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Kinetic-exclusion analysis-based immunosensors versus enzyme-linked immunosorbent assays for measurement of cancer markers in biological specimens

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ABSTRACT

Kinetic exclusion analysis (KinExA)-based immunosensors and enzyme-linked immunosorbent assays (ELISA) have been developed and validated for measurement of five different cancer markers in biological specimens. These markers were: 2′-deoxycytidine (dCyd), 8-hydroxy-2′-deoxyguanosine (8HdG), carbohydrate antigen (CA15-3), α-fetoprotein (AFP), and β-subunit of human chorionic gonadotropin (β-HCG). The KinExA-based assays were conducted on the KinExA™ 3200 instrument. The ELISA assays employed the competitive immunoassay format for dCyd and 8HdG, however they employed the direct sandwich-type format for CA15-3, AFP, and β-HCG. Each assay was validated in terms of its limit of detection, working range, precision profile, and accuracy. The analytical performances of the KinExA-based sensors were found to be superior to the ELISA for the five markers. The data demonstrated that the format of the assay may influence its performance characteristics (sensitivity, precision, etc.), even when exactly the same reagents are employed. The superior performance of the KinExA format is most likely due to: (1) the high surface area of beads containing the immobilized capture in the flow cell of the instrument, (2) the high flow rate of the reagents passing through the beads, which minimizes the diffusion limitations at the reaction surface, and (3) the limited time that the antibody is in contact with the capture reagent. The KinExA-based assays 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 convenience; and (3) providing high sensitivity with a lower limit of detection and better precision than ELISA. The proposed KinExA-based immunosensors are anticipated to have a great value in measurement of the cancer markers where more confident results are needed.

1. Introduction

Cancer is the principal cause of mortality among men and women worldwide [1]. Recent studies revealed that cancer has become an ever-increasing problem [2–4]. The high rate of mortality in cancer patients is attributed to the late diagnosis of the disease, and consequently delayed initiation of the medical treatment by surgery, radiotherapy and/or chemotherapy [5]. Many research studies have been conducted with the general goal of identifying some unique metabolic product or unusual component(s) of malignant cells which can be measured in patient's biological fluids, by appropriate analytical techniques. These components are termed as "cancer markers". These markers could be useful for early detection of the disease state, confirmation of a diagnosis, or monitoring the progress of the disease and the patient's response to therapy. Cancer markers have become very important in providing useful information such as possible cancer type and the stage of patient's progression at a very early time [6–8].

Many authors established many cancer markers of diverse chemical structures. These markers include: modified nucleosides [9], damaged DNA (e.g. 8-hydroxy-2′-deoxy-guanosine [10], proteins (e.g. α-fetoprotein), enzymes (e.g. lactate dehydrogenase, neuron specific enolase, placental alkaline phosphatase, and prostatic acid phosphatase), carbohydrates (e.g. carbohydrate antigen 15-3), and hormonal compounds (e.g. mycoglobulin, human chorionic gonadotropin, and calcitonin) [11]. The analytical technologies that have been used for measuring cancer markers are mostly chromatography [12,13], and to lesser extent immunoassays [14,15]. In general, immunoassays have more importance in measuring cancer markers than the chromatographic methods.

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This importance is attributed to their specificity, high-throughput, and high sensitivity for the analyses of wide range of markers, irrespective the diversity in chemical natures, in complex biological matrices. Furthermore, immunoassays are more compatible with the clinical laboratory setting than chromatography as they do not require pre-treatment of the sample or sophisticated expensive analytical equipment [16]. The most versatile and most commonly used immunoassays are the enzyme-linked immunosorbent assays (ELISA). Many ELISA-based assays have been reported for measuring cancer markers in biological specimens [17–19]. However, in some instances relatively low sensitive assays are created. The inadequate sensitivity for analysis, and/or or long analysis time limit the routine application of ELISA in the clinical analysis of cancer markers.

Immunosensors represent the most technological progress in the field of biochemical and clinical analysis [20]. Immunosensors that involve kinetic exclusion analysis (KinExA) are the most advanced immunosensors. The basic features and operation of these sensors were described by Blake et al. [21]. KinExA-based immunosensors offered many advantages over ELISA-based assays. These advantages include: (1) being rely on a continuous-flow analysis, they are devoid from of mass transport limitation problems encountered in ELISA, (2) avoid the effect of analyte modification on the analytical results as the sensors measure chemically-unmodified analytes in solution, (3) the analysis by KinExA can be used for measuring of wide range of molecules of varying kinetic bindings, (4) the continuous-flow analysis after short time pre-equilibration reduces the total analysis time, and the simplicity of the KinExA operation increases the analysis convenience. These advantages made KinExA-based sensors efficient unique analytical technique.

In the present study, we briefly delineate two different immunoassay formats (KinExA-based immunosensors and ELISA) developed in our laboratory using identical reagents, and compare the analytical performance of both assay formats for five different cancer markers. These markers were: 2′-deoxycytidine (dCyd), 8-hydroxy-2′-deoxyguanosine (8HdG), carbohydrate antigen (CA15-3), α-fetoprotein (AFP), and β-subunit of human chorionic gonadotropin (β-HCG).

2. Experimental

2.1. Instruments

KinExA™ 3200 instrument (Sapidyne Instruments, Inc., Boise, ID, USA) empowered by KinExA Pro 20.0.1.26 software provided with the instrument. Microplate reader (ELx808, Bio-Tek Instruments Inc., Winooski, USA) empowered by KC Junior software, provided with the instrument. FLX500 microplate washer (Bio-Tek Instruments Inc., Winooski, USA). Spectrophotometer (UV-1601 PC, Shimadzu, Kyoto, Japan) double beam with matched 1-cm quartz cells. Sanyo TSE incubator (Sanyo Co Ltd., Japan). Nutating mixer (Taitec, Saitama-ken, Japan). Biofuge Pico centrifuge (Heraeus Instruments, Hanau, Germany). Nanopure II water purification system (Dubuque, IA, USA) was used to make all the solutions described in this study.

2.2. Materials

2′-Deoxycytidine (dCyd), 8-hydroxy-2′-deoxyguanosine (8HdG), α-fetoprotein (AFP), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), bovine serum albumin (BSA), 2,4,6-trinitrobenzene sulfonic acid, horseradish peroxidase labeled goat anti-mouse IgG (HRP-IgG), 3,3′,5,5′-Tetramethylbenzidine (TMB) peroxidase substrate for ELISA were obtained from Sigma Chemical Co. (St. Louis. MO, USA). Highly purified CA15-3 was a product of Biospecific (Emeryville, CA, USA). Anti-CA15-3 mouse monoclonal antibody was obtained from Cal-Bioreagents (San Mateo, CA, USA). Highly purified β subunit of HCG (β-HCG), anti-β-HCG mouse monoclonal antibody, anti-AFP monoclonal antibody was obtained from MyBiosource, LLC (San Diego, CA, USA). Anti-8HdG mouse monoclonal antibody was obtained from Trevigen (Helgerman Court, Gaithersburg, MD, USA). Anti-dCyd monoclonal antibody was available from a previous study [22]. 8-Hydroxyguanosine (8HG) was obtained from Cayman Chemical (Ann Arbor, MI, USA). Polymethylmethacrylate (PMMA) beads (140–170 mesh, 98 μm) 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). AFP ELISA kit was obtained from Calbiotech Inc. (Spring Vally, CA, USA). CA15-3 ELISA kit was obtained from Calbiotech Inc. (Spring Vally, CA, USA). Free β-HCG enzyme immunoassay kit was obtained from BioCheck Inc. (Foster City, CA, USA). BCA reagent for protein assay was obtained from Pierce Chemical Co. (Rockford, IL, USA). ELISA high-binding microwell plates were a product of Corning/Costar, Inc. (Cambridge, MA, USA). Human serum and urine samples were collected from normal healthy volunteers at King Khalid University Hospital (Riyadh, Saudi Arabia), and were kept frozen at −20 °C until analysis. Phosphate buffer saline (PBS) was obtained from Bio-Basic Inc. (Markham, Canada). All other materials were of analytical grade.

2.3. Procedures

2.3.1. Preparation of BSA conjugates for dCyd and 8HdG

Bovine serum albumin (BSA) conjugate of dCyd (dCyd-BSA) was prepared by the procedures previously described by Darwish et al. [14]. Briefly, dCyd was first activated by formation of its hemisuccinate derivatives by its mixing with succinic anhydride in triethylamine: dioxane mixture. The monosuccinyl (3′- and 5′-) and disuccinyl (3′,5′-) were separated from each other by liquid chromatography and their structures were confirmed by ^{1}H NMR spectroscopy. 5′-Succinyl derivative of dCyd was conjugated with BSA by EDC reagent.

BSA conjugate for 8HdG was prepared by conjugating 8-hydroxyguanosine (8HG) with BSA according to a reported procedure [23]. Briefly, 5 mg of 8HG was dissolved in 1 mL of 50 mM sodium metaperiodate and the mixture was incubated for 1 h in the dark. The reaction was stopped by adding 2.5 μL of ethylene glycol. The mixture was mixed with 2 mL of BSA solution (25 g/ in 50 g/L K_2CO_3, pH 9.5), and incubated for 1 h. After adding 2 mL of $NaBH_4$ solution (24 g/L), the reaction mixture was incubated at 4 °C overnight, and the reaction mixture was collected.

The remaining unconjugated dCyd and 8HG residues were removed from their corresponding BSA conjugates by dialyzing the reaction mixtures against PBS. Protein concentration of the conjugates were determined using BCA reagent and the extent of substitution of free amino groups on the BSA protein was determined by estimating the free amino groups on both unreacted BSA and on the protein that has been subjected to the conjugation, by the procedures described by Habeeb [24]. The percentages of dCyd and 8HG residues in their corresponding BSA conjugates were found to be 45.60 and 32.54% for dCyd and 8HG, respectively.

2.3.2. Coating of PMMA beads with cancer markers

PMMA bead vials containing 200 mg (dry weight) of beads were coated with cancer markers. AFP, CA15-3, and β-HCG were coated directly onto the beads, however dCyd and 8HdG were coated via their corresponding BSA conjugates. The coating solutions consisted of 1 mL of PBS containing 580 international units (IU) for CA15-3, and 32.5, 5, 30 and 30 μg mL^{-1} for dCyd-BSA, 8HG-BSA, AFP, and β-

HCG, respectively. 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 were allowed to settle. The supernatant was aspirated from the coating solution and 1 mL of blocking solution (10 mg mL^{-1} 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. The coated and blocked beads could be stored for one week without any noticeable deterioration.

2.3.3. Preparation of DyLight™ 649-conjugated secondary antibody

Secondary antibody working solutions of DyLight™ 649-conjugated goat anti-mouse IgG was prepared by dilution of the stock solution (1.5 mg mL^{-1}) in standard KinExA sample buffer (PBS, 0.02% sodium azide, pH 7.4 with 1 mg mL^{-1} of BSA). The concentrations of these working solutions were 100, 250, 200, 150, and 150 ng mL^{-1} for dCyd, 8HdG, AFP, CA15-3, and β-HCG, respectively. The solutions were prepared fresh at the start of each experiment.

2.3.4. Preparation of samples for analysis by KinExA-based immunosensors

The calibration standard samples were prepared by spiking the standard cancer markers in blank biological specimens collected from healthy volunteers. These samples were plasma, urine serum, and serum for dCyd, 8HdG, AFP, and β-HCG, respectively. The standards were spiked to give concentrations of 2–4400 nM, 0.01–20 ng mL^{-1}, 0.02–77 IU mL^{-1}, 2–500 ng mL^{-1}, and 0.2–200 ng mL^{-1} for dCyd, 8HdG, CA15-3, AFP, and β-HCG, respectively. Each spiked sample was mixed with an equal volume of its corresponding specific primary antibody solution. These solutions were 100, 150, 30, 300, and 4 ng mL^{-1} for dCyd, 8HdG, CA15-3, AFP, and β-HCG, respectively. The solutions were prepared in PBS containing 1 mg mL^{-1} BSA); BSA was added to reduce any subsequent non-specific binding of the primary antibody to the microbeads in the instrument micro-column. The samples were pre-equilibrated by incubation at room temperature for 2 h in case of dCyd, 8HdG, and AFP, 1 h in case of CA15-3, and 30 min in case of β-HCG. Pre-equilibration time was studied by incubating the cancer marker (antigen) with its specific antibody for varying times (0.5–2 h) and the mixtures were subjected to the analysis every 0.5 h so as to attain the best equilibration time for each individual marker. After achieving equilibrium, samples were analyzed by the KinExA instrument.

2.3.5. Analysis by KinExA instrument

Each of 12 sample lines (of total 14 lines) of the KinExA™ 3200 instrument was placed into a sample tube containing the pre-equilibrated mixture of antibody and antigen (cancer marker and its specific antibody). The 13th line was placed into a tube containing the blank (zero concentration of cancer marker and the primary antibody), and the 14th line was placed into a tube that contained the fluorescently labeled goat anti-mouse IgG secondary antibody solution. Analyte-coated beads (200 mg) were diluted to 30 mL with PBS, placed in a bead bottle and loaded into the instrument. All subsequent steps in the assay were performed automatically by the KinExA instrument. Beads were first automatically packed into the capillary flow/observation cell of the instrument. The system was charged twice to produce a bead column of the appropriate height. A camera that monitored the flow/observation cell assisted with this procedure.

In the final assay, aliquots of the beads suspension (in PBS) was drawn over the flow cell of the instrument. These aliquots were 583, 917, 667, 683, and 750 μL for dCyd, 8HdG, CA15-3, AFP, and β-HCG, respectively. The aliquots were drawn at a flow rate of 1 mL min^{-1} for 35, 55, 40, 41, and 45 s dCyd, 8HdG, CA15–3, AFP, and β-HCG, respectively. These conditions produced uniform and reproducible packs for of the coated beads. Aliquots (450 μL for CA15-3 and 500 μL for the other markers) of each equilibrated sample solution was then withdrawn and passed over the micro-column at a rate of 0.25 mL min^{-1}; the time taken was 108 s for CA15–3 and 120 s for the other markers. An automatic buffer wash (333 μL of the PBS) removed unbound primary antibodies and excess soluble cancer marker molecules from the bead pack. Fluorescently labeled goat anti-mouse IgG secondary antibody solutions were drawn past the beads, and unbound labeled secondary antibodies were subsequently removed by drawing 1.5 mL of PBS through the bead-pack over a period of 90 s at a flow rate of 1 mL min^{-1}. 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 bead pack was used for each run. The data were collected by KinExA Pro 20.0.1.26 software provided with the KinExA instrument, 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:

$$F = F_0 - \{(F_0 - F_1)[M]/(IC_{50} + [M])\}$$

where F is the fluorescence signal at a definite known marker concentration $[M]$, F_0 is the fluorescence signal at zero concentration of the marker, F_1 is the fluorescence signal at the saturating concentration of the marker, and IC_{50} is the marker concentration that produces a 50% inhibition of the signal. The concentrations of each marker in its samples were obtained by interpolation on the corresponding standard curve.

2.3.6. Analysis by ELISA

For analysis of dCyd and 8HdG, competitive ELISA procedures were developed in our laboratory. Microwells of the ELISA plates were coated with dCyd-BSA and 8HG-BSA for analysis of dCyd and 8HdG, respectively. Coating was carried out by dispensing 50 μL of each conjugate solution into each well. The conjugate solutions were prepared in PBS at 5 and 2 μg mL^{-1} for dCyd-BSA and 8HG-BSA, respectively. The plates were incubated for 2 h at 37 °C. The plates were washed with PBS containing 0.05% Tween-20 (PBS-T) using microplate washer, and the wells were blocked with 200 μL of 1% BSA in PBS by incubation at room temperature for 1 h. The microplate was washed once with washing buffer just prior to assay. Aliquots (50 μL) of dCyd and 8HdG samples were mixed with equal volumes of their corresponding antibodies (1 μg mL^{-1}, in PBS for both dCyd and 8HdG) and allowed for competitive binding reaction by incubation for 2 h at 37 °C. The plates were washed with PBS-T, and 50 μL of HRP conjugate of secondary goat anti-mouse IgG (HRP-IgG, 1:2,000 in PBS) was added to each well. After incubation for 1 h at 37 °C, the plates were washed with PBS-T and 50 μL of TMB substrate solution was added and the reaction was allowed to proceed for 10 min at 37 °C for color development. The absorbance in each well was measured at 630 nm by the microplate reader. The data were acquisitioned by KC Junior software and transformed to a four-parameter curve fitting using Slide Write software, version 5.011. Values for IC_{50} were those that gave the best fit to the following equation:

$$A = A_0 - \{(A_0 - A_1)[M]/(IC_{50} + [M])\}$$

Where A is the signal at a definite known concentration $[M]$ of each of dCyd and 8HdG, A_0 is the signal at zero concentration of the analyte (dCyd and 8HdG), A_1 is the signal at the saturating concentration, and IC_{50} is the concentration that produces a 50% inhibition of the signal. The standard calibration curves were generated by fitting the data

obtained by analyzing standard concentrations of dCyd and 8HdG in the range of 8.5–4400 and 2–8000 ng mL^{-1}, respectively. The concentrations of dCyd and 8HdG in their samples were then obtained by interpolation on the standard curves.

Analysis of AFP [25], CA15-3 [26], and β-HCG [27] were performed using their commercially available ELISA kits according to the instructions of the manufactures. Both assays were based on the direct solid phase sandwich ELISA. The assay systems utilized unique monoclonal antibodies directed against a distinct antigenic determinant on its corresponding analyte. Mouse monoclonal antibodies were used for solid phase immobilization (on the microwells of the assay plates). Goat anti-analyte antibodies conjugated with horseradish peroxidase (HRP) enzyme were used as second antibodies. The test sample was allowed to react sequentially with the two antibodies, resulting in the free analyte molecules being sandwiched between the solid phase and enzyme-linked antibodies. After two separate incubations, the wells were washed to remove unbound labeled antibodies. A solution of TMB reagent was added and incubated, resulting in the development of a blue color. The color development was stopped with the addition of stop solution changing the color to yellow. Absorbances were measured by plate reader at 450 nm. The concentrations of each analyte were directly proportional to the color intensity in the assay wells.

3. Results and discussion

3.1. Selected cancer markers and their clinical significance

Details of the KinExA instrument and assays procedures have been described elsewhere [21,41,42]. For development of KinExA-based sensors that would have universal applications in measuring cancer markers irrespective their chemical entity, cancer markers of diverse chemical nature were selected for the study described herein. These markers were dCyd (modified nucleoside), 8HdG (damaged DNA), AFP (serum protein), CA15-3 (carbohydrate antigen), and β-HCG (hormonal peptide). The clinical significances of these markers are outlined in the following paragraph.

The plasma level of dCyd was suggested as a marker for monitoring the prognosis of breast cancer patients treated with combined chemotherapeutic agents: cyclophosphamide, methotrexate and 5-fluorouracil [6]. The urinary level of 8HdG has been proposed to be indicative for DNA damage associated with various cancers [28]. 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 [29–31]. On the other hand it has been found that the AFP level in serum is associated with tumor cell growth [32]. CA15-3 is a transmembrane carbohydrate antigen belonging to large family of glycoproteins encoded by the MUC1 gene that are heterogeneously expressed on the apical surface of normal epithelial cell types, including those of the breast. CA15-3 is elevated in a proportion of BC patients with distant metastases [33]. Elevated serum levels of CA15-3 is used to anticipate detection of recurrences in patients with breast cancer, and provides an additional tool in evaluating therapeutic response in patients with advanced disease [34]. Pre-operative levels of CA15-3 have a significant and independent relation to outcome in patients with early breast cancer [35]. Patients with high serum CA15-3 concentrations have a significantly worse prognosis than those with low concentrations, both in terms of disease-free survival and overall survival, probably due to a larger burden of occult disease. CA15-3 measured during follow-up has been consistently shown to predict liver and bone metastases [36]. Human chorionic gonadotropin (HCG) is a glycoprotein hormone produced at very high concentrations by placental trophoblasts during pregnancy. The hormone is present in blood and urine around 7 to 13 days following implantation of the fertilized ovum. Structurally intact HCG molecules consist of two non-covalently linked polypeptide subunits; α- and β-subunits. In normal second-trimester maternal sera, the level of intact HCG range from 20,000 to 50,000 mIU mL^{-1} (1 ng = 15 mIU). Measurement of intact HCG is useful marker for pregnancy. The β subunit of HCG (β-HCG) is always expressed in placental and other trophoblastic tumors [37]. Elevated levels of β-HCG in serum is a strong diagnostic and prognostic marker for trophoblastic tumors of placental and germ cell origin [38]. As well, monitoring of choriocarcinoma therapy is mainly based on β-HCG assay [39]. American Society of Clinical Oncology Clinical Practice Guideline on uses of serum tumor markers in adult males with germ cell tumors panel recommends measuring β-HCG before and after orchiectomy and before chemotherapy for those with extragonadal non-seminomas. They also recommended measuring β-HCG shortly before retroperitoneal lymph node dissection and at the start of each chemotherapy cycle for nonseminoma, and periodically to monitor for relapse [40].

3.2. Optimization of assay conditions for KinExA-based immunosensor

Based on the fact that CA15-3 [43], AFP [44,45], and β-HCG [46] are macromolecular protein in nature molecules, it was anticipated that they could be coated directly on the PMMA beads in the proposed KinExA assay [42]. However, dCyd and 8HdG are small molecules that could not be bound directly to the PMMA beads. Therefore, BSA conjugates for these two markers were prepared and the conjugates were coated onto the PMMA beads.

The assay conditions were optimized; these conditions were: concentration of reagents required for coating onto the beads, concentration of primary antibodies, antigen-antibody pre-equilibration time, concentrations of fluorescent-labeled secondary antibody, its volume, and passing flow rate. Optimization for the antigen-antibody pre-equilibration time was studied by incubation the cancer marker (antigen) with its specific antibody for varying times (0.5–2 h) and the mixtures were subjected to the analysis every 0.5 h so as to attain the best equilibration time for each individual marker. A summary for the optimum conditions for analysis of each particular marker is given in Table 1.

3.3. Validation of the KinExA assays

3.3.1. Calibration curves and detection limits

The calibration curves for determination of the investigated cancer markers by the proposed KinExA-based immunosensors were generated using varying concentrations of each marker (Table 2). The data showed good correlation coefficients ($r = 0.9942–0.9993$) on the four-parameter curve fit. The calibration curve generated for dCyd, as a representative example, is shown in Fig. 1A, and similar curves were obtained for the other cancer markers. The limit of detection (LOD) of the proposed sensor was defined to be the concentration that caused inhibition of 10% of the maximum signal (e.g. at 90% signal). Based on the basis of duplicate measurements, the limits of detection in the assays were found to be 20 nM, 0.05 ng mL^{-1}, 0.2 IU mL^{-1}, 8 ng mL^{-1} and 0.2 ng mL^{-1} for dCyd, 8HdG, CA15-3, AFP, and β-HCG, respectively. These high sensitivities enabled the determination of low concentrations of each marker in its corresponding biological specimens as the normal levels of all the markers are usually higher than the achieved LOD values (Table 2).

Table 1
Optimum conditions for KinExA-based immunosensors for measuring cancer markers.

Marker	Concentration of capturing reagent coated onto PMMA beads (μg mL^{-1})[a]	Volume of beads suspension (μL)/time of drawing (s)	Primary anti-marker antibody (ng mL^{-1})	Secondary fluorescent-labeled antibody (ng mL^{-1})	Volume of sample (μL)/ time of drawing (s)[b]
dCyd	32.5	583/35	100	100	500/120
8HdG	5	917/55	150	250	500/120
CA15-3	580	667/40	30	150	450/108
AFP	30	683/41	300	200	500/120
β-HCG	30	750/45	4	150	500/120

[a] The concentration of CA15-3 was in international unit (IU) mL^{-1}.
[b] Samples were withdrawn at a flow rate of 0.25 mL min^{-1} for all cancer markers.

Table 2
Assay parameters and analytical performances for KinExA-based immunosensors for measuring cancer markers.

Parameter/analytical performance	dCyd	8HdG	CA15-3	AFP	β-HCG
Experimental range[a]	2–4400	0.01–20	0.02–77	2–500	0.2–200
Correlation coefficient	0.9966	0.9952	0.9990	0.9942	0.9993
Working range[a]	20–2200	0.05–10	0.2–77	8–500	0.2–50
Limit of detection[a]	20	0.05	0.2	8	0.2
Normal level[b]	0.4–2.9		< 30	< 25	< 60
Accuracy (%)	98.4–107.4	96.1–100.6	90.7–108.6	93.8–105.8	96.9–105.4
Accuracy (RSD)[c]	2.48–8.40	3.0–6.4	2.05–7.45	2.8–8.1	3.54–6.84
Intra-assay (RSD)	3.6–6.2	3.28–4.89	3.8–5.1	2.5–5.4	3.28–4.89
Inter-assay (RSD)	5.2–7.5	4.40–6.89	5.2–7.2	3.4–7.2	4.4–6.89

[a] Experimental range is the range of concentrations used for generating the calibration curve. Concentrations were in nM for dCyd, IU mL^{-1} for CA15-3, and in ng mL^{-1} for the other markers.
[b] Concentrations were in μM for dCyd, IU mL^{-1} for CA15-3, and in ng mL^{-1} for the other markers.
[c] RSD is the relative standard deviation.

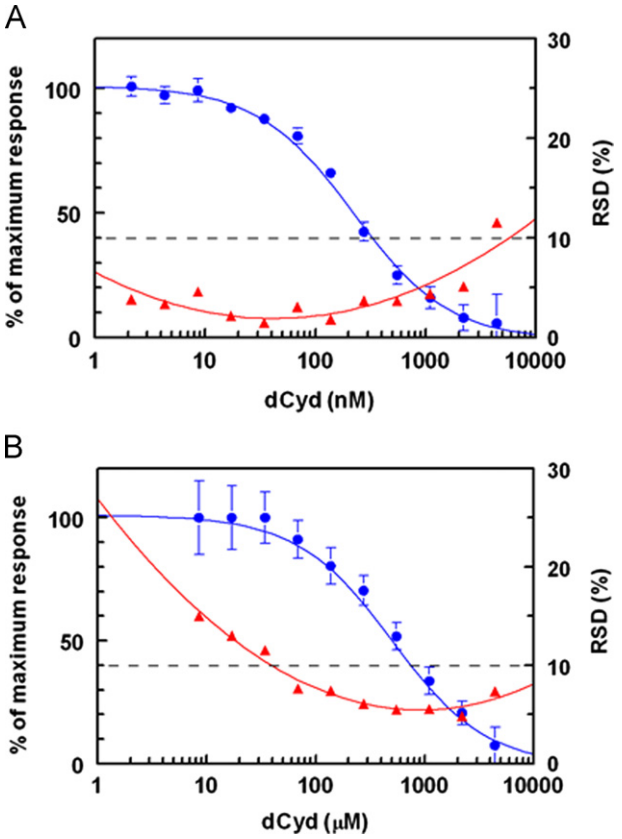

Fig. 1. Calibration curve (●) and precision profile (▲) of the proposed KinExA-based sensor (A) and ELISA (B) for measurement of dCyd.

3.3.2. *Precision profiles*

The precision profiles obtained from the results of the calibration standard samples, assayed in duplicate, were assessed throughout the entire range of each assay. The relative standard deviation (RSD) was used as a measure. The practical working range of each assay was defined as the concentration range that gives RSD values ≤10%. These ranges are given in Table 2. The intra- and inter-assay precisions were tested at three varying concentration levels (low, medium, and high) of each marker. The intra-assay precision was assessed by analyzing three 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 [47], the assays gave satisfactory results; the RSD values were less than 10% for both intra- and inter-assay precisions (Table 2).

3.3.3. *Accuracy and applications*

The accuracy of each assay and its applicability was assessed by the recovery study. Samples were prepared by spiking varying concentrations (throughout the working range) of each assay into three batches of blank biological specimens. The spiked samples were analyzed for their contents of markers, as described in the Experimental Section. The analytical recovery was calculated as the ratio of the found marker concentration to that of the spiked concentration, and the ratio was expressed as percentage. The analytical recovery values were in the range of 90.70 to 108.6% with RSD 2.05 to 8.40% (Table 2). These recovery values, according to the guidelines for immunoassay validation [47], indicated the accuracy of each assay for determination of the corresponding marker in its samples, and absence of endogenous interfering substances in the samples.

3.4. Analytical performance of ELISA assays

For dCyd and 8HdG, competitive ELISA systems were optimized using the same reagents used in developing the KinExA assays (antibodies and BSA conjugate). The most efficient and commonly used ELISA for detection of CA15-3, AFP, and β-HCG are the sandwich type immunoassay by commercial kits. The optimum conditions required to achieve the highest possible sensitivity were given in the Experimental Section. Under these conditions, calibration curves were generated and each assay was validated in terms of its LOD, working range, accuracy, and precision. The analytical performance data for the ELISA assays are given in Table 3.

3.5. Comparison of the KinExA-based immunosensor with ELISA

The calculated limits of detection achieved by ELISA-based assays were found to be 50 μM, 30 ng mL^{-1}, 4 IU mL^{-1}, 4 ng mL^{-1}, and 0.5 ng mL^{-1} for dCyd, 8HdG, CA15-3, AFP, and β-HCG, respectively (Table 3). These values were compared with those of KinExA-based assays: 20 nM, 0.05 ng mL^{-1}, 0.2 IU mL^{-1}, 8 ng mL^{-1}, and 0.2 ng mL^{-1} for dCyd, 8HdG, CA15-3, AFP, and β-HCG, respectively (Table 2). It is obvious that KinExA-based assays have higher sensitivities (lower detection limits). The higher sensitivity of the KinExA-based assays is attributed to the inherent high sensitivity of the competitive immunoassays. In addition, the antibodies and their free antigens (markers) were incubated and allowed to approach binding equilibrium in solution before subsequent exposure of the mixtures to the markers immobilized on the surface of the beads. The time of exposure of each equilibrium mixture to the immobilized capture reagent was kept sufficiently short to insure that negligible dissociation of the free antigen–antibody complexes occurred during the swift passage of the mixture through the beads in the observation cell. Consequently, the immobilized antigen served merely as a tool to separate and quantify only those antibodies in the equilibrium mixture that bore unoccupied binding sites.

Quantifiable and reproducible instrument responses were achieved in the KinExA format by using beads (approx. 10,000/column) with a higher surface to maximize the opportunities for the capture of free antibody area (surface area in KinExA is approx. 260 mm^2 compared to the 64 mm^2 calculated for each microwell in the ELISA format [41]. In addition, the high flow rate of the reagent through the beads minimizes mass transport limitations at the reaction surface, which is encountered problem in ELISA [48,49].

Table 3
Assay parameters and analytical performances for ELISA-based assays for measuring cancer markers.

Parameter/analytical performance	Competitive ELISA		Sandwich ELISA		
	dCyd	8HdG	CA15-3	AFP	β-HCG
Experimental range[a]	8.5–4400	2–8000	1.0–240	2–500	1–100
Correlation coefficient	0.9925	0.9904	0.9953	0.9916	0.9974
Working range[a]	70–4400	25–4000	4.0–2400	4.0–400	0.4–100
Limit of detection[b]	50 (20)	30 (0.05)	4 (0.2)	8 (4)	0.5 (0.2)
Relative sensitivity[c]	2500	600	20	2	2

[a] Experimental range is the range of concentrations used for generating the calibration curve. Concentrations were in μM for dCyd, IU mL^{-1} for CA15-3, and in ng mL^{-1} for the other markers.

[b] Figures in parentheses are the LOD achieved by KinExA-based immunosensors.

[c] Relative sensitivity is the LOD achieved by LOD achieved by ELISA/KinExA-based assay.

The precision profiles of both assay-formats were compared and it was found that KinExa-based assays gave higher precision than ELISA did. This could be explained on the basis that 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 (e.g. imprecise dispensing for the solutions, fluctuations in temperature etc) lead to higher imprecision. However, the use of capturing beads with higher surface area in the KinExA assay 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. 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.

It is well known that ELISA involves multiple steps during the processing of samples; however, in case of KinExA processing of samples is done once at the start of analysis. Also with the use of autosampler when it is available, the throughput of KinExA can be increased. The protocol of the conventional multiple-steps ELISA take ~4–5 h for the analysis and generating the data irrespective of the number of samples; however, in KinExA the total time of analysis depends on the number of samples for analysis. Also there are no multiple step procedures to be undertaken as the system is automated. The limited number of sampling lines in KinExA (13 lines) can be overcome by use of autosampler.

Besides, the proposed KinExA-based immunosensors 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 levels of sensitivity and precision than ELISA. These data demonstrate that the format of the assay may influence its performance characteristics (sensitivity, precision, etc.), even when exactly the same reagents are employed.

4. Conclusions

KinExA-based immunosensors have been developed and validated for the measurement of five different cancer markers (dCyd, 8HdG, CA15-3, AFP, and β-HCG) in biological specimens. The developed sensors were compared with the conventional ELISA. The data demonstrated that the format of the assay may influence its performance characteristics (sensitivity, precision, etc.), even when exactly the same reagents are employed. The superior performance of the KinExA format is most likely due to: (1) the high surface area of beads containing the immobilized capture in the flow cell of the instrument, (2) the high flow rate of the reagent through the beads, which minimizes the diffusion limitations at the reaction surface, and (3) the limited time that the antibody is in contact with the capture reagent.

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 ELISA. The proposed sensors are anticipated to have a great value in measurement of the cancer markers where more confident results are needed.

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