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Selection of Aptamers Specific for Glycated Hemoglobin and Total Hemoglobin Using On-Chip SELEX

Hsin-I Lin, Ching-Chu Wu, Ching-Hsuan Yang, Ko-Wei Chang, Gwo-Bin Lee, and Shu-Chu Shiesh

Blood glycated hemoglobin (HbA1c) levels reflecting average glucose concentrations over the past three months are fundamental for the diagnosis, monitoring, and risk assessment of diabetes. It has been hypothesized that aptamers, which are single-stranded DNAs or RNAs that demonstrate high affinity to a large variety of molecules ranging from small drugs, metabolites, or proteins, could be used for the measurement of HbA1c. Aptamers are selected through an in-vitro process called systematic evolution of ligands by exponential enrichment (SELEX), and they can be chemically synthesized with high reproducibility at relatively low costs. This study therefore aimed to select HbA1c- and hemoglobin (Hb)-specific single-stranded DNA aptamers using an on-chip SELEX protocol. A microfluidic SELEX chip was developed to continuously and automatically carry out multiple rounds of SELEX to screen specific aptamers for HbA1c and Hb. HbA1c and Hb were first coated onto magnetic beads. Following several rounds of selection and enrichment with a randomized 40-mer DNA library, specific oligonucleotides were selected. The binding specificity and affinity were assessed by competitive and binding assays. Using the developed microfluidic system, the incubation and partitioning times were greatly decreased, and the entire process was shortened dramatically. Both HbA1c- and Hb-specific aptamers selected by the microfluidic system showed high specificity and affinity (dissociation constant, K_d=7.6±3.0 nM and 7.3±2.2 nM for HbA1c and Hb, respectively). With further refinements in the assay, these aptamers may replace the conventional antibodies for in-vitro diagnostics applications in the near future.

Introduction

Diabetes mellitus, one of the most common non-communicable diseases worldwide, is the leading cause of blindness, renal failure, and non-traumatic foot/leg amputations. These complications lead to reduced life expectancies and enormous healthcare expenditures, making diabetes one of the most pressing human health issues of the 21st century. A substantial body of evidence has indicated that diabetes-related complications can be prevented or delayed by early diagnosis and strict control of blood glucose levels, which is usually monitored by frequently measuring fasting blood glucose levels. However, glucose levels are readily affected by diet, exercise, stress, and many other factors. Alternatively, glycated hemoglobin (HbA1c) levels in blood, which represent proxies for the average blood glucose values over the preceding 2-3 months, demonstrate less biological variation, and their measurement does not require fasting beforehand. HbA1c is therefore recommended for diabetes screening, diagnosis, and risk assessment.1-3

HbA1c, the predominant form of glycated hemoglobin in human blood, is formed by the non-enzymatic reaction of glucose with the N-terminal valine amino group of the β-chain hemoglobin (Hb). Epidemiological analysis of clinical trial data from the Diabetes Control and Complications Trial (DCCT) in patients with type-1 diabetes4-6 and the United Kingdom Prospective Diabetes Study (UKPDS) in patients with type-2 diabetes7-9 found that HbA1c levels are directly related to the risk of development and progression of diabetic complications. The National Glycohemoglobin Standardization Program (NGSP) was established in the USA to standardize and certify HbA1c methods so that clinical laboratory results could be traceable to the clinical outcomes of the DCCT and UKPDS5,10 studies. Starting from 2010, the American Diabetes Association (ADA) endorses the use of HbA1c for diabetes diagnosis with a cutoff of 6.5% or greater.11,12 Furthermore, HbA1c levels are used to guide choice of therapy for patients with diabetes, with the goals < 7.0% or even < 6.5% recommended for effective treatment of diabetes.12 Moreover, individuals with HbA1c levels of 5.7%-6.4% are referred to as having “pre-diabetes,” indicating an increased risk for the future development of this disease.1-3 Thus, the precise and accurate measurement of HbA1c can allow for the diagnosis of the earlier stages of diabetes, at which point its progression into a serious ailment can be thwarted. However, HbA1c tests are uncommon in small clinics in rural or even suburban settings, and the test costs are relatively high, both of which can limit the degree of usage of such clinical tests. Therefore, a portable HbA1c testing device offering low costs and high accuracy could help facilitate the early diagnosis and proper management of a greater number of diabetic patients.

Currently, HbA1c is measured by chromatography-based assays, antibody-based immunoassays, or enzymatic assays. To differentiate HbA1c from native Hb proteins, several property differences have been exploited, such as the charge differences via cation-exchange chromatography, molecular structure differences via immunoassay or boronate affinity chromatography, and mass differences via mass spectrometry. For the diagnosis of diabetes, the ADA recommends that HbA1c analysis should be performed in a laboratory using NGSP-certified methods.10,12 From the results of the GH-2 survey of HbA1c test conducted by the College of American Pathologists (CAP, USA), which included more than 3000 laboratories, around 65% of participants use immunoassay, with 31 and 4% using cation-exchange chromatography and affinity chromatography, respectively.13 However, chromatography-based methods use the ratio of the HbA1c peak area over the total Hb peak area via photometric measurements of Hb color to estimate the HbA1c concentration (%). Therefore, these methods are subject to interference from hemoglobin variants or chemically-modified hemoglobins which may result in false negative or positive results.15 Furthermore, even though chromatography-based methods for determining HbA1c levels are the current gold standards, they cannot be used in most clinics due to the expense, size, and complexity of the required machinery. Alternatively, immunoassays
using mono- or poly-clonal antibodies to recognize glucose-bound amino acids at the N-terminal of β-chain hemoglobin proteins are less affected by hemoglobin variants. However, antibodies are relatively expensive and demonstrate significant lot-to-lot variations and validation quality control problems. In addition, immunoassays suffer from the reagent instability, particularly with respect to the antibodies themselves, as well as the agglutinators. In certain immunoassay protocols, a separate measurement of total Hb concentration is usually performed by a colorimetric method or is not even required in other cases. Therefore, an alternative method of HbA1c measurement that is less influenced by interfering substances and demonstrates both better reagent stability and lower cost would be highly desirable. A fast and low-cost HbA1c testing system may enable effective primary and/or secondary prevention of diabetes, ultimately reducing the overall risk of complications in patients with diabetes. However, no clinical trial data have supported the efficacy of any currently available, hand-held HbA1c measurement devices for diabetes diagnosis and management, mainly because the instruments developed to date do not meet the required analytical performance characteristics.

Aptamers are oligonucleotide ligands selected from a single stranded DNA or RNA library through a process known as “in vitro” systematic evolution of ligands by exponential enrichment (SELEX) selection. Aptamers bind to a wide range of molecules with affinities and specificities that are comparable to or better than antibodies. Therefore, they have a wide range of applications in diagnostics and therapeutics. The high binding affinity and specificity of aptamers are attributed to their specific three-dimensional shapes. Once the sequence of an aptamer is determined, it can be chemically synthesized with a high degree of purity and reproducibility. Compared to protein antibodies, aptamers have many advantages, such as ability for long-term storage, resistance to thermal denaturation, and batch-to-batch consistency. Despite having this high potential, there are currently few commercially available aptamers. This is because the SELEX process is time-consuming, labor-intensive, and relatively expensive, as it involves repeated incubation, partitioning, and amplification steps and requires a large amount of both costly reagents and samples. This study therefore was aimed to select HbA1c- and Hb-specific aptamers through the SELEX process on an integrated microfluidic chip. The SELEX chip enabled a faster, easier-to-use selection process with less reagent consumption when compared with traditional SELEX procedures. The binding affinity and specificity of the selected aptamers were verified by both competitive and equilibrium binding assays and produced dissociation constants (K) in the nM range with correspondingly high specificity.

Materials and methods

Design of the SELEX chip

To successfully perform the SELEX process in an automated and integrated manner, this study reports a new microfluidic system to execute on-chip aptamer selection with minimal handling. The microfluidic system enables the selection of both Hb-specific and HbA1c-specific aptamers on a single chip. The SELEX chip was designed to select aptamers via the magnetic bead-based SELEX method (Figure 1). The multi-functional microfluidic devices can automatically coordinate the injection, sample preparation, partitioning, and PCR steps required for the SELEX process. Furthermore, the microfluidic system enables the precise and rapid manipulation of a small number of magnetic beads. The use of magnetic beads to screen specific aptamers in microfluidic devices can improve the sensitivity and specificity of the SELEX process.

Compared to traditional methods for performing SELEX, which are labor-intensive and time-consuming, the proposed microfluidic system is easier to-use, lower in cost, and more compact in size.

The schematic illustration of the SELEX chip designed herein is shown in Figure 2 (a). The operating process for screening of aptamers performed on the integrated microfluidic chip is described as follows. First, the pool of ssDNA library was loaded in the middle of the open chamber (incubation chamber). After denaturing the ssDNA at 95°C for 5 min, the ssDNA library was transported to the Hb or HbA1c bead chambers, where epoxy-coated magnetic beads (Dynabeads® M-450 Epoxy, Invitrogen, USA) were pre-coated with Hb or HbA1c at 4°C overnight and blocked by BSA (1% in Tris buffer) overnight. The ssDNA was then incubated with the magnetic beads by activating the micromixer for 5 min. After using an external magnet to collect ssDNA-bead complexes in the chamber, ssDNAs that were either unbound or only weakly bound to the target were washed away by a wash buffer that was transported via a transportation unit located within the incubation chamber. The magnetic beads were collected using an external magnet for 2 min and washed three times with 1 mL PBS. Then PCR reagents and oil were robotically transported into the Hb and/or HbA1c chambers to amplify target-bound ssDNAs. Note that the oil was loaded in order to avoid the evaporation of PCR reagents. With this approach, one complete round of SELEX, including incubation, partitioning, and amplification could be automated in the integrated microfluidic chip within 70 min. In this work, the target-specific aptamers characterized by high affinity and specificity were finally selected after several rounds of SELEX.

An exploded view of the chip is schematically shown in Figure 2 (b). The chip was fabricated by bonding two polydimethylsiloxane (PDMS, Sylgard silicon elastomer, USA) layers to a glass layer (G-Tech Optoelectronics Corp., Taiwan) in a sandwich structure. The bottom PDMS layer was a thin-film liquid channel layer, and the top PDMS layer was a thick-film air chamber layer. The diameter of the loading chamber was 5 mm. The liquid channel layer was about 0.3 mm in thickness, and the microchannel was 0.2 mm in depth. The air chamber layer, which contained the pneumatic components including open-chamber micromixers, suction-type micropumps, and normally-closed microvalves, was about 5 mm in thickness. Compressed air was injected into the air chambers, which were modulated by electromagnetic valves (EMVs) such that the pumping rate and mixing efficiency of the microfluidic devices could be precisely controlled. Detailed information about the operation of the suction-type micropumps, normally-closed microvalves and open-chamber micromixers can be found in our previous works.

Fabrication of the SELEX chip

The PDMS-based SELEX chip was fabricated by polymethylmethacrylate (PMMA) computer-numerical-controlled (CNC) machining and PDMS casting processes so that the single chip could perform the entire SELEX process in an automated format. PDMS is a suitable material for biomedical applications, and especially for fabricating microfluidic chips. PDMS has several advantages, including ease of fabrication, bio-compatibility, high flexibility, and high optical transparency. The PDMS casting process includes solidification and demolding steps. The details are described briefly as follows. First, the elastomer (Sylgard 184A silicon elastomer, USA) and curing agent (Sylgard 184B elastomer curing agent, USA) of the PDMS were thoroughly mixed in a 10:1 mass ratio, and air bubbles were degassed from the PDMS mixture in a vacuum. After mixing, the PDMS was poured into the PMMA
Selection of aptamers by on-chip SELEX

The detailed operating principle of the magnetic bead-based on-chip SELEX is shown in Supplementary Figure 1. The binding between the target proteins and nucleic acid aptamers is mainly via van der Waals interactions and hydrogen bonds. Note that there are also DNA molecules in the library that non-specifically bind to the protein targets via electrostatic interactions. The goal of the selection process is, then, to reduce the number of DNA molecules demonstrating non-specific binding while simultaneously enriching those that can bind to the targets with high affinity and specificity; this is typically achieved by conducting the on-chip SELEX process multiple times.

A schematic illustration of the chambers and the heating/cooling element the SELEX of chip is shown in Figure 2(c). The magnetic bead-based SELEX process, including the incubation, partitioning, and amplification steps, was performed on a single chip. Note that the PCR product was denatured and then used in the next round of the SELEX process. After several repeated rounds, the aptamer(s) with high affinity and specificity to the target protein were selected. In the magnetic bead-based SELEX protocol, magnetic beads were first surface-coated with the target proteins, and then blocked by BSA. Then the DNA pools were denatured thermally. Note that the heating/cooling element was used in this study to perform PCR and denature processes and to avoid the evaporation and damage of the reagents. The ssDNA library contained ~10^15 sequences, each composed of a 40-bp, internally randomized region flanked by two 16-bp primers, was thermally denatured to yield ssDNAs. The aptamer selection process, including incubation, bead partitioning, and aptamer amplification, was performed on the SELEX chip in an automated fashion. The final PCR product enriched from several rounds of the automated SELEX process was then cloned. The binding affinity and the specificity of each aptamer were assessed by a competitive assay. The selected aptamer was sequenced, synthesized, and validated with an ELISA-like assay used to estimate the affinity constant (Kd).

First, PDMS microfluidic channels and chambers were blocked by BSA to prevent non-specific binding onto the PDMS and the glass plate. Magnetic beads were coated with the target proteins (either Hb or HbA1c), and the bead-protein complexes were subsequently blocked with BSA. Thirty-seven µL of target protein-beads (4 x 10^8 beads/mL) and a random ssDNA pool (3 µL of 10 µM) were then loaded into the incubation chamber of the SELEX chip and mixed by the micromixer for 5 min. Those ssDNA ligands with high affinity were bound to the target proteins and hence onto the magnetic beads. Then, an external magnet was applied to attract ssDNA-bead complexes in the chamber. Note that ssDNAs that either failed to bind to the target protein or only weakly bound it were washed away with a 1000 µL wash buffer (0.01 M phosphate buffer, pH 7.4) that was pumped by the micropump through the incubation chamber. PCR reagents (volume = 28 µL) were then
loaded into the reagent chamber to amplify the target-bound ssDNAs. This incubation, partitioning, and amplification process constituted one round of SELEX. Before the next round, the amplified PCR product was thermally denatured into ssDNAs, which were then subjected to the next round of incubation, partitioning, and amplification. The SELEX process was repeated for seven rounds. The target-specific aptamers demonstrating high affinity and specificity to the target proteins were finally selected. In order to select the aptamers that were HbA1c-specific (i.e., did not bind to other Hb molecules), the ssDNA pool was incubated first with HbA1c-coated beads as a negative selection step, and the unbound ssDNA pool was moved to next well to react with HbA1c-coated beads as a positive selection step. As such, the selected aptamers were confirmed to be specific for HbA1c. Screening for Hb-specific aptamers was performed by incubating the ssDNA pool with HbA1c-coated beads as a positive selection step and then with BSA-coated beads as a negative selection step. Five rounds of the SELEX process were conducted with both positive and negative selection processes for the selection of Hb-specific aptamers.

Figure 2: (a) Schematic illustration and layout of the SELEX chip. The dimensions of the chip are 5.1 cm x 4.6 cm. (b) The SELEX chip is composed of two PDMS layers (the thick-film air chamber layer and the thin-film liquid channel layer) and a glass plate. (c) The chambers and heating/cooling element in the SELEX chip.

Experimental setup for on-chip SELEX

The experimental setup was composed of a personal computer with an integrated graphical user interface developed using Visual Basic software (Visual Basic 2005, Microsoft, USA), a custom-made control circuit, a compressed air source (MDR2-1 A/11, Jun-Air Inc., Japan), a vacuum (cULus, Gast Manufacturing, Inc., USA), and several EMVs (SMC Inc., S070n-5BG-32, Taiwan). Micropumps, micromixers, and normally-closed microvalves were activated by compressed air or vacuum. The control circuit provided digital signals to control the EMVs and then activate the microfluidic devices (including micropumps, micromixers, and microvalves) to automatically operate all steps on the SELEX chip.

Preparation of the HbA1c- or Hb-coated magnetic beads

Twenty-five microliters of epoxy-coated magnetic beads (4 x 10⁸ beads/mL, 4.5 µm diameter) were loaded to a 1.5 mL tube. After three washes with 500 µL of phosphate buffer (0.01 M, pH 7.4), 10 µL of Hb (2 µg/µL) (H0267, Sigma, USA) or 10 µL of HbA1c (1 µg/µL) (IFCC-466, IRMM) were added to 500 µL of carbonate buffer, and the mixture was then incubated at 4°C overnight. A blocking buffer (500 µL of 10 mM Tris, 100 mM NaCl, 2 mM CaCl₂ with 1% BSA) was added into the tube to block free spaces on the surface of the Hb-conjugated or HbA1c-conjugated beads, and the solution was incubated at 4°C overnight.

PCR

After the incubation and the partitioning steps, the isolated ssDNA molecules (2 µL) were amplified by PCR. The reaction (28 µL) contained 2.4 µL of deoxynucleotide triphosphates (dNTPs, 0.2 mM), 1.5 µL forward primer (5’-GGCAGGAAAGACAAACA-3’, 0.5 µM), 1.5 µL reverse primer (5’-ACAGCACCCAGACCA-3’, 0.5 µM), 3 µL magnesium chloride (1.5 mM), 19.4 µL deionized water and 0.2 µL Super-Therm Gold DNA polymerase (Bertac Enterprise Co., Ltd., Taiwan). After an initial denaturation step at 94°C for 10 min, thermocycling was performed for 20 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s. The PCR was completed with a final extension step at 72°C for 7 min.

After PCR, the products were examined by gel electrophoresis on 8% polyacrylamide gels, typically at 100 V for 60 min. The gels were subsequently stained with ethidium bromide (50 mg/mL, Sigma, USA) for 5 min and visualized and imaged on an ultraviolet analysis system (302 nm, BioDoc-It 220® Imaging System, UVP Co., USA).

Competitive assay

A competitive assay was performed to examine the binding affinity and specificity of ligands selected from the on-chip SELEX process. At the first step of the assay, each ssDNA ligand selected from the on-chip HbA1c-SELEX was incubated with three types of beads for 5 min. As shown in Figure 3, three types of beads were used: BSA-coated magnetic beads (negative control [N]), HbA1c-coated magnetic beads (positive control [P] and competitive test [C]), and Hb-coated magnetic beads (non-target test [Hb]). The competitive test was subsequently incubated with free HbA1c, which served to compete with the HbA1c-coated beads for binding of the ssDNA ligands. After magnetic separation and washing, all of the four samples (negative control, positive control, competitive test and non-target test) were subjected to PCR amplification. The selected ssDNA ligands from the Hb-specific SELEX process were subjected to a similar competitive assay, but without the non-target test.
For the negative, positive, and non-target tests, 3 µL selected ssDNA and 37 µL protein-coated beads were loaded into the mixing chamber and mixed for 5 min. For the competitive test, 3 µL selected ssDNA, 34 µL protein-coated beads, and 3 µL free target proteins were loaded into the mixing chamber and mixed for 5 min. Then, the magnetic beads were collected using an external magnet and washed three times with 500 µL PBS. Finally, all bead-bound ssDNA ligands were PCR-amplified.

Figure 3. The competitive assay for determining the binding affinity and specificity of the selected aptamer. “P” represents the positive control test that used HbA1c-coated magnetic beads. “C” represents the competitive test that used HbA1c-coated magnetic beads and free HbA1c. The signal of C is expected to be weaker than that of P because a portion of the ssDNA pool that was originally bound to the HbA1c-coated beads would competitively bind to the free HbA1c proteins and be removed by the washing process. “N” represents the negative control test that used only BSA-coated magnetic beads. “Hb” represents the specificity (non-target) test for the HbA1c specific aptamer, which should be characterized by minimal binding of the isolated aptamers to Hb.

Equilibrium binding assay

The binding affinity of the selected DNA aptamers was determined using a magnetic bead-based chemiluminescence assay. Biotinylated DNA aptamers were heated at 95°C for 10 min and cooled down immediately on ice. These single-stranded aptamers were diluted to 100 nM with SSCT buffer (750 mM sodium chloride, 75 mM sodium citrate, and 0.05% Tween-20, pH 7.0) and then mixed with streptavidin-conjugated magnetic beads (Roche Life Science). After blocking with 0.1 M ethanolamine in phosphate buffer for 2 hr, various concentrations (ranging from 0 to 150 nM) of the target proteins (HbA1c or Hb), or other non-target proteins were incubated with these beads at room temperature for 2 hr. After washing with phosphate buffer with 0.01% Tween-20 (pH 7.4), the aptamer-bound beads were trapped with an external magnet (MPC-L, Dynal Inc., Norway) and then incubated with an acridinium ester-labeled antibody (anti-HbA1c GTX42177 or anti-Hb GTX42268 from GeneTex, Taiwan) for 1 hr. The bound complexes were trapped by an external magnet and the chemiluminescence intensity was quantified with an AutoLumat LB 953 Luminometer (EG & G Berthold, Bad Wildbad, Germany). Dissociation constants (Kd) were calculated by non-linear regression analysis using GraphPad Prism 5.0 (La Jolla, CA, USA). Non-target proteins including BSA, human thrombin, and human myoglobin were tested in the binding assay to examine the binding specificity of the selected aptamers.

Immuoassay-based validation of HbA1c- and Hb-specific aptamers

The HbA1c or Hb-specific aptamers were validated using an aptamer-antibody sandwich-like chemiluminescence immunoassay. The HbA1c(or Hb)-specific antisense aptamer was labeled with biotin at the 5'-end. After binding to streptavidin-conjugated magnetic beads, the aptamer was used as the capture ligand in a chemiluminescence immunoassay to validate its feasibility for diagnostic applications. We used commercially available human whole blood HbA1c calibrators (Abbott) with the concentrations 4.0, 5.5, 7.5, 9.0 and 14.5 %HbA1c, which were traceable to the International Federation of Clinical Chemistry (IFCC) reference standards. Briefly, magnetic beads coated with the HbA1c-aptamers were blocked with 100 nM ethanolamine for 2 hr. Blood samples with various concentrations of HbA1c (10 µL diluted 100-fold with deionized water) were incubated with 190 µL of these aptamer-coated beads for 2 hr at room temperature. After washing 2 times with 500 µL wash buffer, the acridinium ester-labeled anti-HbA1c antibody was added and incubated for 1 hr. After a second cycle of washing, the chemiluminescence signals were detected by a luminometer with the addition of 200 µL of 0.1 N HNO3 containing 0.6% H2O2 and 100 µL of 0.75 N NaOH.

Results and discussion

Selection of HbA1c- and Hb-specific aptamers

As shown in Figures 4(a) and 4(b), the partitioned and amplified ssDNA molecules produced after each round of the SELEX were examined by gel electrophoresis. Experimental data showed that HbA1c-specific aptamer (72 bp) was successfully
Table 1. The sequences of the PCR primers and ssDNA library used in this study, as well as the two aptamers selected using the established SELEX chip

<table>
<thead>
<tr>
<th>Primer/Sequence</th>
<th>GTA GCT GTT GGT CTG TGG TGC TGT-3'</th>
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sequences of the PCR primers and ssDNA library used in this study, as well as the two aptamers selected using the established SELEX chip
enriched and selected using the microfluidic SELEX chip developed herein. Note that the HbA1c-specific aptamer should be bound to HbA1c but not other Hb molecules. Therefore, for the selection of an HbA1c-specific aptamer, the ssDNA pool was incubated first with the Hb-coated beads as a negative selection. Then the un-bound ssDNA pool was transported to the HbA1c incubation chamber for the positive selection processes. After 7 consecutive rounds of negative and positive selection processes, high-affinity aptamers that bound to HbA1c only were enriched and isolated. As shown in Figure 4(b), the negative selection results after the sixth round of SELEX had significantly lower ssDNA quantities, as indicated by the trace amount of PCR product in the respective lane. Therefore, the product of the seventh positive selection round was cloned. Afterwards, thirteen clones were selected for subsequent competitive assays, and an HbA1c-specific aptamer with high affinity and specificity was identified (Figure 4(c)). This HbA1c-specific ssDNA aptamer was then sequenced (Table 1).

![Figure 4. The partitioned and amplified DNAs produced after each round of SELEX were examined by gel electrophoresis. Experimental results showed that the HbA1c-specific DNAs were successfully extracted and amplified by the SELEX chip developed herein. After seven rounds of incubating ssDNAs with (a) Hb-coated beads (negative selection) and HbA1c-coated beads (positive selection) or (b) only Hb-coated beads, the high-affinity aptamers bound to HbA1c were successfully enriched and isolated. (c) The binding affinity and specificity of the HbA1c-specific aptamers were verified by a competitive assay. Lane “N” depicts the results of the negative control test in which BSA-coated beads were incubated with individual ssDNAs; Lane “Hb” depicts the results of the non-target test in which individual ssDNAs were incubated with free Hb and then mixed with HbA1c-coated magnetic beads to examine the specificity of the HbA1c aptamer; Lane “P” depicts the results of the positive control test, in which HbA1c-coated magnetic beads were blocked with BSA; Lane “C” depicts the results of the competitive test, in which individual ssDNAs previously incubated with free HbA1c were mixed with HbA1c-coated magnetic beads to compete for the limited number of ssDNAs. “L” in (a) and (b) represent a DNA ladder.](image)

Similarly, an Hb-specific DNA-aptamer was successfully selected by the developed microfluidic SELEX chip. As shown in Figure 5(a), after five rounds of selection, the concentrations of the high affinity aptamers in the pool were significantly enriched. However, non-specific binding was observed (data not shown). In order to reduce the absorption of the selected aptamers on other proteins and improve the specificity of the pool, the selection stringency was increased by decreasing the incubation time and increasing the BSA concentrations from 0.1% to 1%. The non-specific binding decreased through the amplification process (Figure 5(b)). After cloning the selected ssDNA, each candidate was tested individually through the competitive assay. An Hb-specific aptamer with high affinity and specificity was identified (Figure 5(c)) with the sequence listed in Table 1.

![Figure 5. Gel electropherograms for selected Hb-specific aptamers from the SELEX chip. (a) The high affinity ssDNA aptamers were amplified after five consecutive rounds of on-chip SELEX (lanes 1-5). (b) The selection stringency was controlled by decreasing the incubation time and increasing the concentration of the BSA as the SELEX round number increased. The results show that the signal decreased after the fifth round of on-chip SELEX. (c) The binding affinity of the Hb-specific aptamers was assessed by a competitive assay. Lane “P” depicts the results of the positive control test. The bright band in this lane suggests that the aptamer demonstrates strong binding affinity for Hb. Lane “C” depicts the results of the competitive test, and lane “N” depicts the results of the negative control test.](image)

The characterization of the selected aptamers

The secondary structure of the HbA1c-specific and Hb-specific aptamers was predicted by the Zuker algorithm using MFOLD software and is shown in Supplementary Figure 2. The Gibbs free energy values of the HbA1c-specific sense and anti-sense strands were -10.62 and -14.49 kcal/mol, respectively. The Gibbs free energy values of the Hb-specific sense and anti-sense strands were -4.90 and -7.37 kcal/mol, respectively. The predicted secondary structures revealed a different complex stem-loop structure in HbA1c-aptamer which may have a specific affinity to HbA1c.

The HbA1c-specific aptamer selected by the microfluidic system demonstrated a Kd of 7.6±3.0 nM. The binding specificity of the HbA1c aptamer was further verified by incubating it with various concentrations of non-target proteins, including Hb, BSA, thrombin.
Figure 6. Equilibrium binding curves for the selected aptamers with (a) HbA1c and four non-target proteins (p<0.0001, Tukey's HSD test); and (b) Hb and three non-target proteins (p<0.0001, Tukey's HSD test). These selected aptamers exhibited $K_d$ values comparable to those of high-specificity antibodies.

Figure 7. A sandwich-like chemiluminescence immunoassay using the selected aptamers as the capture molecules and acridinium ester-labelled antibodies as the detection molecules. (a) The relationship between the chemiluminescence signal (relative light units [RLU]) and HbA1c concentrations of five commercially available blood standards. The HbA1c concentrations were measured by the IFCC reference method (high performance liquid chromatography-mass spectrometry). (b) The relationship between RLU and Hb concentrations. Error bars represent standard error of the mean of three replicate measurements made on different days.

Conclusions

By utilizing a combination of magnetic beads and microfluidic techniques, we designed and fabricated a SELEX chip for the automatic screening of HbA1c- and Hb-specific aptamers. The developed system required smaller reagent and sample volumes, as well as less processing time. The cost of the screening was therefore reduced. Furthermore, the microfluidics enabled rapid aptamer selection, which only took 70 min per round of SELEX. When compared to traditional methods, the microfluidic device enabled efficient incubation and provided enhanced washing efficiency by using magnetic beads. The selected ssDNA ligands were further examined for their affinity and specificity using competitive and equilibrium binding assays. The dissociation constants of the Hb- and HbA1c-specific aptamer were measured to be 7.6 nM and 7.3 nM, respectively, which are comparable to those of antibodies. Moreover, the Hb- and HbA1c-specific aptamers were validated using a chemiluminescence immunoassay and demonstrated linearity across a clinically relevant range of target protein concentrations. The developed SELEX chip is therefore promising for fast screening of aptamers for diagnosis of diabetes and could be readily modified and adapted to aid in the diagnosis of other human diseases.

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Notes

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