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Immunochemical Determination of Hemoglobin-A1c Utilizing a Glycated Peptide as Hemoglobin-A1c Analogon

Daniela Stöllner[1], Axel Warsinke[1], Walter Stöcklein[1], Rudolf Dölling[2] and Frieder Scheller[1]
[1]Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Str. 24-25, Haus 25 D-14476 Golm
dstoelln@rz.uni-potsdam.de
[2]Biosyntan- Gesellschaft für bioorganische Synthese mbH, Robert-Rössle-Str. 10, D-13125 Berlin

Abstract

We describe the development of a heterogeneous affinity-matrix based immunoassay for the determination of HbA1c which could in future be applicable to analytical devices.

We developed an immunoenzymometric assay (IEMA) where the glycated pentapeptide Val-His-Leu-Thr-Pro (VHLTP) as HbA1c analogon is immobilized either to the surface of a microtiter plate by adsorption or to an amino-modified cellulose membrane by covalent linkage. The immobilized analogon competes together with the HbA1c in the sample for the antigen binding sites of the anti-HbA1c antibodies. Glucose oxidase-labeled antibodies have been used to indicate the antigen-antibody reaction indirectly and enzyme activity was detected optically. Calibration curves for HbA1c were obtained with a linear range of 1,5-10 µg ml^{-1} (23-155 nM). In a mixture of non-glycated and glycated hemoglobin with a total hemoglobin concentration of 30 µg ml^{-1} (465 nM) a linear range was obtained between 5-50 % HbA1c.

Since the glycated peptide shows a high affinity for the anti-HbA1c antibody (K_d = 0,3 nM) only a low contact time (< 1 min) between the modified solid support and the preincubated mixture of HbA1c and anti-HbA1c antibody was required. Regeneration of the affinity-matrix was carried out with 10 mM HCl for 3 min without loss of antibody binding activity.

Introduction

Diabetes mellitus is characterized by chronic hyperglycemia which means an increased blood glucose level of over 150 mg dL^{-1} (8,3 mM) due to defects in insulin production or action [a, b, c]. In order to prevent long-term damage of various organs, e.g. eyes, kidneys, nerves and blood vessels, it is the aim of each diabetes management to keep the blood glucose concentration at ideal levels (80-120 mg dL^{-1} or 4,4-6,7 mM). Conventionally, the blood glucose level is determined at any time as a guide for insulin or oral agents treatment which is carried out easily by the patient himself at home. Since the blood glucose concentration fluctuates rapidly in dependence on the consumed food and insulin injection, a more reliable method to control the overall glycemic status is required. Hemoglobin-A1c (

HbA1c) reflects the average blood glucose control for the past three months according to the lifespan of the red blood cells. It helps the patient and the health care team to judge about the success of the diabetes treatment plan [1].

Hemoglobin, as many other proteins in blood, links up with sugars, such as glucose. HbA1c in particular is formed when its N-terminal valine of the beta-subunit is non-enzymatically glycated by blood glucose [2]. In a person without diabetes less than 5 percent of all hemoglobin molecules are glycated. Since the blood glucose level is elevated up to 3 times in diabetics more hemoglobin gets glycated [3]. The HbA1c level can directly be related to a patient's mean blood glucose level (MBG). If the mean blood glucose concentration varies from 1,4 to 2 mM (25-36 mg ml^{-1}) a change of 1 % of the HbA1c level can be observed . This calculation is based on the linear regression formula reported in [4].

Laboratory tests for HbA1c are based on cation exchange or affinity chromatography, electrophoresis or immunochemical methods [5]. Usually they are fully automated with a high throughput but not suitable for single patient-side measurements.

To provide a better diabetes treatment point-of-care HbA1c devices based on immunochemical methods [6, d] or affinity chromatography [e] were developed. The assay systems consist of disposable cartridges which require only a small drop of finger blood and produce test results within a few minutes [7]. However, only this single parameter can be determined resulting in high expenses since an additional device has to be purchased.

Since biosensor based instruments for clinical parameters, e.g. glucose and lactat, have already found application in physician's offices, an integration of HbA1c measurements into existing analyzers would be desirable. Therefore principles allowing repetitive HbA1c analysis were investigated utilizing either an enzyme or antibodies as the biorecognition element. An electrochemical biosensor based on the enzymatic conversion of fructosyl-valine as model compound for HbA1c by a novel fructosamine oxidase from a marine yeast was reported [8]. However, a time-consuming pretreatment of HbA1c by a protease in order to release the fructosyl-valine residue is necessary.
We focused our research on the development of a heterogeneous immunoassay utilizing the high specificity of antibodies to distinguish between HbA1c, HbAo (nonglycated hemoglobin) and other variants of hemoglobin. In this immunoenzymometric assay (IEMA) a glycated peptide as a HbA1c analogon is immobilized on a solid support and competes together with the HbA1c of the blood sample for the amount of anti-HbA1c antibody (Fig.1). The synthetic glycated peptide has an amino acid sequence corresponding to the first 5 amino acids of the N-terminal hemoglobin sequence of the beta-chain. For the development of a regenerable HbA1c-biosensor based on an affinity matrix the use of a synthetic peptide instead of the whole protein provides more functional stability when elution buffer is applied. Thus, repeated measurements should be possible without loss of antibody binding activity.

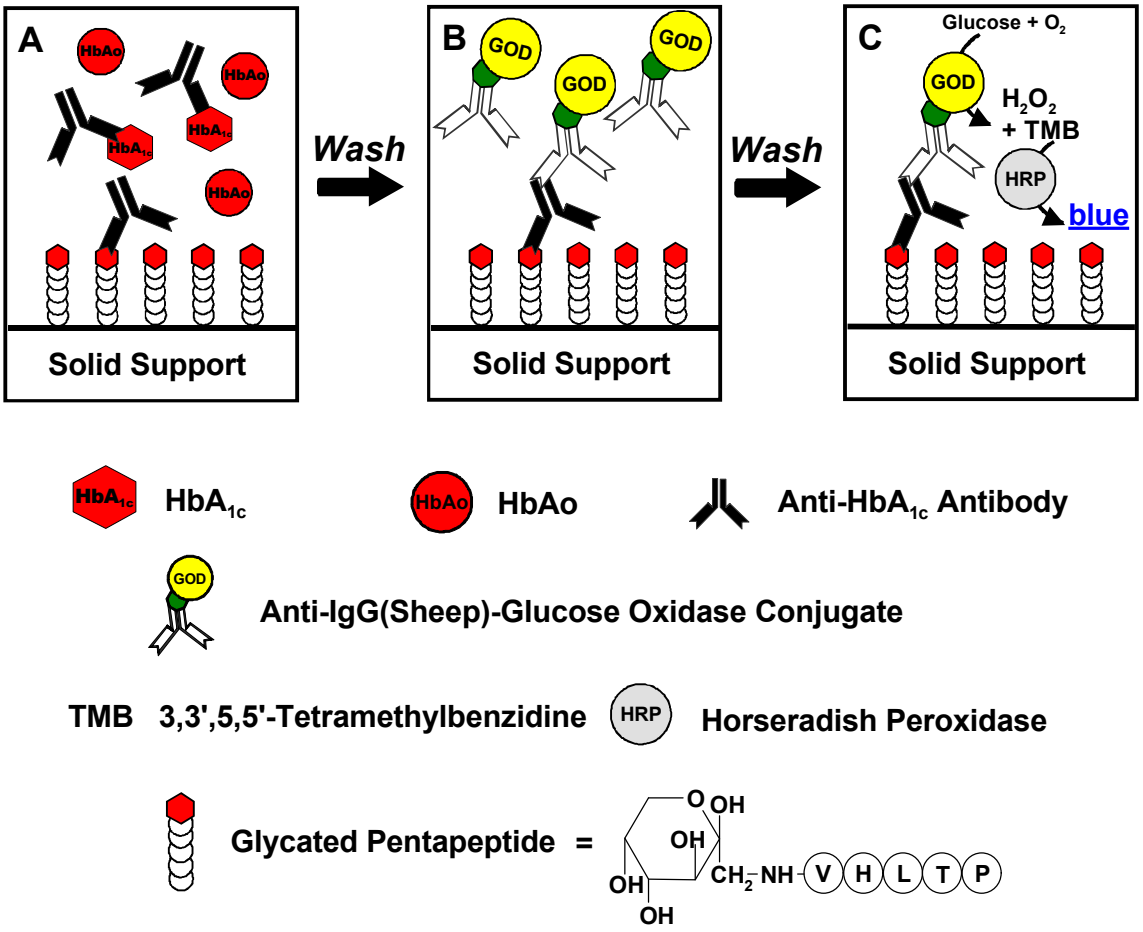

Fig.1 Principle of the HbA1c immunoassay. HbA1c in the sample containing either solely HbA1c or a HbA1c/HbAo mixture competes with the immobilized glycated pentapeptide for the antigen binding sites of the anti-HbA1c antibodies (A). After a washing step the added anti-IgG(sheep)-GOD binds to the anti-HbA1c antibody (B). After a washing step the bound GOD is measured (C).

Here, we show that the synthetic glycated peptide Val-His-Leu-Thr-Pro (VHLTP) can be used as HbA1c analogon in a microtiter plate immunoassay to quantitate HbA1c. Furthermore, we developed a cellulose based affinity matrix which, in future, could act as solid support for a HbA1c-biosensor.

Materials and Methods

Chemicals

Hemoglobin-A1c (HbA1c) was isolated from hemolysates of diabetics according to the procedure described in [9]. A stock solution was made up at 10 mg ml^{-1} in PBS and stored at 4°C.
Ferrous Hemoglobin-Ao (HbAo), horseradish peroxidase (HRP, EC 1.11.1.7), activity 3500 U mg^{-1}, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) were purchased from Sigma (Steinheim, Germany). 1,1'-Carbonyldiimidazole (CDI), N-hydroxysulfosuccinimide (sulfo-NHS) and bovine serum albumin (BSA) were obtained from Fluka (Steinheim, Germany). 1,8-Diamino-3,6-dioxaoctan and triethylamine (TEA) were received from Merck (Darmstadt, Germany). The pentapeptide (VHLTP) was purchased from Bachem (Switzerland). Anti-HbA1c antibodies were from Tinaquant® kit from Roche (Switzerland). Biotin-labeled anti-IgG(sheep) antibodies were from Jackson Immuno Research (U.S.A.). Avidin-labeled glucose oxidase was supplied from Vector Laboratories (U.S.A.). All other chemicals were of analytical grade and obtained from Sigma, Aldrich, Fluka, or Merck.
Cellulose membrane (Nephrophan) was from ORWO (Wolfen, Germany).

Instrumentation

Microtiter plates (Maxisorp) were purchased from Nunc (Danmark). The microtiter plate reader was a Multiscan RC Version 3.0 (Labsystems, Finland). The BIACORE 2000 and sensor chips CM5 were obtained from Biacore AB (Sweden). A RAININ-HPLC system with UV detection (Zinsser Analytic, Germany) with a cation-exchange column (Poros SP/H, 4,6x100 mm, PerSeptive Biosystems, USA) was used.

Preparation of Glycated Pentapeptide

The N-terminal glycated pentapeptide with the amino-acid sequence Val-His-Leu-Thr-Pro (VHLTP) was used as ligand for the anti-HbA1c antibody. It was prepared by suspending 35 µmol of the underivatized Val-His-Leu-Thr-Pro peptide and 180 µmol D(+)glucose in 2 ml pyridine. After incubating at room temperature for 5 days, the reaction mixture was dried in vacuum and the resulting syrup was resuspended in distilled water and dried again. After repeating this procedure 6 times, the product was purified and analysed by cation exchange chromatography and mass spectrometry. The extent of glycation was determined to be at least 99 %.

Preparation of Glycated Pentapeptide-BSA Conjugate

3 mg of the glycated pentapeptide was activated via the carbodiimide method [10] with EDC and sulfo-NHS (molar ratio 1:2,5:6) in DMSO for 30 min and coupled to 4 mg BSA in 0,1 M carbonate buffer at pH 8,5 for 16 h. After gel filtration with PBS-equilibrated NAP-5 columns (Pharmacia Biotech, Sweden), the resulted product was evaluated by testing the binding activity of the anti-HbA1c antibody in comparison to uncoupled BSA. After adsorption of glycated pentapeptide-BSA, the microtiter plates were blocked with PBS containing 3 % BSA and 0,05 % Tween20 (blocking buffer) and first incubated with anti-HbA1c antibodies (0,1-5 µg in blocking buffer), washed again, and finally incubated with a

mixture of 0,12 µg biotin-labeled anti-IgG (sheep) antibodies and 0,5 µg avidin-GOD conjugate. After addition of substrate solution containing 50 mM D(+)glucose, 0,4 mM 3,3‘,5,5‘-tetramethylbenzidine (TMB) and 25 µg ml^{-1} HRP in 0,1 M acetate buffer at pH 5,5, absorbance was measured at 650 nm with the microtiter plate reader.

Hemoglobin-A1c ELISA

Microtiter plates were coated with 4 µg ml^{-1} glycated pentapeptide-BSA (100 µl per well) for 1 h at room temperature and blocked with 3% BSA in PBS for 1 h. HbA1c samples were denatured with 1 M guanidinium hydrochloride by heating up to 56°C for 1 min before mixing with hemoglobin-Ao to give a final hemoglobin concentration of 30 µg ml^{-1}. The HbA1c /HbAo-mixture was preincubated with 1 µg ml^{-1} anti-HbA1c antibody in PBS/3 % BSA for 30 min. Samples containing either solely HbA1c or the HbA1c/HbAo mixture were applied to the wells for 1 min. The following steps were performed according to the protocol of the evaluation of the glycated pentapeptide-BSA conjugate desribed above except that the reaction with glucose and TMB was stopped with 2 N sulfuric acid after 7 min, and the extinction was measured at 450 nm. Each HbA1c concentration was measured 3-fold in separated wells, and the standard deviation was calculated.

Preparation of the Glycated Pentapeptide-Modified Membrane

Cellulose membrane (1 cm diameter) was activated in dry acetone with carbonyldiimidazole (0,636 mg ml^{-1}) for 1 h. After washing with acetone, the membrane was treated with 1 M 1,8-diamino-3,6-dioxaoctan in 1 M carbonate buffer (pH 9,5) for 12 h. Glycated pentapeptide was activated by the carbodiimide method for 30 min with EDC and sulfo-NHS (molar ratio 1:9:4) in 0,01 M phosphate buffer (pH 6,0) and coupled to the amino-modified membrane at 4°C for 16 h. The glycated pentapeptide-modified membrane was washed rigidly and stored in PBS containing sodium azide.

HbA1c-Immunoenzymometric Assay Carried Out on Glycated Pentapeptide-Modified Membranes

The immunoassay on glycated pentapeptide-modified membranes was carried out according to the protocol of the HbA1c ELISA described above.
Dilution of HbA1c and antibodies were made from stock solution in PBS/3% BSA. Glycated pentapeptide-modified membranes were blocked with PBS/3% BSA before incubating with the preincubated (30 min) HbA1c/anti-HbA1c antibody mixture for 5 minutes and with 1,2 µg biotin-labeled anti-IgG (sheep) antibodies and 5 µg avidin-GOD conjugate for 1 h. Bound conjugate was measured by incubating in D(+)glucose in PBS, taking 100 µl aliquots of the reaction solution after 30 min and mixing with TMB/HRP to give a blue colour. Absorption was measured after adding 2 N sulfuric acid at 450 nm. The membranes were regenerated with 10 mM HCl for 3 min.

Results and Discussion

Glycated Pentapeptide as Hemoglobin-A1c Analogon

A heterogeneous HbA1c immunoassay has been developed which uses an immobilized glycated pentapeptide instead of the whole HbA1c molecule for an immunoenzymometric assay (IEMA) configuration. The sequence of the glycated peptide is identically with the first 5 amino-acids of the N-terminal end of the beta-chain of hemoglobin. In addition, a glucose molecule is coupled to the alpha-amino group of valine. In order to check the possibility for utilization in an immunoassay, investigations with BIACORE 2000 concerning affinity of the anti-HbA1c antibody to the glycated peptide were performed. Therefore, the glycated peptide was covalently immobilized via its C-terminal end to the amino groups of a modified CM5 sensor chip surface with high ligand density. K_d values were evaluated by preincubation of the antibodies with different concentrations of glycated peptide, measurement of the noncomplexed antibodies via association curves, and calculation of the K_d values from the inhibition plots according to Karlsson [11] for low molecular weight analytes. For the anti-HbA1c antibody interacting with free glycated pentapeptide the K_d value was determined as 0,3 nM whereas a 250 fold higher K_d value was obtained for the anti-HbA1c antibody interacting with free HbA1c. A K_d value of 75 nM was calculated assuming 1:1 binding for high molecular weight analytes, not regarding possible aggregation, as both the antibody and HbA1c have two binding sites. In conclusion the glycated pentapeptide is recognised more sufficiently by the antibody used. The affinity of the anti-HbA1c antibody to glycated pentapeptide is decreased when the peptide is covalently immobilized onto the sensor chip surface at low ligand density (K_d = 25 nM). The K_d value was derived form k_{on} and k_{off} rates of the antigen-antibody interaction according to BIACORE evaluation software version 3.0, global fitting. However, it was shown that low contact times (less than 1 min) of anti-HbA1c antibody and immobilized glycated pentapeptide are sufficient for a strong and stable binding.

Hemoglobin-A1c ELISA

The well-established ELISA technique was used to develop an immunoenzymometric assay with glucose oxidase labeled antibodies and microtiter plate-adsorbed glycated pentapeptide-BSA conjugate. A calibration curve was measured (absorbances vs. HbA1c concentration) with a useable measuring range between 1,5 and 10 µg ml^{-1} (23-155 nM) HbA1c (Fig.2). Since the principle was tested for its ability of HbA1c analysis HbA1c standards prepared as described in materials and methods were used instead of real blood samples.

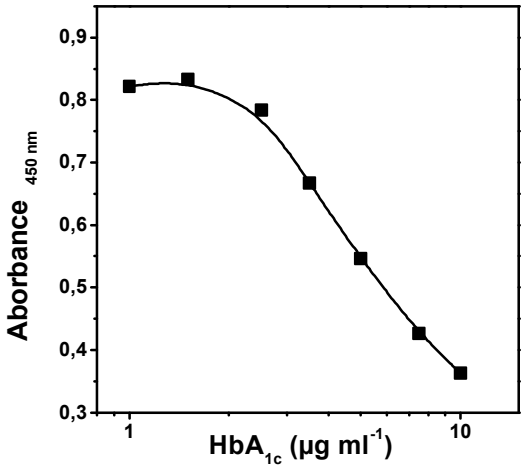

Fig.2 Calibration curve for HbA1c measured with the Hemoglobin-A1c-ELISA.

As expected for competitive assays, there is an inverse dependency of the signal to the antigen concentration. Furthermore HbA1c/ hemoglobin-Ao samples containing 1-100 % HbA1c of total hemoglobin were prepared and assayed. A final concentration of 30 µg ml^{-1} (465 nM) of total hemoglobin was chosen which means a HbA1c concentration of 1,5-6 µg ml^{-1} (23-93 nM) for the clinically relevant range (5-20 %). This should guarantee that the clinically relevant range is included in the useable measuring range obtained in the experiment described above. Indeed it could be shown that the clinically relevant range (5-20 % hemoglobin-A1c) was covered by the measuring interval (5-50 %) of the obtained calibration curve (Fig.3). Hence, the developed ELISA affords a dilution factor of about 5000 for blood samples.

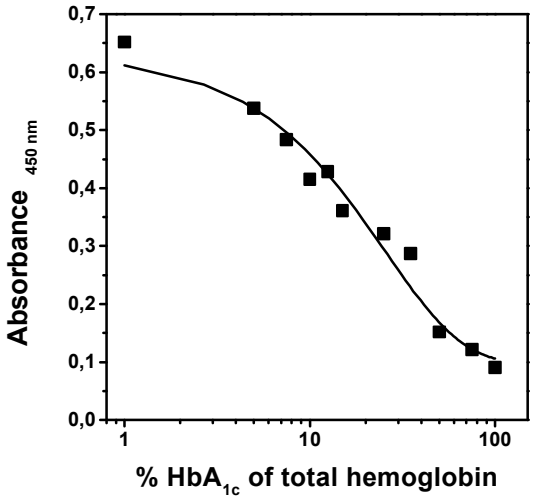

Fig.3 Calibration curve for HbA1c measured with the Hemoglobin-A1c-ELISA, final concentration of total hemoglobin 30 mg ml^{-1} (465 nM).

Hemoglobin-A1c Immunoassay on Modified Cellulose-Membranes

In a next step the hemoglobin-A1c specific immunoreaction was performed on a glycated pentapeptide-modified cellulose membrane as the solid support which was produced by covalent attachment of the ligand to a thin amino-modified cellulose membrane with a MWCO of 20 000. The glycated pentapeptide-modified membrane was incubated with the HbA1c/ anti-HbA1c antibody mixture for 5 min (one membrane per HbA1c sample). So far the detection of the glucose oxidase label was done by optical absorbance spectroscopy. Fig. 4 shows the calibration curve for HbA1c concentrations between 2 and 7,5 µg ml^{-1} which corresponds to 31-116 nM HbA1c. After each measurement the membrane was regenerated with 10 mM HCl for 3 min. The assay was repeated three times, no signal decrease could be obtained.

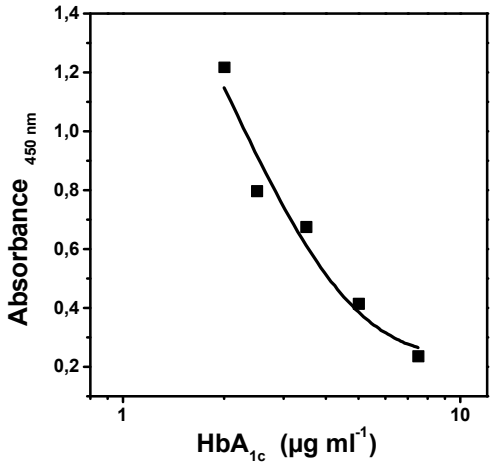

Fig.4 Calibration curve for HbA1c measured with the immunoenzymometric assay (IEMA) carried out on the glycated pentapeptide-modified cellulose membranes. Regeneration after each measurement with 10 mM HCl for 3 min.

As the investigations with BIACORE 2000 showed, repetitive regeneration of the glycated pentapeptide surface is possible more than 20 times without loss in binding activity to the anti-HbA1c antibodies. So far one measuring cycle lasts 2 h. An optimized incubation protocol where an GOD labeled anti-HbA1c antibody instead of the secondary antibody is used and the duration of the preincubation step is reduced will allow measuring cycles of less than 10 minutes.

In conclusion cellulose membrane was chosen as the solid support since it allows a covalent immobilization of an immunoreactant near the electrode surface and excludes proteins (e.g. antibody-enzyme conjugates) from the space between sensor and membrane where they could passivate the electrode. This principle was recently used for the construction of a creatinine immunosensor [12]. The developed glycated pentapeptide membrane is a suitable affinity matrix for biosensor application. In combination with an amperometric transducer this could be a first step towards a regenerable and rapid HbA1c immunosensor.

Acknowledgement

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