Research Article

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Bioanalysis

New analytical application of antibody-based biosensor in estimation of thyroid-stimulating hormone in serum

Background: Conventionally, ELISA is used to measure thyroid-stimulating hormone (TSH) for diagnosis of thyroid disease. In this study, an immunosensor-based, kinetic-exclusion analysis (KinExA) was used for TSH estimation. **Methodology**: A PMMA microbead column coated with TSH antigen is formed inside the flow cell. Samples consisting of mouse anti-TSH monoclonal antibody and TSH antigen complex in solution are passed over the beads and the unbound anti-TSH antibody is captured by the TSH-coated beads, followed by passing fluorescent-labeled antibody over the beads to generate signals for analysis. The limit of detection for the assay was 0.4 mIU l^{-1} and the precision was acceptable. **Conclusion**: The developed sensor was advantageous due to the automated nature and its convenience, without compromising the sensitivity for estimation of TSH.

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Keywords: biosensor • ELISA • thyroid disease • TSH

Tanveer A Wani*[1], Seema Zargar[2], Salma Majid Wakil[3] & Ibrahim A Darwish[1]

[1]Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, PO Box 2457, Riyadh 11451, Saudi Arabia

[2]Department of Biochemistry, College of Science, King Saud University, PO Box 22452, Riyadh 11451, Saudi Arabia

[3]Department of Genetics, King Faisal Specialist Hospital & Research Centre, Riyadh 11211, Saudi Arabia

*Author for correspondence:
Tel.: +966 114 679 841
Fax: +966 114 676 220
twani@ksu.edu.sa

Thyrotroph cells lying in the anterior pituitary secrete a glycoprotein known as thyroid-stimulating hormone (TSH) (approximately 28 KDa) [1,2]. TSH is a heterodimer and consists of α- and β- noncovalently joined subunits. α-subunit is common in four hormones: TSH, LH, FSH and HCG secreted by thyrotroph cells whereas, the specific biological activity of each hormone is provided by the β-subunit of these heterodimers [3,4].

The thyroid gland and its functions are controlled by the circulating TSH via specific receptors on the surface of the gland [5]. Thus, TSH stimulates the thyroid gland to produce thyroxine (T4) and triiodothyronine (T3), and modulate the metabolic processes [6]. Thyroid diagnosis and treatment of the thyroid-related diseases depends on the correct and precise estimation of TSH concentrations in blood. Various assays have been developed for the measurement of circulating TSH and these assays have helped to correlate the altered levels of circulating TSH with altered thyroid function and various metabolic disorders [7].

Radioimmunoassays (RIAs), with a detection limit of 1–2 mIU l^{-1} were the initial TSH assays developed. Even though currently accepted limits for TSH are above the detection limit of RIAs, but they had a limited use only in defining low TSH levels. Insensitivity limited the use of RIAs for TSH in clinical settings [8–11]. Immunoassays involving TSH specific monoclonal antibodies are currently used for estimation of low levels of TSH in blood [12]. Monoclonal antibodies are highly specific and the sensitivity of immunoassays is very high, hence, were used to measure very small amounts of TSH [11]. The principle behind this assay is that the monoclonal antibodies act at two different sites of the TSH antigen. In spite of the fact, different methodologies are used for the immunometric assays, however, primary antibody is generally attached to the β-subunit of the TSH molecule during blood analysis [13].

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A secondary antibody (labeled either with an enzyme, radioactive molecule, a fluorophore or a chemiluminescent molecule) acts as signal generator.

Even though ELISA provides a very high selectivity, there are certain drawbacks with this technique that include frequent washing and pipetting steps, long incubation times and sometimes less sensitivity which is to enhanced by using signal amplifiers. Keeping the above drawbacks in consideration, a new competent analytical methodology is needed for estimation of TSH. Antibody-based biosensors for biochemical estimations is great advancement of analytical technology [14–17]. These immunosensors are devices in which the development of antigen–antibody complex is noticed, and this interaction is converted into a signal via a signal transducer. The concentration of the analyte present is proportional to the signal generated. In this study, a new immunosensor-based assay was developed and validated for estimation of TSH in serum.

In order to measure the interaction of binding partners (antibody/antigen) in a solution, KinExA™ instrument provided an excellent platform [18–20]. In addition to the automation it also has other advantages like high sensitivity to quantify unchanged molecules in solution. In this study, a novel assay was developed to quantify TSH using KinExA platform. The KinExA format uses beads (approximately 10,000/column), thus providing a higher surface area and increases the opportunity to capture the free antibody. The surface area provided by the KinExA is approximately 260 mm^2 compared with the 64 mm^2 calculated for each microwell in the ELISA format [21]. The high flow rate of the reagent through the beads minimizes mass transport limitations at the reaction surface, which is an encountered problem in ELISA [22,23]. During this assay unmodified TSH molecules in solution were quantified instead of antigen or antibody immobilized on solid phase as is done in conventional ELISA assay. The KinExA methodology provides several advantages over ELISA: the system is automated and hence more convenient; antibody immobilization negative effects and mass transport were evaded; KinExA is more sensitive technology compared with ELISA.

Experimental

Instruments

The kinetic exclusion analysis instrument (KinExA 3200) was acquired from Sapidyne Instruments, Inc. (ID, USA). Millipore water purification system, Milli Q Advantage (MA, USA) was used for all water needs. The centrifuge (Biofuge Pico) was a product of Heraeus Instruments (Hanau, Germany). Nutating mixer (EM-36N microtube) was obtained from Taitec (Saitama-ken, Japan).

Materials

TSH and its mouse monoclonal antibody was obtained from MyBioSource (CA, USA). Secondary antibody DyLight™ 649-conjugated AffiniPure goat antimouse IgG were procured from Jackson Immuno Research Laboratories, Inc. (PA, USA). PMMA beads (140–170 mesh, 98 µm) were procured from Sapidyne Instruments, Inc. Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (MO, USA). Serum from healthy human volunteers was obtained from King Khalid University (Riyadh, Saudi Arabia), and stored at -20°C. Phosphate buffer saline (10×) was obtained from Bio-Basic, Inc. (Markham, Canada). All other chemicals used during the experiments were of analytical grade.

Procedures

Preparation of solutions

Blocking solution: 100 mg of BSA was weighed and dissolved in 10 ml of PBS to get a blocking solution concentration of 10 mg ml^{-1}.

Standard KinExA buffer: Standard KinExA sample buffer was prepared by adding 1 mg ml^{-1} BSA, 0.02% sodium azide to PBS and maintaining the pH at 7.4.

The secondary antibody: DyLight 649-conjugated goat antimouse IgG (1.5 mg ml^{-1}) was diluted with standard KinExA sample buffer to get a final concentration of (150 ng ml^{-1}). The secondary antibody solution was prepared fresh.

Coating of PMMA bead with TSH

TSH was directly coated on PMMS beads. Vials containing 200 mg of PMMA bead were coated with TSH. 1 ml of PBS containing 25 µg of TSH was added to the bead vial and kept on a nutating mixer for 2 h at room temperature (25 ± 2)°C. Later the beads were allowed to settle and the supernatant was pipetted out and replaced with blocking solution. The vial was again placed on the nutating mixer for another 1 h. The blocked beads were stored at 4°C in the blocking solution. The coated beads were stable for a period of 1 week.

Preparation of TSH samples

Blank human serum samples were spiked with TSH to acquire a calibration range of 0.05–100 mIU l^{-1}. These spiked samples were added to the tubes containing anti-TSH primary antibody 50 ng ml^{-1} in KinExA buffer. Since a possibility of nonspecific binding of the primary antibody to the microbeads in the column; BSA was added to the KinExA buffer to reduce the nonspecific binding. The resulting samples were filtered with syringe filters having pore size of 5 µm. The samples were loaded in the instrument and the instrument was equilibrated for a period of 1 h before the actual run of samples for analysis by the KinExA instrument.

Analysis on KinExA instrument

There are 13 sample inlets in the KinExA instrument and of these 13 inlets, 12 inlets were used for the aspiration of the samples (containing a definite concentration of TSH and its specific antibody). Inlet 1 contained the highest concentration of calibration standard and the inlet 12 the lowest. Inlet number 13 was used for blank sample which contained only the primary antibody without any antigen. A separate inlet provided in the KinExA instrument was used for aspiration of the secondary antibody (fluorescently labeled goat antimouse IgG antibody). Beads vials coated with TSH were poured in the bead holding bottle and added with 30 ml of PBS and attached to the particle reservoir and stirrer. The beads are charged into the flow cell twice to appropriate bead column height. The flow volume and time of flow of beads were altered to attain the required bead column. The flow cell was monitored with an inbuilt camera installed. The optimum height inside the flow cell was obtained at a flow rate of 1 ml min^{-1} with flow volume of 0.683 ml for 41 s. These optimized conditions were used to obtain a reproducible uniform bead column.

During each sample run 500 µl of equilibrated sample was aspirated for 120 s at flow rate of 0.25 ml min^{-1} and passed over the microbead column and was followed by a buffer wash consisting of of 125 µl of PBS for 30 s to automatically remove the excess of TSH molecules and unbound primary antibody and from the microbead column. In the next step, secondary antibody solution, 500 µl for 120 s (fluorescently labeled goat antimouse IgG) was aspirated and passed over the generated microbead column. This was followed by buffer wash of the microbead column to remove the unbound labeled secondary antibody and this buffer was consisted of 1.5 ml of PBS for 90 s at a flow rate of 1 ml min^{-1}.

Once all the run for the calibrators or unknown samples was completed, a duplicate run for all the samples was performed. KinExA 3200 instrument was run by KinExA Pro 20.0.1.26 software (Sapidyne Instruments, Inc.) provided with the instrument. The generated data were analyzed on Graph Pad Prism 6 (Graph Pad Software, Inc., CA, USA) and was fitted in the following equation to produce a calibration curve:

$$F = F0 - ([F0–F1]\ \{TSH\} / [IC_{50} + \{TSH\}])$$

where, F, F0 and F1 represent fluorescence signal at a definite known concentration of TSH, at zero concentration of TSH and at saturating concentration of TSH. IC_{50} represents the concentration of TSH which causes 50% inhibition of the signal. The unknown sample concentration was determined by interpolation of the standard curve.

Results & discussion

Features & operation of the proposed sensor

The working procedures for KinExA instrument are discussed elsewhere [24–28]. The concentration for the primary antibody for TSH was determined and optimized with the help of signal test. The optimized concentration of the primary antibody was fixed during the analysis with varying concentrations of the TSH antigen. The resulting antigen–antibody complex was passed over the bead microcolumn. Those TSH antibodies whose binding sites were unoccupied bind to the TSH immobilized on the beads and the anti-TSH antibodies whose sites were already occupied could not bind to the beads. The equilibrated TSH antigen–antibody complex is drawn past the bead microcolumn rapidly in order to minimize the contact time of the TSH antibody–antigen complex with the immobilized TSH. This short contact time of the complex with beads ensures that those antibodies whose binding sites are occupied are kinetically excluded and do not disrupt the equilibrium and interact with the immobilized TSH. The buffer wash step which is followed after the drawing of samples past the beads ensures to wash away the soluble reagents past the beads. Quantification of the anti-TSH antibody thus captured on the immobilized TSH can subsequently be achieved by the brief exposure of the immobilized TSH and its antibody complexes to a fluorescently labeled DyLight 649-conjugated AffiniPure goat antimouse IgG antispecies secondary antibody directed against the TSH antibody. The excess secondary antibody is washed away with a buffer wash step. The resulting fluorescence after removal of the excess secondary antibody is monitored with the help of photodiode present in the instrument.

Measurement of TSH by the proposed sensor

Once the bead microcolumn was produced, the instrument starts to generate the response which is a function of time. The response represents the various functions the instrument performs during the intervening period of (0-524 s) (Figure 1). The phase of response from (0–305 s) represent the time from aspiration of the equilibrated sample solution, passing of the sample over the beads and washing of the unbound TSH from the microbead column. The next phase (306–420 s) is drawing fluorescently labeled secondary antibody over the microcolumn and washing away of the excess label with buffer wash (421–524 s). The lowest curves represent the highest concentration of the calibration curve and the highest curve represents the blank sample which zero concentration of antigen and only contains the antibodies for TSH. The square wave was generated because of the transit of the fluorescently labeled secondary antibody when it passes through the bead column.

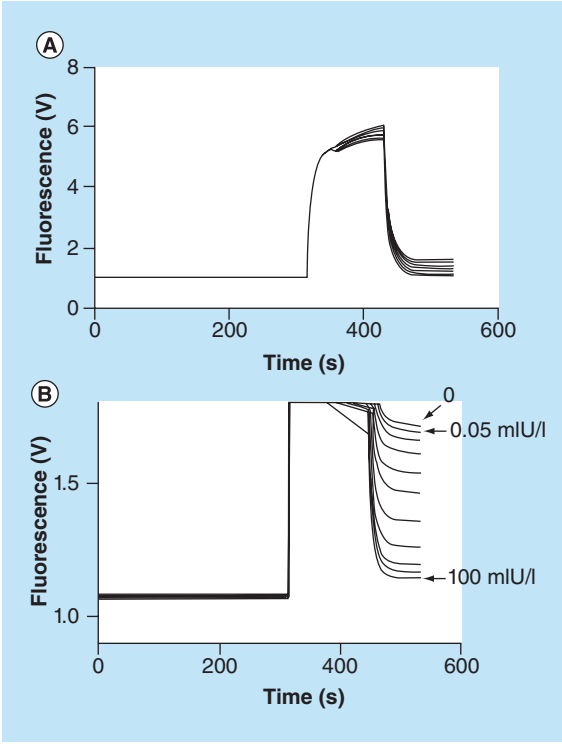

Figure 1. Instrument response as a function of time for various concentrations of thyroid-stimulating hormone. (A) Real raw trend-line fluorescence responses obtained by the KinExA™ instrument for varying concentrations of thyroid-stimulating hormone. **(B)** The same signals, however, they are presented on different scales for the time (s) and fluorescence (V).

The signal failed to return to background, indicating a small nonspecific binding of the fluorescently labeled secondary antibody to the beads. Once the excess when the excess fluorescently labeled secondary antibody was removed by from the beads by buffer wash, the final signal was the summation of the signal from the nonspecifically bound antibody and signal from the labeled secondary antimouse antibody specifically bound to the primary anti-TSH antibody caught on the beads. The calibration curve consisted of equilibrated solution of TSH from zero to saturation (Figure 1). The KinExA format provides a higher surface area 264 mm^2 compared with 64 mm^2 in one well of ELISA plate. The higher surface area maximizes the opportunities for the capture of free antibody. Also, there can be difference while coating the reagent on the microwell plates due to imprecise dispensing for the solutions, fluctuations in temperature, etc. leading to higher imprecision. The KinExA format uses automatic system to dispense the reagents or labels with high precision, thus avoiding the imprecision during experiments. These advancements lead to a better measurable response with low signal-to-noise ratio and better precision of the sensor than conventional techniques. ELISA involves multiple steps during sample processing but in case of KinExA, the sample processing is performed prior to the analysis only. Generally speaking, the multiple steps during the ELISA require approximately 4–5 h for the analysis and data generation without taking into regard the number of samples, whereas in KinExA the total time of analysis depends on the number of samples to be analyzed. The throughput of the KinExA instrument can be increased with the help of autosampler.

Optimization of KinExA signal for measurement of TSH

Optimization of the experimental conditions was performed for KinExA instrument by determining the 'net signal.' Net signal is determined when a certain concentration of primary antibody (anti-TSH antibody) binds to the beads on which TSH is immobilized. When no TSH antigen is added to the anti-TSH antibody solution, the binding sites on the anti-TSH antibody are in a free form to interact with the immobilized TSH coated on the PMMA beads and thus voltage produced by the KinExA instrument is called as '100% signal.' The third type of voltage signal that was determined was the 'nonspecific binding' (NSB). This voltage signal was generated when the solution contained neither the anti-TSH antibodies nor the antigen and the signal was due to the sample buffer and the labeled secondary antibody. The net signal is shown as below:

$$\text{Net signal} = [(\text{Signal } 100\%) - (\text{nonspecific binding})]$$

As is evident from the above equation that the reproducibility of the results depends on the low background noise. The principal reasons behind the high background noise are reagent adsorption to the microcolumn wall and inadequate washing. To reduce the influence of this background noise over the reproducibility of the results, all the 13 inlet lines for samples along with the label line (14th line) were fast rinsed and then kept for overnight wash as per the manufacturer's procedures [19].

Optimization of assay conditions for measurement of TSH by KinExA

As discussed in the introduction section that TSH is 28–30 kDa glycoprotein and consists of two subunits α- and β- which are noncovalently linked to each other, the α-chain consists of 92 amino acid sequence and the β-chain is composed of 118 amino acid sequence. Of these three carbohydrate chains, two (ASN-52 and ASN-78) are attached to the human α-subunit and the third carbohydrate chain is attached to the β-subunit.

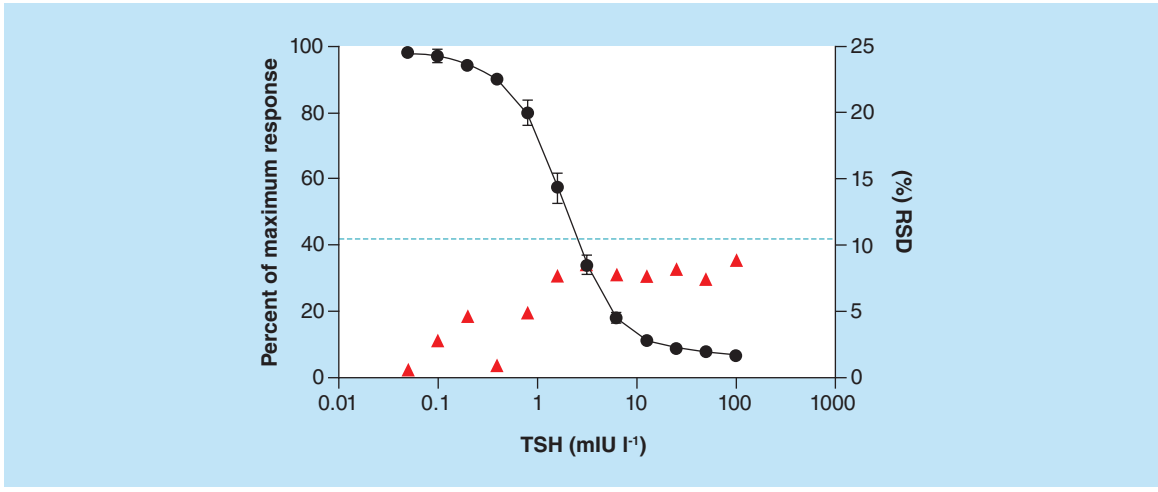

Figure 2. Calibration curve (circles) and precision profile (triangles) of the proposed KinExA-based sensor.

The macromolecular structure of TSH having a molecular weight of 28–30 kDa was expected to get TSH directly coated on the PMMA beads. The successful signal test showed that the TSH immobilized on the PMMA beads and also this immobilization had not altered the binding capacity of the TSH to its specific antibodies.

To determine the optimum concentration of TSH for bead coating, different concentrations of TSH antigen were tried ranging from 15 to 40 µg and the preliminary tests were also performed to get the optimum signal ideal for the analysis. After optimization, the concentration of 25 µg was found to be the best concentration for bead coating and produced the desired signal for analysis. Similarly, optimization was performed for the primary anti-TSH antibody ranging from 30 to 80 ng ml^{-1} and the secondary labeled antimouse IgG antibody ranging from 50 to 200 ng ml^{-1} and the optimum concentrations for the two were found to be 50 and 150 ng ml^{-1}, respectively. The preequilibration time during the analysis was also optimized between the range (0.25–1.5 h) subjecting to the analysis every 15 min to obtain the most suitable equilibration time.

In addition to the optimization of the concentration parameters, many instrumental parameters were also evaluated and optimized. These parameters included the sample volume and the secondary labeled antimouse IgG antibody and their flow rate at which they were drawn past the beads. The optimum values for both these parameters were found to be 500 µl, at a flow rate of 0.25 ml min^{-1}.

Validation of the KinExA assay

Calibration curve & detection limit

The calibration curve was generated for the determination of TSH as in (Figure 2). The calibration curve ranged from 0.05 to 100 mIU l^{-1} with correlation coefficient of 0.9982 on four parameter curve fit. Two runs were performed and the mean of the two runs was used for all the measurements. The limit of detection (LOD) of the immunosensor was that concentration which caused 10% inhibition of the maximum signal. Therefore, the LOD for the proposed sensor was 0.4 mIU l^{-1}.

Precision profile

According to the recommendations of immunoassay validation [29], two separate runs were carried out for the calibration standards. The precision results of the calibration standards revealed %relative standard deviation (RSD) of <10 for the entire calibration range. For intra-

Table 1. Precision of the proposed antibody-based biosensor for measurement of thyroid-stimulating hormone.

(mIU l^{-1})	Intra-assay	Inter-assay
	Mean ± RSD† (mIU l^{-1})	Mean ± RSD (mIU l^{-1})
0.2	0.20 ± 9.67	0.19 ± 9.20
3	3.06 ± 8.33	3.07 ± 6.03
20	19.51 ± 7.47	20.02 ± 7.71

†Values are mean of three determinations ± RSD.
RSD: Relative standard deviation.

Table 2. Analytical recovery of thyroid-stimulating hormone spiked into three different batches of serum samples.

Spiked TSH (mIU l^{-1})	Recovery† (% ± RSD)		
	Batch A	Batch B	Batch C
0.2	100.8 ± 9.09	98.3 ± 9.46	95.0 ± 8.15
0.4	108.3 ± 7.95	107.0 ± 7.73	106.6 ± 8.60
0.8	105.0 ± 6.73	105.2 ± 7.22	102.3 ± 6.34
1.6	94.6 ± 6.94	104.2 ± 7.31	95.2 ± 5.83
3.2	105.6 ± 5.13	106.6 ± 4.10	104.9 ± 5.28
6.4	102.0 ± 3.59	102.9 ± 4.50	101.0 ± 4.43

†Values are mean of three determinations,
RSD: Relative standard deviation.

and inter-assay precisions, three different concentrations of TSH were analyzed. For the intra-assay precision, three replicates for each sample were analyzed and for the inter-assay precision the same samples were analyzed in three different runs. Each run was performed in duplicate. The results obtained with the precision profiling were satisfactory and the RSD for the intra- and inter-assay precision ranged from 7.47 to 9.67% and 6.03 to 9.20%, respectively (Table 1).

Accuracy, selectivity & application of the proposed sensor

Recovery studies were performed to determine accuracy of the proposed sensor. Three different concentrations of serum samples in three different batches of serum were prepared within the calibration range of TSH. Each sample was run three-times and each run was in duplicate. The analytical recovery was defined as the ratio of the actual concentration of TSH found in the sample to that of spiked concentration and the recovery was expressed as percentage. The values for analytical recovery and RSD were between 94.6 and 108.3%, and 3.59 and 9.46% (Table 2). Also, these results showed no interference from any endogenous substances in the serum samples and the method was selective according to the guidelines for immunoassay validation [29]. The proposed method results have almost the same LOD (0.312 mIU l^{-1}) as those of com-

Executive summary

Background

- To develop an alternate immunosensor-based method, other than conventional ELISA for estimation of thyroid-stimulating hormone (TSH) in blood.

Experimental

- The immunosensor was based on kinetic exclusion analysis.
- Kinetic exclusion analysis was carried out with the help of KinExA™ 3200 instrument.

Results & discussion

- The linearity range for the developed and validated immunosensor for estimation of TSH in serum was 0.05 mIU l^{-1} to 100 mIU l^{-1}.
- KinExA format exhibited several advantages over the conventional ELISA technique and these advantages included:
- Automation of the instrument which reduced analysis time and convenience.
- The method was sensitive even without any chemical modification of TSH.
- The analysis of TSH 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 ELISA.

Conclusion

- The immunosensor for the measurement of TSH in serum was developed with the aim of higher sensitivity, faster analysis and convenience of automation of the KinExA instrument. The developed method was quite sensitive and could measure up to 0.4 mIU l^{-1} of TSH in serum. The proposed method had several advantages over the conventional ELISA in evading mass transport limitations and mobility effects, measuring chemically unmodified TSH in solution, high sensitivity with a lower limit of detection. The automated sampling, avoiding multiple pipetting and washing steps as in ELISA reduced the analysis time considerably and increased the convenience in performing the assay. Due to the sensitivity, convenience and automation, the developed sensor can be highly useful in medical diagnostic laboratories.

mercially available ELISA kits and thus can be used as an alternate format for estimation of TSH in serum [30].

Conclusion & future perspective

Kinetic exclusion assay KinExA 3200 instrument is basically an automated flow fluorimeter which has been manufactured with the intention of fast quantification and separation of free unbound antibody-binding sites present in reaction mixtures of free antibody, free antigen and antibody–antigen complexes. Ideally, the assays developed for the estimation of antibody–antigen binding interactions need to be accurate, sensitive and convenient, however only the accuracy being the most essential component of the three. The kinetic exclusion assays have all the three qualities of being accurate, have high sensitivity and convenient because of their automation. In addition to immunoassays, the instrument can be used to determine the equilibrium dissociation constants and individual kinetic constants. In future it is hoped various complex biochemical processes even at molecular levels could be understood by the science community with widespread use of KinExA assays. Also, the KinExA biosensor assays can be highly useful in medical diagnostic laboratories because of their convenience, automation and sensitivity.

Financial & competing interests disclosure

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