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ORIGINAL ARTICLE

Comparison of chemiluminescence enzyme immunoassay based on magnetic microparticles with traditional colorimetric ELISA for the detection of serum α-fetoprotein

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KEYWORDS

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Magnetic microparticles;
Colorimetric enzyme-linked immunosorbent assay

Abstract A chemiluminescence enzyme immunoassay based on magnetic microparticles (MmPs-CLEIA) was developed to evaluate serum α-fetoprotein (AFP) in parallel with traditional colorimetric enzyme-linked immunosorbent assay (ELISA). A systematic comparison between the MmPs-CLEIA and colorimetric ELISA concluded that the MPs-CLEIA exhibited fewer dosages of immunoreagents, less total assay time, and better linearity, recovery, precision, sensitivity and validity. AFP was detected in forty human serum samples by the proposed MPs-CLEIA and ELISA, and the results were compared with commercial electrochemiluminescence immunoassay (ECLIA) kit. The correlation coefficient between MPs-CLEIA and ELISA was obtained with $R^2=0.6703$; however, the correlation between MPs-CLEIA and ECLIA ($R^2=0.9582$) was obviously better than that between colorimetric ELISA and ECLIA ($R^2=0.6866$).

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1. Introduction

Enzyme immunoassay (EIA) is a kind of immunoassay that uses enzyme labeled antibody/antigen to detect antigen/antibody, and is well known in the bio-analytical field [1,2]. The most common detection method of EIA is enzyme-linked immunosorbent assay (ELISA). ELISA was first reported by Engvall and Perlmann in 1971 [3] and has been widely used as a diagnostic tool in clinic [4,5], plant pathology [6] and food industry [7]. However, the need to detect increasingly smaller amounts of target molecules has led to the emergence of more sensitive indicators, such as fluorescence and chemiluminescence.

Since reported by Woodhead in 1985 [8], chemiluminescence immunoassay (CLIA) has been applied broadly to the clinical

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diagnosis and environmental analysis. In initial stages, CLIA generally used chemiluminescent indicator [9,10], such as luminol, isoluminol, acridinium ester and so on, directly labeling antigen (antibody). Although CLIA has improved the analytical sensitivity of immunoassays, direct labeling of chemiluminescent indicator was limited by a relatively short duration of light output, so its application needed robot to assist. Consequently, chemiluminescence enzyme immunoassay (CLEIA) is developed based on enzyme–antibody conjugates using a chemiluminescent substrate, and luminometer was used for measurements. In this way, CLEIA with improved duration of light output is developed quickly in recent years. Further to improve the performance, magnetic microparticles have been applied in CLEIA as separating agent [11–14] and solid phase [15–17] with higher sensitivity and larger detection linear range. Although many reports declared that CLEIA had obvious advantages over ELISA, no detail work has been done so far to compare them in terms of the performance and mechanism.

We have focused on determining the α-fetoprotein (AFP) in human serum. AFP, as a well-established tumor marker relating to hepatocellular carcinoma (HCC) [18], is recommended by the European Association for the Study of the Liver, the British Society of Gastroenterology, the European Group on Tumor Markers, the National Academy of Clinical Biochemistry, and the National Comprehensive Cancer Network. It is necessary for serial AFP measurements together with consideration of sustained increases in AFP even at low concentrations [19,20]. Thus sensitive and time-saving methods for the detection of AFP are an imperative demand in clinical diagnosis and prognosis of HCC.

In our work, magnetic microparticles based CLEIA (MPs-CLEIA) was developed to detect AFP in human serum in parallel with colorimetric ELISA. MPs-CLEIA was compared systematically with colorimetric ELISA in terms of the dosage of capture antibody, detection antibody, and analytical parameters. The advance performance of MPs-CLEIA and the role of magnetic microparticles in MPs-CLEIA were discussed according to the results in this work.

2. Experimental

2.1. Chemicals and immunoreagents

Mouse anti-human AFP monoclonal antibodies (McAbs) and AFP were purchased from Fitzgerald Industries International, Inc. (MA, USA). HRP labeled anti-AFP antibody (HRP-AFP Abs) was offered by Beijing Chemclin. Biotech. Co., Ltd (Beijing, China). Chemiluminescent substrates (Luminol, H_2O_2 and enhancer) were purchased from Monobind Inc. (California, USA). HRP Substrate TMB Solutions for ELISA was purchased from Thermo Fisher Scientific Inc. (Rockford, USA). The magnetic particles (2-μm, 0.1%, w/v) modified with amino groups (MP-Amine) were purchased from Shanghai Allrun Nano Science&Tecnology Co., Ltd. (Shanghai, China). All other relating chemicals were purchased form Sigma-Aldrich (St. Louis, MO, USA).

Highly distilled and deionized water was used throughout. The washing solution was 0.1 M PBS buffer (pH 7.4) with 0.5% (w/v) saline solution and 0.05% (v/v) Tween-20. The AFP stock solution of 100 μg/mL was prepared in 5% bovine serum and stored at ×20 °C. Carbonate buffer was used as coating buffer. Disinfectant equine serum was used as calibrator matrix. The human sera from local hospitals were collected and analyzed without any pretreatment.

2.2. Apparatus

BHP9504 microtiter plate reader for chemiluminescence intensity detection was purchased from Hamamatsu (Hamamatsu Co., Ltd., Beijing, China). ELISA analyzer was purchased from Bio-Rab (USA). C96 MicroWell™ Plates and Immuno™ Tubes (polysorp™) were purchased from Nunc (Roskilde, Denmark). The incubation and shaking procedures at 37 °C were carried out at a thermostatic culture oscillator (ZHWY-100, Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd., Shanghai, China). A microplate mixer (Beijing Xinjingke Biotechnology Co., Ltd., Beijing, China) was employed to blend the solutions in microwells. ADEM-3 microtiter plate washer (Beijing Tuopu Analytical Instrument Co., Ltd., Beijing, China) was used.

2.3. Immunoassay procedure of ELISA

2.3.1. Preparation of anti-AFP McAbs coated C96 MicroWell™ Plates

An aliquot of 100 μL of AFP McAbs (5 μg/mL) was added into each well of C96 MicroWell™ Plates, and then stored at 4 °C overnight. During incubation, anti-AFP McAbs were physically adsorbed to microwells through hydrophobic interaction. Afterwards, the microplates were washed with PBS buffer (with 4% Tween-20) three times to eliminate the free antibodies. Next, bovine serum albumin (BSA) buffer (5%, w/v) of 500 μL were added to block the vacant sites for a period of 12 h at 4 °C. Finally, the prepared microplates were washed and dried off, then stored at 4 °C for further use.

2.3.2. The procedure of colorimetric ELISA

The immunoassay procedure of colorimetric ELISA is displayed in Fig. 1(A). An aliquot of 25 μL AFP calibrators or serum samples, and 50 μL HRP-AFP Abs (dilution ratio of 1:10,000) were added into the microplates stepwise, and incubated for 90 min at 37 °C. After the sandwich reaction, the microplates were washed five times and the remaining solution was removed by gently tapping the microplates against tissue paper. Finally, HRP ELISA substrate was added and the absorbance was measured as optical density (OD).

2.4. Immunoassay procedure of MPs-CLEIA

2.4.1. Preparation of anti-AFP antibody coated MPs (MPs-anti-AFP Abs)

Before coating anti-AFP McAbs to MPs-Amine, MPs-Amine were washed two times with 1 mL 2-(N-morpholino)ethanesulfonic acid (MES) buffer using the magnetic separator. Next, MPs-Amine was resuspended in 0.25 mL MES buffer containing 10 mg (N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride) EDC HCl, and then anti-AFP McAbs were added to the MPs-Amine. Furthermore, the mixture was incubated on a shaker for 2 h at room temperature. After that MPs-anti-AFP McAbs were formed. Finally MPs-anti-AFP McAbs were washed three times and suspended in storage buffer for further use.

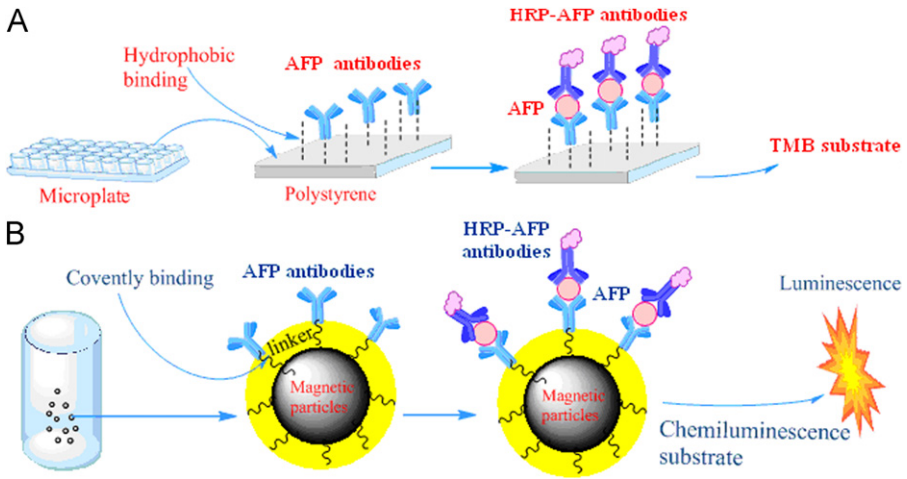

Figure 1 Principles for the evaluation of α-fetoprotein in human serum by HRP-based enzyme immunoassay. (A) Chemiluminescence immunoassay based on magnetic particles; (B) colorimetric ELISA.

2.4.2. The procedure of MPs-CLEIA

The procedure of the MPs-CLEIA is presented in Fig. 1(B). First, an aliquot of 50 μL AFP calibrators or serum samples, 50 μL MPs-anti-AFP McAbs, and 50 μL HRP-AFP Abs (dilution ratio of 1:20,000) were added into Immuno^{TM} Tubes stepwise, and incubated for 30 min with gentle shaking at 37 °C. After the sandwich reaction, total four times washing was performed by using the samarium–cobalt magnet to separate the sandwich complex and the unbound reagent. Finally, 250 μL substrate solution (A and B solutions were mixed together with 1:1 ratio) was added. The mixture was incubated for 5 min at room temperature and the emitted photons as relative light unit (RLU) were measured.

3. Results and discussion

3.1. Optimization of the concentration of immobilized anti-AFP antibodies and evaluating the performances

Anti-AFP McAbs were diluted by 20% bovine serum into 1, 1.5, 2, 2.5, 5, 10 and 20 μg/mL. The concentration of anti-AFP McAbs used in the ELISA and MPs-CLEIA was optimized and compared. As shown in the Fig. 2, OD_{S1}/OD_{S0} and OD_{S5}/OD_{S0} increased with increasing the concentration of coated antibodies and reached a platform at the concentration of 5 μg/mL, while RLU_{S1}/RLU_{S0} and RLU_{S5}/RLU_{S0} reached a platform merely at 1.5 μg/mL. The results indicated that anti-AFP McAbs physically adsorbed on microplates would get saturation at the concentration of 5 μg/mL, and abundant antibodies adsorbed on microwells would damage the bioactivity of antibodies. However, by covalently conjugated to MPs-Amine surfaces, anti-AFP Abs presented a high coating efficiency and the structure of anti-AFP Abs would be resulted in a less distortion compared with anti-AFP Abs physically adsorbed on microwells.

3.2. Optimization and comparison of the concentration of HRP-AFP Abs

The concentration of HRP-AFP Abs is another important factor to the sensitivity and working range. Therefore five dilution

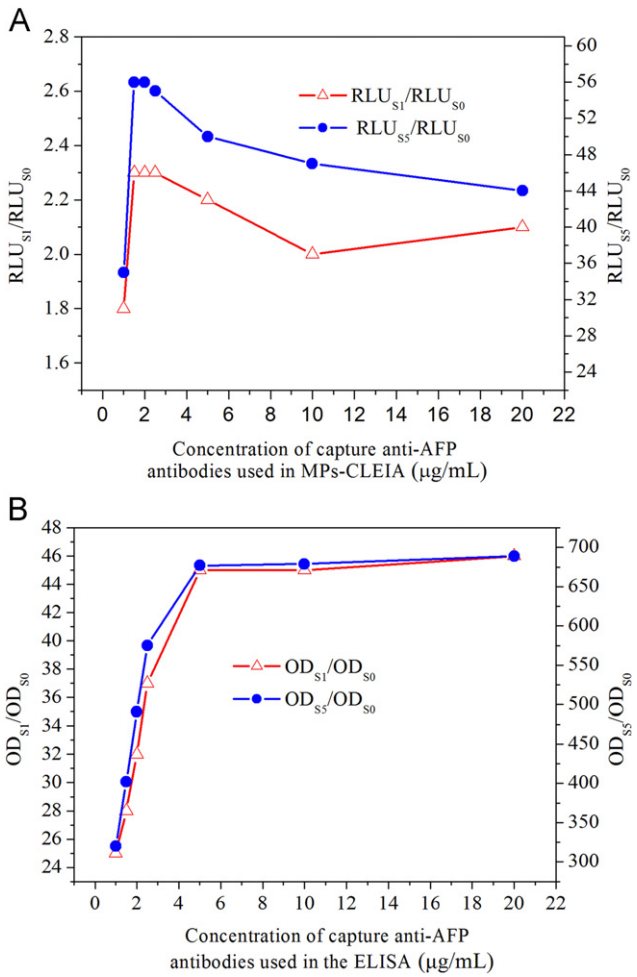

Figure 2 Influence of concentration of anti-AFP McAbs (coating antibodies) used in the ELISA and MPs-CLEIA. RLU was the relative light unit; RLU_{S0} was resulted from determination the concentration of AFP with calibrator S_0, the same with RLU_{S1} and RLU_{S5}. OD was the optical density, such as OD_{S0} was resulted from determination the concentration of AFP with calibrator S_0, the same with OD_{S1} and OD_{S5}.

ratios, 1:4000, 1:8000, 1:10,000, 1:20,000 and 1:40,000, were prepared. The concentration of HRP-AFP Abs used in the ELISA and MPs-CLEIA was optimized and compared by evaluating OD_{S1}/OD_{S0} and OD_{S5}/OD_{S0}, RLU_{S1}/RLU_{S0} and RLU_{S5}/RLU_{S0}, respectively (Fig. 3). OD_{S1}/OD_{S0} increased with the dilution ratio increasing and reached maximum at the ratio of 1:10,000, at the same time OD_{S5}/OD_{S0} increased with the dilution ratio and reached a short platform at the ratio of 1:8000. Therefore, dilution ratio of 1:10,000 is appropriate for the ELISA with a higher sensitivity and better linearity. With similar situation, we found that dilution ratio of 1:20,000 was appropriate for the MPs-CLEIA. The dosage of HRP-AFP Abs for MPs-CLEIA was half of that for colorimetric ELISA. The results could be explained by the fact that detecting emitted luminescence by counting photons coupled with the photomultiplier was much more sensitive than colorimetric ELISA by accumulating absorbance value.

3.3. *Optimization and comparison of immunoreaction time*

The time for the immunoreagents to interact is one of the key parameters determining the sensitivity of the immunoassay. The influence of the immunoreaction time of ELISA and CLEIA was studied. In Fig. 4, RLUs of MPs-CLEIA decreased and the calibration curve got worse after 30 min immunoreaction, so the immunoreaction time for MPs-CLEIA was fixed at 30 min. While, ODs of ELISA reached a platform after 90 min and the calibration curve got a better linearity, so the immunoreaction time for ELISA was 90 min. The less immunoreaction time for MPs-CLEIA was mainly due to that the large specific surface area of MPs exerted an active role in accelerating the reaction. On the other hand, the suspension of MPs during reaction reduced the dilution distance of immunoreagents, and thus speeded up the rate of reaction.

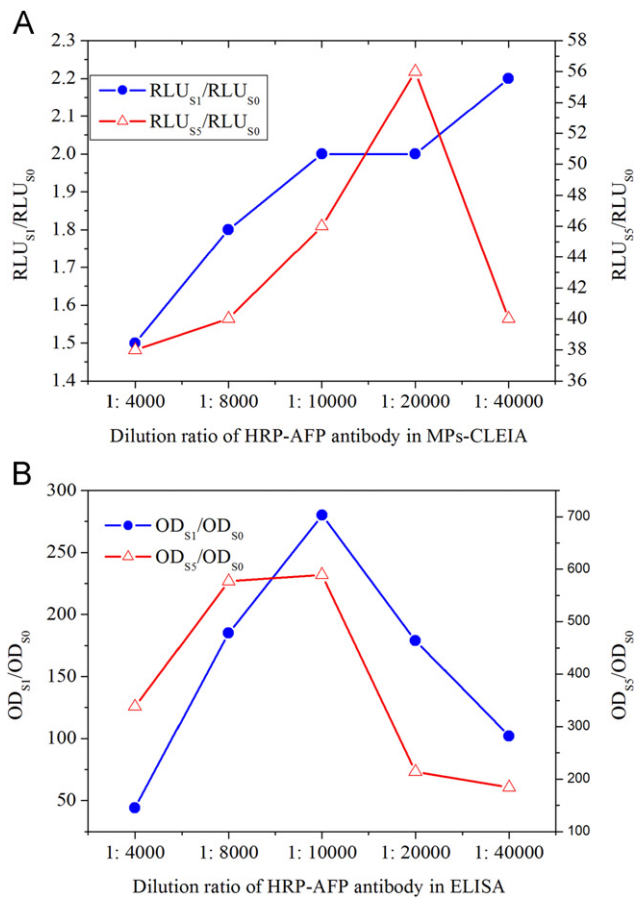

Figure 3 Influence of concentration of HRP-AFP Abs used in the ELISA and MPs-CLEIA. Calibrators of S_0, S_1 and S_5 were applied to the optimization.

3.4. *Analytical parameters*

The calibration, linear range, sensitivity, total assay time of the two methods were studied and compared systematically. The results are listed in Table 1. As can be seen, the linear detection range of MPs-CLEIA increased four times more than that of ELISA, and the total assay time of MPs-CLEIA was much less than ELISA. The immunoassay time of MPs-CLEIA was much shorter than ELISA, which might be due to immunoreactions between antigens and antibodies being carried out in a homogeneous mixture and decreasing diffusion distance. While, AFP antibodies coated in microplates in ELISA were restricted on the coating surface, thus immunoreaction time increased because of diffusion effect.

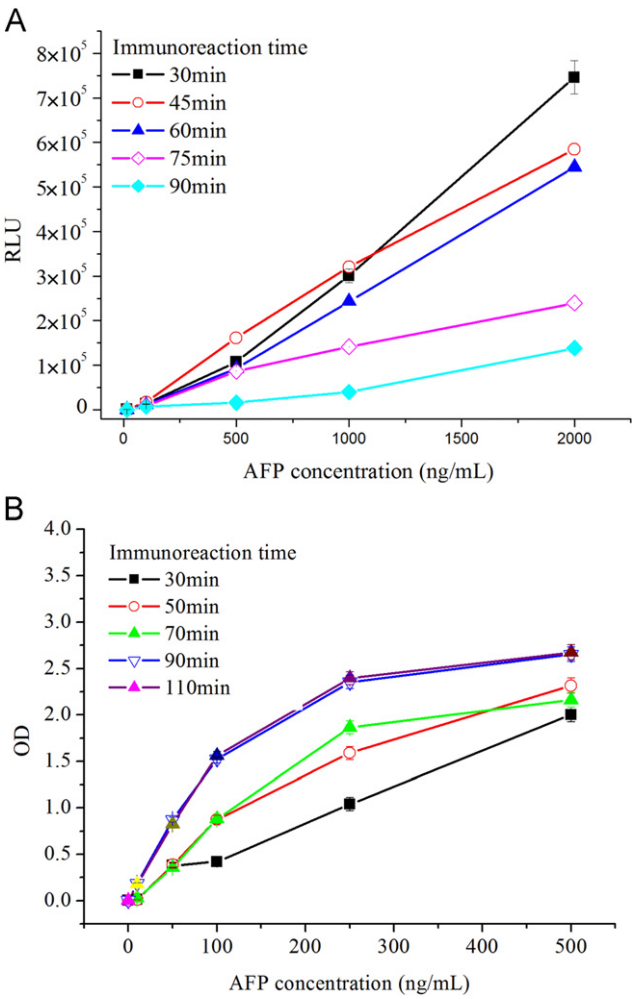

Figure 4 Influence of the immunoreaction time of ELISA and MPs-CLEIA. Three calibrators of S_0, S_1 and S_5 were applied to the optimization.

Table 1 Comparison of some of the analytical parameters.

Method	Calibration curve	Linear range (ng/mL)	Sensitivity (ng/ml)	Immunoassay time (min)	Total assay time (min)
MPs-CLEIA	$Y=1.8432+0.9735X$ ($R^2=0.9980$)	10–2500	0.74	30	50
Colorimetric ELISA	$Y=1.8707+1.2160X$ ($R^2=0.9934$)	15–600	0.82	90	120

Table 2 Recoveries of AFP from human serum samples ($n=3$).

Method	AFP samples (ng/mL)	AFP added (ng/mL)	AFP determined (ng/mL)	Recovery (%)
Colorimetric ELISA	1.55	600	554.87	92
	1.55	110	97.78	87
	1.55	17.5	16.04	86
MPs-CLEIA	1.55	600	587.96	98
	1.55	110	117.78	105
	1.55	17.5	17.85	93

Additionally, the limit of detection (LOD) of MPs-CLEIA (0.74 ng/mL) was slightly lower than that of ELISA (0.82 ng/mL), but the regression line of the MPs-CLEIA calibration cure ($R^2=0.9980$) was better than that of colorimetric ELISA ($R^2=0.9934$), and the slope of the MPs-CLEIA calibration curve (1.319) was higher than that of ELISA (0.9372), tending to show that MPs-CLEIA is more precise and sensitive than ELISA for AFP quantification. The linear-dilution effect test also indicated that MPs-CLEIA was more sensitive and precise over ELISA. One of the key factors for the proposed MPs-CLEIA showing better performances over ELISA about sensitive and precise was that MPs-CLEIA exhibited a larger linear detection range and a higher slope of the calibration curve.

3.5. *Recovery test*

Recovery test is taken by adding quantity of AFP antigen to the normal human serum. Then the real value is detected. Recovery rate $=(R\times H)/A$ 100%. Samples that included high, middle and low value in the detectable range were taken to do the recovery test. The results are shown in Table 2. The recoveries of both methods were between 90% and 105% (between 85% and 105% is well acceptable in immunoassay kit development).

3.6. *Validity*

Linearity-dilution effect is an indicator of validity of the proposed method. A serum sample with high AFP level was diluted stepwise by the calibrator matrix (disinfectant equine serum), and the final diluted samples were detected with the proposed MPs-CLEIA and ELISA. Linearity-dilution curve (Fig. 5) of MPs-CLEIA showed a good linear, while with ELISA the concentration of AFP detected did not fit a linear correlation with the dilution ratios. The results prove that the MPs-CLEIA is reliable in determining AFP with high concentration in serum samples.

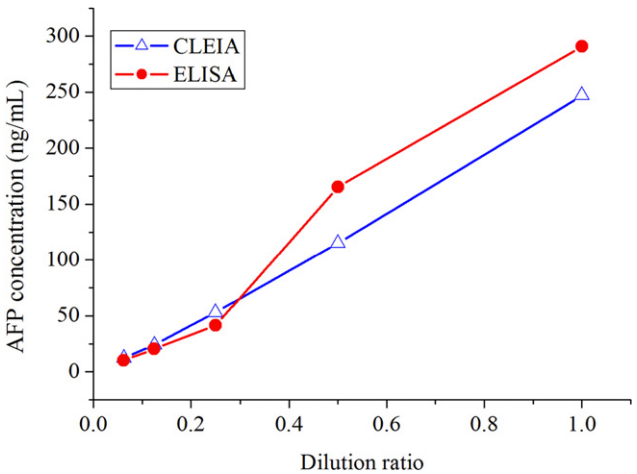

Figure 5 Linearity-dilution effect of ELISA and MPs-CLEIA. The serum sample with high AFP level was diluted stepwise with the calibrator matrix.

3.7. *Determination of AFP in serum samples by CLEIA and ELISA and comparison with commercial ECLIA kit*

The proposed MPs-CLEIA and colorimetric ELISA were applied to evaluate AFP in human serum samples. The results obtained using the proposed method in the determination of AFP in fourty clinical sera samples were compared with those obtained by the commercially available ECLIA kit. As can be seen in Fig. 6, the correlation coefficient between MPs-CLEIA and ELISA was 0.6703, and that between ELISA and ECLIA was 0.6866, while the correlation coefficient between MPs-CLEIA and ECLIA was 0.9582. There was much better agreement between MPs-CLEIA and ECLIA than that between ELISA and ECLIA indicating that not only the bioactivity of antibodies but indicators could influence the detection precision.

4. Conclusion

In the present work, the construction and systemically comparison of MPs-CLEIA with colorimetric ELISA were performed for detection of serum AFP. The MPs-CLEIA was proved to be apparently advantageous over the ELISA in terms of less dosage of immunoreagents, higher dose hook effect and bioactivity of immunoreagents, less assay time and wider linear range. MPs-CLEIA was used to evaluate AFP in human sera samples and a good correlation was obtained when comparing the results with that from a commercial electrochemiluminescence immunoassay kit. All of these indicated that in the clinical diagnosis, the MPs-CLEIA for detecting of AFP was a convenient, economical, and time-saving method for screening, prognosis and monitoring of HCC.

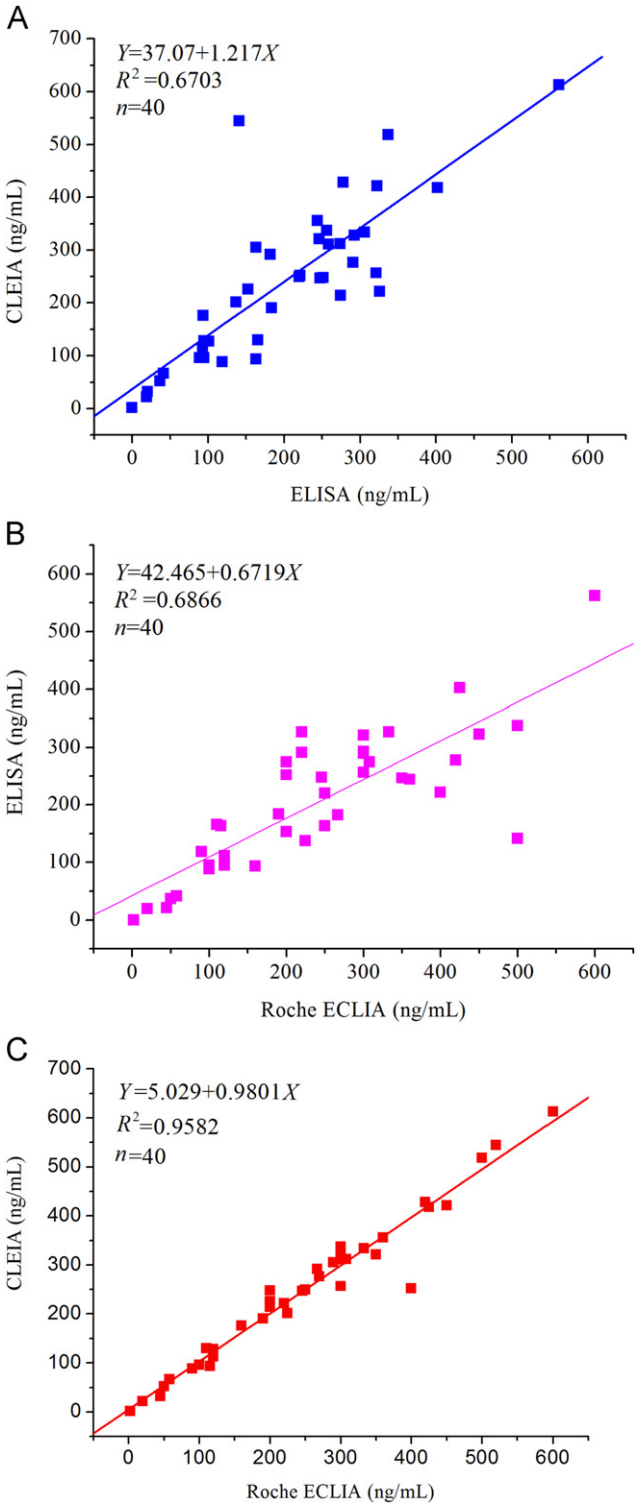

Figure 6 Evaluation of AFP in human serum samples with MPs-CLEIA, ELISA and ECLIA.

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References

[1] R.M. Lequin, Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA), Clin. Chem. 51 (12) (2005) 2415–2418.

[2] W.G. Wood, Immunoassays & co.: past, present, future?—a review and outlook from personal experience and involvement over the past 35 years, Clin. Lab. 54 (11–12) (2008) 423–438.

[3] T. Porstmann, S.T. Kiessig, Enzyme-immunoassay techniques—an overview, J. Immunol. Methods 150 (1–2) (1992) 5–21.

[4] S. Sakamoto, J. Sakoda, O. Morinaga, et al., Development of an enzyme linked immunosorbent assay for direct determination of anticancer drug vitamin K3 in serum, J. Health Sci. 54 (4) (2008) 715.

[5] W. Phrompittayarat, W. Putalun, H. Tanaka, et al., An enzyme-linked immunosorbant assay using polyclonal antibodies against bacopaside I, Anal. Chim. Acta 584 (1) (2007) 1–6.

[6] S. Sakamoto, W. Putalun, R. Tsuchihashi, et al., Development of an enzyme-linked immunosorbent assay (ELISA) using highly-specific monoclonal antibodies against plumbagin, Anal. Chim. Acta 607 (1) (2008) 100–105.

[7] J.D. Thacker, E.S. Casale, C.M. Tucker, Immunoassays (ELISA) for rapid, quantitative analysis in the food-processing industry, J. Agri. Food Chem. 44 (9) (1996) 2680–2685.

[8] J.S. Woodhead, I. Weeks, Chemi-luminescence immunoassay, Pure Appl. Chem. 57 (3) (1985) 523–529.

[9] I. Weeks, I. Beheshti, F. McCapra, et al., Acridinium esters as high-specific-activity labels in immunoassay, Clin. Chem. 29 (8) (1983) 1474–1479.

[10] J.T. Wu (Ed.), Quantitative immunoassay: a practical guide for assay establishment, trouble shooting, and clinical application. AACC Press, Washington, DC, 2000.

[11] X. Wang, J.-M. Lin, X.T. Ying, Evaluation of carbohydrate antigen 50 in human serum using magnetic particle-based chemiluminescence enzyme immunoassay, Anal. Chim. Acta 598 (2) (2007) 261–267.

[12] H. Jin, J.-M. Lin, X. Wang, et al., Magnetic particle-based chemiluminescence enzyme immunoassay for free thyroxine in human serum, J. Pharm. Biomed. Anal. 50 (5) (2009) 891–896.

[13] W. Dungchai, W. Siangproh, J.-M. Lin, et al., Development of a sensitive micro-magnetic chemiluminescence enzyme immunoassay for the determination of carcinoembryonic antigen, Anal. Bioanal. Chem. 387 (6) (2007) 1965–1971.

[14] X. Wang, Q.Y. Zhang, Z.J. Li, et al., Development of high-performance magnetic chemiluminescence enzyme immunoassay for alpha-fetoprotein (AFP) in human serum, Clin. Chim. Acta 393 (2) (2008) 90–94.

[15] Q.Y. Zhang, X. Wang, Z.J. Li, et al., Evaluation of alpha-fetoprotein (AFP) in human serum by chemiluminescence enzyme immunoassay with magnetic particles and coated tubes as solid phases, Anal. Chim. Acta 631 (2) (2009) 212–217.

[16] N. Pamme, Magnetism and microfluidics, Lab Chip 6 (1) (2006) 24–38.

[17] B. JE, Ed. Solid phases in immunoassay. In: Immunoassay. C.T. Diamandis E. Academic Press: San Diego.1996.

[18] C. Sturgeon, Practice guidelines for tumor marker use in the clinic, Clin. Chem. 48 (2002) 1151–1159.

[19] J.M. Llovet, A. Burroughs, J. Bruix, Hepatocellular carcinoma, Lancet 362 (9399) (2003) 1907–1917.

[20] J. Bruix, M. Sherman, Management of hepatocellular carcinoma, Hepatology 42 (5) (2005) 1208–1236.