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DEVELOPMENT OF AUTOMATED FLOW IMMUNOSENSOR BASED ON KINETIC EXCLUSION ANALYSIS FOR MEASUREMENT OF α-FETOPROTEIN IN SERUM

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An automated flow immunosensor has been developed and validated for the measurement of the cancer marker α-fetoprotein (AFP) in serum. The sensor employed the kinetic-exclusion analysis using the KinExA™ 3200 instrument. Various concentrations of AFP were incubated with a fixed amount of mouse anti-AFP monoclonal antibody until the binding reaction reached equilibrium. These solutions were then passed rapidly over AFP coated onto polymethylmethacrylate beads contained in the observation cell of the KinExA instrument. The anti-AFP antibody captured by the beads was quantified by passing a fluorescent-labeled secondary antibody over the beads, and the fluorescence signals were monitored during its flow. Calibration curve was generated by plotting the fluorescence signals that were retained on the beads as a function of AFP concentrations. The assay limit of detection was 8 ng/ml, and the working range of the assay was 8–500 ng/ml. Analytical recovery of serum–spiked AFP was 93.8–105.8 ± 2.8–8.1%. The precision of the sensor was satisfactory; RSD was ≤ 7.2%. The proposed sensor was found to be superior to the existing conventional enzyme-linked immunosorbent assay (ELISA) for AFP. The sensor is anticipated to have a great value in measurement of AFP when a more confident result is needed.

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Keywords: Cancer markers; α–Fetoprotein; Immunosensor; Kinetic exclusion analysis; Enzyme–linked immunosorbent assay.

1. Introduction

Cancer is one of the main causes of mortality worldwide whose cure has not yet been achieved [1,2]. During the tumor process, increased levels of tumor markers in human serum are associated with patients with certain types of cancer [3-5]. α-fetoprotein (AFP) is one of the most extensively used clinical tumor markers. The concentration of AFP in healthy human serum is typically below 25 ng/ml [6,7]. Elevated AFP concentration is used for early indication for some types of cancers such as hepatocellular carcinoma, liver metastasis from gastric cancer, testicular cancer, and nasopharyngeal cancer [8-12]. On the other hand it has been found that the AFP level in serum is associated with tumor cell growth [13]. Therefore, detection of tumor markers that signal the recurrence of cancer is crucial for effective early diagnosis and treatment of cancer, and AFP can directly be used as an important target of tumor therapy to evaluate curative effect, recrudescence, and/or metastasis [14,15].

In clinical laboratories, immunoassays have been mostly used for determination of AFP due to the highly selective nature of the recognition between the AFP and its corresponding antibodies [16-21]. The conventional immunoassays, including immunoradiometric assay (IRMA) [16,17], enzyme-linked-immuonosorbent assay (ELISA) [18], enzyme immunoassays coupled with liquid chromatography [19], capillary electrophoresis [20] or inductively coupled plasma mass spectrometry [21], usually have various limitations such as relying on the labeling of either

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AFP or its antibody, multiple pipetting and washing steps, and sometimes long analysis time. IRMA, besides its radiation hazards, it suffers from the short lives of the radioactive labels, and requires skillful operators.

Immunosensors are new alternative technology that has great interest because they combine the selectivity and sensitivity of traditional immunoassays with the rapid response of the sensor without the need for long incubation or extensive sample manipulation, besides the automation of the analysis. They are also characterized with reduction in size, cost and time of analysis compared to the conventional of immunoassays. Many articles have been published about the development of immunosensors for a wide range of bioactive molecules in the field of biochemical and clinical analysis [22-24]. A variety of immunosensors have been developed for AFP: quartz crystal microbalance [25], enzyme membrane [26], piezoelectric [27], and electrochemical immunosensors employing various types of electrodes [28-31]. However, the flow immunosensors recently have emerged for applications in a variety of new areas [32]. The most promising and outstanding technological progress in the field of flow immunosensor development is the immunosensors employing kinetic exclusion analysis (KinExA). KinExA–based immunosensors measure the amount of free antibody- binding sites in an equilibrium mixture of antibody and antigen. The flow KinExA–based immunosensors have shown significant potential advantages in measurements of wide range of molecules in various applications [32].

In this study, a flow KinExA–based immunosensor assay has been developed and validated for determination of AFP in serum. The proposed sensor assay offered several advantages: (1) the analysis of AFP was done without any modifications, thus avoiding the negative effect of the modification on the analytical results, (2) it avoided the problems of mass transport limitations and mobility effects that are encountered in ELISA, and (3) it provided higher level of convenience by automation of the analysis.

2. Experimental

2.1. Apparatus

The KinExA™ 3200 instrument was obtained from Sapidyne Instruments Inc. (Boise, ID, USA) empowered by KinExA Pro 20.0.1.26 software provided with the instrument. ELx808 microplate reader and FLX50 microplate washer were obtained from Bio-Tek Instruments Inc. (Winooski, USA). The reader was empowered by KC Junior software, provided with the instrument. TSE incubator was obtained from Sanyo Co Ltd. (Osaka, Japan). Nutating mixer was obtained from Taitec (Saitama-ken, Japan). Biofuge Pico centrifuge was obtained from Heraeus Instruments (Hanau, Germany). Nanopure II water purification system (Barnstead/Thermolyne, Dubuque, USA) was used to make all the solutions described herein.

2.2. Materials

α-Fetoprotein (AFP) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis. MO, USA). Anti–AFP monoclonal antibody was obtained from MyBiosource, LLC (San Diego, CA, USA). Polymethylmethacrylate (PMMA) beads (140–170 mesh, 98 mm) were obtained from Sapidyne Instruments Inc. (Boise, ID, USA). DyLight™ 649–conjugated AffiniPure goat anti–mouse IgG secondary antibody was obtained from Jackson ImmunoReserach Laboratories Inc. (West Grove, PA, USA). ELISA high–binding microwell plates were a product of Corning/Costar, Inc. (Cambridge, MA, USA). AFP ELISA kit (Calbiotech Inc., Spring Valley, CA, USA). Human serum samples were collected from normal healthy volunteers at King Khalid University Hospital (Riyadh, Saudi Arabia), and were kept frozen at –20 ºC until analysis. PBS was obtained from Bio-Basic Inc. (Markham, Canada). Other reagent-grade chemicals were obtained from Fisher Scientific Co. (Pittsburgh, PA, USA).

2.3. Procedures

2.3.1. Coating of PMMA beads with AFP. PMMA bead vials containing 200 mg (dry weight) of beads were coated with AFP. The coating solution consisted of 1 ml of PBS containing

30 μg/ml of AFP. Bead vials containing the coating solution were kept on the nutating mixer for 2 h at room temperature. Bead vials were subsequently removed from the mixer and beads allowed settling down. The supernatant was aspirated from the coating solution and 1 ml of blocking solution (10 mg/ml of BSA in PBS) was added to the bead vial. Bead vials were returned to the nutating mixer for an additional 1 h at room temperature. The blocked beads were used immediately or stored at 4 °C in the blocking solution until use.

2.3.2. Preparation of AFP samples. The AFP samples were prepared by spiking blank human serum with AFP (2–500 ng/ml). Each spiked sample was mixed with an equal volume of anti-AFP antibody solution (300 ng/ml in PBS) containing BSA at a concentration of 1 mg/ml in the reaction mixture; BSA was added to reduce any subsequent non-specific binding of the primary antibody to the PMMA beads in the instrument microcolumn. The samples were pre-equilibrated by incubation for 2 h at room temperature. After achieving equilibrium, samples were analyzed by the KinExA instrument.

2.4. Analysis by KinExA instrument

Each of 12 sample lines (of total 14 lines) of the KinExA instrument was placed into a sample tube containing the pre-equilibrated mixture of antigen and antibody (AFP and its specific antibody). The 13^{th} line was placed into a tube containing the blank (zero concentration of AFP and the primary antibody, and the 14^{th} line was placed into a tube that contained the fluorescently labeled secondary antibody solution. AFP-coated beads (200 mg) were diluted to 30 ml with PBS, placed in a bead bottle and loaded into the capillary micro-column of the KinExA^{TM} 3200 instrument. All subsequent steps in the assay were performed automatically by the Instrument. Beads were first automatically packed into capillary flow/observation cell by the KinExA^{TM} 3200. The system was charged twice to produce a bead micro-column of the appropriate height. A camera that monitored the flow/observation cell assisted with this procedure. In the final assay, a 683 μl aliquot of a suspension of the beads in PBS was drawn over the flow cell at a flow rate of 1 ml/min for 41 sec. These conditions produced a uniform and reproducible pack for coated beads. A 500 μl of each equilibrated sample solution was then withdrawn and passed over the micro-column for 120 sec at a rate of 0.25 ml/min. An automatic buffer wash (333 μl of the PBS) removed unbound primary antibody and excess soluble AFP molecules from the beadpack. Fluorescently labeled goat anti-mouse IgG secondary antibody solution (200 ng/ml) was drawn past the beads, and unbound labeled secondary antibody was subsequently removed by drawing 1.5 ml of PBS through the bead-pack over a period of 90 sec at a flow rate of 1 ml/min. The secondary antibody bound to the beads was quantified by measuring the difference in fluorescence intensity at the beginning and end of each sample run. Each calibrator or unknown was run in duplicate, and a fresh beadpack was used for each run. The data were collected by the KinExA Pro 20.0.1.26 software and transformed to a four-parameter curve using the fitting programs in SlideWrite, version 5.011 (Advanced Graphics Software, Inc., Rancho Santa Fe, CA, USA). A calibration curve was generated by fitting the data to the following equation: $F = F_0 - \{(F_0 - F_1)[AFP] / (IC_{50} + [AFP])\}$

Where F is the fluorescence signal at a definite known concentration of AFP [AFP], F_0 is the fluorescence signal at zero concentration of AFP, F_1 is the fluorescence signal at the saturating concentration of AFP, and IC_{50} is the AFP concentration that produces a 50% inhibition of the signal. The concentrations of AFP in the samples were obtained by interpolation on the calibration curve.

3. Results and discussion

3.1. Features and operation of the proposed sensor

Details of the KinExA instrument have been described elsewhere [33-36]. The basic features and operation of the proposed KinExA–based immunosensor are illustrated schematically in Fig. 1.

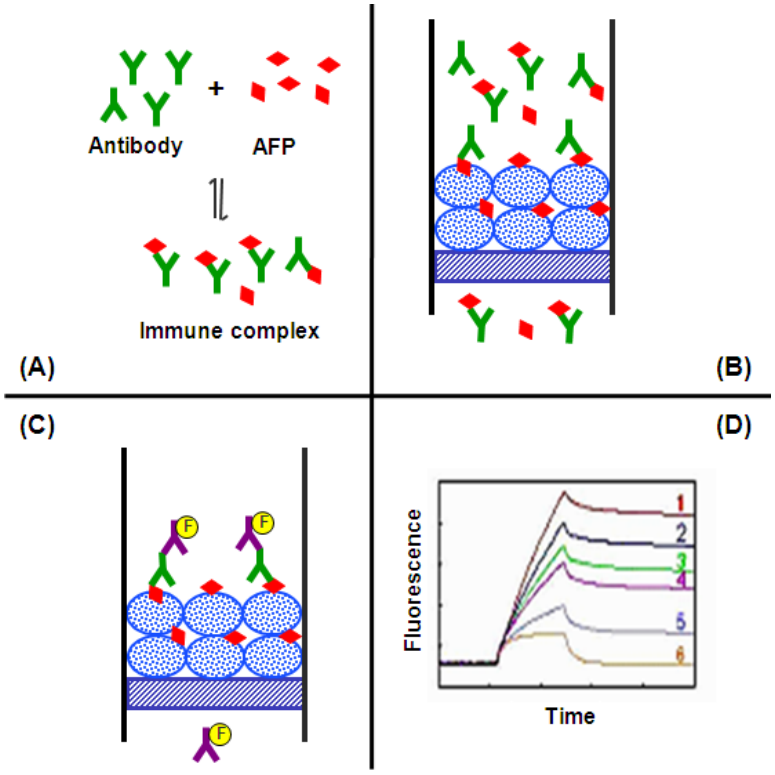

Fig. 1. Format for KinExA-based immunosensor for measurement of AFP.

In this study, a fixed amount of anti-AFP antibody was mixed with varying concentrations of AFP (Fig.1A) and a small volume of the reaction mixture was then rapidly passed through the packed bed of PMMA beads coated with AFP (Fig. 1B). Anti−AFP antibodies with unoccupied binding sites were available to bind to the immobilized AFP (coated on the surface of the PMMA beads); antibodies with binding sites occupied by soluble AFP analyte were not. Exposure of the AFP−antibody immune complexes to the immobilized AFP was sufficiently brief to ensure that negligible dissociation of the immune complexes occur during the time of exposure to the beads. Those antibody molecules whose binding sites were occupied by AFP molecules were thus kinetically excluded from interacting with the immobilized AFP. The soluble reagents were removed from the beads by an immediate buffer wash. Quantification of the antibody captured on the immobilized AFP could subsequently be achieved by the brief exposure of the particles to a fluorescently labeled secondary antibody directed against the primary antibody (Fig. 1C), followed by measurement of the resulting fluorescence from the particles after removal of excess unbound reagents (Fig. 1D). Fluorescence signals were monitored continuously by a photodiode in the optical unit of the instrument [32,37]. Curve 1 corresponds to zero AFP concentration; curve 6 corresponds to a saturating AFP concentration. Curves 2 – 5 are the concentrations of AFP between zero and saturation.

3.2. Measurement of AFP by the proposed sensor

Data acquisition was initiated immediately following the establishment of the beads microcolumn, and the instrument responses as a function of time for various concentrations of AFP are shown in Fig. 2. The instrumental response from 0 to 165 sec corresponds to the background signal generated while the unlabeled equilibrium mixture is exposed to and washed out of the bead-packed microcolumn. The beads were then exposed to a solution of fluorescently-labeled secondary antibody (165–280 sec), and excess unbound labeled secondary antibody was removed from the beads with a buffer wash (280–385 sec). When the equilibrium mixture contained a saturating concentration of free AFP (curve 6 corresponding to 500 ng mL^{-1}), the instrument

response approximated a square wave corresponding to the fluorescence of the secondary antibody during its transient passage through the beads in the observation cell. The signal failed to return to background, indicating a small non-specific binding of the fluorescently-labeled secondary antibody to the beads. When AFP was omitted from the equilibrium mixture (curve 1 corresponding to zero concentration of AFP), the instrument response from 165 to 280 sec reflected the sum of two contributions: the fluorescence of unbound secondary antibody in the interstitial regions among the beads and that of the labeled secondary antibody that had bound to the primary antibody captured by the AFP immobilized on the beads. Binding of the secondary antibody was an ongoing process that produced a positive slope in this portion of the curve. When the excess unbound secondary antibody was washed from the beads, the signal that remained was the sum of that from the non-specifically bound antibody plus that of the labeled secondary anti-mouse antibody specifically bound to the primary anti-AFP antibody captured on the beads. Equilibrium mixtures comprised of AFP present at concentrations intermediate between those of zero and saturation (Fig. 2) thus provided intermediate instrument responses from which a calibration curve for measurement of AFP could be generated.

3.3. Optimization of assay conditions for measurement of AFP by KinExA

AFP is a glycoprotein with a molecular mass of about 68,000 Daltons consisting of single polypeptide chain comprised of 590 amino acid residues [37,38]. Based on the fact that AFP is a macromolecular protein in nature, it was anticipated that it could be coated directly on the PMMA beads in the proposed KinExA assay. Preliminary experiments indicated that the direct coating of AFP proceeded successfully and it did not affect its binding activity to its specific antibody. The parameters that could affect the assay performance were optimized. The optimum concentration of AFP required for coating onto the beads was 30 µg/ml. The most appropriate concentration of the primary anti–AFP was found to be 300 ng/ml. The optimum concentration of the secondary labeled anti–mouse IgG antibody was found to be 200 ng/ml. Volumes of the samples and secondary labeled antibody flowing over the solid-phase (beads coated with AFP) were found to be 500 µl, in both cases, at a flow rate of 0.25 ml/min. The KinExA data that have been generated under these conditions are shown in Fig. 2.

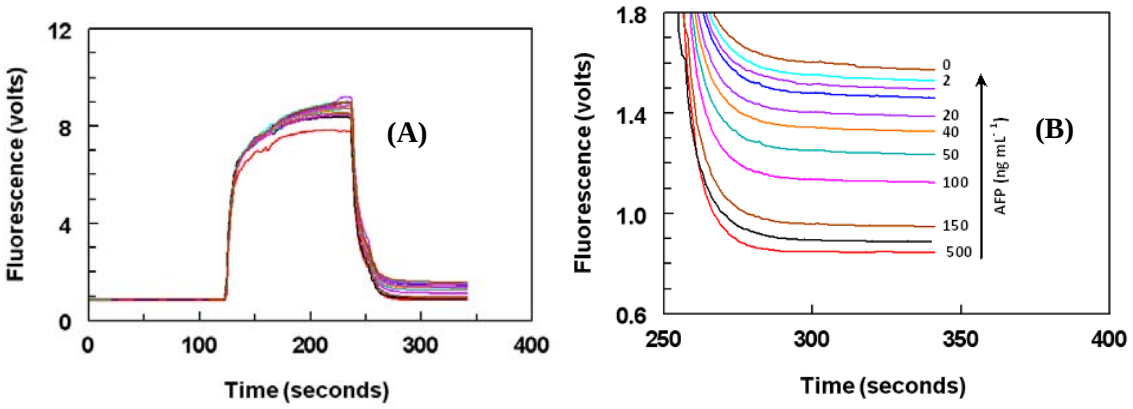

Fig. 2. (A): Real raw trend-line fluorescence responses obtained by the KinExA™ instrument for varying concentrations of AFP. (B): The last 85 sec of each curve from which the calibration curve was generated, were presented on different scales for the time and fluorescence.

3.4. Validation of the proposed KinExA-based sensor

3.4.1. Calibration curve and detection limit. The calibration curve for determination of AFP by the proposed sensor is shown in Fig. 3. The data showed good correlation coefficient (r = 0.9942) on the four-parameter curve fit. The limit of detection (LOD) of the proposed sensor was defined to be the AFP concentration that caused inhibition of 10% of the maximum signal (e.g. at 90% signal). LOD was found to be 8 ng/ml, and the working range of the assay at RSD ≤ 10% was 8–500 ng/ml.

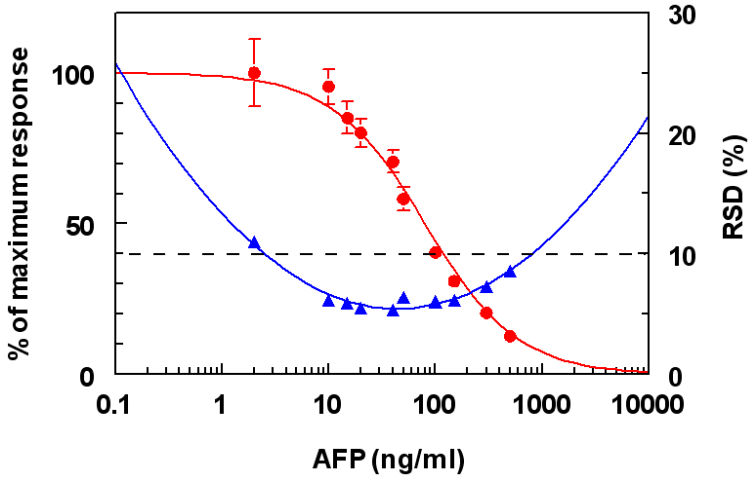

Fig. 3. Calibration curve (●) and precision profile (▲) of the proposed sensor.

3.4.2. Precision profile. The assay precision profile obtained from the results of calibration standard samples, assayed in duplicate, is also shown in Fig. 3. The RSD values were less than 10% throughout the entire working range of the assay. The intra- and inter-assay precisions were tested at three varying levels of AFP. The intra-assay precision was assessed by analyzing 3 replicates of each sample in a single run and the inter-assay precision was assessed by analyzing the same samples, as duplicates, in 3 separate runs. According to the recommendations of immunoassay validation [39], the assay gave satisfactory results; the RSD was 2.5–5.4 and 3.4–7.2% for the intra- and inter-assay precision, respectively (Table 1).

3.4.3. Accuracy and recovery studies. The accuracy of the proposed sensor and its applicability was assessed by the recovery study. Samples were prepared by spiking varying concentrations (20–400 ng/ml) of AFP into 3 batches of blank serum samples. The spiked samples were analyzed for their contents of AFP, as described in the Experimental Section. The analytical recovery was calculated as the ratio of the found AFP concentration to that of the spiked concentration, and the ratio was expressed as percentage. The analytical recovery values were 93.8–105.8% with RSD 2.8–8.1%. These recovery values, according to the guidelines for immunoassay validation [39], indicated the accuracy of the proposed method for determination of AFP in serum samples, and absence of endogenous interfering substances in the plasma samples.

Table 1. Precision of the proposed sensor and existing ELISA for measurement of AFP

AFP (ng/ml)	KinExA-based sensor		ELISA	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
20	18.6 ± 5.4[a]	21.5 ± 7.2	20.4 ± 6.2	19.5 ± 8.4
80	82.5 ± 4.8	77.8 ± 3.8	81.2 ± 5.4	78.6 ± 5.2
400	405.0 ± 2.5	412.4 ± 3.4	397.9 ± 5.8	403.8 ± 7.5

[a] Values are mean (in ng/ml) of 3 determinations ± relative standard deviation (RSD).

3.5. Comparison of the proposed KinExA-based sensor with ELISA

In order to compare the proposed KinExA-based sensor with the commercially available validated ELISA kit [40], serum samples were spiked with AFP at known concentrations and were analyzed by both methods. The analysis by KinExA was performed as described in the Experimental Section, and the analysis by ELISA was performed according to the instructions of the kit manufacturer [40]. The concentrations measured by ELISA were plotted versus their corresponding values that have been measured by the proposed sensor. Regression analysis of the results was performed, and the results revealed the good agreement between the accuracy of the two methods, as indicated from the good correlation coefficient (r) of the regression equation:

KinExA = 4.119 + 1.068 ELISA (r = 0.9935). As well, the precisions of the two assays were compared at the same AFP concentration levels (Table 1). The results indicated that KinExA-based sensor had better precision (lower RSD values) than ELISA (Table 1). The precision in ELISA depends mainly upon the uniformity in the quantity of the coated reagent from well to well in a microwell plate. Any interference in this uniformity could arise from the experimental manipulations lead to higher imprecision. However, the use of capturing beads with higher surface area in the KinExA assay [34] made the assay precision dependent only on the concentrations of the primary and secondary-labeled antibodies and maximizes the opportunities for the capture of more free antibodies [41]. These reagents were dispended automatically with high precision by the KinExA instrument. This led to an improved quantifiable response with low signal-to-noise characteristics and better precision of the proposed KinExA-based sensor than ELISA. Besides, the proposed KinExA-based sensor exhibited three noteworthy advantages over ELISA: (1) avoiding the problems of mass transport limitations, and mobility effects, (2) KinExA analysis with automated sampling increase the assay throughput and convenience; and (3) providing higher level of precision than ELISA.

4. Conclusions

An automated flow immunosensor employing the KinExA format has been developed and validated for the measurement of the cancer marker AFP in serum at concentrations as low as 8 ng/ml. The developed sensor was comparable with the existing validated ELISA, in terms of the accuracy. In addition, the proposed KinExA assay format exhibited three noteworthy properties compared with ELISA: (1) avoiding the problems of mass transport limitations, and mobility effects, (2) KinExA analysis with automated sampling increase the assay throughput and convenience; and (3) providing high sensitivity with a lower limit of detection and better precision than the existing ELISA. The proposed sensor is anticipated to have a great value in measurement of AFP where a more confident result is needed; it would be very beneficial in making proper medical decisions on the patient status. This information may ultimately limit the mortality rate among cancer patients.

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