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Graphene based aptasensor for glycated albumin in diabetes mellitus diagnosis and monitoring

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ABSTRACT

We selected and modified DNA aptamers specifically bound glycated human serum albumin (GHSA), which is an intermediate marker for diabetes mellitus. Our aptamer truncation study indicated that the hairpin-loop structure with 23 nucleotides length containing triple G-C hairpins and 15-nucleotide loop, plays an important role in GHSA binding. Fluorescent quenching graphene oxide (GO) and Cy5-labeled G8 aptamer were used in this study to develop simple and sensitive graphene based aptasensor for GHSA detection. The limit of detection (LOD) of our aptasensor was 50 μg/mL, which was lower than other existing methods. In addition, with the nuclease resistance system, our GHSA detection platform could also be used in clinical samples. Importantly, our approach could significantly reveal the higher levels of GHSA concentrations in diabetes than normal serums. These indicate that our aptasensor has a potential for diagnosis and monitoring of diabetes mellitus.

1. Introduction

Measuring combination of blood sugar and glycated hemoglobin (HbA1c) is a way to monitor the diabetes progression. However, HbA1c level in the red blood cell keeps constant during its life cycle (120 days), which is somehow too long to be monitored in severe cases. In addition, in condition effecting red blood cell survival or hemoglobin production (hemolytic anemia, thalassemia, abnormal hemoglobins etc.), HbA1c level can be unreliable (Singhamatr et al., 2010; Srisurin, 2011; Paroni et al., 2007; Fitzgibbons et al., 1976; Hashimoto et al., 2008; Inaba et al., 2007). Therefore, monitoring the intermediate indicator outside the red blood cell could improve the way to control diabetes progression and treatment.

Human serum albumin (HSA) is multifunctional and the most abundant protein in plasma, which the life cycle of 20-25 days. The multifunction of HSA is associated with its structure that allowed to bind and transport a number of metabolites such as fatty acids, metal ions, bilirubin and some drugs (Wood, 1986; Anguizola et al., 2013). HSA is subjected to non-enzymatic glycation by excess circulating sugar, resulting in the formation of glycated human serum albumin (GHSA) and interfering normal HSA functions (Iberg and Fluckiger, 1986). Also, the glycation was observed to cause the malfunction in HSA (Awang et al., 2016). The pathogenic implication of GHSA formation can be observed in glucose metabolism of both adipocyte cells and skeletal muscle cells in diabetic patients (Unoki et al., 2007). It has been reported that typical diabetic GHSA levels are 2–5 times higher than normal values (Roohk and Zaidi, 2008). Thus, GHSA is considered as a marker for diabetes mellitus.

Aptamers are short single stranded-DNA, RNA or peptide (< 100 bases/amino acids) which bind target molecule as good as monoclonal antibodies. They can be selected from the random nucleotide pool by a process called systematic evolution of ligands by exponential enrichment (SELEX). Higashimoto and his colleagues found some DNA aptamers that bound GHSA and blocked the toxic effect of GHSA in the retinal pericyte cells, which may provide a useful tool for *in vivo* detection and inhibition of GHSA (Higashimoto et al., 2007). However, there is still no report using those aptamers for GHSA detection or other applications.

Here, we report the selection and modification of DNA

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aptamers bound GHSA for development of diabetes mellitus detection and monitoring. The high binding affinity aptamers were used in combination with graphene oxide (GO), to develop new method for GHSA detection. Our aptasensor platform could effectively detect elevated GHSA concentration in diabetes serums compared to normal serums, which can be applied for diabetes mellitus detection and monitoring.

2. Experimental section

2.1. Aptamer selection

Preparation and selection of DNA aptamer were processed by using the systemic evolution of ligand by exponential enrichment (SELEX), which was modified from the previous reports (Higashimoto et at., 2007; Sefah et al., 2010) (Selection detailed is in SI). After five rounds selection, aptamers bound GHSA was PCR amplified and cloned into pCR®4-TOPO® plasmid. Then plasmids consisting selected aptamer sequences were transfected into Top 10 competent cells, isolated and verified by DNA sequencing.

2.2. Secondary structure prediction

Secondary structures of selected DNA Aptamers were predicted using the Mfold web server (Zuker et al., 2003; SantaLucia et al., 1998; Peyret et al., 2000). Parameters used in this experiment were (1) the folding temperature was 25 °C (2) ionic condition at 137 mM [Na^+] with correction type was oligomer (3) the DNA sequence was linear (4) percent sub-optimality was 5 (5) an upper bound on the number of computed folding was 50.

2.3. DNA binding assay

Electrophoretic mobility shift assay (EMSA) was used for study binding of selected aptamers and GHSA protein. Secondary structure of ssDNA aptamer was denatured by heating at 65 °C for 5 min, followed by cooling on ice for 1 min before doing the experiment. After secondary structure denaturation, 2–10 ng of the biotinylated ssDNA aptamer was incubated with 0.4 μg or 0.2 μg GHSA protein or 0.6 μg HSA in 10 μL 1xPBS buffer at 25 °C for 1 h. The resulting complex was analyzed on 8% polyacrylamide gel and transferred to nylon membrane (Amersham Hybond-N+; GE Healthcare). Visualization of the biotinylated DNA on the membrane was performed using Phototope®-Star Detection Kit (New England Biolabs).

2.4. ELISA-like assay with aptamer (ELASA)

50 μL of 1 μg GHSA or HSA protein in 0.05 M carbonate buffer (pH 9.6) were coated on 96-well plate by incubation at 4 °C for 12 h. After discarding the excess protein, the immobilized proteins were washed 5 times with 1xPBST (0.5% Tween) and 200 μL of 2% tryptone in 1xPBST (0.5% Tween) was added. After incubation at 37 °C for 1 h, the excess tryptone was removed by washing with 1xPBST (0.5%Tween). Then 40 ng biotinylated aptamer in 50 μL 1xPBST (0.5% Tween) was added and incubated at 25 °C for 1 h, before washing with 1xPBST. After adding 50 μL of 1:1000 dilution HRP conjugated streptavidin in 1xPBST and incubation at 25 °C for 1 h, the excess protein was removed by washing with 1xPBST. Then 50 μL TMB was added and incubated at 25 °C for 30 min. The reaction was stopped by adding 50 μL of 0.6 M H_2SO_4 and the OD450 nm was subsequently measured by using spectrophotometer.

2.5. Fluorescent measurement of GO-aptamer approach

GO monolayer powder (Supplement Information) was dissolved in sterile water to make 5.0 mg/mL stock solution and sonicated for at least 30 min. To form GO-aptamer complex, 2 μL of 5.0 mg/mL GO was mixed with 2 μL of 10 μM aptamer for 5 min at room temperature in the dark. Then 2 μL of GHSA with varied concentrations and 196 μL sterile PBS were added and incubated at 25 °C for another 30 min in the dark before measured fluorescent signal (630/670 nm) using fluorometer (SpectraMax, Molecular Device). In case of HSA and clinical samples, varied HSA or serum dilutions were added instead of GHSA, then followed the same protocol as previously described.

3. Results and discussion

3.1. Selection and characterization of aptamers bound GHSA

DNA aptamers bound GHSA were selected from the DNA aptamer library of $\sim 10^{15}$ using SELEX process. After DNA analysis of 25 selected aptamers, the result showed 18 aptamer sequences with the GC content of 49–70% and the length of 49–53 nucleotides (Table S1). Some selected aptamers were 1–3 nucleotides error from the expected length perhaps due to the insertion or deletion mutations during the PCR amplification in the selection process. After using Mfold program (Zuker, 2003; SantaLucia, 1998) to predict ssDNA secondary structures, we found $\Delta G \leq -3.66$ kcal/mol from all selected aptamers. Once comparing all secondary structures, G8 aptamer with the hairpin loop and the lowest ΔG of -4.08 kcal/mol was chosen for further binding and truncation study.

The electromobility shifted assay (EMSA) and ELISA-like assay with aptamer (ELASA) were used to confirm the binding and specificity of the G8 aptamer. The result from EMSA, which is the common experiment for DNA-protein binding study, showed shifted band when G8 aptamer was incubated with GHSA protein and non-shifted band when G8 aptamer was incubated with HSA (Fig. 1**a**). Similar results were observed from the ELASA experiment, in which, normalized OD450 nm from G8 aptamer bound GHSA protein was significantly higher than that from G8 aptamer bound HSA (Fig. 1**b**). Although the result from the ELASA experiment was shown 15% normalized OD450 nm from G8 aptamer bound HSA protein, this non-specific binding was weak and could be eliminated by increased incubation temperature and added more washing steps. These results suggest that G8 aptamer specifically bound GHSA in condition described here.

According to the Mfold prediction, G8 aptamer formed secondary structures with 2 hairpin loops at the 5′ end and the long flexible region at the 3′end as shown in Fig. 2**a**. The small loop at the 5′end containing 2 G-C base pairs, which was not stable at room temperature. Therefore, we purposed that only the big hairpin loop plays a role in GHSA binding. To test our hypothesis, the small hairpin loop and the flexible region were deleted (Fig. 2**b, c, d, e and f**) and GHSA binding properties of truncated aptamers were determined. Results from both EMSA and GO-aptamer method (detail is in the next section) showed that 5-, 10- and 15-nucleotides (del5, del10 and del15) except 20-nucleotide deletion (del20) bound GHSA. Moreover, after removing of the small loop at the 5′ end (5del11and3del15), GHSA binding was still observed (Table S2). Computer simulation results predicted that 5del11and3del15 bound subdomain IIIB. The binding position on the GHSA protein will be further studied. These results suggested that the big hairpin loop structure with 23 nucleotides length containing triple G-C hairpins and 15-nucleotide loop, plays an important role in GHSA binding.