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Accepted Manuscript

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PII: S0003-2670(18)30296-4

DOI: 10.1016/j.aca.2018.02.056

Reference: ACA 235772

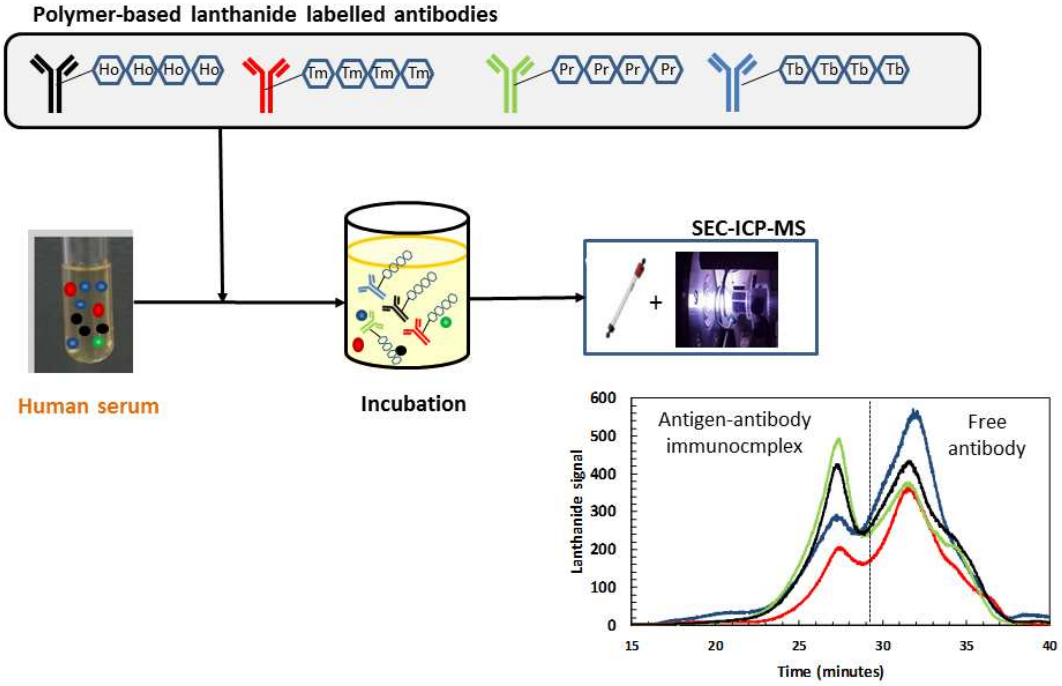
To appear in: *Analytica Chimica Acta*

Received Date: 6 November 2017

Revised Date: 19 February 2018

Accepted Date: 20 February 2018

Please cite this article as: E. Pérez, K. Bierla, G. Grindlay, J. Szpunar, J. Mora, R. Lobinski, Lanthanide polymer labels for multiplexed determination of biomarkers in human serum samples by means of size exclusion chromatography-inductively coupled plasma mass spectrometry, *Analytica Chimica Acta* (2018), doi: 10.1016/j.aca.2018.02.056.



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

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Abstract

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

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

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

1. Introduction

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

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

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

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

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

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

2. Experimental

2.1. Regents and materials

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

Mouse IgG subclass 1 (IgG_1) antihuman monoclonal antibody (mAb) for α-CEA (clone 1C11) and mouse IgG_1 antihuman mAb for α-CA 125 (clone X325) were purchased from Gene Tex (Irvine, CA). Mouse IgG_1 antihuman mAb for α-sErbB2 (clone 5J297) was obtained from antibodies-online (Aachen, Germany) and mouse IgG_1 antihuman mAb for α-CA 15.3 (clone M002204) was from LifeSpan BioSciences (Seattle, WA). The antibody solutions should not contain additives, such as bovine serum albumin (BSA) or gelatin, because the latter could be labelled as well and cause interferences. Upon reception, mAb were divided into single working aliquots and stored at -20ºC.

MAXPAR™- polymer -Ab labelling kits were obtained from Fluidigm (Les Ulis, France). Human Albumin Albutein™ 20% was purchased from Grifols Biologicals Inc. (Los Angeles, CA). 1,4,7,10 – Tetraazacyclododecane - 1,4,7 - tris(aceticacid) - 10-maleimido - ethylacetamide (DOTA) was obtained from Macrocyclics (Dallas, TX). Tris (2-carboxyethyl) - phosphine hydrochloride (TCEP), Trizma™ base, lanthanide chlorides ($HoCl_3$, $TbCl_3$, $TmCl_3$, $PrCl_3$) with natural isotopic abundance, ammonium acetate (≥ 98%, for molecular biology), monosodium phosphate, disodium phosphate, ethylenediaminetetraacetic acid disodium salt (EDTA), dimethyl sulfoxide (DMSO), sodium chloride and

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

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

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

2.2. Buffers

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

2.3. Serum samples

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

2.4. Instrumentation

2.4.1. Size Exclusion Chromatography

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

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

2.4.2. Inductively Coupled Plasma Mass Spectrometry

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

2.5. Antibody labelling procedure

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

2.5.1. Partial reduction of the antibody

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

2.5.2. Antibody labelling via the polymer labelling kit

The mAb was labelled following the protocol of the reagent supplied. Briefly, the polymer was pre-loaded with a lanthanide for 30 - 40 min at 37ºC. Then, the mAb was conjugated with the lanthanide - loaded polymer for 1 h at 37ºC. The excess of the ligand was removed from the mAb solution by 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 37ºC. Then, the lanthanide (III) ion was made
230 react with DOTA for 30 min at 37ºC. 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 – 20ºC 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™ Protein Assay Kit*. The *DC™* (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*

A 0.15 µL volume of all mAbs conjugated with the labelled polymer and 3µL of mAbs labelled with the DOTA-chelate complexes were diluted up to 5 mL with 3.5% V V^{-1} nitric acid for the determination of the labelling degree of the mAbs. An external calibration series from 1 ng mL^{-1} to 1 µg mL^{-1} was prepared using a rare earth multielemental standard solution. Samples were analyzed by ICP-MS using the operating conditions listed in Table 1.

2.7. Immunoassay procedure

A human serum aliquot (120 µL) was incubated overnight at 4ºC with a mixture of labelled mAbs 2 µg mL^{-1} or 10 µg mL^{-1}, for the polymer and DOTA-chelate labels, respectively, and subsequently, analyzed by SEC-ICP-MS.

The incubation was performed at 4ºC in order to avoid protein degradation.

3. Results and discussion

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

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

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

Next, with the aim to verify the pAb activity and the immunocomplex formation, a 10 µg mL^{-1} mouse IgG_1 Ab (antigen) solution in ammonium acetate was incubated overnight at 4ºC with pAb labelled with either ^{165}Ho polymer or ^{165}Ho DOTA chelate complex at nominal concentration of 10 µg mL^{-1} and, then, the mixture obtained was analyzed by SEC-ICP-MS (Fig. 2). In the case of using the polymer reagents (Fig. 2.A), the elution profile shows, in addition to those shown in Fig. 1, two new peaks at 17 and 31 min, respectively. Given that

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

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

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

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

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

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

3.2.1. Optimization of polymer-labelled antibodies synthesis

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

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

Initially, a molar excess rate of 60 of TCEP (concentration recommended by the reagent supplier) relative to mAb molarity was tested but no immunocomplexes were registered for all the mAb tested. Similar findings were observed for 20-fold molar excess, suggesting that the reduction of the disulfide bridges was too harsh leading to a denaturation of the mAbs. These results were totally unexpected taking into account polymer manufacturer recommendations and previous data reported by Waentig et al. [14]. As in the IgG_1 subclass the 2 heavy chains are connected in the hinge region by 2 disulfide bonds [16] and each disulfide bridge needs at least to be reduced by 2 protons from the TCEP, the molar excess of TCEP was further reduced ranging from 2 to 8-fold. For a molar excess of TCEP lower than or equal to 8-fold, the chromatographs obtained showed the four peaks previously mentioned (free lanthanide-labelled polymer, unreacted labelled mAb, LMW and HMW immunocomplexes) thus showing that the mAbs conserved their binding properties. The elution time for all peaks was similar to that previously pointed out. In general, with a decreasing amount of TCEP, the intensities of the different immunocomplexes also decreased. Because of signal differences between 4-fold and 8-fold molar excess of TCEP were lower than 5% and the

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

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

3.2.2. Influence of the incubation medium on immunocomplex formation

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

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

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

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

3.2.3. Optimization of the concentration of the polymer-labelled antibody

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

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

Fig. 3 shows the chromatograms obtained after incubation overnight at 4ºC of a human serum sample spiked with 50 ng mL^{-1} CEA and with the corresponding ^{165}Ho polymer-labelled mAb at a nominal concentration of 6 ng mL^{-1} or 2 µg mL^{-1}. As expected, the Ag:Ab ratio employed was critical on immunocomplex formation. Thus, incubating the antigen with the polymer-labelled mAb at a nominal concentration of 6 ng mL^{-1}, just the HMW immunocomplex was formed and no LMW immunocomplex signal was detectable. The opposite behavior was observed for the polymer-labelled mAb at a nominal concentration of 2 µg mL^{-1}.

Alternatively, human serum samples containing concentrations from 5 to 50 ng mL^{-1} of CEA were incubated with the corresponding ^{165}Ho polymer-labelled mAb at the nominal concentrations of 6 ng mL^{-1} or 2 µg mL^{-1} (Table 2). Interestingly, the HMW immunocomplex signal did not increase at increasing antigen concentration when the polymer-labelled mAb nominal concentration was 6 ng mL^{-1}. Nevertheless, the LMW immunocomplex signal did show a linearly increased response for a polymer-labelled mAb nominal concentration of 2 µg mL^{-1}. The fact that, in the former case, the assay dose response had a maximum is related to the Hook effect [21] and it is caused by excessively high concentrations of antigen saturating all of the available binding sites of the Ab without forming complexes. Consequently, the immunocomplex formation is not favored and the SEC-ICP-MS signal decreases instead of increasing. This 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 µg 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

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

Due to the lack of a certified biomarker reference material for CEA, sErbB2, CA 15.3 and CA 125 antigen, the method accuracy was evaluated comparing the results of the proposed analytical procedure with those obtained using an accurate well-established procedure (i.e., the routine immunoassay procedure employed in the hospital where the serum samples were obtained). Thus, the reference method for CEA, CA 15.3 and CA 125 was based on an heterogenous sandwich-type immunoassay with electrochemiluminescence detection [23-25] whereas the sErbB2 analytical procedure was based on ELISA sandwich-type immunoassay kit [26]. To perform this comparison, a 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 methos 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
616 **Acknowledgments**
617 The authors would like to thank the Generalitat Valenciana (Project
618 GV/2014/138) for the financial support of this work as well as Dr. Gloria Peiró,
619 from the Hospital General Universitario of Alicante, and Francisco M. Marco,
620 from the University of Alicante, for their support and fruitful discussions. E.
621 Pérez also thanks the University of Alicante – Spain for the fellowship
622 (UAFPU2011).

623

624

625 **Appendix A. Supplementary data**

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

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 µg mL^{-1}, column: Superose 6 Increase 10/300 GL.

632

633 **Fig. 2.** SEC-ICP-MS chromatograms obtained after incubation of a mouse IgG_1
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 µg mL^{-1}; antigen concentration: 10 µg 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

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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

Table 2. Influence of the CEA concentration on the immunocomplexes integrated signals after incubation with ^{165}Ho polymer-labelled mAb at nominal concentrations of 6 ng mL^{-1} or 2 µg 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

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

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

Biomarkers	LoD	LoQ	Linear dynamic range	Linear regression* $y=m(t\cdot s_m)\cdot[biomarker]+b(t\cdot s_b)$	r^2
CEA	0.06 ng mL^{-1}	0.18 ng mL^{-1}	0.06– 100 ng mL^{-1}	$y= 4.3(\pm0.3)\cdot10^5\cdot[CEA]+4(\pm8)10^4$	0.998
sErbB2	0.5 ng mL^{-1}	1.5 ng mL^{-1}	0.5 – 300 ng mL^{-1}	$y= 5.8(\pm0.4)\cdot10^4\cdot[sErBb2]+8.9(\pm0.2)10^4$	0.998
CA 15.3	0.6 IU mL^{-1}	1.8 IU mL^{-1}	0.6 – 300 IU mL^{-1}	$y= 1.0(\pm0.4)\cdot10^5\cdot[CA\ 15.3]+0(\pm7)10^5$	0.994
CA 125	0.5 IU mL^{-1}	1.5 IU mL^{-1}	0.5 – 300 IU mL^{-1}	$y= 1.13(\pm0.08)\cdot10^6\cdot[CA\ 125]+1(\pm4)10^5$	0.998

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

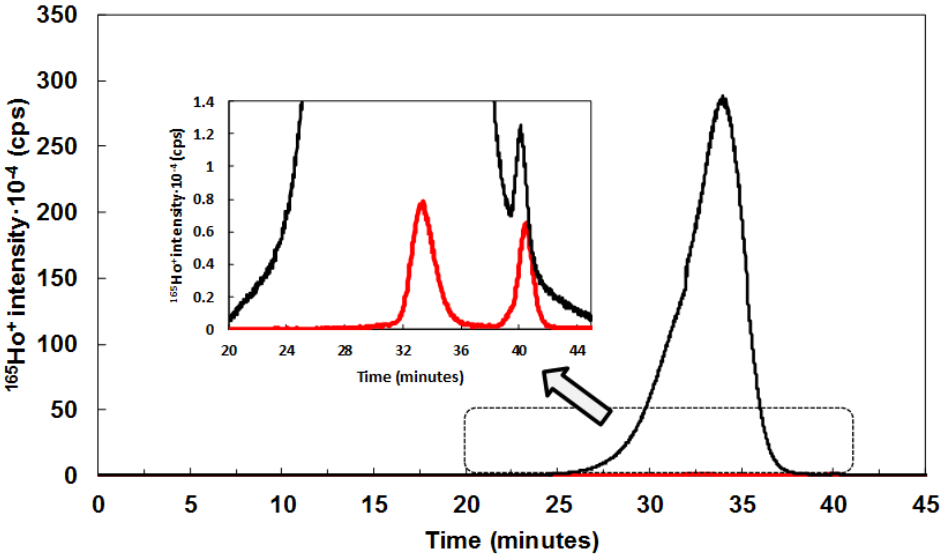
		SEC-ICP-MS		Reference immunoassay procedure[$]	
Antigen	**[Antigen]$_{spiked}$**	**[Antigen]$_{calc}$***	**Recovery (%)**	**[Antigen]$_{calc}$***	**Recovery (%)**
CEA	3.5 ng mL^{-1}	3.4±0.4 ng mL^{-1}	103±11	3.35±0.16 ng mL^{-1}	96±5
	16 ng mL^{-1}	16.6±0.3 ng mL^{-1}	96±2	15.4±0.9 ng mL^{-1}	97±6
	32 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

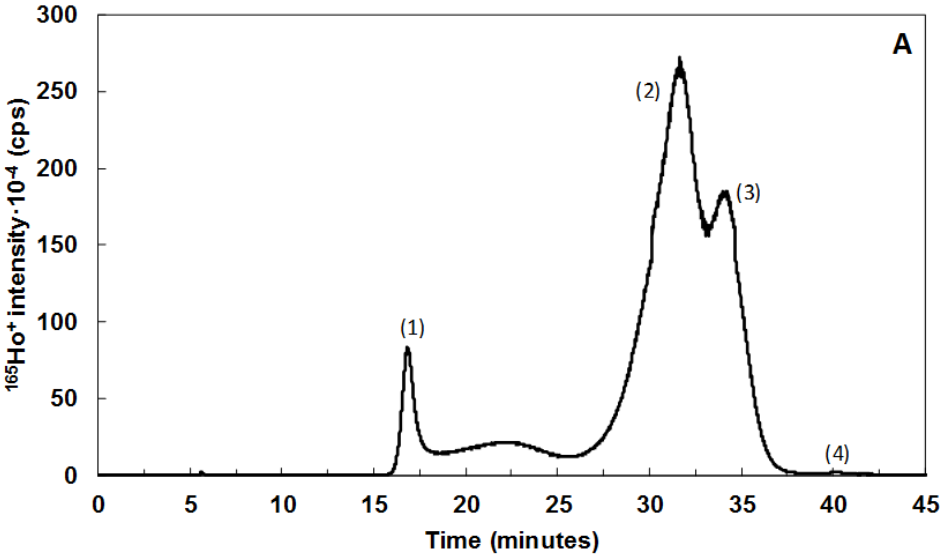
*mean ± t·s·n-1/2, n = 3, P = 95%

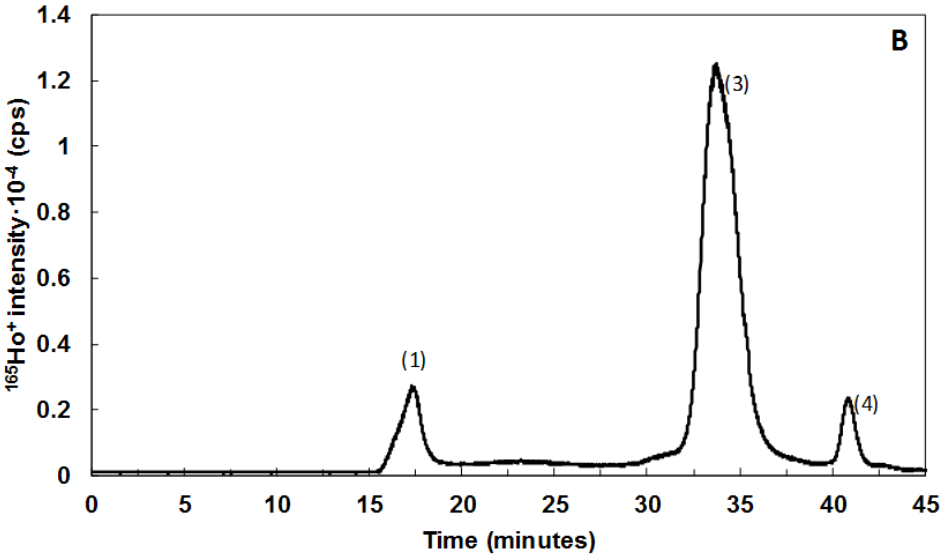
[$] Electro-chemiluminescence immunoassay: CEA, CA 15.3, CA 125; ELISA: sErbB2

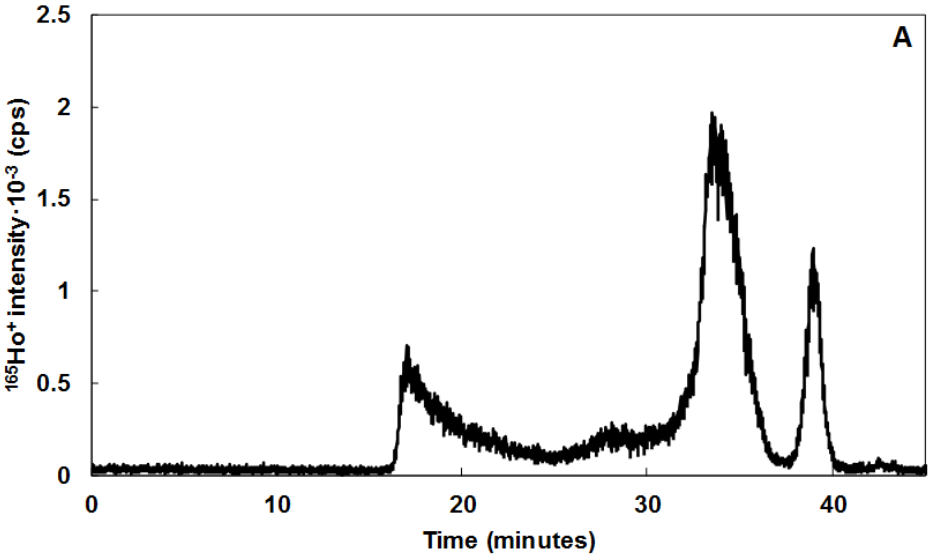
Table 5. Comparison of different methods for CEA, sErbB2, CA 15.3 and CA 125 analysis.

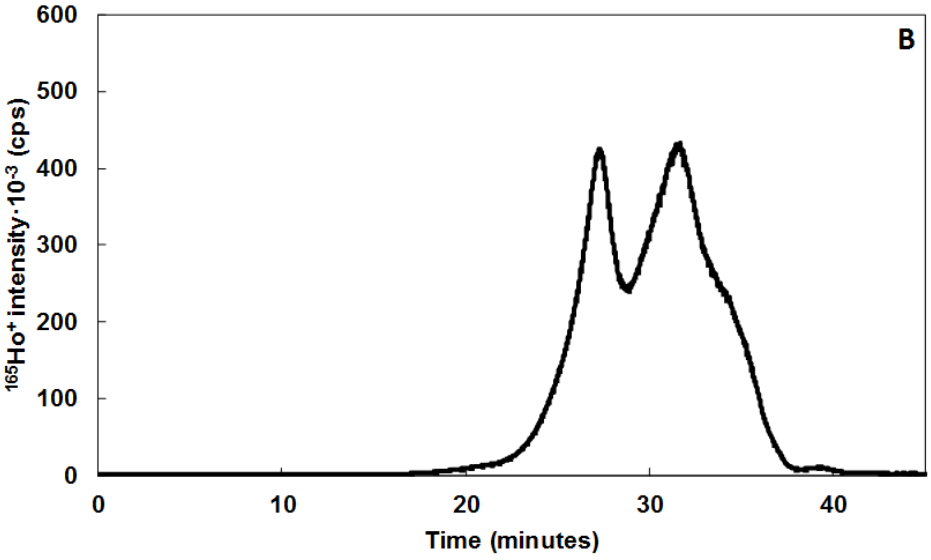
Analytical method	Target protein	LOD	Concentration range	Reference
ICP-MS based magnetic immunoassay	CEA	0.05 ng mL^{-1}	0.2–50 ng mL^{-1}	[27]
Chip-based magnetic immunoassay-ETV-ICP-MS	CEA	0.06 ng mL^{-1}	0.2-50 ng mL^{-1}	[28]
Commercial ELISA kit	CEA	0.2 ng mL^{-1}	0.2 - 250 ng mL^{-1}	Abcam (Cambridge, 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-chemiluminiscence	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