JAAS

TECHNICAL NOTE

View Article Online
View Journal | View Issue

Detection of HIV-1 p24 antigen using streptavidin–biotin and gold nanoparticles based immunoassay by inductively coupled plasma mass spectrometry

Cite this: *J. Anal. At. Spectrom.*, 2014, **29**, 1477

Qian He,[ab] Zhenli Zhu,*[a] Lanlan Jin,[a] Lu Peng,[c] Wei Guo[a] and Shenghong Hu[ab]

A sensitive assay for detection of HIV-1 p24 antigen by inductively coupled plasma mass spectrometry (ICP-MS) was developed using a biotin–streptavidin (BA) system and gold nanoparticles (Au NPs) based immunoassay. In this immunoassay, the p24 antigen was firstly captured by an immobilized anti-HIV-1 p24 monoclonal antibody. After immunoreactions with the biotinylated anti-p24 polyclonal antibody and Au NPs-labeled streptavidin, diluted HNO_3 (5%, v/v) was used to dissociate Au NPs, which were then introduced to the ICP-MS for measurements. Under the optimized conditions, the calibration graph for the p24 antigen was linear in the range of 7.5–75 pg mL^{-1} with a detection limit of 1.49 pg mL^{-1} (3σ, $n = 5$). The relative standard deviation (RSD) for three replicate measurements of 37.5 pg mL^{-1} of the p24 antigen was 3.7%. Other proteins, such as human IgG, human HSA, human CEA and human AFP, did not obviously interfere with the assay for p24 antigen. This method was also applied to measure p24 concentrations in artificially positive human serum samples. Compared with the biotin–streptavidin enzyme-linked immunosorbent assay (BA-ELISA) method for p24 antigen detection, the ICP-MS linked immunoassay process deals with Au NPs – tagged instead of enzyme-conjugated antibodies, making it free of toxic enzyme substrate reagents. In addition, it also simplifies the experimental process and saves the experimental time, since the color rendering steps are omitted. The proposed approach provides a sensitive method for HIV-1 p24 antigen determination.

Received 21st January 2014
Accepted 22nd May 2014

DOI: 10.1039/c4ja00026a

www.rsc.org/jaas

1 Introduction

Human immunodeficiency virus (HIV) infection, which causes acquired immunodeficiency syndrome (AIDS) in humans, is considered pandemic by the World Health Organization (WHO).[1] Two closely related types of HIV, designated HIV-1 and HIV-2, have been identified. HIV-1 has a higher toxicity than HIV-2 and is by far the most common cause of AIDS. Detection of HIV-1 p24 antigen will reduce the diagnostic window between the time of human immunodeficiency virus (HIV) infection and laboratory diagnosis than detection of anti-HIV antibody. Therefore, detection of HIV-1 p24 antigen in serum or plasma is one of the traditional methods for early diagnosis of HIV-1 infection.

Several methods have been developed to determine p24 antigen, including boosted enzyme-linked immunosorbent assay (ELISA),[2–5] real-time immuno-polymerase chain reaction (IPCR),[6] magnetic immuno-chromatography (MICT),[7] immunosensing,[8,9] biobarcode amplification (BCA) assay[10,11] and colorimetric detection with the naked eye,[12] *etc.* Among these, ELISA is the most common method for the detection of HIV-1 p24 antigen due to its specificity, simplicity, and low cost over various instrumental methods.[13] However, the conventional HIV-1 p24 ELISA is relatively insensitive and, thus, has limited clinical usefulness. The biotin–streptavidin (BA) system, which is based on the high affinity of streptavidin for biotin (affinity constant = 10^{15} mol^{-1}), offers the possibility of improving the sensitivity of immunoassays, which makes ELISA more effective.[14] Streptavidin binding to biotin is specific enough to ensure that the binding is directed only to the target of interest. Biotin is a small molecule (244.31 Da) that, when introduced into biologically active macromolecules in most cases, does not affect their biological activity, *e.g.*, enzymic catalysis or antibody binding.[15] Thus, the BA system has been introduced into enzyme immunoassays by most commercial ELISA kits for p24 antigen determination. Nonetheless, the BA-ELISA method remains time-consuming and is often limited by moderate sensitivity.

Element tagged immunoassays combined with inductively coupled plasma mass spectrometry (ICP-MS) detection have become an emerging technique in the analysis of biosamples.[16,17] Compared with traditional immunoassays, ICP-MS linked immunoassay deals with element-tagged instead of radioisotope-tagged or enzyme-conjugated antibodies, making it

[a]*State Key Laboratory of Biogeology and Environmental Geology, China University of Geosciences (Wuhan), Wuhan, 430074, China. E-mail: zhuzl03@gmail.com; zlzhu@cug.edu.cn; Fax: +86-27-6788-3456; Tel: +86-27-6788-3452*

[b]*Faculty of Earth Sciences, China University of Geosciences (Wuhan), Wuhan, 430074, China*

[c]*Wuhan Institute of Virology, Chinese Academia of Science, Wuhan, 430071, China*

free of radioactive isotope or toxic enzyme substrate reagents.[18] In the past few years, several reports on ICP-MS linked immunoassay using element-labeled reagents for the detection of various biomolecules have been published. For example, Eu^{3+},[19–22] Ru^{2+},[23] and lanthanide[24–26]–chelate antibody conjugates have been used to develop both direct competitive and non-competitive immunoassays. Another distinguishing feature of ICP-MS linked immunoassay is that high sensitivity could be easily obtained by the use of the nanoparticles (NPs) tags instead of metal ions, due to large quantities of detectable atoms in each NP tag.[27,28] The nanomaterials that have been used in label-based immunoassays by ICP-MS include metal NPs (*e.g.* Au,[27,29–33] Ag), semiconductor NPs (quantum dots (QDs)), *e.g.* PbS,[34] TiO_2 (ref. 35) and so on. Moreover, the ICP-MS linked immunoassays also enable multiplexed and absolute quantification of proteins based on elements or NP labeling,[36–41] *etc.*

In this work, we developed a sensitive assay with a BA system and Au NPs based immunoassay by ICP-MS to detect HIV-1 p24 antigen in human serum. After a typical sandwich-type immunoreaction with anti-HIV-1 p24 monoclonal antibody, p24 antigen, biotinylated anti-p24 polyclonal antibody and Au NPs-labeled streptavidin, the captured Au NPs were released and quantitatively detected by ICP-MS to indirectly determine the p24 antigen concentration. The immunoreaction conditions of the diluted ratio of Au NPs-labeled streptavidin and biotinylated anti-p24 polyclonal antibody were optimized. The specificity of the proposed ICP-MS linked immunoassay was also investigated. Furthermore, it had also been applied to measure p24 concentrations in artificially positive human serum samples. Concentrations of p24 in human serum samples determined by the ICP-MS linked immunoassay were also cross validated with the results of BA-ELISA.

2 Experimental

2.1 Apparatus

Gold measurements were performed using an ICP-QMS (Agilent 7700x, Tokyo, Japan). The optimized parameters are listed in Table 1. The BA-ELISA results were obtained by measuring the absorbance at 450 nm using a microplate reader (Multiskan MK3, Thermo, USA).

Table 1 Operating parameters for ICP-QMS

Parameter	Description
ICP RF power (W)	1400
Carrier gas flow rate (L min^{-1})	0.69
Diluted gas flow rate (L min^{-1})	0.52
Sample uptake rate (mL min^{-1})	1.0
Integrated time (s)	1.5
Acquisition time (s)	9.7
Sampling depth (mm)	8
Replicates	3
Isotope used	^{197}Au
Internal standard used	^{115}In

2.2 Reagents and materials

HIV-1 p24 antigen standards, biotinylated anti-p24 polyclonal antibody (origin concentration, 1.5 μg mL^{-1}) and 96-well ELISA microplates were obtained from the Wuhan Institute of Virology, Chinese Academy of Sciences (Wuhan, China). Bovine serum albumin (BSA) was purchased from Wuhan Chu-ChengZhengMao Science and Technology Engineering Co. Ltd. (Wuhan, China). Tween 20 was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Au NPs (15 nm)-labeled streptavidin (origin concentration, 400 μg mL^{-1}) was purchased from Beijing Biosynthesis Biotechnology Co. (Beijing, China). Human immunoglobulin G (IgG), human serum albumin (HSA), human alpha-fetoprotein (AFP) and human carcinoembryonic antigen (CEA) were purchased from Uscn Life Science Inc. (Wuhan, China). Unless otherwise stated, all the other reagents used in this study were of analytical grade and obtained from Sinopharm Chemical Reagent Beijing Co. Ltd. (Beijing, China).

Control blood samples from healthy persons were provided by volunteers of China University of Geosciences (Wuhan, China). Serum samples were prepared from whole blood by centrifuging at 10 000 × *g* for 5 min in a serum separation vial and then stored as aliquots at −20 °C until analysis.

2.3 Buffers

The buffers and solutions used were:

(1) phosphate buffer (PBS): 8.0 g of NaCl, 3.58 g of $Na_2HPO_4 \cdot 12H_2O$, 0.27 g of KH_2PO_4 and 0.2 g of KCl dissolved in 1 L distilled water (pH 7.4);

(2) coating buffer: 1.59 g of Na_2CO_3 and 2.93 g of $NaHCO_3$ in 1 L distilled water (pH 9.6);

(3) blocking buffer: 5% (w/v, g L^{-1}) BSA in PBS. The blocking solution was stored at 4 °C and used within a week;

(4) washing solution (PBST): 0.24 g of KH_2PO_4, 2.9 g of $Na_2HPO_4 \cdot 12H_2O$, 8.0 g of NaCl, 0.2 g of KCl and 0.1% Tween 20 (v/v) in 1 L distilled water (pH 7.4);

(5) assay solution: 1% (w/v, g L^{-1}) BSA in PBS.

All buffers were prepared using water (18.2 MΩ cm^{-1}) from a Milli-Q water purification system (90005-02, Labconco waterpro ps, Canada).

2.4 Immunoassay protocol

The immunoassay was conducted by following the typical procedure for the sandwich-type immunoreaction (Fig. 1). Initially, a polystyrene 96-well microtiter plate was coated using 100 μL of anti-HIV-1 p24 monoclonal antibody (diluted to 10 μg mL^{-1} with coating buffer) and incubated at 4 °C overnight. The unbound antibody was washed twice with 300 μL of washing solution (PBST) to remove any unbound antibody, and the uncoated active sites of the polystyrene substrate were saturated with 125 μL of blocking buffer, in which BSA was used as a blocking agent to minimize nonspecific adsorption of the antigens in the next step. 75 μL of diluted HIV-1 p24 antigen standards or serum samples and 25 μL of biotinylated anti-p24 polyclonal antibody were pipetted into the wells together and

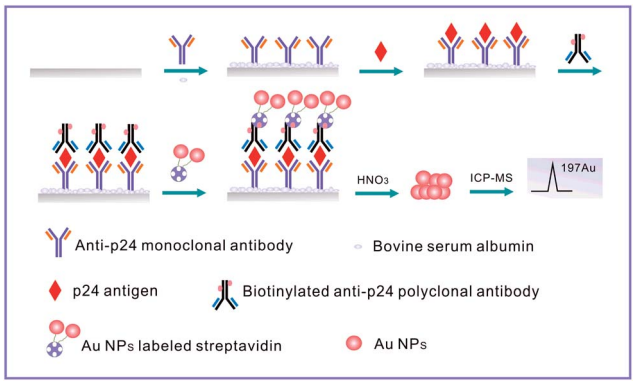

Fig. 1 Schematic diagram of the sensitive assay with the BA system and Au NPs based immunoassay for p24 antigen determination by ICP-MS.

incubated for 1 hour at 37 °C. The unbound antigen and antibody were removed from the plate with 300 μL of washing solution (five times). Then 100 μL of Au NPs-labeled streptavidin was added to each well and incubated for 30 min at 37 °C followed by washing five times with PBST. Finally, a 200 μL aliquot of 5% (v/v) HNO_3 solution was added to each well and shaken for 10 min to dissociate Au NPs from the immunoassay complex. Samples were diluted to 4 mL with 5% HNO_3 solution and introduced to the ICP-MS for determination by a peristaltic pump. The ^{197}Au intensities by ICP-MS were proportional to the concentrations of HIV-1 p24 antigen in the sample. External calibration was used for the quantitative determination of HIV-1 p24 antigen. In was used as an internal standard element of Au to correct the fluctuation of the instrument.

3 Results and discussion

3.1 Effect of diluted ratio of Au NPs-labeled streptavidin

During the sandwich immunoassay, the dilution ratio of the Au-NPs labeled antibody is a key factor affecting the detection sensitivity and the nonspecific binding of Au-NPs labels.[42] In this work, the influence of the diluted ratio of Au NPs-labeled streptavidin on the Au signal/background intensity ratio (S/B) was investigated from 1 : 800 to 1 : 100 to obtain a favorable S/B value (Fig. 2). The Au signal intensities were all investigated with a p24 antigen concentration of 75 pg mL^{-1}. The Au background intensities had been tested as described in Section 2.4, but using assay solution instead of p24 antigen. It was found that the Au signal and background intensities increased with the decrease of the diluted ratio of Au NPs-labeled streptavidin. When the diluted ratio was lower than 1 : 400, the Au background intensities increased sharply and even higher than the Au signal intensities. Au-NPs labels in high concentration are usually used to provide high sensitivity for the high concentration range of the analyte. However, nonspecific binding of Au-NPs mainly derived from charge attraction, hydrophobic absorption and dative binding is increased accordingly with the increase of Au-NPs label concentration.[30] Thus, the background intensities caused by nonspecific binding of Au-NPs in this

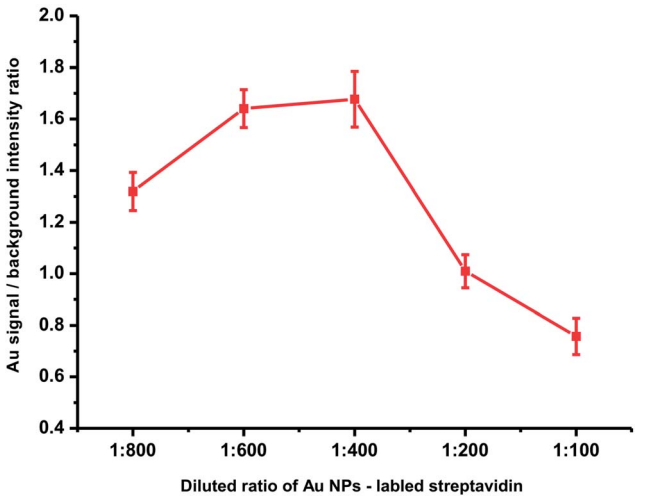

Fig. 2 Effect of Au NPs-labeled streptavidin diluted ratios. Concentration of p24 antigen and diluted ratio of biotinylated anti-p24 polyclonal antibody, 75 pg mL^{-1} and 1 : 100; the error bars in the figure represent standard deviations of the results.

paper also increase with the increase of Au-NPs label concentration. As shown in Fig. 2, the maximal S/B value was obtained at the diluted ratio of 1 : 400. Therefore, a diluted ratio of 1 : 400 was chosen for the subsequent experiments.

3.2 Effect of diluted ratio of biotinylated anti-p24 polyclonal antibody

The diluted ratio of the biotinylated anti-p24 polyclonal antibody was also optimized in Fig. 3. The Au signal intensities were also investigated with a p24 antigen concentration of 75 pg mL^{-1}. It can be seen that the Au signal intensities did not change significantly when the diluted ratio of biotinylated anti-p24 changed from 1 : 10 000 to 1 : 100, but the Au background

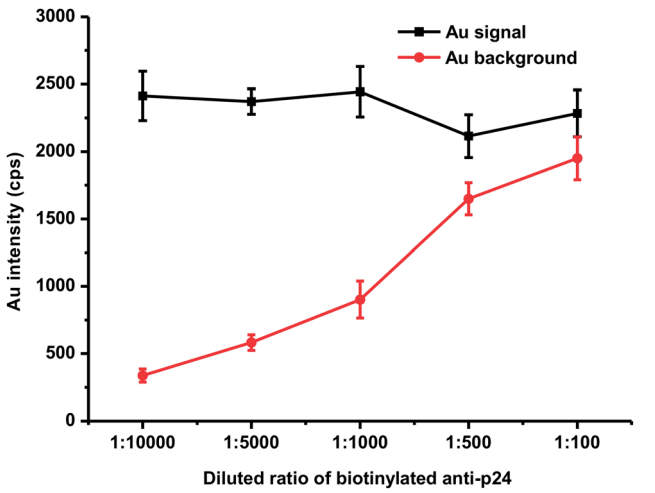

Fig. 3 Effect of biotinylated anti-p24 diluted ratios. Concentration of p24 antigen and diluted ratio of Au NPs-labeled streptavidin, 75 pg mL^{-1} and 1 : 400; the error bars in the figure represent standard deviations of the results.

intensities with assay solution instead of p24 increased obviously with the decrease of the diluted ratio of biotinylated anti-p24 and the maximal ratio of Au signal intensity and an Au background intensity of nearly 8 was obtained at the diluted ratio of 1 : 10 000. The small decrease of the Au signal might be due to the experimental error. Considering that the concentration of biotinylated anti-p24 with a higher diluted ratio may not be enough to cover the antigen, thus the diluted ratio higher than 1 : 10 000 was not investigated. Therefore, a diluted ratio of 1 : 10 000 for biotinylated anti-p24 was chosen for later studies.

3.3 Specificity of the immunoassay

Four proteins in place of p24 antigen were tested with the same experimental procedure for the specificity study (Fig. 4). These tested proteins are either abundant (*e.g.* human IgG, human HSA) or pathogenic proteins (*e.g.* human CEA and human AFP) in serum. The Au net-signal intensities here were calculated by subtracting the Au background intensities with assay solution from the Au signal intensities tested by different proteins. It can be seen that only the p24 could be recognized in the sandwich-type immunoreaction. Human AFP, human CEA, human IgG or human HSA did not significantly interfere with the determination of p24. It indicated that our proposed method had good specificity toward the target protein. Since the tested concentrations of the CEA, AFP, IgG and HSA are close or higher to the concentrations in real healthy adult serum, this ICP-MS linked immunoassay is capable of efficiently detecting p24 antigen in human serum.

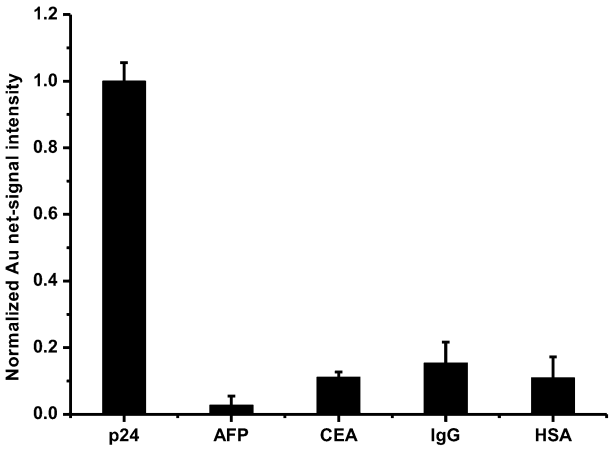

Fig. 4 Specificity for the determination of p24 antigen using the proposed immunoassay. Concentration of p24, 75 pg mL^{-1}; concentration of AFP and CEA, 100 ng mL^{-1}; concentration of IgG, 10 mg mL^{-1}; concentration of HSA, 100 mg mL^{-1}; diluted ratio of biotinylated anti-p24 polyclonal antibody and Au NPs-labeled streptavidin, 1 : 10 000 and 1 : 400; the error bars in the figure represent standard deviations of the results.

3.4 Analytical performance

Under the optimal conditions, the analytical performance of the developed ICP-MS linked immunoassay for the detection of p24 antigen had been carried out. As shown in Fig. 5a, the Au signal intensities were linear with the concentration of p24 antigen in the range from 7.5 to 75 pg mL^{-1} with a correlation coefficient of 0.995. The precision for three replicate measurements of 37.5 pg mL^{-1} p24 antigens was 3.7% (the relative standard deviation, RSD). The detection limit (LOD, 3σ) of the developed method for p24 antigen was 1.49 pg mL^{-1}, where σ is the standard deviation of five repetitive measurements of assay solution blank, corresponding to 4.65 amol of absolute molar mass. In addition, the analytical merit of BA-ELISA for p24 antigen determination was also studied in our work (Fig. 5b). The linear range of p24 antigen was found between 15 and 150 pg mL^{-1} with a correlation coefficient of 0.997 and the LOD (3σ) of p24 antigen was 10.6 pg mL^{-1}. The narrow linear dynamic range of BA-ELISA might be caused by the nonspecific adsorption of HRP-labeled streptavidin that occurs on the surface of the microtiter plate. A comparison of analytical performance of the present method with those of other methods for the determination of p24 antigen is given in Table 2. The LOD of the ICP-MS linked immunoassay has around 1 order of magnitude improvement compared to that of the amperometric immunosensor[44] and

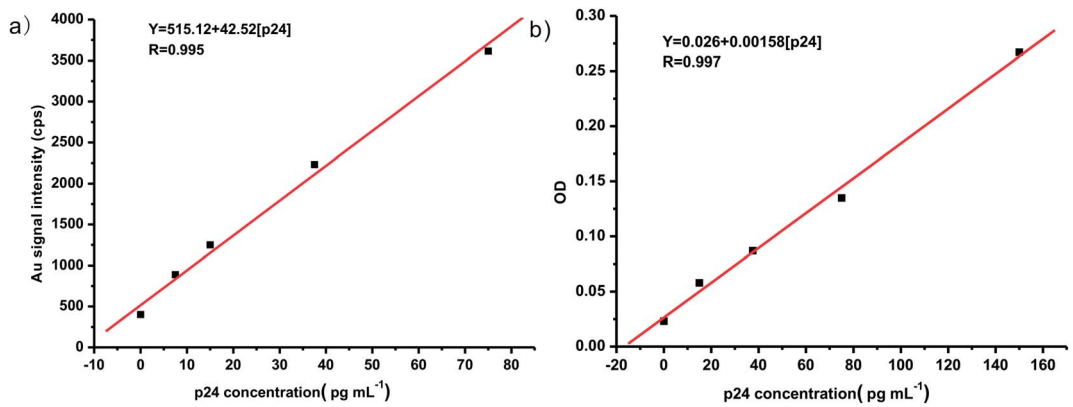

Fig. 5 Dependence of Au signal intensity by ICP-MS linked immunoassay (a) and OD value with BA-ELISA (b) on p24 antigen concentration. Conditions of ICP-MS linked immunoassay: diluted ratio of biotinylated anti-p24 polyclonal antibody and Au NPs-labeled streptavidin, 1 : 10 000 and 1 : 400; conditions of BA-ELISA: biotinylated anti-p24 polyclonal antibody with no dilution.

Table 2 Comparison of performances of the proposed ICP-MS linked immunoassay with other different approaches for the determination of p24 antigen

Approaches	Limit of detection (pg mL^{-1})	Ref.
Cytometric bead-based assay	0.43	43
Magnetic immuno-chromatography	15–30	7
Nanofunctionalized surfaces in a capacitive immunosensor	7.9×10^{-8}	8
Biobarcode amplification assay with europium NPs	0.5	10
An amperometric immunosensor with gold nanoparticles, multi-walled carbon nanotubes and an acetone extracted propolis film	6.4	9
Plasmonic ELISA with the naked eye	1×10^{-6}	12
An amperometric immunosensor with polyelectrolyte/gold magnetic nanoparticles	50	44
ICP-MS linked immunoassay with Au NPs	1.49	This work
BA-ELISA	10.6	

magnetic immuno-chromatography,[7] although it is still higher than the capacitive immunosensor[8] and plasmonic ELISA,[12] indicating that this method still needs to improve sensitivity in biological application.

3.5 Application

For the analysis of spiked serum samples by our proposed method, p24 antigen was spiked into 5000 and 10-fold diluted healthy adult serum samples with assay solution and reached final concentrations of 37.5 and 75 pg mL^{-1}, separately. Samples were analyzed in triplicate, and the average signal response was used to calculate the p24 concentration from the standard curve obtained prior to analysis. The percentage recoveries were calculated and are summarized in Table 3. Better recoveries seen in the highly diluted samples may be indicative of a "washing out" of matrix effects caused by nonspecific adsorption. Therefore, dilution was necessary for real human serum determination.

The appropriate validation of a new analytical method is always the most crucial stage of its development. The most valuable evaluating protocol is usually by comparing the developed method with a different method that is commonly used in routine analysis or accepted as a reference method for a given analyte and matrix.[9] In the present work, BA-ELISA was selected since it is widely used in conventional p24 determination. However, since the patient serum with AIDS was difficult to get in our lab, a preliminary evaluation of the validity of the proposed ICP-MS linked immunoassay in artificially positive human serum samples was performed. We added 30–80 pg mL^{-1} p24 standards to normal human serum (diluted 10-fold) for simulation and determined both by ICP-MS linked immunoassay and BA-ELISA for a comparison. The results are shown in Table 4. It was demonstrated that results from our method agreed well with those from BA-ELISA, indicating that the present method could be applied to real clinical samples.

Table 3 Recoveries of spiked p24 antigens in serum samples

Dilution	p24 spiked (pg mL^{-1})	Recovery (%)
5000	37.5	92.0 ± 8.0^a
	75	96.6 ± 10.2
10	37.5	119.6 ± 3.6
	75	123.3 ± 11.2

[a] Standard deviation ($n = 3$).

Table 4 Analytical results of HIV-1 p24 antigens in artificially positive serum samples by ICP-MS linked immunoassay and BA-ELISA

Sample	ICP-MS linked immunoassay	BA-ELISA
1	44.6 ± 1.6^a	35.3 ± 1.5
2	92.2 ± 8.7	65.5 ± 3.7

[a] Standard deviation ($n = 3$).

4 Conclusion

A feasibility study for sensitive analysis of HIV-1 p24 antigen using a sensitive assay with a BA system and Au NPs-based ICP-MS immunoassay was demonstrated. Owing to the Au NPs amplification process, the new detection approach achieved a high detection sensitivity of 4.65 amol. Another advantage of the proposed strategy is elimination of enzymatic reactions, making it free of toxic enzyme substrate reagents. Moreover, the reagents are either biotinylated or labeled with streptavidin; thus, the systems are easily adapted to detection of other biomolecules. This ICP-MS linked immunoassay is promising for sensitive detection of other proteins. Further studies also can focus on how to decrease the nonspecific adsorption ratio of Au NPs and further improve their detection sensitivity by using single particle detection mode.

Acknowledgements

We gratefully acknowledge the financial support from the National Nature Science Foundation of China (no. 21375120, 21175120 and 41173018), the R&D Special Fund for Public Welfare Industry of Hubei province (2012DCA19001), and the Fundamental Research Funds for the Central Universities (CUG120117 and CUG120502), China University of Geosciences (Wuhan).

References

1 M. G. Rossmann, *Proc. Natl. Acad. Sci. U. S. A.*, 1988, **85**, 4625–4627.
2 D. Nadal, J. Böni, C. Kind, O. E. Varnier, F. Steiner, Z. Tomasik and J. Schüpbach, *J. Infect. Dis.*, 1999, **180**, 1089–1095.
3 B. Ledergerber, M. Flepp, J. Böni, Z. Tomasik, R. W. Cone, R. Lüthy and J. Schüpbach, *J. Infect. Dis.*, 2000, **181**, 1280–1288.
4 J. Schupbach, *AIDS Rev.*, 2002, **4**, 83–92.
5 A. Tehe, C. Maurice, D. L. Hanson, M. Y. Borget, N. Abiola, M. Maran, D. Yavo, Z. Tomasik, J. Böni and J. Schüpbach, *J. Clin. Virol.*, 2006, **37**, 199–205.
6 J. M. Barletta, D. C. Edelman and N. T. Constantine, *Am. J. Clin. Pathol.*, 2004, **122**, 20–27.
7 S. Workman, S. K. Wells, C.-P. Pau, S. M. Owen, X. F. Dong, R. LaBorde and T. C. Granade, *J. Virol. Methods*, 2009, **160**, 14–21.
8 K. Teeparuksapun, M. Hedström, E. Y. Wong, S. Tang, I. K. Hewlett and B. Mattiasson, *Anal. Chem.*, 2010, **82**, 8406–8411.
9 F. Kheiri, R. Sabzi, E. Jannatdoust, E. Shojaeefar and H. Sedghi, *Biosens. Bioelectron.*, 2011, **26**, 4457–4463.
10 S. Tang and I. Hewlett, *J. Infect. Dis.*, 2010, **201**, S59–S64.
11 H. Dong, J. Liu, H. Zhu, C.-Y. Ou, W. Xing, M. Qiu, G. Zhang, Y. Xiao, J. Yao and P. Pan, *Virol. J.*, 2012, **9**, 180–186.
12 R. de La Rica and M. M. Stevens, *Nat. Nanotechnol.*, 2012, **7**, 821–824.
13 Y. Zhang, X. Gao, A. Gao and M. Fan, *Food Anal. Methods*, 2012, **5**, 1214–1220.
14 P. Ginel, J. Margarito, J. Molleda, R. Lopez, M. Novales and W. Bernadina, *Res. Vet. Sci.*, 1996, **60**, 107–110.
15 E. P. Diamand and T. K. Christopoulos, *Clin. Chem.*, 1991, **37**, 625–636.
16 E. Razumienko, O. Ornatsky, R. Kinach, M. Milyavsky, E. Lechman, V. Baranov, M. A. Winnik and S. D. Tanner, *J. Immunol. Methods*, 2008, **336**, 56–63.
17 C. Giesen, L. Waentig, U. Panne and N. Jakubowski, *Spectrochim. Acta, Part B*, 2012, **76**, 27–39.
18 Y. Lu, W. Wang, Z. Xing, S. Wang, P. Cao, S. Zhang and X. Zhang, *Talanta*, 2009, **78**, 869–873.
19 C. Zhang, F. Wu, Y. Zhang, X. Wang and X. Zhang, *J. Anal. At. Spectrom.*, 2001, **16**, 1393–1396.
20 C. Zhang, F. Wu and X. Zhang, *J. Anal. At. Spectrom.*, 2002, **17**, 1304–1307.
21 M. Careri, L. Elviri, A. Mangia and C. Mucchino, *Anal. Bioanal. Chem.*, 2007, **387**, 1851–1854.
22 M. W. Yang, Z. W. Wang, L. Fang, J. P. Zheng, L. J. Xu and F. F. Fu, *J. Anal. At. Spectrom.*, 2012, **27**, 946–951.
23 R. Liu, Y. Lv, X. Hou, L. Yang and Z. Mester, *Anal. Chem.*, 2012, **84**, 2769–2775.
24 R. Liu, X. Hou, Y. Lv, M. McCooeye, L. Yang and Z. Mester, *Anal. Chem.*, 2013, **85**, 4087–4093.
25 L. Waentig, N. Jakubowski, S. Hardt, C. Scheler, P. H. Roos and M. W. Linscheid, *J. Anal. At. Spectrom.*, 2012, **27**, 1311–1320.
26 M. Terenghi, L. Elviri, M. Careri, A. Mangia and R. Lobinski, *Anal. Chem.*, 2009, **81**, 9440–9448.
27 C. Zhang, Z. Zhang, B. Yu, J. Shi and X. Zhang, *Anal. Chem.*, 2002, **74**, 96–99.
28 V. I. Baranov, Z. Quinn, D. R. Bandura and S. D. Tanner, *Anal. Chem.*, 2002, **74**, 1629–1636.
29 S. Hu, R. Liu, S. Zhang, Z. Huang, Z. Xing and X. Zhang, *J. Am. Soc. Mass Spectrom.*, 2009, **20**, 1096–1103.
30 R. Liu, Z. Xing, Y. Lv, S. Zhang and X. Zhang, *Talanta*, 2010, **83**, 48–54.
31 R. Liu, X. Liu, Y. Tang, L. Wu, X. Hou and Y. Lv, *Anal. Chem.*, 2011, **83**, 2330–2336.
32 F. Li, Q. Zhao, C. Wang, X. Lu, X.-F. Li and X. C. Le, *Anal. Chem.*, 2010, **82**, 3399–3403.
33 P. Jarujamrus, R. Chawengkirttikul, J. Shiowatana and A. Siripinyanond, *J. Anal. At. Spectrom.*, 2012, **27**, 884–890.
34 B. Chen, B. Hu, P. Jiang, M. He, H. Peng and X. Zhang, *Analyst*, 2011, **136**, 3934–3942.
35 H. Cho and H. Lim, *J. Anal. At. Spectrom.*, 2013, **28**, 468–472.
36 S. Hu, S. Zhang, Z. Hu, Z. Xing and X. Zhang, *Anal. Chem.*, 2007, **79**, 923–929.
37 S. Zhang, C. Zhang, Z. Xing and X. Zhang, *Clin. Chem.*, 2004, **50**, 1214–1221.
38 Z. A. Quinn, V. I. Baranov, S. D. Tanner and J. L. Wrana, *J. Anal. At. Spectrom.*, 2002, **17**, 892–896.
39 J. M. Liu and X. P. Yan, *J. Anal. At. Spectrom.*, 2011, **26**, 1191–1197.
40 X. W. Yan, M. Xu, L. M. Yang and Q. Q. Wang, *Anal. Chem.*, 2010, **82**, 1261–1269.
41 S. C. Bendall, E. F. Simonds, P. Qiu, E. D. Amir, P. O. Krutzik, R. Finck, R. V. Bruggner, R. Melamed, A. Trejo, O. I. Ornatsky, R. S. Balderas, S. K. Plevritis, K. Sachs, D. Pe'er, S. D. Tanner and G. P. Nolan, *Science*, 2011, **332**, 687–696.
42 J. Gu and M. Dandrea, *Am. J. Anat.*, 1989, **185**, 264–270.
43 A. Biancotto, B. Brichacek, S. S. Chen, W. Fitzgerald, A. Lisco, C. Vanpouille, L. Margolis and J.-C. Grivel, *J. Virol. Methods*, 2009, **157**, 98–101.
44 N. Gan, J. Hou, F. Hu, L. Zheng, M. Ni and Y. Cao, *Molecules*, 2010, **15**, 5053–5065.