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Short Communication

Piezoelectric Immunosensor for Tissue Transglutaminase Antibodies Determination for Celiac Disease Diagnostic and Comparison with ELISA Method

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Celiac disease (CD) is a hereditary disease characterized by occurrence of IgA class antibodies against tissue transglutaminase (anti-tTG) in serum. In this work, we compared standard Enzyme-Linked Immunosorbent Assay (ELISA) with a quartz crystal microbalance (QCM) measuring system for determination of anti-tTG. The new immunosensor is based on immobilized tTG in an open conformation (open-tTG) and optimized for assay of IgA anti-tTG with limit of detection 13.3 RU/ml and good specificity and stability over time. Testing serum samples of patients suffering CD showed suitability of the prepared immunosensor for practical performance. Compare to ELISA requiring skilled personnel and expensive equipment, the newly constructed measuring system for measurement of QCM sensor is cheap, easy to prepare and does not require any special skill thus it can be introduced into any laboratory or general practitioner office.

Keywords: Celiac disease; immunosensor; piezoelectric sensor; IgA antibodies; human tissue transglutaminase

1. INTRODUCTION

Piezoelectric effect was first described at the end of 19th century by Currie brothers. It´s based on principle that piezoelectric crystal generates voltage when it is pressed and vice versa. During application of voltage on piezoelectric crystalThe mechanical deformations produce oscillations during application of voltage on piezoelectric crystal. Measure oscillation frequency is a routine process and the change of oscillations is directly proportional to mass bound on surface of crystal according to Sauerbrey equation [1].

Big effort has been focused on development of biosensors for applications in various fields e.g. environmental control [2-4], food control [5-8], clinical diagnostics [9,10] etc. Demand on sensitive method led researchers for construction of QCM biosensors which were used in many applications [11-13]. QCM biosensors allow direct detection of biochemical interaction without following labeling and moreover, fast response, easy handling and portability are the other advantages known for the use of QCM sensors. QCM sensors can be divided into immuno-, enzyme-, DNA-, and haptasensors according type of the used receptor macromolecule [14]. Currently, QCM immunosensors belong to the most applied gravimetric biosensors and many of QCM immunosensors have been described in recent years [15-17]. Main benefits of QCM-based immunosensor are considered in simplicity and price as initial cost is lower comparison to standard immunoanalytical methods (e.g. ELISA).

CD is a hereditary autoimmune disease caused by gluten intolerance. HLA alleles DQα1*0501/DQβ1*0201 are playing important role in etiology of this enteropathy. Nowadays, CD presents one of the most frequent hereditary diseases in the general population [18]. Abdominal pain, nausea and diarrhea belong to main symptoms of CD. Diagnosis is based on serological assay of anti-tTG in IgA class which is sensitive and specific marker of CD diagnosis. In the case of IgA deficiency, there is possibility to determinate anti-tTG in IgG class completed with assay of antibodies against deaminated gliadin peptide (anti-DGP). Important part of diagnosis is positive histological finding in duodenum which together with serology confirms disease and in opposite way, positive histology should be confirmed by serological testing [19-22].

Standard method for quantification of anti-tTG is ELISA although there is possibility to quantify anti-tTG by other immunochemical and other methods as well [23,24]. Most of the methods use tTG as an antigen, recently, open-tTG was used in fabrication of QCM-immunosensor for determination of anti-tTG in CD diagnostic [25] and authors claimed that it better simulates conditions in body during inflammation than the use of tTG. Here, we present piezoelectric immunosensor with bound open-tTG for determination of IgA anti-tTG and compare it with standard ELISA protocol. Unavailability of commercial system for measuring piezoelectric crystal frequency in our country led us to construct of our measuring system which is cheap and easy to prepare in any laboratory.

2. MATERIAL AND METHODS

2.1. Chemicals

Human tTG in open conformation (250 µg/ml) was supplied by Zedira (Darmstadt, Germany), anti-tTG 2 IgG antibody produced in rabbit, anti-rabbit IgG (tagged with 6 nm gold particle) produced in goat was obtained from Abcam (Cambridge, UK). ELISA kit for anti-tTG IgA assay was supplied by EUROIMMUN (Lübeck, Germany). Immunoglobulin A from human serum, 11-mercaptoundecanoic acid (11-MUA), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), bovine serum albumin (BSA), sodium chloride, calcium chloride, ethylenediaminetetraacetic acid, DL-dithiothreitol, Tween-20, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), phosphate buffer saline (PBS) pH 7.4 (138 mmol/l NaCl and 2.7 mmol/l KCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (96%) was obtained from Lach-Ner (Neratovice, Czech Republic). Enzyme buffer (pH 7.2) was prepared according supplier

recommendation, i.e. 20 mmol/l Tris-HCl, 150 mmol/l sodium chloride, 10 mmol/l calcium chloride, 1 mmol/l ethylenediaminetetraacetic acid and 1 mmol/l dithiothreitol. Washing buffer was prepared from phosphate buffer saline pH 7.4 with addition of Tween 20 up to final concentration 0.05 % w/w.

2.2. QCM measuring system fabrication

All measurements were performed by own prepared measuring system (Fig. 1) consisted from rigid base with two horizontally placed springs put on copper thorns with 20 mm distance between and connected with oscillation generator (International Crystal Manufacturing Company, Oklahoma City, OK, USA) and frequency counter (Aim and Thurlby Thandar instruments, Huntingdon, UK) capturing frequency in 0.3 sec interval. Parameters of the used springs are follows: length 28 mm, diameter 4 mm, thickness of wire 0.2 mm, toughness 600 N/m. QCM with basic oscillation frequency 10 MHz, 20 mm in diameter with 7 mm gold electrode on chromium core on the both sides and 166 µm thick were supplied by Krystaly (Hradec Kralove, Czech Republic).

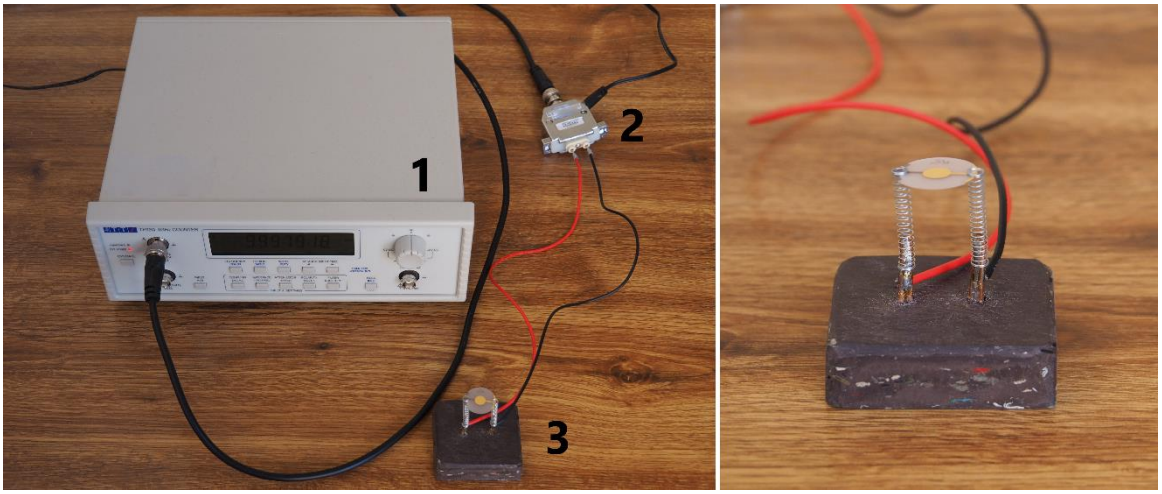

Figure 1. Complete arrangement of the prepared QCM measuring system – left picture, 1 – Frequency counter, 2 – Oscillation generator, 3 – Base with chrome springs. Detail of crystal inserted in the springs – right picture.

2.3. Immunosensor preparation

In the first step of immunosensor preparation, QCM crystal surface was cleaned by immersing it into 96 % v/v ethanol for 30 min. Crystal was then placed into solution of 15 mmol/l 11-MUA ethanol solution and shaken for 23 h (510 rpm) on PST-60HL shaker (Biosan, Riga, Latvia) to create self-assembled monolayer (SAM). After that, crystal was washed by ethanol to remove unbound 11-MUA. Then, created SAM was activated by 100 µl of 0.1 mol/l EDC and 0.1 mol/l NHS mixture for 1 h. After activation step, 30 µl of 25 µg/ml open-tTG was applied onto sensor surface for another hour and then blocking of remaining functional groups of MUA and EDC/NHS was performed by 15 min shaking (380 rpm) of QCM crystal with enzyme buffer. Blocking of non-specific surface interactions was done by 6 % BSA solution shaken for 1 hour. After the blocking steps, QCM surface was shaken in washing buffer

for 5 min (380 rpm) and frequency of dry crystal was measured. 30 µl of IgA anti-tTG or sample were then introduced onto sensor surface for 1 h. Then, electrode was shaken in washing buffer for 10 min (380 rpm) to remove unbound antibodies and 30 µl of secondary antibody was added for another hour to amplify measured signal. After washing step (5 min, 380 rpm), oscillation frequency of dry crystal was measured and difference before and after adding of antibodies was calculated. This difference is directly proportional to concentration of anti-tTG in the solution. Scheme of the prepared immunosensor is depicted on Figure 2.

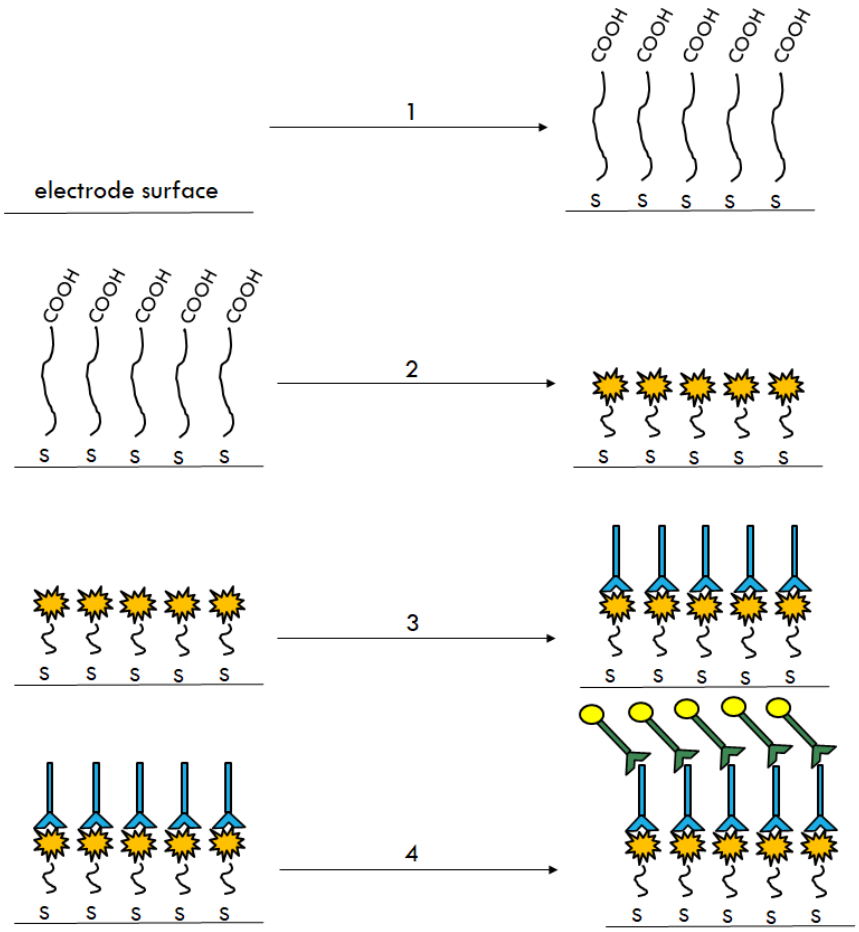

Figure 2. Scheme of the immunosensor preparation. 1 – Preparation of SAM layer on bare electrode surface using 11-MUA, 2 – Enzyme bonding to carboxy group via mixture of EDC/NHS, 3 – Antibodies bonding, 4 – Signal amplification by secondary antibody.

2.4. Determination of anti-tTG by ELISA

Determination of IgA anti-tTG was done by commercially available ELISA kit, all samples were assayed in triplicate. Briefly, 100 µl of positive/negative control, calibrator or diluted sample was pipetted into tTG precoated plate and incubated for 30 min. After washing step (each washing step = 3x300 µl of washing buffer), 100 µl of enzyme conjugate was added and incubated for another 30 min followed by washing step. Finally, 100 µl of chromogen-substrate solution was added and reaction was stopped by adding 100 µl of stop solution after 15 min and absorbance was measured in 450 nm on microplate spectrophotometer Epoch (BioTek, Winooski, VT, USA).

2.5. Data processing

All measured data were processed and graphs created in Origin software (OriginLab, Northampton, MA, USA). Differential in frequency (Δf) was obtained by subtracting crystal frequency before adding certain reagent and crystal frequency after the reaction.

3. RESULTS AND DISCUSSION

3.1. QCM measuring system fabrication and optimization

For measuring of oscillation frequencies, we developed measuring system consisted from oscillation generator, frequency counter and rigid base with horizontally placed springs. The springs holding immunosensor were placed on copper thorns protruding from base with soldered electric cables. Frequency of QCM crystal was measured as follows: QCM crystal was placed between springs, operator count to five and wrote down frequency from counter display.

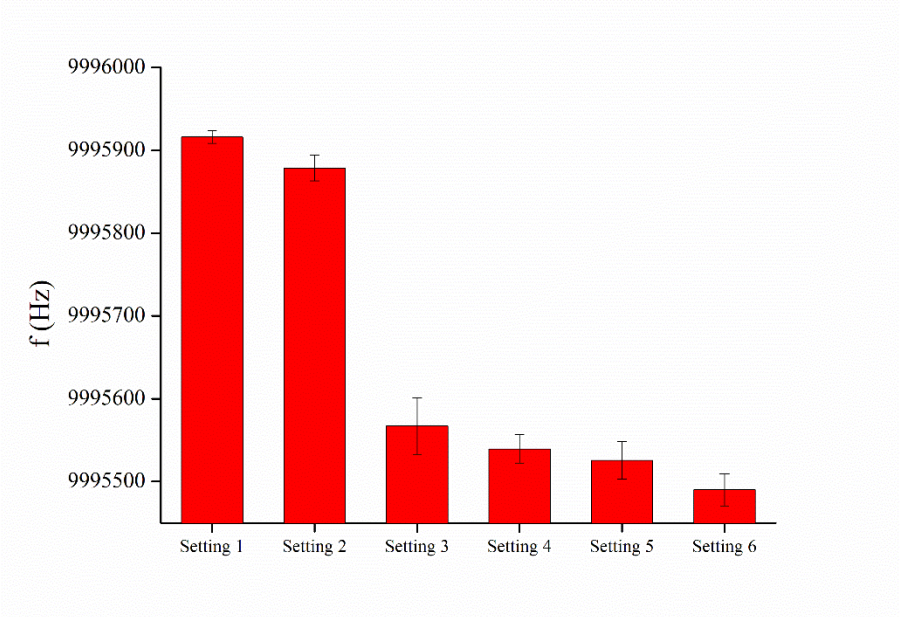

Figure 3. Optimization of QCM measuring system with different spring settings. Each setting was measured with the same QCM crystal with gold electrode, n = 10 ± SD.

It is possible to let crystal stabilized however this approach is more time consuming when stabilization lasts minutes. Optimization of system was carried out using six different settings of springs, (1) independently placed spring, (2) independently placed spring with height adjustment during measurement, (3) two mechanically connected springs, (4) two mechanically connected springs with height adjustment during measurement, (5) two soldered springs, (6) two soldered springs with height adjustment during measurement. Aim of height adjustment during measurement was to assess influence of spring movement to signal quality. The decrease of QCM crystal frequency due to imperfect contact of springs with thorns and probably the mechanical stress of QCM crystal was the result of height adjustment. Settings (3) and (4) is not applicable due to disconnection of mechanically connected springs during measurement, moreover crooked connection between the springs made problems with QCM

crystal inserting, spring disconnection in particular. In settings (5) and (6), disconnection was not occurred; however, signal intensity was not satisfying. Best results were achieved in settings (1) and (2), nevertheless setting (2) provided bigger standard deviations than (1), thus setting (1) was chosen as the best option and used for further measurements (Fig. 3).

3.2. Immunosensor preparation

Another piezoelectric immunosensor for determination of anti-tTG was already introduced [25]; however, the cited work used flow wet system. Here dry system was proposed thus viscosity of solvent is not necessary to be considered and frequency changes can be calculated according Sauerbrey equation [1]. Thiolic compounds form SAM layers on gold, silver and other metallic surfaces [26,27]. Various thiolic compounds have been studied in preparation of SAM layers on gold surface [28-30]. Because 11-MUA has been used in previous papers dealing with biosensors preparation, we decided to use it as well [25,31,32]. Concentration of 11-MUA was optimized in range from 5 to 25 mmol/l. Results shown that frequency is independent on concentration of 11-MUA, therefore for further measurements 15 mmol/l of 11-MUA were chosen (Fig. 4). As can be seen in the graph, even pure ethanol causes frequency shift, which is caused by impurities having affinity to gold [33]. After activation of carboxy group in 11-MUA by mixture of EDC and NHS, open-tTG was dropped onto sensor surface with concentration range varying from 20 to 40 µg/ml. The optimization is depicted as figure 5.. It is obvious that it is not necessary to give high loads of enzyme because in low concentrations of antibodies performance of the sensor will not improve, moreover it can lead to antibodies aggregation in their high levels [25]. Calibration of anti-tTG in IgA class were performed in dilution ranges from 20 to 200 RU/ml using standard solutions of IgA antibodies from ELISA kit (Fig. 6). Limit of detection was calculated to be 13.3 RU/ml. Although IgA antibodies have bigger diagnostic value in CD diagnosis, in small percentage of patients IgA deficiency may occur and IgG antibodies have relevance as well [34]. Therefore, alternative calibration of IgG anti-tTG were performed in dilution ranges from 15 to 125 µg/ml (Fig. 7) and labeling of anti-tTG was done by secondary antibody tagged by gold nanoparticle (6 nm) diluted ten times. Limit of detection was set to be 4.83 µg/ml. Selectivity of the sensor was tested and no cross reactivity with other IgA antibodies and albumin as a main blood protein was observed (Fig. 8). Long-term stability of prepared immunosensor was also tested and showed to be stable at least two months at 4 °C (Fig. 9).

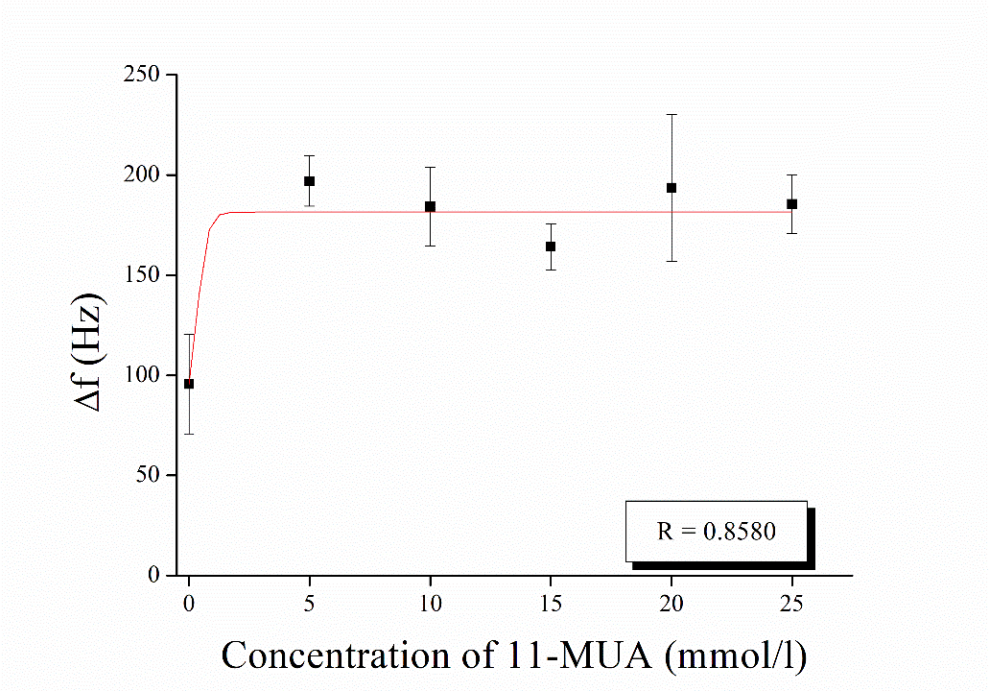

Figure 4. Optimization of SAM layer using 11-MUA, n = 3 ± SD. Negative control (ethanol only) is included in the graph.

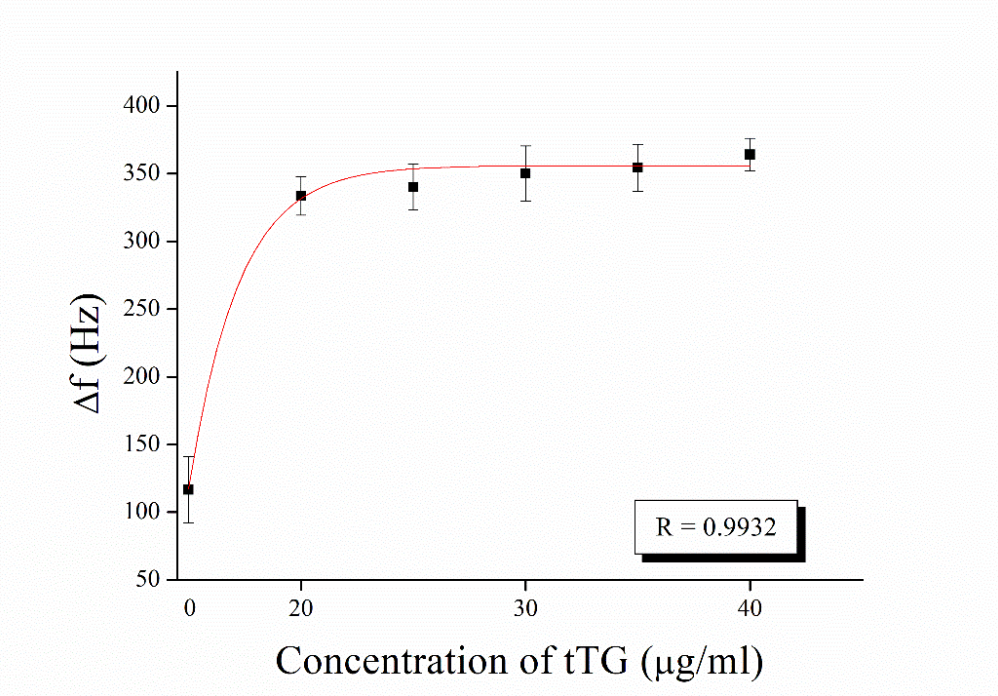

Figure 5. Optimization of tTG concentration, n = 3 ± SD. Negative control (0 µg/ml tTG) is included in the graph.

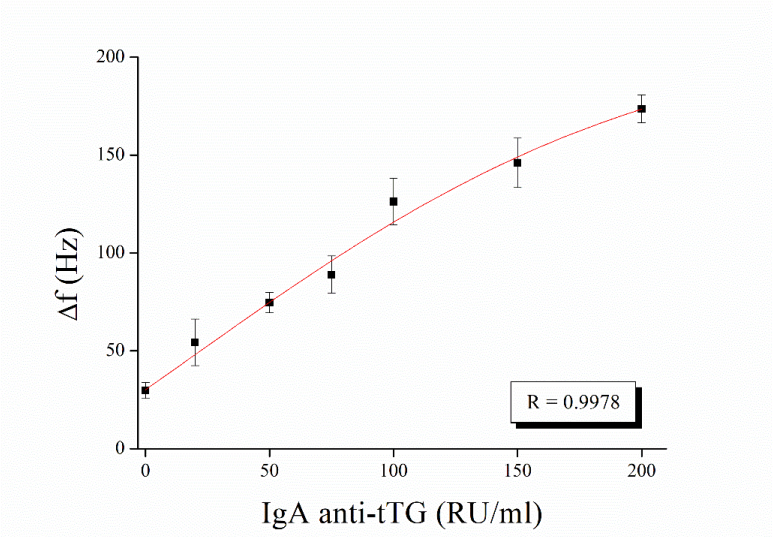

Figure 6. Calibration curve of IgA anti-tTG, n = 3 ± SD.

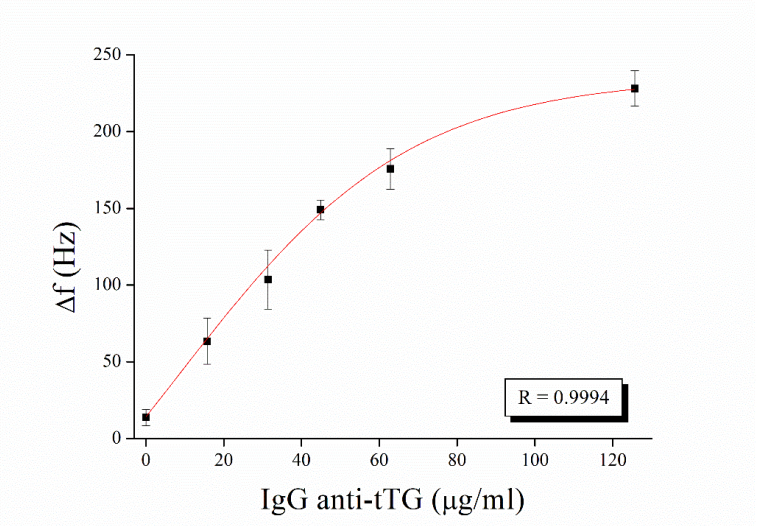

Figure 7. Calibration curve of IgG anti-tTG, n = 3 ± SD.

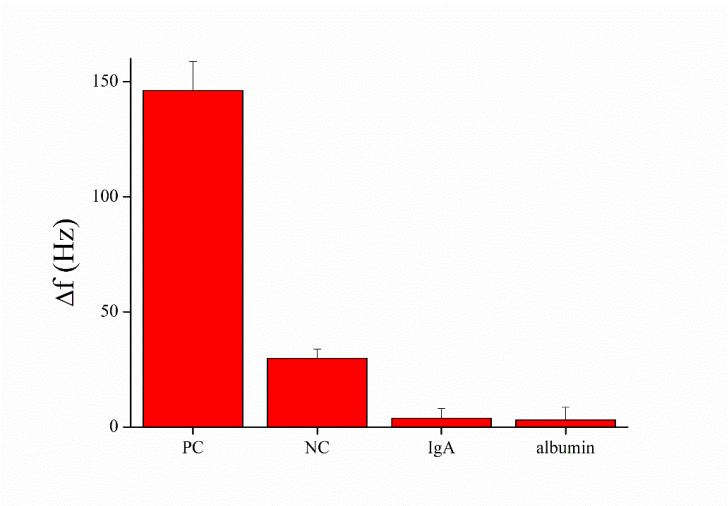

Figure 8. Selectivity of the sensor for IgA anti-tTG, n = 3 ± SD. PC = positive control, NC = negative control.

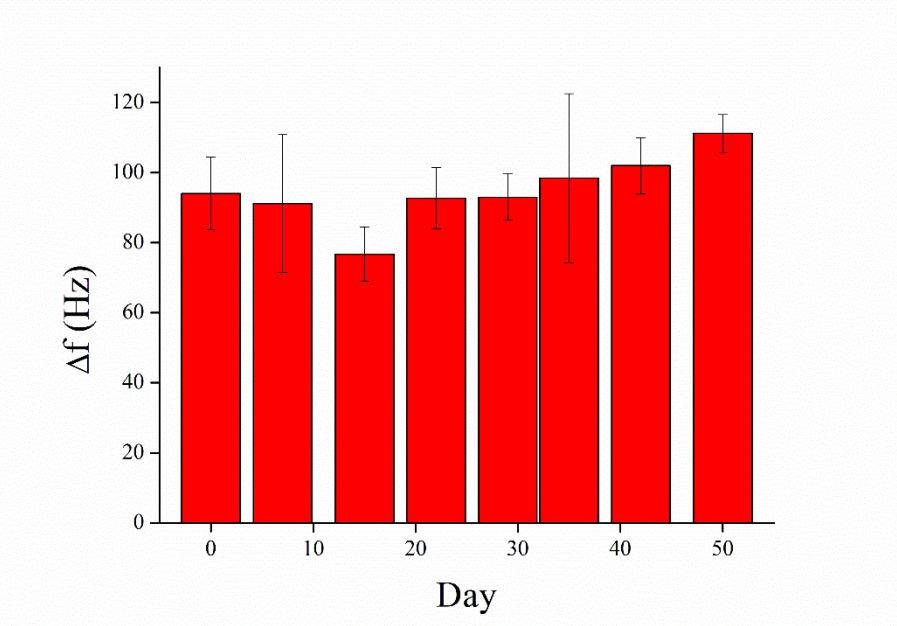

Figure 9. Long-term stability of the prepared immunosensor, n = 3 ± SD.

3.3. Real samples measurement and method validation

Method using QCM sensor was validated by measuring of real serum samples collected from patients suffering CD. All used samples were also assayed by standard ELISA method and correlated with QCM method (Fig. 10). From correlation graph, it can be learned that both methods are equally sensitive in assay of anti-tTG. On the other hand, there is a limitation of QCM method in higher serum concentrations of anti-tTG that could be given by electrode surface and amount of enzyme bounded onto electrode surface (Fig. 11). During measuring of real samples, significant matrix effect was observed. It was minimized by increasing concentration of BSA in blocking step of remaining surface of gold electrode from 2 % to 6 % and prolonging time from 30 min to 1 hour compare to use of standard antibodies solution. However, it is impossible to shrink it to zero as serum still contains big amount of proteins and the goal is to use it without any additional treatment or dilution. Therefore, negative control provides frequency change around 160 ± 20 Hz thus samples providing higher frequency change should be considered as positive. In absolute numbers, this frequency change is equal to 96 RU/ml of anti-tTG, on the other hand considering acute phase of disease this sensitivity is sufficient as much higher concentration of anti-tTG can be expected.

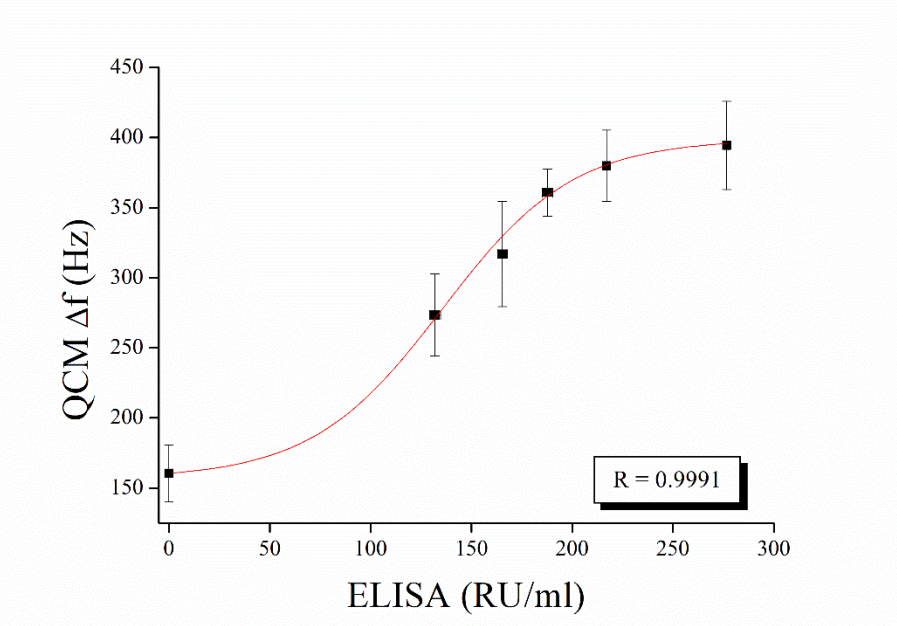

Figure 10. Correlation of the new QCM method with standard ELISA protocol, n = 3 ± SD.

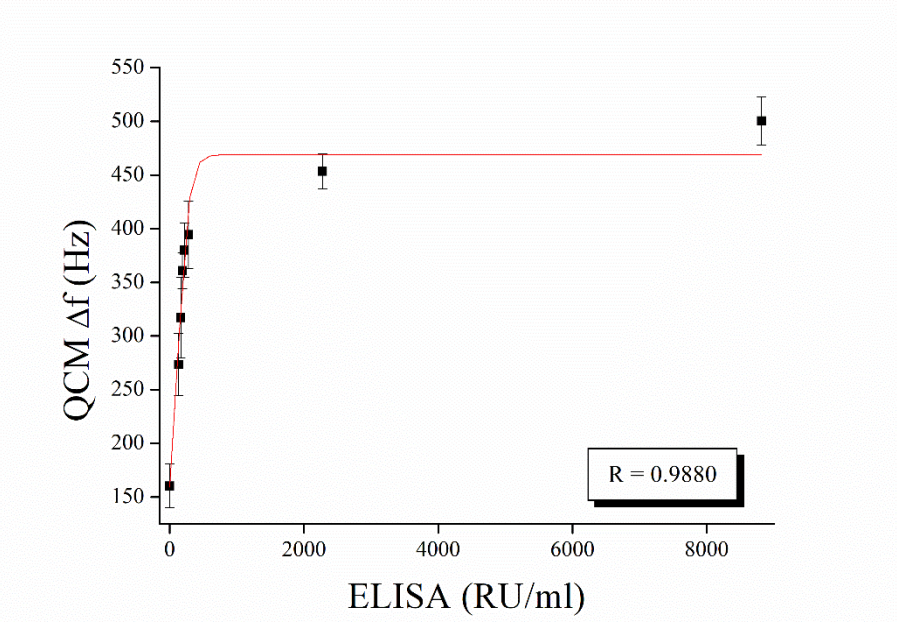

Figure 11. Correlation of the new QCM method with standard ELISA protocol, n = 3 ± SD.

4. CONCLUSION

Determination of anti-tTG antibodies in IgA class together with histological findings create main clues in diagnosis of CD. In this paper, we present immunosensor based on open-tTG for determination of anti-tTG in IgA class with detection limit 13.3 RU/ml. It was tested on real samples of patients suffering CD and proved sensitivity and selectivity for IgA antibodies. New method was validated by ELISA and immunosensor showed stability at least two months. Main advantage of the here presented

method is cheap and easy to prepare measuring system thus it can be introduced into any laboratory or general practitioner´s office as a quick tool for diagnostic in suspicion of CD.

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References

1. M. Pohanka, *Int. J. Electrochem. Sci.*, 12 (2017) 496.
2. Q. Lang, L. Han, C. Hou, F. Wang and A. Liu, *Talanta*, 156-157 (2016) 34.
3. J. Warner and S. Andreescu, *Talanta*, 146 (2016) 279.
4. C. I. L. Justino, A. C. Duarte and T. A. P. Rocha-Santos, *Sensors (Basel, Switzerland)*, 17 (2017) 2918.
5. M. S. Thakur and K. V. Ragavan, *J. Food Sci. Technol.*, 50 (2013) 625.
6. G. Murugaboopathi, V. Parthasarathy, C. Chellaram, T. P. Anand and S. Vinurajkumar, *Biosci. Biotechnol. Res. Asia*, 10 (2013) 711.
7. B. van der Gaag, S. Spath, H. Dietrich, E. Stigter, G. Boonzaaijer, T. van Osenbruggen and K. Koopal, *Food Control*, 14 (2003) 251.
8. I. Palchetti and M. Mascini, *Anal. Bioanal. Chem.*, 391 (2008) 455.
9. H. R. S. Lima, J. S. da Silva, E. A. de Oliveira Farias, P. R. S. Teixeira, C. Eiras and L. César C. Nunes, *Biosens. Bioelectron.*, 108 (2018) 27.
10. C. I. L. Justino, A. C. Duarte and T. A. P. Rocha-Santos, *Trends Anal. Chem.*, 85 (2016) 36.
11. H. Jin, X. Tao, B. Feng, L. Yu, D. Wang, S. Dong and J. Luo, *Vacuum*, 140 (2017) 101.
12. M. Pohanka, P. Skládal and O. Pavliš, *J. Immunoass. Immunoch.*, 29 (2007) 70.
13. N. Liu, J. Han, Z. Liu, L. Qu and Z. Gao, *Anal. Methods*, 5 (2013) 4442.
14. T. N. Ermolaeva, E. N. Kalmykova and O. Y. Shashkanova, *Russ. J. Gen. Chem.*, 78 (2008) 2430.
15. M. Pohanka, *Talanta*, 178 (2018) 970.
16. N. M. do Nascimento, A. Juste-Dolz, E. Grau-García, J. A. Román-Ivorra, R. Puchades, A. Maquieira, S. Morais and D. Gimenez-Romero, *Biosen. Bioelectron.*, 90 (2017) 166.
17. C. Crosson and C. Rossi, *Biosens. Bioelectron.*, 42 (2013) 453.
18. D. Sblattero, I. Berti, C. Trevisiol, R. Marzari, A. Tommasini, A. Bradbury, A. Fasano, A. Ventura and T. Not, *Am. J. Gastroenterol.*, 95 (2000) 1253.
19. A. Rubio-Tapia, I. Hill, C. Kelly, A. Calderwood and J. Murray, *Am. J. Gastroenterol.*, 108 (2013) 656.
20. B. Lebwohl, D. S. Sanders and P. H. R. Green, *Lancet* (2017).
21. C. Catassi and A. Fasano, *Am. J. Med.*, 123 (2010) 691.
22. A. Aita, E. Rossi, D. Basso, G. Guariso, D. Bozzato, M. Pelloso, M. Pescarin, C.-F. Zambon, F. Navaglia, E. Greco, M. Gasparetto, P. Fogar, A. Padoan, S. Moz and M. Plebani, *Clin. Chim. Acta*, 421 (2013) 202.
23. F. Benkebil, C. Combescure, S. I. Anghel, C. Besson Duvanel and M. G. Schäppi, *World J. Gastroenterol.*, 19 (2013) 5111.
24. M. Giannetto, M. Mattarozzi, E. Umiltà, A. Manfredi, S. Quaglia and M. Careri, *Biosens. Bioelectron.*, 62 (2014) 325.

25. A. Manfredi, M. Mattarozzi, M. Giannetto and M. Careri, *Sens. Actuator B-Chem.*, 201 (2014) 300.
26. M. H. Schoenfisch and J. E. Pemberton, *J. Am. Chem. Soc.*, 120 (1998) 4502.
27. J. C. Love, D. B. Wolfe, R. Haasch, M. L. Chabinyc, K. E. Paul, G. M. Whitesides and R. G. Nuzzo, *J. Am. Chem. Soc.*, 125 (2003) 2597.
28. C. Jung, O. Dannenberger, Y. Xu, M. Buck and M. Grunze, *Langmuir*, 14 (1998) 1103.
29. A. Tlili, A. Abdelghani, S. Hleli and M. A. Maaref, *Sensors (Basel, Switzerland)*, 4 (2004) 105.
30. S. A. Jadhav, *Cent. Eur. J. Chem.*, 9 (2011) 369.
31. R. Mossanha, M. K. Ramos, C. S. Santos and C. A. Pessoa, *J. Electrochem. Soc.*, 162 (2015) B145.
32. A. Ahmad and E. Moore, *Analyst*, 137 (2012) 5839.
33. K. Barbe, M. Kind, C. Pfeiffer and A. Terfort, *Beilstein J. Nanotechnol.*, 5 (2014) 1254.
34. A. Fasano and C. Catassi, *N. Engl. J. Med.*, 367 (2012) 2419.