Digest Journal of Nanomaterials and Biostructures Vol. 8, No. 1, January - March 2013, p. 69 - 79

NOVEL AUTOMATED FLOW-BASED IMMUNOSENSOR FOR MEASUREMENT OF THE BREAST CANCER PROGNOSTIC MARKER 2`-DEOXYCYTIDINE IN PLASMA

I.A. DARWISH*, A.M. ALANAZI, T.A. WANI, N.Y. KHALIL, M.A. HAMIDADDIN

Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

A novel automated flow-based immunosensor has been developed and validated for the measurement of the breast cancer prognostic marker 2`-deoxycytidine (dCyd) in plasma. The sensor employed the kinetic-exclusion analytical technology using the KinExA™ 3200 instrument. Various concentrations of dCyd were incubated with a fixed amount of mouse anti-dCyd monoclonal antibody until binding reaction reached equilibrium. These solutions were then passed rapidly over dCyd-bovine serum albumin conjugate (dCyd-BSA) coated onto polymethylmethacrylate beads contained in the observation cell of the KinExA instrument. The free anti-dCyd antibody was bound to the immobilized dCyd-BSA, however the unbound reagents were removed from the beads bed by flushing the system with phosphate-buffered saline. Fluorescent-labeled secondary antibody was passed rapidly over the beads bed, and the fluorescence was recorded during the flow of the secondary antibody through the beads. The calibration curve was generated by plotting the fluorescence signals that were retained on the beads as a function of dCyd concentrations. The assay limit of detection was 20nM, and the working range of the assay was 20—2200 nM. The analytical recovery of plasma-spiked dCyd was 94.8–107.4 ± 2.5–8.4%. The precision of the sensor was satisfactory; RSD was 3.6–6.2 and 5.2–7.5% for the intra- and inter-assay precision, respectively. The analytical performance of the proposed sensor was found to be superior to the conventional enzyme-linked immunosorbent assay for dCyd. The proposed sensor is anticipated to have a great value in measurement of dCyd where a more confident result is needed.

(Received November 2, 2012; Accepted December 18, 2012)

Keywords: Breast cancer; Prognostic markers; 2`-Deoxycytidine; Immunosensor; Kinetic Exclusion Analysis; Enzyme-linked immunosorbent assay

1. Introduction

Breast cancer (BC) is the first leading cause of deaths among women, accounting for approximately 15% of all cancer-related deaths. According to new reports, the number of BC patients may increase substantially [1-3]. Besides the crucial problem of BC incidence, the mortality rate among the patients is also high. The high rate of mortality in BC patients is attributed to the late diagnosis of the disease, and consequently the delayed initiation of the medical treatment by surgery, radiotherapy and/or mostly chemotherapy. Besides, the delayed initiation of the treatment program is usually associated with poor prognosis [4-9]. Early detection and proper monitoring for the prognosis of BC is the urgent need of today to decline the rate of mortality among BC patients [10]. Identification of a compound that can be used as a marker for the early diagnosis of the disease and/or the prognosis of the patient to the therapeutic program would be clinically very valuable and ultimately reduce the rate of mortality among BC patients.

2'-Deoxycytidine (dCyd) level in plasma was suggested as a marker for monitoring the

*Corresponding Author: idarwish@ksu.edu.sa

prognosis of BC patients treated with combined chemotherapeutic agents: cyclophosphamide, methotrexate and 5-fluorouracil [11]. In particular, the measurement of dCyd in plasma is challenging because of its low concentrations and large number of interfering structurally related substances. The analytical technologies that have been used for measuring dCyd and/or other nucleosides are mostly chromatography [12-20], and to a lesser extent enzyme-linked immunosorbent assay (ELISA) [21-23]. Chromatographic methods are usually associated with major drawbacks: (1) they usually give discrepancies in the quantization results because of the incomplete resolution of dCyd from its interfering structural analogues and the multiple steps required for pre-treatment of the samples, (2) complex and expensive instrumentation (e.g. chromatography with tandem mass detectors) is required to achieve the required selectivity, and (3) these methods are not suited for screening large number of specimens in clinics and/or hospitals, particularly in national screening programs. ELISA [22,23] offered higher level of selectivity, lesser sample pre-treatment procedures, and lower instrumental cost, however it suffers from multiple pipetting and washing steps, and sometimes long incubation periods. For these reasons, serious research is required for the development of new alternative more efficient analytical technology for measuring the low concentrations of dCyd in plasma.

Immunosensors represent the most promising and outstanding technological progress in the field of biochemical and clinical analysis [24-28]. These sensors are analytical devices composed of an immunochemical recognition element directly interfaced to a signal transducer, which together relate the concentration of an analyte to a measurable response.

The present study describes, for the first time, the development of a novel immunosensor for measuring the plasma levels of dCydby interfacing its immunochemical reaction to the KinExA^{TM}3200 instrument for the flow kinetic-exclusion analysis. The proposed sensor offered several advantages over the reported ELISA: (1) avoiding the negative effect of modification on the analytical results, (2) avoiding the problems of mass transport limitations and mobility effects, (3) achieving higher sensitivity than ELISA, and (4) providing higher level of convenience by employing the flow automated analysis.

2. Experimental

Materials

2`-Deoxycytidine (dCyd), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), bovine serum albumin (BSA), 2,4,6-trinitrobenzene sulfonic acid, horseradish peroxidase labeled goat anti-mouseIgG (HRP-IgG), 3,3`,5,5`-Tetramethylbenzidine (TMB) peroxidase substrate for ELISA were obtained from Sigma Chemical Co. (St. Louis. MO, USA). Polymethylmethacrylate(PMMA) beads (140–170 mesh, 98 mm) were obtained from Sapidyne Instruments Inc. (Boise, ID, USA). DyLight^{TM} 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). Centricon-30 filter (Amicon, Inc., Beverly, MA, USA). BCA reagent for protein assay was obtained from Pierce Chemical Co. (Rockford, IL, USA). Luna C18 (250 mm × 4.6 mm, 5 μm) reversed phase HPLC column was obtained from Phenomenex (Torrance, CA, USA). HPLC-grade solvents and other reagent-grade chemicals (Fisher Scientific Co., Pittsburgh, PA, USA). Human plasma samples were collected from normal healthy volunteers at King Khalid University Hospital (Riyadh, Saudi Arabia), and they were kept frozen at –20 ºC until analysis. Phosphate-buffered saline (PBS) was obtained from Bio-Basic Inc. (Markham, Canada). All other materials were of analytical grade. All water was purified by Nanopure II water purification system.

Apparatus

KinExA^{TM} 3200 instrument (Sapidyne Instruments, Inc., Boise, ID, USA) empowered by KinExA Pro 20.0.1.26 software provided with the KinExA 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 (Barnstead/Thermolyne, Dubuque, IA, USA). HPLC apparatus consisted of a Shimadzu system (Shimadzu Corporation, Kyoto, Japan) equipped with two solvent delivery systems (LC-20AD VP) with FCV-12AH high-pressure flow channel changeover valve, DGU-20AS on-line degasser, SIL-20A auto-sampler, CTO-20A column oven, SPD-20A UV–vis detector, and CBM-20A system controller.

Procedures

Preparation of anti-dCyd antibody and dCyd-BSA conjugate

Mouse monoclonal anti-dCydantibody was generated by fusing SP2/0-Ag14 mouse myeloma cells with spleen cells from BALB/c mice immunized with keyhole limpet hemocyanin protein conjugated with dCyd. The procedures for isolation, purification and characterization of this antibody have been previously described by Darwish [29]. dCyd-BSA conjugate was prepared by the procedures previously described by Darwish*et al*. [23]. 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 carbodiimidereagent (EDC). The remaining unconjugated dCyd residues were removed from its protein conjugate by buffer exchange using a Centricon filter. Protein concentration of the conjugate was 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 [30].

***Coating of PMMA beads with dCyd-BSA* conjugate**

PMMA bead vials containing 200 mg (dry weight) of beads were coated with dCyd-BSA. The coating solution consisted of 1 mL of PBS containing 32.5µg $mL^{-1}$of dCyd-BSA. Bead vials containing the coating solution were kept on nutating mixer for 2 h at room temperature to allow coating of the beads. Bead vials were removed from the mixer and beads allowed settling down. The supernatant was aspirated from the coating solution and 1mL of blocking solution (10 $mgmL^{-1}$ of BSA in PBS) was added to the bead vial. Bead vials were then kept again on nutating mixer at room temperature for 1 h. The blocked beads were used immediately or stored at 4°C in the blocking solution until use in the analysis. The coated and blocked beads could be stored for one week without any noticeable deterioration.

Preparation of DyLight^{TM} 649-conjugated secondary antibody

Secondary antibody working solution (100$ngmL^{-1}$) of DyLight^{TM} 649-conjugated goat anti-mouse IgG was prepared by proper dilution of the stock concentrated solution (1.5 $mgmL^{-1}$) in standard KinExA sample buffer (PBS, 0.02% sodium azide, pH 7.4 with 1 $mgmL^{-1}$of BSA). The solution was prepared fresh at the start of each experiment.

Preparation of dCyd samples

The dCyd samples were prepared by spiking blank human plasma with dCyd(2–4400nM).Each spiked sample was mixed with equal volume of anti-dCyd antibody solution (100 $ngmL^{-1}$ in PBS) containing BSA at a concentration of 1 $mgmL^{-1}$ 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 KinExA instrument.

Analysis byKinExA instrument

Each of 12 injection lines (of total 14 lines) of the KinExA instrument connected to the rotary selector valve was placed into the sample tube containing the pre-equilibrated (dCyd and its

specific antibody), the 13th line into the tube containing blank solution (zero concentration of dCyd and the primary antibody, and the 14th line into the fluorescently labeled secondary antibody solution. dCyd-BSA-coated beads were loaded in the capillary micro-column of the KinExA™ 3200 instrument. Beads were charged twice to produce appropriate bead-packed column height (above the minimum mark and near the set point mark) in the flow cell of the instrument with the help of flow-cell camera by drawing of 583 µL of a suspension of the beads in PBS at a flow rate of 1 $mLmin^{-1}$ for 35 sec. These conditions produced a uniform and reproducible pack for coated beads. A 500µL of each sample solution were withdrawn and passed through the beads micro-column for 120 seconds at a rate of 0.25 $mLmin^{-1}$ during each sample run. Each sample was run in duplicate, followed by 333 µL of the PBS buffer to wash out unbound primary antibody and excess dCyd molecules. The fluorescently labeled goat anti-mouse IgG secondary antibody solution (100 $ngmL^{-1}$) was drawn past the beads. Unbound labeled secondary antibody was subsequently removed by flowing 1.5 mL of PBS through the bead-pack over a period of 90 sec at a flow rate of $1mLmin^{-1}$. The bound secondary antibody was quantified by measuring the fluorescence intensity. The data were acquisitioned by the KinExA Pro 20.0.1.26 software and transformed to a four-parameter curve fitting using Slide Write software, version 5.011 (Advanced Graphics Software, Inc., Rancho Santa Fe, CA, USA). Calibration curve was generated by fitting the data to the following equation:

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

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

Analysis by ELISA

Microwells of the ELISA plates were coated with dCyd-BSA (5µg mL^{-1} in PBS, 50 $\mu Lwell^{-1}$) by incubation 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 3% BSA in PBS by incubation at 37 °C for 1 h. Aliquots (50 µL) of dCydsamples were mixed with equal volumes of anti-dCyd antibody (1µg mL^{-1}). Aliquot (50 µL) of the mixture was added to each well of the plate 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-IgG (1:2,000 in PBS) was added to each well. After incubation for 1 h, 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 colour 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: $A = A_0 - \{(A_0 - A_1)[dCyd] / (IC_{50} + [dCyd])\}$, Where A is the signal at a definite known concentration of dCyd, A_0 is the signal in the absence of dCyd, A_1 is the signal at the saturating concentration of dCyd, and $IC_{50}$is the dCyd concentration that produces a 50% inhibition of the signal. The concentrations of dCyd in the samples were then obtained by interpolation on the standard curve.

3. Results and discussion

Features and operation of the proposed sensor

The basic features and operation of the proposed KinExA-based immunosensor are illustrated schematically in Fig. 1. Details of the KinExA instrument and assays procedures have been described elsewhere [24,31-34]. In this study, a fixed amount of anti-dCyd antibody was mixed with varying concentrations of dCydand a small volume of the reaction mixture was then rapidly passed through the packed bed of PMMA beads coated with dCyd-BSA conjugate. Anti-dCyd antibodies with unoccupied binding sites were available to bind to the immobilized dCyd(coated on the surface of the PMMA beads); antibodies with binding sites occupied by

soluble dCyd analyte were not. Exposure of the dCyd-antibody immune complexes to the immobilized dCydwas 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 dCyd molecules with slow unimolecular dissociation rate constants were thus kinetically excluded from interacting with the immobilized dCyd. The soluble reagents were removed from the beads by an immediate buffer wash. Quantification of the antibody captured on the immobilized dCydcould subsequently be achieved by the brief exposure of the particles to a fluorescently labeled secondary antibody directed against the primary antibody, followed by measurement of the resulting fluorescence from the particles after removal of excess unbound reagents. The fluorescence signals were monitored continuously by a flow cell camera facing the beads-packed microcolumn.

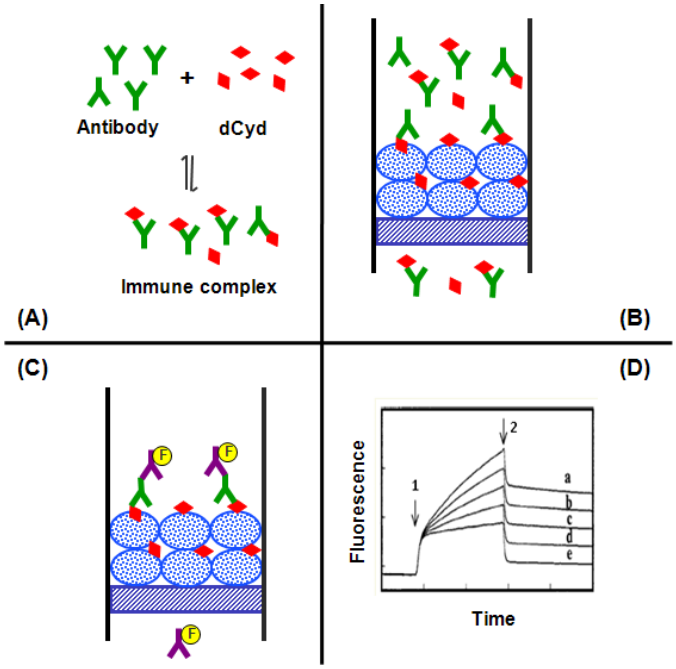


Fig. 1.Format for KinExA-based immunosensor for measurement of dCyd. (A) dCyd and its antibody are mixed and allowed to reach the equilibrium. (B)

Measurement of dCyd 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 are shown in Fig. 2 for various concentrations of dCyd. 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 dCyd (most bottom curve corresponding to 4400 nM), the instrument response approximated a square wave corresponding to the fluorescence of the secondary antibody during its transient passage past 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 dCyd was omitted from the equilibrium mixture (most top curve corresponding to zero concentration of dCyd), 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 dCyd 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-dCydantibody captured on the beads. Equilibrium mixtures comprised of dCyd 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 dCyd could be generated.

Optimization of KinExA signal for measurement of dCyd

Preliminary experiments were conducted to determine the "net signal" of the KinExA instrument when a particular concentration of free anti-dCyd antibody binds to a solid phase (PMMA coated with dCyd). "100% signal" was the voltage given by the instrument when all the binding sites on the primary antibody molecule in solution were in the free form and were completely available for binding to dCyd antigen. Non-specific binding was the voltage given by the instrument when there were no any binding sites in solution (only sample buffer and labeled secondary antibody). The net analytical signal was determined by subtracting non-specific binding signal from the 100% signal. To generate reproducible results, it was important to keep the background signal at a constant level since the processed signal was the difference between background and final signal. Adsorption of the reagents onto the column wall and insufficient washing were a principal cause for a background increase. Beginning the assay runs with the most dilute samples and progressing to higher concentrations could help to minimize the problem. Overnight washing of inlet lines, before the analysis, with PBS-Talso reduced the background increase and alleviated the problem [35].

Optimization of assay conditions for measurement of dCyd by KinExA

dCyd, being a small molecule, its direct immobilization onto the PMMA beads was not possible [36]. Therefore, a protein conjugate of dCyd was required via which dCyd could be immobilized onto the beads. dCyd-BSA conjugate was successfully prepared and the extent of conjugation was found to be 35.8% of the total amino group residues on BSA. The optimization of the assay parameters that has been conducted in this study are summarized in the following paragraph.

In order to select the most appropriate concentration of dCyd-BSA required for coating onto the PMMA beads, the beads were coated with varying concentrations of dCyd-BSA (12.5–37.5μg mL^{-1}) and the analysis was performed using each concentration. It was found that the best analytical signal was obtained when the beads coated with 32.5μg $mL^{-1}$of dCyd-BSA conjugate. This concentration was used in all the subsequent experiments. In order to select the most appropriate concentration of the primary anti-dCyd, varying concentrations (50—200ng mL^{-1}) was used in the analysis, and the optimum concentration was found to be 100 $ngmL^{-1}$. The optimum concentration of the secondary labeled antibody was found to be 100 $ngmL^{-1}$. Volumes of the samples and secondary labeled antibody flowing over the solid-phase (beads coated with dCyd-BSA) were found to be 500 μL, in both cases, at a flow rate of 0.25 $mLmin^{-1}$. The KinExA data that have been generated under these conditions are given in Fig.2.

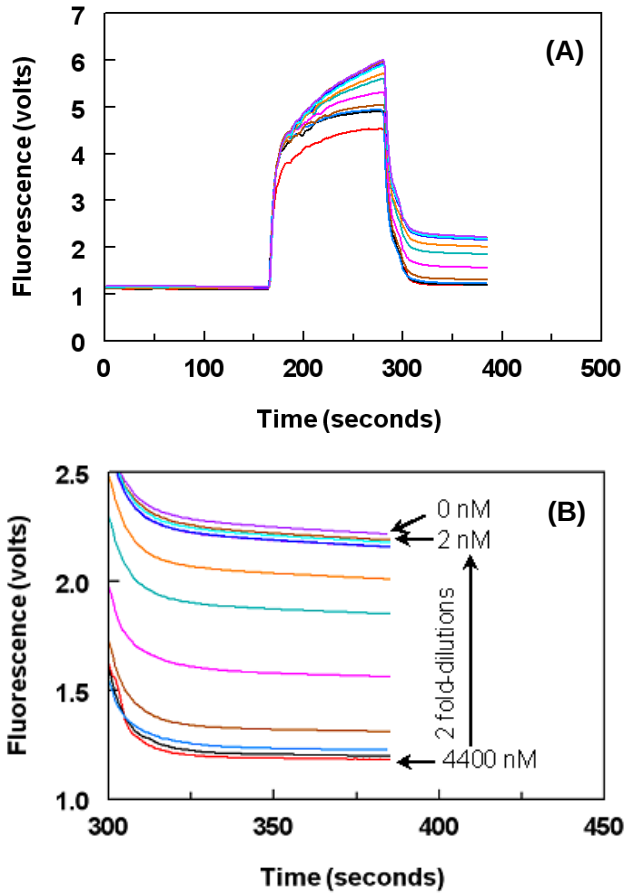


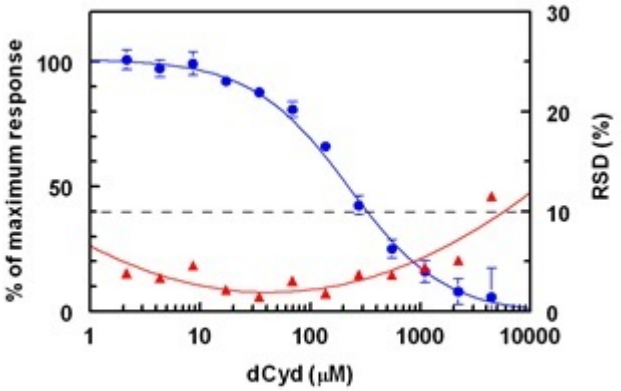
Fig.2. (A): Real raw trend-line fluorescence responses obtained by the KinExA™ instrument for varying concentrations of dCyd (2—4400 nM). (B): The same signals, however they are presented on different scales for the time (seconds) and fluorescence (volts).

Validation of the proposed KinExA-based sensor

Calibration curve and detection limit

The calibration curve for determination of dCyd by the proposed sensor is shown in Fig. 3A. This curve was generated using dCyd concentrations in the range of 2—4400nM. The data showed good correlation coefficient (r = 0.9966) on the four-parameter curve fit. The limit of detection (LOD) of the proposed sensor, defined as the dCyd concentration that cause inhibition of 10% of the maximum signal (e.g. at 90% signal). Based on the basis of duplicate measurements, the limit of detection was found to be 20nM, and the working range of the assay at RSD ≤ 10% was 20—2200 nM. This LOD was comparable to that achieved by Darwish *et al.* [23], however by using a mouse monoclonal antibody with much lower affinity than the rat monoclonal antibody that has been used in the previous study [23]. The high sensitivity of the proposed sensor enabled the determination of low concentrations of dCyd in diluted plasma. The analysis of diluted plasma samples by the proposed KinExA-based sensor avoided the problems of mass transport limitations, and mobility effects that are encountered in the analysis by the conventional sensitive ELISA.

(A)



(B)

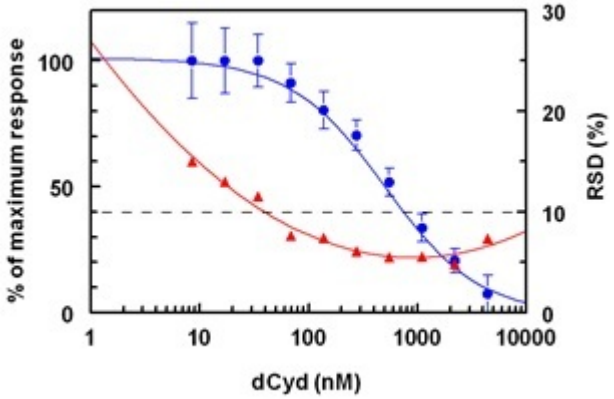


Fig. 3.Calibration curve (●) and precision profile (◢) of the proposed KinExA-based sensor (A) and ELISA (B) for measurement of dCyd.

Precision profile

The assay precision profile obtained from the results of calibration standard samples, assayed in triplicate, is shown in Fig. 3A.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 dCyd. 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 [37], the assay gave satisfactory results; the RSD was 3.6– 6.2 and 5.2–7.5% for the intra- and inter-assay precision, respectively (Table 1).

Table 1.Precision of the proposed KinExA-based sensor for measurement of dCyd

dCyd (nM)	Intra-assay		Inter-assay
	Mean ± RSD[a] (nM)		Mean ± RSD (nM)
40	38.6 ± 6.2		41.2 ± 7.5
200	204.5 ± 5.1		197.5± 5.2
800	795.0 ± 3.6		814.2± 5.9

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

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 (50–800nM) of dCyd into 3 batches of blank plasma samples. The spiked samples were analyzed for their contents of dCyd, as described in the Experimental Section. The analytical recovery was calculated as the ratio of the found dCyd concentration to that of the spiked concentration, and the ratio was expressed as percentage. The analytical recovery values were 94.8–107.4% with RSD 2.48–8.40% (Table 2). These recovery values, according to the guidelines for immunoassay validation [34], indicated the accuracy of the proposed method for determination of dCyd in plasma samples, and absence of endogenous interfering substances in the plasma samples.

Table 2. Analytical recovery of dCyd spiked into three different batches of plasma samples.

Spiked dCyd(nM)	Recovery (% ± RSD)[a]		
	Batch A	Batch B	Batch C
50	104.6± 7.4	107.4 ±6.3	105.4 ± 8.4
100	106.4± 6.5	96.9± 4.4	102.4± 6.8
200	103.8± 2.5	98.2± 4.7	104.6 ± 3.6
400	102.3±5.4	103.5±6.4	105.8±5.1
800	97.2± 4.6	94.8 ± 8.3	95.9± 4.7

[a] Values are mean of 3 determinations; RSD is the relative standard deviation.

Comparison of the proposed KinExA-based sensor with ELISA

In this study, ELISA system was optimized for dCyd using the same reagents (anti-dCyd antibody and dCyd-BSA conjugate). The assay system involved the conventional antigen-capturing immunoassay format [38]. In this format, a competitive binding reaction occurred between dCyd, in the sample, and the immobilized dCyd–BSA (on microwells of ELISA plate) for the binding sites on the anti-dCyd antibody. The anti-dCyd antibody bound to the immobilized dCyd-BSA was quantified with peroxidase-labeled anti-mouse IgG second antibody and TMB as a peroxidase chromogenic substrate. The concentration of dCyd in the sample was quantified by its ability to inhibit the binding of the anti-dCyd antibody to the immobilized dCyd–BSA and subsequently the colour intensity was inversely proportional to the concentration of dCyd in the original sample solution. The optimum concentrations of dCyd–BSA and anti-dCyd antibody required to achieve the highest possible sensitivity were determined by checkboard titration [39]; these conditions were given in the Experimental Section. Under these conditions, the calibration curve that has been generated at dCyd concentrations range of 8.5–4400 μM (Fig. 3B), and the LOD was found to be50μM, neither of which was comparable with those obtained by the KinExA format described herein. KinExA had at least 2500-fold more sensitivity than the ELISA, which agreed with a previous study [24]. The higher sensitivity of KinExA, compared with ELISA, was attributed the co-functional inherent advantages of KinExA. As the concentration of the primary antibody decreases, the signal-concentration curve is shifted to lower concentrations (e.g. higher sensitivity) until becoming maximal when the antibody concentration equal its dissociation constant (K_D) value [40]. For ELISA, higher concentration of primary antibody than K_D value is usually demanded to achieve a detectable signal. However, in KinExA, even a very low concentration of antibody can give adequate signal strength by the accumulation from the continuous sample flow. This permits the achievement of the highest possible sensitivity, which is limited by the affinity of the antibody to the antigen, but not by the instrument. In addition, the working conditions of KinExA(rapid flow of the pre-equilibrated antigen-antibody mixture over the immobilized antigen in the observation cell) do not allow the dissociation of the antigen-antibody immune complexes because the immobilized antigen served merely as a tool for separation, but not as an effective competing reagent for the antibody binding sites. This enables detecting only free antibodies that bore unoccupied binding sites, in the equilibrium mixture, thus achieving higher sensitivity. Furthermore, the instrument response in the KinExA format was acquisitioned on capturing beads column (approximately 10,000column^{-1}) with a higher surface

(260 mm^2) than does the ELISA microwell plate (64 mm^2well^{-1}) [32]. This higher surface area of the capturing beads, in KinExA format, maximizes the opportunities for the capture of more free antibodies, leading to an improved quantifiable response with low signal-to-noise characteristics and acceptable reproducibility [37]. This obviously evidenced from better precision profile of KinExA-based sensor than that of ELISA (Fig. 4); the RSD values over the working assay range of KinExA-based sensor was ≤ 5 compared with values > 5 in ELISA. 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 protocol and other manipulations (change in the temperature and periods of incubation, and dispensing the reagents) lead to higher imprecision. However, the use of capturing beads with higher surface area, in KinExA format, makes the precision dependent only on the concentrations of the primary and secondary-labeled antibodies, which are dispended automatically with high precision by the KinExA instrument. For this reason the KinExA-based sensor showed better precision profile than ELISA.

4. Conclusions

A novel automated flow-based immunosensor employing the KinExA format has been developed and validated for the measurement of the BC prognostic marker dCyd in plasma at concentration as low as 20nM. The developed sensor was compared with the conventional ELISA. The data demonstrated that the format of the assay may influence its performance characteristics (sensitivity, working range, etc.), even when exactly the same reagents are employed. 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 sensor is anticipated to have a great value in measurement of dCyd where a more confident result is needed, contributes in further evaluation of dCyd as a prognostic marker for breast cancer chemotherapy, and elucidating the role of dCyd in various biological and biochemical systems

Acknowledgment

The work was supported by National Program for Science and Technology (NPST) by King Saud University, Project Number 08-NAN306-2.

References

[1] H.M. Heneghan, N. Miller, A.J. Lowery, K.J. Sweeney, M.J. Kerin, J. Oncol. Article ID 950201 (2010).
[2] C.R. James, J.E. Quinn, P.B. Mullan, P.G. Johnston, D.P. Harkin, Oncologist **12**, 142 (2007).
[3] E.M. Ibrahim, A.A. Zeeneldin, B.B. Sadiq, A.A. Ezzat, Med. Oncol. **25,**387(2008).
[4] O. Khatib, M. Aljurf, Hematol. Oncol.Stem Cell.Ther.**1,** 44 (2008).
[5] K. Zabicki, J.A. Colbert, F.J. Dominguez, M.A. Gadd, K.S. Hughes, J.L. Jones, M.C. Specht, J.S. Michaelson, B.L. Smith, Ann. Surg. Oncol. **13**,1072 (2006).
[6] M.A. Bollet, B. Sigal-Zafrani, V. Mazeau, A. Savignoni, A. de la Rochefordière, A. Vincent-Salomon, R. Salmon, F. Campana, Y.M. Kirova, R. Dendale, A. Fourquet, Radiother. Oncol.**82,** 272(2007)
[7] T. Aryandono, Harijadi, Soeripto, Asian Pac. J. Cancer Prev. **7**, 451 (2006).
[8] U.W. Jayasinghe, R. Taylor, J. Boyages, ANZ. J. Surg. **75**, 762(2005).
[9] C.S. Foo, D. Su, C.K. Chong, H.C. Chng, K.H. Tay, S.C. Low, S.M. Tan, ANZ. J. Surg. **75**, 566 (2005).
[10] H. Alexander, A.L. Stegner, C. Wagner-Mann, G.C. Du Bois, S. Alexander , E.R. Sauter, Clin. Cancer Res. **10**, 7500 (2004).
[11] M. Yoshioka, M. Abu-Zeid, T. Kubo, M. El-Merzabani, Biol. Pharm. Bull. **17**, 169 (1994).

[12] A. Bond, E. Dudley, F. Lemiere, R. Tuytten, S. El-Sharkawi, A.G. Brenton, E.L. Esmans, R.P. Newton, Rapid Commun. Mass Spectrom. **20**, 137 (2006).
[13] H.M. Liebich, S. Muller-Hagedorn, F. Klaus, K. Meziane, K.R. Kim, A. Frickenschmidt, B. Kammerer, J. Chromatogr. A **1071**, 271 (2005).

[14] T. Yamamoto, K. Higashino, S. Tamura, H. Fujioka, Y. Amuro, T. Hada, Anal. Biochem. **170,** 387 (1988).
[15] A.K. Goodenough, H.A. Schut, R.J. Turesky, Chem. Res. Toxicol. **20**, 263 (2007).

[16] C.G. Zambonin, A. Aresta, F. Palmisano, G. Specchia, V. Liso, J. Pharm. Biomed. Anal. **21,** 1045 (1999).
[17] H.M. Liebich, S. Muller-Hagedorn, M. Bacher, H.G. Scheel-Walter, X. Lu, A. Frickenschmidt, B. Kammerer, K.R. Kim, H. Gerard, J. Chromatogr. B: Anal. Technol. Biomed.Life Sci. **814**, 275 (2005).
[18] J. Zhao, B. Todd, G. Fleet, J. Chromatogr. A **673**, 167 (1994).
[19] E.P. Petryaev, S.V. Moshshinskaya, V.A. Timoshchuk, O.I. Shadyro, Zh. Anal. Khim. **44**, 950 (1989).
[20] N. Hiroki, N. Hitoshi, S. Mikihiko, O. Yosuke, Anal. Sci. **8**, 345 (1992).

[21] K. Omura, K. Hirose, M. Itoh, T. Akizawa, M. Yoshioka, J. Pharm. Biomed. Anal **15**, 1249 (1997).
[22] I. Darwish, S. Emara, H. Askal, N. El-Rabbat, T. Akizawa,M. Yoshioka, Anal. Chim. Acta **404**, 179 (2000).
[23] I.A. Darwish, A.M. Mahmoud, T. Aboul-Fadl, A.A. Al-Majed, N.Y. Khalil, Anal. Chim. Acta **632**, 266 (2009).
[24] D.A. Blake, R.M. Jones, R.C. Blake, A.R. Pavlov, I.A. Darwish, H. Yu, Biosens. Bioelectron.**16**,799 (2001).
[25] L. Campanella, E. Martini, M. Tomassetti, J. Pharm. Biomed. Anal. **53,** 186 (2010).
[26] C. Tlili, L.N. Cella, N.V. Myung, V. Shetty, A. Mulchandani, Analyst **135,** 2637 (2010).
[27] L.M. Bonanno, T.C. Kwong, L.A. DeLouise, Anal. Chem. **82**, 9711 (2010).
[28] A.W. Kusterbeck, D.A. Blake, Flow Immunosensors, F.S. Ligler, C.R. Taitt (Ed.), Optical Biosensors: Today and Tomorrow, 2nd edition, Elsevier, 2008, pp. 243-285.
[29] I.A. Darwish, Ph.D. Thesis, Immunoanalytical Method for Cytidine Derivatives as Cancer Markers, Assiut University, Egypt, 1998.
[30] A.F. Habeeb, Anal. Biochem.**14**, 328 (1966).
[31] D.A. Blake, P. Chakrabarti, M. Khosraviani, F.M. Hatcher, C.M. Westhoff, P. Goebel, D.E. Wylie, R.C.II Blake, J. Biol. Chem. **271**, 27677 (1996).
[32] R.C. Blake, A.R. Pavlov, D.A. Blake, Anal. Biochem.**272**, 123 (1999).
[33] M. Khosraviani, R.C.II Blake, A.R. Pavlov, S.C. Lorbach, H. Yu, J.B. Delehanty, M.W. Brechbiel, D.A. Blake, Bioconjug. Chem. **11**, 267 (2000).
[34] D.A. Blake, A.R. Pavlov, H. Yu, M. Khosraviani, H.E. Ensley, R.C.II Blake, Anal. Chem. Acta **444**,3 (2001).
[35] KinExA Technology operation manual, draft 1.0.8.0 direct to systems using software version 2.0.1.26, Sapidyne Instruments Inc, Boise, ID, USA, 2010.
[36] S. Sachetelli, C. Beaulac, J. Lagace, Biochim. Biophys.Acta **1379**, 35 (1998).
[37] J.W. Findlay, W.C. Smith, J.W. Lee, G.D. Nordblom, I. Das, B.S. DeSilva, M.N. Khan, R.R. Bowsher, J. Pharm. Biomed. Anal. **21**, 1249 (2000).
[38] I.A. Darwish, Int. J. Biomed. Sci. **2**, 217 (2006).
[39] E. Engvall, Meth. Enzymol. **70**, 419 (1980).
[40] N. Ohmura, S.J. Lackie, H. Saiki, Anal. Chem. **73**, 3392 (2001).