Highly sensitive fluorescent detection of 25-HydroxyvitaminD3 using truncated affinity-improved DNA aptamers

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Abstract

Vitamin D insufficiency is closely related to various kinds of metabolic diseases. Acted as a marker of vitamin D status, 25-HydroxyvitaminD3 detection possesses important practical significance. In this study, highly sensitive fluorescent detection of 25-HydroxyvitaminD3 using truncated affinity-improved aptamers were developed, based on fluorescence intensity changes of PicoGreen (PG) generated upon binding to double-stranded DNA (dsDNA) formed through hybridization of aptamer and corresponding complementary strand. Four truncated aptamers were obtained by intercepting the small hairpin loop as the functional domain and retaining double helix structural domains of different lengths that exist in the selected original 25-HydroxyvitaminD3 aptamer. Under the optimized PG concentration, we conducted comparison experiments for affinity and specificity of these four truncated aptamers. Among them, the shortest aptamer with only 21 bp, D3-4, was found to show the highest affinity and specificity to 25-HydroxyvitaminD3, with the limit of detection of 0.04μg/mL, which solved the problem that original long aptamer could not applied for this fluorescent detection of 25-HydroxyvitaminD3. The truncated 25-HydroxyvitaminD3-specific aptamer with highly enhanced affinity performs promising application in sensitive detection of 25-HydroxyvitaminD3.

Introduction

Vitamin D plays a vital role in bone health through regulation of calcium and phosphorus homeostasis, which can facilitate intestinal calcium absorption and provide calcium necessary for bone mineralization.[1,2] Prolonged and severe deficiency of vitamin D leads to rickets (in children) and osteomalacia (in adults).[3,4] Besides, epidemiological studies have increasingly found that vitamin D deficiency link with a wide range of non-communicable diseases such as obesity, hypertension, diabetes, heart failure.[5-7] Vitamin D is synthesized when exposed to ultraviolet radiation (UVR) through the skin epidermis (vitamin D_3), or absorbed from food or supplements (vitamin D_2 and vitamin D_3).[8] The vitamin D_3 is hydroxylated by the liver enzyme 25-hydroxylase to 25-HydroxyvitaminD_3 [25(OH)D_3, cholecalciferol], half-activated form of vitamin D_3. When the certain tissue in our body needs vitamin D function, 25(OH)D_3 is converted by 1-α-hydroxylase to 1,25-dihydroxyvitamin D_3[1,25$(OH)_2D_3$, calcitriol], which is a fully activated form.[9,10] As the major circulating form of vitamin D, 25-HydroxyvitaminD_3 is considered to be the indicator of vitamin D status.[11] The serum vitamin D_3levels can be assessed through measuring the concentration of 25-HydroxyvitaminD_3.[12]

The standard quantitative detection methods for vitamin D_3 levels mainly include high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA).[13,14] HPLC method requires tedious pretreatments, sophisticated equipments and trained personnel, giving rise to increasing analysis time. Although with good sensitivity and selectivity, ELISA may suffer from matrix interference, false

positive results and large deviations in qualitative results. Besides, some commercial antibody kits are applied for 25-HydroxyvitaminD_3 detection, however with the antibody limitations of high cost, complicated storage condition and short shelf life.[12] In comparison, aptamers (Apt), highly specific oligonucleotides, can bind to a variety range of target ligands and are used as an alternative to antibodies.[15-18]

Aptamers are single-stranded DNA (ssDNA) or RNA molecules which are selected in vitro by systematic evolution of ligands via an exponential enrichment (SELEX). They can bind to various target ligands ranging from small molecules, peptides, proteins to cells with high affinity and specificity.[19] Compared with traditional antibodies, aptamers possess notable advantages including high binding capability, excellent stability, target diversity, low cost, simple synthesis and easy modification.[20,21]Aptamer-based detection system are non-immunogenic, low toxicity and stable to temperature and pH changes.[22] However, many selected original aptamers with long sequences possess poor affinity and are limited in application. Thus, truncation of aptamers is valuable work, for that many aptamers lying in the corner can be used with high performance after truncation.

Lee et al., have developed novel aptamers for detecting 25-Hydroxyvitamin$D_3$by graphene oxide-based systemic evolution of ligands by exponential enrichment (GO-SELEX) and found the aptamer VDBA14 (5'-AGCAGCACAGAGGTCATGGGGG-GTGTGACTTTGGTGTGCCTATGCGTGCTACGGAA-3', 56 bases) showing specific affinity to 25-HydroxyvitaminD_3, with the limit of detection of 1 μM.[12] In order to increase the aptamer affinity to its target and avoid large steric hindrance of long aptamer sequence, a few simple or heuristic approaches towards the truncation of aptamers were reported, other than addition to optimize the length of the random library used in the SELEX process.[23] In this study, we have heuristically truncated the VDBA14 to four aptamers with different sequences by intercepting the small hairpin loop and retaining double helix regions of different lengths that exist in VDBA14 aptamer, while maintaining higher affinity to 25-HydroxyvitaminD_3. A universal PicoGreen-based fluorescence strategy for the quantitative detection of 25-HydroxyvitaminD_3 was designed, that the fluorescent signal of PG generated upon the double-stranded DNA (dsDNA) formed between the aptamer and its complementary strand. Coupled with the simple PG-based fluorescent assay, the analytical results indicated that the truncated aptamers showed higher affinity than the original aptamer, and exhibited great potential for sensitive detection of 25-HydroxyvitaminD_3.

Materials and methods

Materials and reagents

All the oligonucleotide sequences used in this study were synthesized and then purified through HPLC by Shanghai Sangon Biotechnology Co., Ltd (Shanghai, China), and sequence information were listed in Table 1. The lyophilized powder of aptamers and complementary strands were dissolved in ultrapure water to 10μM and then diluted to 0.5μM in binding buffer (100 mM NaCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, pH 7.6). The PicoGreen (PG) dsDNA reagent was purchased from Thermo Fisher Scientific (Shanghai, China). 25-HydroxyvitaminD_3 monohydrate was obtained from Sigma-Aldrich International GmbH. Vitamin C and Vitamin B12 were purchased from TMstandard (Beijing, China). Folic acid was obtained from Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China). All other reagents were of analytical grade, and the ultrapure water used throughout the experiments was purified by Milli-Q Academic system (Millipore, Molsheim, France). The fluorescence intensities were recorded with a Tecan Infinite 200 multifunctional microplate reader (Tecan Austria GmbH, Austria), with the excitation and emission wavelengths of 480 nm and 520 nm, respectively.

Truncation for 25-HydroxyvitaminD_3 aptamer

The original aptamer with long oligonucleotide sequences may bind analogues of its targets through primer region or additional fragments, resulting in compromised selectivity.[24] It is significant to confirm the target binding region where the aptamer undergoing conformation change upon target binding. Thus, truncation of the non-binding region for original aptamer may generate aptamers with better performances in consideration of affinity, selectivity and cost. Several studies reported on aptamer engineering approaches were focused on the G-quadruplex region, secondary structure (stem-loop) and stem-loop multiplication, on account of

stem-loop known to be the binding motif for most proteins.[23] Based on molecular shape and functional base distribution of 25-HydroxyvitaminD_3, the loop regions of original aptamer were assumed to be the functional domain for binding to 25-HydroxyvitaminD_3target. In this study, the original VDBA14 aptamer was truncated on the basis of retaining small hairpin loops. Then the experiments for affinity and specificity were performed to ascertain the functional and structural domains of original aptamer.

Principle of the fluorescent assay

PicoGreen (PG) reagent is an asymmetric cyanine dye that does not fluoresce when free. However, the fluorescence of this commercially available dye increases by more than 1000-fold when upon binding to dsDNA, while no significant fluorescence change can be observed after binding to ssDNA.[25] Here, we performed a universal PG-based fluorescence strategy for the quantitative detection of 25-HydroxyvitaminD_3. The sensing mechanism of the method was illustrated in Figure 1. In the control group, dsDNA was formed through hybridization of 25-HydroxyvitaminD_3-specific aptamer and corresponding complementary strand, then PG dye inserted into the minor groove of dsDNA duplex, that resulting in significant enhancement of fluorescence.[26,27] In the test group, specific binding of 25-HydroxyvitaminD_3 and aptamers occurred prior to dsDNA formation, and the small amount of remaining free aptamers reacted with complementary strands, which generating a decrease in fluorescence intensity compared to the control group. Thus, quantitative analysis of 25-HydroxyvitaminD_3 can be accomplished by monitoring the fluorescence intensity changes of PG dye.

Screening of aptamers for 25-HydroxyvitaminD_3

The screening of four truncated aptamers (D3-1, D3-2, D3-3 and D3-4) and the original aptamer (VDBA14) for 25-HydroxyvitaminD_3were carried out in this work, based on the analysis foundations of affinity and specificity. Although dissociation constant (KD) can be applied to evaluate affinity between aptamers and target ligands, it fails to describe specificity and cross-reactivity. Therefore, we performed the aptamers screening for 25-Hydroxyvitamin$D_3$with high affinity and specificity by means of a simple fluorescent strategy using commercial PG dye. Through comparison for the affinity and specificity of the four truncated aptamers and the original aptamer, suitable aptamer with highest affinity and best specificity to 25-HydroxyvitaminD_3 was identified.

Fluorescent detection of 25-HydroxyvitaminD_3

To determine the sensitivity of this method, different concentrations of 25-HydroxyvitaminD_3 standard solution were progressively diluted from 10μg/mL in binding buffer (100 mM NaCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, pH 7.6). 10μg/mL 25-HydroxyvitaminD_3 was obtained through dissolving the lyophilized powder of 25-HydroxyvitaminD_3monohydrate in methanol. 25μL of 0.5μM aptamer solution and 50μL of different concentrations of 25-HydroxyvitaminD_3 were successively added into a microplate well and incubated at room temperature for 40 min. Afterwards, 25μL of 0.5μM complementary strand solution was added to the microplate well and incubated for 10min. Then 10μL 10×PG solution was added, and after 3min incubation, the fluorescent intensities were recorded with a multifunctional microplate reader. Each concentration was performed in triplicate to obtain average values for analysis.

Results and discussion

Aptamer truncations and sequence designs

With the aim to ascertain the binding sites of original VDBA14 aptamer and to obtain the truncated sequences with increased affinity, the secondary structure analysis of original aptamer is essential. In this study, two secondary structures of the original aptamer for 25-HydroxyvitaminD_3(VDBA14) were obtained from mfold software program (shown in Figure 2A and Figure 2B).

Based on the first secondary structure of original VDBA14 aptamer, we designed two truncated aptamers (denoted as D3-1 and D3-2) by rationally intercepting the sequences containing small hairpin loop and retaining double helix structural domains of different lengths. In the double helix region of D3-1 and D3-2,

the G-T mismatched pairs were replaced by G-C base pairs, and one A-T base pair was replaced by G-C base pair in D3-2 aptamer to strengthen the stability of the whole structure (displayed in Figure 2C and 2D). There is an alternative small loop in another secondary structure of original VDBA14 aptamer (Figure 2B), and then D3-3 and D3-4 (Figure 2E and Figure 2F) were obtained by substitution of this small loop with loop regions in D3-1 and D3-2. The secondary structures of all aptamers predicted by the mfold program were shown in Figure 2, and the sequences for 25-HydroxyvitaminD_3 aptamers and corresponding complementary strands used in this study were listed in Table 1.

Optimization of PG concentration for different aptamers

The fluorescent assay of 25-HydroxyvitaminD_3 was based on a combination of PG dye and aptamer/complementary strand-formed dsDNA. Thus, the concentration of PG fluorescent dye is an important parameter influencing the detection performance. The PicoGreen dsDNA reagent was diluted 5-folds, 10-folds, 20-folds, 30-folds and 40-folds by 1×TE buffer, that diluted PG reagents were labeled as 5×, 10×, 20×, 30× and 40×. After adding 25μL of 0.5μM aptamer solution and 50μL binding buffer to a microplate well, 25μL of 0.5μM complementary strand was added and incubated at room temperature for 10min. Then 10μL of 5×, 10×, 20×, 30× and 40×PG solution were added into the mixture at the last reaction section. As displayed in Figure 3, the fluorescence intensities gradually increased with the increase of PG concentration. For VDBA 14, D3-3 and D3-4 aptamers, the fluorescence intensities reached basically stable peak and did not change obviously when 10×PG were added, indicating that 10× PG were enough to mark dsDNA after binding of aptamers and complementary strands. For D3-1 and D3-2 aptamers, increasing the PG concentration did not lead to an obvious intensity increase when adding 20×PG. Thus, the selected PG concentrations for VDBA 14, D3-1, D3-2, D3-3 and D3-4 were 10×PG, 20×PG, 20×PG, 10×PG and 10×PG, respectively.

Screening of 25-HydroxyvitaminD_3-specific aptamers

In this study, we compared the affinity of original aptamer and four truncated aptamers to 25-HydroxyvitaminD_3 through PG-based fluorescent assay, and selected the aptamers with high affinity for further detection of 25-HydroxyvitaminD_3. The comparison results for aptamer affinity were shown in Figure 4A, in which ΔF stand for the decreased value of fluorescence intensity when aptamer specifically bound to 25-HydroxyvitaminD_3, and F_0 stand for the fluorescence intensity in the absence of 25-HydroxyvitaminD_3. Demonstrated in Figure 4A, compared with VDBA14 aptamer, four truncated aptamers of D3-1, D3-2, D3-3 and D3-4 gave rise to significant decrease of fluorescence intensity, indicating these four truncated aptamers possessed higher affinity to 25-HydroxyvitaminD_3 than original VDBA14 aptamer. In particular, D3-3 and D3-4 had strongest affinity to 25-HydroxyvitaminD_3 with distinct fluorescence intensity decrease than D3-1 and D3-2. Therefore, D3-3 and D3-4 aptamers were used for following specificity evaluation.

Specificity of the selected aptamers for 25-HydroxyvitaminD_3

To evaluate the specificity of D3-3 and D3-4 aptamers, the fluorescence intensity changes were induced against 25-HydroxyvitaminD_3 and its analogues including vitamin C, vitamin B12 and folic acid at the same concentration of 10μg/mL. As shown in Figure 4B, the ΔF (F-F_0) of 25-HydroxyvitaminD_3 was only up to less than two-fold higher than that of vitamin B12 when applying D3-3 aptamer as the recognition sequence. The decrease of fluorescence intensity produced by D3-4 aptamer were most obvious while similar fluorescence intensity changes appeared for other three non-specific targets, demonstrating D3-4 bound to 25-HydroxyvitaminD_3 the strongest and had only slight binding with other analogues. By comparison, D3-3 had poorer specificity than D3-4 to 25-HydroxyvitaminD_3, then D3-4 was selected as the 25-HydroxyvitaminD_3-specific aptamer with high affinity and specificity, in which the hairpin loop was the functional domain binding to target and the double helix region was the structural domain.

Sensitivity of the fluorescent assay

Under the above optimized conditions, the sensitivity experiment of PG-based fluorescent assay for 25-HydroxyvitaminD_3 using D3-4 were conducted, that through obtaining fluorescence intensities at different concentrations of 25-HydroxyvitaminD_3. Figure 5 displayed the linear relationship of the fluores-

cence intensity (F) vs 25-HydroxyvitaminD_3 concentration, presenting linear decreases in F with increasing 25-HydroxyvitaminD_3concentrations ranging from 0.04μg/mL to 0.8μg/mL. The increased concentrations of 25-HydroxyvitaminD_3 caused binding of targets to more aptamers, so that less remaining aptamer/complementary strand DNA duplexes were formed. The decrease in DNA duplexes and intercalated PG lead to gradual diminution in the fluorescence intensity. A good linear relationship between the fluorescence intensity and the target concentration was observed in Figure 5, with a correlation coefficient of 0.968 and a low detection limit of 0.04μg/mL. Compared to the results of VDBA14 aptamer in relevant reference reported by Lee et al. (2017), the truncated D3-4 aptamer performed higher affinity and lower detection limit to 25-HydroxyvitaminD_3.

Conclusions

In this study, highly sensitive fluorescent detection of 25-HydroxyvitaminD_3 using truncated aptamers with different sequences were developed. By intercepting the sequences containing small hairpin loop and retaining double helix structural domains of different lengths that exist in original aptamer (VDBA14), we obtained four truncated aptamers for 25-HydroxyvitaminD_3detection based on the fluorescent intensity changes of PG dye generated upon binding to aptamer/ complementary strand-formed dsDNA duplex. After optimization of PG concentration, we screened the truncated aptamers through comparing the affinity and specificity to 25-HydroxyvitaminD_3 target, among which D3-4 aptamer was selected for 25-HydroxyvitaminD_3 detection. The linear dynamic range were from 0.04μg/mL to 0.8μg/mL with a correlation coefficient of 0.968. The proposed fluorescent assay method accurately quantified 25-HydroxyvitaminD_3, attributed to high affinity of truncated aptamer to 25-HydroxyvitaminD_3 and ultra-sensitivity of PG to trace dsDNA. Therefore, the truncated aptamer (D3-4) with increased affinity and reduced base numbers than original aptamer in this work is expected to be utilized for sensitive 25-HydroxyvitaminD_3 detection with promising application.

In addition, the affinities of many aptamers to complementary strands are higher than the affinities to corresponding targets due to the long sequences of aptamers. Thus, the applications of some methods based on the competitive principle of target and complementary strand are limited. In this study, affinity of aptamer to target was improved by the truncation of original 25-HydroxyvitaminD_3 aptamer, which provided truncation reference for other long-chain aptamers.

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Conflicts of Interest

The authors acknowledge that there is no conflict of interest in this article.

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Table

Table 1 25-HydroxyvitaminD_3 aptamers and complementary strands used in this study.

Description	Sequence (5'-3')
VDBA14 aptamer	AGCAGCACAGAGGTCATGGGGGGTGTGACTTTGGTGTGCCTATGCGTGCTACC
VDBA14 complementary strand	TTCCGTAGCACGCATAGGCACACCAAAGTCACACCCCCCATGACCTCTGTGCTC
D3-1 aptamer	GAGGTCACGGGGGGTGTGACCTCGG
D3-1 complementary strand	CCGAGGTCACACCCCCCGTGACCTC
D3-2 aptamer	GAGGGCACGGGGGGCGTGCCTT
D3-2 complementary strand	AAGGCACGCCCCCCGTGCCCTC
D3-3 aptamer	GAGGTCACGTGTGTGTGACCTCGG
D3-3 complementary strand	CCGAGGTCACACACACGTGACCTC
D3-4 aptamer	GAGGGCACGTGTGCGTGCCTT
D3-4 complementary strand	AAGGCACGCACACGTGCCCTC

Figure captions

Figure 1. Schematic illustration of PG-based fluorescent detection for 25-HydroxyvitaminD_3. The PicoGreen dye inserted into the minor groove of dsDNA duplex when forming dsDNA through hybridization of 25-HydroxyvitaminD_3-specific aptamer and corresponding complementary strand, that generating significant fluorescence enhancement. In the test group, specific binding of 25-HydroxyvitaminD_3 and aptamers occurred prior to dsDNA formation, and small amount of remaining free aptamers reacted with complementary strands.

Figure 2. The secondary structures of original aptamer and truncated aptamers. The secondary structures of original VDBA14 aptamer (A and B) and truncated aptamers including D3-1 (C), D3-2 (D), D3-3 (E) and D3-4 (F) were obtained from mfold software program. While maintaining higher affinity to 25-HydroxyvitaminD_3, the VDBA14 were heuristically truncated to four aptamers with different sequences by intercepting the small hairpin loop and retaining double helix regions of different lengths that existed in

VDBA14 aptamer.

Figure 3. The fluorescence intensities change depending on PG concentrations. The concentration of PG fluorescent dye is an important parameter influencing the detection performance. For VDBA 14, D3-3 and D3-4 aptamers, the fluorescence intensities reached basically stable peak and did not change obviously when 10×PG were added, indicating that 10×PG were enough to mark dsDNA. For D3-1 and D3-2 aptamers, increasing PG concentration did not lead to obvious intensity increase when adding 20×PG.

Figure 4. Affinity and specificity evaluation of aptamers. A) Affinity evaluation of aptamers with different sequences to 25-HydroxyvitaminD_3. Through comparison, D3-3 and D3-4 gave rise to significant fluorescence intensity decrease, indicating these two truncated aptamers possessed higher affinity than original VDBA14 aptamer. B) Specificity evaluation of D3-3 and D3-4 for 25-HydroxyvitaminD_3 compared to VC, VB12 and FC. By comparison, D3-3 had poorer specificity than D3-4 to 25-HydroxyvitaminD_3.

Figure 5. Calibration plot with fluorescence intensity against different concentrations of 25-HydroxyvitaminD_3 . The calibration plot with fluorescence intensity of PG/aptamer duplex mixture against different concentrations of 25-HydroxyvitaminD_3 from 0.04μg/mL to 0.8μg/mL were displayed. Linear decreases in fluorescence intensity (F) were appeared with increasing 25-HydroxyvitaminD_3concentrations, for the reason that increased concentrations of 25-HydroxyvitaminD_3 caused binding of targets to more aptamers and less remaining aptamer/complementary strand DNA duplexes were formed.