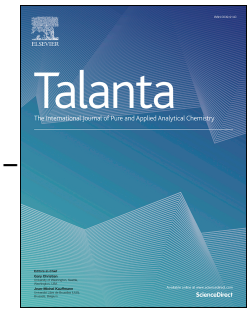

Novel approach to the measurement of antithyroglobulin antibodies in human serum – application of the Quartz Crystal Microbalance sensors

Lidija S. Vrhovac, Sonja A. Šelemetjev, Saša Vatić, Aleksandar Mitrović, Jelica R. Milošević, Aleksandar.Đ. Lolić, Anđelo D. Beletić, Natalija Đ. Polović

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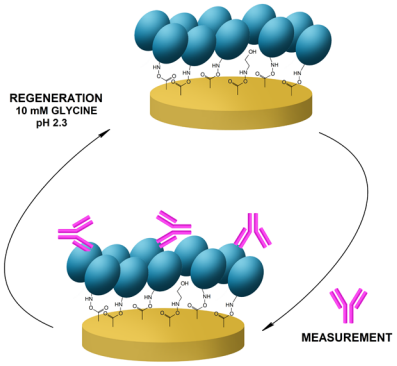
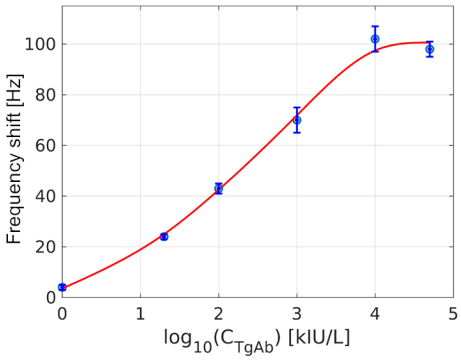
Credit Author Statement

LSV did Methodology, part of the investigation (QCM), parts of visualization and wrote parts of the Original draft
SAŠ did part of the investigation (RIA), wrote parts of the Original draft and provide the resources
SV did parts of visualisation and wrote parts of the Original draft
SAM provided the resources
JRM did validation
AĐL did formal analysis, and Review & Editing
ADB did Review & Editing
NĐP did Conceptualization, Methodology, provide the resources and Review & Editing





Title: Novel approach to the measurement of antithyroglobulin antibodies in human serum – application of the Quartz Crystal Microbalance sensors

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Abstract

Measurement of antithyroglobulin antibodies (TgAb) is an inevitable laboratory tool in the management of thyroid gland diseases. Currently available immunoassays still have limitations underlying the necessity of the introduction of fast, sensitive, and label-free technologies. Our aim was to develop a method for TgAb measurement in human serum based on the quartz crystal microbalance (QCM) technology. We immobilized thyroglobulin on the surface of Attana LNB Carboxyl sensor chip®, prepared standard curve covering the range of 1-50000 kIU/L, and established optimal measurement conditions. The validation included determination of the detection limit (LOD), functional sensitivity, linearity, precision, as well as the comparison with the results of the radioimmunoassay (RIA). The LOD and functional sensitivity were 4.2 kIU/L and 4.7 kIU/L, respectively. The method was linear in the range of 20 - 10000 kIU/L. The regression equation for comparison with RIA was $C_{QCM} = 1.0056 \bullet C_{RIA} - 24.2778$, whereby no significant proportional or systematic difference was present. There was a good agreement with RIA in the classification of patients according to the clinical significance of the results. The developed method has advantages over currently available assays in terms of better LOQ, a higher upper limit of linearity, and precision.

The characteristics of the developed method unambiguously show that the application of the QCM biosensors offers a highly reliable novel approach for the measurement of TgAb in human serum.

Keywords: Thyroglobulin, antibodies, human serum, biosensor, quartz crystal microbalance

Introduction

Thyroglobulin (Tg), the most abundant protein of thyroid follicles, has an essential role in the biosynthesis of thyroid hormones [1]. Antibodies produced against thyroglobulin (TgAb) are one of the most important biomarkers of autoimmune diseases of the thyroid gland [2] and some types of thyroid gland cancers [3].

Throughout the last couple of decades, the methodology for TgAb measurement has significantly evolved, achieving a reduced time of analysis and improved sensitivity. Pivotal measurements of serum TgAb began in the middle of the last century [4]. Those assays were qualitative and based on complement fixation, passive particle agglutination, immunodiffusion, and indirect immunofluorescence tests. During the 1970s, these techniques were surpassed by semiquantitative passive hemagglutination tests [4]. Later on, more sensitive competitive radioimmunoassays were developed, using ^{125}I labeled thyroglobulin. Having superior sensitivity than previously used assays, radioimmunoassays are still in use in clinical laboratories [5–8]. In the last decades, most clinical laboratories have moved on to automated, non-isotopic, non-competitive assays [2, 9, 10]. Nevertheless, these methods are characterized by significant variability in limits of detection and quantitation, reference values, as well as the unsatisfactory concordance [2].

For these reasons, there is a significant demand for fast, sensitive, and label-free assays for the measurement of TgAb. The quartz crystal microbalance (QCM) technology offers reliable opportunities for their development. The surface of the quartz crystal is chemically modified, which allows immobilization of a high spectrum of compounds. The frequency of vibration of quartz crystal depends on the total mass of the sensor crystal, meaning that in cases of the addition or loss of mass from the sensor surface, the frequency changes. Since the sensor system detects the mass change, there is no need for compound labeling. So far, QCM detection systems were adapted for the analysis of volatile organic compounds and toxic gases [11]. Other than that, QCM biosensors for different proteins have been developed, among which are: serum albumin [12], prostate-specific antigen [13, 14], C-reactive protein [15] and epidermal growth factor receptor [16].

In this article, we describe the development and validation of a method for the rapid measurement of TgAb in human serum based on QCM.

Materials and Methods

Materials and Equipment

Biosensor measurements were performed on Attana 200® (Attana®, Stockholm, Sweden). The instrument is automated, and all components, including running and evaluation software, are provided by the manufacturer. Degasser, temperature controllers, and pumps with the flow-rate range 1-150 μL/min are integral parts of the instrument. Sensor chips and all chemicals needed for immobilization, namely 1-ethyl-3-(3 dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysulfosuccinimide (sulfo-NHS) and ethanolamine solutions, were purchased from Attana (Attana®, Stockholm, Sweden). Tg, TgAb (rabbit polyclonal antihuman Tg), and serum samples were obtained from the Institute for Application of Nuclear Energy (INEP), University of Belgrade (Belgrade, Serbia). PreciControl ClinChem Multi 1 (lyophilized control based on human serum) was purchased from Roche® Systems Ltd (Oswestry, United Kingdom). All other chemicals were commercial products of analytical grade and were used without further purification.

Immobilization of Tg

Thyroglobulin was immobilized on the Attana LNB Carboxyl sensor chip. The sensor chip used has AT-cut crystals (quartz cut at an angle 35° 15’ from the Z-axis) and the fundamental frequency of around 10 MHz. Chip was re-hydrated in the running buffer (PBS/Tween 20, 50 mM phosphate buffer saline containing 0.005% Tween 20) for 1 hour. Immobilization was performed at 22°C with a flow rate of 10 μL/min. The sensor chip surface was activated with EDC/sulfo-NHS according to the manufacturer’s instructions with some modifications. Briefly, the water solution of 0.1 M EDC and 0.05 M sulfo-NHS was injected twice over the sensor chip for 450 s. Thyroglobulin solution (0.1 μg/mL) in saline solution was 9 times diluted with 50 mM acetate buffer pH 3.5 to shift the pH. The obtained solution with protein concentration 900 ng/mL was injected over the sensor chip for

450 s in the volume of 75 µL. This step was repeated three times. The surface was then blocked with 1 M ethanolamine throughout two 300 s long steps. The sensor chip was rinsed twice with a regeneration buffer (10 mM glycine, pH=2.3) for 20 s, and after that, the new baseline was recorded in the running buffer.

The sensor coverage by thyroglobulin was calculated using the Sauerbrey equation, which describes the relationship between the change in frequency and the change in adsorbed mass [17]:

$$\Delta m = \frac{-\Delta f A \sqrt{(\rho_q \mu_q)}}{2 f_0^2} \quad (1)$$

where Δm (gcm^{-2}) is the adsorbed mass per unit area, Δf the frequency change, A the area of the gold surface (0.159 cm^2), f_0 the fundamental mode resonant frequency of the crystal (~10 MHz), ρq the density of quartz (2.648 gcm^{-3}) and μq the shear modulus of quartz (2.947x1011 gcm^{-1}s^{-2}).

TgAb detection assay – Standard series/curve

Standard solutions of TgAb (1, 20, 100, 1000, 10000 and 50000 kIU/L) were prepared in 75% commercial control human serum with the addition of PBS and Tween 20 (to a final concentration of 0.125%). Standard solutions were centrifuged (14000 rpm, 4°C, for 1 h) and then injected with a flow rate of 20 μL/min for 105 s (Figure 1). Commercial human serum (75%) in PBS/Tween 20 was used as blank. After each injection sensor chip surface was regenerated using a regeneration buffer (20 s, 20 μL/min). Measurements were performed in triplicates.

Limits of blank and detection, functional sensitivity and precision

Limit of detection (LOD) was determined according to CLSI Protocol EP17-A [18]. First, the limit of blank (LOB), defined as the mean of the blank sample +1.645 times the standard deviation (SD), was calculated. LOD is defined as LOB + 1.645 times the SD of the lowest analyte concentration. The functional sensitivity as the lowest TgAb concentration measured with the coefficient of variation (CV) ≤ 20% [2].

Repeatability was tested by consequently measuring the same sample of defined concentration (500 kIU/L) within the linear range (20; 100; 1000 and 10000 kIU/L) for five times within the same day, during 20 days period according to CLSI Protocol EP5-A2 [19]. Reproducibility was evaluated by repeating the measurement of the same four samples in triplicates every four months during 13 months period (the first, fifth, ninth, and thirteenth month) [19]. The sensor chip was sealed with PARAFILM® M (Sigma-Aldrich, St. Louis) and stored in a humid environment at 4°C between measurements.

Concordance between RIA and QCM assay

TgAb in human sera samples (N=15) were measured both with standard radioimmunoassay (RIA) and QCM assay.

This study was performed on the leftover of the anonymized sera specimens collected for routine analysis, so the informed consent was not necessary. The study was approved by the Ethics Committee of INEP (No. 02-114/1) according to the guidelines (No.# 02-832/1), which conforms with the Helsinki Declaration, 1975 (revised 2008).

RIA protocol

The assay is based on the binding of serum TgAb with ^{125}I-labeled human Tg, resulting in the formation of the radioactively labeled immune complexes, which are then precipitated with 9% polyethylene glycol (PEG). The unbound molecules of labeled Tg are removed by aspiration and rinsing. The measured radioactivity is directly proportional to the concentration of TgAb. At the same time as tested serums were analyzed, standards of defined concentration of TgAbs were used for standard curve creation. Concentrations of TgAb standards were equal to those in QCM assay, only the bovine serum was used as the diluent [20].

QCM protocol

Patients' sera samples were diluted with PBS/Tween20 to the final concentration of sera 75% and Tween 20 of 0.125%. The mixture was centrifuged at 14000 rpm, 4°C, for 1 h. Measurements were performed under the same conditions as described for standard solutions.

Concordance was statistically evaluated with Bland-Altman, Passing-Bablok, and Inter-rater (Kappa) agreement tests, using MedCalc® v.12 software.

Results and discussion

Immobilization of Tg

Before immobilization, the LNB Carboxyl sensor chip was re-hydrated in the running buffer, and the baseline was recorded for 2 hours (until the change in frequency was lower than 1 Hz within 10 minutes). Chemistry of immobilization is shown in Figure 1A. Exact recorded signals are shown in Figure 1B. To ensure sufficient Tg immobilization, the activation step was performed twice (signals 1 and 2), and protein binding three times (signals 3-5). Blocking of the surface was performed twice (signals 6 and 7), as well as the regeneration step (signals 8 and 9). Immobilization response, R_L was determined from the presented graph.

Experimental frequency change, Δf was 255 Hz, indicating the mass of immobilized thyroglobulin was 179 ng when calculated using the Sauerbrey equation [17]. Obtained mass suggests successful immobilization as 202 ng was applied to the crystal in three binding steps, and the total efficiency of immobilization is 88%.

TgAb detection assay

The developed assay is based on the mechanism of binding between TgAb and Tg immobilized on the sensor chip, resulting in the change of oscillation frequency, proportional to TgAb concentration. Dissociation of bonded antibodies is achieved by rapid pH change from 7.4 to 2.3,

which leads to the breakdown of salt bridges stabilizing the binding complex due to protonation of negatively charged amino acid residues. Even though low pH is known as a destabilizing for protein native fold, the irreversible changes on protein surface require aggregation [21], and in the case of proteins immobilized on a solid support, this effect is prevented, and proteins are stabilized [22]. Thus, short contact with 50 mM glycine buffer pH 2.3 resulted only in the dissociation of antibodies without affecting immobilized protein. After dissociation, the same Tg chip can be used for another measurement (Figure 2). Representative sensorgrams of 4 different samples in duplicates are shown in Figure S1. The height of each peak represents the signal corresponding to the frequency change of vibration of sensor crystal. Constant baseline (less than 1 Hz of change in 10 minutes) between measurements indicates efficient regeneration of the thyroglobulin sensor chip surface. We performed over 350 measurements with the same chip with satisfactory behavior, baseline frequency, and response.

Detection of TgAb

Figure 3 shows the calibration curve obtained by detecting frequency changes of six anti-thyroglobulin antibody standards dissolved in 75% commercial standard human serum containing 0.125% Tween 20 after retracting frequency change of the blank.

LOD, functional sensitivity and linearity

The LOD and the functional sensitivity of the assay are quoted in Table 1. Detection signal, ν increases linearly with the logarithm of TgAb concentration according to the equation: $v = 28.7495 \cdot log(C_{TgAb}) - 14.2874$, in a wide concentration range of 20 - 10000 kIU/L ($R^2 = 0.998$) (Figure S2) and the CV between 4 and 7% across the range.

Precision

The results of the precision evaluation are presented in Table 1 as coefficients of variation (intra- and inter- corresponding to repeatability and reproducibility, respectively). The signals obtained for four samples during 13 months period are presented in Figure 4. The stability of detected signals, with similar precisions within the linear range, proves that the same sensor chip can be reused for multiple measurements over at least 1 year. These results also support the thesis of thyroglobulin stability during short regeneration steps in low pH.

The content of Table 1 indicates that the developed method has better performances than currently used assays regarding LOD and LOQ, as well as a higher linearity range. Data about precision, comparable with already available assays, can introduce an additional advantage because the QCM technology allows for the multiple reuses of sensor chips. Also, the developed method can be easily automated, but even with manual measurements, it is rather fast. In this work, the turn-around time was about 10 minutes, so the turn-around time cannot be reliably compared with automated assays in clinical laboratories. However, the manual preparation of the biosensor and the sample pretreatment might be considered as limitations.

Comparison between RIA and QCM

Bland-Altman difference plot (Figure 5A) shows that the relative difference between methods was acceptable for the majority of samples. Even in exceptions, the result of bot assays was far below the threshold of 100 kIU/L (RIA). Passing-Bablok regression line (Figure 5B) had slope (95% confidence interval (CI)) of 1.0056 (0.8957 to 1.1481) and intercept (95% CI) -24.2778 (-35.9259 to 1.2553) which excluded the presence of a significant proportional or systematic difference. Finally, the inter-rater agreement test gave kappa value (95% CI) of 0.865 (0.611-1.000), which indicates good agreement between the results of RIA and QCM in the context of classification of patients using the threshold of 100 kIU/L.

Conclusion

The characteristics of the developed method unambiguously show that the application of the QCM biosensors offers a highly reliable novel approach for the measurement of TgAb in human serum. The QCM based assay has advantages over the currently available ones in terms of better LOQ, a higher upper limit of linearity, and precision. Also, there is a good agreement with RIA in the classification of patients according to the clinical significance of the results.

Acknowledgements

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Table 1. Analytical performances of different TgAb immunoassays. LOD-Limit of Detection, LOQ-Limit of Quantitation, RIA-Radioimmunoassay, NC CLIA- Non-competitive chemiluminescence immunoassay, NC FIA- Non-competitive fluoroimmunoassay, QCM- quartz crystal microbalance, *Functional sensitivity defined as TgAb concentration with total CV≤20%, nd - not declared

Assay type		LOD (kIU/L)	LOQ (kIU/L)	Assay range (kIU/L)	Precision (%)		Reference
					intra	inter	
RIA		6.0	30	nd	8.3	12.8	[20]
NC CLIA	Assay N^{o}1	10	30*	10-500	2.9-5.5	1.8-2.0	[2]
	Assay N^{o}2	2.2	nd	20-3000	3.2-4.9	4.6-5.8	[2]
	Assay N^{o}3	5	10	5-5000	2.3-3.2	4.4-8.9	[2]
	Assay N^{o}4	5.152	5.152	5.152-3000	1.8-4.6	nd	[2]
	Assay N^{o}5	10	nd	10-2800	2.8-9.1	5.2-9.8	[2]
NC FIA		12	nd	12-4794	3.3–5.6	2.6–6.5	[2]
QCM		4.2	4.7*	20-10000	1.3-4.2	2.1-4.6	This article

Figure captions

Figure 1. A) Schematic representation of thyroglobulin immobilization on LNB Carboxyl sensor chip. B) Frequency change recorded during immobilization (Steps 1 and 2 – activation; 3-5 thyroglobulin binding; 6 and 7 – blocking with ethanolamine and 8 and 9 – regeneration).

Figure 2. A) Schematic representation of the assay principle. B) Representative sensorgram of one sample. Binding of antibodies from the sample to thyroglobulin immobilized to chip surface induces frequency change that can be recorded (1). Full dissociation of antibodies is achieved by pH shift, regenerating the surface for another measurement (2).

Figure 3. Calibration curve obtained with a series of TgAb solutions (1–50000 kIU/L) (mean value ± standard deviation of triplicates).

Figure 4. Measured signals for four samples (mean value ± standard deviation of triplicates). Measurements were performed over 13 months.

Figure 5. A) Bland-Altman plot of differences between RIA and QCM. B) Passing-Bablok regression line for comparison of RIA and QCM.

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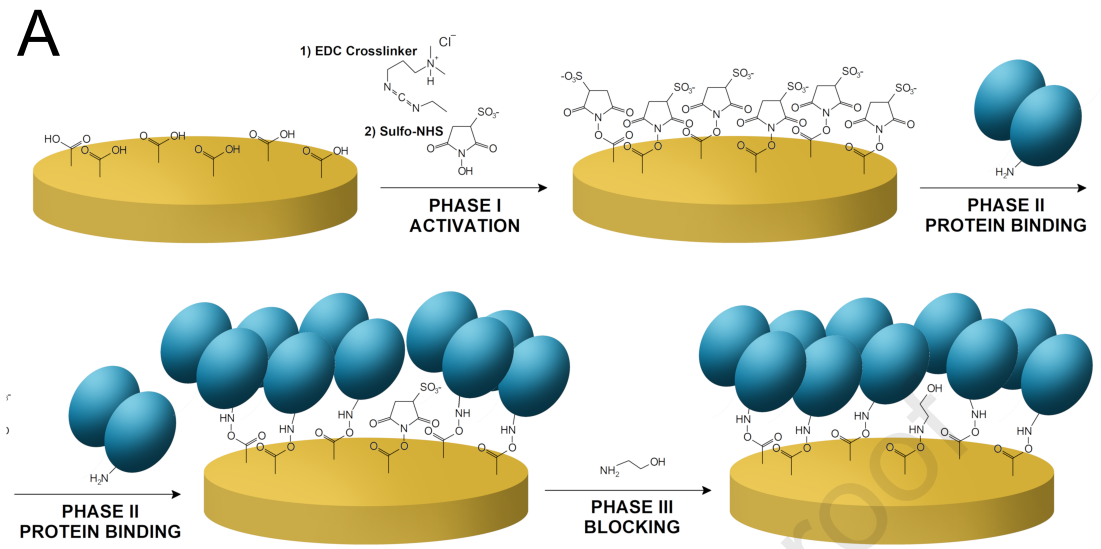

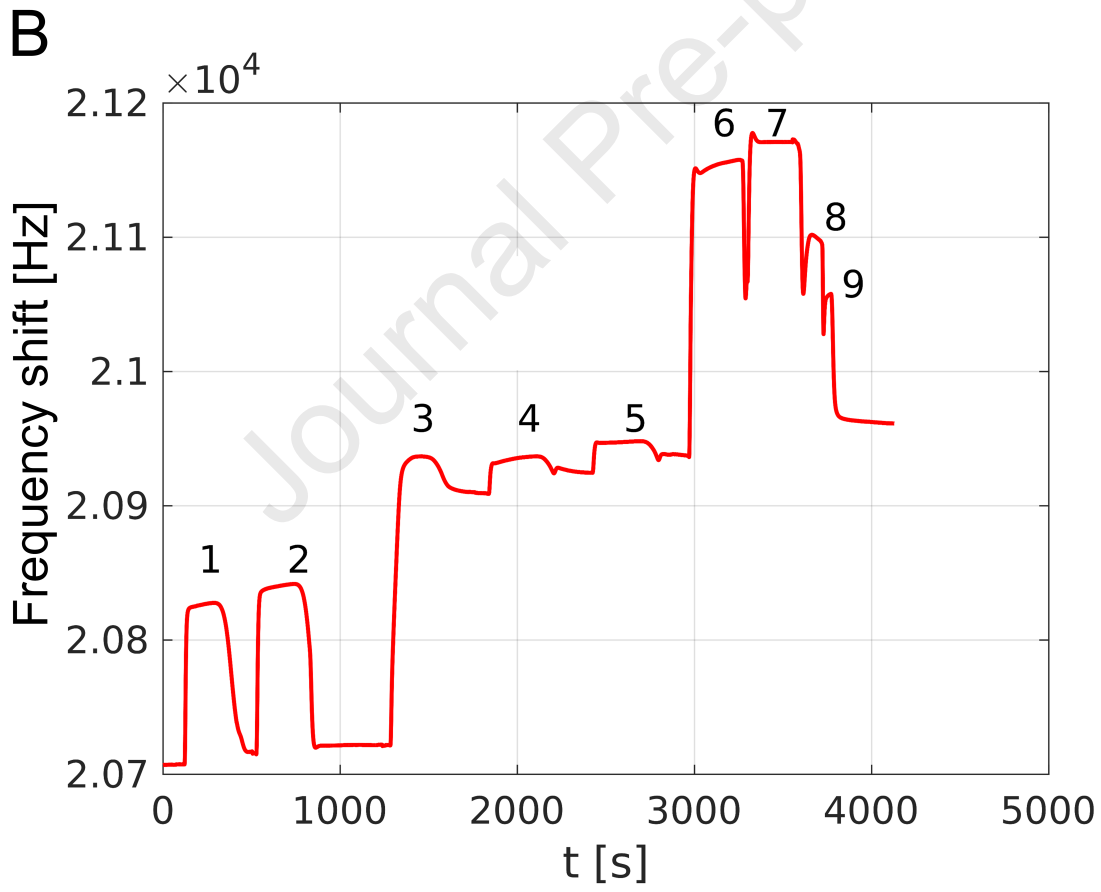

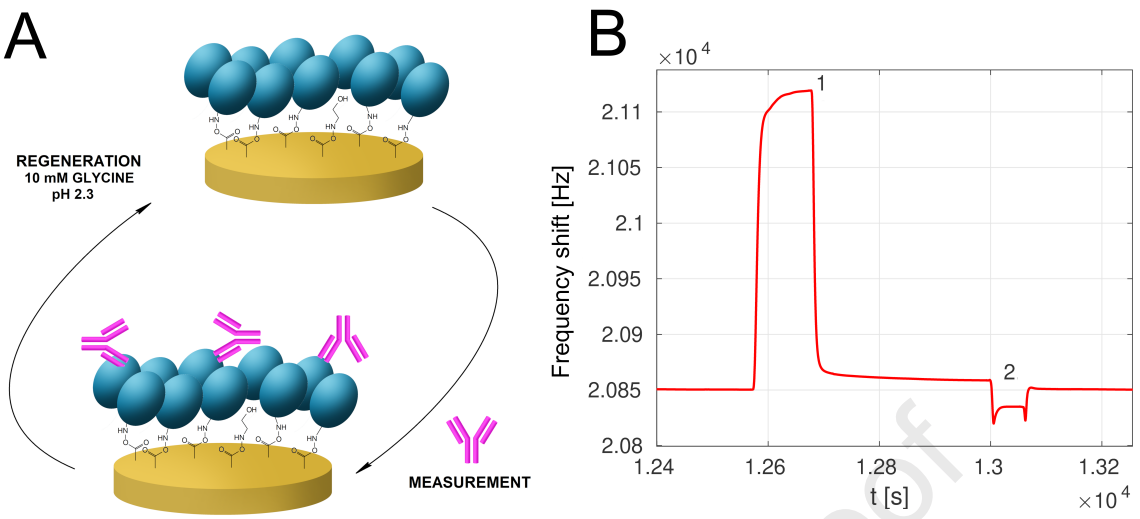

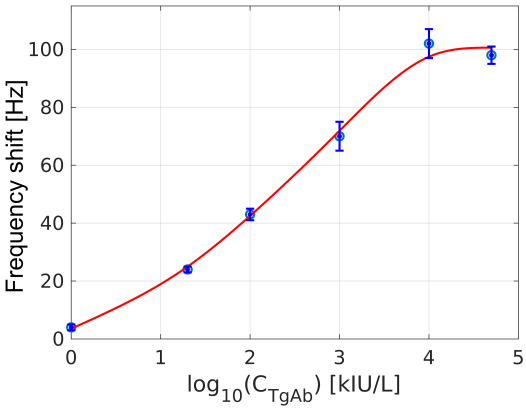

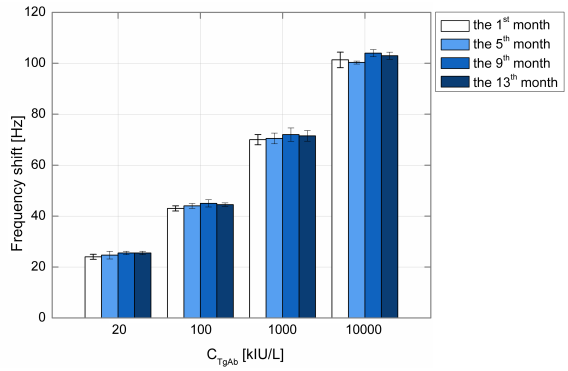

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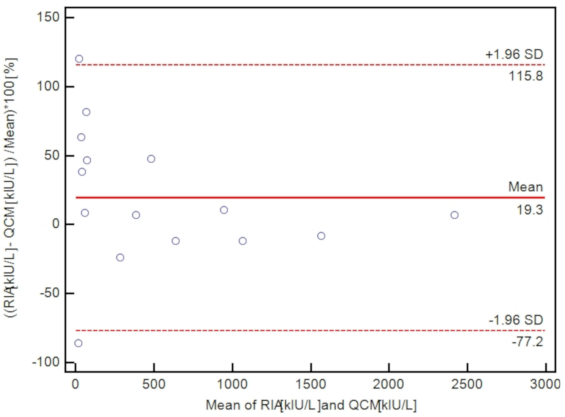

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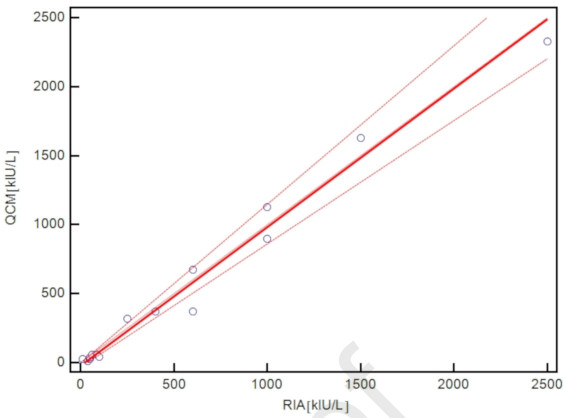

Highlights:

- A novel QCM biosensor method for TgAb measurement is established
- Measurement results are in good correlation with the standard RIA method
- The assay shows low LOD, extended upper limit of linearity and high precision
- The method is applicable for human sera analysis

Declaration of interests

X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: