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*Article*
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# A Label-Free Optical Nanobiosensor for Measurement of HbA1c via Portable Reader

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**Abstract**: Access to accurate diagnostic tools is one of the essential challenging improvements in global health assessment. Currently, most existing diagnostic tools are targeted for use in developed countries and do not meet the health needs of developing and underdeveloped countries. When they are presented as a solution to global health problems, they are expensive and complex. One solution is using portable diagnostic methods like point-of-care tests (POCTs). One of the most widely used POCTs instruments is the portable glucometer, which is used today by a large group of diabetic and non-diabetic patients to control blood sugar. Although the common view of this device is called glucometer, according to the basic knowledge and technology of the electrochemical mechanism of this device, it can be used for identifying various markers by engineering and designing via an appropriate condition. This study aims to design and develop a new nanomolecular diagnostic method to quantify HbA1c using a glucometer as an electrochemical-optical reader with several advantages compared to the other existing methods for this purpose. Here, we design and describe a portable and label-free optical method with high reproducibility in performance using aptamer-conjugated magnetic nanoparticles (MNPs) for capturing and specific detection via measurement with a glucometer.

**Keywords:** aptamer; magnetic nanoparticles; biosensor; glucometer; colorimetric assay.



## 1. Introduction

Diagnosis and treatment of diseases have always been the main focus of research in the field of biomedicine and have attracted the interest in the field of medicine [1]; because early diagnosis of the disease provides an opportunity for timely and appropriate treatment and dramatically plays an effective role in reducing the mortality rate from the disease [2]. Currently, most existing diagnostic methods are targeted for use in developed countries and do not meet the health needs of developing and underdeveloped countries. When they are presented as a solution to global health problems, they are expensive and complex [3, 4]. For example, many of these diagnostic methods require moderate facilities and training that are not available in large areas of non-developing countries [5]. Although most of these molecular diagnostic methods provide the sensitivity and specificity needed to diagnose a wide range of diseases, they have their complexity and require efficient, trained personnel and sophisticated tools and techniques [6]. Lack of efficient and expert personnel and access to complex diagnostic facilities and tools due to its high cost, the need to design diagnostic systems that are simple, fast, cost-effective, point of care, and done automatically feels strongly [7, 8]. The

growing number of reports on global health indicates the need for better and more accessible diagnostic tools for measuring human health biomarkers and detecting a variety of infectious diseases in deprived areas. The ideal diagnostic method, according to the standards of the World Health Organization, is a method that has an appropriate and acceptable level of sensitivity and specificity, is simple, reproducible, and cost-effective in terms of technique, and is able to adapt to any weather conditions and point of care use [9, 10]. Due to the increase in various diseases in underdeveloped and developing countries due to lack of timely diagnosis resources and lack of POCT, disease control and management have decreased. The main purpose of POCTs is to provide the diagnosis with simple and fast test results [11]. One of the most widely used POCT instruments today is the portable electrochemical glucose meter (glucometer), used by a large group of diabetic and non-diabetic patients to control blood sugar. One of the most widely used POCT instruments is the portable glucometer, used today by a large group of diabetic and non-diabetic patients to control blood sugar [12, 13]. Although the common view of this device is called glucometer, according to the basic knowledge and technology of the electrochemical mechanism of this device, it can be used to identify various markers by engineering and designing appropriate conditions based on nanotechnology.

For this reason, new nanotechnology-based diagnostic systems have been proposed to respond to the need for POCTs. Introducing nanotechnology into diagnostics to design accurate, fast, and cost-effective diagnostic methods based on molecular principles can be an important step toward improving global health [14]. In recent decades, nanoparticles have found significant importance in improving the performance and efficiency of nanotechnology in modern biomedical sciences and other fields of biotechnology. Nanoparticles can be engineered using a similar scale of biomolecules such as oligonucleotides, peptides, proteins, and cells to provide a variety of hybrid nanomaterials for the development of bio-analysis tools [15, 16].

A diagnostic system consists of at least three important parts: the target molecule recognition element, the signal transducer, and the appropriate platform for high loading of the recognition element to improve sensitivity [17]. Among these, various nanomaterials such as metal nanoparticles, semiconductor nanocrystals such as quantum dots and carbon nanomaterials, silica nanoparticles, and magnetic nanoparticles with special properties such as high aspect ratio and optical/electrical/magnetic and catalytic properties make them good candidates for signal transduction or even a suitable substrate for high capacity loading of recognition elements such as aptamers or antibody in the construction of new biosensors [18]. Sensing systems based on hybrid nanomaterials have unique properties that are significant for bioassay and medical diagnostic applications. In particular, the surface-to-volume ratio of nanomaterials provides a high potential for increasing the loading capacity of the recognition molecule at the nanomaterial surface, thereby increasing and amplifying the sensing signal several times, as well as the process of identifying the target molecule [19]. Meanwhile, magnetic nanoparticles provide the possibility of effective magnetic separation to remove unwanted molecular agents from the environment. This factor leads to the reproducibility of the reaction, reduces the error rate in quantifying the desired molecule, and increases the accuracy and sensitivity of the diagnostic reaction [20]. Therefore, the challenge of providing diagnostic methods and accurate control of diabetes remains in the research efforts as a stimulus [21]. Early diagnosis of diabetes and regular examination of blood glucose levels are essential factors in preventing health complications caused by this disease. Portable glucose meters are available to measure daily glucose levels and control diet and insulin intake. However, users

of these glucose meters acknowledge some of their shortcomings. For example, measuring blood glucose levels three to four times a day is recommended by physicians to patients. Glucose, which occurs naturally over the course of a day, can be affected by environmental factors and user error [22, 23]. The importance of these problems is highlighted when a clinician uses the results of daily glucose measurements using glucometers to diagnose diabetes and determine the relationship between lifestyle and the amount of medication used. Since the level of HbA1c in the blood responds to the long-term marker of diabetes without fluctuations in glucose levels, it has recently been concluded that the best marker for long-term glycemic control is HbA1c [24]. The Committee of International Experts (with members appointed by the American Diabetes Association, the European Union for the Study of Diabetes, and the International Diabetes Federation) decided in 2009 to introduce the HbA1c test as the first marker to diagnose diabetes [25]. The current standard laboratory methods for detecting HbA1c are mainly based on load difference (i.e., chromatography), structure (i.e., inclination assay or immunoassay such as ELISA), or enzymatic assays aimed at distinguishing between HbA1c and primary Hb. According to a study of effective methods for measuring HbA1c levels conducted by the American School of Pathology, safety methods (~ 65%) are the most common methods for measuring this biomacromolecule. Then, cation exchange chromatography (~ 31%) and inclination chromatography (~ 4%) are among the applied methods in this direction, respectively [26]. Diabetes occurs in people; the current WHO report estimates that 80% of deaths from type 2 diabetes occur in low- and middle-income countries where the HbA1c test market is under-resourced. In these countries, low-cost HbA1c monitoring tools for personalized medicine are crucial in providing timely treatments that slow the progression of the disease and minimize the devastating health, social, and economic consequences for those affected [6]. The development of inexpensive, user-friendly methods for testing HbA1c reduces operating costs, increases public access to HbA1c testing [27], and improves disease management by monitoring the level of HbA1c in a patient's blood. High-performance liquid chromatography (HPLC) and ion exchange and propensity chromatography are generally used to perform HbA1c tests. Still, their long test time and high operating cost are unsuitable for on-site diagnostic methods [6]. Numerous immunoassay methods have been designed to quantify HbA1c using monoclonal and polyclonal antibodies, such as the direct and sandwich ELISA methods that are commercially available. However, the separate measurement kits of these two factors (due to the long laboratory phase, the cost of consumption, and the inaccuracy of the percentage values of the target molecule) have not created a desire for the audience in clinical laboratories. In addition, safety assessments suffer from the instability of antibodies, the high production cost, and the structural and functional changes in each production line (batch to batch variation) that limit the clinical usefulness of these methods. Aptamers are now recognized as a viable alternative to immunoassay systems. Aptamers are short oligonucleotide single strands of DNA or RNA that can be used as antibody substitutes in the role of new biosensor detectors and are selected using the SELEX process in vitro and can cover a wide range of different types of molecules. Aptamers are used as a suitable alternative to antibodies in many bioassays operating systems due to their specific tendency to target molecules and high stability in vitro, ease of administration to various groups with high production capacity, and cost-effectiveness [22].

Compared to various biosensors such as microarrays, surface plasmon resonance, and chemical luminescence, electrochemical and optical-based biosensors containing specific aptamers are of great interest for detecting HbA1c [28]. These electrochemical sensors are

considered compatible with miniaturization in portable instruments for on-site detection. However, these designs can meet the demand for low-cost HbA1c measurements, due to the need for a professional user for two-step measurement [29]. Measurement (for total hemoglobin and glycosylated hemoglobin), precise preparation, and accurate purification of blood before testing and interpretation and evaluation of results that require professional personnel, the cost of performing this method increases. This study aims to design and develop a new nanomolecular diagnostic method to quantify HbA1c via glucometer as an optical-electrochemical reader for colorimetric analysis (Figure 1).

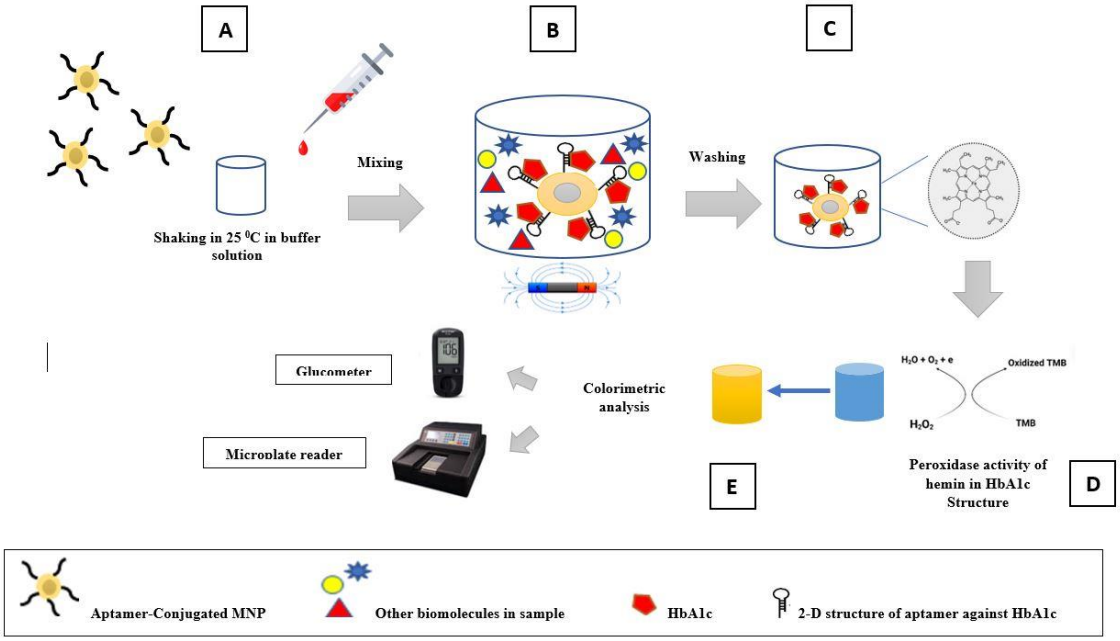



**Figure 1**. Schematic illustrated the nanomolecular detection method for detection and measurement of HbA1c in a blood sample using glucometer; (**A**) Adding HbA1c aptamer-conjugated MNPs to blood sample; (**B**) Specific and non-specific complexes among the aptamer-MNPs and HbA1c and other biomolecules in a blood sample; (**C**) Isolation of the specific complexes of the aptamer-conjugated MNPs and HbA1C molecules after capturing the specific complexes via a magnetic field and waste the non-specific complexes through a washing step; (**D**) Activating the peroxidase activity of the hemin part in the captured HbA1c and converting TMB-$H_2O_2$ to oxidased TMB, $H_2O$, $O_2$, and free-electron; (**E**) Color changing of the substrate-chromogen and its analysis with microplate reader and glucometer.

## 2. Materials and Methods

### *2.1. Chemicals and instruments.*

BIO-RP-purified DNA oligonucleotide with amino group modification in 3'end of sequence as modified specific aptamers for HbA1c and Hb were synthesized by Bioneer Corporation (Korea). Starch-coated iron oxide ($Fe_3O_4$), magnetic nanoparticles (fluidMAG), and magnetic separator plates were purchased from Chemicell (Berlin, Germany). Cyanogen bromide, $NaHCo_3$, phosphate buffer saline (PBS), bovine serum albumin (BSA), and sodium azide were obtained from Sigma-Aldrich, Germany. 3, 3', 5, 5' tetramethylbenzidine (TMB)/$H_2O_2$ ready to use, and stop reagent ($H_2SO_4$) for the TMB substrate were provided from Sigma -Aldrich, Germany. HbA1C control-high and HbA1C control-low (purchased from Pishtazteb Company, Tehran, Iran). UV-Vis spectra were given using a UV-Vis spectrophotometer (WPA Biowave II, UK). The Accu-Chek® Active Blood Glucose Meter

was purchased from Roche Company (Switzerland). Microplate readers were given using DANA-3200 ELISA READER, Garnimed Company, Tehran, Iran.

*2.2. Selection of specific aptamer sequences against HbA1c and total Hb.*

Many aptamers against HbA1c and total hemoglobin (tHb) have been previously reported (Table 1). We use the aptamers identified against HbA1c and tHb in the literature review to do this. Then, using the folding determination software, the desired and suitable dedicated aptamer for the proposed detection method has been selected in this study. This prediction was made using proprietary online software RNA-Structure (which makes it possible to study and predict secondary structures of RNA and DNA/ https://rna.urmc.rochester.edu/RNAstructure.html) that provides the ability to predict and simulation of the secondary structure of oligo-aptamers in collisions with a target molecule. Thus, the final 2-D structures of the aptamer folded by the sequence of specific oligo-aptamers are modeled by this program, and finally, the most suitable aptamer is selected.

**Table 1**. Aptamer sequences against tHb and HbA1c.

| Clone number | Aptamer sequence (5'-3') | Reference |
|---|---|---|
| G11 | CGACACCAGCACACAGACCCGAGACACACGTCAGATCAACAGCGACCGTATCATTGGTTG | [22] |
| G18 | GGCCACAGCAGCCAGTACACCCACCCACCAGCCCCGTCAACGACCTGAACCTGCCCTGTGTG | [22] |
| G15 | ACGCACACCAGAGACAAGTAGCCCCCCAAACGCGGCCACGGAACGCAGCACCTCCATGGC | [22] |
| G20 | GGGGACACAGCAACACACCCACCCACCAGCCCCAGCATCATGCCCATCCGTCGTGTGTG | [22] |
| G23 | GGACACGGCAAAGGGGTATAGCCTACCGGACCGTGGACATGGAATTGTGTGCTGCGTGG | [22] |
| G23 | GGACACGGCAAAGGGGTATAGCCTACCGGACCGTGGACATGGAATTGTGTGCTGCGTGG | [22] |
| G15 T1 | ACGCACACCAGAGACAAGTAGCCCCCCAAACGCG-FAM | [30] |
| G15 T2 | GCCACGGAACGCAGCACCTCCATGGC-FAM | [30] |
| G15 T3 | ACGCATAGCCCCCCAAACGCGGCCACGGAACGCAGCACCTCCATGGC- FAM | [30] |
| G20 T1 | ACACACCCACCCACCAGCCCCAGCATCATGCCCATCCGTCGTGTGT-FAM | [30] |
| G20 T2 | ACCCACCCACCAGCCCCAGCATCATGCCCATCCGTCGT-FAM | [30] |
| G20 T3 | ACCCACCCACCAGCCCCAGCATCATGCC-FAM | [30] |
| * | GGC AGG AAG ACA AAC ACA TCG TCG CGG CCT TAG GAG GGG CGG ACG GGG GGG GGC GTT GGT CTG TGG TGC TGT | [26] |
| ** | GGC AGG AAG ACA AAC ACC AGG TGA GGG AGA CGA CGC GAG TGT TAG ATG GTA GCT GTT GGT CTG TGG TGC TGT | [26] |

*2.3. Investigation of HbA1C peroxidase activity.*

Previous research has shown the peroxidase activity of the hemin prosthetic group of the human hemoprotein that is able to catalyze the oxidation of a large variety of substrates through a reaction with hydrogen peroxide [31]. The following test was performed to evaluate the peroxidase activity of HbA1c**.**

10 and 20 µl of HbA1c control with 5.5% concentration based on NGSP (National Glycohemoglobin Standardization Program) percentage concentration with 40 µl TMB / $H_2O_2$ (as a substrate/ chromogen for peroxidase reaction) were added to plate wells in duplicate tests for each concentration. In order to remove the background color caused by HbA1c, the same concentration of HbA1c in PBS buffer was used. The plate was incubated at room temperature with gentle shaking for 30 minutes, and then $H_2So_4$ as a reaction stop reagent was added to the same volume of TMB/$H_2O_2$. The color changes due to the peroxidase activity of the complex in the presence of HbA1c were evaluated, and the adsorption of the reaction solution at 450 nm was determined with a microplate reader. The following equations were used to convert values of HbA1c concentration in different units through NGSP percentage and IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) parameters.

$$Molar\left(\frac{\mu mol}{L}\right)\ to\ IFCC(\frac{mmol}{L})HbA1c \rightarrow \left(\frac{mmol}{mol}\right) = \frac{HbA1c\ \left(\frac{\mu mol}{L}\right)}{Hb\ \left(\frac{\mu mol}{L}\right)} \times 1000$$

$$IFCC\ \left(\frac{mmol}{mol}\right) to\ NGSP\ (\%) \rightarrow HbA1c\ (\%) = (IFCC\ \times 0.09148) + 2.152$$

*2.4. Bioconjugation of the aptamer to MNPs.*

To prepare aptamer-conjugated magnetic nanoparticles, the amino group modified aptamers were attached to the hydroxyl group of starch-coated MNPs according to the manufacturer's instructions (Figure 2). Briefly, fluidMAG nanoparticles (25 mg/ml) were washed once with 1 ml of activation buffer (0.2 mm sodium hydrogen carbonate buffer, pH 8.4) on a magnetic separator, then dispersed in 0.25 ml of activation buffer. 50 ml of CNBr was added to the nanoparticles and mixed by vortexing. The tube was placed in ice-cold water10 for minutes. The resulting nanoparticles were rapidly washed twice with 10 ml PBS; then, the activated nanoparticles were dispersed in 0.25 ml PBS. The aptamers were added to the activated nanoparticles and suspension on mixed on a shaker for 2 hours at room temperature; then, the nanoparticles were washed 3 times with 1 ml PBS, and finally, the nanoparticles were suspended in blocking / storage buffer (PBS, 0.1% BSA, 0.05% sodium azide) by vortexing [32].

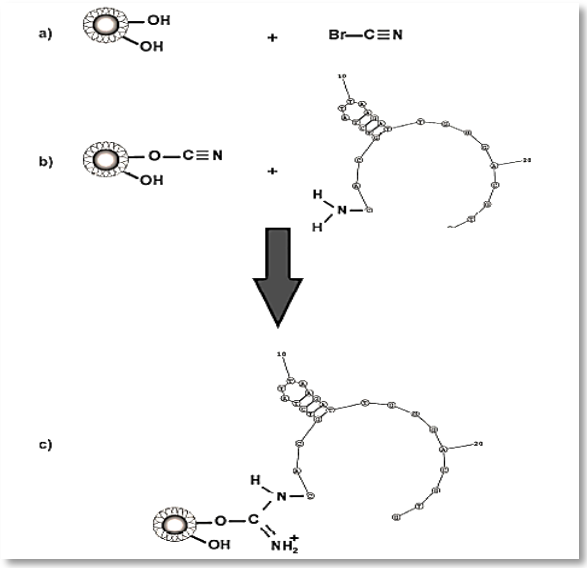



**Figure 2.** Schematic for the bioconjugation of the amino-modified aptamer to starch-coated magnetic nanoparticles [32]; (**a**) Activation of hydroxyl groups of the starch-coated MNPs using cyanogen bromide; (**b**) Adding activated the MNPs to amino-modified aptamers; (**c**) Aptemr-conjugated MNPs.

*2.5. UV-visible spectroscopy of aptamer-conjugated MNPs.*

The UV-vis absorption spectra of the MNPs and aptamer-conjugated MNPs were obtained using the Hellma TrayCell cuvette. A droplet (volume of 3 µl) was used for each sample, and the spectrum was saved using Biochrom PVC software.

*2.6. Evaluation of aptamer-conjugated MNP against HbA1c.*

To ensure the specific recognition function of hemoglobin by conjugated aptamer to magnetic nanoparticles, the performance of hemoglobin peroxidation by the prosthetic group

was investigated immediately after incubation and formation of aptamer and hemoglobin complex.

To perform this test, 40 µL of aptamer-conjugated MNP (1 mg/ml) was added to 3 plate wells, and respectively, 10 µL PBS as a non-specific binding (NSB) test to the first well, 10 µL and 20 µL of HbA1c standard were added to second and third plate wells. The reaction mixture consisting of aptamer-conjugated MNP and HbA1c was incubated for 15 minutes at room temperature in constant and continuous shaking to the occurrence of suitable conditions and collision for the formation of the hemoglobin-nanoconjugate complex. The resulting nanocomplex were quickly washed twice with 1 ml PBS under a magnetic field, and then complex formed between nanoconjugate and HbA1c were dispersed in 0.25 ml PBS. Then, TMB-$H_2O_2$ as a substrate-chromogenic solution was added to the mix and incubated at room temperature with gentle shaking for the same time, and then $H_2SO_4$ as a reaction stop reagent was added to the same volume of TMB/$H_2O_2$. The color changes due to the peroxidase activity of the complex in the presence of HbA1c were evaluated. The adsorption of the reaction solution at 450 nm was determined with a microplate reader and active blood glucose meter (ABG meter).

*2.7. Determining the limit of detection and drawing standard curve.*

To determine the nanomolecular detection method's detection limit (LoD), a serial dilution from control hemoglobin was prepared with a dilution coefficient of 1 to 10 in the PBS buffer. Then, to determine the minimum detectable HbA1c using aptamer-conjugated MNPs, 10 µl from dilutions prepared from standard hemoglobin (1, 0.8, 0.4, 0.2, 0.0 v/v) were added to the amount of 40 µl nanoconjugate (1mg/ml) concentration solution in the well plates. The reaction mixture consisting of aptamer-conjugated MNPs and HbA1c was incubated for 15 minutes at room temperature in constant and continuous shaking to create suitable conditions and collision for forming the hemoglobin-nanoconjugate complex. The resulting nanocomplex were quickly washed twice with 1 ml PBS under a magnetic field, and then complex formed between nanoconjugate and HbA1c were dispersed in 0.25 ml PBS. Then, TMB-$H_2O_2$ as the substrate-chromogenic solution was added to the mix and incubated at room temperature with gentle shaking for the same time, and then $H_2SO_4$ as a reaction stop reagent was added to the same volume of TMB/$H_2O_2$. The color changes due to the peroxidase activity of the complex in the presence of HbA1c were evaluated, and the adsorption of the reaction solution at 450 nm was determined with a microplate reader and ABG meter. In all designed assays for HbA1c measurement, total Hb measurement was performed specifically using Hb-specific aptamer-conjugated MNPs with the same condition for the reaction because the determination of HbA1c concentration in the patient sample is always measured as a ratio of HbA1c concentration to the total hemoglobin.

*2.8. Measurement of HbA1c in human blood samples with the proposed method.*

Human blood samples were measured to evaluate the performance of the nanomolecular detection method based on aptamer-conjugated MNPs for detection of HbA1c. To perform this test, 10 µl of blood sample was added to 40 µl of aptamer-conjugated MNPs (1mg/ml) concentration solution. The reaction mixture consisting of aptamer-conjugated MNPs and HbA1c was incubated for 15 minutes at room temperature in constant and continuous shaking to create suitable conditions and collision for forming the hemoglobin-

nanoconjugate complex. The resulting nanocomplex were quickly washed twice with 1 ml PBS under a magnetic field, and then complex formed between nanoconjugate and HbA1c were dispersed in 0.25 ml PBS. Then, TMB-$H_2O_2$ as the substrate-chromogenic solution was added to the mix and incubated at room temperature with gentle shaking for the same time, and then $H_2SO_4$ as a reaction stop reagent was added to the same volume of TMB/$H_2O_2$. The color changes due to the peroxidase activity of the complex in the presence of HbA1c were evaluated, and the adsorption of the reaction solution at 450 nm was determined with a microplate reader and ABG meter.

*2.9. The evaluation criteria of the nanomolecular detection method with a glucometer.*

In order to evaluate the results obtained from aptamer and aptamer-based magnetic nanoprobe, the Pearson correlation coefficient ($r$) has been used (https://www.statisticshowto.com/probability-and-statistics/correlation-coefficient-formula/). This criterion is expressed as the following relation:

$$r = \frac{\sum(x_M - x_G)(y_M - y_G)}{\sqrt{\sum(x_M - x_G)^2 \sum(y_M - y_G)^2}}$$

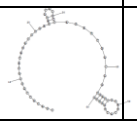
where $x_M$ and $y_M$ are the microplate reader results average and glucose meter results average, respectively. Moreover, $x_G$ and $y_G$ are the individual microplate reader and the glucose meter results, respectively. The standard curve trendline slopes were also used to identify nanoparticle conjugation's role in the detection probe's performance via aptamer-based magnetic nanoprobe vs. ELISA kit as a standard gold method for HbA1c concentration measurement in the laboratory.

## 3. Results and Discussion

*3.1. Selection of specific aptamers against HbA1c and tHb.*

2-D structure of all sequences of HbA1c and tHb aptamers (Table 2) were studied in RNA-Structure software in structure-forming exposed to the target molecule. Demonstrated results of aptamer structure simulation in web-based software showed that the aptamer sequence (G-20 for HbA1c and G-15 for tHb) seems to be the best aptamer sequences for our work because these have been able to show a more suitable steam-loop structure with small stem and a large loop than other sequences cause the more structural stability. These aptamers in most articles have the highest specificity and affinity to HbA1c and tHb and have more standard structures (Table 3). The 2-D simulated structure of aptamers is given below.

**Table 2.** HbA1c and tHb aptamer sequences and 2-D structure simulations by RNA structure.

| Clone Number | Aptamer sequence (5'-3') | Kd (nM) HbA1c | Kd (nM) Hb | Folding | Energy (Kcal/mol) | Refrence |
|---|---|---|---|---|---|---|
| G11 | CGACACCAGCACACAGACCCGAGAC ACACGTCAGATCAACAGCGACCGTA TCATTGGTTG | 108 | NB |  | -2.1 | [22] |
| G18 | GGCCACAGCAGCCAGTACACCCACC CACCAGCCCCGTCAACGACCTGAAC CTGCCCTGTGTG | 95 | NB |  | -2.1 | [22] |

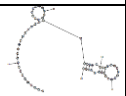
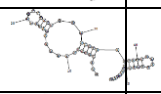
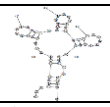
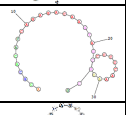
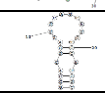
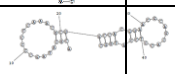
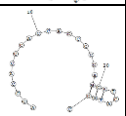
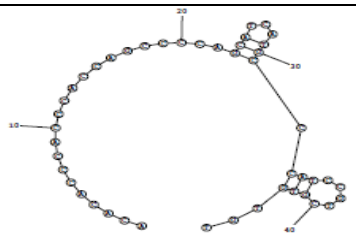
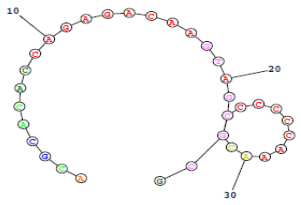
| Clone Number | Aptamer sequence (5'-3') | Kd (nM) HbA1c | Kd (nM) Hb | Folding | Energy (Kcal/mol) | Refrence |
|---|---|---|---|---|---|---|
| G15 | ACGCACACCAGAGACAAGTAGCCCC CCAAACGCGGCCACGGAACGCAGCA CCTCCATGGC | 3.3 | 2.8 |  | -4.5 | [22] |
| G20 | GGGGACACAGCAACACACCCACCCA CCAGCCCCAGCATCATGCCCATCCGT CGTGTGTG | 2.8 | NB |  | -3.4 | [22] |
| G23 | GGACACGGCAAAGGGGTATAGCCTA CCGGACCGTGGACATGGAATTGTGT GCTGCGTGG | 303 | NB |  | -6.4 | [22] |
| G23 | GGACACGGCAAAGGGGTATAGCCTA CCGGACCGTGGACATGGAATTGTGT GCTGCGTGG | 303 | NB |  | -5.8 | [22] |
| G15 T1 | ACGCACACCAGAGACAAGTAGCCCC CCAAACGCG-FAM | 1.1 | 1.8 |  | 2.8 | [30] |
| G15 T2 | GCCACGGAACGCAGCACCTCCATGG C-FAM | - | - |  | -3.0 | [30] |
| G15 T3 | ACGCATAGCCCCCCAAACGCGGCCA CGGAACGCAGCACCTCCATGGC-FAM | 9 | 10.3 |  | -5.2 | [30] |
| G20 T1 | ACACACCCACCCACCAGCCCCAGCA TCATGCCCATCCGTCGTGTGT-FAM | 0.2 | - |  | -1.3 | [30] |
| G20 T2 | ACCCACCCACCAGCCCCAGCATCAT GCCCATCCGTCGT-FAM | 14.4 | - |  | -1.1 | [30] |
| G20 T3 | ACCCACCCACCAGCCCCAGCATCAT GCC-FAM | 31.5 | - |  | -1.1 | [30] |

**Table 3.** Finalized specific aptamers for HbA1c and t Hb detection in this study.

| Name | Seq | Kd | 2-D structure | Target |
|---|---|---|---|---|
| **G-20 T1** | 5'-ACACACCCACCCACCAGCCCCAGCATCATGCCCATCCGTCGTGTGT-3' | 0.2 |  | HbA1c |
| **G-15 T1** | 5'-ACGCACACCAGAGACAAGTAGCCCCCCAAACGCG-3' | 1.1 |  | tHb |

*3.2. Evaluation of tHb peroxidase activity.*

The results of testing the effect of hemoglobin peroxidase activity in the presence of TMB as a substrate-chromosome solution show that the family of hemoproteins, including hemoglobin in the blood, due to the presence of the same prosthetic group in its structure, can mimic the activity of peroxidase activity. The background color of the reaction changed from pink (due to the presence of the same groups in the hemoglobin structure) to blue after the peroxidase activity and TMB oxidation reactions (Figure 3). By adding the stop reagent of

reaction ($H_2So_4$), the reaction turns yellow, and the results of the optical density (OD) reading at 450 nm using the microplate reader are given in the table below (Table 4).

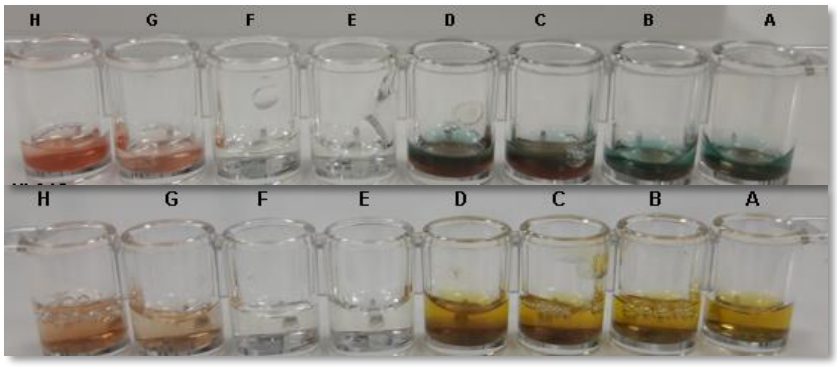



**Figure 3.** Peroxidase activity of tHb in TMB solution (substrate-chromogen). The top row shows the reaction mixture before stopping the reagent addition, and the bottom row shows the color change of the peroxidase reaction after stopping the reagent addition.

**Table 4.** Results of the optical density (OD) reading in 450 nm after stop reagent addition using the microplate reader.

| | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** |
|---|---|---|---|---|---|---|---|---|
| **HbA1c** | 10µl | 10µl | 20µl | 20µl | 0 | 0 | 10µl | 20µl |
| **PBS** | 0 | 0 | 0 | 0 | 20µl | 10µl | 40µl | 40µl |
| **TMB/$H_2O_2$** | 40µl | 40µl | 40µl | 40µl | 40µl | 40µl | 0 | 0 |
| **OD (450 nm)** | 1.216 | 1.301 | 1.174 | 1.331 | 0.245 | 0.286 | 0.600 | 0.826 |

*3.3. UV-visible spectroscopy of aptamer-conjugated MNPs.*

Figure 4 indicated the UV-Vis spectra of MNPs after and before conjugation to amino-modified aptamers. MNPs demonstrated a peak at 380 ± 5 nm using UV-Vis spectroscopy. Also, aptamers showed a peak at 260 nm; however, two peaks at 260 nm and 380 ± 5 nm were seen from aptamer-conjugated MNPs. These two peaks confirmed efficient interaction between amino-modified aptamers and starch functionalized MNPs.

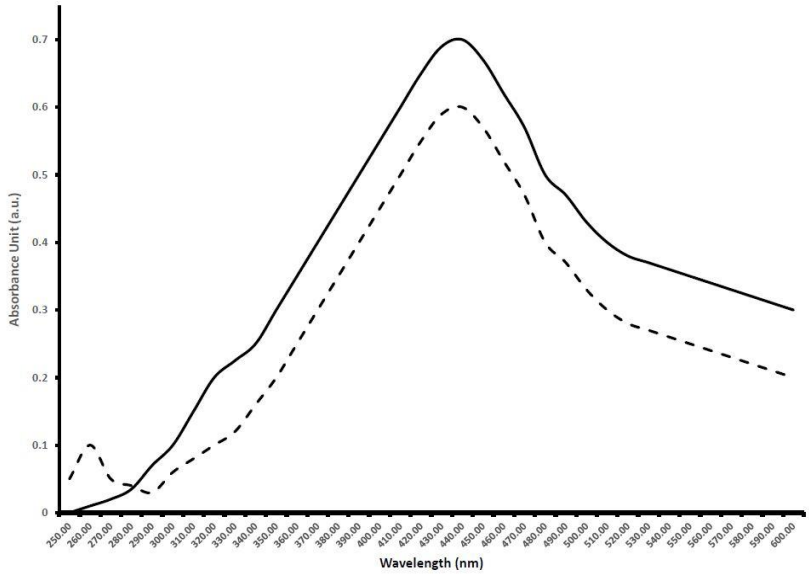



**Figure 4**. Uv-vis spectra of aptamer-conjugated MNP with Uv-vis spectroscopy.

*3.4. Evaluation of aptamer-conjugated MNPs against HbA1c.*

Peroxidase activity of two separate reactions was performed using aptameric nanoconjugates to identify and measure total hemoglobin and hemoglobin A1C in substrate solution. As can be seen in the image, the intensity of the color changes in the total hemoglobin detection reaction using the aptameric conjugate is greater than the hemoglobin detection reaction using the HbA1c aptameric conjugate (Figure 5). This indicates that after the washing process under a magnetic field and removal of total hemoglobin except for HbA1c that specific aptamer-conjugated MNPs entrapped, only HbA1c remained in the mix of residual reaction, which is part of the percentage of total hemoglobin in the blood, and therefore has less intensity of color changes than the total hemoglobin reaction and shows that Due to the contribution of hemoglobin in the sample, the severity of peroxidase is also reduced and confirms the relationship between the concentration of HbA1c and the severity of peroxidase activity, which confirms the proper performance of a specific aptameric-conjugate for each hemoglobin.

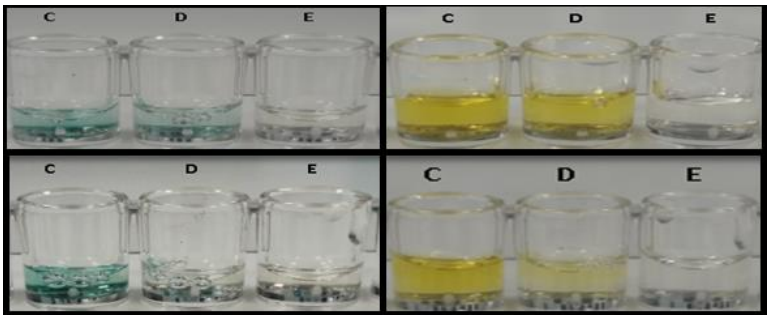



**Figure 5.** Evaluation of the performance of nano-conjugated specific aptamer for Hb and HbA1c. The top row shows the color change of the peroxidase reaction before and after stop reagent addition to the reaction mixture containing aptamer-conjugated MNPs for total Hb, and the bottom row shows the color change of the peroxidase reaction before and after stop reagent addition to the reaction mixture contain aptamer-conjugated MNPs for HbA1c.

**Table 5.** the optical density (OD) reading in 450 nm after stop reagent addition using the microplate reader.

| | **C** | **D** | **E(NSB)** |
|---|---|---|---|
| **MNP-Apt.G15 (tHb)** | 40µl nanoprobe 20µl total Hb | 40µl nanoprobe 10µl Hb | 40µl nanoprobe 10µl PBS |
| **OD** | 0.933 | 0.720 | 0.210 |
| **MNP-Apt.G20 (HbA1c)** | 40µl nanoprobe 20µl Hb | 40µl nanoprobe 10µl Hb | 40µl nanoprobe 10µl PBS |
| **OD** | 0.645 | 0.451 | 0.225 |

*3.5. Limit of detection and standard curves.*

As can be seen in the image obtained from the plate wells, as the concentration of total hemoglobin and hemoglobin A1c increases, the intensity of the yellow dye increases, followed by the intensity of the blue dye created in the reaction mixture, indicating the proper performance of aptameric nanoconjugates to identify the target molecules (Figure 6). The intensity of the color changes in the total hemoglobin detection reaction using the aptameric conjugate is greater than the hemoglobin detection reaction using the HbA1c aptameric conjugate. This indicates that after the washing process under a magnetic field and removal of total hemoglobin except for HbA1c that specific aptamer-conjugated MNP entrapped, only HbA1c remained in the mix of residual reaction, which is part of the percentage of total hemoglobin in the blood, and therefore has less intensity of color changes than the total hemoglobin reaction and shows that Due to the contribution of hemoglobin in the sample, the

severity of peroxidase is also reduced and confirms the relationship between the concentration of HbA1c and the severity of peroxidase activity, which confirms the proper performance of a specific aptameric-conjugate for each hemoglobin.

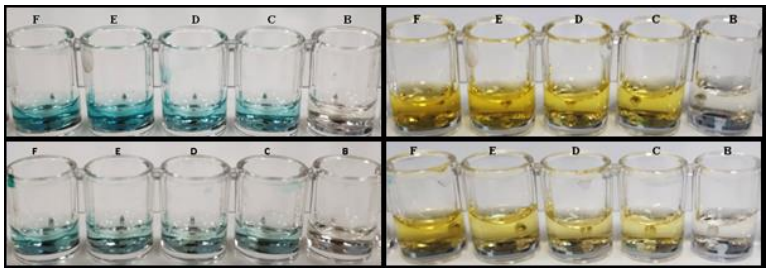



**Figure 6.** Limit of detection of hemoglobin and hemoglobin A1c via the aptamer-conjugated MNPs. The top row shows the color change of the peroxidase reaction before and after stop reagent addition to the reaction mixture containing aptamer-conjugated MNPs for total Hb, and the bottom row shows the color change of the peroxidase reaction before and after stop reagent addition to the reaction mixture contain aptamer-conjugated MNPs for HbA1c.

The optical density reading results using a microplate reader and ABG meter (Tables 6 and 7) also confirm the OD increase in proportion to the increase in concentration in both total hemoglobin and hemoglobinA1c (Figures 7 and 8). Using the results of this step, a standard curve was drawn to determine the concentration of unknown samples.

**Table 6.** Optical density (OD) reading in 450 nm after stop reagent addition using the microplate reader.

| | B(NSB) | C | D | E | F |
|---|---|---|---|---|---|
| **MNP-Apt.G20 Control-low** | 0 | 0.2x HbA1c<br>22.5<br>1.1% | 0.4x HbA1c<br>44.6mg/dl<br>2.2% | 0.8x HbA1c<br>89.2<br>4.4% | 1x HbA1c<br>111.5<br>5.5% |
| **OD** | 0 | 0.154±0.1 | 0.240±0.1 | 0.284±0.1 | 0.306±0.1 |
| **MNP-Apt.G20 Control-high** | 0 | 0.2x HbA1c<br>39.5 mg/dl<br>1.7% | 0.4x HbA1c<br>79 mg/dl<br>3.4% | 0.8x HbA1c<br>158 mg/dl<br>6.8% | 1x HbA1c<br>197.5 mg/dl<br>8.5% |
| **OD** | 0 | 0.211±0.1 | 0.263±0.1 | 0.350±0.1 | 0.394±0.1 |

**Table 7.** Optical density (OD) reading using ABG meter after stop reagent addition.

| **Concentration mg/dl(%)** | 0 | 22.5(1.1) | 39.5(1.7) | 44.6(2.2) | 79(3.4) | 89.2(4.4) | 111.5(5.5) | 158(6.8) | 197.5(8.5) |
|---|---|---|---|---|---|---|---|---|---|
| **MNP-Apt.G20** | 23 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |

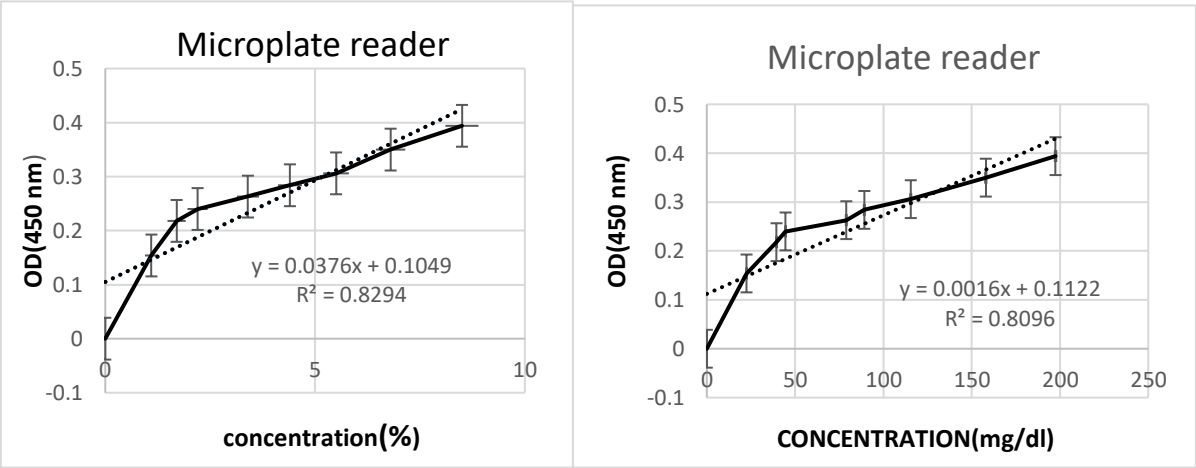



**Figure 7.** Standard curves for HbA1c drawn using HbA1C aptamer (left) and HbA1C aptamer-MNPs (right) measured with a microplate reader.

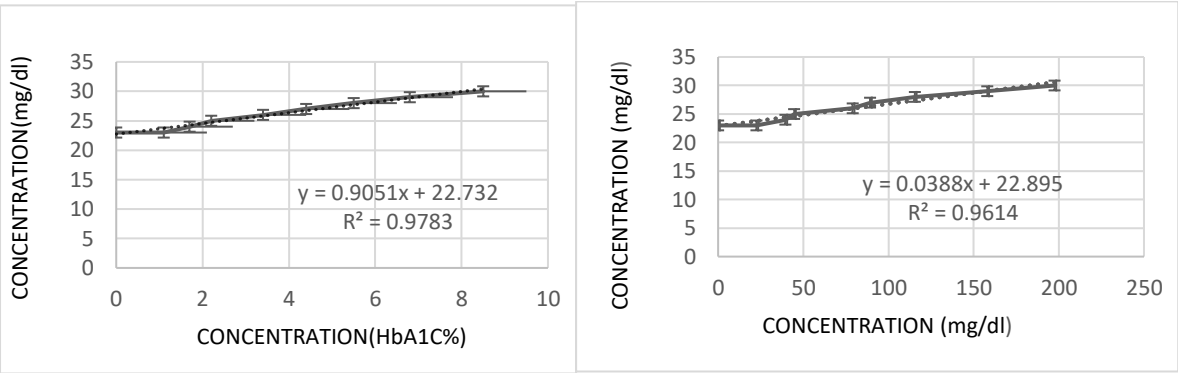



**Figure 8.** Standard curves for HbA1c drawn using HbA1C aptamer (left) and HbA1C aptamer-MNPs (right) measured with by portable reader.

*3.6. Measurement of human samples with the proposed method.*

Ten human blood samples that have already been measured in a clinical laboratory for measuring hemoglobinA1c using the ELISA method have also been tested using a nanomolecular method based on aptamer-conjugated MNPs. This was done to compare the accuracy of the nanomolecular method proposed in this study. The results show that the method of measuring HbA1c using a glucometer as an optical reader and the nanomolecular detection method proposed in this study can be used as suitable methods (Table 8, Figure 9). The results show that the method of measuring hemoglobinA1c using a glucometer as an optical read can be used as a suitable point of care method for measuring HbA1c (Figure 10).

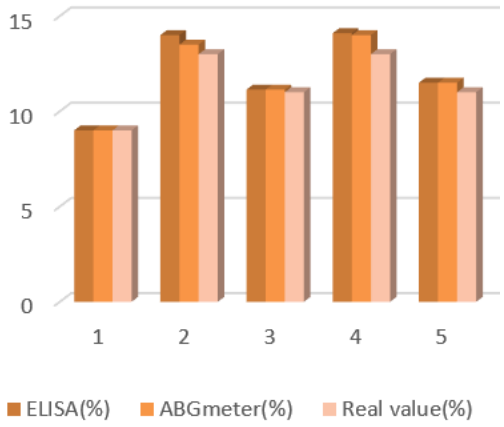



**Figure 9.** Comparison of the results obtained from the ELISA method with the proposed methods on clinical samples.

**Table 8.** Comparison of the results obtained from the ELISA method with the proposed method on clinical samples.

| Patients | Microplate reader (mg/dl) | Microplate reader (%) | Portable reader (mg/dl) | Portable reader (%) | Real value (%) |
|---|---|---|---|---|---|
| **1** | 210 | 9 | 208 | 9 | 9 |
| **2** | 324 | 14 | 311 | 13.5 | 13 |
| **3** | 257 | 11.14 | 265 | 11.14 | 11 |
| **4** | 327 | 14.1 | 318.5 | 14 | 13 |
| **5** | 265 | 11.5 | 260 | 11.5 | 11 |

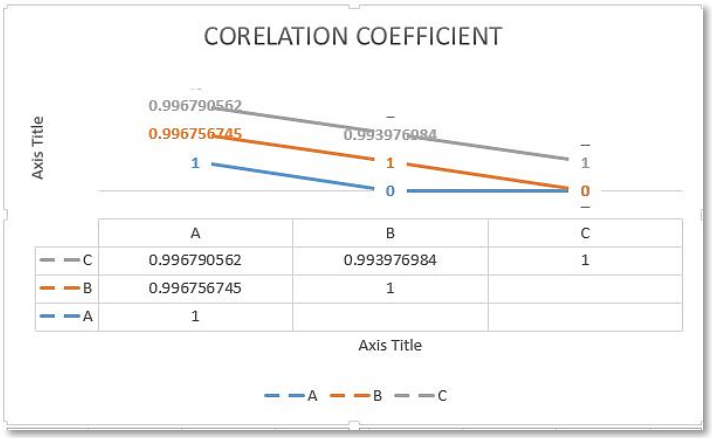



**Figure 10.** Comparison of the results obtained from the ELISA method with the proposed method on clinical samples using Pearson equation.

The obtained results demonstrated that this developed assay has several advantages compared to other existing methods, including 1) using aptamers as a specific identifier of the target molecule. Significantly reduces the cost of diagnostic procedures; 2) High stability and ease of operation due to the use of aptamers as antibody substitutes; 3) Only a few microliters of the patient's blood sample are used for measurement; 4) Aptamer diagnostic template without the need for enzymatic or color label helps reduce costs and improve the performance of aptamers; 5) Due to the use of the electrochemical reading system to identify the target molecule, this method will have the ability to miniature in future studies; 6) The use of magnetic nanoparticles functionalized with a specific aptamer eliminates the possibility of removing unwanted agents in the biological sample and improves the accuracy of quantification of the target molecule. This assessment happens with a glucometer (a good and accessible device) as an optical and electrochemical reader for the colorimetric measurement of HbA1c.

## 4. Conclusions

The development of inexpensive, user-friendly methods for testing HbA1c reduces operating costs, increases public access to HbA1c testing, and improves disease management by monitoring the level of HbA1c in a patient's blood [33, 34]. We designed a portable, label-free-optical method for measuring blood glycosylated hemoglobin (HbA1c) using aptamer-conjugated MNP. Detection of Hb and HbA1c from whole blood was successfully demonstrated. With more tests on the reliability of this detection process, this platform may become a practical method for long-term monitoring of diabetes. However, in the optical method, it can be said that it has a similar function to the usual laboratory methods, and its advantage over these methods is its portability, high response speed, and simplicity and as an economically promising tool (Table 9). Compared to bench-top antibody assays (e.g., ELISA), reagent consumption can be reduced by 75%, while the analysis time can be dramatically reduced from 3.5 hours to 30 minutes.

Furthermore, two parallel assays (Microplate reader and portable reader) can be performed based on colorimetric detection simultaneously on this plate; thus, the time can be reduced to half. The measurements of Hb using a similar approach were undergone. The HbA1c and tHb-specific aptamers were then applied to detect HbA1c % using a colorimetric method based on aptamers array on the MNP platform, showing remarkable sensitivity and selectivity.

The aptasensor array platform was validated using standard human whole blood samples and demonstrated linearity over a wide concentration range. We believe that the developed platform is superior to current methodologies due to the simplicity, stability, and lower cost, which will facilitate the early and accurate diagnosis of diabetes.

**Table 9**. Comparison of the performance of the electrochemical sensor proposed in this study with other HbA1C sensors.

| HbA1c/tHb | Recognition Element | Transducer | Detector | LoD | Ref |
|---|---|---|---|---|---|
| HbA1c specific aptamer/tHb specific aptamer attached GO surface | Specific Aptamer | Fluorescent | Fluorospectrometer | <5.7% | [31] |
| HbA1c specific aptamer/tHb specific aptamer attached on Magnetic beads | Specific Aptamer labeled fluorescent dye | Fluorescent | Optical detection module | 46 mg/dl | [33] |
| HbA1c specific aptamer/tHb specific aptamer attached on Magnetic beads | aptamer-based sandwich assay | Fluorescent | laser excitation and optical detection modules | < 7% | [34] |
| Thiol-modified forms of the specific aptamers immobilized on gold nanoparticles (AuNPs)-modified array electrodes | Aptamer-conjugated gold nanoparticles (AuNPs)-modified array electrodes | Voltammetric aptasensors | cyclic voltammetry (CV) and electrochemical impedance spectroscopy | 0.2 ng/ml | [22] |
| HbA1c specific aptamer/tHb specific aptamer attached ON MNP | Specific aptamer-conjugated MNP | Colorimetric | Spectroscopy Portable reader | 1.5-3% | This study |

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## Conflicts of Interest

The authors declare no conflict of interest.

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