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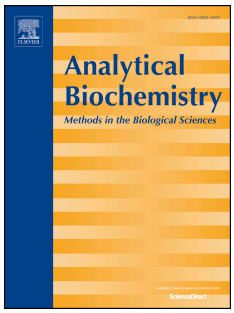

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High affinity truncated DNA aptamers for the development of fluorescence based progesterone biosensors

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Abstract

Aptamers have shown a number of potential applications in sensing and therapeutic due to the high affinity and specificity towards their target molecules. Not all the nucleotides in the full length aptamers are involved in the binding with their targets. The non-binding domain of the aptamer may affect the binding affinity of the aptamer-target complex. Mapping the aptamer binding region could increase the affinity and the specificity. In this paper, we designed aptamer-based fluorescence sensors from a truncated progesterone (P4) aptamer. Then, fluorescein and quencher labelled aptamer complementary oligonucleotide sequences were hybridized to the truncated aptamer at different sites to form duplex structures. We used fluorescence-quencher pair displacement assay upon progesterone binding for the determination of P4. One of the truncated sequences have shown high binding affinity with 16 fold increase in the dissociation constant, K_d (2.1 nM) compared to the original aptamer. The aptasensor was highly selective for P4 against similar compounds such as 17-β estradiol, bisphenol-A and vitamin D. The sensor has been applied for the detection of P4 in spiked tap water and in urine samples showing good recovery. This new developed aptamer-based fluorescence biosensor can be applied in food, pharmaceutical, and clinical industries.

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1. Introduction

Food products and drinking water contamination with endocrine-disrupting chemicals (EDCs) may have a serious effect on hormonal functions such as metabolism, biosynthesis, etc [1]. Progesterone (P4) is a small hydrophobic steroid hormone secreted from corpusluteum, which plays a major role in mammalian pregnancy, animal growth and development. Progesterone level is used as an indicator for early pregnancy. Many clinical assays are currently used to measure hormonal levels in a variety of body fluids. The progesterone concentration is 1 ng/ ml in serum during the pre-ovulation period, 20 ng/ml in the mid cycle and more than 300 ng /ml at the pregnancy period. Elevated levels of P4 leads to headache, breast tenderness, stomach upset, constipation, diarrhea, body pain, tiredness, virginal discharge and urinal infections [2]. Consumption of high levels of progesterone in cow milk may case breast and lung cancers [3, 4] and it affect the gonadotropin (GnRH) releasing hormone secretion in males [5]. When high amount of P4 is consumed, the body retains certain amount and the rest is released to the environment as waste. Therefore, it is highly important to monitor P4 levels in environmental and clinical samples.

Several methods are applied for the detection of P4. Instrumental analysis methods such as high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC-MS) are highly sensitive. However, they need well-trained operators, high cost and not suitable for field applications. Immunoassays like enzyme linked immunosorbent assay (ELISA), radioimmune assays, electrophoresis-chemiluminescence and non-competitive audiometric assays are used for the detection of P4 in different samples [6-8]. However, the limited stability and high cost of the immunoassays are still major challenges.

Recently, there is an increasing interest towards aptamers as alternatives to antibodies. Aptamers are widely used for sensor development for the detection of various compounds for clinical, food and environmental applications. Aptamers bind their target specifically with high affinity (K_ds in the nanomolar to picomolar range). They can be synthesized easily, highly stable and their production cost is very low. Aptamers are single stranded DNA (ssDNA) or RNA, consisting typically of 40 to 100 nucleotides. At suitable conditions, aptamers form unique 3D structure due to the intra molecular forces of attractions such as hydrogen bonding, hydrophobic and Wan der walls interactions between the nucleotides. This unique secondary or tertiary structures of the aptamer forms a binding packet that fits the target molecule and form a stable target-aptamer complex. Aptamers for various analytes have been reported including metal ions [9], proteins [10], bacteria [11], viruses [12] and small molecules [13-15]. The in vitro selection of aptamers are usually screened from a pool of DNA or RNA libraries using systematic Evaluation of Ligands by Exponential Enrichment (SELEX) method.

The goal of applying aptamers in bioanalysis is to report the presence of target without any complicated steps. Fluorescence detection is one of the commonly used analytical ways using aptamers. The aptamer results from the classical SELEX does not usually exhibit intrinsic fluorescence. The fluorescent molecule is introduced at the position where the aptamer undergo the target induced conformation change [16, 17] or fluorescent nucleotides in the library pool are introduced during the in vitro selection of aptamer [17]. However, it is not straight forward to identify the region where the confirmation change occurs. Moreover, if there is no significant change in the fluorescence intensity upon target binding, the sensitivity of the detection assay will be low. Therefore, it is important to know the target binding region where the aptamer undergo conformation change upon target

binding. By truncating the non-binding region, the aptamer may form better conformation to make a stronger aptamer-target complex. Many approaches have been used for truncating the aptamers. For example, the secondary structure of the Tat HIV protein aptamer can be split into two parts. A molecular beacon (in which the fluorophore and quenchers are attached to both ends) and a complementary sequence to the stem of the beacon. In the presence of target the beacon undergo conformation change which leads to a physical separation of the fluorophore and quencher resulting in a change in the fluorescence signal [18].

Here, we have developed a fluorescence-based aptasensor for the detection of P4 via structure switching mechanism. In this study, we designed truncated aptamers/DNA duplex structures by annealing fluorescein-labelled (FDNA) and quencher-labelled DNA (QDNA) complementary sequences at different sites. Originally, in the designed duplex structures the fluorophore and quencher will be in close contact to each other leading to minimal fluorescence intensity. However, in the presence of P4, an increase in the fluorescence intensity is recorded due to the conformation change of the aptamer upon target binding which leads to FDNA and/or QDNA dissociation. This is due to the favourable formation of the aptamer-P4 complex than the DNA duplex form. Knowing the minimal binding sequence within the aptamer, a biosensing platform for the detection of P4 was established.

2. Experimental

2.1. Materials and Methods

Progesterone, 17-β estradiol, bisphenol, vitamin D, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris base), sodium chloride, ethylenediaminetetraacetic acid (EDTA), sodiumazide, potassium phosphate, sodium hydroxide, sodium chloride, potassium chloride, urea, sodium phosphate hydrochloric acid and magnesium chloride were purchased form Sigma-Aldrich (Saint-Louise, MO, USA). HPLC purified labelled and unlabelled

oligonucleotides (Table 1) were purchased from Metabion International (Planegg, Germany). A solution of 50 mM tris-HCL (pH 7.5), 150 mM NaCl and 5mM $MgCl_2$ is used as binding buffer. The stock solution of P4, 17-β estradiol, bisphenol A and vitamin- D was prepared in DMSO and further dilutions were made in binding buffer. The DNA were dissolved in ultra pure Milli-Q water to make the stock solutions and stored at -20°C until further use. The DNA solutions used in the experiments were diluted in binding buffer. The fluorescein-labelled oligonucleotides were protected from the light while performing the experiments.

2.2. Fluorescence Measurements

The fluorescence of the fluorescein labelled complementary aptamer oligonucleotides and the aptamer-beacon has been measured using Nanodrop ND3300 fluorospectrometer (Thermo Scientific, Canada) and Molecular device F5 fluoromax microtiter plate reader (Sunnyvale CA, USA) using 96 well fluorescence plates. The samples were excited at 470±10 nm and the emission was monitored at 519 nm. All the measurements were recorded in binding buffer at room temperature.

2.3. Designing the aptamer sequences

The truncated aptamers were designed based on the secondary structure of PG13 full length aptamer selected in our previously published work [15]. The secondary structure was obtained from the mfold software. Two designs were done; displacement of a complementary sequence and molecular beacon. The complementary sequences, 5'-fluorescein labelled and 3'-dabcyl quencher labelled oligonucleotides were designed for the fluorescence-based competitive displacement assays in which the fluorescein quencher pair are in close contact upon hybridization with the truncated aptamer. The PG13 aptamer was truncated into two

different domains as shown in the Table .1 The aptamer- beacon was designed from the original full length aptamer with addition of few nucleotides either 5'or 3'end. Aptamer-beacons are similar to molecular beacon in which the 5'and 3'end nucleotides are complementary to each other to form stem-loop secondary structure. In this design, the truncated aptamer oligonucleotides were labelled with fluorescein and dabcyl quencher at 5' and 3' ends, respectively

2.4. Competitive fluorescence displacement and Aptabeacon Assays

For the fluorescence displacement assay, a mixture of 500 nM solutions of each truncated DNA aptamer and equal concentrations of their 5'fluorescein-labelled and 3'dabcyl-labelled complementary sequences in binding buffer were kept at 90°C . Then the solution was kept at 4°C for 10 min. followed by keeping at room temperature for 10 min. 100 nM of hybridized oligonucleotide () were incubated with different concentrations of P4 ranging from 0 to 250 nM for 45 min. and the fluorescence intensity of each sample was measured. The saturation curve was obtained for each aptamer sequence and the K_d values were calculated using non-linear regression fitting.

The selectivity of the sensors has been tested by cross reacting the sensors with 100 nM solutions of similar structural and functional compounds such as bisphenol A, 17-β estradioland vitamin D.

The aptamer-beacons were heated to 95°C for 5 min, 4°C for 10 min and room temperature for 10 min. Various concentration of progesterone was incubated with 100 nM solution of aptamer beacon and the fluorescence intensity of each samples were recorded.

For real samples experiment, concentration range from 0 to 80 ng/ml of P4 was spiked in tap water and in artificial with the composition of 0.17M NaCl, 0.08M KCl , 0.52M NaH4PO4and 700mg/L urea [19]. The percentage recovery was determined from the increase in the fluorescence intensity of the sensors.

3. Results and Discussion

Previous studies have shown that most of the aptamer sequences resulting from SELEX screening are hardly to be integrated directly in biosensor platforms [20]. Therefore, post-SELEX optimization of the selected aptamer is usually required in order to regulate the aptamer sequence to a specific sensing platform. It is also believed that not all nucleotides in the aptamer sequence are involved in target binding. These non- binding domains may destabilise the aptamer-target complexes [21-23]. In our previous work, P4-binding aptamers were successfully selected from a 60 mer DNA library contains 2×10^{15} sequences of oligonucleotides by in vitro selection [15]. The full length PG13 aptamer which showed the highest affinity (K_d=35 nM) and conformation change upon target binding, have been utilized for the detection of P4 using a label-free impedimetric method [15]. It was also found that hybridizing complementary DNA (10 mers) sequences at the different regions of PG13 aptamer has improved the binding affinity towards P4. The maximum affinity was observed when the position 40 to 50 from 5'end was hybridized [15]. This may indicate that this region of the aptamer is not involved in the P4 binding (non-binding domains).

3.1. Aptamer truncations and displacement designs

With the aim to ascertain the binding site of the PG13 aptamer and to find the shortest sequence with the highest binding affinity, we designed here two truncated aptamers by rationally splitting the sequence into two halves (PG13T1 and PG13T2). Then, fluorescent-quencher complementary DNA reporters were designed for each truncated part to form DNA duplexes. The PG13T1 truncated sequence was hybridized with a fluorescein-labelled short

complementary fragment (PG13C1) as well as a dabcyl labelled fragment (PG13C2). Similarly, the PG13T2 sequence was hybridized with a fluorescein labelled fragment (PG13C3) and a dabcyl labelled fragment (PG13C4). The fluorescein and quencher reporters are designed to be in close proximity when the duplex DNA is formed which should lead to quenching of the fluorescence signal. However, upon target binding, certain binding region of the aptamer is expected to undergo a conformational change which may result in a full or partial displacement of one or both complementary sequences (Scheme 1). This can be eventually detected by the increase in the fluorescence intensity due to the increased distance between the fluorescein and dabcyl labels.

Fig. 1A and B shows the fluorescence spectra of the fluorescein-labelled fragments before (black curve) and after (red curve) hybridization with the truncated aptamers as well as after P4 binding (blue curve). As shown in Fig. 1A, when equal concentrations of PG13T1 and its complementary sequences PG13C1 and PG13C2 were hybridized, 70 % of the fluorescence intensity was quenched compared with the free fluorescein-labelled PG13C1. The quenching of the fluorescence confirms the success of the hybridization step which leads to bringing the fluorescein and dabcyl labels in close contact in the duplex sensor probe as we expected. However, after incubating the duplex probe with the P4, no significant change in the fluorescence intensity was observed, indicating that no displacement in both complementary probes has occurred. These results suggest that the PG13T1 fragment may not be involved in the target binding.

On the other hand, as shown in Fig.1B, when PG13T2 and its complementary sequences PG13C3 and PG13C4 were hybridized, the fluorescence intensity was also quenched similar to the case with PG13T1. However, upon target binding, a dramatic increase in the fluorescence intensity was seen indicating that the fluorescein and dabcyl labels were forced apart from each other presumably due to displacement in one or both

complementary fragments. These results confirm that the truncated sequence PG13T2 is involved in the target binding.

3.2. Molecular beacon design

As explained above, it was confirmed that PG13T2 aptamer contains the binding site to P4. Two possible regions can be involved in binding: 1) region 39 to 52 which was hybridized with PG13C3 or 2) region 27 to 48 which was hybridized with PG13C4. In order to gain more insight into this, we designed other truncated sequence by cutting the sequence at 49 from the 5'end to eliminate the second region (27-48). Fortunately, we found that this truncation forms a hairpin structure as shown in the mfold secondary structure prediction (Fig. 2). Therefore, we added few nucleotides at both ends to complete the stem and we modified both ends with fluorecein and quencher to form a molecular beacon (PG13MB). We then tested PG13MB after incubation with P4. No significant change in the fluorescence intensity was detected in this case, indicating that the first region (39-52) was not involved in the target binding and therefore, the stem sequence of the molecular beacon was not opened. In fact, these results are in good agreement with our previously published work that showed better binding affinity when the site 40-50 was blocked by hybridization [15].

Fig. 3 shows the percentage changes in the fluorescence intensity for the three tested truncated sequences (PG13T1, PG13T2 and PG13MB) after binding with P4. Only PG13T2 hybridized with PG13C3 and PG13C4 have shown high fluorescence signal change, concluding the involvement of the region 27 to 48 in the target binding. It is worth mentioning that the aptamer binding domain is not necessarily to be too long. Miaral et al. has used an aptamer with 11 nucleotides which showed specific binding to β-conglutin with three orders of magnitude enhancement in the dissociation constant [24].

3.3. Fluorescence-based displacement progesterone aptasensor

The design which showed the highest fluorescence signal change after P4 binding was then exploited for the detection of P4. A titration with different concentrations of P4 was done and the fluorescence measurements were recorded. As shown in Fig. 4A, a saturation curve was obtained with different P4 concentrations. The dissociation constant of the PG13T2-P4 complex calculated using non-linear regression fitting of the obtained curve was found to be 2.2 nM. This K_d is 16 times less that the value obtained for the non-truncated PG13 aptamer reported in our previous work [15], confirming the enhancement of the binding affinity of the aptamer after truncation.

Fig. 4B represents the calibration curve of the P4 aptasensor (a plot of the % change of the fluorescence intensity before and after binding $((i-i^o)/i^o\%)$ versus the logarithm of the P4 concentration). A linear relationship was obtained in the range from 10 to100 ng/ml. The detection limit of the P4 aptasensor was calculated to be 110 pg/ml based on 3SD/b, where SD is the standard deviation of the aptasensor probe when no analyte was added and b is the slope of the straight line. This detection limit indicates the high sensitivity of the developed assay compared with previously published aptasensor [15] and immunosensors [25-27]. Recently X-aptamers selected against progesterone was used for the development of nanoSPRi-aptosensors. The limit of detection of this sensor was 1.575 ng/ml in PBS buffer [28]. However, our method is 10 times more sensitive (110 pg/ml) than the SPR method. Moreover, in the SPR assay, the detection of the target in the real sample was not demonstrated whereas our method is applicable for the progesterone detection in water and urine. Colorimetric methods based on the aggregation of gold nanoparticle progesterone aptasensors have been reported recently with similar sensitivity [29,30]. These methods depend on the aggregation of gold nanoparticles influenced by the concentration of NaCl and surfactant. However, the real samples usually contain salt and liposomes (surfactants) which limit the sensitivity of the progesterone detection. In 2007, Yuan et al has reported an SPR

based biosensor for the detection of P4. The P4 was conjugated to ovalbumin (OVA) with an oligoethylene glycol (OEG) and form self-assembled monolayer on the sensor surface. This gold nanoparticle linked antibody -based biosensor was able to detect 4.9 pg/ml of P4 [31]. However, this method was applied only in the HBS buffer containing 0.3 M NaCl and not in real samples. Moreover, it is not straight forward for the field applications.

3.4. Selectivity of the progesterone aptasensor

The selectivity of the Pg13T2 duplex aptasensor to P4 was tested against molecules which share similar chemical structures and functions, such as 17-β estradiol, bisphenol-A and vitamin D. As shown in the Fig.5, there is no detected significant increase in the fluorescence intensity with these molecules compared with the high response obtained with the P4, indicating the selectivity of the developed aptasensor for P4.

3.5. Testing the progesterone aptasensor in spiked water and urine samples

In order to check the performance of the PG13T2 sensor in real samples, spiking of P4 in tap water and urine has been done. Table 2 and 3 show good recovery percentages of P4 from tap water and urine without significant interference from other components in the sample. This result suggests the possible applicability of the developed aptasensor assay in real samples.

4. Conclusions

We have developed aptamer-based competitive displacement assay for the detection of progesterone with high affinity and specificity. The dissociation constant of the aptamer-P4 was enhanced by 16 fold after deleting the non-binding region from the original aptamer. The aptasensor assay developed using the truncated sequence PG13T2 has shown high sensitivity with a detection limit of 110 pg/ml without significant cross reactivity with structurally

related compounds such as E2, BPA and Vitamin-D. From these results, we could conclude that the truncation of the aptamer has significantly improved the affinity of the aptamer and consequently the sensitivity of the fluorescence assay. Finally, the aptasensor assay has shown high recovery percentage of progesterone from spiked tap water and urine.

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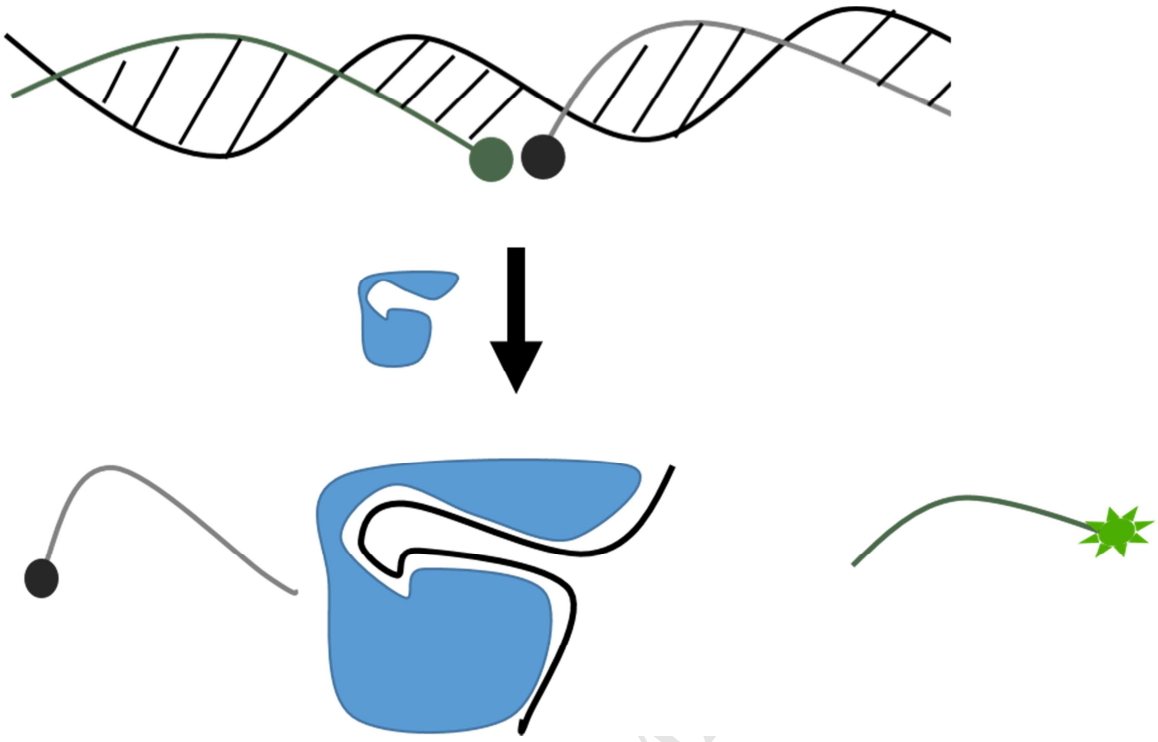
Scheme.1



Scheme.1 : Detection assay design: (A) A tripartite fluorescence-quenching dsDNA duplex assembly composed of fluorescein (green) and Dabcyl (gray) labelled complementary oligonucleotides with unlabelled DNA aptamer (Black). In the presence of target molecule (Blue), the dsDNAduplex undergo a conformation change in the aptamer and the fluorescein labelled DNA and/or quencher labelled DNA were released which and enhances the fluorescence.

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Fig.1

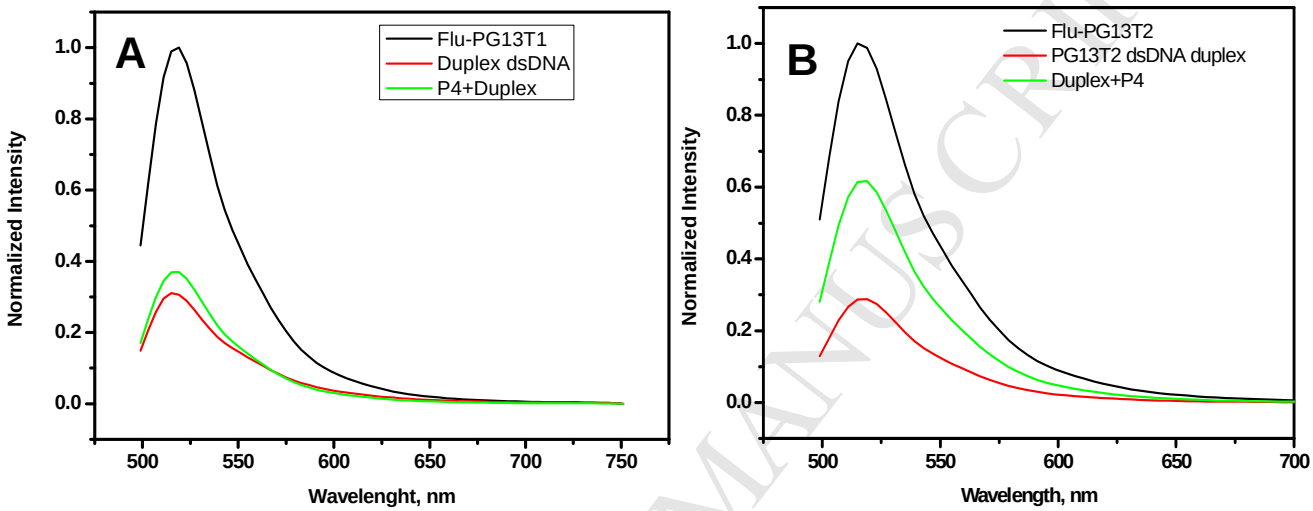

Fig. 1. The fluorescence spectra of the fluorescein and dabcyl labelled cDNA hybridized with PG13T1 (A) and PG13T2 (B). Black curve is the spectra of the fluorecein-labelled complementary fragments, red curve: after the duplex formation and the blue curve is after P4 binding.

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Fig. 2

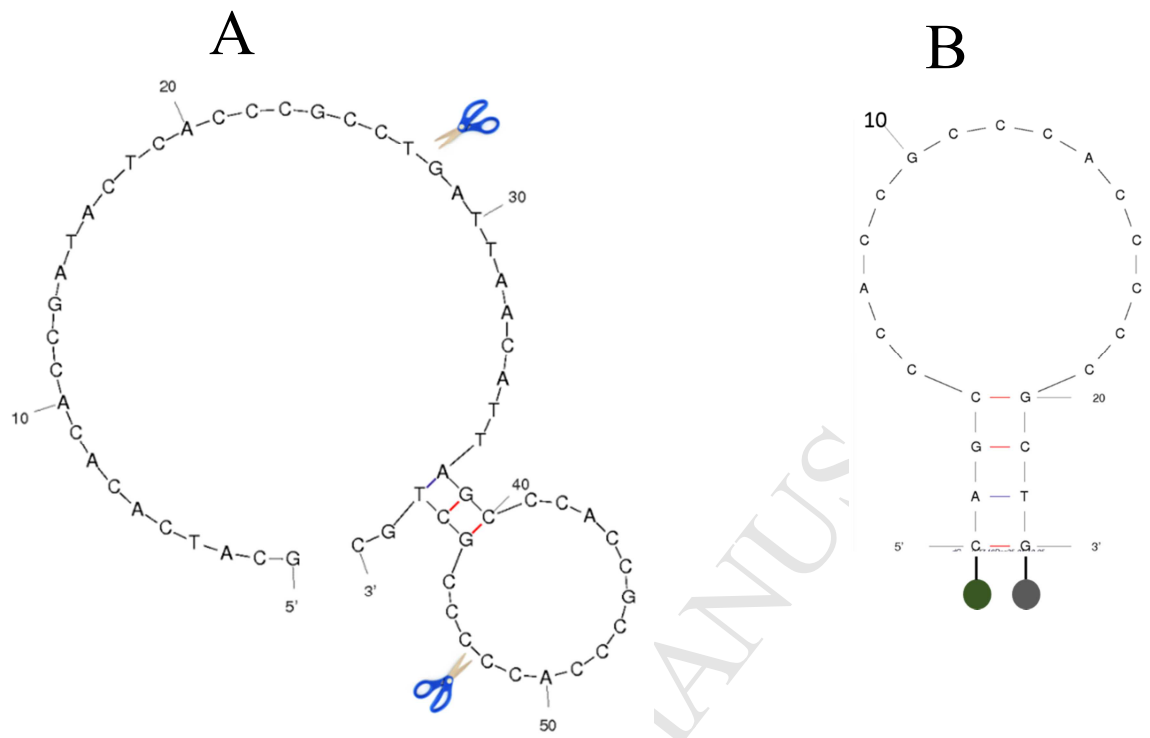

Fig. 2 . The secondary structure of original PG13 aptamer obtained form mfold software program. (A)The nucleotide 27-52 region used for the displacement studies (B) The nucleotides from 37 to 59 in the PG13 were truncated which, form stem-loop structured molecular beacon. At the 37th nucleotide, the T was replaced by G to make stable beacon. The 5'and 3' ends of the stem was labelled with fluorescein (green) and dabcyl (gray)respectively.

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Fig. 3

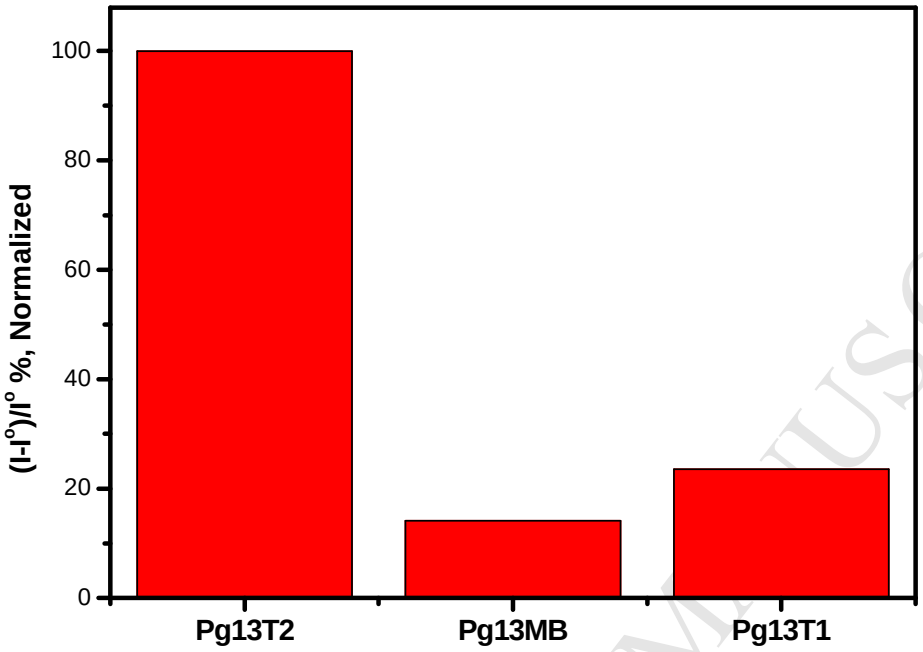

Fig.3. the relative change in the fluorescence intensity of truncated aptamers obtained from various regions of the original aptamer. Pg13T2 shows the largest increase in the fluorescence intensity in the presence of 100 nM P4. No significant change in the fluorescence intensity of Pg13T1 and PG13MB in the presence of P4 was observed.

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Fig. 4

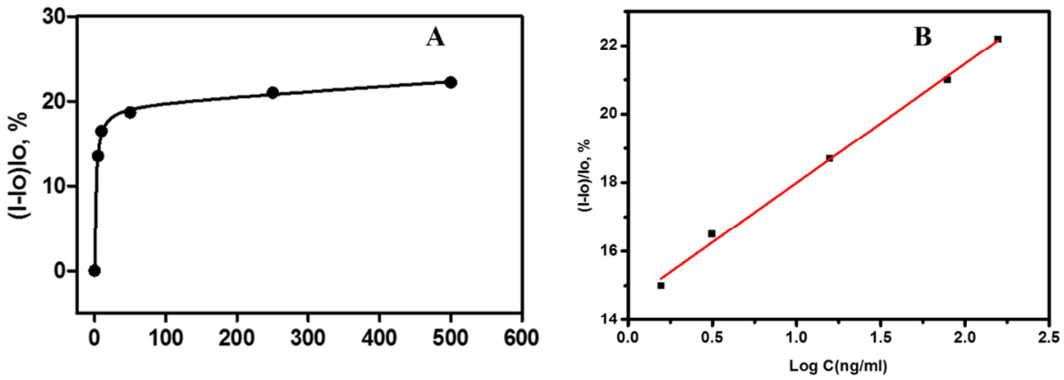

Fig 4. (A) The binding affinity curve of PG13T2 truncated dsDNA aptamer duplexed upon progesterone binding. (B) The calibration curve plotted as the change in the fluorescence intensity versus logarithm of P4 concentration.

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Fig. 5

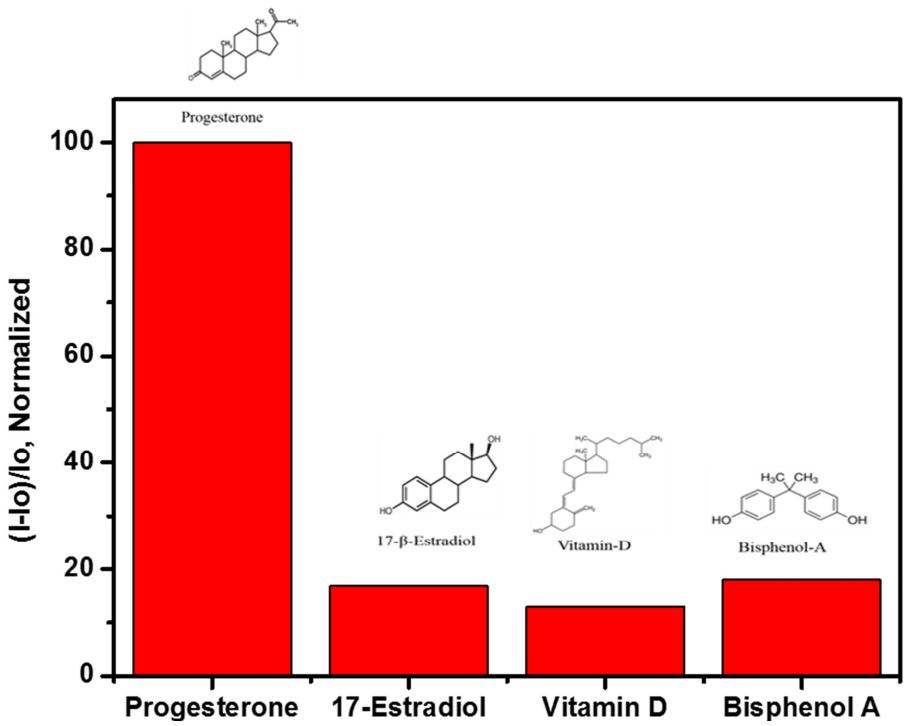

Fig.5 The cross reactivity of the progesterone aptamer against 17-bestradiol, vitamin-D, Bisphenol A

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Table 1 Original and truncated aptamers sequences

Name	Sequences
Pg13	GCATCACACACCGATACTCACCCGCCTGATTAACATTAGCCCACCGCCCACCCCCGCTGC
PG13T1	GCATCACACACCGATACTCACCCGCCTGAT
PG13C1	Flu-5'-CGGTGTGTGATGC
PG13C2	GGCGGGTGAGTAT-3'-Dabcyl
PG13T2	GATTAACATTAGCCCACCGCCCACC
PG13C3	Flu-5'-CTAATGTTAATC
PG13C4\	TGGGCGGTGGG-3'-Dadcyl
PG13MB	Flu-5"CAGCCCACCGCCCACCCCCGCTG-3"-Dabcyl

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Table .2 Application of PG13T2 in detection of P4 from tap water

Spiked P4 (nM)	Found Concentration (nM)	Recovery (%)	SD
100	95.2	95.2	12.03
250	221.5	88.6	10.19
400	357.43	89.35	9.35

Table .3 Application of PG13T2 in detection of P4 from artificial urine samples

Spiked P4 (nM)	Found Concentration (nM)	Recovery (%)	SD
100	97.3	97.3	8.23
250	233.2	93.28	10.31
400	348.69	87.17	11.61

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