Talanta 97 (2012) 499–504

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Novel automated flow-based immunosensor for real-time measurement of the breast cancer biomarker CA15-3 in serum

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ARTICLE INFO

Article history:
Received 8 December 2011
Received in revised form
7 May 2012
Accepted 8 May 2012
Available online 16 May 2012

Keywords:
Breast cancer
Biomarkers
CA15-3
Immunosensor
KinExA
ELISA

ABSTRACT

A novel automated immunosensor assay has been developed for real-time measurement of the breast cancer biomarker CA15-3 in serum. The assay employed the kinetic-exclusion analytical technology of the KinExA™ 3200 instrument. Polymethylmethacrylate (PMMA) beads coated with CA15-3 were used as capturing reagent, mouse anti-CA15-3 monoclonal antibody was used as primary antibody, and the fluorescence was monitored and recorded during the flow of the fluorescent-labeled antibody through the beads. The fluorescence signal retained on the beads was plotted versus CA15-3 concentration to generate a calibration curve. The concentrations of CA15-3 in the samples were then obtained by interpolation on the curve. The assay limit of detection was 0.2 IU mL^{-1}. This highly sensitive automated system allowed rapid and reliable quantification of CA15-3 without any matrix effect; analytical recovery of serum-spiked CA15-3 was 90.7%–108.6% $\pm$ 2.05%–7.45%. The precision of the sensor was satisfactory; relative standard deviation (RSD) was 3.8%–5.1% and 5.2%–7.4% for the intra- and inter-assay precision, respectively. The analytical performance of the proposed sensor was superior to the non-competitive sandwich immunoassays for CA15-3. The automated analysis by the sensor facilitated the processing of a large number of specimens, and the new sensor-based assay is anticipated to have a great value in measurement of CA15-3.
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1. Introduction

Cancer is the most devastating disease in the world, causing about 7.6 million deaths (around 13% of all deaths) in 2008. Deaths from cancer worldwide are projected to continue to rise to over 11 million in 2030 [1,2]. Breast cancer (BC) is the leading cause of death among women. It is responsible for approximately 15% of all cancer-related deaths, and the incidence of BC may increase substantially in the future [3–5]. BC affects women of all ages and the mortality rate among the patients is also high. The high rate of mortality in BC patients can be attributed in part to the late diagnosis of the disease, and consequently the delayed initiation of the medical treatment by surgery, radiotherapy and/or chemotherapy. The late diagnosis/treatment of BC is usually associated with poor prognosis [6–10]. Early detection and proper monitoring of patient status during therapy is urgently needed to decrease the rate of mortality among BC patients [11]. Identification of biomarkers that can be used for the early diagnosis of the disease and/or the prognosis of the patient in therapeutic programs would be clinically very valuable and ultimately reduce the rate of mortality among BC patients.

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. MUC1 has a core protein mass of 120–225 kDa. It extends 200–500 nm beyond the surface of the cell. The protein is anchored to the apical surface of many epithelia by a transmembrane domain. Beyond the transmembrane domain is a SEA domain that contains a cleavage site for release of the large extracellular domain. The extracellular domain includes a 20 amino acid variable number tandem repeat domain. These repeats are rich in serine, threonine and proline residues, which permit heavy O-glycosylation. MUC1 is cleaved in the endoplasmic reticulum into two pieces, the cytoplasmic tail including the transmembrane domain and the extracellular domain. These domains tightly associate in a non-covalent fashion [12].

CA15-3 is elevated in a proportion of BC patients with distant metastases [13]. Though current American Society of Clinical Oncology [14] and National Comprehensive Cancer Network guidelines [15] do not recommend its use for surveillance purposes, CA15-3 is helpful in monitoring of treatment of women with BC. Elevated serum levels of CA15-3 are used to anticipate detection of recurrences in patients with BC, and provide an additional tool in evaluating therapeutic response in patients with advanced disease [16]. Pre-operative levels of CA15-3 have a significant and independent relation to outcome in patients with early BC [17]. Patients

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http://dx.doi.org/10.1016/j.talanta.2012.05.005

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 [18].

Enzyme-linked immunosorbent assay (ELISA) is the technique most commonly used for measurement of CA15-3 [19–23]. This assay system is based on the principles of a binding to reagents immobilized on a solid-phase and employs the spectroscopic detection of a colored reagent. The traditional protocol of ELISA for CA15-3 utilizes a monoclonal antibody directed against a distinct antigenic determinant on the CA15-3 molecule. This antibody, when immobilized on a solid phase (i.e., microwells of assay plate) can be used to capture CA15-3 from the serum. A secondary antibody conjugated to an enzyme is used as a signal generator. The test sample is allowed to react sequentially with the two antibodies, resulting in the CA15-3 molecules being sandwiched between the solid-phase and enzyme-linked antibodies. In general, ELISA offers a quite high level of selectivity, and requires minimal sample pre-treatment procedures; however it suffers from multiple pipetting and washing steps, long incubation periods, and sometimes quite low sensitivity unless signal enhancer is used. For these reasons, more efficient analytical technology is required for measurement of CA15-3 in serum.

Immunosensors represent the most promising and outstanding technological progress in the field of biochemical and clinical analysis [24–27]. 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-based assay for serum levels of CA15-3. This assay interfaces the binding of CA15-3 to a monoclonal antibody with a fluorescent signal generated the by KinExA™ instrument, which employs the principle of kinetic exclusion analysis (a). The KinExA-based assay was designed to measure the unmodified CA15-3 molecules in solution, rather than employing the antigen- or antibody-immobilized solid phase used in the conventional ELISA. The new sensor-based assay offered several advantages over the existing ELISA: (1) it avoided the negative effect of antibody immobilization on the analytical results; (2) it bypassed the problems of mass transport and mobility that arise when the antibody or antigen is immobilized on a solid support; (3) the assay achieved a higher sensitivity than ELISAs reported here and elsewhere (b); and (4) the assay provided higher levels of convenience by employing an automated sensor system.

2. Experimental

2.1. Instruments

The KinExA™3200 instrument was obtained from Sapidyne Instruments Inc. (Boise, ID, USA). A microplate reader (ELx 808 IU,) and automatic microplate strip washer (ELx 50/8) were provided by Bio-Tek Instruments Inc. (Winooski, WI USA). An EM-36N microtube nutating mixer was a product of Taitec (Saitama-ken, Japan). The Biofuge Pico centrifuge was obtained from Heraeus Instruments (Hanau, Germany). A Nanopure II water purification system (Dubuque, IA, USA) was used to make all the solutions described in this study.

2.2. Materials

Highly purified CA15-3 was a product of Biospecific (Emeryville, CA, USA), human breast tumor marker mouse monoclonal antibody (anti-CA15-3) was obtained from Cal-Bioreagents (San Mateo, CA, USA). DyLight™ 649-conjugated AffiniPure goat anti-mouse IgG Secondary antibody was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). An anti-CA15-3 antibody conjugated to horseradish peroxidase was obtained from MyBiosource, LLC (San Diego, CA, USA). PMMA beads (140–170 mesh, 98 μm) were provided by Sapidyne Instruments Inc. (Boise, ID, USA). Bovine serum albumin (BSA) was a product of Sigma Chemical Co. (St. Louis, MO, USA). Human serum samples were collected from normal healthy volunteers at King Khalid University Hospital (Riyadh, Saudi Arabia), and were kept frozen at −20 °C until analysis. PBS was obtained from Bio-Basic Inc. (Markham, Canada). All other materials were of analytical grade.

2.3. Procedures

2.3.1. Coating of PMMA beads with CA15-3

PMMA bead vials containing 200 mg (dry weight) of beads were coated with CA15-3. The coating solution consisted of 1 mL of PBS containing 580 international units (IU) of CA15-3. 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 centrifuged. 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.

2.3.2. Preparation of DyLight™ 649-conjugated secondary antibody

Secondary antibody working solution (150 ng mL^{-1}) 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 solution was prepared fresh at the start of each experiment.

2.3.3. Preparation of CA15-3 samples

The calibration standard samples were prepared by spiking blank human serum with CA15-3 to obtain concentrations of 0.02–77 IU mL^{-1}. Each spiked sample (1 mL) was mixed with an equal volume of anti-CA15-3 primary antibody solution (30 ng mL^{-1} 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 for 1 h at room temperature. After achieving equilibrium, samples were analyzed by the KinExA instrument.

2.3.4. Analysis on 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 antibody and antigen (CA15-3 and its specific antibody). The 13th line was placed into a tube containing the blank (zero concentration of CA15-3 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. CA15-3-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 3200 Instrument. Beads were first automatically packed into the capillary flow/observation cell by the KinExA™ 3200. 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,

a 667 μL aliquot of a suspension of the beads in PBS was drawn over the flow cell at a flow rate of 1 mL min^{-1} for 40 s. These conditions produced a uniform and reproducible pack for CA15-3-coated beads. A 450 μL of each equilibrated sample solution was then withdrawn and passed over the micro-column for 108 s at a rate of 0.25 mL min^{-1}. An automatic buffer wash (333 μL of the PBS) removed unbound primary antibody and excess soluble CA15-3 molecules from the bead pack. An aliquot (500 μL) of fluorescently labeled goat anti-mouse IgG secondary antibody solution (150 ng mL^{-1}) 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 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 triplicate, 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:

$$F = F_0 - (F_0 - F_1)[\text{CA15-3}]/(IC_{50} + [\text{CA15-3}])$$

where F is the fluorescence signal at a definite known concentration of CA15-3 [CA15-3], F_0 is the fluorescence signal at zero concentration of CA15-3, F_1 is the fluorescence signal at the saturating concentration of CA15-3, and IC_{50} is the CA15-3 concentration that produces a 50% inhibition of the signal. The concentrations of CA15-3 in the samples were obtained by interpolation on the standard curve.

2.3.5. Analysis by sandwich ELISA format

Fifty microliters of anti-CA15-3 antibody solution (2 μg mL^{-1}; prepared in PBS) was introduced into each well of the microplate wells and the plate was incubated at 37 °C for 2 h. After incubation, the plates were washed with PBS. The wells were blocked by receiving 100 μL of BSA (1%, w/v in PBS) and incubation at 37 °C for 1 h. The plates were washed with PBS as described above, and 50 μL of the CA15-3 samples (spiked serum or standards samples) was added to each well. After incubation for 1 h at 37 °C, the plates were washed with PBS, and 50 μL anti-CA15-3 antibody labeled with peroxidase enzyme (1 μg mL^{-1} in PBS) was added to each well. After incubation for 1 h, the plates were washed with PBS and the color signals were developed using TMB substrate, and the generated signals were related to the CA15-3 concentration in the sample using a four-parameter curve fit: $F = F_0 + \{(F_0 - F_1)[\text{CA15-3}]/(IC_{50} + [\text{CA15-3}])\}$

3. Results and discussion

3.1. Features and operation of the proposed sensor

Details of the KinExA instrument and assays procedures have been described elsewhere [24,28–31,35]. The basic features and operation of the proposed KinExA-based immunosensor are illustrated schematically in Fig. 1.

In this study, a fixed amount of anti-CA15-3 antibody was mixed with varying concentrations of CA15-3 and a small volume of the reaction mixture was then rapidly passed through the packed bed of CA15-3-coated beads. CA15-3-specific antibodies with unoccupied binding sites were available to bind to the immobilized CA15-3 (coated on the surface of the beads), antibodies with binding sites occupied by soluble CA15-3 analyte were not. Exposure of the soluble antibody-CA15-3 complex to the immobilized

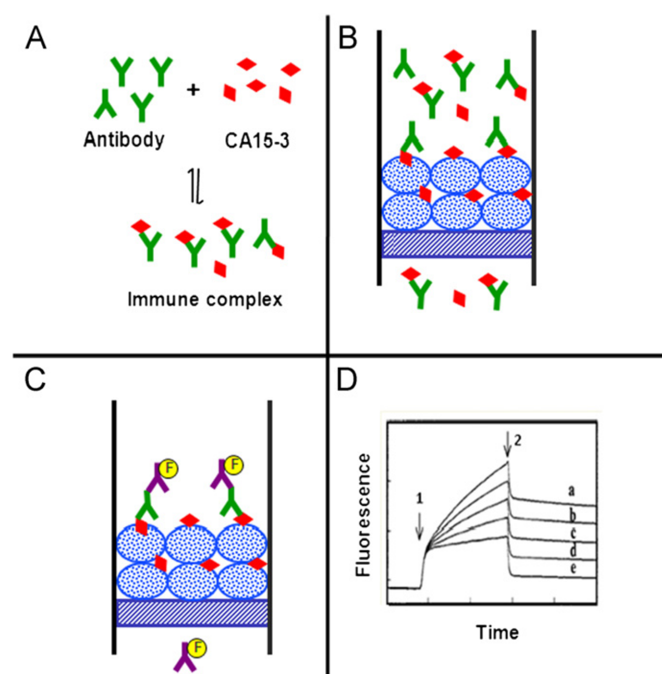

Fig. 1. Format for KinExA-based immunosensor for measurement of CA15-3. (A) CA15-3 and its antibody are mixed and allowed to reach the equilibrium. (B) The equilibrium mixture of antibody, free CA15-3, and antibody–antigen immune complex is passed rapidly through the microcolumn over the beads that have been coated with CA15-3, and held in the observation cell of the KinExA instrument by a microporous screen. A portion of those antibody molecules with free binding sites is captured by the column, while antibody bound to CA15-3, and free CA15-3 are washed through the beads. (C) Fluorescently labeled anti-mouse IgG antibody is used for detection of anti-CA15-3 antibody bound to the beads. Fluorescence is continuously monitored and recorded via a PC interface. (D) Raw data curves from the KinExA instrument. Curve a corresponds to zero CA15-3 concentration; curve e corresponds to a saturating CA15-3 concentration. Curves b–d are the concentrations of CA15-3 between zero and saturation. Arrow 1 indicates to the introduction of fluorescently labeled secondary antibody; and arrow 2, cessation of labeled secondary antibody and initiation of a second buffer wash.

CA15-3 was sufficiently brief to ensure that negligible dissociation of the immune complex occurs during the time of exposure to the beads. Those antibody molecules whose binding sites were occupied by CA15-3 molecules were thus kinetically excluded from interacting with the immobilized CA15-3. The soluble reagents were removed from the beads by an immediate buffer wash. Quantification of the antibody captured on the immobilized CA15-3 could 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. Fluorescence was monitored continuously by a photodiode in the optical unit of the instrument [32,35].

3.2. Measurement of CA15-3 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 CA15-3 are shown in Fig. 2.

The instrumental response from 0 to 155 s corresponds to the background signal generated while the unlabeled equilibrium mixture is exposed to and washed out of the bead-packed micro-column. The beads were then exposed to a solution of fluorescently labeled secondary antibody (156–270 s), and excess unbound labeled secondary antibody was removed from the beads with a buffer wash (271–375 s). When the equilibrium

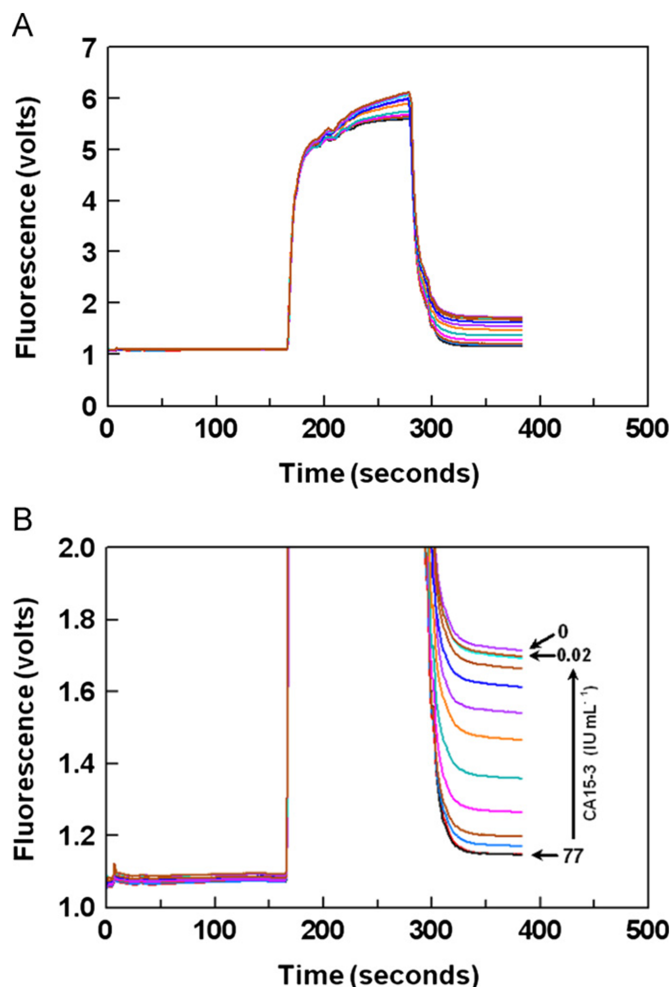

Fig. 2. (A): Trend-line fluorescence responses obtained by the KinExA™ instrument for varying concentrations of CA15-3 (0.02–77 IU mL^{-1}). (B): The same data, however the vertical axis (fluorescence intensity) was from 1 to 2 V.

mixture contained a saturating concentration of free CA15-3 (lowest curve, corresponding to 77 IU 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 soluble CA15-3 was omitted from the equilibrium mixture (top curve, corresponding to zero concentration of CA15-3), the instrument response from 156 to 270 s 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 CA15-3 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 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-CA15-3 antibody captured on the beads. Equilibrium mixtures comprised of CA15-3 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 CA15-3 could be generated.

3.3. Optimization of KinExA signal for measurement of CA15-3

Preliminary experiments were conducted to determine the "net signal" of the KinExA instrument when a particular concentration of free anti-CA15-3 antibody binds to a solid phase (PMMA coated with CA15-3). "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 CA15-3 antigen. Non-specific binding was the voltage given by the instrument when there were no binding sites in solution (only sample buffer and labeled secondary antibody). The net analytical signal was determined by subtracting the 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-T also reduced the background increase and alleviated the problem [31]. However, during assay optimization, the background increased despite use of these measures. The problem was eliminated by introducing PBS-T through the 14th sample injection line immediately after back-flushing the beads.

3.4. Optimization of assay conditions for measurement of CA15-3 by KinExA

As mentioned above (in Introduction), CA15-3 is a macromolecular protein in nature, thus 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 CA15-3 proceeded successfully and it did not affect its binding activity to its specific antibody.

The optimization of assay parameters that has been conducted in this study can be are summarized as follows: (1) In order to select the most appropriate concentration of CA15-3 required for coating onto the beads, the beads were coated with varying concentrations of CA15-3 (250–1000 IU mL^{-1}) and the analysis was performed using each concentration. It was found that the best analytical signal was obtained when the beads were coated with 580 IU mL^{-1} of CA15-3. This concentration was used in all the subsequent experiments. (2) In order to select the most appropriate concentration of the primary anti-CA15-3, varying concentrations (10–100 ng mL^{-1}) were used in the analysis, and the optimum concentration was found to be 30 ng mL^{-1}. (3) The optimum concentration of the secondary labeled anti-mouse IgG antibody was found to be 150 ng mL^{-1}. (4) Volumes of the samples and secondary labeled antibody flowing over the solid-phase (beads coated with CA15-3) were found to be 450 and 500 μL, respectively, at a flow rate of 0.25 mL min^{-1}. The KinExA data that have been generated under these conditions are shown in Fig. 2.

3.5. Validation of the KinExA assay

3.5.1. Calibration curve and detection limit

The calibration curve for determination of CA15-3 by the proposed sensor is shown in Fig. 3. This curve was generated using CA15-3 concentrations in the range of 0.02–77 IU mL^{-1}. The data showed good correlation coefficient ($r=0.9990$) on the four-parameter curve fit. The limit of detection of the proposed sensor was defined to be the CA15-3 concentration that caused inhibition of 10% of the maximum signal (e.g. at 90% signal). Based on the

basis of duplicate measurements, the limit of detection in the assay was found to be 0.2 IU mL^{-1}. This high sensitivity enables the determination of low concentrations of CA15-3 in diluted serum as the normal serum level of CA15-3 is usually less than 30 IU mL^{-1}. The analysis of CA15-3 in diluted serum samples by the proposed KinExA-based sensor avoids the problems of mass transport limitations and mobility effects that are encountered in the analysis by the conventional sandwich ELISA.

3.5.2. 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 CA15-3. 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 [34], the assay gave satisfactory results; the RSD was 3.8%–5.1% and 5.2%–7.4% for the intra- and inter-assay precision, respectively (Table 1).

3.5.3. Accuracy and application of the proposed sensor

The accuracy of the proposed sensor and its applicability was assessed by the recovery study. Samples were prepared by spiking varying concentrations (0.3–4.8 IU mL^{-1}) of CA15-3 into 3 batches of blank serum samples. The spiked samples were analyzed for their contents of CA15-3, as described in the Experimental Section. The analytical recovery was calculated as the ratio of the found CA15-3 concentration to that of the spiked concentration, and the ratio was expressed as percentage. The analytical recovery values were 90.7%–108.6% with RSD 2.05%–7.45% (Table 2). These recovery values, according to the guidelines for immunoassay validation [34], indicated the accuracy of the proposed method for determination of CA15-3 in serum samples, and absence of endogenous interfering substances in the serum samples.

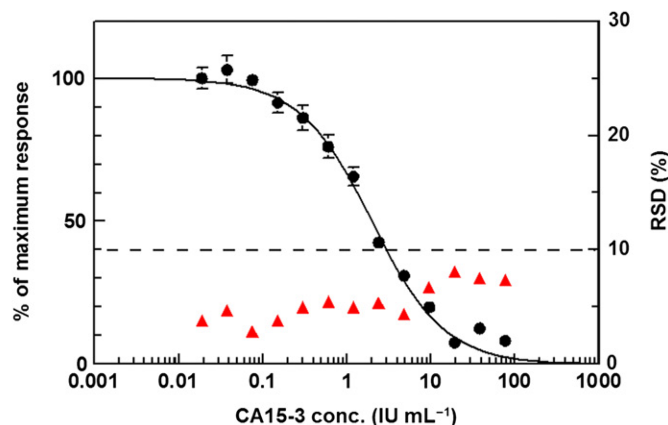

Fig. 3. Calibration curve (●) and precision profile (▲) of the proposed KinExA-based sensor for measurement of CA15-3. Varying concentrations of CA15-3 were mixed with anti-CA15-3 antibody (30 ng mL^{-1}). The reaction mixtures were further manipulated as described in the Experimental Section. The values plotted are mean ± SD of three determinations.

Table 1
Precision of the proposed KinExA-based sensor for measurement of CA15-3.

CA15-3 (IU mL^{-1})	Intra-assay Mean ± RSD[a] (IU mL^{-1})	Inter-assay Mean ± RSD (IU mL^{-1})
0.5	0.48 ± 5.1	0.51 ± 7.2
2	2.05 ± 4.5	1.94 ± 6.8
10	10.21 ± 3.8	9.86 ± 5.2

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

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

In this study, the ELISA was optimized for CA15-3 using the same antibodies as were used in the KinExA, to compare the sensitivities of the assays. The ELISA utilized a non-competitive sandwich assay format [23], which is the most commonly used format for the measurement of CA15-3. The features of this format are shown in Fig. 4.

The sample that contains CA15-3 is incubated and allowed to bind to an immobilized anti-CA15-3 antibody that recognizes a distinct antigenic determinant on the intact CA15-3 molecule. After a wash step, a labeled-anti-CA15-3 antibody, that recognizes another antigenic determinant on CA15-3 molecule, is added to the solid phase on which the immune complex has been formed. In ELISA, the wells are washed and a color signal is generated by a chromogenic enzyme substrate (Fig. 5).

The limit of detection was 2IU mL^{-1} for ELISA, compared to 0.2 IU mL^{-1} for the proposed KinExA. The working assay ranges were 3–200 mL^{-1} for ELISA, compared to 0.3–20 IU mL^{-1} for KinExA. It is obvious that ELISA gave less sensitive however wider assay range than KinExA.

In the sandwich ELISA, CA15-3 and the immobilized antibody should be incubated together until no further changes occur in the binding of CA15-3 to the immobilized antibody, and to allow the CA15-3 molecules in sample solution to overcome the mass

Table 2
Analytical recovery of CA15-3 spiked into three different batches of serum samples.

Spiked CA15-3 (IU mL^{-1})	Recovery[a](% ± RSD)		
	Batch A	Batch B	Batch C
0.3	105.8 ± 6.28	97.1 ± 5.82	100.2 ± 7.45
0.6	108.6 ± 5.21	101.7 ± 4.42	107.9 ± 6.21
1.2	105.4 ± 3.85	94.5 ± 3.98	105.1 ± 4.48
2.4	90.7 ± 4.12	99.3 ± 5.42	98.4 ± 3.58
4.8	99.4 ± 2.05	91.4 ± 4.51	100.1 ± 2.49

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

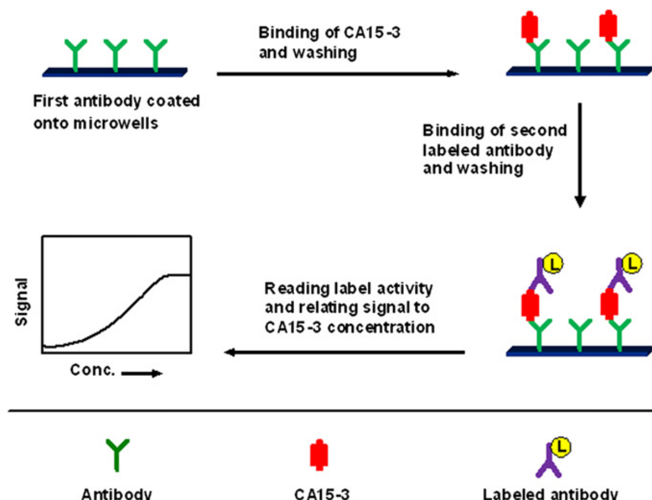

Fig. 4. Schematic diagram for the sandwich-ELISA for measurement of CA15-3.

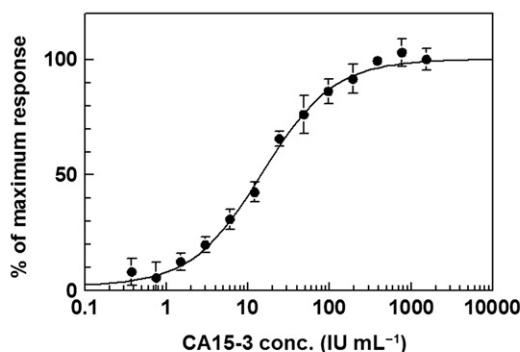

Fig. 5. Calibration curves for sandwich-ELISA for measurement of CA15-3. The involved procedures were described in the Experimental Section. The values plotted are mean ± SD of three determinations.

transport limitations attendant with the binding to the immobilized antibody.

The KinExA-based assay described herein (Fig. 2) represents an antigen inhibition assay where free and immobilized version of the antigen compete for a limited number of binding sites on the antibody. This format has the inherent high sensitivity of the competitive immunoassays. In addition, the antibody and free CA15-3 antigen were incubated and allowed to approach binding equilibrium in solution before subsequent exposure of the mixture to the immobilized CA15-3 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 CA15-3 antigen served merely as a tool to separate and quantify only those antibodies in the equilibrium mixture that bore unoccupied binding sites. Since the immobilized CA15-3 antigen has limited time to compete for antibody binding sites, the KinExA format created a more sensitive assay with a lower limit of detection than the reported ELISA [19–23]. 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) [29]. In addition, the high flow rate of the reagent through the beads minimizes mass transport limitations at the reaction surface. Furthermore, the entire protocol of the ELISA, including coating and blocking of the assay plates, required ~6 h, compared with ~1.5 h for KinExA.

4. Conclusions

A novel automated flow-based immunosensor employing the KinExA format has been developed and validated for the real-time measurement of the breast cancer biomarker CA15-3 in serum at concentrations as low as 0.2 IU mL^{-1}. The assay exhibited four noteworthy properties compared with the existing conventional ELISA: (1) avoiding the problems of mass transport limitations, and mobility effects, (2) measuring chemically unmodified CA15-3 in solution, thus avoiding the effect of modification on the analytical results, (3) providing high sensitivity with a lower limit of detection than the existing ELISA, (4) KinExA analysis with automated sampling reduced the total analysis time and increased assay convenience. The application of the proposed sensor is anticipated to be very beneficial in making proper medical decisions on the patient status. This information may ultimately limit the mortality rate among BC patients.

Acknowledgment

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

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