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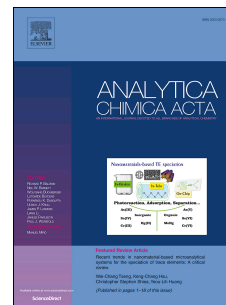
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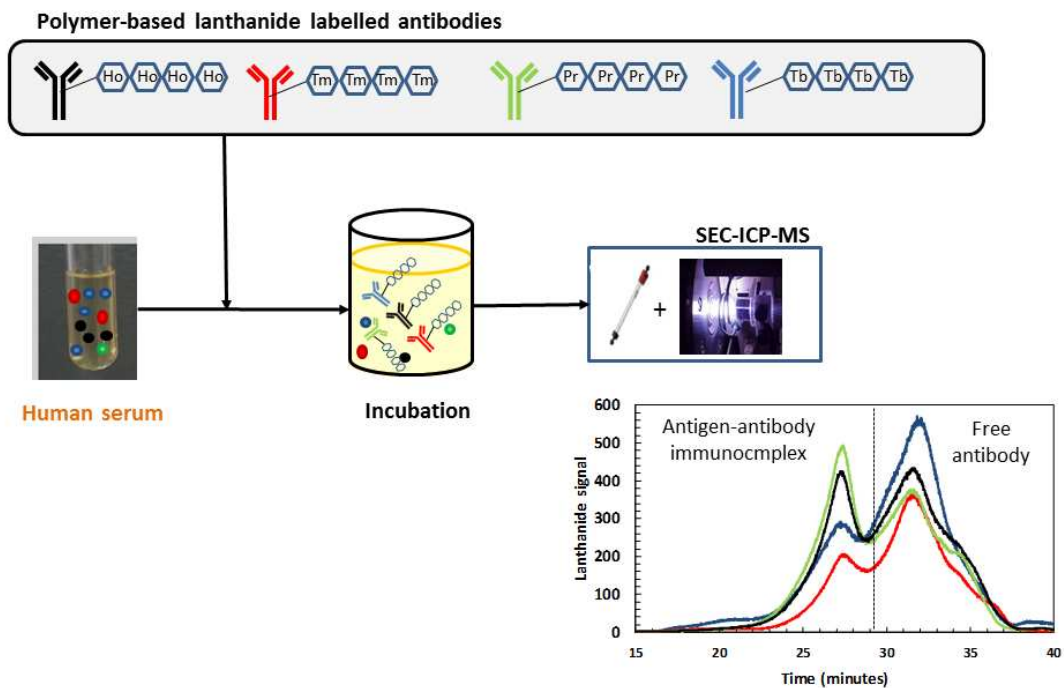
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1 **Lanthanide polymer labels for multiplexed determination of biomarkers in**
2 **human serum samples by means of size exclusion chromatography-**
3 **inductively coupled plasma mass spectrometry**

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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 (IgG₁) antihuman monoclonal antibody (mAb) for
109 α -CEA (clone 1C11) and mouse IgG₁ antihuman mAb for α -CA 125 (clone
110 X325) were purchased from Gene Tex (Irvine, CA). Mouse IgG₁ antihuman
111 mAb for α -sErbB2 (clone 5J297) was obtained from antibodies-online (Aachen,
112 Germany) and mouse IgG₁ antihuman mAb for α -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 MAXPARTM- polymer -Ab labelling kits were obtained from Fluidigm (Les
118 Ulis, France). Human Albumin AlbuteinTM 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), TrizmaTM base, lanthanide chlorides (HoCl₃, TbCl₃, TmCl₃, PrCl₃) 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⁻¹ nitric acid were
128 purchased from Panreac (Barcelona, Spain). Rare earth 100 µg mL⁻¹ Complete
129 Standard was provided by Inorganic Ventures (Lakewood, Colorado) and DCTM
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 AmiconTM 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 EppendorfTM 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^{-1} using 100 mM
156 ammonium acetate (pH 6.8) as mobile phase and sample injection volume of
157 $100 \text{ }\mu\text{L}$. 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; $10 \text{ mm} \times 300 \text{ mm} \times 8.6$
160 μm average beads size) with the approximate bed volume of 24 mL and an
161 optimum separation range of $5\text{-}5000 \text{ kDa}$ for globular proteins and a Superdex
162 200 HR 10/300 (cross-linked agarose and dextran composite stationary phase;
163 $10 \text{ mm} \times 300 \text{ mm} \times 8.6 \mu\text{m}$ average beads size) with the approximate bed
164 volume of 24 mL and an optimum separation range of $10\text{-}600 \text{ kDa}$ 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 ($M_r 2000 \text{ kDa}$),
167 thyroglobulin ($M_r 669 \text{ kDa}$), ferritin ($M_r 440 \text{ kDa}$), aldolase ($M_r 158 \text{ kDa}$),
168 ovalbumin ($M_r 44 \text{ kDa}$), ribonuclease A ($M_r 13.7 \text{ kDa}$) and ubiquitin from bovine
169 ($M_r 8.6 \text{ kDa}$) 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 $M_r \geq 440 \text{ kDa}$ and $y = -0.170x + 7.754$; $r^2 =$
173 0.998 for proteins which $M_r \leq 440 \text{ kDa}$). 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 μ 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 μ 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⁻¹. 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}Ho , ^{159}Tb ,
237 ^{169}Tm and ^{141}Pr , respectively, following both labelling methods described above.
238 Element-labelled mAbs were stored at -20°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 *DCTM Protein Assay Kit*. The *DCTM* (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 μL volume of all mAbs conjugated with the labelled polymer and
251 3 μL of mAbs labelled with the DOTA-chelate complexes were diluted up to 5 mL
252 with 3.5% V V⁻¹ nitric acid for the determination of the labelling degree of the
253 mAbs. An external calibration series from 1 ng mL⁻¹ 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 μL) 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 ¹⁶⁵Ho 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 ¹⁶⁵Ho 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}Ho polymer or ^{165}Ho
276 DOTA using the Supereose 6 Increase 10/300 GL column. Irrespective of the
277 labelling approach selected, two ^{165}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}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}Ho polymer or free ^{165}Ho DOTA chelate complexes. As
284 can be seen in Fig. 1, in the case of using ^{165}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}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 IgG₁ Ab (antigen) solution in ammonium acetate
294 was incubated overnight at 4°C with pAb labelled with either ^{165}Ho polymer or
295 ^{165}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}Ho DOTA labelled pAb (Fig. 2.B) was different to that
312 obtained using the ^{165}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}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}Ho DOTA labelled pAb over the use of
318 ^{165}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}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}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}Ho , ^{159}Tb , ^{169}Tm and ^{141}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 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 IgG₁ 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°C with the maximum concentration of wished-to be determined
431 antigens (namely: 50 ng mL^{-1} CEA, 100 ng mL^{-1} sErbB2, 100 IU mL^{-1} CA 15.3
432 and 100 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 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 ng mL^{-1} CEA, 7 ng
466 mL^{-1} sErbB2, 15 IU mL^{-1} CA 15.3 and 13 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⁻¹ CEA and with the
478 corresponding ¹⁶⁵Ho polymer-labelled mAb at a nominal concentration of 6 ng
479 mL⁻¹ or 2 µg mL⁻¹. 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⁻¹, 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⁻¹.

485 Alternatively, human serum samples containing concentrations from 5 to
486 50 ng mL⁻¹ of CEA were incubated with the corresponding ¹⁶⁵Ho polymer-
487 labelled mAb at the nominal concentrations of 6 ng mL⁻¹ or 2 µg mL⁻¹ (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⁻¹. Nevertheless, the LMW immunocomplex signal did show a
491 linearly increased response for a polymer-labelled mAb nominal concentration
492 of 2 µg mL⁻¹. 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 μg
510 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⁻¹, [sErbB2]
536 < 15 ng mL⁻¹, [CA 15.3] < 30 IU mL⁻¹, [CA 125] < 35 IU mL⁻¹) 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}Ho polymer reagents (black line) and ^{165}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 IgG₁
634 antibody solution with a goat polyclonal antimouse IgG antibody (pAb) labelled
635 with (A) ^{165}Ho polymer reagents and (B) ^{165}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 ng mL^{-1} CEA and with its corresponding ^{165}Ho
643 polymer labelled mAb at a nominal concentration of: (A) 6 ng mL^{-1} or (B) 2 μg
644 mL^{-1} . Column: Superose 6 Increase 10/300 GL.

645

646

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- 766
767

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

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

2

3

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

	CEA concentration (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* $y=m(t\cdot s_m)\cdot[\text{biomarker}]+b(t\cdot s_b)$	r^2
CEA	0.06 ng mL ⁻¹	0.18 ng mL ⁻¹	0.06– 100 ng mL ⁻¹	$y= 4.3(\pm 0.3)\cdot 10^5\cdot[\text{CEA}]+4(\pm 8)10^4$	0.998
sErbB2	0.5 ng mL ⁻¹	1.5 ng mL ⁻¹	0.5 – 300 ng mL ⁻¹	$y= 5.8(\pm 0.4)\cdot 10^4\cdot[\text{sErBb2}]+8.9(\pm 0.2)10^4$	0.998
CA 15.3	0.6 IU mL ⁻¹	1.8 IU mL ⁻¹	0.6 – 300 IU mL ⁻¹	$y= 1.0(\pm 0.4)\cdot 10^5\cdot[\text{CA 15.3}]+0(\pm 7)10^5$	0.994
CA 125	0.5 IU mL ⁻¹	1.5 IU mL ⁻¹	0.5 – 300 IU mL ⁻¹	$y= 1.13(\pm 0.08)\cdot 10^6\cdot[\text{CA 125}]+1(\pm 4)10^5$	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

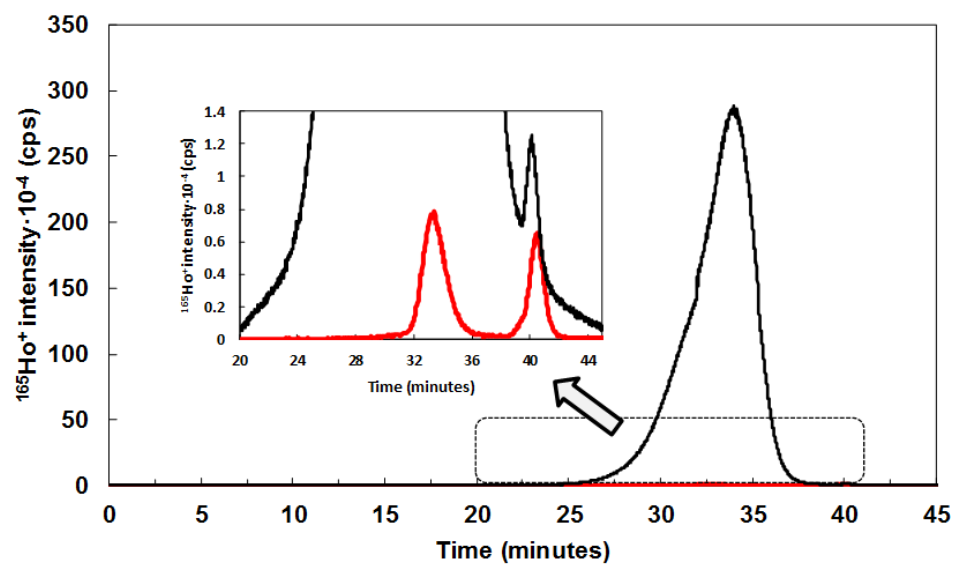
Antigen	[Antigen] _{spiked}	SEC-ICP-MS		Reference immunoassay procedure [§]	
		[Antigen] _{calc} [*]	Recovery (%)	[Antigen] _{calc} [*]	Recovery (%)
CEA	3.5 ng mL ⁻¹	3.4±0.4 ng mL ⁻¹	103±11	3.35±0.16 ng mL ⁻¹	96±5
	16 ng mL ⁻¹	16.6±0.3 ng mL ⁻¹	96±2	15.4±0.9 ng mL ⁻¹	97±6
	32 ng mL ⁻¹	33±2 ng mL ⁻¹	98±5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

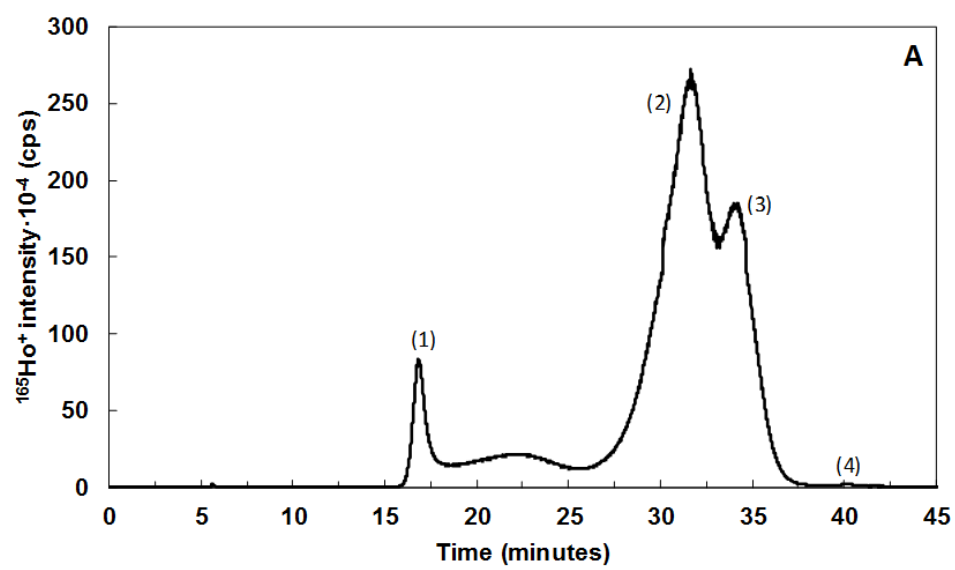
*mean ± t·s·n-1/2, n = 3, P = 95%

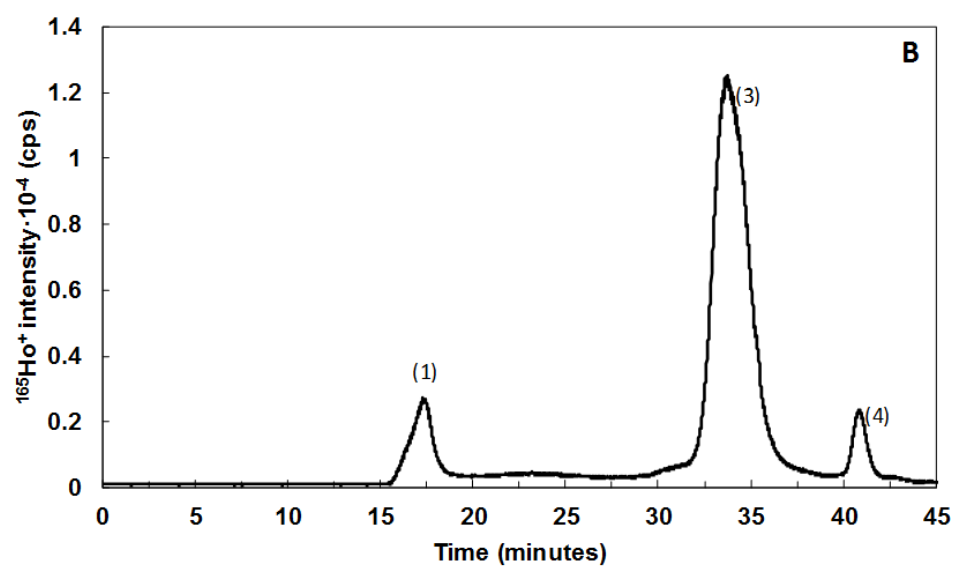
§ Electro-chemiluminescence immunoassay: CEA, CA 15.3, CA 125; ELISA: sErbB2

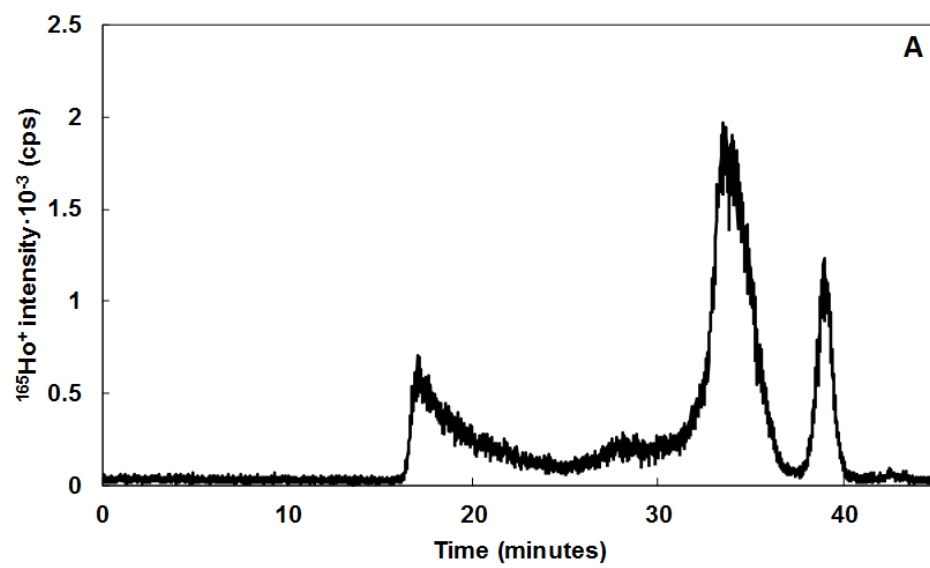
Table 5. Comparison of different methods for CEA, sErbB2, CA 15.3 and CA 125 analysis.

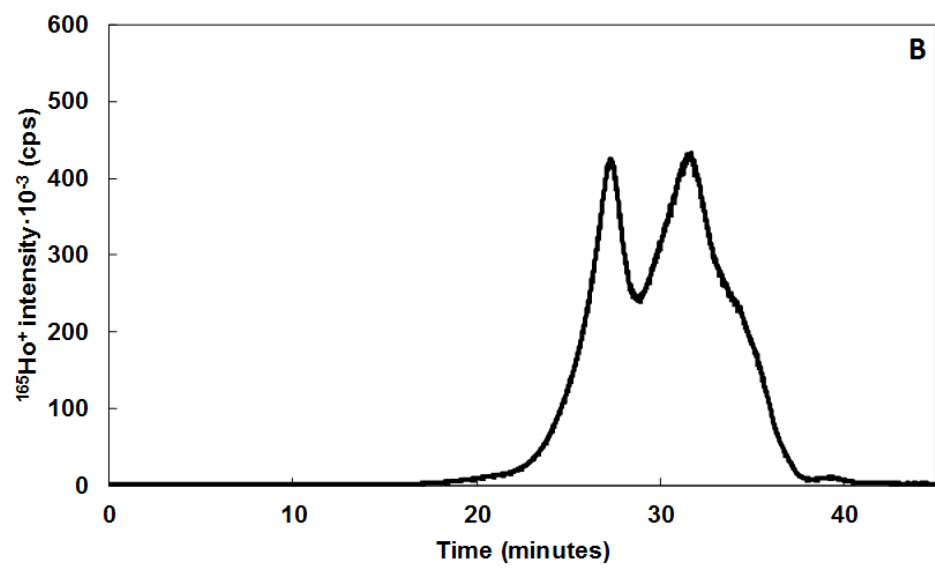
Analytical method	Target protein	LOD	Concentration range	Reference
ICP-MS based magnetic immunoassay	CEA	0.05 ng mL ⁻¹	0.2–50 ng mL ⁻¹	[27]
Chip-based magnetic immunoassay-ETV-ICP-MS	CEA	0.06 ng mL ⁻¹	0.2-50 ng mL ⁻¹	[28]
Commercial ELISA kit	CEA	0.2 ng mL ⁻¹	0.2 - 250 ng mL ⁻¹	Abcam (Cambridge, U.K.)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











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