BIOINTERFACES BASED ON IMMOBILIZED BORONIC ACID WITH SPECIFITY TO GLYCATED PROTEINS

Jan Přibyl and Petr Skládal
National Center for Biomolecular Research, Masaryk University, Kotlářská 2, Brno, Czech Republic

Keywords: Glycated hemoglobin, aminophenylboronic acid, biosensors, quartz crystal microbalance, heterogeneous affinity assay, microtitre plate.

Abstract: Development of bioanalytical assays for determination of glycated hemoglobin content in blood samples is reported. First, a combined biosensor setup for determination of total and glycated hemoglobin content was successfully developed and tested. The effect of various operating parameters, such as ionic strength, flow rate and instrumental set-up was optimized. The total hemoglobin content was analyzed by measuring of absorbance of the hemoglobin-cyanide derivative at 540 nm. Only one standard (calibrator), diluted in various proportions, was necessary for the method calibration. The full range of HbA_{1c} content (4 to 15 %) presented in blood can be analyzed. Only 1 µl of blood was required for analysis. The developed method was successfully evaluated for analysis of blood samples collected from diabetic patients. Next, the heterogeneous affinity assay performed in a microtitre plate with an immobilized boronic acid is described. This assay is based on ELISA (Enzyme-Linked Immunosorbent Assay) principle; however stable chemiselective ligand is used in this case. The content of glycated hemoglobin is determined according to its peroxidase activity after attachment to immobilized boronic acid derivative; the total hemoglobin concentration is measured as an absorbance at 405 nm.

1 INTRODUCTION

Diabetes mellitus is a group of diseases characterised by high levels of blood glucose resulting from defects in insulin production, insulin action, or both. Diabetes can be associated with serious complications and premature death. Diabetes was the sixth leading cause of death in USA in 2000. (National Diabetes Information Clearinghouse, http://diabetes.niddk.nih.gov). The steps to control the disease and lower the risk of complications should be taken. In this way, blood and urine glucose analysis, cholesterol reduction and blood pressure control should be mentioned. Analysis of glycated hemoglobin (HbA_{1c}) helps to monitor the long-term progression of diabetes without influence of the short-term fluctuations of blood glucose. The fraction of HbA_{1c} is usually indicated as percentage of its presence in the total hemoglobin content. The content of glycated hemoglobin in blood should substitute the term “glycemia”; values lying under 7% indicate good health state of patient and effective practicing of the proposed therapy (Marshall and Barth, 2000).

Glycated hemoglobin (GHb) refers to a series of minor hemoglobin components which are stable adducts formed by reaction of hemoglobin primary aminogroups with various sugars. Hemoglobin HbA_{1c} is a stable glucose adduct to the N-terminal group of the β-chain of HbA_0. In current opinion, concentration of the hemoglobin variant HbA_{1c} is considered to be the only specific and stable indicator of long-term diabetes progress. Neither the whole glycated fraction of hemoglobin (HbA_1) nor fructosamine can be any longer used in the disease diagnosis.

A wide range of methods for analysis of the glycated hemoglobin (either HbA_1 or HbA_{1c}) has been reported recently. However, only few of them were based on biosensor approach and mostly the chromatographic approaches were employed. From the area of biosensor development, especially two concepts should be mentioned. The first one was based on selection of ligands from the hexapeptide combinatorial library for binding the glycated terminus of hemoglobin β-chain; thus found hexaptides exhibited high specifity and stability (Chen et al., 1998). However, only the preliminary study was performed, additional testing of this

Přibyl J. and Skládal P. (2008).
BIOINTERFACES BASED ON IMMOBILIZED BORONIC ACID WITH SPECIFITY TO GLYCATED PROTEINS.
In *Proceedings of the First International Conference on Biomedical Electronics and Devices*, pages 107-112
DOI: 10.5220/0001051501070112

affinity molecules and integration to some analytical method (e.g. affinity chromatography) is essential for correct evaluation. A monolayer of boronic acid conjugate with 11-mercaptoundecaonic acid immobilised on the surface of gold nanoclusters was used as recognition element in another study (Valina-Saba et al., 1999). An easy approach was reported, when the precipitation reaction between boronic groups on the particle surface and glycated protein (horse-radish peroxidase) was visible. Unfortunately, the application for determination of HbA_{1c} content in whole blood, which is rather complex mixture, was not tested.

Boronic acid shows ability to bind covalently to either 1,2- or 1,3-diols and thus forms five- or six-membered cyclic esters. 3-aminophenylboronic acid (APBA) binds in this way to the *cis*-diols of saccharides, glycated proteins or nucleic acids (Pickup et al., 2005). The formation of a boronate ester is usually described as a two step reaction; the planar boron group initially reacts with hydroxyl (pH>7.0 is essential) to form tetrahedral boronate anion, which subsequently binds reversibly to the positively charged carbon atoms in the diol-containing structure (Ito et al., 2003). This kind of ester formation designates boronic acid and its derivatives to be used as the affinity recognition elements in variety of applications, such as construction of sensors for saccharides with piezoelectric (Lau et al., 2000) and surface plasmon resonance (Kugimiya and Takeuchi, 2001) transducers or fluorescent (Kataoka et al., 1995) detection. Boronic acid derivatives immobilized in the matrix of columns have formed the basis of a new field of chromatographic techniques designated for analysis and separation of sugars and glycated proteins. This area is commonly known as boronate affinity chromatography (Bongartz and Hesse, 1995).

The aim of presented study was to continue our previous attempts with boronate-modified sensors for sugars (Přibyl and Skládal, 2005) in order to develop an innovative, easy to handle and cost effective but reliable biosensor set-up with high stability and reproducibility for determination of glycated hemoglobin in blood samples. The designed system contains two parts, one performs the analysis of HbA_{1c} using a piezoelectric sensor modified with phenylboronic acid, and the second one is designed for a photometric determination of total Hb. The absolute concentration of these blood components differs in each sample. The percentage of HbA_{1c} presence will be determined as a ratio of these two concentrations; $(conc_{HbA1c}/conc_{Total\ Hb})$ x 100%.

Another interesting method for detection of glycated hemoglobin is reported, too. This bioanalytical method called AHA (Affinity Heterogeneous Assay) employs microtitre plates consisting of the wells covered with aminophenylboronic acid. The AHA assay allows determination of total hemoglobin as well as glycated fraction of hemoglobin in blood samples, similarly as the biosensor based method.

2 EXPERIMENTAL

2.1 Chemicals and Reagents

Chemicals were obtained from Sigma (St. Luis, USA) and used as received without any further purification. Microtitre plates with chemically reactive surface (NUNC Immobilizer Amino) were from Nunc (Copenhagen, Denmark).

The special solutions were prepared, stored and used as officially recommended (International Committee for Standardization in Haematology, 1978) for analysis of total hemoglobin content in blood samples.

2.2 Instrumentation

Measurements with the piezoelectric biosensor were performed using 10 MHz, AT-cut quartz crystals (ICM, Oklahoma City, OK, USA) with gold-coated smooth quartz discs (electrode area, 0.8 cm^2).

In the center of the system, there was placed a PMMA-made flow-trough cell (internal volume 10 μl) from NanoQ (Brno, Czech Rep.) with the piezoelectric biosensor mounted between two silicon rubber O-rings. The cell was supplied with flowing liquid via two stainless steel tubes (i.d., 0.5 mm). Sensor was connected to MultiLabPlus instrument (MultiLab) combining oscillator with high resolution frequency counter.

Handling of liquids and samples was performed by the FIAlab 3500b instrument (Alitea, Seattle, WA, USA).

Optical part for determination of total hemoglobin content was located in front of the biosensor cell. The detector consisted of a Z-type flow-trough absorption cell (optical path, 10 mm) supplied with flowing liquids trough the Teflon tubing and standard visible light source and optical fibre spectrophotometer from Ocean Optics (Dunedin, FL, USA).

2.3 Immobilization Procedure

2.3.1 Affinity Biosensor Fabrication

Matrix based layer was prepared when 2% solution of polyethylene imine (PEI) in methanol (3 µl) was used to activate the gold surface. The APBA layer was attached through the glutaraldehyde linker (8%, 8 hours, 4 ^{o}C) In the last step, the recognition layer was stabilised by the reduction of Schiff bonds with 10 mg/ml solution of sodium borohydride (2 hours).

The thiocompound-APBA conjugates were prepared in order to modify the gold biosensor surface with a monolayer of boronate groups. In the first step, the carboxygroup of mercapto-terminated (on the opposite side of chain) acids was activated with carbodiimide (3 hours, 99 ^{o}C). Conjugation of aminophenylboronic acid to bellow mwntioned thiocompounds was performed during the next step: DTSP, 11-MUA, 16-mercaptohexadecanoic acid and lipoic acid (3 hours, 99 ^{o}C). Final products exhibited light-yellow color and were stored at -20 ^{o}C prior use.

A monolayer of boronic groups was prepared, when the freshly cleaned gold electrodes were incubated with 15 µl of the conjugate for 24 hours at laboratory temperature in a closed chamber.

Gold surface modified with 11-mercaptoundecanoic acid and the freshly cleaned gold electrode were used as reference surfaces. For comparison of binding specifity to the matrix-modified surfaces, the polyethylene imine layer was attached to the piezosensor.

2.3.2 Specific Modification of Microtitre Plate

The 'Amino Immobilizer' microtitre plate from Nunc shows ability to bind covalently any molecule containing primary aminogroup. 10 mg/mL solution of aminophenylboronic acid (APBA) in 50 mM carbonate buffer pH=9.5 was used to cover the microtitre plate with boronic groups. The solution of APBA was kept overnight under ambient temperature in order to cover the wells of plate with boronic groups. After 4-times repeated washing (PBS pH=7.4), the unreacted surface group were saturated with glycine (25 mg/mL in PBS buffer pH=7.4) during 2 hours reaction performed under ambient temperature. After thorough washing with PBS, the plates were dried in the air stream of ambient temperature (4 hours). Such modified plates can be long-term stored in a well sealed box (4 ^{o}C) without any significant loosing of their binding activity.

2.3.3 Biosensor Setup - Measuring Procedure

A similar protocol was used for all experiments:

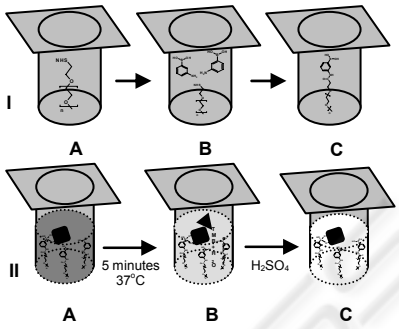

Figure 1: I – Immobilization of the APBA molecule to the activated surface of microtitre plate (A, activated surface; B, APBA solution added; C, APBA-modified surface. II – Procedure of total and glycated hemoglobin determination in boronic acid modified plate (A, well filled with diluted blood sample – total hemoglobin determination during binding of GHb to the surface; B, peroxidase activity of bound GHb is used to oxidase a substrate; C, reaction stopped and the overall activity measured).

after 5 min of the base-line signal stabilisation with the carrier buffer, the flow of sample - glycated hemoglobin dissolved in the carrier buffer (alternatively supplied by sorbitol solution) followed for 7 min. For the next 7 min, the flow cell was washed with the carrier buffer in order to equilibrate the signal (non-specifically adsorbed molecules dissociated from the biosensor surface). Injection of 200 mM aquatic solution of HCl for 120 seconds disintegrated the complex formed between glycated hemoglobin and monolayer of boronic acid groups; complex [GHb-matrix immobilized APBA] dissociated spontaneously. Washing with working buffer for a few minutes followed (new base-line stabilization) prior performing the next measuring cycle.

2.3.4 AHA Analysis

Another way of determination of total hemoglobin in blood samples is, comparing to the previously employed conversion to a cyanomethemoglobin, its conversion to alcalic hematin. The carbonate buffer pH=9.0 was used for this purpose, when the blood samples were diluted 400-times in this medium, the most of hemoglobin molecules was converted to the hematin. This can be quantified by measuring of absorbance at 405 nm. Moreover, the alcalic pH is an optimal value to support the affinity interaction between the boronic group and GHb in a solution.

The blood solution (in a carbonate buffer) was left to interact with the surface immobilized boronic groups for 60 minutes, at ambient temperature, in a closed box. The total hemoglobin content was quantified as change of A_{405} during 20 minutes following the reagent addition. Afterwards, the wells were washed 4-times with PBS buffer pH=7.4 and the peroxidase substrate solution, containing 0.075% hydrogen peroxide and 105 ug/mL of tetramethylbenzidine in 50 mM acetate buffer pH=4.5 (solution freshly prepared before each experiment). The enzymatic reaction releasing intensive blue color was left to proceed for 5 minutes in a dark box heated to 37 ^{o}C. The reaction was stopped by addition of 50 uL of 1 M H_2SO_4 solution to each well. The color of solution in wells turns yellow immediately. The amount of the enzymatic reaction product was measured as absorbance at 405 nm in a microtitre plate reader.
The absorbance of the whole blood solution corresponds to a total hemoglobin in a sample, the enzymatic activity of bound hemoglobin (measured as A_{405} in a next step) is proportional to a glycated hemoglobin content. The percentage of GHb presence in the total hemoglobin can be easily calculated by simple dividing of those two values.

3 RESULTS AND DISSCUSSION

3.1 Biosensor based Experiments

The amount of boronic groups deposited on the surface of piezoelectric sensors was first monitored during the immobilisation procedure. The deposited mass was calculated according to Saurbray equation from the difference of resonant frequency during deposition. These results indicate that the highest amount of boronic groups was coupled to the biosensor surface via 3,3′-Dithiodipropionic acid di(N-hydroxysuccinimide ester (DTSP), 11-mercaptoundecanoic acid (11-MUA) and mainly inside the polyethylene imine structure.

However, the evidence of optimal affinity for samples containing glycated hemoglobin provided the comparative experiments. Within those, the eight types of prepared biosensors with either specific or reference surfaces were consequently mounted into the flow-trough cell and the response to GHb sample (410 μg/ml, constant concentration) was monitored. The experiments were done in duplicate with each sensor; the equal scheme of experiment was used in all cases. The lowest response provided the Gold-PEI-GA-APBA sensor (together with the appropriate reference one). Therefore these were tested for their ability to bind sorbitol (low molecular compound containing vicinal diol group) in concentration of 10 mg/ml (in phosphate buffer pH=9.0). Response of the specific sensor (229.6 Hz) together with the reference one (19.8 Hz) showed correctness of theoretical predictions. Low density of boronate groups presented on the top of PEI-matrix (low affinity to glycated hemoglobin) allowed only low binding of glycated hemoglobin. Moreover, the difference between specific and non-specific response (66.5 vs. 44.6 Hz, respectively) was the next, and probably main, reason to exclude the PEI-GA-APBA recognition layer from further use in GHb analysis.

As it was commonly considered the boronic acid-diol interaction is not substantially affected by ionic strength of environment. However, most recent publications (Zhong et al., 2004) indicated a substantial increase of boronate affinity to diol group in low ionic strength solutions (co-solute concentrations up to 0.25 M). Determination of the ionic strength effect on glycated hemoglobin interaction with immobilised boronic groups was not the principal aim of our study. However, the examination of influence of the used various reagents on interaction were carried out prior to the final analysis. The ionic strength of tested reagents proceeded from 0.9 to 84.3 mM (Modified Drabkin reagent and 50 mM phosphate buffer, respectively), pH was in range 7.4 - 9.6. Low ionic strength, i.e. use of Modified Drabkin Reagent, supports the affinity interaction. This result well correlates with findings of other authors.

The biosensor Gold-MUA-APBA and the previously optimized conditions (peristaltic pump; flow rate of 100 μl/min; Modified Drabkin reagent as the working medium and the 2 min regeneration of sensing surface with 200 mM HCl) were used in all calibration experiments. The presented method shows the advantage of calibration using only one standard solution – blood sample with defined content of glycated hemoglobin. After dilution in various proportions, thus obtained standards were used for calibration. The response of the piezoelectric biosensor as well as photometric sensor was increasing with increasing concentration of glycated and total Hb, respectively. The percentage of glycated hemoglobin was calculated as the glycated hemoglobin / total Hb ratio (x 100%). Both values (glycated and total hemoglobin concentration, respectively) were taken from the calibration curves, constructed as the response of biosensor and photometric sensor to concentration of glycated and total hemoglobin, respectively. The biosensor can not be calibrated only using samples containing various percentage of glycated hemoglobin, the

amount of total hemoglobin should be considered, too.

A blood sample of diabetic patient with high content of glycated hemoglobin (14.3%; determined by the ion-exchange HPLC) was used for calibration of our setup. The set of six samples for calibration of analyser was prepared by dilution of blood with the Modified Drabkin reagent in the following sequence: 300, 375, 500, 600, 1000 and 2875-times. Thus prepared samples were placed to the autosampler and after 15 min of preincubation (including the base-line stabilisation) were consequently measured. The combined calibration graph was constructed using the responses of photometric and piezoelectric sensors in the time 5 min (Fig. 2).

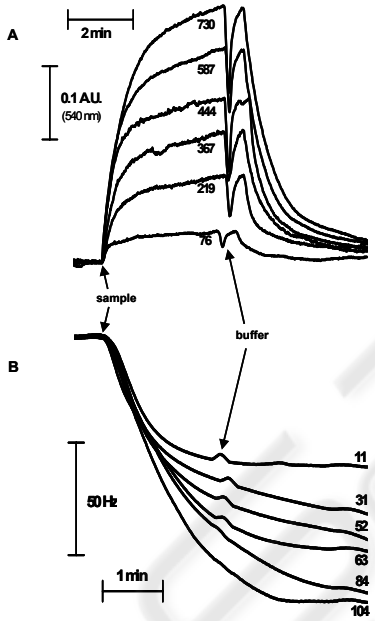

Figure 2: Calibration of a combined setup, upper curves show absorbance changes due to the different hemoglobin content in samples, the curves below were recorded as a result of binding of various concentration of glycated hemoglobin.

3.2 AHA Experiments

The AHA method was optimized according its ability to bind glycated hemoglobin. Buffers of various pH (7 and 9, respectively) were used to maximize the surface affinity to GHb. Although there was found higher adsorption of glycated hemoglobin at pH=7, the next experiment showed the low specifity of binding at this pH. On the other hand, use of buffer of pH=9.0 provides quite a lower capacity of the surface, however, the binding is highly specific (Fig. 3). The reference surface, covered only with glycine, was employed to compare specifity of binding.

In the further experiments the assay was calibrated by use of hemoglobin standard solution (total Hb calibration) and blood sample (with known GHb content, determined by a standard method), both diluted in various ratio in order to get at least five points in a calibration graph. The total hemoglobin assay provided a linear response in range 10 – 1000 μg/mL; the GHb analysis can be performed in the concentration range varying between 10 and 40 μg GHb/mL.

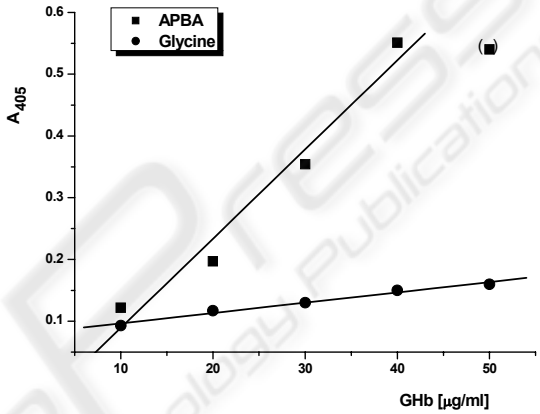

Figure 3: Measured enzymatic activity as result of binding of glycated hemoglobin to reference (modified with glycine) and specific surface (modified with aminophenylboronic acid, APBA). Experiments performed at pH=9.0.

4 CONCLUSIONS

Two methods for analysis of hemoglobin A_{1c} are presented. First, a combined biosensor for determination of glycated and total hemoglobin in blood is reported. The amount of total hemoglobin is measured in flow-through photometric sensor, concentration of the glycated fraction is subsequently monitored by its binding to the APBA-modified piezoelectric biosensor (higher content cause higher damping of resonant frequency).

The other method is based on ELISA principle (AHA, Affinity Heterogeneous Assay) in either direct or indirect arrangement. Boronic acid derivative, capturing the glycated fraction of hemoglobin, is immobilized on the surface of the microtitre plate. Amount of glycated hemoglobin is visualized by measuring of its peroxidase activity. Total hemoglobin concentration is measured photometrically at 405 nm.

Both methods present promising approach in diagnosis of glycohemoglobin. The first one presents fully automatic, low-cost instrument, the other one offers the possibility to monitor simultaneously HbA_{1c} content in 96 blood samples within a relatively short time (2 hours).

Possible use of those methods in routine analysis of blood samples and their comparison with conventional methods (HPLC) was shown, too.

ACKNOWLEDGEMENTS

The work was supported by the grant no. KJB401630701 of the Grant Agency of the Czech Academy of Science.

REFERENCES

Bongartz, D., Hesse, A., 1995. Selective extraction of quercetrin in vegetable drugs and urine by off-line coupling of boronic acid affinity chromatography and high-performance liquid chromatography. J. Chromatogr., B. 673, 223-230.

Chen, B., Bestetti, G., Day, R.M., Turner, A.P.F., 1998. The synthesis and screening of a combinatorial peptide library for affinity ligands for glycosylated hemoglobin. Biosens. Bioelectron. 13, 779-785.

International Committee for Standardization in Haematology, 1978. Recommendations for reference method for hemoglobinometry in human blood (ICSH Standard EP 6/2: 1977) and specifications for international hemoglobincyanide reference preparation (ICSH Standard EP 6/3: 1977). J. Clin. Pathol. 31, 139-143.

Ito, H., Kono, Y., Machida, A., Mitsumoto, Y., Omori, K., Nakamura, N., Kondo, Y., Ishihara, K., 2003. Kinetic study of the complex formation of boric and boronic acids with mono- and diprotonated ligands. Inorg. Chim. Acta 344, 28-36.

Kataoka, K., Hisamitsu, I., Sayama, N., Okano, T., Sakurai, Y., 1995. Novel sensing system for glucose based on the complex formation between phenylborate and fluorescent diol compounds. J. Biochem. 117, 1145-1147.

Kugimiya, A., Takeuchi, T., 2001. Surface plasmon resonance sensor using molecularly imprinted polymer for detection of sialic acid. Biosens. Bioelectron. 16, 1059-1062.

Lau, O.W., Shao, B., Lee, M.T.W., 2000. Affinity mass sensors: determination of fructose. Anal. Chim. Acta 403, 49-56.

Marshall, S.M., Barth, J.H., 2000. Standardization of HbA_{1c} measurements: a consensus. Ann. Clin. Biochem. 37, 45-46.

Pickup, J.C., Hussain, F., Evans, N.D., Rolinski, O.J., Birch, D.J.S., 2005. Fluorescence-based glucose sensors. Biosens. Bioelectron. 20, 2555–2565.

Přibyl, J., Skládal, P., 2005. Quartz crystal biosensor for detection of sugars and glycated hemoglobin, Anal. Chim. Acta 530, 75-84.

Valina-Saba , M., Bauer , G., Stich , N., Pittner , F., Schalkhammer T., 1999. A self assembled shell of 11-mercaptoundecanoic aminophenylboronic acids on gold nanoclusters. Mat. Sci. Eng. C 9, 205-209.

Zhong, H., Li, N., Zhao, F., Li, K., 2004. Determination of proteins with Alizarin Red S by Rayleigh light scattering technique. Talanta 62, 37–42.