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Novel colorimetric enzyme immunoassay for the detection of carcinoembryonic antigen

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ABSTRACT

We developed a highly sensitive colorimetric enzyme immunoassay for the detection of carcinoembryonic antigen (CEA). This method employed gold nanoparticles (AuNPs) as carriers of anti-CEA antibody labeled with biotin, which served as an affinity tag for streptavidin-horseradish peroxidase (streptavidin-HRP) binding. Using this strategy, about 12 HRP molecules were coated onto each AuNP. Through "sandwich-type" immunoreaction, the AuNP–anti-CEA–HRP complex was brought into the proximity of magnetic microparticle. As a result, HRP molecules confined at the surface of the "sandwich" immunocomplexes catalyzed the enzyme substrate and generated an optical signal. The spectrophotometric measurement confirmed effective signal amplification. The signals were linearly dependent on CEA concentrations from 0.05 to 50 ng mL^{-1} in a logarithmic plot, with a detection limit of 48 pg mL^{-1}. Intra- and inter-assay coefficients of variation were <8.5%. The CEA concentrations of serum specimens assayed by the developed immunoassay showed consistent results in comparison with those obtained by a conventional enzyme-linked immunosorbent assay. The developed method thus proved its potential use in clinical immunoassay of CEA.

1. Introduction

The detection of tumor biomarker levels in human serum is very important in clinical diagnosis [1]. The tumor-associated antigen carcinoembryonic antigen (CEA) is one of the most widely used tumor biomarkers. It has been reported that CEA is over-expressed in various tumors: colorectal, stomach, pancreas, liver, ovarian, breast, prostate, thyroid, bladder, kidney, and lung [2–4]. Therefore, higher-than-normal values of CEA may indicate the presence of tumors. The CEA level in serum is also related to the stage of tumor, so it can be used as a marker to directly evaluate curative effects, recrudescence, and metastasis [5,6]. Detection of the CEA level in serum therefore plays an important part in the initial diagnostic evaluation and follow-up examination during therapy.

Various analytical methods and strategies have been exploited for CEA determination, including colorimetric enzyme immunoassays [7–9], electrochemical immunoassays [10–12], radioimmunoassays [13,14], and fluoroimmunoassays [15–17]. Of these, colorimetric enzyme immunoassays have been well recognized to be the dominant technology currently used due to their advantages in applying visible radiation, nondestructive operation, and rapid signal generation and reading. Various colorimetric enzyme immunoassays have been developed for the determination of serum CEA [7,8,18–20]. Although advances in conventional colorimetric enzyme immunoassays (e.g. enzyme-linked immunosorbent assays (ELISAs)) can provide relatively high sensitivities and low detection limits, more sensitive detection methods are still needed to fulfill the requirements of early diagnosis in cancer monitoring.

Recently, the use of nanomaterials in immunoassays has shown great promise for ultra-sensitive detection of proteins. Gold nanoparticle (AuNP)/biomolecule conjugates have found application as amplification tags in a wide range of bioanalytical applications [21–23]. In the present study, we described a colorimetric enzyme immunoassay based on AuNPs to detect CEA in human serum. AuNP was employed as a carrier for numerous biotinylated anti-CEA antibodies. Streptavidin-HRP molecules were then immobilized at the surface of the AuNP via the biotin–avidin bridge. Using this strategy, each AuNP could accumulate about 12 HRP molecules. Colorimetric enzyme immunoassays based on an AuNP complex designed in this way can offer remarkably improved sensitivity for CEA measurement.

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2. Experimental

2.1. Chemicals and materials

Carboxyl-functionalized 1-μm-diameter magnetic microparticles (MMP) were purchased from Dynal Invitrogen Corp. Carcinoembryonic antigen and antibodies were purchased from Meridian Life Science Inc. 3-sulfo-*N*-hydroxysuccinimide ester (BAC-SulfoNHS), hydrogen tetrachloroaurate trihydrate ($HAuCl_4{\cdot}3H_2O$), trisodium citrate dihydrate, sodium chloride (NaCl), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 2-(*N*-morpholino) ethane sulfonic acid (MES), streptavidin-horseradish peroxidase (streptavidin-HRP), and 3,3,5,5-tetramethylbenzidine (TMB)-H_2O_2 were purchased from Sigma–Aldrich Corp.

The buffer solutions used in these experiments are as follows. Blocking&storage buffer was 10 mM phosphate sodium buffer solution, pH 7.4, 1% BSA, 2.5% sucrose, 0.1% PEG8000. Assay buffer was 10 mM phosphate sodium buffer solution, pH 7.4, 5% BSA, 2.5% sucrose, 0.1% PEG8000. Washing buffer was 10 mM phosphate sodium buffer solution, pH 7.4, 0.1% BSA, 0.05% Tween-20. All solutions were prepared using Milli-Q water from a Millipore system.

2.2. Biotinylation of the anti-CEA detection antibodies

The anti-CEA detection antibodies were biotinylated according to the manufacture's protocol. In brief, antibody solution was dialyzed against 0.1 M phosphate buffer, pH 7.2 to remove the sodium azide. Dissolved the BAC-SulfoNHS with 0.1 M phosphate buffer, pH 7.2 to give a concentration of 10 mg mL^{-1}, and immediately added this solution to antibody solution with gentle stirring for 30 min at room temperature. The biotinylated antibody solution was extensively dialyzed against 10 mM PBS, pH 7.4. Finally, BSA was added to the resulting biotinylated antibody solution to a final concentration of 0.1%, and stored at −70 °C before use. The concentration of the biotinylated antibody was determined by UV/vis absorbance at 280 nm.

2.3. Preparation of AuNP–anti-CEA–HRP complex

AuNPs were synthesized by citrate reduction method [24]. Briefly, an aqueous solution of 1 mM $HAuCl_4$ (250 mL) was boiled with rapid stirring, and added a solution of 38.8 mM trisodium citrate (25 mL) to the boiling $HAuCl_4$ solution. After a continuous boiling for an additional 15 min, the solution was slowly cooled to room temperature and filtered through a 0.22 μm cellulose nitrate filter. The synthesized AuNPs were characterized by using transmission electron micrograph (TEM) and UV/vis spectroscopy.

The AuNP–anti-CEA–HRP complex was prepared according to a previously published procedure [25,26] with a few modifications. The pH value of AuNPs solution was adjusted to 8.5 by adding 0.2 M K_2CO_3. An aqueous solution of the minimum biotinylated anti-CEA antibody determined by AuNPs aggregation test plus 20% (7.8 μg) was added to 200 μL of AuNPs solution. Then, the mixed solution was incubated for 1 h at room temperature under gentle shaking, and PEG8000 was added to a final concentration of 0.5% for stabilizing the AuNPs. The excess of biotinylated antibody was removed by centrifugation at 9000 rpm for 50 min at 4 °C. Subsequently, the AuNPs were suspended and streptavidin-HRP was added to the solution to a final concentration of 0.6 μM. After 2 h incubation at room temperature, the unbound streptavidin-HRP was removed by repeated centrifugation and rinsing with blocking&storage buffer. The precipitated gold conjugates were resuspended in 100 μL of blocking&storage buffer and stored at 4 °C before use.

2.4. Preparation of capture antibody-coated MMPs

Anti-CEA capture antibodies were immobilized onto the carboxyl-functionalized MMPs following the manufacture's protocol. Carboxyl-functionalized MMPs were washed twice with 25 mM MES buffer, pH 6. The MMPs were then activated with EDC (50 mg mL^{-1}) and NHS (50 mg mL^{-1}) in MES buffer for 30 min at room temperature. The activated MMPs were washed twice with 25 mM MES, pH 6. Subsequently, a solution of anti-CEA capture antibody was added to the collected MMPs in a ratio of 50 μg antibodies to 1 mg MMPs, and the solution was mixed for 30 min at room temperature. The MMPs were magnetically collected and incubated with 50 mM Tris–HCl buffer, pH 7.4 for 15 min to passivate the residual activated carboxyl groups. Then, the coated MMPs were washed again and stored in blocking&storage buffer at 4 °C for further use.

2.5. Procedure for analysis of CEA

In a typical experiment, 50 μL of capture antibody-coated MMPs (0.2 mg mL^{-1}) were added to a 200 μL eppendorf tube. The MMPs were washed with assay buffer, and magnetically collected. 50 μL of assay buffer containing various concentrations of CEA standard or 1% diluted human serum samples were added and incubated with the MMPs for 30 min at 37 °C with gentle shaking. After incubation, the magnetic beads were magnetically collected and rinsed with washing buffer twice. Then, 50 μL of the AuNP–anti-CEA–HRP complex (0.9 nM) in assay buffer were added and the mixture was incubated for 30 min at 37 °C with gentle mixing, followed by five washing steps with washing buffer. 100 μL of TMB-H_2O_2 solution was added to each tube, and these tubes were incubated at 37 °C for 15 min with gentle shaking. Color development was stopped by adding 100 μL of 0.5 M sulfuric acid. After the magnetic separation, the absorbance at 450 nm of the supernatant was read with a microplate reader.

3. Results and discussion

AuNPs (size, 13 nm) were made from the chemical reduction of $HAuCl_4$ by sodium citrate. It is known that the stability of AuNPs is dependent upon the concentration of electrolytes in solution, and that high concentrations of salt can induce aggregation of colloids, accompanying a color change from red ($\lambda_{max} \sim 520$ nm, A_{520}) to blue (A_{580}). AuNPs protected by the citrate ligand are relatively unstable. They tend to aggregate irreversibly in tens of millimolar NaCl. AuNPs functionalized with biomolecules, e.g. antibody, are much more stable and can survive even molar concentrations of NaCl [25]. Therefore, the salt-induced colloidal gold aggregation test was used to optimize the antibody concentration and pH value of the AuNP solution used for coating. For the biotinylated anti-CEA detection antibody, the minimum antibody concentration to stabilize colloidal gold was 32.5 μg for 1 mL of AuNPs (Fig. S1a), and the optimal pH value of AuNPs solution was 8–9 (Fig. S1b).

In this work, AuNPs were employed as carriers of HRP-labeled anti-CEA antibody. Fig. 1b shows the TEM image of the complexes AuNP–anti-CEA–HRP. Compared with bare AuNPs (Fig. 1a), a shadow coating surrounding the dark Au core was clearly observed in the AuNP complexes, which represented the protein layer on the surface of AuNPs. As shown in Fig. 1c, the UV/vis spectrum of AuNPs solution (solid line) exhibited a characteristic plasmon absorption peak at 520 nm. After modification of HRP-labeled anti-CEA antibody on the surface, the UV/vis spectrum of the AuNP complex showed a small surface plasmon shift from 520 nm to 528 nm (dash line). It is well-known that the peak position of the surface plasmon band of AuNPs is correlated with particle size and the local chemi-

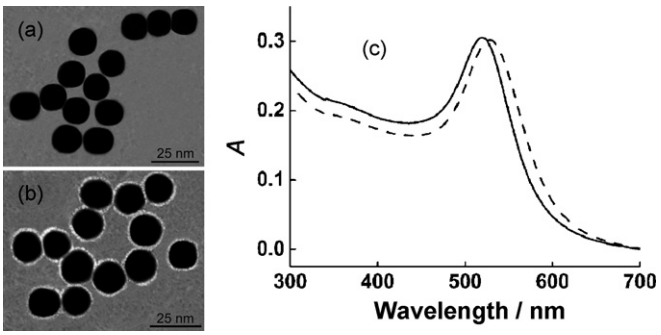


Fig. 1. TEM images of AuNPs before (a) and after (b) coating with HRP-labeled anti-CEA antibody. (c) UV/vis spectra of AuNPs solution before (solid line) and after (dash line) coating with HRP-labeled anti-CEA antibody.

cal environment [27,28]. The shift after modification of AuNPs with HRP-labeled anti-CEA antibody was attributed to changes in the particle size and the dielectric nature surrounding the AuNPs due to the presence of protein. Both results suggested successful immobilization of HRP-labeled anti-CEA antibody onto the AuNPs.

To determine the amount of active HRP on each AuNP–anti-CEA–HRP complex, bare AuNPs solution and the AuNP complex dispersion were reacted with the HRP substrate TMB and H_2O_2. The AuNP complex had the activity of HRP, and produced a soluble product with a characteristic absorbance peak at 450 nm, whereas the bare AuNP did not (Fig. S2a). The result further confirmed that HRP molecules were successfully linked onto the biotinylated anti-CEA antibody-coated AuNPs. The absorbance value produced by the AuNP complex dispersion was compared with a calibration curve constructed with HRP after subtracting the background absorbance of an equivalent dispersion of bare AuNPs (Fig. S2b). The concentration of active HRP in the stock AuNP complex dispersion was 275 ± 17 nM. The concentration of AuNP was 11.3 ± 0.2 nM based on the absorption of Au NP at 520 nm [29]. The concentration of the AuNP complex was about 22.6 nM. From these data, the number of active HRP molecules per AuNP complex was 12 ± 1.

Scheme 1b illustrates the procedure of the colorimetric enzyme immunoassay of CEA based on the AuNP complex and MMP. According to the principle of the assay, experimental parameters such as the amount of capture antibody-coated MMP and the AuNP complex would affect the signal response. First, we studied the effect of the MMPs. Response signals increased with increasing MMPs concentration, and a maximum was attained at

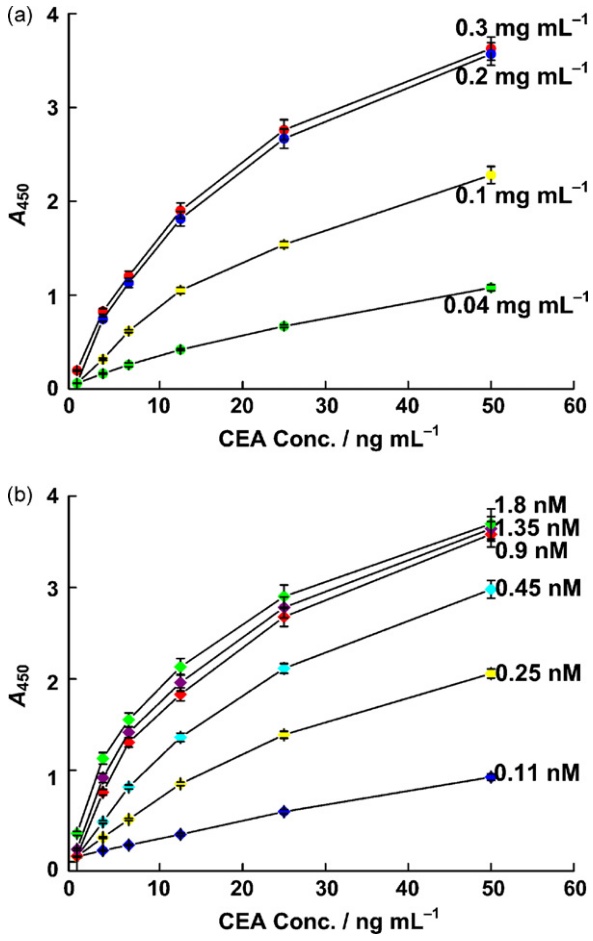
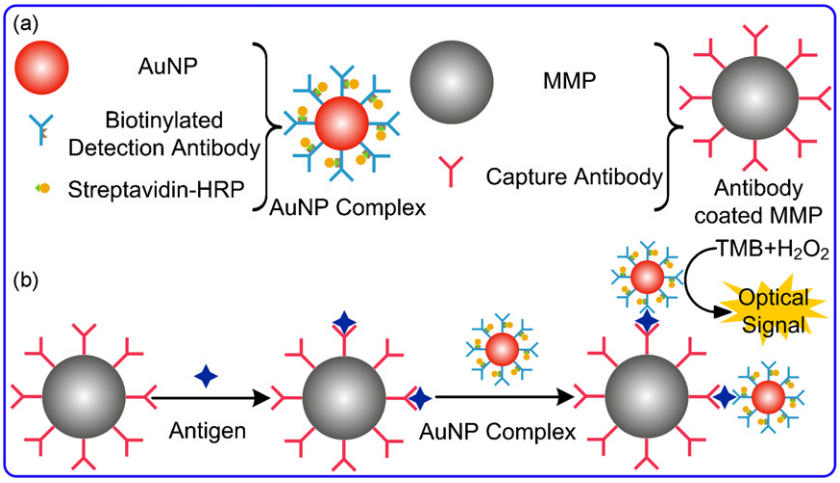


Fig. 2. (a) Optimization of MMPs concentration. The concentration of the AuNP complex was 1 nM. (b) Optimization of the concentration of the AuNP complex. MMPs concentration was 0.2 mg mL^{-1}. Other conditions: blocking buffer, 10 mM PBS, 1% BSA, 2.5% sucrose, 0.1% PEG8000; assay buffer, 10 mM PBS, 5% BSA, 2.5% sucrose, 0.1% PEG8000; incubation time, 30 min at 37 °C; washing buffer, 10 mM PBS, 0.1% BSA, 0.05% Tween-20; signal developer, TMB; color developing time, 10 min at 37 °C. Error bars are based on standard deviation with $n = 3$.

0.2 mg mL^{-1} (Fig. 2a). Further increase in MMPs concentration, e.g. 0.3 mg mL^{-1}, had very little additional beneficial effect. However, using 0.3 mg mL^{-1} of MMPs resulted in a high nonspecific signal because the high value of A_{450} was observed when no antigen



Scheme 1. Schematic diagrams of the experimental system. (a) Preparation of the AuNP–anti-CEA–HRP complex and the antibody-coated MMP. (b) The colorimetric enzyme immunoassay processes based on the AuNP complex and MMP.

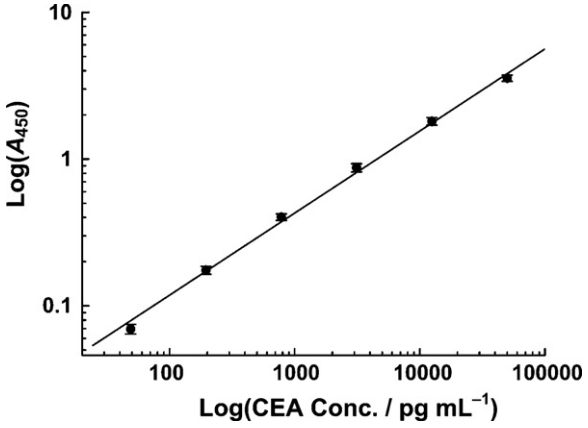


Fig. 3. Calibration plot of the AuNP complex-based colorimetric enzyme immunoassay for determination of CEA standards. Experimental conditions are as detailed in Fig. 2. Error bars are based on standard deviation with $n = 4$.

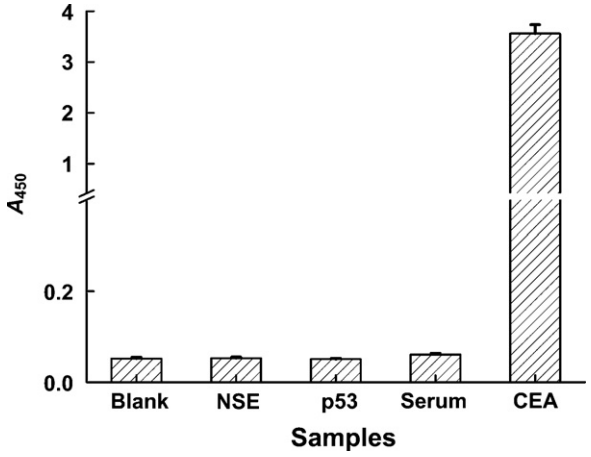


Fig. 4. The AuNP complex-based colorimetric enzyme immunoassay for the detection of blank control sample, 400 ng mL^{-1} of NSE, 400 ng mL^{-1} of p53, 1% diluted normal human serum, and 50 ng mL^{-1} of CEA. NSE and p53 refer to nonspecific antigens, and CEA is the target protein. Error bars are based on standard deviation with $n = 3$.

was used in the assay. Therefore, 0.2 mg mL^{-1} of MMPs was chosen in subsequent experiments. Fig. 2b shows optimization of the AuNP complex concentration. As the AuNP complex concentration increased, the values of A_{450} increased accordingly. A high negative control signal was obtained when a high AuNP complex concentration (1.35 nM or 1.8 nM) was employed, which significantly lowered the signal-to-noise ratio. Therefore, 0.9 nM of the AuNP complex was used for subsequent experiments.

Under optimal experimental conditions, we examined the performance of the proposed immunoassay with different concentrations of CEA standard. The plot of the logarithm of A_{450} versus the logarithm of CEA concentration is shown in Fig. 3. It could be seen that the linear range for the detection of CEA was from 0.05 to 50 ng mL^{-1}. The linear regression equation was $\log y = 0.51897 \log x - 2.04168$ ($R^2 = 0.99827$). The CEA concentration in the serum samples was obtained quantitatively via the linear regression equation. The detection limit (defined as 3SD above the zero-dose response) was 48 pg mL^{-1}. This was comparable with those of other nanolabeled-based immunoassays for CEA [30–34]. The sensitivity of the classic CEA ELISA kit sold by Bio-Quant (San Diego, CA, USA; www.bio-quant.com) was 0.64 ng mL^{-1}. The higher sensitivity exhibited by the AuNP complex-based immunoassay was attributed to using AuNP as a signal amplifier. Each AuNP could accumulate about 12 HRP molecules owing to its high surface-to-volume ratio. Thus one immunoreaction event could bring multiple HRP molecules, leading to large amplification of signals.

To investigate the specificity of the proposed immunoassay, the AuNP complex was employed to detect other nonspecific proteins. A high concentration of neuron-specific enolase (NSE), p53 and 1% diluted normal human serum (known CEA concentration 31 pg mL^{-1}) produced signals only as low as the blank control (Fig. 4). These results clearly demonstrated that the specificity of the proposed immunoassay was satisfactory, and 1% diluted human serum was used for subsequent experiments.

The proposed immunoassay was applied to determine CEA level in human serum. To examine the precision of the method for the determination of CEA in human serum, three concentrations of CEA standard were spiked into normal human serum and analyzed. The intra-assay precision of the proposed immunoassay was measured by assaying the CEA levels of three spiked human serum samples ($N = 5$) within one assay time, and using the freshly prepared solution of AuNP–anti-CEA–HRP complex. The variation coefficients (CVs) of the intra-assay were 4%, 5.9% and 4.9% at 2.5, 10, and 40 ng mL^{-1} of CEA, respectively (Table 1). Similarly, the inter-assay precision was examined by determination of the same three spiked human serum samples on three different days and using for each assay time a new freshly prepared AuNP–anti-CEA–HRP solution. Assessing the inter-assay precision was very important because it also took into account variability in preparation of the AuNP complex. The inter-assay CVs were 8.3%, 7.1% and 7.9% at 2.5, 10, and 40 ng mL^{-1} of CEA, respectively (Table 1), which were acceptable values for inter-assay assessment. The accuracy of the proposed immunoassay was also evaluated. The recovery for CEA was 91.8–106.5%. To further investigate the application of this method for clinical analysis, we examined

Table 1
Precision of the AuNP complex-based colorimetric enzyme immunoassay.

Assay type	Measured CEA/ng mL^{-1}	CV (%)
Intra-assay[a]	2.5 ± 0.1	4.0
	10.2 ± 0.6	5.9
	40.5 ± 2.0	4.9
Inter-assay[b]	2.4 ± 0.2	8.3
	9.8 ± 0.7	7.1
	40.4 ± 3.2	7.9

[a] $N = 5$ within an assay time.
[b] $N = 9$ in three assay times.

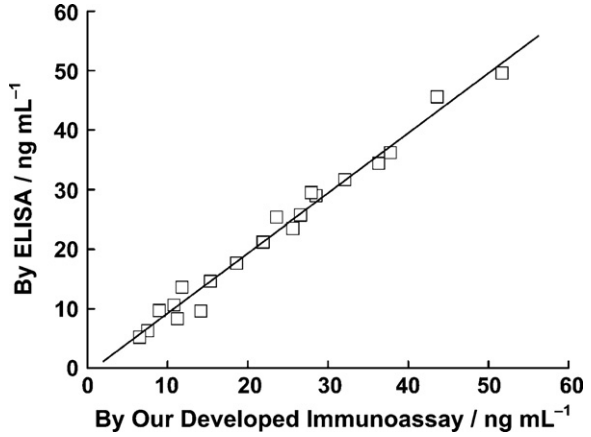


Fig. 5. Comparison of the results obtained using the developed immunoassay and the conventional ELISA method for the determination of CEA in 20 samples of human serum. A regression equation of the line: $y = 1.00855x - 0.85583$ was obtained with a correlation coefficient of 0.98293 ($p > 0.05$).

20 specimens of human serum using the proposed immunoassay and standard ELISA method. Fig. 5 describes the correlation of the results obtained by the proposed immunoassay and standard ELISA method. The regression equation of the line given by the ELISA method versus our proposed immunoassay was $y = 1.00855x - 0.85583$ (x-axis, the proposed immunoassay; y-axis, ELISA) with a correlation coefficient of 0.98293. It indicated that there was no significant difference between the results obtained by two methods. This confirmed that the proposed immunoassay showed good applicability for real-sample detection.

4. Conclusions

We developed a colorimetric enzyme immunoassay to detect CEA in which AuNPs were used as carriers of HRP-labeled anti-CEA detection antibody, and MMPs were used as supporting substrates for anti-CEA capture antibodies. The AuNP–anti-CEA–HRP complex loaded a high amount of HRP amplification enzyme, thus the proposed immunoassay based on the AuNP complex exhibited improved sensitivity as compared with a classic CEA ELISA kit. This feature, as well as its convenient magnetic separation, shorten assay time made it a promising alternative to conventional CEA ELISA methods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.03.014.

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