Int. J. Electrochem. Sci., 14 **(2019)** 11340 – 11348, doi: 10.20964/2019.12.16

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

QCM Biosensor for Measurement of Glycated Hemoglobin

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Received: 24 June 2019 / *Accepted:* 16 August 2019 / *Published:* 29 October 2019

In the body, glycated hemoglobin is spontaneously created by a reaction between hemoglobin and glucose. The glycation reaches only minimal scale in health people but it is much higher as the glucose level is increased. It typically serves as a marker in diabetes mellitus. Chromatography, mass spectrometry and immunoassays are the standard methods for glycated hemoglobin measurement in the current clinical praxis. In this work, piezoelectric biosensor is proposed as a tool for glycated hemoglobin measurement. The biosensor contained immobilized antibodies against glycated hemoglobin in a stable film composed from iron oxide nanoparticles. Standard solution of glycated hemoglobin and human blood samples were used for the prepared biosensor characterization. Non glycated hemoglobin and albumin served for interference testing. The biosensor exerted quite good limit of detection highly under expected physiological level: 0.045 mg/ml and coefficient of determination 0.968 for the assay was achieved. The assay by biosensor fully correlated to standard ELISA (coefficient of determination 0.948). Non-glycated hemoglobin and albumin in concentrations expected in blood or blood plasma did not caused interference in the assay. The piezoelectric biosensor seems to be a promising tool for routine assay of glycated hemoglobin. Due to overall simplicity, no application of specific reagents or sample manipulation, the biosensor can be easily used in field conditions or in the environment of small medical facilities.

Keywords: affinity; antibody; biosensor; biorecognition; blood; diabetes; glucose; glycated hemoglobin; hemoglobin; immunoassay; label free; piezoelectric; quartz crystal microbalance; QCM

1. INTRODUCTION

Glycated hemoglobin is a product of spontaneous reaction between hemoglobin and glucose. In health people, the glycation reaches only minimal scale but diabetics with not well controlled glucose level suffer from glycation of various proteins including hemoglobin which is quite sensitive to the glycation. When expressed amount of glycated hemoglobin per the non-glycated one, health people exert approximately under 42 mmol/mol respectively 6.0 %. Range 42 – 47 mmol/mol respectively 6.0 to 6.4

% is considered as suspect of diabetes and value 48 mmol/mol and more (6.5 % and over) is typical for diabetes mellitus [1,2].

The glycation is a chemical procedure in which hemiacetal oxygen (respective aldehyde oxygen in the non-cyclic form of glucose) undergo reaction with free amino groups of proteins producing Schiff bases, chemical products of glycation in body are called advanced glycation end products known under acronym AGEs. Albumin is also a protein that become widely glycated [3]. Glycated hemoglobin is called hemoglobin A1c or HbA1c and it is differing from the standard hemoglobin by presence of β-N-1-deoxy fructosyl in hemoglobin structure. Glycated hemoglobin can be measured by mass spectrometry [4-8], chromatography techniques [9,10] and immunochemical methods like Enzyme-Linked Immunosorbent Assay [11]. In the recent time, small, hand held analyzers like biosensors are popular for purpose of personalized medicine and lowering of costs and demands on laboratory equipment [12-14]. Biosensors for determination of glycated hemoglobin draw attention and would be a promising alternative to the standard methods in the future [15-17].

In this study, an immunoassay based on biosensor composed from Quartz Crystal Microbalance (QCM) sensor and an antibody against glycated hemoglobin is proposed as a diagnostic tool suitable for a simple measuring of glycated hemoglobin. The biosensor should provide full applicability for processing of blood samples but designed as a disposable one step assay that can be performed by people without education in laboratory analysis and without necessity of another laboratory equipment. Scheme of the assay by the constructed biosensor and its diagnostical principle is depicted as figure 1.

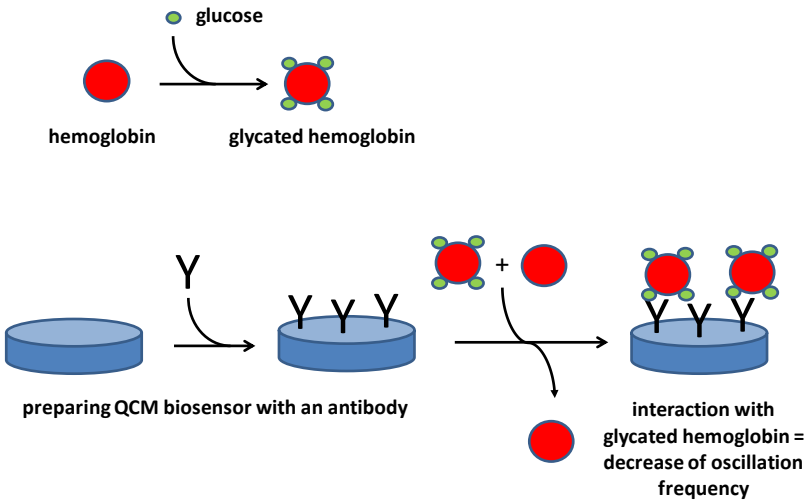

Figure 1. Principle of the assay, in the first step glycated hemoglobin is created in diabetics. The biosensor interacts with glycated hemoglobin but not with the standard hemoglobin, decrease of oscillation frequency follows.

2. MATERIALS AND METHODS

2.1. Preparing of biosensor for glycated hemoglobin assay

10 MHz QCM sensors sized 20 mm and thick 0.166 mm with circled 7 mm gold electrodes were achieved from company Krystaly (Hradec Kralove, Czech Republic; http://www.krystaly.cz/en/). The

sensors were washed in pure ethanol (96 % v/v) for 30 minutes and then let dry. Gold electrode were chemically activated by 50 µl of cysteamine (Sigma-Aldrich; St. Louis, MO, USA) 50 mg/ml applied in a wet chamber and let to incubate for five hours. After that, QCM sensors were rinsed with deionized water and let to dry. In the next step, gold electrodes activated by cysteamine were further modified by glutaraldehyde. 50 µl of glutaraldehyde (Litolab; Chudobin; Czech Republic) solved in deionized water up to concentration 5 % w/w was given per one electrode and let to incubate in a wet box for 5 hours and then rinsed and dried as in the previous step. The activated surface of QCM was covered with 50 µl of a polyclonal antibody against human hemoglobin A1c. The antibody was a polyclonal one produced from sheep and containing purified IgG isotype of immunoglobulins. It was purchased from Abcam (Cambridge, United Kingdom). The antibody was diluted 1:1000 by phosphate buffered saline pH 7.4 prior to use. The antibody solution was let to incubate with electrode surface in a wet chamber for 12 hours and then rinsed with deionized water and let dry in the same way like in the previous steps.

In a separate step, magnetic nanoparticles composed from iron oxide (II,III), sized 30 nm and containing carboxylic acid on their surface served as a stabilizer of the antibody layer on the QCM sensor surface. The nanoparticles were purchased from Sigma-Aldrich and they were suspended in phosphate buffered saline pH 7.4 from the manufacturing process, solid content was 5 % of the total weight. New batch of magnetic nanoparticles was repeatedly washed by phosphate buffered saline pH 7.4, separated by an external magnet and refilled by the same volume of buffer as was removed in the previous step. Finally, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (Litolab) in concentration 20 µg per µl of phosphate buffered saline pH 7.4 was used for resuspending of magnetic nanoparticles. The suspension was shaken for 30 minutes and then magnetic particles were separated and resuspended in phosphate buffered saline pH 7.4.

The nanoparticles activated by N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride were given in an amount 50 µl on a QCM electrode and let to interact in a wet chamber for 12 hours. Finally, the surfaces were rinsed and as a final step, 50 µl of bovine serum albumin 10 mg/ml was given per electrode, let to incubate in a wet chamber for five hours. After final washing by deionized water, the biosensors were finished and prepared for use.

2.2. Biosensor performance

Frequency of the biosensors´ oscillations were measured by frequency counter UZ 2400 (Grunding, Nuremberg, Germany) after plug into 10 MHz oscillation circuit composed from ICM Level Oscillator (ICM, Oklahoma City, OK, USA). After that, sample sized 50 µl was spread over biosensor electrode and let to incubate for 10 minutes then washed with deionized water with 0.1 % w/w Tween 20 and dried under laboratory conditions. After drying, oscillation frequency was measured again and difference of oscillations before and after sample application Δf was determined.

Human glycated hemoglobin of A1c type (Abcam), normal human hemoglobin containing no glycated variant (Sigma-Aldrich) and human serum albumin (Sigma-Aldrich) solved in phosphate buffered saline pH 7.4 served as samples beside blood samples from human volunteers. The calibration range of glycated hemoglobin was following: 0 (pure phosphate buffered saline), 0.0390, 0.0781, 0.156,

0.313, 0.625, 1.25, 2.50, 5.00, 10.0, 20.0 and 40.0 mg/ml. Four blood samples provided from Faculty Hospital (Hradec Kralove) were used for validation purposes. The blood samples were anonymized and manipulation was allowed by ethical committee. The blood was achieved as a non-clotting one kept in tubes with lithium heparin (Dialab, Radotin, Czech Republic).

2.3. Standard ELISA

Human glycated Hemoglobin (HbA1c) ELISA Kit by Abbexa (Cambridge, United Kingdom) was used for validation of biosensor by assaying of the same samples like biosensor. Total level of hemoglobin was determined by Human Hemoglobin ELISA Kit (Sigma-Aldrich). The kits were processed in the way how recommended by protocols attached to the kit by manufacturers.

2.4. Data processing

All samples were assayed five times. Limit of detection was calculated for each method using the rule that it is the point on calibration curve equal to triplicate of noise value (signal to noise is equal to three: S/N = 3). Comparison of data from samples and control assays was evaluated by analysis of variance (ANOVA) on probability levels 0.05 and 0.01. The data processing including ANOVA test was made in software Origin 9 OriginLab Corporation, Northampton, MA, USA).

3. RESULTS AND DISCUSSION

In the first step, the biosensors were tested for their ability to measure glycated hemoglobin. Number of standard samples were prepared for this purpose, they contained various glycated hemoglobin concentration in phosphate buffered saline pH 7.4. Calibration curve achieved by assay of the given concentrations is depicted as figure 2. The assay by biosensor exerted good sensitivity to glycated hemoglobin up to 20 mg/ml interpolation to the concentration 40 mg/ml and extrapolation of the experimental data showed reduced sensitivity for samples containing higher concentration of glycated hemoglobin than 20 mg/ml. Coefficient of determination r^2 equal to 0.968 was found for the calibration. Limit of detection equal to 0.045 mg/ml was achieved considering the rule S/N = 3. Regarding to sensitivity, the assay appears to be suitable for real samples because limit of detection is deeply under expected concentration of glycated hemoglobin in real samples. Considering the threshold for glycation 42 mmol/mol, it is expected that up to 7 mg/ml of glycated hemoglobin can be presented in a blood sample.

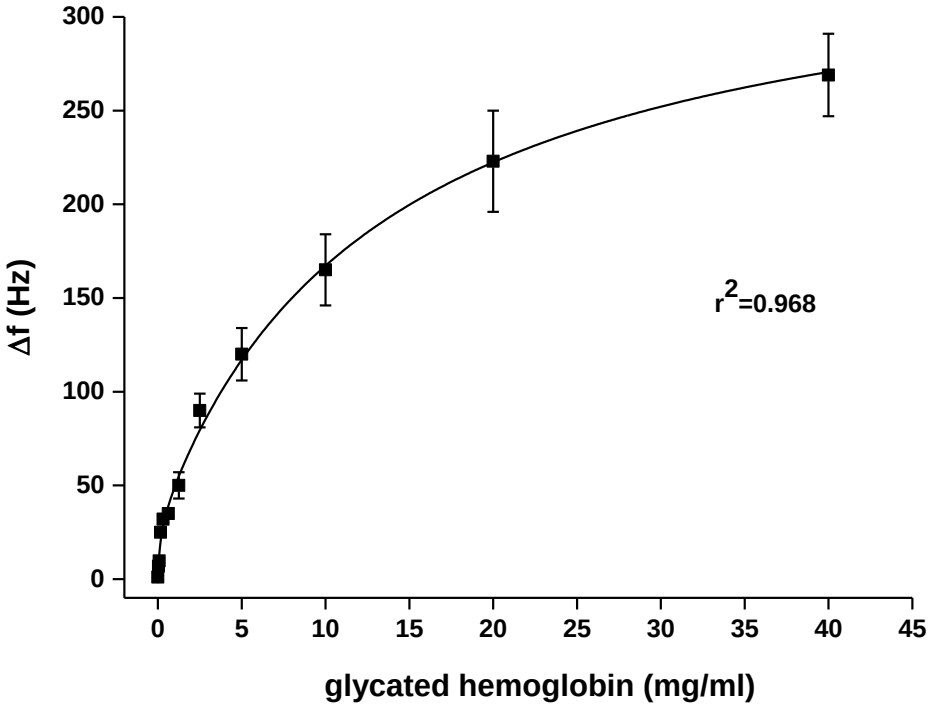

Figure 2. Calibration of QCM biosensor for glycated hemoglobin assay. Error bars indicate standard deviation for n = 5.

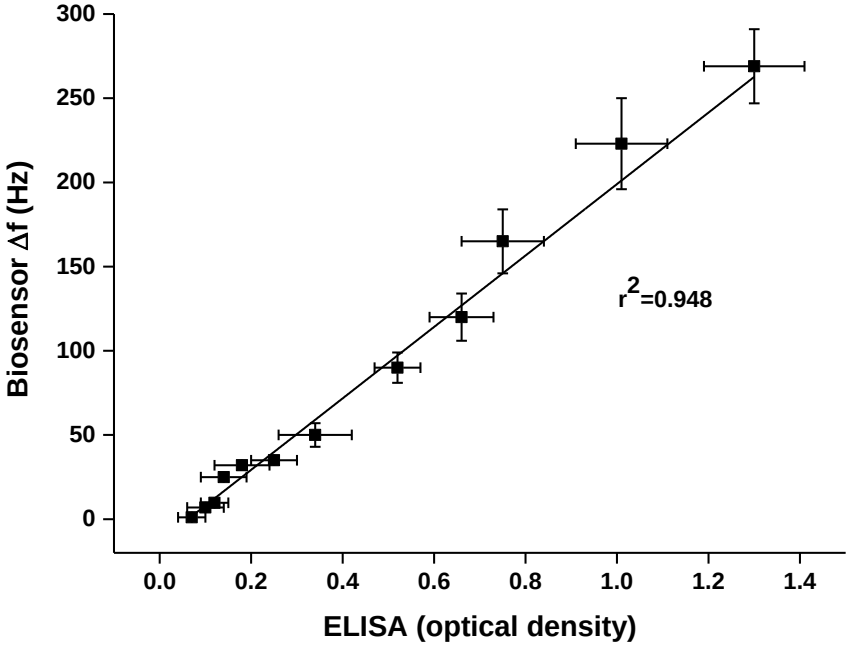

Figure 3. Validation of glycated hemoglobin assay by biosensor to standard ELISA method. Concentration range of glycated hemoglobin 0, 0.0390, 0.0781, 0.156, 0.313, 0.625, 1.25, 2.50, 5.00, 10.0, 20.0 mg/ml was used for the validation purpose. Error bars indicate standard deviation for n = 5.

The standard samples used for the calibration purposes were also analyzed by the standard ELISA and correlated mutually. The data from correlation of biosensor results to ELISA are presented in figure 3. When the two assays compared, coefficient of determination equal to 0.948 was achieved. It can be stated that the both methods exert good applicability to assay glycated hemoglobin and no relevant differences can be learned from the correlation. Though the assay by biosensor could be concluded that it does not bring improvements comparing to ELISA, the contrary is true. While the performance exerts similar results like ELISA, the samples are analyzed by a significantly simpler manner where no specific reagents are needed and no pretreatment of sample is necessary as well.

Interferences were determined by analyzing of human serum albumin and hemoglobin in the same manner like the samples with glycated hemoglobin were measured. The expected concentration of albumin in a human blood sample is 35 – 55 mg/ml and hemoglobin can be presented up to the concentration 175 mg/ml. The Expected physiological concentration of glycated hemoglobin is 5 mg/ml in health people. The concentration of interferents exceeding physiological level (hemoglobin 200 mg/ml, albumin 100 mg/ml), the closest concentration of glycated hemoglobin in the calibration curve which was above limit o detection (0.0781 mg/ml) and lower physiological concentration of glycated hemoglobin (5 mg/ml) were tested and results from the test can be found in figure 4. The signal caused by glycated hemoglobin 0.0781 mg/ml was significantly differing on the probability level P 0.05 to the signals by albumin and hemoglobin. The signal caused by glycated hemoglobin in concentration 5 mg/ml was differing on the probability level P 0.01 to signals by albumin and hemoglobin.

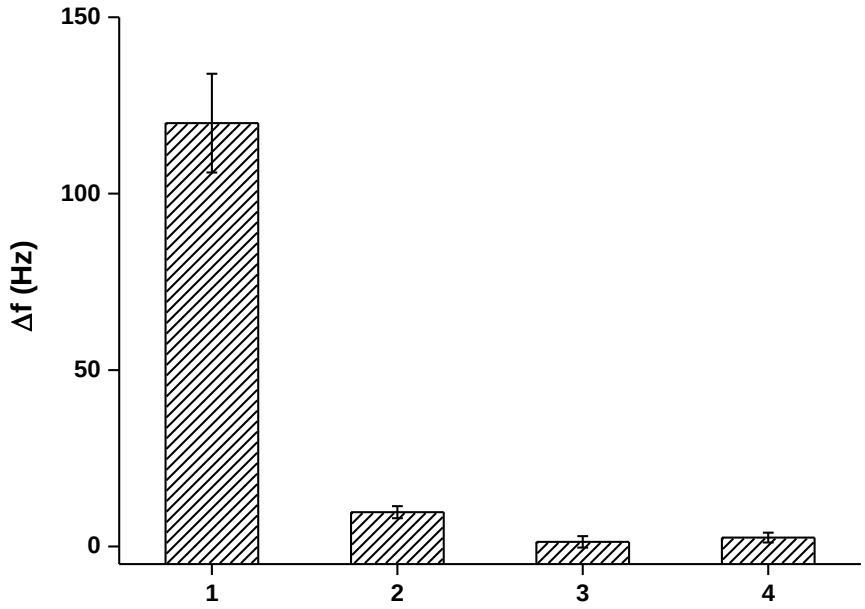

Figure 4. Interference testing by assay of hemoglobin 200 mg/ml and albumin 100 mg/ml (columns 3 and 4) for a biosensor-based assay. Glycated hemoglobin 5 mg/ml was applied as a positive control (column 1). In the column 2, there is concentration of glycated hemoglobin above limit of detection (0.0781 mg/ml). Error bars indicate standard deviation for n = 5.

Four real samples were analyzed in order to check applicability of the biosensor as a tool of glycated hemoglobin assay and the assay was also compared with ELISA method. Total hemoglobin ELISA kit served for the both assays to provide information about hemoglobin and data from this kit were used for calculation of glycation percent. The found results are presented as figure 5. When results from the two methods compared, no statistically significant difference was revealed (the both probability levels 0.05 and 0.01 were tested by ANOVA). It can be concluded that the biosensor provides similarly plausible data like the standard ELISA.

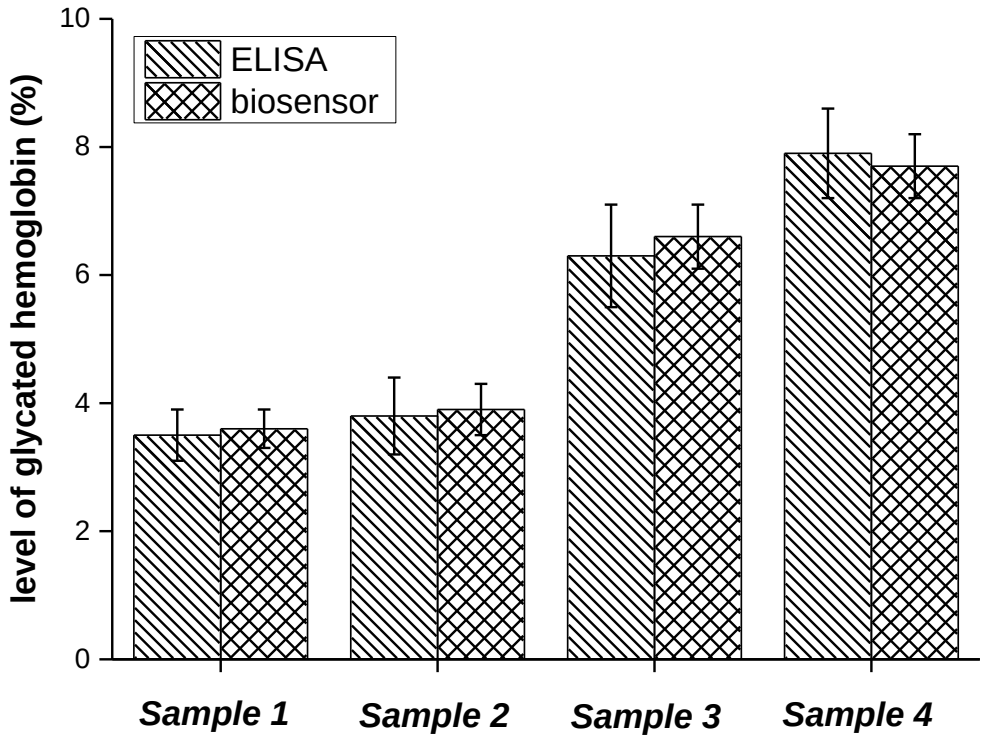

Figure 5. Assay of four real human blood samples by biosensor and ELISA. Error bars indicate standard deviation for n = 5. There is no statistical difference between the two assays (P = 0.05).

The prepared biosensor is intended as a simple tool for the determination of glycated hemoglobin in blood or other biological samples. The sensitivity to the glycated hemoglobin is adequate to expected levels in blood samples. Further improvements focused on limit of detection are not advantageous because even the reached limit of detection 0.045 mg/ml is significantly under typical physiological concentrations (around 5 mg/ml). The quite low volume of a sample (50 µl) and no use of specific reagents (label free assay) are the major advantages of the assay by the biosensor. Overall simplicity makes this biosensor highly competitive. On the other hand, there should be also mentioned recent works on this field where advanced types of biosensors were also published. Screen printed electrodes modified

with nanoflowers [18], molecularly imprinted polymer sensor [19], surface plasmon resonance based biosensor [20], optical photonic crystal based ring resonator biosensor [21], prism coupled waveguide sensor with nanostructured recognition platform [22], fluorescent nanosheets with affinity to glycated hemoglobin [23] are another sensor and biosensor devices with good applicability potential. Specifications of the here introduced biosensor are surveyed in table 1. The here presented biosensor can be further combined with the previously presented one for the total hemoglobin [24]. Data from the two biosensors can be used for glycation level determination.

Table 1. Specifications of the biosensor.

Limit of detection for hemoglobin	0.045 mg/ml
Coefficient of determination for calibration	r^2=0.968
Coefficient of determination for validation to ELISA	r^2=0.948
Hemoglobin and albumin interference	not significant for albumin 100 mg/ml and hemoglobin 200 mg/ml

4. CONCLUSION

The biosensor presented in this paper represent a simple tool suitable for a full-value detection of glycated hemoglobin in biological samples. It can be combined with the previously reported biosensor assaying the total hemoglobin [24] in order to provide data for calculation of glycation level. It is expected that this platform can be suitable for small laboratories or medical facilities but the biosensor as well as the whole assay is so simple in its conception that it can be performed even in home or field conditions. It represents an alternative to the more elaborative standard immunoassays. Practical impact of the here presented results is expected.

ACKNOWLEDGEMENTS
This work was supported by a Ministry of Defence of the Czech Republic - long-term organization development plan Medical Aspects of Weapons of Mass Destruction of the Faculty of Military Health Sciences, University of Defence.
Ms. Jitka Zakova is highly acknowledged for laboratory assistance.

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