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Magnetic particle-based time-resolved fluoroimmunoassay for the simultaneous determination of α-fetoprotein and the free β-subunit of human chorionic gonadotropin

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In this paper, a novel time-resolved fluoroimmunoassay (TRFIA) protocol using magnetic particles for the simultaneous determination of α-fetoprotein (AFP) and the free β-subunit of human chorionic gonadotropin (free β-hCG) in human serum is described. The new approach uses magnetic particles as an immobilization matrix and means of separation, while the luminescent europium and samarium chelates are used as probes. The proposed method was evaluated *via* a single-step, sandwich-type TRFIA immunoassay of AFP and free β-hCG as model analytes in serum. With the advantages of magnetic particles, the TRFIA immunoassay exhibited a wide dynamic range for AFP of 0.1–750 ng mL^{-1}, with a lower detection limit of 0.05 ng mL^{-1}. The dynamic range for free β-hCG was 0.16–450 ng mL^{-1}, with a lower detection limit of 0.08 ng mL^{-1}. Satisfactory specificity, reproducibility, and recovery of the immunoassay were demonstrated. Good correlations were obtained in the analysis of 446 human serum samples between the proposed method and a commercial TRFIA kit. These results demonstrate the feasibility and potential of the new method as a rapid and highly sensitive immunoassay that could be developed into a platform for multi-analyte determinations in clinical practice.

Introduction

Increasing attention has recently been focused on the development of immunoassay methods for quantifying two or more analytes simultaneously in one sample in a single assay.[1–3] Such simultaneous assays have obvious advantages, including rapid analysis, high throughput, small analyte volume, and low cost. Various multi-analyte methods using enzymes, radiolabels, fluorescent or quantum dots as labels and a number of non-labeled methods have been developed in molecular biology, food safety, and clinical research.[4–8] Despite progress, none of these assays has achieved widespread use, probably because they are technically complex, costly, labor-intensive and time-consuming. Thus, exploring new strategies for sensitive and convenient multi-analyte detection with high throughput in clinical diagnosis is of great interest.

The time-resolved fluoroimmunoassay (TRFIA) using lanthanide chelates as labels is an ultrasensitive immunoassay that has been widely used since it was first established by Pettersson and Eskola in the 1980s.[9] The labels used in this immunoassay are rare earth metals such as europium (Eu^{3+}), samarium (Sm^{3+}), and terbium chelates, which can generate strong fluorescence with long decay times, large Stokes shifts and sharp emission profiles.[10,11] As the chelates of these lanthanides have fluorescence peaks at different wavelengths and are clearly distinguishable from one another, the combined use of several chelates enables double or even multiple-label immunoassays to simultaneously quantitate several indicators contained in a single sample. Because of its unique advantages, such as high sensitivity, large linear dynamic range, easy automation, low susceptibility to matrix interference, and simultaneous multi-labeling, TRFIA has received significant attention over recent years and many attempts have been made to develop such immunoassays for bioanalysis.[12–14]

Magnetic particles have been successfully employed in many areas of research, including cell separation, biomolecule detection, DNA extraction and various immunoassay methodologies.[15–19] Magnetic particles with bioactive molecules such as antibodies are very useful tools for immunoassays but a literature survey revealed few reports on the application of magnetic particles in TRFIA. Therefore, we attempted to develop a TRFIA detection method that could screen for two parameters at once, taking the concentrations of α-fetoprotein (AFP) and the free β-subunit of human chorionic gonadotropin (free β-hCG), which

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are determined in maternal serum during the second trimester of pregnancy as markers for Down syndrome,[20–24] as a model system.

Unlike conventional TRFIA, a specific anti-analyte antibody was covalently coupled to the surface of magnetic particles rather than immobilized on the surface of 96-well microplates by physical adsorption. The available sites for antigen binding are symmetrical and the mass transfer distance of analytes to the immobilized antibody is greatly reduced, with minor spatial hindrance, by this approach, and consequently, antibody–antigen binding equilibrium can be achieved rapidly. Magnetic particles modified with chemically functional groups provide a relatively large surface area, which enables more antibodies to be coupled efficiently to the surface. Also, the immunocomplex can be separated conveniently from unbound components by application of a magnetic field. These advantages enable an effective assay with short analysis time, broad dynamic range, and low reagent and labor consumption.

In the present study, a novel immunoassay format for the simultaneous measurement of two components in human serum is described, which is characterized by the use of magnetic particles as a solid-phase and the detection of Eu and Sm chelates. We applied the new TRFIA method to the assay of AFP and free β-hCG concentrations in the serum of second trimester pregnant women, as markers for Down syndrome, to examine the feasibility of the method. Compared with the results from single- or dual-label assays of AFP and free β-hCG, the proposed method possesses apparent advantages of high sensitivity, short analysis time and large linear ranges. Hence, this strategy has significant promise and could be further developed for practical clinical detection of further important biomarkers.

Experimental section

Reagents and apparatus

Bovine serum albumin (BSA), 4-morpholineethanesulfonic acid (MES), *N*-hydroxysulfosuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), proclin-300 and Tween-20 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sephadex G-50 was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). All other chemicals used were of analytical reagent grade and ultra-pure water obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used throughout the experiments.

Monoclonal anti-AFP antibodies (H45301M and H45610M), AFP (A15108H) and free β-hCG standards (A81455M) were obtained from Meridian Life Science (Memphis, TN, USA). Monoclonal anti-free β-hCG antibodies (10-C25A and 5012) were obtained from Fitzgerald Industries International Inc. (Concord, MA, USA) and Medix Biochemicals (Kauniainen, Finland). Magnetic particles (1101GA-03) were obtained from JSR Life Sciences (Tokyo, Japan). A 1420 Multi-label Counter (Victor3™) for spectral analysis of fluorescent chelates was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA, USA). Eu^{3+} and Sm^{3+}-labeled kits, AFP/free β-hCG commercial dual kit (B067-101) and enhancement solution were obtained from Perkin-Elmer Wallac (Turku, Finland).

Buffer solutions used in the study were coating buffer (0.1 mol L^{-1} MES, pH 5.0); labeling buffer (50 mmol L^{-1} Na_2CO_3–$NaHCO_3$, containing 0.9% NaCl, pH 9.0); assaying buffer (25 mmol L^{-1} Tris–HCl, containing 0.02% BSA, 0.09% NaCl, 0.05% Tween-20 and 0.05% proclin-300, pH 7.8); elution buffer (50 mmol L^{-1} Tris–HCl, containing 0.9% NaCl and 0.05% proclin-300, pH 7.8); washing buffer (50 mmol L^{-1} Tris–HCl, containing 0.9% NaCl, 0.2% Tween-20 and 0.05% proclin-300, pH 7.8), and blocking buffer (5% BSA, pH 7.0).

Coating conjugate preparation

Covalent conjugation between magnetic particles and anti-AFP antibody (H45301M) was carried out as described in our previous work.[25] Briefly, 500 μL of magnetic particles (20 mg mL^{-1}, 2.0×10^9 magnetic particles mL^{-1} in H_2O) was suspended in 500 μL coating buffer. Then, 25 μL of EDC (10 mg mL^{-1}) and 40 μL of NHS (10 mg mL^{-1}) freshly prepared were added into the above magnetic particles suspension and the resultant mixtures were incubated at room temperature under gentle stirring to activate the carboxylic acid groups on the surface of the magnetic particles. After incubation for 30 min, the activated magnetic particles were magnetically isolated, followed by rinsing with coating buffer three times. Subsequently, 100 μg specific anti-AFP antibody in 1 mL coating buffer was added to the activated magnetic particles. The reaction proceeded at room temperature for 18 h under gentle stirring and the mixtures were then rinsed four times with assaying buffer to remove unbound antibody using magnetic separation. The resultant magnetic particles were resuspended in 1 mL blocking buffer at room temperature for another 3 h to eliminate nonspecific binding effects and block the remaining active groups. After a final rinsing with assaying buffer, the magnetic particles–antibody conjugates (7.2 μg mg^{-1}) were resuspended in assaying buffer and stored at 4 °C until use.

The anti-free β-hCG antibody (5012) was conjugated to magnetic particles (6.6 μg mg^{-1}) using a similar method.

Labeling of antibody

Antibody labeling with Eu and Sm chelates was carried out using a method based on our previous work with minor modifications.[25,26] Briefly, anti-AFP antibody (H45610M) was dialyzed in labeling buffer overnight at room temperature and dissolved in labeling buffer to a final concentration of 1 mg mL^{-1}, then 0.2 mg Eu^{3+}-N^1-(*p*-isothiocyanatob-enzyl)-diethylenetriamine-N^1,N^2,N^3,N^4-tetraacetic acid (DTTA) was added. After reaction with continuous slow stirring for 18 h at room temperature, the labeled antibody was separated from the free chelate and aggregated antibodies using gel filtration on a Sephadex G-50 column (1.5 cm × 40 cm) and eluted with elution buffer. The concentration of Eu^{3+}-labeled anti-AFP antibody was determined by spectrophotometry at 280 nm and calculated using the equation:

$$c\ (\text{mg mL}^{-1}) = \{A_{280} - (0.008 \times [\text{Eu}^{3+}])\}/1.43.$$

Sm^{3+}-labeled anti-free β-hCG (10-C25A) was prepared similarly, except that 0.2 mg of Eu^{3+}–DTTA was replaced with 0.5 mg of Sm^{3+}–DTTA. The molar ratio of Eu^{3+} to mAb was determined to be 8.7. The molar ratio of Sm^{3+} to the mAb was determined to be 8. Finally, aliquots of the labeled antibody were stored at 4 °C and were stable for 12 months after adding 0.2% BSA as stabilizer.

Samples

Four hundred and forty six maternal serum samples collected from pregnancy weeks 9–14 were kindly provided by Guangdong Women and Children's Hospital (Guangdong, China) and were stored at −20 °C until use. The Ethical Committee of Science and Technology Department of the Southern Medical University approved collection of these samples.

Time-resolved fluoroimmunoassay protocol

The proposed immunoassay for the simultaneous determination of AFP and free β-hCG was performed based on a sandwich-type immunoassay format by combining a TRFIA assay and immunomagnetic separation, and is shown schematically in Fig. 1. Initially, 25 μL of standards or samples were added to test tubes, then 50 μL of magnetic particles coated with anti-AFP antibody, 50 μL of magnetic particles coated with anti-free β-hCG antibody and 100 μL of Eu^{3+}-labeled anti-AFP antibody/Sm^{3+}-labeled anti-free β-hCG antibody were added sequentially into the analytes. The mixtures were then incubated at room temperature for 30 min with continuous gentle stirring. Subsequently, the formed sandwich immunocomplexes were attracted to the sidewall of the test tubes and separated from free substances by the application of a samarium–cobalt magnet. After removing the free substances and rinsing with washing buffer four times, 200 μL of enhancement solution was added and then the immunocomplexes were resuspended in enhancement solution and the mixtures incubated for 5 min at room temperature with stirring. Finally, the fluorescence signal was measured using a Victor3™ 1420 Multi-label Counter (the mode of europium and samarium dual label). The fluorescence of Eu^{3+} was measured at an excitation wavelength of 340 nm and an emission wavelength of 615 nm. The Sm^{3+} fluorescence was measured at an excitation wavelength of 340 nm and an emission wavelength of 642 nm.

Statistics

Sample means and standard deviations (SD) were determined using Microsoft Excel. Standard curves were obtained by plotting the logarithm of fluorescence intensity (y) against the logarithm of analyte concentration (x) and fitting a logistic equation using Origin Pro7.5 (Microcal, USA): $\log(y) = A + B \times \log(x)$. Pearson's linear regression was used to display the linearity and correlations. Serum samples measured using the proposed method and a commercial TRFIA kit were analyzed and compared using the paired Student's t-test by SPSS 13.0 (Chicago, IL, USA), and $P < 0.05$ was considered statistically significant.

Results and discussion

Optimization of the sandwich assay

In this study, we introduced a new TRFIA assay based on a conventional sandwich-type immunoassay using magnetic particles. Antibodies were immobilized on the surface of magnetic particles rather than 96-well microplates, and a one-step reaction was carried out. The analytical performance was significantly influenced by several reaction parameters, including reaction temperature, incubation time, concentration of magnetic particles and the dilution ratios of Eu^{3+}or Sm^{3+}-labeled antibody.

It is well known that temperature has a great effect on the immunoreaction between antigens and antibodies. Typically, immunoreactions are carried out at either room temperature (25 °C) or at 37 °C, so we chose these two temperatures as controls. Standards at five different concentrations were tested with the same reaction conditions at different temperatures. The results indicate that there was no significant difference between the standard curves of free β-hCG from data at 25 °C and 37 °C (not present), although the fluorescence intensity of AFP at 37 °C was slightly higher than at 25 °C. However, to simplify operations and instruments, a temperature of 25 °C was considered as the optimal reaction temperature for practical applications.

Because the incubation times can dramatically influence the immunoreaction process and immunoassay sensitivity, the effect of incubation times was studied over the range 5 to 60 min by measuring AFP (400 ng mL^{-1}) and free β-hCG (200 ng mL^{-1}) standards, as shown in Fig. 2. Apparently, the fluorescence intensity of the two standard curves achieved equilibrium after incubation for 30 min, and longer incubation times did not noticeably change fluorescence intensity. Compared with the data from previous studies, the required incubation times using our method were dramatically decreased and detection efficiency was improved.[27–30] Such a decrease in incubation time can possibly be attributed to the homogeneous suspension of magnetic particles and low steric hindrance allowing antigen accessibility. Finally, within the time range considered, 30 min was selected as the optimum reaction time in subsequent work.

The sensitivity of the proposed method for the AFP/free β-hCG was closely related to the concentration of magnetic particles coated with antibodies in the sandwich immunoassay approach. As shown in Fig. 3, a higher fluorescence intensity was achieved with an initial increase in the amount of magnetic particles and this then remained unchanged with an increase in the magnetic particle concentration. This saturation behavior may have occurred because the antibody immobilized on the surface of the magnetic particles captured the maximum amount of analytes. Therefore, a concentration of 300 μg mL^{-1} magnetic particles coated with AFP and 400 μg mL^{-1} magnetic particles coated with free β-hCG were chosen as the optimal concentrations.

Usually, the dilution ratios of Eu^{3+}or Sm^{3+}-labeled antibody also partially affect the sensitivity of an immunoassay. Thus, various dilution ratios of Eu^{3+}or Sm^{3+}-labeled antibody were investigated, as shown in Fig. 4. As the dilution ratio of Eu^{3+}or

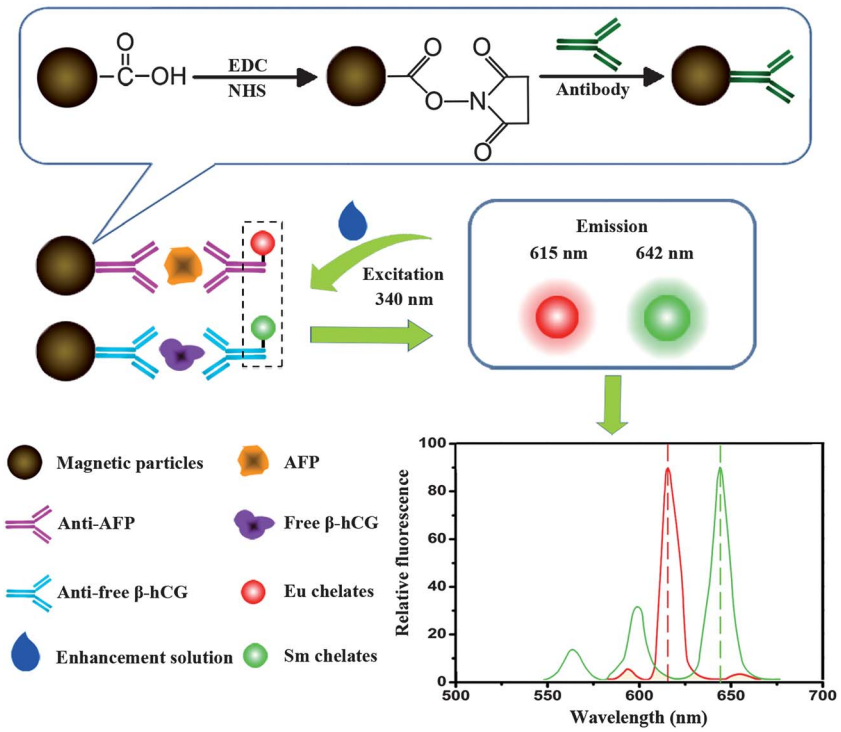

Fig. 1 Diagram of the magnetic particle-based TRFIA method for the simultaneous determination of AFP and free β-hCG.

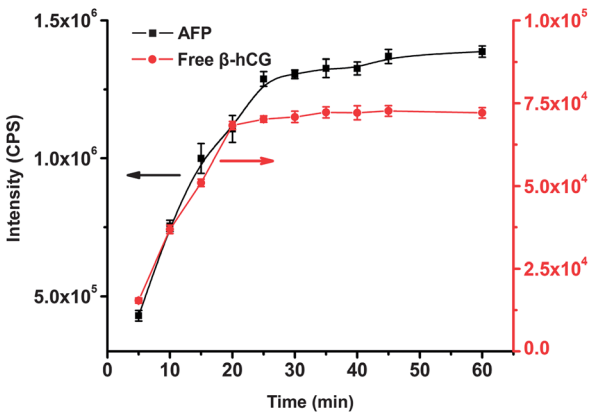

Fig. 2 Influence of incubation time on fluorescence intensity. Experimental conditions: The curves correspond to a series of incubation times (from 5 to 60 min), 25 μL of AFP/free β-hCG standards (400 and 200 ng mL^{-1}), 50 μL of magnetic particles (500 μg mL^{-1}), 100 μL of Eu^{3+} (or Sm^{3+})-labeled antibody (dilution ratio of 1 : 25).

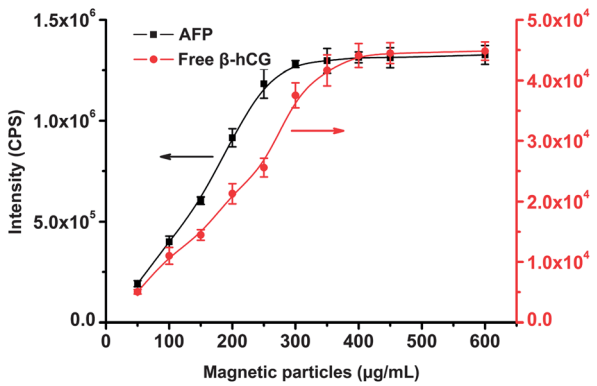

Fig. 3 Influence of the concentration of magnetic particles on fluorescence intensity. Experimental conditions: the curves correspond to a series of concentrations of magnetic particles (50 to 600 μg mL^{-1}), 25 μL of AFP/free β-hCG standards (400 and 200 ng mL^{-1}), 100 μL of Eu^{3+} (or Sm^{3+})-labeled antibody (dilution ratio of 1 : 25), incubation for 30 min at room temperature.

Sm^{3+}-labeled antibody decreased (a lower dilution ratio relative to a higher concentration), the fluorescence intensity increased and trended towards a maximum value at a dilution ratio of 1 : 75/1 : 50. A dilution ratio of 1 : 50/1 : 25 was selected finally.

Performance of the time-resolved fluoroimmunoassay

The new TRFIA method was validated in terms of the response linearity and limits of detection using a one-step immunoassay format. Under optimized experimental condition, a series of AFP/free β-hCG standards with different concentrations (0/0, 1.85/3.7, 7.7/10.5, 30/30, 125/100, and 750/400 ng mL^{-1}) were measured simultaneously and the calibration curves for AFP/free β-hCG are illustrated in Fig. 5. The calibration curves for AFP (Fig. 5A) showed a linear relationship over the concentration range of 0.1–750 ng mL^{-1}, with a correlation coefficient of 0.9970. The linear regression equation was adjusted to $\log(y) = 0.9931 \log(x) + 3.5172$, with a lower detection limit of 0.05 ng mL^{-1} AFP (defined as the concentration corresponding to blank fluorescence intensity plus two standard deviations, $n = 20$). Similarly, we obtained calibration curves for free β-hCG

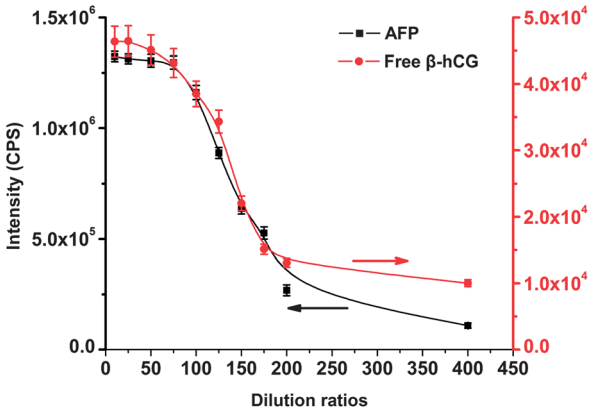

Fig. 4 Influence of the dilution ratio of Eu^{3+} (or Sm^{3+})-labeled antibody on fluorescence intensity. Experimental conditions: the curves correspond to a series of dilution ratios of Eu^{3+} (or Sm^{3+})-labeled antibody (from 1 : 10 to 1 : 400), 25 μL of AFP/free β-hCG standards (400 and 200 ng mL^{-1}), 50 μL of magnetic particles (500 μg mL^{-1}), incubation for 30 min at room temperature.

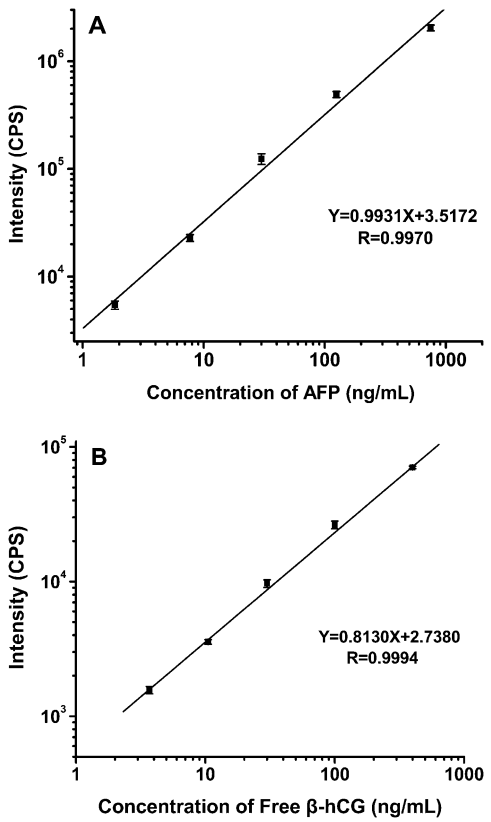

Fig. 5 Calibration curves for the magnetic particle-based TRFIA method for the simultaneous measurement of AFP (A) and free β-hCG (B) standards. Fluorescence intensity and standard deviations were calculated from a set of five measurements.

(Fig. 5B), fitted by $\log(y) = 0.8130 \log(x) + 2.7380$ with a correlation coefficient of 0.9994, which also responded linearly over a wide concentration range, between 0.16 and 400 ng mL^{-1}, with a lower detection limit of 0.08 ng mL^{-1}. No high-dose hook effect was observed within these linear ranges.

Such low detection limits and wide linear ranges are comparable with or better than other detection methods previously reported. A comparison with several methods is shown in Table 1. The superior performance may be attributed to the relatively high surfaces of the magnetic particles, which promotes the efficient immobilization of antibodies. Also, the magnetic particles were dispersed uniformly throughout the reaction mixture (*i.e.* a highly homogeneous suspension), which would enhance the chances of interaction between the antigen and antibody. Consequently, rapid antigen–antibody interaction equilibrium would be achieved. Importantly, we were able to complete the entire analysis within 40 min, which is briefer than the methods described previously. Thus, the proposed immunoassay method can conveniently attain highly sensitive detection, suggesting it could be further extended to the detection of other biomolecules at low levels and over wide assay ranges in clinical serum samples.

Specificity, reproducibility and recovery

The specificity of the TRFIA was evaluated over a wide spectrum of possible interferents, namely, carcinoembryonic antigen (CEA), carcinoma antigen 125 (CA125), cancer antigen 15-3 (CA15-3), cancer antigen 19-9 (CA19-9), luteinizing hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH) and hCG. The cross-reactivity percentages for each interfering compound are shown in Table 2. The results show that the presence of the potential interferents at relatively high concentrations had negligible effects on the simultaneous assay for AFP/free β-hCG, clearly verifying the high specificity of the proposed immunoassay.

The reproducibility of the immunoassay method was estimated by intra- and inter-assay coefficients of variation (CV) using maternal serum controls at three concentrations. The experiments were run on 1 day for intra-assay CVs and the same batch of reagents on different days for inter-assay CVs. Results are shown in Table 3. It was found that the intra-assay variations were 2.6–5.1% for AFP and 4.1–4.7% for free β-hCG, while the inter-assay variations were 1.9–5.1% for AFP and 4.7–5.0% for free β-hCG, demonstrating an acceptable reproducibility (Table 3).

Recovery was evaluated using given amounts of AFP or free β-hCG standards (50 ng) by spiking into the above-mentioned maternal serum controls at various analyte levels. As shown in Table 4, the recoveries of the analyte varied from 96.0 to 106.0%, indicating that the recovery of the proposed immunoassay was satisfactory.

Clinical serum samples

The reliability of the immunoassay system for clinical applications was further investigated by analyzing 446 clinical serum samples from second trimester pregnancies. Results were compared with those obtained from a commercially available TRFIA kit using linear regression analysis, as shown in Fig. 6A and B. The correlation between the two methods was investigated using *t*-tests for comparison, and no significance differences were observed. A good agreement between both analytical

Table 1 Comparison of sensitivity for the determination of AFP and free β-hCG by other, previously reported methods[a]

Analysts	Methods	Detection limit (ng mL^{-1})	Linear range (ng mL^{-1})	Total assay time (min)
AFP	EIA (ref. 31)	0.16	0.32–100.0	>120
	LSPCF (ref. 32)	0.10	2.33–143.7	60
	AIS (ref. 28)	5.00	15.0–350.0	40
	TRFIA (ref. 27)	0.07	0.10–100.0	>120
	ICP-MS (ref. 34)	1.20	4.60–500.0	—
	Proposed method	0.05	0.10–750.0	30
Free β-hCG	TRFIA (ref. 30)	0.04	7.30–525.0	>120
	TRFIA (ref. 33)	0.20	1.00–200.0	90
	ICP-MS (ref. 34)	1.70	5.00–170.0	—
	Proposed method	0.08	0.16–450.0	30

[a] Enzyme immunoassay: EIA, amperometric immunosensor: AIS, localized surface plasmon coupled fluorescence: LSPCF, inductively coupled plasma mass spectrometry: ICP-MS.

Table 2 Effect of potentially interfering substances on the determination of AFP and free β-hCG

Interfering substance	Concentration (ng mL^{-1})	AFP ($n = 5$)		Free β-hCG ($n = 5$)	
		Observed	Cross-reactivity (%)	Observed	Cross-reactivity (%)
LH	250	0.029	0.02	0.272	0.11
FSH	200	0.061	0.03	0.169	0.08
TSH	300	0.051	0.02	0.235	0.07
HCG	2000	0.092	0.00	7.000	0.35
CEA	1000	0.165	0.02	0.119	0.01
CA125	600	0.236	0.04	0.112	0.02
CA15-3	500	0.234	0.05	0.136	0.03
CA19-9	500	0.139	0.03	0.142	0.03

Table 3 Inter-assay and intra-assay stability of the proposed method

Sample	Add (ng mL^{-1})	Intra-assay precision[a] ($n = 10$)		Inter-assay precision[a] ($n = 30$)	
		Observed (ng mL^{-1})	CV (%)	Observed (ng mL^{-1})	CV (%)
AFP	11.5	11.02 ± 0.51	4.6	11.00 ± 0.21	1.9
	30.5	30.76 ± 0.80	2.6	30.73 ± 0.82	2.6
	78.5	76.81 ± 3.90	5.1	76.30 ± 3.17	5.1
Free β-hCG	11.1	10.93 ± 0.49	4.5	11.21 ± 0.56	5.0
	34.0	33.42 ± 1.36	4.1	33.76 ± 1.63	4.7
	96.2	95.91 ± 3.47	4.7	95.80 ± 4.65	4.8

[a] Mean value ± standard deviation.

Table 4 Recoveries determined using the proposed method *via* spiking three different concentration levels of maternal serum controls into AFP and free β-hCG standards

Sample	Original (ng mL^{-1})	Spiked (ng mL^{-1})	Observed[a] (ng mL^{-1})	Recovery (%)
AFP	50	11.5	60.69 ± 1.56	98.7
	50	30.5	77.29 ± 3.55	96.0
	50	78.5	132.86 ± 3.13	103.4
Free β-hCG	50	11.1	64.71 ± 1.63	106.0
	50	34.0	81.52 ± 0.32	97.1
	50	96.2	154.29 ± 0.35	105.5

[a] Mean value ± standard deviation.

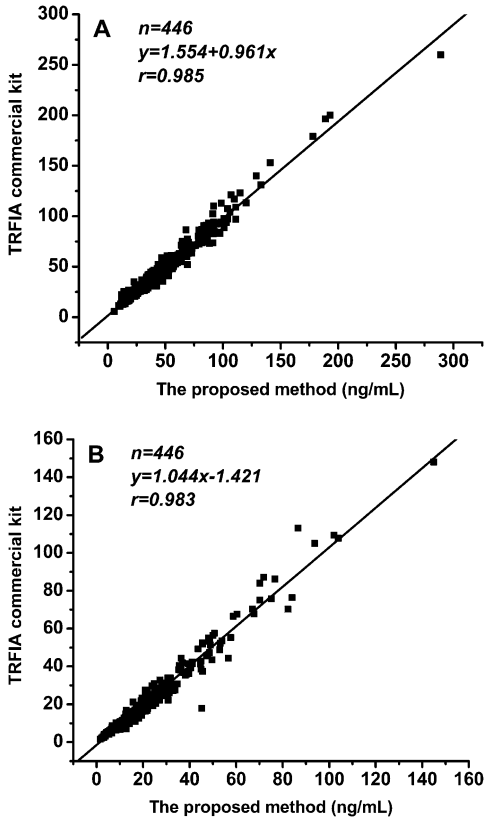

Fig. 6 Plot of the results obtained using the proposed method *versus* those obtained using TRFIA commercial kits for AFP (A) and free β-hCG (B) detection in 446 serum samples from second trimester pregnancies.

methods was observed, with r values of 0.985 for AFP and 0.983 for free β-hCG. The regression line was fitted to $y = 0.961 + 1.554x$ or $y = 1.044x - 1.421$, respectively, where x stands for the AFP or free β-hCG concentrations estimated by the proposed method and y stands for those obtained using the TRFIA kit. The results indicate that the magnetic particle-based TRFIA has good potential application for the simultaneous determination of AFP and free β-hCG in real sample analyses.

Furthermore, attempts to improve Down syndrome detection rates have been ongoing in our research and one potential avenue has focused on the measurement of unconjugated estriol[22,24] by labeling terbium. We expect that these important and well-established biomarkers for second trimester pregnancy serum screening for Down syndrome can be examined by the present homogenous multiple immunoassay.

Conclusion

The present work has resulted in the development and validation of a TRFIA method for the simultaneous determination of AFP and free β-hCG by immobilizing primary antibodies on the surface of magnetic particles. The application of magnetic particles as both the immobilization matrix and a separation tool effectively improves sensitivity and significantly reduces the analysis time via a homogenous format. The proposed immunoassay method possesses several advantages over previous methods, including high sensitivity, broad dynamic assay ranges, short analytical time, and cost-effectiveness. Additionally, the method shows good properties for detection of AFP and free β-hCG with acceptable reproducibility, specificity and recovery. Moreover, the combination of TRFIA and magnetic particles may provide an interesting alternative tool for the simultaneous determination of other biomarkers in clinical laboratories. The extension of the assay to the simultaneous detection of other multiple analytes is currently under investigation.

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