Sensitive targeted multiple protein quantification based on elemental detection of Quantum Dots

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ABSTRACT

A generic strategy based on the use of CdSe/ZnS Quantum Dots (QDs) as elemental labels for protein quantification, using immunoassays with elemental mass spectrometry (ICP-MS), detection is presented. In this strategy, streptavidin modified QDs (QDs-SA) are bioconjugated to a biotinylated secondary antibody (b-Ab₂). After a multi-technique characterization of the synthesized generic platform (QDs-SA-b-Ab₂) it was applied to the multiparametric quantification of five proteins (Transferrin, Complement C3, Apolipoprotein A1, Transthyretin and Apolipoprotein A4) at different concentration levels in human serum samples. It is shown how this generic strategy does only require the appropriate unlabeled primary antibody for each protein to be detected avoiding the need to optimize every specific QDs-to-protein bioconjugation for every particular target protein. Results obtained were validated with those obtained using UV-Vis spectrophotometry and commercial ELISA Kits. As expected, ICP-MS offered one order of magnitude lower DL (0.23 fmol absolute for transferrin) than the classical spectrophotometric detection (3.2 fmol absolute). ICP-MS precision and detection limits, however turned out to be compromised by procedural blanks. The full analytical performance of the ICP-MS-based immunoassay proposed was assessed for detection of transferrin (Tf), present at the low ng mL⁻¹ range in a complex “model” synthetic matrix, where the total protein concentration was 100 µg mL⁻¹. Finally, ICP-MS detection allowed the quantitative control of all the steps of the proposed immunoassay, by computing mass balances obtained, and the development of a faster indirect immunoassay format where the plate wells were directly coated with the whole protein mixture sample.

KEYWORDS

Quantum Dots, immunoassay, labeling, elemental mass spectrometry, protein quantification,x

1. INTRODUCTION

Quantum Dots (QDs) are inorganic nanoparticles with outstanding photoluminescent properties [1]. They are very attractive as highly valuable novel fluorophores to improve bioanalytical applications based on biolabeling and bioimaging methodologies [2,3]. So far, however, such methods are still far from being well established for quantitative bioanalytical measurements. In fact, the use of QDs as labels for reliable biomolecule quantification requires a strict control of the synthesis and the surface-modification processes [4]. In this context, previous works demonstrated that inductively coupled plasma mass spectrometry (ICP-MS) can play a pivotal role in the assessment of CdSe/ZnS QDs synthesis [5], solubilization and bioconjugation for their eventual use in reliable quantitative bioassays [6–9].

Non-isotopic standard immunoassays are widely considered as one of the methods of choice for the quantification of target proteins in complex mixtures as they offer a combination of sensitivity, specificity and cost-effectiveness. Nowadays, UV-Vis spectrophotometry is the reference detection technique in ELISA immunoassays. In this context, recent developments in biofunctionalized nanoparticles (NPs) have allowed significant improvements, including better sensitivity, specificity, multiplexing ability and response time [10,11]. In addition, the use of NPs as labels allows easy coupling to optical, electrochemical, piezoelectric, electrical, and mechanical devices as transduction means [12,13]. In most of those applications, the well-established specificity and high affinity of biotin (b) noncovalent interaction with streptavidin (SA) provide the basis for developing selective assay systems, particularly useful for antibodies [14]. However, covalent modification of either carboxyl or amine groups in the constant fraction of the primary antibodies can adversely affect their binding properties. In these cases, a general strategy consists of the use of a SA-coated NP labeled with biotinylated secondary antibodies (b-Ab₂) able to recognize the primary one (Ab₁), which in turn targets specifically the protein under study. Such functionalization has been already reported for

QDs in some bioanalytical applications [15–17]. A rough estimation of the CdSe/ZnS QDs-SA stoichiometry using gel electrophoresis has been reported [18]. Interestingly, the b-SA system could be established in this way as a linker system for detecting different targets (e.g. proteins), providing a significant signal amplification along with an additional degree of flexibility [19,20].

On the other hand, ICP-MS has been successfully introduced in the latest years as a complementary tool for sensitive protein detection [21] and quantification at the trace and ultratrace levels [22]. One of the most promising applications in this sense relies on the use of element-labeled immunoassay strategies [23]. Several elemental labeling strategies have been applied for that purpose [24]. Among them, the use of NPs as elemental labels for antigens or antibodies stands out as a most promising strategy since that allows the introduction of hundreds of atoms per protein molecule. Of course, such “element amplification” can significantly lower the protein detection limit by ICP-MS. Several metal NPs, including AuNPs [25,26], AgNPs [27], Ag deposited on AuNPs [28], PbS-NPs [29] or even metal oxides, have been tested for this purpose so far. However, QDs appear to exhibit superior properties as elemental labels due to the possibility to provide complementary fluorescence, a feature which can be critical to control possible NP degradations [8] and to open the door to imaging applications. In addition, CdSe QDs allow multiplexing analysis, labeling each Ab with one of the numerous isotopes available (i.e. 8 from Cd and 5 from Se). Surprisingly, the number of reported immunoassays based on QDs as elemental labels for protein quantification is very limited so far [30–32]. A common disadvantage of all such reported strategies is the need of individual and tedious optimization of the QDs bioconjugation to every specific target (antigen/protein) or primary antibody. On the other hand, a remarkable advantage of ICP-MS over conventional spectrophotometric detection in quantitative immunoassays is ICP high tolerance to sample matrix (matrix effects can produce a severe

influence on the luminescent emission of bioconjugated QDs as demonstrated by Trapiella-Alfonso[33] and Giesen [23].

We describe here the development and in-depth characterization of a generic immunoassay platform, based on QDs and ICP-MS detection, for “amplified” protein quantifications which rules out the need for specific bioconjugation procedures for each target protein. The flexibility and the robustness of the proposed approach have been tested for the multiparametric determination of serum proteins [34]. Results were validated using the mass balances attained by ICP-MS and after their critical comparison to those obtained by commercial ELISA kits. Finally, the analytical performance characteristics have been assessed for Transferrin using both sandwich and indirect immunoassay formats.

2. MATERIAL AND METHODS

2.1 Reagents, Solutions and Materials.

All experiments were carried out with analytical grade chemical reagents used as received without further purification. Deionised ultrapure water (18.2 MΩ/cm) was obtained with a Milli-Q system (Millipore, Bedford, MA).

CdSe/ZnS Qdot® 565 streptavidin conjugate (QDs-SA) was acquired from Invitrogen (Karlsruhe, Germany). Agarose D1 Medium EEO from Pronadisa (Madrid, Spain), Tris-Borate EDTA Buffer (TBE) from Sigma-Aldrich (St. Gallen, Switzerland), Glycerol 85% from Merck (Darmstadt, Germany) and Bromophenol Blue from Sigma-Aldrich were used in Gel Electrophoresis (GE). Iminobiotin agarose and spin columns screw caps were purchased from Thermo Fisher Scientific (Bremen, Germany). Sodium chloride from Merck, ammonium acetate from Panreac (Barcelona, Spain) and Acetic Acid (≥ 99.5%) from Sigma-Aldrich. Plastic syringes (1 mL) from BD (NJ, USA). Ammonium bicarbonate (grade puriss.) from Sigma-Aldrich and ammonium solution (28-30%) from Merck were used as size

exclusion chromatography mobile phase. A full reagent list is presented in the Supporting Information.

2.2 Instrumentation.

Agarose Gel Electrophoresis (GE). QDs-SA and QDs-SA-b-Ab₂ bioconjugates were electrophoresed in an electrophoresis apparatus (Mini-Sub Cell GT system Bio-Rad, Hercules, CA, USA) connected to a power supply Power Pac 300 (Bio-Rad).

Size Exclusion Chromatography (SEC). A column Superdex 200 10/300 GL (GE Healthcare Bio-Sciences AB, Staffanstorp, Sweden) was selected to demonstrate the successful chemical union between the QDs-SA derivate and b-Ab₂. A dual piston liquid chromatography pump (Shimadzu LC-10AD, Shimadzu Corporation, Kyoto, Japan) was used, as well as two different detectors (Fluorescence and ICP-MS). Sample injections were performed using a Rheodyne valve, model 7125 (Cotati, CA, USA) fitted with a 50 µL injection loop, and a Hamilton syringe of 250 µL.

Fluorescence Spectrometer. QDs detection was achieved with the Varian Cary Eclipse Fluorescence Spectrometer (Varian Iberica, Madrid, Spain) equipped with a xenon discharge lamp (peak power equivalent to 75 kW), a Czerny-Turner monochromator and photomultiplier tube detector (Model R-298).

ICP-MS instrument. Quadrupole-based Agilent 7700x (Agilent Technologies, Tokyo, Japan) was used as elemental detector of SEC. Elemental measurements were carried out on Triple Quad ICP-MS (Agilent 8800 ICPQQQ, Tokyo, Japan) for determination of Cd in the immunoassays, due to the high sensitivity required. The sample introduction system consisted in both cases of a concentric nebulizer with double-pass glass spray chamber Scott type (Glass Expansion, Romainmotier, Switzerland).

Fluorescence and ICP-MS optimized conditions are given in Tables S-1 and S-2, respectively.

QDs-based ELISA. Flat-bottomed Microtest™ 96-well ELISA plates (BD Falcon, BD Biosciences, Bedford, MA, USA) were used in all immunoassays. Plates were read at 450 nm on an ELx800 plate reader (BIOTEK Instruments, Inc., Winooski, VT, USA). An ultrasonic bath (Ultrasons, 9 L, JP Selecta, Barcelona, Spain) was used to facilitate the acid digestion.

2.3 Procedures.

Preparation of the QDs-labeled b-Ab₂ bioconjugates (QDs-SA-b-Ab₂). Equal volumes of QDs-SA (66 nM) and b-Ab₂ (13.2 nM) solution both in PBS-1% BSA were mixed at room temperature with constant magnetic stirring for 2 hours (a molar ratio of 5:1). The detailed description of characterization methods used for QDs-SA and QDs-SA-b-Ab₂ and the protocols of QDs-based ELISA for Tf determination with spectrophotometric and elemental detection are presented in the Supporting Information.

The optimized immunoassay developed for Tf determination was applied to Complement C3 (C3), Apolipoprotein A1 (APOA1), Transthyretin (TTR) and Apolipoprotein A4 (APOA4) in serum samples. Optimized concentrations for capture and detection antibodies are shown in Table S-3. Serum samples were diluted to obtain a concentration in the linear range of the calibration curve for each protein.

3. RESULTS AND DISCUSSION

3.1 Characterization of QDs and their bioconjugates.

The commercial QD-streptavidin conjugate (QD-SA) used throughout this work consists of a nanometer-scale core of a semiconductor material (CdSe), coated with an additional semiconductor shell (ZnS) and showing a fluorescence emission maximum centered at 565 nm. This QD is surface-modified with a polymer layer that allows it to be water soluble and further conjugated to 5-10 streptavidin units. The final conjugate has a size close to a large macromolecule or protein (~15–20 nm diameter). As such nanomaterial is functionalized

175 with SA, the QDs can specifically bind appropriate biotinylated antibodies (Ab) which, in
176 turn, would recognize the desired target proteins in complex samples.

177 In order to develop a generic approach a biotinylated secondary Ab (b-Ab₂), able to bind to
178 the heavy chain of a primary Ab (Ab₁), was selected. Thus, the recognition capacity of Ab₁
179 against the target protein would not be significantly affected. Such generic quantitative
180 platform (QDs-SA-b-Ab₂) could be used against different protein targets just by changing the
181 corresponding Ab₁ used. Then, the targeted protein(s) would be indirectly quantified by ICP-
182 MS determination of the Cd (or Se) present in the nanoparticle tag. The number of Cd atoms
183 introduced per protein molecule (in the corresponding bioconjugate) will determine the final
184 sensitivity achievable with ICP-MS. External Cd calibration, after acidic digestion of an
185 aliquot of the original QDs-SA commercial conjugate solution, showed that the number of
186 atoms of Cd per QD nanoparticle was 153 ± 15 (n=6).

187 The chemical stability of the conjugation of SA to QD in the commercial QDs-SA was tested
188 using an iminobiotin agarose affinity column where Cd was quantified by ICP-MS in the
189 loaded, washed and retained fractions, in order to obtain an accurate elemental mass balance.
190 The observed results have been plotted in Figure S-1. As can be seen, most of the Cd (80 ± 5
191 %, n=3) was retained and then eluted in the elution step, confirming a chemically stable
192 binding between functional SA and QDs and their capacity to bind biotin molecules.

193 For further characterizing such QDs-SA and their subsequent functionalization with the b-
194 Ab₂ a multi-technique approach was assayed. We should stress here that while an excess of
195 b-Ab₂ could interfere later on the immunoanalysis results, free QDs would not because they
196 should be washed out during the different washing steps during the immunoassay. Therefore
197 the use of an excess of QDs-SA, during the incubation step with b-Ab₂, was preferred. At this
198 point should be stressed that final quantitative results would be independent of the actual
199 stoichiometry QDs-SA to b-Ab₂ obtained as it would apply in the same way to calibration
200 standards and samples.

Size Exclusion Chromatography (SEC) coupled with integrated ICP-MS and fluorescence detection was tried first. Chromatographic profiles obtained for QDs-SA and for QDs-SA-b-Ab₂ bioconjugates are shown in Figure 1 using the two different detectors. In order to evaluate the column recovery of the NPs, flow injection analysis (FIA) with the species under study (QDs-SA and QDs-SA-b-Ab₂) was carried out. Recoveries turned out to be quantitative in both cases, $101 \pm 4 \%$ and $107 \pm 8 \%$, respectively (detailed data are presented in Table S-4). Interestingly, the original QDs-SA sample eluted in a single well-defined peak at 10 min. This result seems to indicate that the species distribution previously observed using affinity chromatography (Figure S-1) was most likely due to QD steric effects hampering SA interaction with immobilized biotin residues for some of the QD-SA conjugates ($20 \pm 1 \%$, $n=3$).

As clearly shown by both detection modes, the final bioconjugate (solid line) eluted first, at a higher hydrodynamic region as the original QDs-SA species (dashed line). As expected, some excess of the original QDs-SA could be also detected at the original elution volume (10 mL in Figure 1). Interestingly, the peak corresponding to the final bioconjugate was thinner than the peak corresponding to the excess of QDs-SA. The absence of free b-Ab₂ in the resulting solution was further confirmed because any fluorescence peak was detected at its original elution volume (14 mL, see Figure 1B). The significant decrease of the fluorescent emission intensity observed in Figure 1B for the QDs-SA-b-Ab₂ bioconjugate, in comparison to the injected original QDs-SA species, could be due to the fact that the biomolecules bound to the QD can alter its fluorescence properties [16]. Of course, elemental detection (Figure 1A) does not suffer from this effect and that fact enabled us to derive some quantitative conclusions from the elemental profile (solid line, Figure 1A). It is noteworthy that results demonstrated that $64 \pm 5 \%$ of the injected QD eluted as QD-SA-b-Ab₂ while $36 \pm 2 \%$ eluted as excess of the original QD-SA (10 mL).

---Preferred site for Figure 1---

The bioconjugation of QDs-SA to b-Ab₂ was further assessed using agarose gel electrophoresis (GE). As can be seen in Figure 2, a significant separation between the different QDs-derived species was observed: whereas the free negatively charged QDs, used as reference [30], run to the top of the gel (lanes 1,2,7 and 8), the original QDs-SA (lanes 3,4) run slower and fell into the middle region of the gel. The long tail observed is likely due to the different number of SA associated to the QDs (between 5 and 10), indicating considerable size dispersion. Conversely, the QDs-SA-b-Ab₂ bioconjugate mixture (lanes 5, 6) hardly entered the gel (narrow lines at the bottom), due to the size enlargement originated by the incorporation of the b-Ab₂. Again, a significant excess of QDs-SA (bands in the middle of the gel) could be detected in the QDs-SA-b-Ab₂ samples. Interestingly, the results showed that reaction of the b-Ab₂ with the high excess of different QDs-SA conjugates seems to be selective. The QDs surrounded by a higher number of SA molecules reacted more efficiently with the low number of b-Ab₂ molecules available. This is the reason why the low part of the band observed for the original QDs-SA (lanes 3 and 4) disappeared in the lanes corresponding to the QDs-SA-b-Ab₂ reaction mixture (lanes 5 and 6). In fact, this effect of Figure 2 could also explain the different peak widths observed previously using SEC (Figure 1).

---Preferred site for Figure 2---

A last proof of successful bioconjugation was obtained by resorting again to the iminobiotin agarose affinity columns. Results are shown in Figure S-1 and illustrate the behavior of the two species, QDs-SA and QDs-SA-b-Ab₂. The injection of the QDs-SA-b-Ab₂ bioconjugation mixture demonstrated by ICP-MS that the elution of the vast majority (85 ± 4

%, n=3) of the QDs appears in the load fraction. It seems that the bioconjugate between the big Ab₂ and the SA molecules surrounding the QD, hampered the interaction of the remaining free SA sites in the QDs-SA-b-Ab₂ with the immobilized iminobiotin residues of the column. As previously observed by SEC and GE, a significant QDs-SA excess could be quantified in the eluting fraction. It is worth stressing that, as indicated above, the steric effects were already responsible for a significant amount (20 ± 1 %), of the original QDs-SA injected molecules eluting without interaction in the load fraction.

3.2 Development of QDs-based sandwich ELISA with parallel spectrophotometric and elemental detection.

The protein Transferrin (Tf) was selected as first target protein for optimization of the proposed QDs-based immunoassay. In order to check the eventual detrimental effect on the recognition capabilities of the generic b-Ab₂ for anti-Tf Ab₁, after labeling with the QDs-SA, we resorted to a simple spectrophotometric immunoassay. The UV-Vis signals obtained using both, QDs-labeled (QDs-SA-b-Ab₂) and free b-Ab₂, were compared. The observed change of the final color was lower than 10% (labeling with the QDs-SA did not produce a significant effect on the Ab recognition capabilities).

The well-established UV-Vis absorption detection was used as the reference technique for validation of the here investigated ICP-MS detection. As shown in Figure 3A, the wells of the microtiter plates were coated with the capture anti-Tf Ab and incubated with the samples. After subsequent incubations with solutions containing the specific Ab₁ anti-Tf, the generic QDs-SA-b-Ab₂ bioconjugate and finally, the IgG tertiary antibody complexed with peroxidase (HRP-Ab₃), the spectrophotometric detection at 450 nm was carried out. After such measurements, ELISA plates were subjected to the appropriate sample treatment in order to carry out the parallel ICP-MS Cd determination from corresponding QDs cores (see Scheme S-1).

---Preferred site for Figure 3---

The analytical performance characteristics of the QDs-based non-competitive sandwich immunoassay for Tf detection, using both detection techniques, were then evaluated. For that purpose, a series of standard aqueous solutions with different Tf concentrations ranging from 15 to 0.63 ng mL⁻¹ were prepared. A typical non-competitive ELISA standard curve was obtained using spectrophotometric detection, which confirmed a successful development of the proposed immunoassay. A similar trend was also observed for the ICP-MS elemental Cd detection. The obtained analytical performance characteristics, presented in a comparative manner, are given in Table 1, first and second columns.

---Preferred site for Table 1---

Cd blank turned out to be a critical parameter here (higher blanks are likely due to unspecific adsorptions of QDs occurring during the procedural steps typical in the ELISA plate). The blank compromised clearly ICP-MS achievable detection limits, accuracy and precision. Therefore, the Cd blank was appropriately characterized (n=6) and subtracted from the measured ICP-MS signal [30].

In order to assess the precision, each calibration point was carried out in triplicate. Average relative standard deviation (% RSD) obtained along the calibration curve, corresponding to the intraassay repeatability, was higher using ICP-MS (14%) than spectrophotometric detection (7%, see Table 1). Most likely the poorer precision with the ICP-MS is derived from spread of QDs-size distribution. This affects significantly the actual number of Cd ions per NP and therefore the ICP-MS detection. In addition, as stated above, Cd blanks were significant and limited the final precision of the analytical measurement.

The detection limits were evaluated on the basis of the signal-to-noise ratio (3σ IUPAC criterium) and are also given in Table 1. For the spectrophotometric detection, DL was found to be 2.51 ± 0.92 ng mL⁻¹ of Tf (3.2 ± 1.2 fmol), a figure of the same magnitude reported so far for colorimetric commercial transferrin ELISA quantification kits [35]. ICP-MS NPs amplified elemental sensitivity provided a one order of magnitude better DL, 0.18 ± 0.06 ng mL⁻¹ of Tf (0.23 ± 0.08 fmol), in the order of magnitude of the most sensitive QDs-based ICP-MS immunoassays published so far [24,31].

Adequate regression coefficients (R^2) of the linear region of the calibration curves were obtained for UV (0.991) and ICP-MS (0.996). However, substantial differences were observed when evaluating the linear range in each case: by spectrophotometric detection such linear range is always limited in the upper edge for the maximum absorbance signal, specially when longer times for color development are used to measure low and high content protein samples in the same ELISA plate. Conversely, typical instrumental ICP-MS linear range extends up to 9 orders of magnitude. This, in principle, should allow favorable simultaneous detection of very low and high protein levels without the need of tedious dilution steps (as long as the working range of the antibodies allows it).

Another important aspect, if compared to spectrophotometric detection, is that ICP-MS enables us to assess the absolute quantitative distribution of Cd, all along the different steps of the immunoassay. Thus mass balances were performed by measuring the Cd content in the original QDs-SA-b-Ab₂ solution used (taken as reference), in the washed and final solutions as well for some representative samples at the high, medium and low analyzed transferrin levels (triplicates at 0.63, 3 and 15 ng mL⁻¹ of Tf). As can be observed in the last row of Table 1, the obtained Cd recovery was always close to 100%, confirming the capabilities of ICP-MS to provide quantitative control over the different steps common in proteomics sample preparation strategies [36].

3.3 Multiparametric immunoassay in real sample analysis.

The robustness and multiparametric potential of the developed approach was evaluated by the determination of 5 different proteins (Tf, C3, APOA1, TTR and APOA4) in serum samples. Quantitative results obtained were validated by comparison with those obtained using commercial ELISA kits with spectrophotometric detection. As can be seen in Table 2, both set of results showed a satisfactory matching in most cases all along the protein concentration range covered by the five proteins under study (almost 2 orders of magnitude). Furthermore, analyzed proteins showed a very broad MW range (from 30 to 187 KDa for APOA1 and C3, respectively) and physico-chemical properties. A remarkable fact is that the individual optimization and characterization of the QDs bioconjugations to the corresponding primary antibodies, which would involve a lot of time and effort (due to possible functionality losses, steric effects ...), can be saved by the use of the generic platform QDs-SA-b-Ab₂. In this case, its application to any desired target protein just involves the selection of the appropriate primary Abs.

---Preferred site for Table 2---

3.4 Target protein quantification at the ultratrace level.

The general analytical performance characteristics of the developed approach for highly sensitive protein determinations was finally assessed using a synthetic “model” protein matrix (100 µg mL⁻¹ of total protein see Supporting Information) fortified with increasing trace amounts of Tf as target protein (1.25, 7.5, 50 and 120 ng mL⁻¹ of Tf.). As shown in Table 3, Tf recoveries obtained were very satisfactory and matched the results obtained by the standard ELISA method in samples with the two higher Tf concentrations. However, only ICP-MS was able to provide reliable quantitative results (with recoveries again close to 100%) for the very low Tf level samples, indicating the high potential of this approach for ultratrace target protein quantifications.

3.5 Matrix effects evaluation with ICP-MS detection

ICP-MS tolerance to matrix effects is well known and so was used here for faster non-competitive indirect ELISA with the developed QDs-based generic platform. This alternative would be extremely useful when the described sandwich ELISA scheme is not feasible because the capture step is not possible. This could be the case for small targeted biomolecules having only one binding site with the antibody [37] or for recently discovered targets having only one commercially available host-species antibody.

As shown in Figure 3, all the steps of such indirect ELISA format were the same as the sandwich format, except that the plate was coated directly with the samples. In other words, all serum proteins present were fixed over the surface of the plate and this renders later specific Ab₁ recognition, and subsequent detection, much more difficult. As expected, the huge excess of matrix proteins severely interfered the detection of Tf traces when the spectrophotometric reference method was used for detection while the ICP-MS measurement was not significantly affected. Notably, a similar strong matrix effect had been already observed for the direct quantification of a steroid hormone in raw milk using a QDs-based immunoassay with fluorescence detection [33]. In fact, here the spectrophotometric detection provided only two points showing an analytical signal superior to the blank. Therefore, analytical figures of merit of the more conventional detection could not be evaluated using this indirect format, while observed indirect ELISA with ICP-MS detection analytical characteristics are shown in the last column of Table 1. As expected, the DL obtained (1.17 ± 0.18 ng mL⁻¹ of Tf) was not as low as that obtained using a capture antibody but still provided similar precision and quantitative recoveries. It is interesting to note that for ICP-MS detection the Ab₁ recognition capacity seems still satisfactory even in such untreated complex

matrices (something unattainable using spectrophotometry as detection technique). Of course, the most significant benefits of such indirect approach, related to the most sensitive sandwich format, were the shorter analysis time (2 hours less), as one of the incubating steps is avoided, the simpler sample manipulation and a significant money saving because the capture antibody is not necessary.

4. CONCLUSIONS

In summary, we present here a novel generic immunoassay platform for multiparametric quantification of proteins, which makes use of the advantages provided by QDs labelling amplification and ICP-MS detection. It is based on the labeling of secondary antibodies and has been validated by the multiparametric quantification of five proteins, previously quantified by standard ELISA kits, at different concentration levels in human serum. This approach provides a way out to the need for the cumbersome and specific bioconjugation of the QDs to the corresponding primary antibody for every target analyte (protein). The in-depth characterization of the proposed QDs-SA-b-Ab₂ generic platform, carry out by using chromatography, gel electrophoresis and affinity, revealed that: (i) no excess of free b-Ab₂ (that would affect the accuracy of the quantitative results) was present in the final solution, (ii) up to 64% of the original QDs-SA was derivatized into QDs-SA-b-Ab₂ and (iii) the QDs-SA conjugates with higher SA stoichiometry reacted more efficiently with the limited number of b-Ab₂ available.

The precision associated to the sandwich-type immunoassay using ICP-MS detection (10-15% RSD) was limited by procedural blanks. In any case, ICP-MS provides one order of magnitude lower DL (0.23 fmol of protein) than standard spectrophotometric detection. More importantly, ICP-MS detection allowed the quantitative control of the several immunoassay steps via appropriate mass balances.

Finally, the potential of the elemental amplification by the QD-labeling along with the high sensitivity of the ICP-MS detection, was demonstrated by the successful determination of Tf at the 1 ng mL⁻¹ level, while only samples containing more than 50 ng mL⁻¹ could be quantified using spectrophotometric detection. Another strong point of the ICP-MS detection is the robustness of the signal, as demonstrated by the satisfactory results obtained after skipping the capture step in the bioassay. Conversely, spectrophotometric detection was seriously hampered by concomitant matrix proteins. This could be interesting for the fast and simpler quantification of target analytes with only one binding site available (e.g. haptens) as well as for newly discovered biomarkers for which different primary antibodies are not available yet.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at

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FIGURE CAPTIONS

Figure 1. SEC-ICP-MS (**A**) and SEC-Fluorescence at 565 nm (**B**) chromatograms from the original QDs-SA (dashed line) and functionalized QDs-SA-b-Ab₂ (solid line) species.

Figure 2. Gel electrophoresis of the QDs and their bioconjugates. The samples in lanes 1, 2, 7 and 8 correspond to negatively charged free QDs used as reference. The samples in lanes 3-4 and 5-6 correspond to QDs-SA and QDs-SA-b-Ab₂, respectively

Figure 3. Generic Platform for Quantum Dot-based sandwich immunoassays. ELISA sandwich format (**A**) and ELISA indirect format (**B**).

Figure 1

Figure 2

A.

B.

ELISA PLATE

Legend:

- Ab capture: anti-Transferrin
- Transferrin
- Protein in the matrix
- Ab₁: anti-Transferrin
- Ab₂: biotinylated anti-Ab1
- QDs-SA: elemental label
- Ab₃: HRP-anti-mouse IgG
- Substrates: TMB and H₂O₂
- Coloured product

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Table 1. Analytical Characteristics of the different QDs-based immunoassays. The first and second columns correspond to the sandwich ELISA with spectrophotometric and elemental detection respectively. The third column corresponds to the indirect ELISA with elemental detection. Uncertainty corresponds to 1 standard deviation.

Parameter	Sandwich (Spectr.)	Sandwich (ICP-MS)	Indirect (ICP-MS)
DL (ng mL ⁻¹)	2.51 ± 0.92	0.18 ± 0.06	1.17 ± 0.18
Repeatability RSD (%)	7	14	17
R ² (Linearity)	0.991	0.996	0.993
Mass Balance (%)	-----	96 ± 9	96 ± 6

Table 2. Quantitative results obtained for Tf, C3, APOA1, TTR and APOA4 in the real serum samples analyzed using the QDs based generic approach with ICP-MS detection compared with the commercial ELISA Kit with spectrophotometric detection. Uncertainty corresponds to 1 SD (n=3) in the case of the ICP-MS strategy. In the case of the commercial ELISA kit, a single analysis was performed and uncertainty corresponds to the value provided by the supplier (10 % RSD).

Sample	mg mL ⁻¹ of Tf		mg mL ⁻¹ of C3		mg mL ⁻¹ of APOA1		mg mL ⁻¹ of TTR		mg mL ⁻¹ of APOA4	
	kit	ICP-MS	kit	ICP-MS	kit	ICP-MS	kit	ICP-MS	kit	ICP-MS
M1	4.4 ± 0.4	3.8 ± 0.8	3.8 ± 0.4	2.7 ± 0.5	2.8 ± 0.3	1.6 ± 0.2	1.9 ± 0.2	1.5 ± 0.2	0.082 ± 0.008	0.078 ± 0.008
M2	2.7 ± 0.3	3.4 ± 0.4	3.0 ± 0.3	3.1 ± 0.5	1.4 ± 0.2	1.2 ± 0.1	0.60 ± 0.06	0.68 ± 0.07	0.037 ± 0.004	0.031 ± 0.004
M3	3.9 ± 0.4	5.6 ± 1.0	3.5 ± 0.4	2.9 ± 0.3	2.0 ± 0.2	1.3 ± 0.3	1.1 ± 0.1	1.1 ± 0.2	0.060 ± 0.006	0.05 ± 0.01
M4	3.3 ± 0.3	3.0 ± 0.8	3.0 ± 0.3	3.6 ± 0.5	1.2 ± 0.1	1.3 ± 0.3	0.62 ± 0.06	0.52 ± 0.09	0.042 ± 0.004	0.05 ± 0.01
M5	3.1 ± 0.3	3.4 ± 0.6	3.3 ± 0.3	3.2 ± 0.5	2.4 ± 0.2	1.4 ± 0.2	0.80 ± 0.08	0.9 ± 0.1	0.037 ± 0.004	0.040 ± 0.009
M6	3.5 ± 0.4	4.4 ± 0.8	2.9 ± 0.3	2.4 ± 0.2	1.9 ± 0.2	1.8 ± 0.4	0.82 ± 0.08	0.7 ± 0.2	0.033 ± 0.003	0.037 ± 0.009

Table 3. Recovery (%) found for the complex model samples (total protein content of 100 $\mu\text{g mL}^{-1}$) fortified with Tf at very low concentration levels and analyzed using the generic approach and sandwich ELISA formats, with parallel spectrophotometric and ICP-MS detection (1 SD, n=3).

Sample (Tf, ng mL ⁻¹)	Spectrophotometry	ICP-MS
120	105 $\pm$ 7 %	94 $\pm$ 4 %
50	89 $\pm$ 12 %	95 $\pm$ 7 %
7.5	---- (< QL)	105 $\pm$ 8 %
1.25	---- (< QL)	92 $\pm$ 10 %