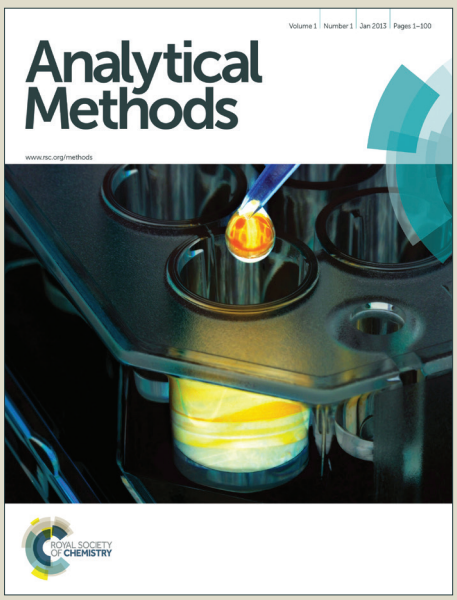
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Gold nanoparticle-based immunosensor for chemiluminescence detection of hepatitis B surface antigen

Soheila Sabouri[a], Hedayatollah Ghourchian[a,b]*, Mostafa Shourian[a], Mahdi Boutorabi[c]

[a] Laboratory of Microanalysis, Institute of Biochemistry and Biophysics, University of Tehran, P.O. Box 13145 1384, Tehran, Iran

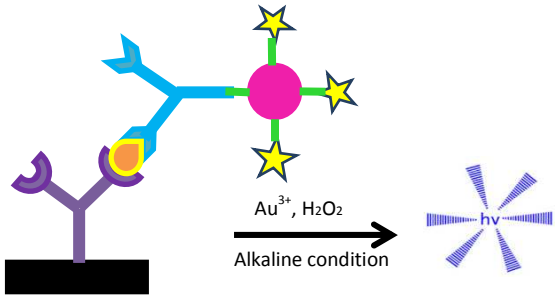
[b] Nanobiomedicine Center of Excellence, Nanoscience and Nanotechnology Research Center, University of Tehran, Tehran, Iran

[c] Pishtaz Teb Diagnostics Company, Tehran, Iran

To whom correspondence should be addressed.

H. Ghourchian, E-mail: hadi@ibb.ut.ac.ir, Tel: +98 21 61112621, Fax: +98 21 66404680

Table of content entry



- $HAuCl_4$ acted more efficient than the other catalysts.
- Modified GNPs caused signal amplification and high specificity toward HBsAg.

Abstract

A sensitive immunoassay method was developed for detection of HBsAg. A chemiluminescence label was prepared based on co-immobilization of secondary antibody and luminol on gold nanoparticles. HBsAg was targeted by a primary antibody, immobilized in polystyrene wells and the secondary antibody, co-immobilized on luminol-gold nanoparticles. After formation of the immune sandwich, the luminescence intensity was recorded in the presence of hydrogen peroxide as oxidant agent and Au^{3+} as an efficient catalyst for luminol oxidation. HBsAg was detected in the linear concentration range from 0.12 to 30 ng/mL and the detection limit of 14 pg/mL. The proposed method has successfully applied to determine the HBsAg in patient real serums. This method has excellent precision, detection limit and wide linear range, with low cost and user friendly. With these features, proposed method is suitable for measuring antigen in patient serums.

Keywords:

Chemiluminescence; Immunoassay; Immunosensor; Biosensor; Hepatitis B surface antigen; Gold nanoparticles

Introduction

Most of traditional analytical methods are not efficient to monitor the important markers in body fluids at low concentrations. In 1959, an immunoassay method was used for radiolabeling of antigen or antibody to detect insulin [1] however it was not safe and the produced bio-conjugate had short half-life [2]. To overcome these defects, photo- and chemi-luminescence were developed [3]. Generally, luminescence is a term used to describe light emission that occurs when a luminant molecule in excited state relaxes to its ground state. Unlike photo-luminescence, in chemiluminescence the energy is provided through a chemical reaction rather than a photo-excitation process. So, in chemiluminescence the problems such as source instability and backgrounds noise are resolved [4]. Furthermore, chemiluminescence labeling which is commonly used in immunoassay has advantages such as fast light emission [5], high sensitivity and safety, controllable emission rate, and low cost. In addition, conjugation makes the chemiluminant reagents more stable and also the luminometers based on photomultiplayer tube are available [6]. So, this is why the chemiluminescent methods are commonly used in routine clinical analysis and clinical research, as well. These methods have ultra-sensitive detection limits and used in broad range of analytical applications such as immunoassay and DNA probe assays [7]. For instance these methods are routinely used in automated immunoassay analyzers, commercialized by several diagnostic companies [8-15].

Hepatitis B virus (HBV) is one of the most widespread virus causes acute and chronic infection cirrhosis and liver cancer [16,17]. Since, the incubation period of HBV is from 1 to 6 months, early diagnosis of HBV is vital for saving patient's health. Basic markers of virus replication in serum include S1 proteins of the hepatitis B surface antigen (HBsAg) and hepatitis B envelop antigen, which are secreted by infected hepatocytes. HBsAg appears during infection and is used

mainly to screen for the infection [17,18]. The fast and high accuracy of HBV diagnosis is the main purpose of diagnostic systems [19].

In the present work, a sandwich type immunoassay system was used for detection of HBsAg. In the first step, monoclonal antibodies were immobilized in the polystyrene well; then, by adding sample containing antigen, the target binds to the primary antibody. This interaction was detected by using a secondary antibody labeled with gold nanoparticles (GNPs) bearing luminol molecules [20]. GNPs exhibit predominant capabilities to be used as a biological label for biosensor applications [21,22]. Increasing in signal indicates the increase of secondary antibody and analyte concentration [23,24]. Anti-HBsAg as secondary antibody (Ab_2) and luminol as a chemiluminant label were covalently immobilized on GNPs surface (Ab_2/GNPs/Luminol). In the presence of $HAuCl_4$ as catalyst and H_2O_2 as oxidant agent, HBsAg was detected.

Results and Discussion

Characterization of GNPs

Usually metallic nanoparticles in solution attract each other via strong van der Waals forces at short distances [25]. By adsorbing citrate molecules on GNPs, negatively charged ions are produced on nanoparticle surface so that the electrostatic repulsion will be enough to keep the particles separated. This modification preserves the nanoparticles from aggregation. The size of obtained GNPs was determined by DLS. For GNP size determination, it was preferred to dilute the nano-particle suspension in deionized water rather than in PBS. Perhaps in PBS, the repulsion of GNPs is reduced and therefore, GNPs might be aggregated. This is why the color of GNPs in buffer was turned in to black. So, GNPs were diluted in deionized water with 1:2 ratio of GNPs/water, respectively. The average diameter of diluted GNPs was 19.6 nm (in the range

from 18.0 to 26.7 nm) while, 99.24% of population had the diameter of 20.28 nm. Since the value of extinction coefficient for GNPs with diameter of 20 nm is 1.57×10^{9} M^{-1} cm^{-1} [26], the concentration of GNPs with the approximate diameter of 20 nm was estimated to be 0.5 nM.

The modification of GNPs' surface was controlled by UV-Vis spectroscopy. The presence of Tween-20 is helpful because it prevents the aggregation of GNPs in buffers and improves chemisorbing of MUA on GNP surface [27]. The UV-Vis absorption intensity of GNPs at 521 nm was about 0.8 when diluted in deionized water (1:2). But it was reduced to about 0.4 when coated with MUA and decreased to about 0.2 when the secondary antibody and luminol were co-immobilized on GNPs-MUA (Data not shown). Absorbance intensity at 521 nm was little more than that we expected. The high intensity could probably be due to the stabilization of GNPs by citrate molecules and preventing their aggregation by Tween-20, as well. These results are well-matched with those reported in the literature [27]. Covering the GNPs by MUA changes the dielectric constant of gold colloids at micro-environment. Therefore, one expects a change on extinction coefficient of GNPs' surface plasmon resonance [25]. The dielectric layer around GNPs also caused a red shift on surface plasmon resonance. Consequently, the coverage on GNPs not only reduces the absorbance intensity but also brings about a red shift from 521 to 530 nm (Data not shown). By co-immobilization of Ab_2 and luminal on GNPs-MUA, the large bio-molecules even more prevent the surface plasmon resonance. This is why we observed more red shift from 530 to 535 nm. Both red shift and reducing the absorption intensity indicated that GNPs were covered with biomolecules successfully.

Order of immobilization of Ab_2 and luminol on GNPs

The experimental results showed that chemiluminescence intensity can also be affected by the order of immobilization of luminol or Ab_2 on GNP surface. To examine this parameter, three experiments were designed and the results were compared. In three test tubes, equal amount of chemically activated GNPs were added. Then, in the first tube luminol and in the second one Ab_2 were added. After incubation at 4 ºC for 30 min, in the first tube Ab_2 and in the second tube luminal and in the third one a mixture of luminol and Ab_2, were added and incubated at 4 ºC (in dark) for 12 hours with gentle shaking. Finally, the co-immobilized Ab_2 and luminol on GNPs (Ab_2/GNP/L), prepared in three test tubs was examined by using the above mentioned procedures of "Immune complex formation" and "Chemiluminescence measurements". The results showed that the Ab_2/GNP/L in first tube, in which firstly luminol and then Ab_2 were attached on GNP surface, illustrated the highest intensity of luminescent emission (Fig. 1). It seems that in such an order, 30 min pre-incubation of GNPs with luminol makes an opportunity for these small molecules to attach on GNPs sufficiently and then in the next 12 h incubation a very limited amount of Ab_2 will attach to GNPs. While, in two other test tubes the self-assembly of large Ab_2 molecules within the first step, probably prevents the efficient absorption of luminol on GNPs, during the second step.

Optimization of conditions

To examine the effect of pH on immobilization of luminol and then Ab_2 on GNPs, at first luminol (in CBS 0.1 M with certain pH) and then after 30 minutes Ab_2 (in PBS 0.1 M with the same pH) was added to GNPs. The thus prepared Ab_2/GNP/L was examined by using the procedures of "immune complex formation" and "chemiluminescence measurements". Comparison of the chemiluminescence intensity produced by Ab_1-Ag-Ab_2/GNP/L immune

sandwich at different pH revealed that the most powerful signal was obtained at pH 9 (Fig. 2A). Therefore, this pH was chosen as optimum pH for the next experiments.

Time and temperature optimization for Ag-Ab interactions was performed at two stages: Ag-Ab_1 and Ag-Ab_2 complex formation. In order to determine the optimum incubation time and temperature for Ag-Ab_2 interaction, Ab_1 was immobilized on plates and incubated with the sample containing HBsAg. Then the complex (Ab_1-Ag) was incubated with Ab_2/GNP/L at different times. After completion of immune sandwich (Ab_1-Ag-Ab_2/GNP/L), chemiluminescence intensity was recorded in the presence of H_2O_2 and $HAuCl_4$. As shown in Fig. 3b, the best incubation time and temperature for Ag-Ab_2 interactions was 45 min and 25 ºC, respectively. In an independent experiment and in the same condition as mentioned for Fig. 2B, it was revealed that the optimum incubation time and temperature for Ag-Ab_1 interaction were 30 min and 37 ºC, respectively (data not shown).

Catalyst effect

To compare the effect of different catalyst on immunoassay, chemiluminescence intensity of sandwich was recorded in the presence of different catalysts ($AgNO_3$, $CoCl_2$, $HAuCl_4$ and hemoglobin: Hb). After completion of immune sandwich (Ab_1-Ag-Ab_2/GNP/L) at optimized conditions the chemiluminescence intensity was recorded. Fig. 3A shows the significant catalytic effect of $HAuCl_4$ relative to $AgNO_3$, $CoCl_2$ and Hb.

Beside, the result of optimization test for catalyst concentration revealed that the chemiluminescence intensity was increased by increasing the $HAuCl_4$ concentration up to 0.1% w/v and then it reaches to a plateau (Fig. 3B). Therefore, concentration of 0.1% was selected as optimum catalyst concentration.

Calibration curve

Under the optimized conditions, the calibration curve for determination of HBsAg was plotted. As shown in Fig. 4, the chemiluminescence intensity (I) increased linearly with increasing HBsAg concentration in the range from 0.12 to 30 ng/mL. The detection limit (DL) at signal to noise ratio of 3 (S/N = 3) was calculated according to the Equation (1):

$$DL = 3.3\ \sigma/S \quad (1)$$

Where, σ is standard deviation of the response and S is the slope of calibration curve [28]. The regression equation for HBsAg calibration curve was: I = 6065.4 [HBsAg] - 156.95 (R = 0.995, n = 3), and the relative standard deviation of the biosensor response at the concentration of 0.12 ng/mL was 0.25% for three successive measurements. Based on Eq. 1, the detection limit was calculated to be as low as 14 pg/ml, at signal/noise ratio of 3.

To evaluate the feasibility of the immunosensor, its analytical parameters were compared with those obtained by other chemiluminescence immunosensor, reported in the literature for the detection of various antigens. The GNPs bearing luminol caused signal amplification and Au^{3+} as non-enzymatic catalyst could improve the sensitivity. Also, these avoided any complicated reaction or extra functionalizing process, making the method low-cost, easy to operate and time-saving. As seen in Table 1, under the optimum condition, chemiluminescence intensity has a linear correlation with the concentration of HBsAg within the concentration range of 0.12-30 ng/mL and a lower detection limit than the other immunosensors.

Specificity and feasibility for real samples

Generally, immunoassay methods are very specific due to high affinity of antibody toward antigen. But, the bio-conjugated Ab_2/GNP/L which was used in the immunoassay may lead to change in Ag-Ab affinity. Therefore, the specificity of HBsAg toward the prepared label (Ab_2/GNP/L) was examined. BSA, which present in blood at high level, and also some antigens with similar structure to HBsAg were used for the specificity test. In Fig. 5, the specificity of prepared label (Ab_2/GNP/L) toward BSA and different antigens such as HBsAg, HCV-core24, HCV-NS_3, HCV-NS_4 and HCV-NS_5 were compared. The results revealed that Ab_2/GNP/L responded to HBsAg more significant than either BSA or other antigens.

In order to investigate the feasibility of the proposed immunosensor for determination of HBsAg in real sample, two human serums from two different patients were examined by the proposed immunosensor. The samples were received from Noor Laboratory, Zanjan, Iran. At first, in the clinical lab, the concentration of HBsAg in real samples was determined to be 0.27 and 2.7 ng/ml by using standard ELISA method (Dia.pro kit, Italy). Then, the luminescence intensities of these human serums were recorded by the proposed immunosensor to be 1574 and 15819. In Table 2, the analytical parameters of linear range, RSD and detection limit obtained by the proposed immunosensor and ELISA method were summarized. As seen these is a satisfactory consistency between the data obtained by ELISA method and immunosensor. This indicates the feasibility of the proposed immunosensor for determination of HBsAg in clinical analysis.

Experimental

Chemicals and apparatus

Luminol (5-amino 2,3-dihydro 1,4-phthalazinedione), H_2O_2 (30% solution) and three sodium citrate ($Na_3C_6H_5O_7$) were purchased from Merck (Germany). Gold (III) chloride hydrate ($HAuCl_4$), bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 11-mercapto undecanoic acid (MUA), 2-morpholinoethansulfonic acid monohydrate (MES), nonionic surfactant polyoxyethylene-20 sorbitan monolaurate (Tween-20) and absolute ethanol were purchased from Sigma-Aldrich. HBsAg, anti-HBsAg antibodies (primary: Ab_1 and secondary: Ab_2) and the polystyrene micro-well coated by Ab_1 were provided by *Pishtaz Teb Zaman Co*. (Tehran, Iran). All chemicals used without further purification. Also in all experiments the solutions were prepared using double distilled deionized water.

UV-Visible spectroscopy was performed by Spectrophotometer (Cary 100 bio, Varian, Australia). Dynamic light scattering (DLS, 90 plus Brookhaven Instruments Corporation, USA) was used for size determination. The micro centrifuge (Sigma, USA) was used to separate GNPs at different conditions. The samples were centrifuged at 14000 rpm for 30 min at 4 ºC. The chemiluminescence emissions were recorded at 425 nm by fluorescence micro plate reader (H4, Bio Tech Co, USA). All of the immune tests were carried out in 96-well plates. By completion of the immune sandwiches 10 µl of $HAuCl_4$ and 10 µl of H_2O_2 were added to each well. After shaking, the emissions intensity was recorded.

Luminol stock solution (10 mM) was prepared in 100 mM sodium carbonate buffer (pH 9), and kept at 4 °C for 48 hours in dark condition. Independently, $HAuCl_4$, $2H_2O$ (0.1% w/v solution) was prepared and used as catalyst. It was stable for several months in refrigerator.

Synthesis of gold nanoparticles

GNPs suspension was prepared according to the literature [34] with a slight modification. Briefly, an appropriate volume of 1% tri-sodium citrate was added into aqueous solution of $HAuCl_4$ (0.1%) with temperate stirring at 70 °C for about 50 min. Then, the temperature was reduced till room temperature while the solution stirred gently. The resulting particle suspension was cooled calmly and stocked in a brown bottle at 4 °C. The size of GNPs in suspension was measured by DLS and UV-Vis spectroscopy. The GNP samples was prepared by diluting the stock suspensions in pure water and used as label.

Co-immobilization of secondary antibody and luminol on GNPs

For chemisorption of MUA on GNPs, 1 mL of GNPs was gently added to 2 mL of phosphate buffer solution (PBS, 10 mM, pH 8) containing MUA (3 mM) and Tween-20 (0.2 mg/mL). The mixture was incubated at room temperature for 30 min. After chemisorption of MUA on GNPs, the GNP conjugates (GNP-MUA) were washed with buffer and centrifuged at 14000 rpm for 30 min. Following the careful removing of supernatant, containing excess MUA and Tween 20, the precipitate was re-suspended in buffer.

For activation of the carboxylic acid functional groups assembled on GNPs surface, the GNP-MUA, prepared in previous section, was added to MES buffer solution (1 mM, pH 5.5) containing 50 μL of EDC (50 mM) and 50 μL of NHS (50 mM) and then, the mixture was incubated at room temperature for 20 min. Thereafter, the modified GNPs were washed three times by centrifugation at 14000 rpm for 30 min and the precipitate was re-suspended in PBS. The activated carboxylic acid groups on GNP surface were ready to react with primary amine groups on either luminol or Ab_2.

Since the primary amine groups of luminol molecules play no role in chemiluminescence, they were used for covalent linkage to GNPs. Therefore, at first 100 µL of luminol solution (50 mM) and then (after 30 min) 25 µL of Ab_2 (20 mg/mL) were added to activated GNPs. The mixture was incubated for 12 hours at dark in cold room (at 4 ºC) with gentle shaking. At the end, the mixture was washed three times and separated by centrifugation (14000 rpm, 30 min) to discard free molecule from those immobilized on GNPs. The prepared bio-conjugation was diluted in PBS (0.01 M, pH 7.4) and stored at 4 ºC.

Immune complex formation and chemiluminescence measurements

100 µL of the sample solution (HBsAg, 20 ng/mL in PBS 0.01 M, pH 7.4) was added to each well of micro-plate (polystyrene micro-well coated by Ab_1) and incubated at 37 ºC for 30 min. Then all of the wells were washed three times with washing buffer (PBS, pH 7.4, 0.1 M and Tween 20, 45 µM). Finally 50 µl of luminal-Ab_2 coated GNPs (Ab_2/GNP/L) was added to each well and after incubation at 25 ºC in dark for 45 min, the completed immune complex was washed three times with washing buffer. Then, sodium carbonate buffer (SCB, pH 9, 0.1 M) was added to the completed immune complex and then $HAuCl_4$ (10 µl, 0.1% w/v) as catalyst and H_2O_2 (10 µl, 10^{-3} M) was injected to the solution. After a quick mixing, the emitted chemiluminescence light was recorded.

Conclusion

A simple, fast and sensitive chemiluminescence immunosensor was developed with wide linear range for detection of HBsAg. Comparison of the catalyst performances for luminol oxidation showed that $HAuCl_4$ acts more efficient than the other catalysts ($AgNO_3$, $CoCl_2$, and Hb).

Designing the bio-conjugated label was so simple that primary amine groups existed in luminol and antibody could be self-assembled on GNP surface. On the other hand, application of GNPs as carriers for bearing luminol and secondary antibody caused both signal amplification and high specificity toward HBsAg. Moreover, Au^{3+} as a non-enzymatic catalyst could improve the sensitivity. Consequently, the prepared immunosensor would be a low-cost, easy to operate and time-saving method which seems to have enough potential to be developed for clinical immunoassays.

Acknowledgments

Financial supports provided by the Research Council of the University of Tehran, and Iran National Science Foundation (INSF) are gratefully appreciated.

References

[1] R.S. Yalow, S.A. Berson, Assay of plasma insulin in human subjects by immunological methods, Nature, 1959, **184**, 1648-1649.

[2] R. Ekins, F. Chu, J. Micallef, High specific activity chemiluminescent and fluorescent markers: their potential application to high sensitivity and 'multi-analyte' immunoassays, J. Biolumin. Chemilumin, 1989, **4**, 59-78.

[3] S. Udenfriend, L.D. Gerber, L. Brink,S. Spector, Scintillation proximity radioimmunoassay utilizing 125I-labeled ligands, Proc. Natl. Acad. Sci. USA, 1985, **82**, 8672-8676.

[4] P.E. Stanley, A survey of more than 90 commercially available luminometers and imaging devices for low-light measurements of chemiluminescence and bioluminescence, including instruments for manual, automatic and specialized operation, for HPLC, LC, GLC and microtitre plates. Part 2: Photographs, J. Biolumin. Chemilumin 1992, **7**, 157-169.

[5] A.L. Grosvenor, C.L. Crofcheck, K.W. Anderson, D.L. Scott, S. Daunert, Calibration of micropipets using the bioluminescent protein aequorin, Anal. Chem, 1997, **69**, 3115-3118.

[6] D.J. Anderson, B. Guo, Y. Xu, L.M. Ng, L.J. Kricka, K.J. Skogerboe, D.S. Hage, L. Schoeff, J. Wang, L.J. Sokoll, D.W. Chan, K.M. Ward, K.A. Davis, Clinical chemistry. Anal. Chem, 1997, **69**, 165R-229R.

[7] L.J. Kricka, Clinical applications of chemiluminescence. Anal. Chim. Acta, 2003, **500**, 279-286.

[8] K.M. O'Connor, G. Svehla, S.J. Harris, M.A. McKervey, Calixarene-based potentiometric ion-selective electrodes for silver, Talanta, 1992, **39**, 1549-1554.

[9] G. Merenyi, J. Lind, T.E. Eriksen, Luminol chemiluminescence, chemistry, excitation, emitter. J Biolumin. Chemilumin, 1990, **5**, 53-56.

[10] M.M. T.Kawasaki, A.Tsuji, Chemiluminescence high-performance liquid chromatography using N-(4-aminobutyl)-N-ethylisoluminol as a precolumn labelling reagent, J. Chromatog. A,1985, **328**, 121-126.

[11] H.Y.K. Nakashima, N. Kuroda, S. Akiyama, Evaluation of lophine derivatives as chemiluminogens by a flow-injection method. Anal. Chim. Acta, 1995, **303**, 103-107.

[12] M.L. Grayeski, E.J. Woolf, Effect of cyclodextrin solutions on chemiluminescence of 10,10'-dimethyl-9,9'biacridinium nitrate, J. Lumin, 1985, **33**, 115-121.

[13] G. Zomer, J. F.C Stavenuiter, Chemiluminescence labels, old and new, Anal. Chim. Acta, 1989, **227**, 11-19.

[14] F. McCapra, Chemical generation of excited states: the basis of chemiluminescence and bioluminescence, Method. Enzymol, 2000, **305**, 3-47.

[15] L. P Palilis, A. C Calokerinos, Analytical applications of chemiluminogenic reactions, Anal. Chim. Acta, 2000, **413**, 175-186.

[16] Y. Chen, W. Wu, Determination of low level HBsAg in serum by microparticle enzyme immunoassay, Hepatobiliary Pancreat Dis Int, 2002, **1**, 262-264.

[17] C.J. Oon, W.N. Chen, K.T. Goh, S. Mesenas, H.S. Ng, G. Chiang, C. Tan, S. Koh, S.W. Teng, I. Toh, M.C. Moh, K.S. Goo, K. Tan, A.L. Leong, G.S. Tan, Molecular characterization of hepatitis B virus surface antigen mutants in Singapore patients with hepatocellular carcinoma and hepatitis B virus carriers negative for HBsAg but positive for anti-HBs and anti-HBc. J Gastroenterol, Hepatol. 17 Suppl ,2002 , S491-S496.

[18] D.A. Frank, K. Bresnahan, B.S. Zuckerman, Maternal cocaine use: impact on child health and development, Curr. Probl. Pediatr, 1996, **26**, 57-70.

[19] A. Perdikaris, N. Alexandropoulos, S. Kintzios, Development of a novel, ultra-rapid biosensor for the qualitative detection of hepatitis B virus-associated antigens and anti-HBV, based on "membrane-engineered" fibroblast cells with virus-specific antibodies and antigens, Sensors (Basel) 9 , 2009, 2176-2186.

[20] I. Chaiken, S. Rose, R. Karlsson, Analysis of macromolecular interactions using immobilized ligands, Anal. Biochem, 1992, **201**, 197-210.

[21] C. Hu, N. Gan, Z. He, L. Song, A novel chemiluminescent immunoassar for microcystin (MC) detection based on gold nanoparticles label and its application to MC analysis in aquatic environmental samples, International Journal of Environmental Anal. Chem, 2007, **88**, 267-277.

[22] S. Chah, M.R. Hammond, R.N. Zare, Gold nanoparticles as a colorimetric sensor for protein conformational changes, Chem. Biol, 2005, **12**, 323-328.

[23] M. H. Van Regenmortel, D. Altschuh, J. Chatellier, L. Christensen, N. Rauffer-Bruyère, P. Richalet-Secordel, J. Witz, G. Zeder-Lutz, Measurement of antigen-antibody interactions with biosensors. J. Mol. Recognit, 1998, **11**, 163-167.

[24] M.H. Van Regenmortel, Thermodynamic parameters in immunoassay, Clin. Chem. Lab. Med, 1998, **36**, 353-354.

[25] D. Li, Q. He, Y. Cui, L. Duan, J. Li, Immobilization of glucose oxidase onto gold nanopartcles with enhanced thermostability, Biochem. Bioph. Res. Co, 2007, **355**, 488-493.

[26] J. Yguerabide, and E.E. Yguerabide, Light-scattering submicroscopic particles as highly fluorescent analogs and their use as tracer labels in clinical and biological applications, Anal. Biochem, 1998, **262**, 157-176.

[27] K. Aslan, V. H. Perez-Luna, Surface modification of colloidal gold by chemisorption of alkanethiols in the presence of nonionic surfactant, Langmuir, 2002, **18**, 6059-6065.

[28] K. Du, Y. Zhou, L. Wang, Y. Wang, Fluorescent-tagged no phosphate and nitrogen free calcium phosphate scale inhibitor for cooling water systems, J. Appl. Polym. Sci, 2009, **113**, 1966-1974.

[29] X. Yang, Y. Guo, A. Wang, Luminol/antibody labeled gold nanoparticles for chemiluminescence immunoassay of carcinoembryonic antigen, Anal. Chim. Acta, 2010, **666**, 91-96.

[30] Y. Wu, Y. Zhuang, S. Liu, L. He, Phenyl boronic acid immunoaffinity reactor coupled with flow injection chemiluminescence for determination of α-fetoprotein, Anal. Chim. Acta, 2008, **630**, 186-193.

[31] H.S. Zhuang, Q.E. Wang, G.N. Chen, J.L. Huang, Study on the relationships between chemiluminescent properties and molecular structures of some biacridine probes, Spectrosc. Spect. Anal. 2004, **24**, 1514-1517.

[32] G. Shi, B.J. Tang, X. Wang, L.X. Zhao, J.M. Lin, Micriplate chemiluminescent enzyme immunoassay for the quantitative analysis of free prostate-specific antigen in humman serum, Chin. J. Anal. Chem, 2007, **35**, 1541-1547.

[33] H. Fukada, A. Haga, T. Fujita, N. Hiramatsu, C.V. Sullivan, A. Hara, Development and validation of chemiluminescent immunoassay for vitellogenin in five salmonid species, Comp. Biochem. Physiol. A Mol. Integr. Physiol, 2001, **130**, 163-170.

[34] G. Frens, Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions, Nat. Phys. Sci, 1973, **241**, 20-22.

Figure Legend

Fig. 1 Chemiluminescence intensity recorded by immune sandwich formation (Ab_1-Ag-Ab_2/GNP/L, containing 20 ng/mL HBsAg) in the presence of H_2O_2 (10^{-3} M) as oxidant agent and $HAuCl_4$ (0.1% v/w) as catalyst. The order of Ab_2 and luminol attachment on GNP surface was as follows: (a) Ab_2 and luminol at the same time were added to GNPs. (b) At first Ab_2 and then after 30 minutes, luminol was added to GNPs. (c) At first luminol and then after 30 minutes, Ab_2 was added to GNPs. Luminol was dissolved in SCB (pH 9, 0.1 M) and Ab_2 was diluted in PBS (pH 9, 0.1 M). The columns in the inset represent the mean value of three independent assays.

Fig. 2 **A**) pH optimization for co-immobilization of Ab_2 and luminol on GNPs surface. At first luminol (0.1 M in CBS with a certain pH) and then after 30 minutes Ab_2 (0.1 M in PBS with the same pH) was added to GNPs at different pHs. Finally, chemiluminescence intensity was recorded by following the procedure mentioned in Experimental Section for "immune complex formation" in presence of 25 ng/mL HBsAg and "chemiluminescence measurements". **B)** Time optimization for Ag-Ab_2/GNP/L interaction at 25 (▲) and 37 (●) ºC. Ab_1 was immobilized on plates and incubated with the sample containing HBsAg (5 ng/mL). Then the complex (Ab_1-Ag) was incubated with Ab_2/GNP/L at different times: 5, 10, 20, 30, 45, 60 and 75 minutes. After completion of immune sandwich (Ab_1-Ag-Ab_2/GNP/L), chemiluminescence intensity was recorded in the presence of H_2O_2 (10^{-3} M) as oxidant agent and $HAuCl_4$ (0.1% v/w) as catalyst. Each point represents the mean value of three independent assays.

Fig. 3 **A**) Comparison between the effects of different catalysts on immunoassay. Chemiluminescence intensity of sandwich containing HBsAg (10 ng/mL) was recorded in the presence of H_2O_2 (10^{-3} M) and different catalysts at their optimized concentration: $AgNO_3$ 10^{-5} M [30], $CoCl_2$ 0.01 M [31], $HAuCl_4$ 0.1% w/v, Hb 150 mg/ml) in SCB (100 mM, pH 9), at room temperature and 45 min incubation. **B**) Optimization of catalyst concentration. Chemiluminescence intensity of sandwich in presence of 20 ng/mL HBsAg was recorded in the presence of H_2O_2 (10^{-3} M) and 10 µl of $HAuCl_4$ at different concentrations. Each point represents the mean value of three independent assays.

Fig. 4 Calibration curve of immunosensor toward HBsAg. Inset shows the linear range. The intensity was recorded in the presence of different concentrations of HBsAg. The immune sandwich was formed in optimum conditions (SCB buffer pH 9, 10 µl H_2O_2 10^{-3} M, and 10 µl $HAuCl_4$ 0.1% w/v). Each point represents the mean value of three measurements.

Fig. 5 Specificity of biosensor towards HBsAg, HCV-core, HCV-NS3, HCV-NS4, HCV-NS5 antigens and BSA. Intensity of immune sandwich was recorded in the presence of 25 ng/mL of antigens, (in SCB buffer, pH 9), 10 µl of H_2O_2 (10^{-3} M), and 10 µl of $HAuCl_4$ (0.1% w/v). Each point represents the mean value of three independent assays.

Scheme 1 (A) The process for functionalization of gold nanoparticles. (I) Formation of MUA self-assembled mono-layer on GNPs in the presence of Tween 20. (II) Activation of carboxylic acid groups via EDC/NHS binding. (III) Attachment of luminol and then secondary antibody (anti HBsAg) to the activated GNPs. (B) The process for immune sandwich formation and chemiluminescence measurement. (I) HBsAg was added to the wells covered with Ab_1, then to remove the unbound antigens the wells were washed by washing buffer. (II) The GNPs labeled with secondary antibody and luminol (Ab_2/GNP/L) were added to the well to complete the immune sandwich. (III) In the presence of catalyst ($HAuCl_4$) and oxidation agent (H_2O_2) the luminal molecules were exited and emission was recorded at 425 nm.

Table 1. Comparison of the analytical parameters obtained by present work and those reported in the literature based on chemiluminescence immunoassay using different catalysts.

Antigen Type	**Linear range** (ng/ml)	**Detection limit** (ng/ml)	**Catalyst**	**References**
CEA	5-20	0.1	HRP and Co^{2+}	[29]
AFP	10-100	3.5	HRP	[30]
CEA	10-100	0.53	DMDSBA	[31]
FPSA	0.15-20	0.1	ALP	[32]
Vg	1.56-8000	0.03	HRP	[33]
HBsAg	0.12-30	0.014	Au^{3+}	Present work

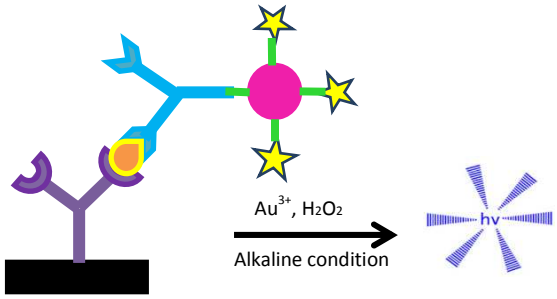
CEA: Carcinoemberyonic antigen, AFP: α-fetoprotein, FPSA: Free prostate-specific antigen, Vg: Vitellogenin, HRP: Horse radish peroxidase, DMDSBA: 10,10'-dimethyl-3,3'-disulfo-9,9'-biacridine, ALP: Alkaline phosphatase

Table 2. Comparison of designed immunosensor responses and the results obtained by ELISA method for determination of HBsAg in real human serum samples.

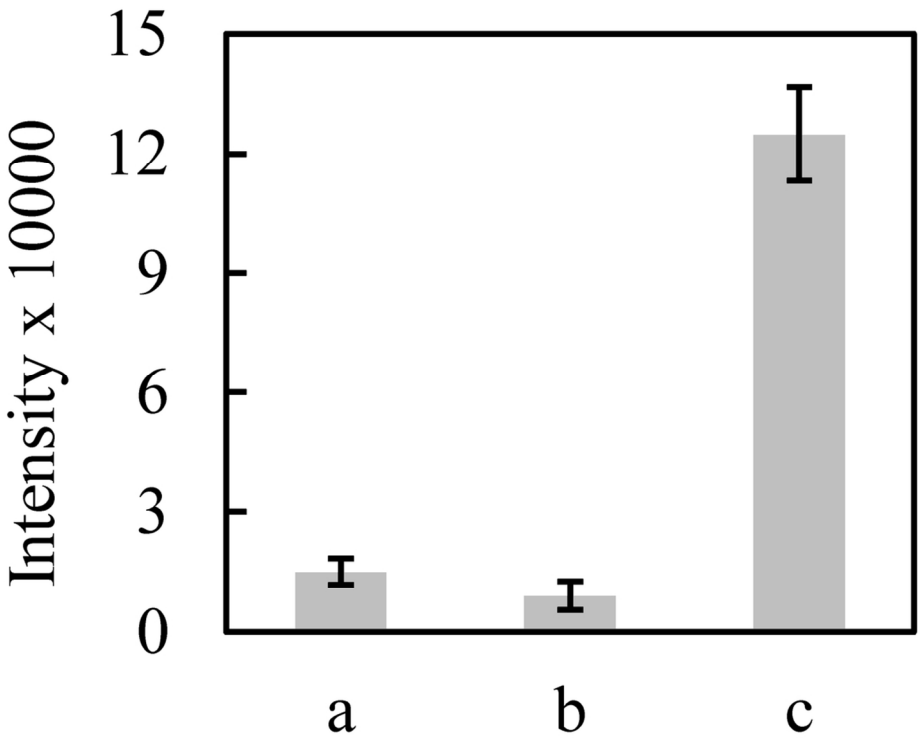
	Sample 1 (ng/mL)	**Sample 2 (ng/mL)**	**Linear range (ng/mL)**	**Detection limit (ng/mL)**
ELISA*	0.27±0.02	2.70±0.02	1-100	0.1
Present Immunosensor**	0.28±0.06	2.63±0.06	0.12-30	0.014

*The results obtained by conventional ELISA method (https://www.diapro.it/index.php/products/elisa/hepatitis/hepatitis-b/HBsAg-detail)
**The results are the mean values of three measurements obtained by designed immunosenor.

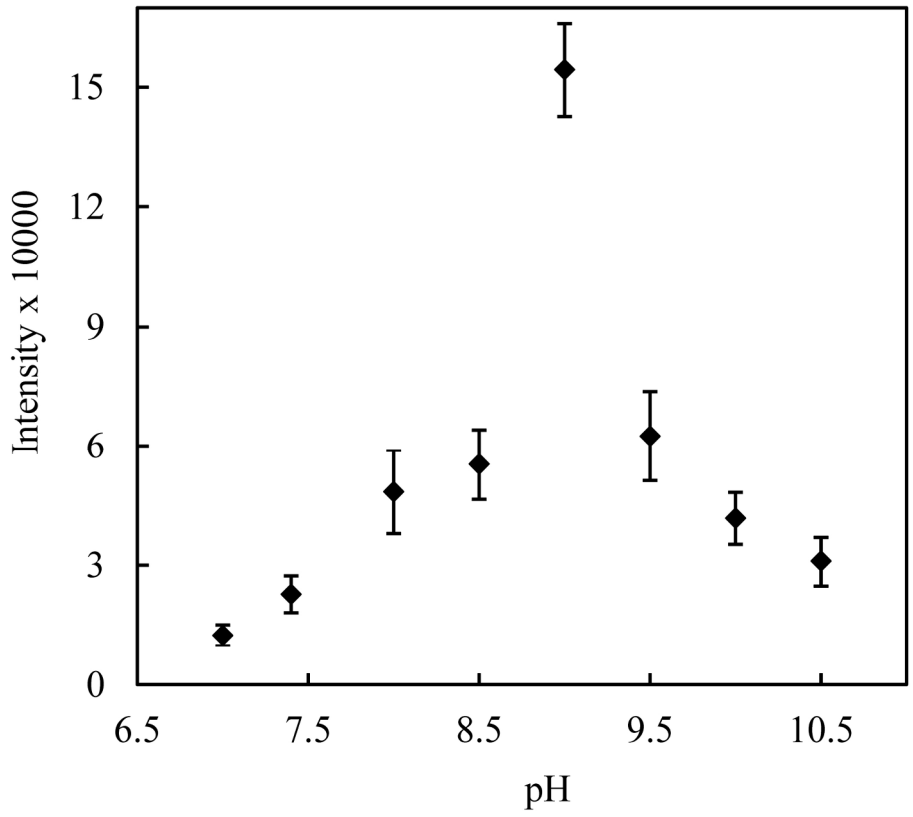
Table of content entry



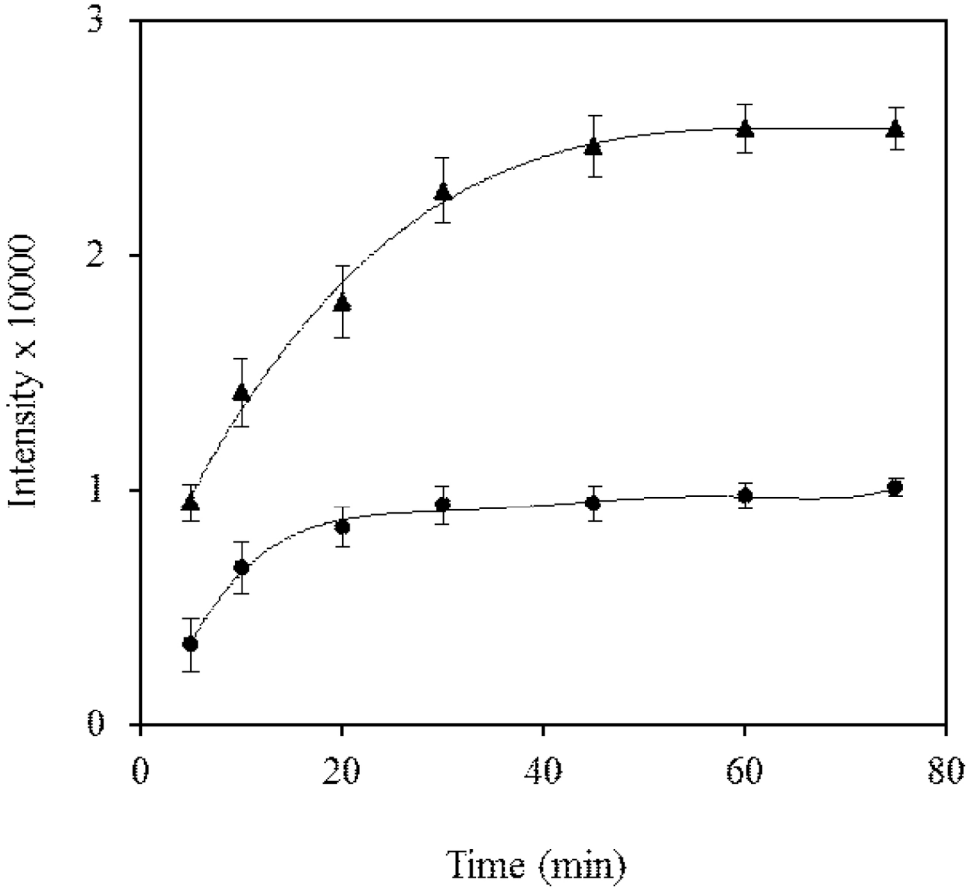
- $HAuCl_4$ acted more efficient than the other catalysts.
- Modified GNPs caused signal amplification and high specificity toward HBsAg.



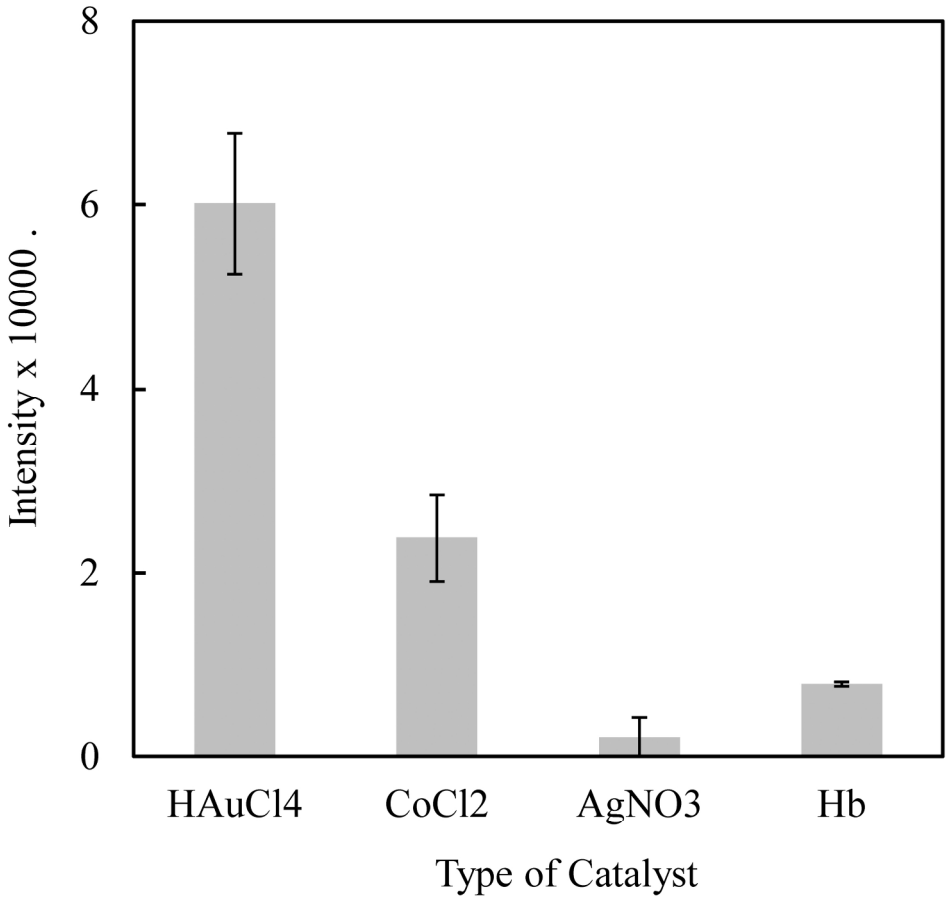
53x45mm (600 x 600 DPI)



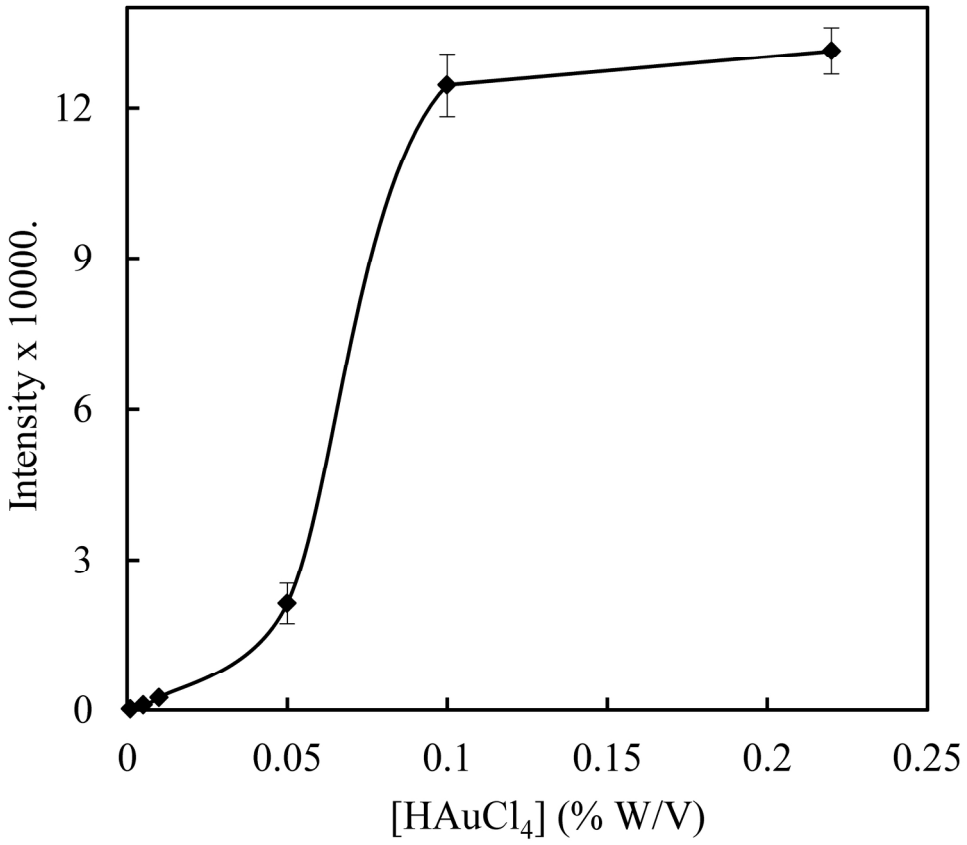
97x85mm (600 x 600 DPI)



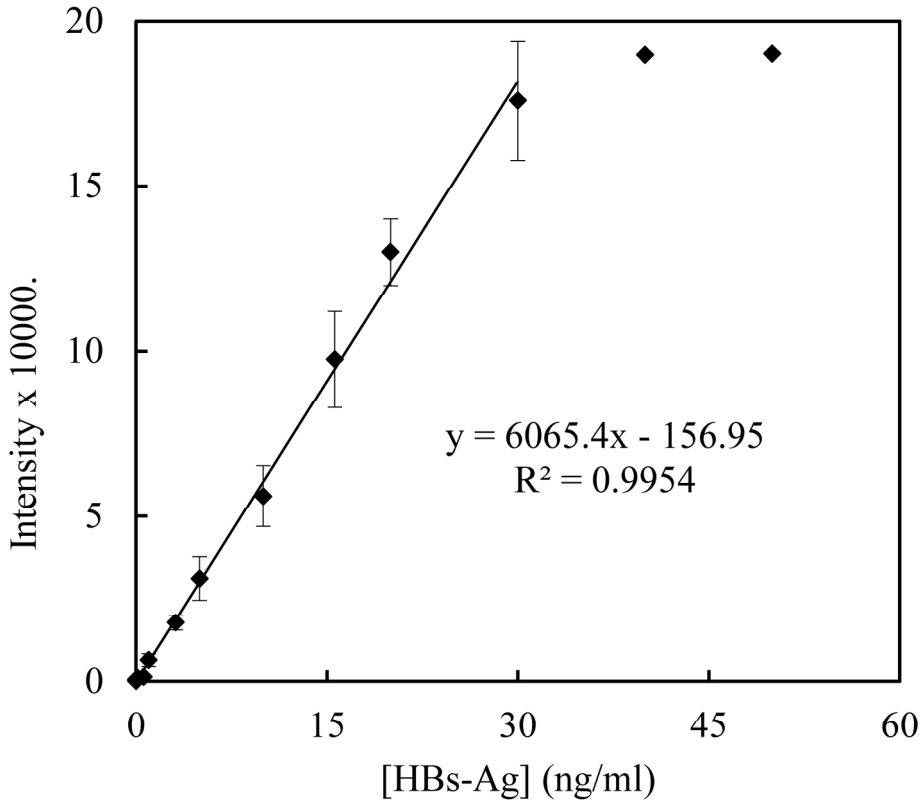
96x88mm (600 x 600 DPI)



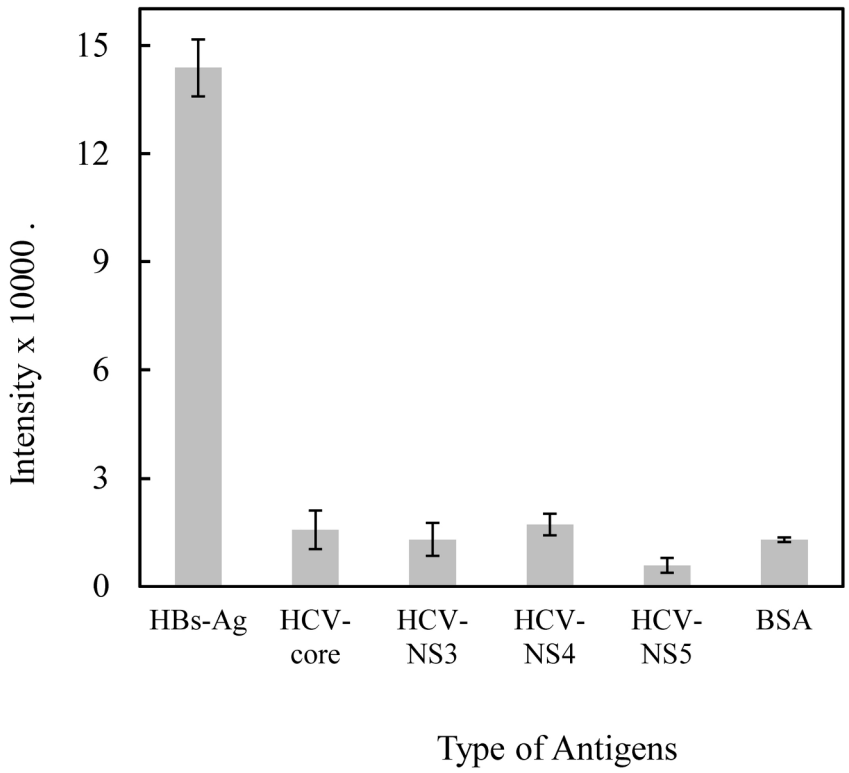
100x96mm (600 x 600 DPI)



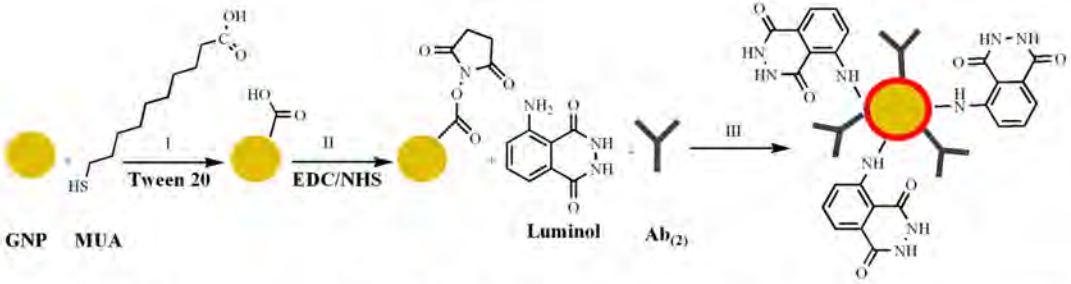
97x89mm (600 x 600 DPI)

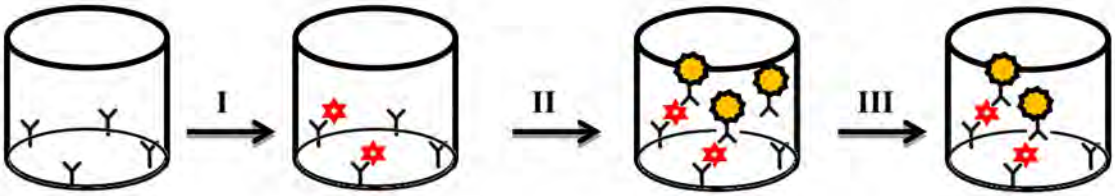


94x80mm (600 x 600 DPI)



100x83mm (600 x 600 DPI)





GNPs: Gold nanoparticles

Carboxylated GNPs

Ab2/GNP/L

HBsAg