RESEARCH ARTICLE

Antibody Conjugated Gold Nanoparticles for Detection of Small Amounts of Antigen Based on Surface Plasmon Resonance (SPR) Spectra

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ABSTRACT

In this paper, a fast and sensitive localized surface plasmon resonance (LSPR) based biosensor was developed and the optimization of gold – antibody conjugates through investigation of different parameters were performed. Gold nanoparticles (AuNPs) with a size of ~20 nm were synthesized via chemical reduction of $HAuCl_4$ with trisodium citrate as reducing and stabilizing agent. The impacts of pH of gold colloids and antibody concentrations on conjugation of electrostatically absorbed antibodies on the AuNPs surface were evaluated. The diverse amounts of antigens were added to the selected gold – antibody conjugate and the calibration curve and limit of detection of the system were successfully obtained. The UV- Vis and DLS outputs were utilized to prove the efficiency and repeatability of the system. As a result, the designed biosensor shows a convincing LOD of 400 ng/ml of antigen. It is suggested that electrostatic absorption strategy of AuNPs with negative charge to the positively charged antibodies can be an efficient methodology. Results showed an effective LSPR system for detection of small amounts of antigen in short time as well as with high accuracy.

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INTRODUCTION

Recently, because of numerous progress in the field of nanotechnology, nanomaterials possess specific applications for creating *in vitro* bio-diagnostic instruments (1, 2). Special characteristics of nanomaterials including surface area to volume ratio, powerful signal production and adjustable surface chemistries make them a great candidate for biological and chemical detections (3). Large surface of nanomaterials permits them to carry diverse range of bioreceptors such as antibodies, enzymes, DNAs and organic dyes and permitting the detection of tiny amounts of analytes (4). Up till now, lots of studies based on different nanomaterials such as carbon nanotubes, AuNPs, magnetic nanoparticles and quantum dots have been done to develop *in vitro* diagnostic systems for detection of biomarkers at different levels. Among those mentioned biosensors, AuNPs based biosensors have attracted outstanding research interest because of their unique physical and chemical characteristics such as localized surface plasmon resonance (LSPR) (5), surface enhanced Raman scattering (SERS) and so on (6). LSPR is known as a spectroscopic technique, originated from collective oscillation of conduction electrons at the interface of noble metal nanoparticles-dielectric medium caused by an incident light (7). When the frequency of incident light matches with the natural frequency of surface electrons, the resonance phenomenon occurs. Subsequently, distinct absorption peak in the visible region can be seen along with the strong electromagnetic fields (8). The LSPR wavelength and width depend

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on significant parameters such as particle diameter, shape, local environment and coating of the surface (9-12). The impacts of these factors can be seen with the color change of the solution or red shift of the absorption band. The influence of the above mentioned factors on the LSPR response, can be applied for detecting proteins, oligonucleotides etc. The most conventional metallic nanoparticles for LSPR apparatus are gold and silver nanoparticles. On the whole, the safety and surface stability of AuNPs are strongly better than silver nanoparticles (7, 13). In general, LSPR sensing technique occurs in colorimetric sensing as a result of absorption band shift (14). It is known that, binding of specific analytes like antibody on the surface of AuNPs can alter the local dielectric environment of AuNPs and result in a LSPR peak shift. This shift can be measured by UV-visible spectrophotometer (15). The colorimetric analysis is another readout technique, which is originated from aggregation of AuNPs that is induced by reaction of certain analytes (16-18). The considerable red shift of LSPR leads to color change from red to blue or even purple (13, 19-21). Up to now, the eminent strategy for detection of protein is known as immunoassay, which depends on antigen-antibody interactions and diversity of transducers including fluorophores, enzymes, tags, labels and DNA barcodes, etc. Among these transduction analytes, conventional ELISA permits detection of proteins by color change which can be diagnosed with naked eye and does not requires complicated tools. ELISA acquires advantages such as sensitivity, and low cost of analysis (22, 23); however, it includes major limitations. ELISA requires several steps of incubation and washing cycles that are difficult and time-consuming, on the other hand, results cannot be produced again and proteins have impact on enzyme activity. An ELISA assay often needs up to 24 hours for analysis; an important part of assay time is consumed by incubation step. Extra difficulties may be faced when an ELISA is utilized to screen antibodies to be incorporated into emerging AuNPs-based immunoassays. Conjugation of the antibody to AuNPs might affect the bioactivity of the antibody which will not be evaluated by ELISA. An alternative method to detect and characterize Abs that overcomes the limitations of ELISA is required (24). In a study by Pieper-Fürst et al., 20 nm AuNPs were synthesized and electrostatically conjugated to the matrix metalloproteinase-2 (MMP-2) Abs and the limit of detection (LOD) of the system was 0,5 pM (25). In other study by Liu et al., 20 nm AuNPs were electrostatically attached to single-chain fragment variable Ab containing cysteine (scFv-cys) and the LOD was 1,7 nM (26). In a study by Springer et al., the SPR system based on covalent attachment of 30 nm AuNPs to carcinoembryonic antigen (CEA) was developed and the final LOD was 0,1 ng/ml (27).

In this work, a rapid screening method for recognizing antibody-antigen binding specificity and affinity was developed using AuNPs and UV-visible spectroscopy as a principal characteristic method. Different parameters that have impacts on conjugation of antibody on the gold surface such as pH of gold colloid and different concentrations of antibody were investigated in advance. After reporting the optimum conjugates, for diagnosing the feasibility of the system, different amounts of antigen for sensing systems sensitivity and limit of detection were added and induced aggregation of the system. The formation of aggregates is detected as a decrease in absorption peak by UV-visible spectroscopy and an increase in hydrodynamic diameter by the DLS instrument. All these steps were evaluated by transmission electron microscopy (TEM), UV-visible spectroscopy combined with dynamic light scattering (DLS) as a helper instrument. The LOD of the system was determined 400 ng/ml. Calibration curve for antigens was constructed to rank the specificity and LOD of system. This procedure is single-step and wash-free and the screening time reduced dramatically in comparison to 24 hours by ELISA.

EXPERIMENTAL

Materials

Tetrachloroauric acid trihydrate 99.5% ($HauCl_4 .3H_2O$), Sodium citrate dehydrate ($Na_3C_6O_7\text{-}2H_2O$) as reducing agents were purchased from Merck Company. Bovine serum albumin was purchased from Sigma-Aldrich (St.Luis.MO). Deionized distilled water was utilized. Rabbit polyclonal anti IgG antibodies specific to IgG protein and human IgG protein were purchased from RojanAzmaTeb Institute (Karaj,Iran).

Synthesis and characterization of AuNPs

AuNPs were synthesized by reducing tetrachloroauric acid with trisodium citrate (28). All laboratory glassware and magnetic stir bars utilized in the synthesis of AuNPs were completely washed in aqua regia (HCl/HNO_3 3:1, v/v), washed

with distilled water, and then oven-dried before usage to avoid undesirable nucleation during the synthesis, and also aggregation of gold colloid solution. A 0.25mM HAuCl4.3H2O solution (50 mL) was boiled with forceful stirring in a 250mL round bottom flask provided with a condenser to maintain a constant volume of the reaction mixture. A 140 Mm trisodium citrate solution (0.25 mL) was then added rapidly to the boiling solution, that leads in a color change from pale yellow to dark red, demonstrating the formation of AuNPs. The solution was left for 10 min at boiling temperature and then discarded from the heating mantle. Stirring was continued for another 15min (28).UV-Vis absorption spectra of AuNPs were recorded by using a Cecil (Cambridge, United Kingdom) Ultraviolet-visible spectrophotometer with an optical resolution of 0.01nm full width at half maximum (FWHM). The spectrum response was taken from 200 to 900 nm with 1 nm steps in a 45×12.5×12.5 mm path quartz cuvette. Morphology of the AuNPs was characterized by imaging analysis using transmission electron microscopy at an operating voltage of 200 kV.

Optimization of pH of the AuNPs colloid and optimization of the polyclonal Ab concentration for AuNPs-polyclonal Ab conjugation

The optimization of gold-antibody conjugates was accomplished as follows: 300 µl of 20 nm AuNPs suspension was added to separate cell culture plates (9 plates in total). In separated plate the pH of colloidal gold was measured and it was obtained between 4 and 5. As a result, the pH of colloidal gold sol was adjusted to 5-5.5; 5.5- 6 and 6-6.5 by adding 10, 12 and 14 µl of 0.1 M sodium hydroxide into each well, respectively. Diverse amounts of antibody (2.7, 3.8 and 5.7 µg/ml) were added to each pH adjusted well. The 1st gold colloid sample with pH 5-5.5 contained 30 µl of 2.7, 3.8 and 5.7 µg/ml of antibody, the 2nd gold colloid sample with pH 5.5-6 contained 30 µl of 2.7, 3.8 and 5.7 µg/ml of antibody and the 3rd group of gold colloid sample with pH 6-6.5 contained 30 µl of 2.7, 3.8 and 5.7 µg/ml of antibody. All the solutions were mixed well for an hour at 25 ^{0}c temperature. After an hour the peak shift and hydrodynamic diameter of antibody-conjugated AuNPs was measured via UV-visible spectrophotometer and DLS instrument respectively. Next, all the solutions underwent the addition of 10 µl of 10% NaCl to verify the stability of AuNPs conjugates in saline environment. The conjugation process for each solution was measured by DLS and UV-visible spectroscopy and the changes in absorption peak after conjugation of antibody to the AuNPs were detected.

Bradford colorimetric assay

Bradford assay is a well-known colorimetric assay for investigating the quantity of protein in a suspension(29). To determine the amount of antibody that attached on the AuNPs, at first, a standard calibration curve based on different concentrations of IgG antibody was obtained. To reach this aim, 1 ml of Bradford reagent was added to 100 µl of antibody with concentrations of 1, 2, 4 and 8 µg/ml separately and the absorption of different samples was read in 595 nm via UV-Vis spectroscopy. This step had been done for other two times. Based on the read absorption, the standard calibration curve was drawn. Next, the antibody-conjugated AuNPs with 3.8 µg/ml of IgG antibody with pH 5.5-6 centrifuged at 8500 rpm for 20 min. After centrifugation, supernatant that contained unbounded antibodies was removed. For determining the amount of unbound antibodies, 1 ml of Bradford reagent was added to 100 µl of supernatant and the absorption was read in 595 nm via UV-Vis spectroscopy.

Preparation and characterization of AuNPs-Ab

60 µl of 0.1 M sodium hydroxide was added to 1500 µl AuNPs to adjust the pH to 5.5-6. Aliquots of 150 µl antibody solution with the concentration of 3.8 µg/ml were added to the PH adjusted AuNPs and the solution was mixed for an hour in 25^{0}c temperature. After an hour, aliquots of 25 µl BSA solution with the concentration of 400 µg/ml were added to the suspension. BSA helps blocking nonspecific binding sites and also contributes to better stabilization of the sol (24). After that, excess antibody was removed via centrifugation at 8500 rpm for 20 min. Next, the supernatant was removed and the conjugate was re-suspended in 1700 µl of DI water to preserve electrolyte balance of antibody conjugated AuNPs after centrifugation.

Immunoassay protocol

Antibody conjugated AuNPs solution with volume of 1700 µl was separated into 340 µl of solution and was transferred into 5 microtubes. In each microtube some aliquots of human IgG with concentrations of 0.2, 0.4, 0.5, 1, 2, 4 and 8 µg/ml were added and allowed to incubate for 30 min at

room temperature. The aggregation process was confirmed by UV-visible spectrophotometry and DLS. The schematic of this procedure was shown in Fig. 1.

Evaluating the specificity of antigen detection

Furthermore, in order to evaluate the specificity of antigen detection with antibody conjugated AuNPs, a control group consisted of antibody conjugated AuNPs that described in immunoassay protocol section was prepared and 30 µl of Human serum albumin (HSA) with concentrations of 0.4, 0.5, 1, 2 and 4 µg/ml was added and the samples were characterized via UV-Vis spectroscopy.

RESULTS AND DISCUSSION

synthesis and characterization of AuNPs

Fig. 2 represents the UV-Visible absorption spectrum and TEM image of GNPs. It was shown that the mean particle diameter for the synthesized AuNPs was 20 nm. UV-Vis spectra of 20 nm AuNPs exhibited extinction peak at 528 nm.

Adsorption of proteins onto AuNPs surface

An important concern related to AuNPs-based immunoassay is the stability of antibody-AuNPs conjugates in biological environments with ionic strength. Antibodies can be immobilized on the gold surface through a cross-linker or direct adsorption to the surface. Without considering the immobilization strategy, the conjugate requires to be protected from aggregation caused by salt. In this project, the direct adsorption was applied. Direct adsorption to construct antibody-AuNPs conjugates is an undemanding method needing minimal expertise in synthesis; therefore, this method is an easy method to screen antibodies. The performance of diverse subclasses of antibodies

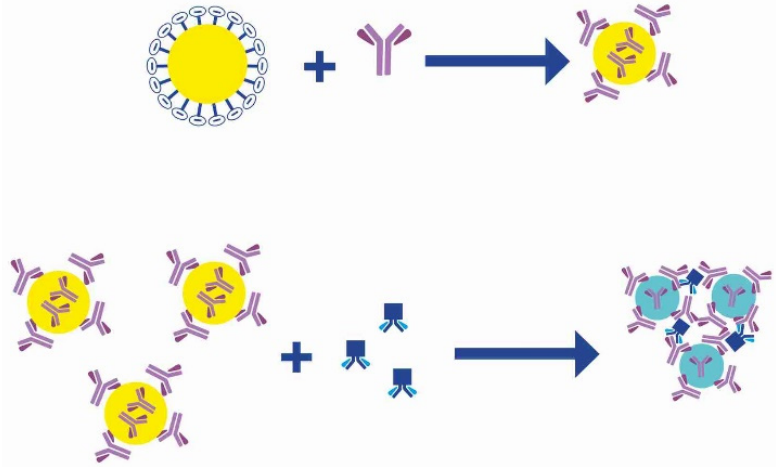

Fig. 1. A schematic diagram of experimental procedure. ( ) citrate capped AuNPs, ( ) antibody, ( )antigen.

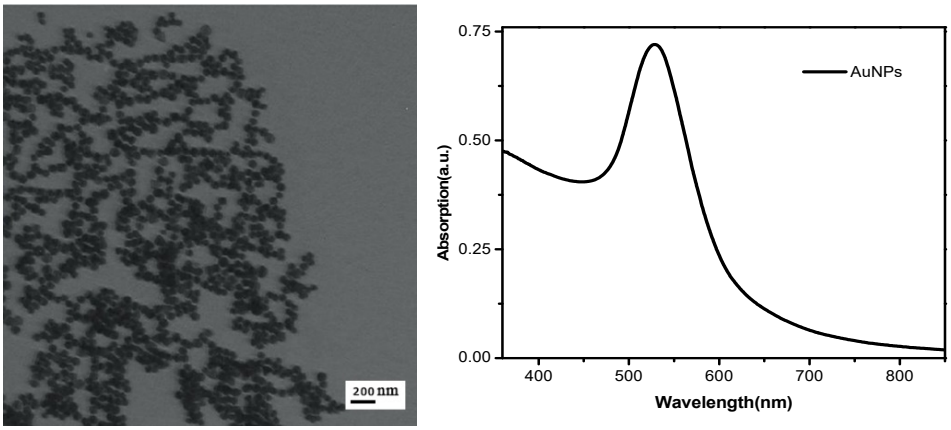

Fig. 2. TEM image and UV-Visible spectra of colloidal AuNPs with average diameter of 20nm.

in the direct conjugation are definite (30). The direct adsorption of protein onto the gold surface is a sophisticated process depends on several parameters such as concentration, isoelectric point of protein and pH. The isoelectric point of IgG antibody is around 7, so the antibody can preserve its configuration and bioactivity in the pH near to the antibody isoelectric point and this pH is an optimal pH for protein adsorption (31). For optimization of this process, the pH range of gold colloid was chosen between 5 to 7 and different concentrations of IgG antibody from 2.7 to 5.7 µg/ml were added to pH adjusted gold colloids. The top of the Fig. 3, represents the extinction spectra of antibody conjugated AuNPs with different gold colloids pH and antibody concentrations after addition of 0.1 M sodium chloride solution were recorded and the stable conjugate was selected. At the bottom of the Fig. 3, the normalized spectra of antibody conjugated AuNPs with different gold colloids pH and antibody concentrations after addition of 0.1 M sodium chloride solution were recorded.

It was shown that the colloidal gold with pH 5-5.5 and different concentrations of antibody after addition of NaCl had unstable spectra compared to bare AuNPs and it was aggregated. The colloidal gold with pH 5.5-6 and 6-6.5 with diverse concentrations of 2.7, 3.8 and 5.7 µg/ml of antibody was stable compared to bare AuNPs and had fewer red shift and decrease in absorption peak. Furthermore, the colloidal gold with pH 5.5-

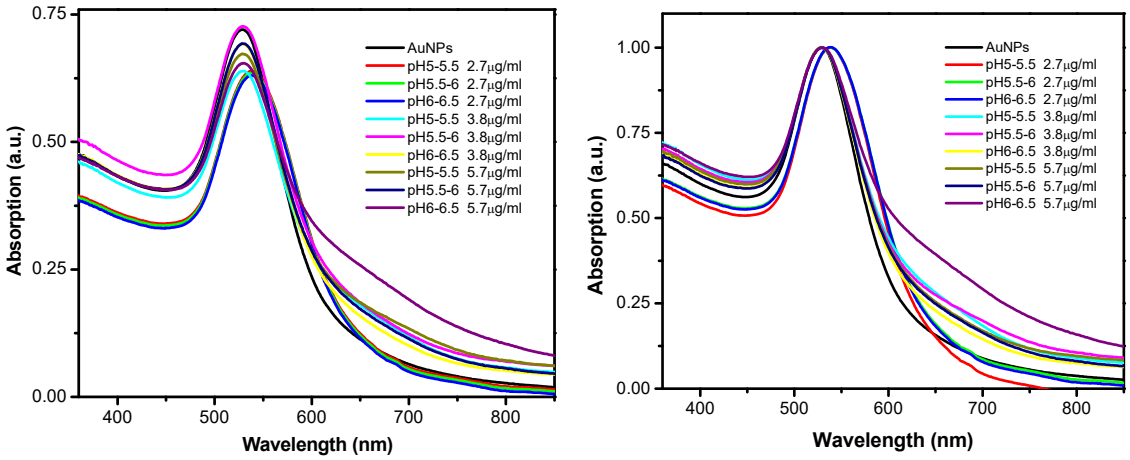

Fig. 3. UV-VIS spectra of colloidal gold and the IgG antibody with titration procedure at different pH of colloidal gold and diverse concentrations of antibody after addition of NaCl (top) and Normalized UV-VIS spectra of colloidal gold and IgG antibody with titration procedure at different pH of colloidal gold and diverse concentrations of antibody after addition of NaCl (bottom).

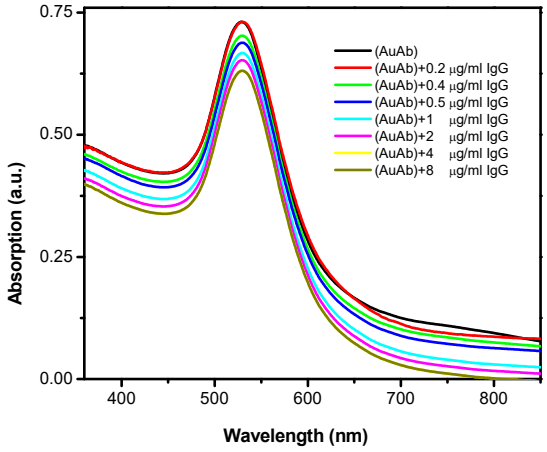

Fig. 4. UV- visible spectra of AuNPs-antibody conjugates after addition of 0.2, 0.4, 0.5, 1, 2, 4 and 8 µg/ml of IgG protein.

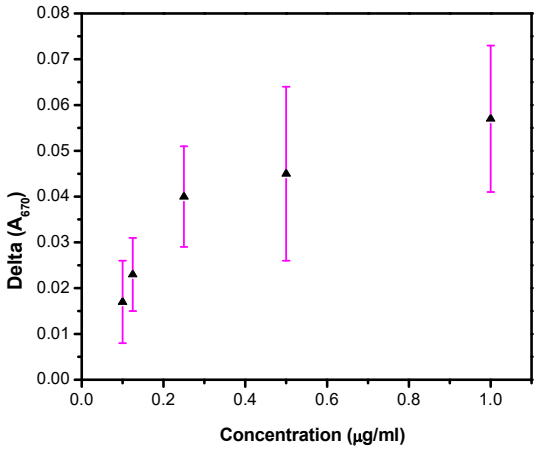

Fig. 5. Calibration curve describing the average intensity in 670 nm wavelength. A limit of detection of 0.4 µg/ml of IgG protein was achieved.

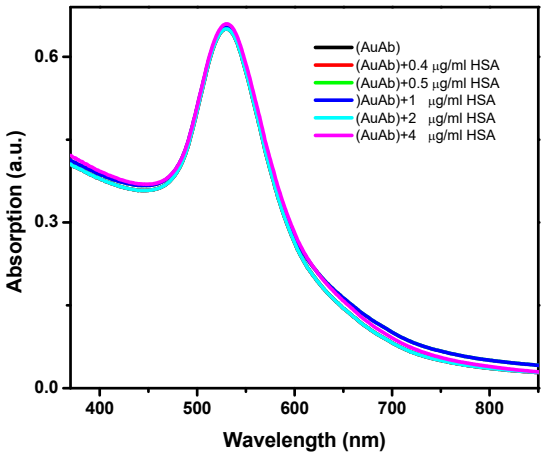

Fig. 6. UV-Vis spectra of AuNPs-antibody conjugates after addition of different concentrations of HSA protein

6 and antibody concentration of 3.7 µg/ml was chosen as a stable antibody-AuNPs conjugate that maintained the stable conjugate and had the least red shift and increase or decrease in absorption peak with the minimum concentration of antibody.

Evaluation of the AuNPs-antibody conjugation efficiency with Bradford assay

Subsequent to AuNPs-antibody conjugation with Bradford assay, antibody concentration in supernatant after centrifugation was determined to be 1.6 µg/ml. Antibody concentration before centrifugation was 4 µg/ml, so it can be inferred that approximately 60% of antibodies were attached to the surface of AuNPs and the conjugation efficiency was 60%.

IgG immunoassay results

The experimental results in Fig. 4 demonstrate that the addition of 0.2 µg/ml of IgG protein to the antibody conjugated AuNPs maintained no response compared to the antibody conjugated AuNPs but subsequently after addition of 0.4 µg/ml of IgG protein, the sensing started and decrease in absorption spectra was tangible. This procedure elongated up to addition of 4 µg/ml of antigen and subsequently after addition of 8 µg/ml of antigen, the system saturated and the plateau state was achieved.

According to these results, the limit of detection of the biosensor based on AuNPs conjugated IgG antibody for detection of IgG protein was obtained to be 0.4 µg/ml. The calibration curve based on absorption intensity changes in 670 nm for concentrations of 0.2, 0.4, 0.5, 1, 2 and 4 µg/ml of IgG protein was shown in Fig. 5.

Analysis of the specificity of antibody-antigen interaction

In order to evaluate the specific detection of IgG protein with AuNPs-antibody conjugates, a control group of AuNPs conjugated IgG antibody was prepared and different concentrations of 0.4, 0.5, 1, 2 and 4 µg/ml of human serum albomin (HSA) were added. In Fig. 6 the non-responding curve of AuNPs conjugated IgG antibody against different concentrations of HSA protein was shown.

CONCLUSIONS

In summary, the main goal of this project was to optimize of AuNPs -antibody conjugates and accordingly, essential factors such as colloidal gold pH and antibody concentration which had impact on the optimized AuNPs-antibody conjugates were investigated. This protocol had the ability to be used in repetitive tests with high turnovers. This LSPR biosensor based on AuNPs - antibody conjugate shows a satisfactory response to IgG protein in the concentration range of 0.4 – 4 µgml^{-1}. It is anticipated that this LSPR biosensor can be utilized for detecting various proteins by selected Au-antibody conjugates, which makes the new sensor capable of detecting diverse proteins. Consequently, this system is reliable and facile for detection of antigen due to: 1) easy preparation of AuNPs-antibody conjugates. 2) detection of IgG protein is not durable and long lasting process. 3) the results of each step have repeatability. 4) specific performance in protein detection.

CONFLICTS OF INTEREST

The author declare that there is no conflicts of interest regarding the publication of this manuscript.

REFERENCES

1. Haes AJ, Chang L, Klein WL, Van Duyne RP. Detection of a Biomarker for Alzheimer's Disease from Synthetic and Clinical Samples Using a Nanoscale Optical Biosensor. Journal of the American Chemical Society. 2005;127(7):2264-71.
2. Kim J, Oh SY, Shukla S, Hong SB, Heo NS, Bajpai VK, et al. Heteroassembled gold nanoparticles with sandwich-immunoassay LSPR chip format for rapid and sensitive detection of hepatitis B virus surface antigen (HBsAg). Biosensors and Bioelectronics. 2018;107:118-22.
3. Konvalina G, Haick H. Sensors for Breath Testing: From Nanomaterials to Comprehensive Disease Detection. Accounts of Chemical Research. 2013;47(1):66-76.
4. Zhou W, Gao X, Liu D, Chen X. Gold Nanoparticles for In Vitro Diagnostics. Chemical Reviews. 2015;115(19):10575-636.
5. Zeinoddini M, Azizi A, Bayat S, Tavasoli Z. Localized Surface Plasmon Resonance (LSPR) Detection of Diphtheria Toxoid Using Gold Nanoparticle-Monoclonal Antibody Conjugates. Plasmonics. 2017;13(2):583-90.
6. Boisselier E, Astruc D. Gold nanoparticles in nanomedicine: preparations, imaging, diagnostics, therapies and toxicity. Chemical Society Reviews. 2009;38(6):1759.
7. Hutter E, Fendler JH. Exploitation of Localized Surface Plasmon Resonance. Advanced Materials. 2004;16(19):1685-706.
8. Kelly KL, Coronado E, Zhao LL, Schatz GC. The Optical Properties of Metal Nanoparticles: The Influence of Size, Shape, and Dielectric Environment. ChemInform. 2003;34(16).
9. Burda C, Chen X, Narayanan R, El-Sayed MA. Chemistry and Properties of Nanocrystals of Different Shapes. Chemical Reviews. 2005;105(4):1025-102.
10. Su KH, Wei QH, Zhang X, Mock JJ, Smith DR, Schultz S. Interparticle Coupling Effects on Plasmon Resonances of Nanogold Particles. Nano Letters. 2003;3(8):1087-90.
11. Tokarev I, Minko S. Tunable plasmonic nanostructures from noble metal nanoparticles and stimuli-responsive polymers. Soft Matter. 2012;8(22):5980.
12. Haes AJ, Duyne RPV. Preliminary studies and potential applications of localized surface plasmon resonance spectroscopy in medical diagnostics. Expert Review of Molecular Diagnostics. 2004;4(4):527-37.
13. Haes AJ, Zou S, Schatz GC, Van Duyne RP. A Nanoscale Optical Biosensor: The Long Range Distance Dependence of the Localized Surface Plasmon Resonance of Noble Metal Nanoparticles. The Journal of Physical Chemistry B. 2004;108(1):109-16.
14. Wu S, Li D, Wang J, Zhao Y, Dong S, Wang X. Gold nanoparticles dissolution based colorimetric method for highly sensitive detection of organophosphate pesticides. Sensors and Actuators B: Chemical. 2017;238:427-33.
15. Petryayeva E, Krull UJ. Localized surface plasmon resonance: Nanostructures, bioassays and biosensing—A review. Analytica Chimica Acta. 2011;706(1):8-24.
16. Rosi NL, Mirkin CA. Nanostructures in Biodiagnostics. Chemical Reviews. 2005;105(4):1547-62.
17. Vilela D, González MC, Escarpa A. Sensing colorimetric approaches based on gold and silver nanoparticles aggregation: Chemical creativity behind the assay. A review. Analytica Chimica Acta. 2012;751:24-43.
18. Lin M, Pei H, Yang F, Fan C, Zuo X. Applications of Gold Nanoparticles in the Detection and Identification of Infectious Diseases and Biothreats. Advanced Materials. 2013;25(25):3490-6.
19. Liu D, Qu W, Chen W, Zhang W, Wang Z, Jiang X. Highly Sensitive, Colorimetric Detection of Mercury(II) in Aqueous Media by Quaternary Ammonium Group-Capped Gold Nanoparticles at Room Temperature. Analytical Chemistry. 2010;82(23):9606-10.
20. Li L, Li B, Cheng D, Mao L. Visual detection of melamine in raw milk using gold nanoparticles as colorimetric probe. Food Chemistry. 2010;122(3):895-900.
21. Lee J-S, Han MS, Mirkin CA. Colorimetric Detection of Mercuric Ion (Hg2+) in Aqueous Media using DNA-Functionalized Gold Nanoparticles. Angewandte Chemie. 2007;119(22):4171-4.
22. Voller A, Bidwell D, Bartlett A. Enzyme immunoassays in diagnostic medicine: Theory and practice*. Bulletin of the World Health Organization. 1976;53(1):55.
23. O'Sullivan MJ, Bridges JW, Marks V. Enzyme Immunoassay: A Review. Annals of Clinical Biochemistry: An international journal of biochemistry and laboratory medicine. 1979;16(1-6):221-39.
24. Lai YH, Koo S, Oh SH, Driskell EA, Driskell JD. Rapid screening of antibody–antigen binding using dynamic light scattering (DLS) and gold nanoparticles. Analytical Methods. 2015;7(17):7249-55.
25. Pieper-Fürst U, Kleuser U, Stöcklein WFM, Warsinke A, Scheller FW. Detection of subpicomolar concentrations of human matrix metalloproteinase-2 by an optical biosensor. Analytical Biochemistry. 2004;332(1):160-7.
26. Liu Y, Liu Y, Mernaugh RL, Zeng X. Single chain fragment variable recombinant antibody functionalized gold nanoparticles for a highly sensitive colorimetric immunoassay. Biosensors and Bioelectronics. 2009;24(9):2853-7.
27. Špringer T, Homola J. Biofunctionalized gold nanoparticles for SPR-biosensor-based detection of CEA in blood plasma. Analytical and Bioanalytical Chemistry. 2012;404(10):2869-75.
28. Turkevich J, Stevenson PC, Hillier J. A study of the nucleation and growth processes in the synthesis of colloidal gold. Discussions of the Faraday Society. 1951;11:55.
29. Bradford M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry. 1976;72(1-2):248-54.
30. Geoghegan WD, Ambegaonkar S, Calvanico NJ. Passive gold agglutination. An alternative to passive hemagglutination. Journal of Immunological Methods. 1980;34(1):11-21.
31. Verkleij AJ, Leunissen JL. Immuno-gold-labeling in cell biology: Crc Press; 1989.