\text{ng mL}^{-1}$ )* |              |              |              |
|---------------|--|--------------|--------------|--------------|
| Immunocomplex | 5  | 15           | 30           | 50           |
| HMW           | 34100±200                                  | 36000±600    | 34100±200    | 32000±700    |
| LMW           | 4391000±2000                               | 4624000±5000 | 5089000±2000 | 5589000±3000 |

6 \*mean  $\pm t \cdot s \cdot n^{-1/2}$ ,  $n = 3$ ,  $P = 95\%$

- 7 **Table 3.** Limit of detection, LoQ, dynamic range and linearity of CEA, sErbB2, CA 15.3 and CA125 biomarkers in SEC-ICP-MS  
 8 using the polymer labelling kit.

| Biomarkers     | LoD                      | LoQ                      | Linear dynamic range          | Linear regression*<br>$y=m(t\cdot s_m)\cdot[\text{biomarker}]+b(t\cdot s_b)$ | $r^2$ |
|----------------|--------------------------|--------------------------|-------------------------------|--|-------|
| <b>CEA</b>     | 0.06 ng mL <sup>-1</sup> | 0.18 ng mL <sup>-1</sup> | 0.06– 100 ng mL <sup>-1</sup> | $y= 4.3(\pm 0.3)\cdot 10^5\cdot[\text{CEA}]+4(\pm 8)10^4$                    | 0.998 |
| <b>sErbB2</b>  | 0.5 ng mL <sup>-1</sup>  | 1.5 ng mL <sup>-1</sup>  | 0.5 – 300 ng mL <sup>-1</sup> | $y= 5.8(\pm 0.4)\cdot 10^4\cdot[\text{sErBb2}]+8.9(\pm 0.2)10^4$             | 0.998 |
| <b>CA 15.3</b> | 0.6 IU mL <sup>-1</sup>  | 1.8 IU mL <sup>-1</sup>  | 0.6 – 300 IU mL <sup>-1</sup> | $y= 1.0(\pm 0.4)\cdot 10^5\cdot[\text{CA 15.3}]+0(\pm 7)10^5$                | 0.994 |
| <b>CA 125</b>  | 0.5 IU mL <sup>-1</sup>  | 1.5 IU mL <sup>-1</sup>  | 0.5 – 300 IU mL <sup>-1</sup> | $y= 1.13(\pm 0.08)\cdot 10^6\cdot[\text{CA 125}]+1(\pm 4)10^5$               | 0.998 |

9 n= 5, P= 95%

**Table 4.** Analysis of biomarker spiked human serum samples by means of SEC-ICPMS using the polymer labelling kit and commercial heterogenous-based immunoassays

| Antigen | [Antigen] <sub>spiked</sub> | SEC-ICP-MS                             |              | Reference immunoassay procedure <sup>§</sup> |              |
|---------|-----------------------------|--|--------------|--|--------------|
|         |                             | [Antigen] <sub>calc</sub> <sup>*</sup> | Recovery (%) | [Antigen] <sub>calc</sub> <sup>*</sup>       | Recovery (%) |
| CEA     | 3.5 ng mL <sup>-1</sup>     | 3.4±0.4 ng mL <sup>-1</sup>            | 103±11       | 3.35±0.16 ng mL <sup>-1</sup>                | 96±5         |
|         | 16 ng mL <sup>-1</sup>      | 16.6±0.3 ng mL <sup>-1</sup>           | 96±2         | 15.4±0.9 ng mL <sup>-1</sup>                 | 97±6         |
|         | 32 ng mL <sup>-1</sup>      | 33±2 ng mL <sup>-1</sup>               | 98±5         | 34.0±1.4 ng mL <sup>-1</sup>                 | 106±4        |
| sErbB2  | 4 ng mL <sup>-1</sup>       | 4.0±0.2 ng mL <sup>-1</sup>            | 101±6        | 4.5±0.3 ng mL <sup>-1</sup>                  | 112±6        |
|         | 20 ng mL <sup>-1</sup>      | 19±1.2 ng mL <sup>-1</sup>             | 105±7        | 22.2±1.8 ng mL <sup>-1</sup>                 | 111±9        |
|         | 60 ng mL <sup>-1</sup>      | 57±3 ng mL <sup>-1</sup>               | 106±6        | 65±5 ng mL <sup>-1</sup>                     | 109±8        |
| CA 15.3 | 11 IU mL <sup>-1</sup>      | 11.6±0.4 IU mL <sup>-1</sup>           | 95±3         | 12.1±1.1 IU mL <sup>-1</sup>                 | 111±10       |
|         | 40 IU mL <sup>-1</sup>      | 41±5 IU mL <sup>-1</sup>               | 98±3         | 42.6±1.4 IU mL <sup>-1</sup>                 | 107±3        |
|         | 68 IU mL <sup>-1</sup>      | 69±4 IU mL <sup>-1</sup>               | 98±5         | 66±3 IU mL <sup>-1</sup>                     | 98±5         |
| CA 125  | 10 IU mL <sup>-1</sup>      | 10.2±0.8 IU mL <sup>-1</sup>           | 98±8         | 10.7±0.4 IU mL <sup>-1</sup>                 | 107±4        |
|         | 35 IU mL <sup>-1</sup>      | 34.8±0.5 IU mL <sup>-1</sup>           | 100±2        | 35.0±0.3 IU mL <sup>-1</sup>                 | 100.0±0.8    |
|         | 65 IU mL <sup>-1</sup>      | 67±2 IU mL <sup>-1</sup>               | 97±3         | 63±3 IU mL <sup>-1</sup>                     | 97±4         |

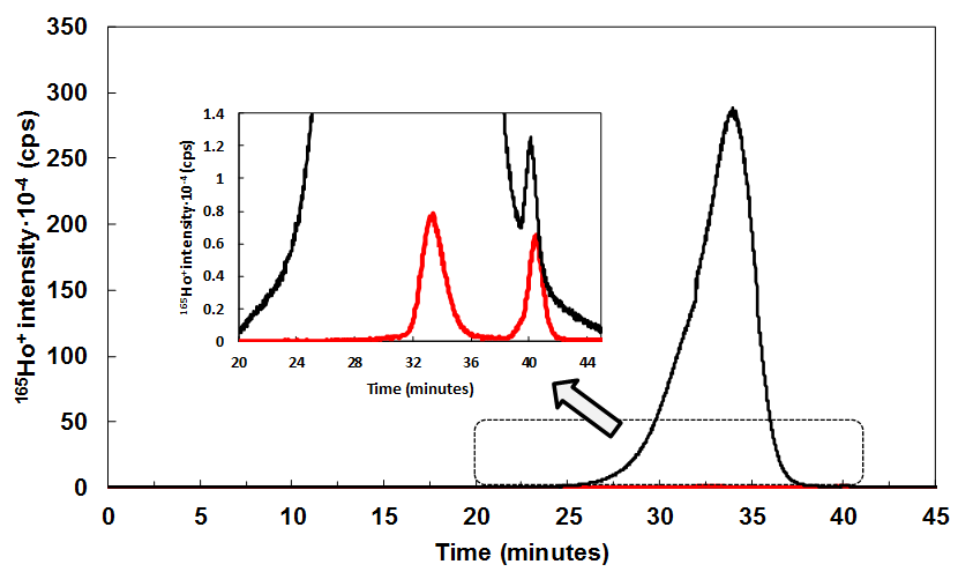
\*mean ± t·s·n-1/2, n = 3, P = 95%

§ Electro-chemiluminescence immunoassay: CEA, CA 15.3, CA 125; ELISA: sErbB2

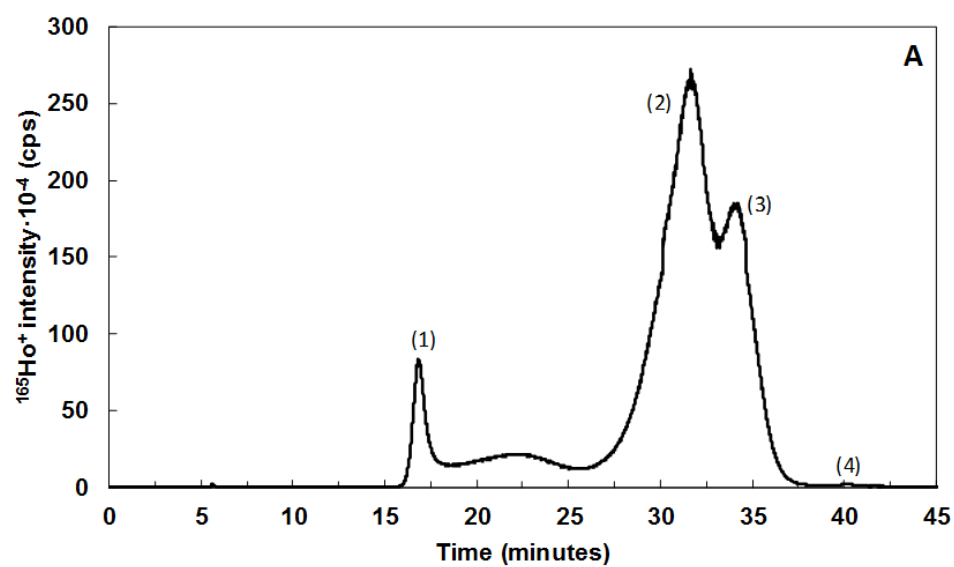
**Table 5.** Comparison of different methods for CEA, sErbB2, CA 15.3 and CA 125 analysis.

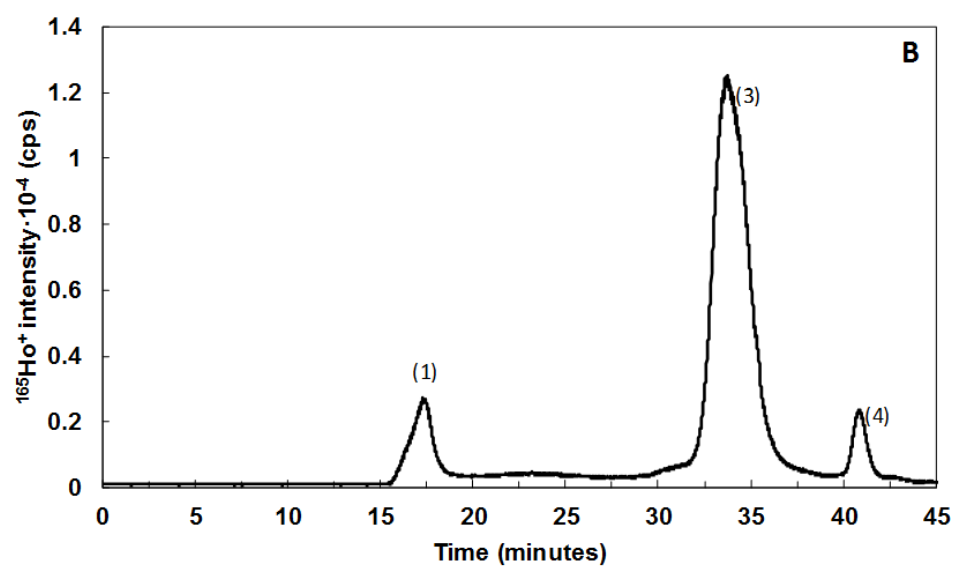
| Analytical method                          | Target protein | LOD                       | Concentration range                           | Reference                      |
|--|----------------|---------------------------|---|--------------------------------|
| ICP-MS based magnetic immunoassay          | CEA            | 0.05 ng mL <sup>-1</sup>  | 0.2–50 ng mL <sup>-1</sup>                    | [27]                           |
| Chip-based magnetic immunoassay-ETV-ICP-MS | CEA            | 0.06 ng mL <sup>-1</sup>  | 0.2-50 ng mL <sup>-1</sup>                    | [28]                           |
| Commercial ELISA kit                       | CEA            | 0.2 ng mL <sup>-1</sup>   | 0.2 - 250 ng mL <sup>-1</sup>                 | Abcam (Cambridge, U.K.) [29]   |
| Commercial ELISA kit                       | sErbB2         | 0.008 ng mL <sup>-1</sup> | 0.008 - 2 ng mL <sup>-1</sup>                 | Abcam (Cambridge, U.K.) [26]   |
| Commercial ELISA kit                       | CA 15.3        | 5 IU mL <sup>-1</sup>     | 5 - 240 IU mL <sup>-1</sup>                   | Abcam (Cambridge, U.K.) [30]   |
| Commercial ELISA kit                       | CA 125         | 5 IU mL <sup>-1</sup>     | 5 - 400 IU mL <sup>-1</sup>                   | Abcam (Cambridge, U.K.) [31]   |
| Chemiluminescent immunoassay               | CEA            | 0.12 ng mL <sup>-1</sup>  | 0.5–100 ng mL <sup>-1</sup>                   | [32]                           |
| Electro-chemiluminescence                  | CEA            | 0.2 ng mL <sup>-1</sup>   | 0.2-1000 ng mL <sup>-1</sup>                  | Roche (Base, Switzerland) [24] |
| Electro-chemiluminescence                  | CA 15.3        | 1.0 IU mL <sup>-1</sup>   | 1.0-300 IU mL <sup>-1</sup>                   | Roche (Base, Switzerland) [25] |
| Electro-chemiluminescence                  | CA 125         | 1.2 IU mL <sup>-1</sup>   | 1.2-5000 IU mL <sup>-1</sup>                  | Roche (Base, Switzerland) [26] |
| Amperometric magnetoimmunosensor           | sErbB2         | 0.03 ng mL <sup>-1</sup>  | 0.1–32.0 ng mL <sup>-1</sup>                  | [33]                           |
| Gold nanorod-based plasmonic sensor        | CA 15.3        | -                         | 0.0249 - 0.2387 IU mL <sup>-1</sup>           | [34]                           |
| Optical microresonators                    | CA 125         | -                         | Limit of linearity of 10 IU mL <sup>-1</sup>  | [35]                           |
| Fluorescence spectroscopy                  | CA 125         | -                         | Limit of linearity of 500 IU mL <sup>-1</sup> | [36]                           |

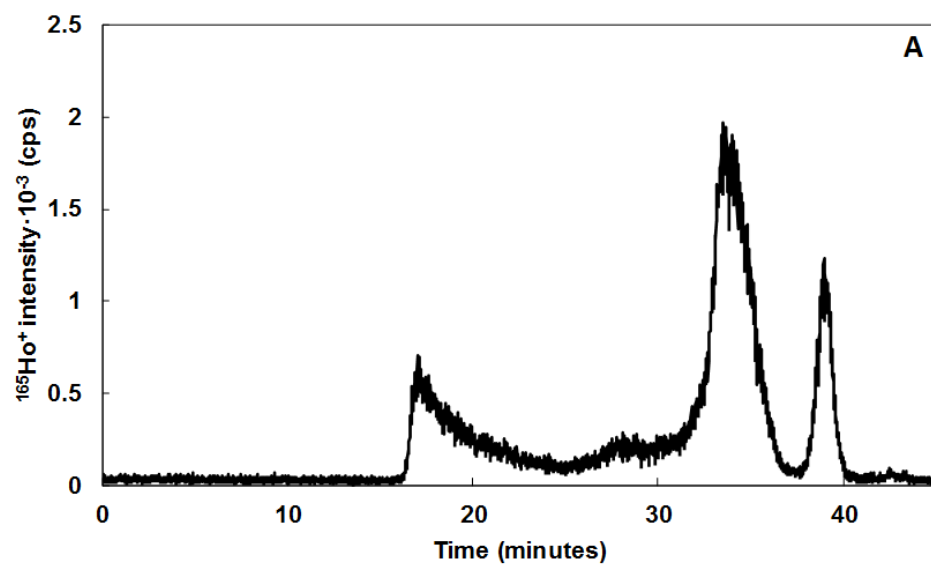


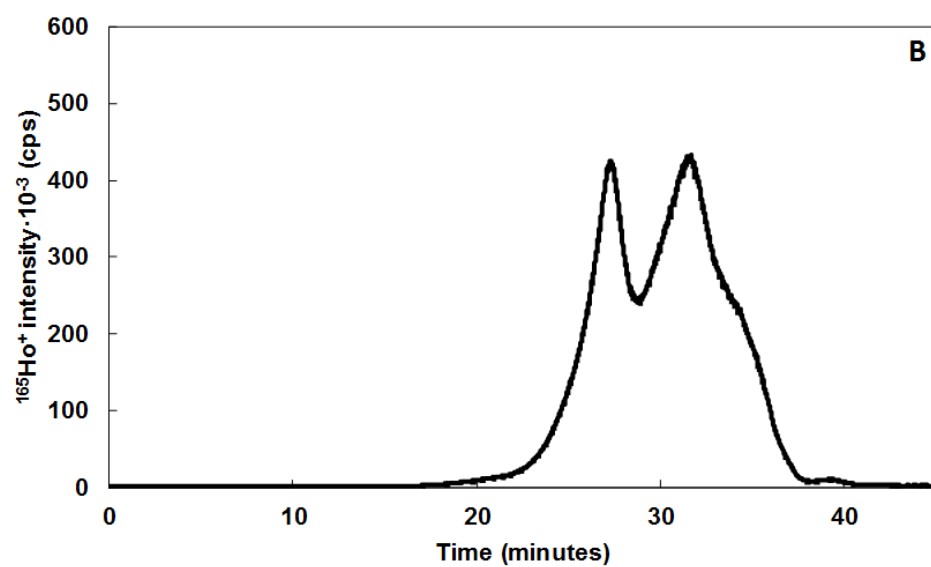


ACCEPTED MANUSCRIPT









**Highlights**

1. Polymer lanthanide labelling is used for the first time in homogenous immunoassay
2. The labelling strategy proposed allows multiplex biomarker analysis with SEC-ICPMS
3. Sensitivity and LoDs are improved regarding previously reported DOTA-labelling