〈Original Article〉

Rapid and sensitive detection of alpha-fetoprotein by a magnetically promoted shake-free immunoassay employing fluorescent magnetic nanobeads

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Summary Recently, a rapid and sensitive fluorescent sandwich immunoassay was reported with an adaptation that magnetically promoted immunoreaction using fluorescent magnetic nanobeads. This immunoassay includes a shake-washing step to wash out the unbound fluorescent magnetic nanobeads. Here, to simplify the washing process and to enhance the wash efficiency, we modified the washing process by applying magnetic assistance. The modified washing process, which was magnetically assisted and shake-free, was easier to perform and it increased the signal-to-noise ratio in the detection of a model biomarker alpha-fetoprotein (AFP). The limit of detection and the limit of quantification of serum AFP were 15.4 pg/mL and 23.5 pg/mL, respectively. The total assay time was within 10 min. Consequently, the proposed immunoassay with shake-free washing allowed simple, rapid, and sensitive detection of the antigen. Because of these advantageous features, the modified immunoassay is highly likely to find application in the development of point-of-care devices.

Key words: Immunoassay, Rapidity, Simplicity, Fluorescence, Magnetic nanobeads

1. Introduction

Sensitive measurement of biomarkers is of great importance in biomedical research, disease monitoring, and clinical diagnosis[1-3]. In particular, much attention has been recently directed to rapid and sensitive diagnostics in the point-of-care (POC) settings. For instance, cardiac biomarkers and diabetes biomarkers have been detected with POC devices that allow on-the-spot diagnosis, thereby facilitating more prompt clinical decision-making[4-6]. Moreover, the demand for POC testing of other biomarkers, such as markers of measles, sepsis, and cancer, is increasing for early diagnosis, timely treatment monitoring, reducing

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diagnostics costs, and for alleviating patient stress[7-10].

Sakamoto et al.[11] recently reported a remarkably rapid and sensitive fluorescent immunoassay system that utilizes submicrometer-sized polymer-coated fluorescent magnetic beads (FF beads). This immunoassay is similar to microplate-based sandwich-type enzyme-linked immunosorbent assay (ELISA); however, it adopts magnetically promoted antigen-antibody binding (magnetic promotion) on a planar surface. The magnetic promotion, the most significant feature of the immunoassay, dramatically speeds up binding of the FF beads coated with detection antibody to the solid-phase captured antigen within 1 min (Fig. 1 (a)). After shake-washing the unbound FF beads, the fluorescent intensity of the bound FF beads is measured by fluorometry without the use of additives such as enhancement reagents. Sakamoto et al.[11] used this immunoassay system to successfully detect both brain natriuretic peptide (BNP) in human plasma samples and prostate specific antigen (PSA) in human serum samples with the limit of detection (LOD) of 5.0 pg/mL within 5 min of specimen addition. However, further simplification of the immunoassay is necessary for application to a POC device. In addition, higher sensitivity will contribute to more precise detection. Moreover, detection of a wide range of biomarker proteins is required to ensure wider applicability of the immunoassay.

In the present study, for simplifying the procedure and for enhancing the sensitivity, we modified the shake-washing method to a magnetically assisted shake-free washing method (magnetic washing) (Fig. 1 (b)). The performance of the modified FF beads-based rapid immunoassay was validated by the detection of alpha-fetoprotein (AFP), a 70,000-Da liver tumor biomarker.

2. Materials and methods

2.1. Materials

In this study, we used normal human serum without clot (BBI Solutions, Cardiff, UK), affinity magnetic beads with NH_2 groups (FG beads NH_2; Tamagawa Seiki Co., Ltd., Iida, Japan), Europium (Ⅲ) thenoyltrifluoroacetonatetrihydrate [$Eu(TTA)_3 \cdot 3H_2O$]; Acros Organics, Gelgium〕, Tri-*n*-octylphos-

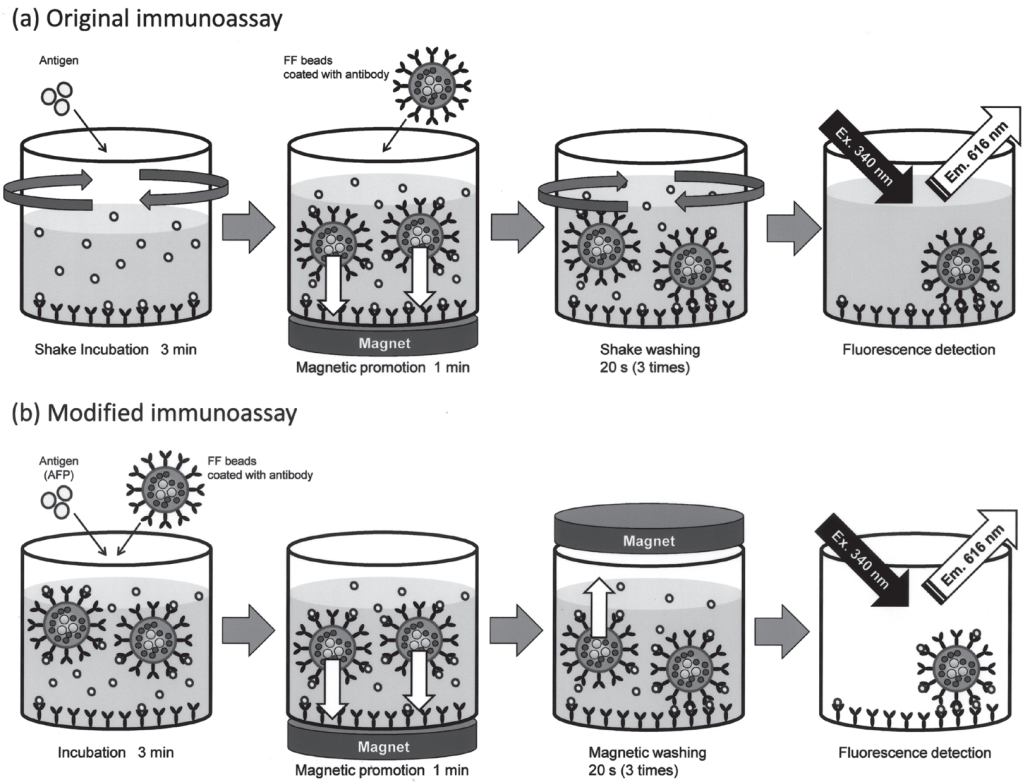

Fig. 1 Scheme of FF beads-based rapid immunoassay with magnetic washing.

phine oxide (TOPO; Strem Chemicals Inc., Massachusetts, US), AFP (BBI solutions, Cardiff, UK), Anti-AFP mouse monoclonal antibodies (4A3 and 5H7; HyTest Ltd., Turku, Finland), Lumipulse AFP-N standard solution (Fujirebio Inc., Tokyo, Japan), Sulfosuccinimidyl-4-(*N*-maleimidemethyl) cyclohexane-1-carboxylate (Sulfo-SMCC; Thermo Fisher Scientific Inc., Massachusetts, US), and black-colored 96-well polystylene microtitration plate (FluoroNunc F16 Black Maxisorp; Thermo Fisher Scientific Inc., Massachusetts, US).

The magnet plate used in this study was prepared as follows: ninety-six cylinder-shaped neodymium magnets (6-mm diameter, 10-mm long, 488 mT surface magnetic flux density) were embedded in a plastic plate (10-mm thick), with the 9-mm pitch arrayed in 8 × 12 form. The magnetic poles of 8 magnets, which formed 1 column, were set in the same direction, and the adjacent columns were set in a different magnetic pole direction.

2.2. Preparation of FF beads

FF beads were prepared as described previously by Hatakeyama et al.[12] Briefly, a suspension of FG beads with NH_2 groups (100 mg) was incubated with 60 mL of acetone containing 200 μmol of $Eu(TTA)_3 \cdot 3H_2O$ and 400 μmol of TOPO with vigorous shaking for 1 h at room temperature in the dark. Then, 60 mL of distilled water was added and acetone was evaporated under vacuum at 40℃. The beads were washed with washing buffer (50 mM HEPES containing 0.3% Tween 20; pH 7.9). Finally, the prepared FF beads were dispersed in distilled water and stored at 4℃ in the dark.

2.3. Maleimidation of the surface of FF beads

The primary amino groups on the FF beads were converted into maleimide groups by utilizing a hetero cross-linking reagent. To the FF beads suspension (2 mg of FF beads in 465 μL of 50 mM phosphate buffer; pH 7.0), 350 μg of Sulfo-SMCC dissolved in distilled water (35 μL of 10 mg/mL) was added, and the mixture was incubated for 1 h at room temperature in the dark. After incubation, the beads were washed three times with 50 mM phosphate buffer (pH 6.0). Finally, the maleimide FF beads were adjusted to 8 mg/mL concentration with 50 mM phosphate buffer (pH 6.0) and stored at 4℃ in the dark until use.

2.4. Immobilization of anti-AFP antibody on FF beads

To 92 μg of anti-AFP mouse monoclonal antibody 4A3 dissolved in 450 μL of 50 mM acetate buffer (pH 5.0), 50 μL of 125 mM 2-aminoethanethiol hydrochloride aqueous solution was added, and the solution was incubated at 37℃ for 1 h. After desalting using microtubes equipped with a filter, the SH group-exposed IgG fragments were covalently conjugated onto the maleimide FF beads as follows: to 78 μg of the SH group-exposed IgG fragments dissolved in 170 μL of 50 mM phosphate buffer (pH 6.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 780 μg of maleimide FF beads suspension (98 μL) and 133 μL of 50 mM phosphate buffer (pH 6.0) containing 1 mM EDTA were added, and the mixture was incubated at 4℃ for 15 h in the dark. Then, to this reaction mixture, 50 μL of 50 mg/mL 2-mercaptoethanol aqueous solution was added, and the solution was incubated at room temperature for 30 min in the dark to mask the remaining maleimide groups on the beads surface. Next, the beads were washed three times each with 500 μL of 50 mM phosphate buffer (pH 6.0). Finally, the FF beads coated with antibody 4A3 were adjusted to 5 mg/mL concentration with 50 mM phosphate buffer (pH 6.0) and stored at 4℃ in the dark until use.

2.5. FF beads-based rapid sandwich immunoassay for detecting AFP

Each well of a black-colored polystyrene 96-well microplate was sensitized with 5 μg/mL of anti-AFP antibody 5H7 (50 μL) in phosphate-buffered saline (pH 7.4) at 4℃ overnight. After removal of the antibody solution, 200 μL of blocking solution (25 mM Tris-HCl containing 150 mM KCl, 2.5 mM EDTA, 0.1% Tween 20, 1% skim milk; pH 7.9) was added. The blocking treatment was conducted at 4℃ for over 1 day. Each concentration of AFP (40 μL) in the blocking solution and 40 μg/mL of the FF beads coated with antibody 4A3 in the blocking solution

(10 μL) were simultaneously added to the same wells sensitized with the antibody 5H7 and incubated for 3 min at room temperature. After incubation, the magnet plate was set on the bottom surface of the microplate and magnetic promotion was conducted for 1 min at room temperature. Then, the wells were washed three times with the washing buffer (25 mM Tris-HCl containing 150 mM KCl, 0.1% Tween 20; pH 7.9). For shake-washing, the microplate was set on a microplate shaker (Nissin, Tokyo, Japan) to shake for 20 s in each washing step. For the magnetic washing, the magnet plate was placed on the top surface of the microplate for 20 s in each washing step. Finally, time-resolved fluorescence measurements were performed by using a plate reader (ARVO X4; PerkinElmer, Massachusetts, US). The wavelength of excitation and emission were 340 nm and 616 nm, respectively.

2.6. Linearity

The linearity of the modified FF beads-based rapid immunoassay was estimated by the measurement of the serum AFP samples. The serum AFP samples were prepared by serial dilution of an AFP-free serum containing 80,000 pg/mL of purified AFP with the blocking solution. For AFP-free serum preparation, a pooled normal serum was mixed with FG beads (Tamagawa Seiki Co., Ltd., Iida, Japan) coated with anti-AFP rabbit polyclonal antibody PG-012 (Nippon Biotest Laboratories Inc., Tokyo, Japan) and incubated overnight at 4℃. The beads which captured endogenous AFP were removed by centrifugation and filtration.

2.7. Intra-imprecision, total imprecision, and sensitivity

For the analysis of intra-imprecision, five different concentrations of serum AFP samples (14.8, 128, 2730, 14,100, and 70,300 pg/mL) were prepared by adding purified AFP protein to the AFP-free serum. Using the above samples, five assays were simulta-

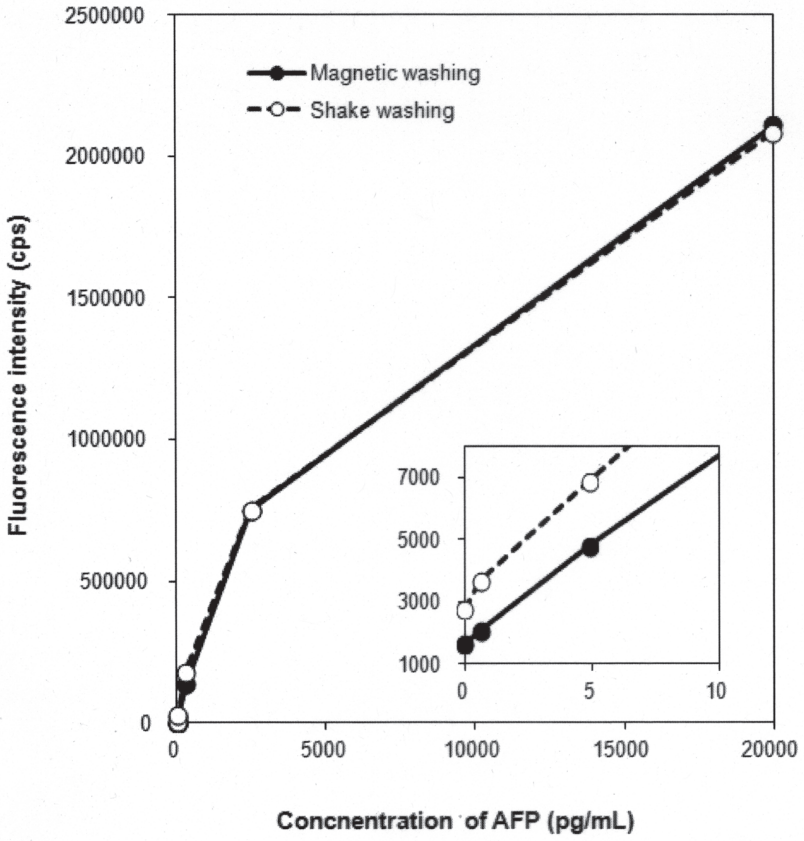

Fig. 2 Calibration curves for FF beads based-rapid immunoassay with using washing method of shake washing and magnetic washing. All data are expressed as mean of 4 measurements.

neously performed (n=5) and the coefficient of variation (CV) at each AFP concentration was calculated by dividing the standard deviation (SD) at each AFP concentration by the prepared AFP concentration. For the analysis of total-imprecision and sensitivity [the LOD and the limit of quantification (LOQ)], six different concentrations of serum AFP samples (12.4, 15.4, 124, 2780, 14,200, and 74,400 pg/mL) and a blank sample were prepared. Fifteen measurements (n=5 per day for 3 days) were performed and the CV of the 15 measurements was similarly calculated. The LOD and LOQ were also evaluated using the results of the 15 measurements.

3. Results

3.1. Comparison of the washing method of FF beads-based rapid immunoassay

We detected purified AFP (0.6-20,000 pg/mL) using the modified FF beads-based rapid immunoassay with magnetic washing (Fig. 1(b)). Immunoassay with shake-washing was performed as a control experiment. The calibration curves obtained in the analysis are depicted in Fig. 2. In both the shake-washing and magnetic washing, the intensities of the fluorescent signals depended on the concentrations of AFP and no 'hook' effect was observed, even at the highest AFP concentration (20,000 pg/mL). The fluorescent intensity of the blank sample was lower for magnetic washing than for shake-washing. The signal-to-noise ratio at 20,000 pg/mL (the fluorescence intensity at 20,000 pg/mL divided by the intensity at the blank) was approximately 750 for shake-washing and 1,300 for magnetic washing methods. The assay time was within 10 min of sample addition for detection using the plate reader.

3.2. Linearity, sensitivity, and imprecision

To verify the linearity, sensitivity, and imprecision of the modified FF beads-based rapid immunoassay with magnetic washing, serum AFP samples were used. Fig. 3 shows the linearity of dilutions. For evaluation, the recovery rates of the measured values to the expected values were 93.2-118.0% at the

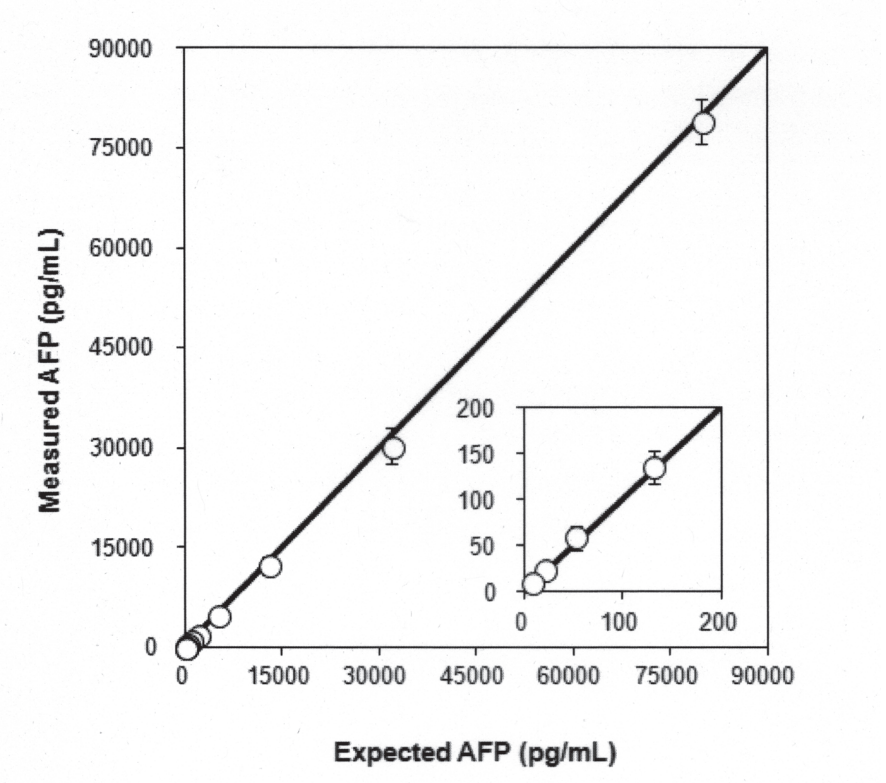

Fig. 3 Linearity of serum AFP detection by using FF beads-based rapid immunoassay with magnetic washing. All data are expressed as mean of 3 measurements. Error bars correspond to 2SD of 3 measurements.

Table 1 Intra-assay imprecision and total imprecision of serum AFP detection

Intra-imprecision (n = 5)			Total-imprecision (n = 15)		
Prepared AFP conc. (pg/mL)	SD	CV (%)	Prepared AFP conc. (pg/mL)	SD	CV (%)
70300	3200	4.6	74400	4874	6.6
14100	1290	9.1	14200	1075	7.6
2730	206	7.5	2780	219	7.9
128	7	5.5	124	11	8.9
14.8	2	13.5	15.4	2	13.0

expected AFP concentration of 8.4-80,000 pg/mL. The LOD was defined as the lowest AFP concentration that generated a mean minus 2 SD signal, which is greater than the mean plus 2SD signal of the blank control. The LOQ was defined as the lowest AFP concentration measurable at a CV <10%. Per the definitions, the LOD and LOQ were 15.4 pg/mL (0.22 pM) and 23.5 pg/mL (0.34 pM), respectively. The LOD and LOQ values were significantly lower than the cut-off value of 20,000 pg/mL used in the current clinical AFP detection[13]. Thus, the modified immunoassay enabled a highly sensitive detection of serum AFP within 10 min. For the estimation of intra-assay imprecision, the serum AFP samples were assayed in 5 replicates within the same day. The CVs of the intra-assay were 4.6-13.5% (Table 1). For the estimation of total imprecision, the serum AFP samples were assayed in 5 replicates over 3 days (n = 15). The CVs of total assay were 6.6-13.0% (Table 1). Both intra-assay imprecision and total imprecision showed low CV, which indicates clinical applicability of this assay.

4. Discussion

In this study, we demonstrated a very rapid and sensitive detection of a tumor biomarker AFP by using the modified FF beads-based rapid immunoassay. To simplify the original procedure, we replaced the shake-washing method with a magnet washing method at the washing step after performing magnet promotion. In this setting, the fluorescent signals that depend on the concentration of AFP (0-20,000 pg/mL) were successfully detected (Fig. 2). Thus, it was confirmed that shake-washing step can be replaced by the magnetic washing step, which is a simpler washing method. In addition, magnetic washing increased the signal-to-noise ratios of the detection because it could decrease the background fluorescent signal. This indicates that the magnetic washing step washed out the unbound FF beads more effectively than the shake-washing step. The magnetic washing step can be more efficient if the magnets are placed closer to the surface of the washing solution.

After confirming the performance of the modified immunoassay with purified AFP samples, we examined its sensitivity, linearity, and imprecision with serum AFP detection. The estimated LOD (15.4 pg/mL; 0.22 pM) was significantly low compared with that of clinically used AFP immunoassay systems. For instance, the LOD of a μTAS-type immunoassay system employing electrophoresis, one of the highly sensitive AFP detection systems, is 100 pg/mL (1.43 pM)[14]. The LOQ was 23.5 pg/mL and the linearity reached up to 80,000 pg/mL. Therefore, the dynamic range was more than three orders of magnitude. The intra-assay CVs and total-assay CVs were sufficiently low for the clinical application of this system (Table 1). In addition, it was confirmed that AFP, a 70,000-Da protein, could be successfully detected in addition to relatively small or middle molecular-weight proteins such as BNP and PSA. Although further investigations such as interference

tests with endogenous substrates are required to establish this modified method, we suggest that the proposed, modified immunoassay will provide a more rapid, sensitive, and simple detection system of biomarkers in clinical settings than the currently available detection systems.

In conclusion, we successfully developed a modified FF beads-based rapid immunoassay that includes a simpler and more effective shake-free method than the original immunoassay. The shake-free magnetic washing step facilitates an increase in the signal-to-noise ratio. Furthermore, the total assay time of this immunoassay is within just 10 min. Moreover, AFP, a new biomarker for the immunoassay system, could be successfully detected by the proposed method. Thus, it is highly likely that the proposed modified immunoassay will find application in the development of a new POC system with optimum rapidity, sensitivity, and simplicity.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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