A New Strategy for Highly Sensitive Immunoassay Based on Single-Particle Mode Detection by Inductively Coupled Plasma Mass Spectrometry

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A highly sensitive immunoassay is proposed based on time-resolved inductively coupled plasma mass spectrometry with nanoparticles as tags to antibody. Instead of using traditional integral mode detection, the transient signals induced by the flash of ions in the plasma torch from the ionization of nanoparticles tagged on antibody were recorded in a time-resolved mode. Since, under certain conditions, the frequency of transient signals is directly correlated to the concentration of nanoparticle tags, the concentration of nanoparticle-tagged antibodies can be quantified by the frequency of transient signals. With the present instrument setup, gold nanoparticle (Au-NP) tags, as small as about 15 nm in diameter, can be detected. This protocol is evaluated for a competitive immunoassay and the linear range for α -fetoprotein is 0.016–6.8 $\mu\text{g/L}$ (between 20 and 80% inhibition). The limit of quantification is 0.016 $\mu\text{g/L}$ (20% inhibition, IC_{20}) with a relative standard deviation of 4.2% (20% inhibition, 4 replicates) for α -fetoprotein. The present strategy provides a sensitive readout method for nanoparticle tags, which is quite promising for numerous applications in immunoassay, DNA hybridization, and other biological analyses. (J Am Soc Mass Spectrom 2009, 20, 1096–1103) © 2009 American Society for Mass Spectrometry

Various highly sensitive methods for detecting trace biomolecules have been established, over the past several years, by using nanoparticles as tags [1–5]. Gold nanoparticles (Au-NPs) are one kind of outstanding nanoparticle tags that have been extensively used in the biological area. Various readout methods have been adopted to detect Au-NP tags including colorimetric [6–9], surface-plasmon resonance [10–14], and electrochemical methods [15] based on their color change during the formation of aggregates, their surface-plasmon oscillation, or electrochemical activity. Au-NP tags can also be detected indirectly by surface-enhanced Raman scattering [16], a quenching effect on fluorescence of various phosphors [17, 18], or enhancement effect on chemiluminescence [19]. Although these above-cited readout methods have their own advantages and features, special physical or chemical properties of nanoparticle materials are required that limit the number

of available nanoparticle tags for biological applications. A universal readout method for nanoparticle tags will inevitably enlarge the scope of nanoparticle tag candidates.

The use of inductively coupled plasma mass spectrometry (ICP-MS) as a readout method does not require nanoparticles to possess the optical, electric, electrochemical, magnetic, or other special properties since ions from the atoms of the elements are directly detected by this instrument. The nanoparticles tagged on biological molecules could be detected by ICP-MS, which has been demonstrated by Baranov et al. [20], Merkoci et al. [21], and our group [22, 23] in recent years. An important advantage of ICP-MS for nanoparticle detection is that high sensitivity could be obtained because of large quantities of detectable atoms in each nanoparticle tag. Another advantage is reduced matrix effects from other components of the biological sample. Potential multiplexing detection is a distinguishing feature in ICP-MS-based immunoassay since signals from element tags are essentially nonoverlapping and all the elements can be simultaneously ionized at the same condition [24, 25].

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However, the high sensitivity of the ICP-MS method for detection of nanoparticle tags has not been sufficiently demonstrated in previous works because the nanoparticles were detected by using traditional integral mode detection as that of analytes dissolved in solution. Those methods could only be able to achieve the detection limits of enzyme-linked immunosorbent assay (ELISA) and not as sensitive as time-resolved fluorescence immunoassay (TRFIA), thus limiting their applications in immunoassay, especially in early cancer diagnosis.

The feasibility of using single-particle analysis for nanoparticle suspensions detection by ICP-MS has been studied by Degueldre et al. [26]. The transient signals induced by the flash of ions arising from the ionization of nanoparticles in the plasma torch can be detected and measured by the mass spectrometer individually so long as only one transient appears during a given dwell time. The single-particle ICP-MS analysis has been tested for nanoparticles of TiO_2 , Al_2O_3 , ZrO_2 , ThO_2 , and Au-NPs in water [26–30]. When the nanoparticles were used as tags in bioassay, this single-particle mode ICP-MS analysis affords the possibility of developing highly sensitive immunoassay and nucleic acids analysis strategies [31].

In the present study, a highly sensitive immunoassay has been proposed based on single-particle mode detection by ICP-MS in a time-resolved analysis (TRA) mode with Au-NPs serving as model tags. The behavior of Au-NPs with an average diameter of about 20, 45, and 80 nm by ICP-MS in the TRA mode has been investigated. The antibodies tagged with Au-NP tags have been quantitatively detected based on the good correlation between the frequency of transient signals and the concentration of antibodies. To illustrate the performance of ICP-MS-based single-particle mode detection to immunoassay, a competitive heterogeneous immunoassay has been carried out for the determination of α -fetoprotein with Au-NP-tagged mouse-anti-human α -fetoprotein antibody.

Experimental

Instrument

An X Series ICP-MS (Thermo Electron Co., Winsford, Cheshire, UK) was used for the present experiments. The instrument was equipped with a glass concentric nebulizer and an impact bead spray chamber for aerosol generation and filtration. The shield torch was used to improve the sensitivity of signal. A Y-piece was constructed in the flow manifold to continuously add the Au-NP suspension into the blank water stream. The flow rates of the water stream and colloid suspension were 0.5 mL/min. The transient signals, induced by the ionization of Au-NPs in plasma torch, were obtained by ICP-MS operated in TRA mode. Only the isotope of ^{197}Au was monitored in the experiment to maximize the data sampling frequency. The duration of the flash for a

single Au-NP was about 200–400 μs [32]. Although a shorter dwell time would prolong the dynamic range for each scan and enhance the signal-to-noise for each transient, the dwell time of 10 ms was chosen, considering that the intrinsic delay time of the instrument for each transient was 0.9 ms. The 60-s duration for each scan was used throughout the experiments. In all, 6000 data points were acquired for each temporal profile. The instrument was tuned with multielement standard solution (10 $\mu\text{g/L}$ of Li, Co, In, Ce, and U) for consistent sensitivity and minimum oxide levels; the operating parameters were then optimized by using a 10 $\mu\text{g/L}$ Au solution. The operating parameters of ICP-MS instrument are listed in Table 1. The intensity of the signal was measured in counts/10 ms and the pulse frequency was in pulse number/min.

Reagents

The α -fetoprotein (AFP) was purchased from the Jiangsu Institute of Nuclear Medicine (Wuxi, China). Mouse-anti-human AFP antibody and bovine serum albumin (BSA) were purchased from Beijing Biosynthesis Biotechnology Co. (Beijing, China). Polystyrene 96-well microtiter plates (Nikon, Sino American Biotechnology Co., Beijing, China) were used to perform the immunoreactions. Pure water with a conductivity of $18.2 \text{ M}\Omega \text{ cm}^{-1}$ was used throughout this experiment.

Preparation of Au-NP Suspension

Au-NP suspension was prepared according to the literature with a slight modification [33]. Briefly, an appropriate volume of 1% trisodium citrate was added into a boiling aqueous solution of HAuCl_4 (0.01%) with vigorous stirring. The solutions were kept at the boil for 10 min. The resulting Au-NP suspension was cooled and stocked in a brown bottle at 4 °C. Three Au-NP suspensions were measured by transmission electron microscopy and the average diameters of 20, 45, and 80 nm, respectively. The calculated particle concentrations of

Table 1. Operating parameters of ICP-MS

Parameter	Value
RF power (W)	1400
Cool gas flow (L/min)	13
Auxiliary gas flow (L/min)	0.8
Nebulizer gas flow (L/min)	0.85
Sample uptake rate (mL/min)	0.5
Water stream flow rate (mL/min)	0.5
Torch	Shield torch
Cones	Nickel, HPI design
Data acquisition mode	Time-resolved analysis (TRA)
Dwell time (ms)	10
TRA duration time (s)	60
Resolution	Standard
Analogue detector voltage (V)	2130
PC detector voltage (V)	3650

Au-NPs of 20-, 45-, and 80-nm Au-NP suspensions were approximately 5.91×10^{11} , 5.19×10^{10} , and 9.24×10^9 particles/mL, respectively. The Au-NP suspensions used for detection were diluted from the stock suspensions with pure water after sonicating for 5 min.

Preparation of Au-NP–AFP Antibody Complex

AFP antibody–Au-NP complex was prepared according to the literature with a slight modification [34]. Briefly, AFP antibody and Au-NP suspension were adjusted to pH 8.2 with 0.1 mol/L K_2CO_3 solution. The minimum tagged amount was determined using a flocculation test. The AFP antibody, with 10% extra amount to the minimum tagged amount, was added to 1 mL Au-NP suspension with stirring for 10 min and followed by incubating at room temperature for 1 h. A 1-mL suspension of Au-NP was tagged with 25 μ g of AFP antibody; 5% BSA and 1% polyethylene glycol (PEG, 20 kDa) were added to a final concentration of 1% and 10% of total volume to avoid the flocculation of Au-NP–AFP antibody complex.

Optimization of Immunoreaction Conditions

The optimum antigen concentration coated on the microwell plates and the optimum concentration of Au-NP–tagged antibody were determined by checkerboard titration. Purified AFP antigen was diluted by buffer (0.05 mol/L sodium carbonate/bicarbonate, pH 9.6) to the concentrations of 0.05, 0.10, 0.25, 0.5, 1.0, 2.5, 5.0, 25.0, and 50.0 mg/L; 200- μ L aliquots of each solution were added to the wells of microwell plates separately and incubated at 37 °C for 1 h and then overnight at 4 °C. After incubation, the plate was washed three times with phosphate-buffered saline (PBS) washing buffer containing 0.1% Tween-20 (PBS-T, pH 7.4) to remove unbound antigens. The unbound sites were blocked with 200 μ L of 3% BSA in 0.01 mol/L Tris-HCl (1.21 g/L of Tris, 10.10 g/L of NaCl, and 0.25 mL/L of HCl) by incubation at 37 °C for 1 h. After washing the plate three times with PBS-T, 200 μ L of the Au-NP–tagged AFP antibody diluted to 0.05, 0.5, 2.5, 5.0, 50, and 500 μ g/L was added to each well of the plates separately and allowed to incubate in the microwells at 25 °C for 1 h. Then, the plate was washed three times with PBS-T to remove excess Au-NP–tagged AFP antibody. Finally, 250 μ L of 2% nitric acid solution was added to each well to release the tags. The 2% nitric acid denatured the protein but did not dissolve the Au-NPs. The obtained suspensions were diluted to 5 mL for ICP-MS determination. The optimum antigen and tagged antibody concentrations were obtained based on the frequency of the flashes of ^{197}Au and the concentrations of Au-NP–tagged AFP antibody, which was used for further testing of the competitive immunoassay procedures.

Competitive Immunoassay Procedure

Figure 1 illustrates the general protocol of this competitive assay for the determination of AFP by using Au-NP tags. In brief, standard solutions of AFP antigen were diluted to 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 μ g/L; 100 μ L of each solution was added into different wells, followed by the addition of 100 μ L of 10 μ g/L Au-NP–tagged AFP antibody. The solution was incubated for 1 h at 37 °C. During this incubation, the AFP in the solution competes with the AFP immobilized on the well for binding sites of Au-NP–tagged AFP antibody. The subsequent assay procedures were performed as described earlier.

Data Analysis

The sensitivity was estimated by the concentration of half-maximal inhibition (IC_{50}), which was obtained by plotting a standard inhibition curve as a function of analyte concentrations. Inhibitory concentration (IC) was calculated by the following expression:

$$IC = 100 \times [(f_i - f_b)/(f_0 - f_b)]$$

where f_b is the frequency of uncoated antigen and named as nonspecific adsorption to protein, f_0 is the frequency in the absence of competitive antigen, and f_i is the frequency with various concentrations of competitive antigen. The concentrations of analyte in the samples were then obtained by interpolation on the standard curve.

Results and Discussion

Comparing the Signals of Au-NPs, Au^{3+} Solution, and Pure Water

Au-NPs with average size of 45 nm in diameter were studied to confirm whether a conventional ICP-MS instrument could be used for determination of extremely diluted nanoparticle suspensions in TRA mode. Figure 2a–c shows the signals recorded by ICP-MS in TRA mode for Au-NP suspension, Au^{3+} solution dissolved from the Au-NP suspension, and pure water,

Figure 1. Schematic diagram of the competitive immunoassay for AFP with Au-NP tags.

Figure 2. Comparison of the signals obtained by ICP-MS in a time-resolved analysis mode from Au-NP suspension, Au³⁺ solution, and pure water. (a) Au-NP suspension of 45 nm average size (10^7 times dilution of the stock solution, $\sim 5.19 \times 10^3$ particles/mL). (b) Au³⁺ solution (20 ng/L). (c) Pure water. Data acquisition mode: time-resolved analysis (TRA). Dwell time: 10 ms. Duration time: 60 s.

respectively. Transient signals from the Au-NP suspension were obviously obtained, as shown in Figure 2a. No obvious pulse signal intensity higher than 10 counts/10 ms was observed from Au³⁺ solution (Figure 2b), although the concentration of Au was higher than that of

Au-NP suspension. The reason is that each Au-NP consists of a large number of gold atoms ($\sim 2.8 \times 10^6$ gold atoms for 45-nm diameter), forming a heterogeneous solution with concentrated gold atoms in different locations of solution. The Au-NP is ionized separately in the plasma torch, forming a flash of ions that can be detected by mass spectrometer in TRA mode. Furthermore, the Au-NP as a single entity is not diluted by the nebulizing gas, thus remaining detectable even with extreme dilution and slight dispersion after atomization. When the solution is extremely diluted, the concentration of the analyte is not sufficient to produce detectable signals because of the limitation of the present ICP-MS instrument, giving only the baseline noise similar to the signals of blank solution (Figure 2c). From the experimental results and the preceding discussion, we concluded that only particles could be detected as transient signals by ICP-MS in TRA mode at extremely low concentration.

Characteristics of the Signals Obtained from Au-NPs

The characteristics of the transient signals obtained from Au-NP suspensions at different concentrations were investigated. It was found that the frequency of the transient signals decreased with the dilution of those suspensions, whereas the average intensities of transient signal retained their original counts. As shown in Figure 3a1–a3, the number of the transient signals in 60 s decreased from 747 to 6 when the suspension of 80 nm in diameter was diluted from 9.24×10^4 to 9.24×10^2 particles/mL. However, the average intensities of transient signal remained roughly 190 counts/10 ms before and after dilution. The invariable signal intensities during the dilution of the suspensions further confirmed that nanoparticles were detected as a single entity. Similar results were also obtained for Au-NP suspensions with the size of 45 and 20 nm (Figure 3b1–b3, c1, and c3).

The intensities of transient signals were dependent on the size of Au-NPs and larger Au-NPs gave stronger transient signals, as shown in Figure 3a–c. The average intensities were 190, 30, and 7 counts/10 ms for Au-NPs with average size of 80, 45, and 20 nm, respectively. The extraordinarily high transient signals that appeared occasionally during the detection were caused by the aggregation of Au-NPs or multiple particles in one 10-ms measurement period and did not affect the statistical results. The minimum detectable size of an Au-NP is around 15 nm in the present work based on three times the standard deviation of the blank signal. Au-NPs of 20-nm size are close to the detection limit; thus a relatively larger deviation was observed and a miscount of smaller particles in the monodispersed suspension may occur during the detection. Thus a

Figure 3. Transient signals obtained from Au-NP with different size and concentration by ICP-MS in TRA mode. (a1) 10^5 times dilution ($\sim 9.24 \times 10^4$ particles/mL) of an Au-NP suspension with 80-nm average size. (a2) 10^6 times dilution ($\sim 9.24 \times 10^3$ particles/mL) of an Au-NP suspension with 80-nm average size. (a3) 10^7 times dilution ($\sim 9.24 \times 10^2$ particles/mL) of an Au-NP suspension with 80-nm average size. (b1) 10^6 times dilution ($\sim 5.19 \times 10^4$ particles/mL) of an Au-NP suspension with 45-nm average size. (b2) 10^7 times dilution ($\sim 5.19 \times 10^3$ particles/mL) of an Au-NP suspension with 45-nm average size. (b3) 10^8 times dilution ($\sim 5.19 \times 10^2$ particles/mL) of an Au-NP suspension with 45-nm average size. (c1) 10^6 times dilution ($\sim 5.91 \times 10^5$ particles/mL) of an Au-NP suspension with 20-nm average size. (c2) 10^7 times dilution ($\sim 5.91 \times 10^4$ particles/mL) of an Au-NP suspension with 20-nm average size. (c3) 10^8 times dilution ($\sim 5.91 \times 10^3$ particles/mL) of an Au-NP suspension with 20-nm average size. Data acquisition mode: time-resolved analysis (TRA). Dwell time: 10 ms. Duration time: 60 s.

45-nm size of Au-NPs was chosen as the optimum for single-particle detection.

Correlation of Signal Frequency and Concentration of Au-NPs

The frequency of the transient signals is proportional to the number concentration of Au-NPs, which could be used as the foundation of a quantitative determination of nanoparticle tags. As shown in Figure 4b, for Au-NPs of 45 nm, the signal numbers in 60 s were linearly

dependent on its number concentration in the range of 5.19×10^2 and 5.19×10^4 particles/mL, with a correlation coefficient (R) of 0.9999. As shown in Figure 4a and c, the linear ranges were from 9.24×10^2 to 9.24×10^4 particles/mL for an Au-NP of 80 nm with $R = 0.9998$ and from 5.91×10^3 to 5.91×10^5 particles/mL for an Au-NP of 20 nm with $R = 0.9828$. The results indicated that the frequency of transient signals was linearly dependent on the number concentrations of Au-NPs in the suspensions. In the present study, as low as 5.19×10^2 particles/mL gave about 5 pulses in

Figure 4. The frequency of transient signal as a function of number concentration of Au-NP suspension with different size. The sizes of Au-NP were (a) 80, (b) 45, and (c) 20 nm. The concentrations of Au-NP suspension with size of 45 nm were 5.19×10^4 , 5.19×10^3 , and 5.19×10^2 particles/mL (10^6 , 10^7 , and 10^8 times dilution of the stock solution). These were 9.24×10^4 , 9.24×10^3 , and 9.24×10^2 particles/mL (10^5 , 10^6 , and 10^7 times dilution) for Au-NP of 80 nm and were 5.91×10^5 , 5.91×10^4 , and 5.91×10^3 particles/mL (10^6 , 10^7 , and 10^8 times dilution) for Au-NP of 20 nm.

60 s with 45-nm size, which means if one particle is detected for a 60-s scan, the Au-NP concentration was around 10^2 particles/mL.

Optimization of the Immunoassay Conditions

To optimize the concentration of AFP for coating the microwell plates, a direct immunoassay for a checker-board titration was performed. Microwell plates were coated with purified AFP antigen with various concentrations and the Au-NP tagged AFP antibody was conjugated directly with the immobilized AFP. Figure 5 shows the effects of AFP concentration for coating on the signal frequencies of various concentrations of Au-NP-tagged AFP antibody. The immunoreactivity of the Au-NP-tagged AFP antibodies with the immobilized AFP antigen was characterized by the frequency of flashes of the ^{197}Au signal. The optimal concentration of 5.0 mg/L for coated AFP antigen was selected for further investigation, considering both sensitivity and reagent-saving.

To optimize the concentration of Au-NP-tagged AFP antibody, a competitive immunoassay was performed in the presence of free AFP at various concentrations. Based on the inhibition curves of AFP antigen with various concentrations of Au-NP-tagged AFP antibodies, the most sensitive assay was obtained using IC_{50} as a measure. When the immobilized AFP concentration was 5.0 mg/L, the IC_{50} for AFP antigen was 0.28, 2.3, and 2.4 $\mu\text{g/L}$ for AFP antibody conjugated with Au-NPs at concentrations of 0.01, 0.1, and 1.0 mg/L, respectively. Therefore, 10 $\mu\text{g/L}$ of the Au-NP conjugate was selected.

Performance of the Analytical Method

A competitive immunoassay with different concentrations of AFP standard solutions was carried out. The frequency of the transient signals is inversely proportional to the concentration of AFP in the solution. The inhibition curves for AFP from 0.0 to 1000 $\mu\text{g/L}$ and the error bar were plotted in Figure 6. The working range

Figure 5. The effects of AFP concentration for coating on the immunoreactivity with various Au-NP-tagged AFP antibody. The concentration of Au-NP-tagged AFP antibody was 0 ([Itrif]), 0.05 (▽), 0.5 (▼), 2.5 (△), 5.0 (▲), 25 (□), 50 (○), and 500 (●) mg/L. The frequency of signal was plotted as a function of coated concentration of purified AFP antigen. The dotted vertical line is the value of 5 mg/L Au-NP-tagged AFP antibody.

Figure 6. The calibration curves for AFP antigen immunoassay. The standard inhibition was plotted with serially diluted solution of AFP antigen at the concentration of 0.0, 0.0001, 0.001, 0.005, 0.01, 0.05, 0.1, 1.0, 5.0, 10, 50, 100, and 1000 $\mu\text{g/L}$. 1.0 mg/L AFP antigen for coating and 10 $\mu\text{g/L}$ Au-NP conjugate AFP antibodies was used for the competition reaction. [The error bars represent confidence intervals of three duplicate detections with confidence level of 95% ($P = 0.05$).]

was around 0.016–6.8 $\mu\text{g/L}$ (between 20 and 80% inhibition). The limit of detection, defined as the concentration that produces a 20% inhibition of the signal (IC_{20}), was 0.016 $\mu\text{g/L}$ of AFP. A relative standard deviation (RSD) of 4.2% can be obtained at 20% inhibition for four replicates.

A comparison of analytical performance of the present method with that of the other immunoassay methods for the determination of AFP is given in Table 2. It is obvious that the limit of detection in the present method was much lower than that of previous ICP-MS immunoassays. It is also comparable to that of time-resolved fluorescent immunoassay and chemiluminescence immunoassay for the determination of AFP.

Assay of Human Serum Samples

Sensitive detection of AFP is essential in clinical laboratories because increased levels are associated with a number

Table 3. Analytical results of clinical serum samples

Sample	This method, $\mu\text{g/L}$		
	mean ($n = 3$)	RSD (%) ^a	CL, $\mu\text{g/L}$ ^b
Serum 1	1.36	10.3	1.54
Serum 2	1.88	6.9	1.98
Serum 3	2.88	13.2	2.83
Serum 4	4.71	4.9	4.84
Serum 5	7.48	11.4	7.86

^aRelative standard deviation ($n = 3$).

^bCL, chemiluminescence immunoassay was performed by Beijing 301 Hospital.

of conditions including primary hepatocellular carcinoma, nonseminomatous testicular carcinomas, ovarian carcinomas, and other epithelial tumors [35, 36]. Clinical human serum samples were collected from Beijing 301 Hospital (Beijing, China). Results of the proposed method agree with those of clinical chemiluminescent immunoassay, indicating that the present method could be used to determine the AFP with sufficient accuracy and precision (Table 3).

Conclusions

A new strategy for highly sensitive immunoassay, based on ICP-MS detection in single-nanoparticle mode, has been proposed for the determination of AFP in serum with Au-NP-tagged antibody. In addition to its high sensitivity, the present detection strategy does not necessarily require the nanoparticles to possess special physical or chemical properties, thus extending the availability of nanoparticle tags for biological applications. One of the potentials of the present strategy is that nanoparticles, even single cells tagged with elemental tags, could be detected individually with improved instrument construction. Further development of this method may advance with the realization of simultaneous detection of multiple proteins at extremely low concentration with suitable multiple nanoparticle labels.

Table 2. Comparison of various immunoassay methods for AFP determination^a

Tag	Immunoassay format	Analytical method	Limit of detection ($\mu\text{g/L}$)	Reference
Au	Microwells/competitive-type	Single-particle mode ICP immunoassay with element tagged	0.016	This method
Eu ³⁺	Microwells/Sandwich-type	ICP-MS immunoassay with element tagged	0.22	20
Eu ³⁺	Microwells/Sandwich-type	ICP-MS immunoassay with element tagged	1.2	25
BHHCT-Eu ³⁺	Microwells/Sandwich-type	Time-resolved Fluoroimmunoassay	0.07	37
BPTA-Tb ³⁺	Microwells/Sandwich-type	Time-resolved fluoroimmunoassay	0.042	38
ALP	Microwells/Sandwich-type	Chemiluminescence immunoassay	0.040	39
ALP	Microwells/Sandwich-type	Amperometric enzyme immunoassay	0.07	40
ITCI	Microwells/Sandwich-type	Electrochemiluminescence immunoassay	2.0	41

^aAu, Au nanoparticles; BHHCT- Eu³⁺, Eu³⁺ chelate of 4,4'- bis (1",1",1",2",2",3"-heptafluoro-4",6"-hexanedion-6'-yl)-chlorosulfo-o-terphenyl; BPTA-Tb³⁺, Tb³⁺ chelate of N,N,N₁,N₁-(2,6-bis(3'-aminomethyl-1'-pyrazolyl)-4-phenyl pyridine) tetrakis (acetic acid); ALP, alkaline phosphatase; ITCl, isothiocyanato isoluminol.

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