Applied
NANOMEDICINE

2023
3(1), 554

Evaluation of sensing ability of aptamers towards β-casomorphin-7 peptide (BCM-7) in different assay formats

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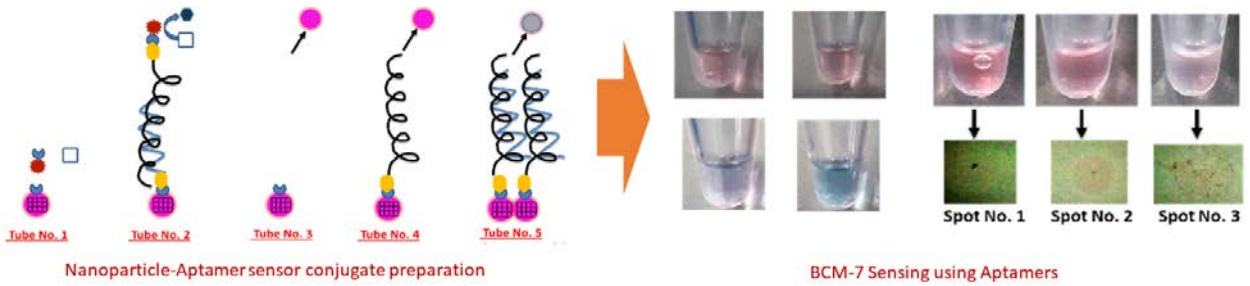
Submitted on: 25-Nov-2022, Accepted and Published on: 21-Dec-2022

Article

ABSTRACT



Aptamers are useful ligand molecules for the recognition of target molecules. Aptamers, as well as target molecules, are modified for their use in developing assay protocols for targets. Assay format uses gold nanoparticles or magnetic beads or 96-well plate or membrane surface for interaction between aptamer and target molecules. The present investigation is aimed to evaluate the recognition potential of modified aptamers towards β-casomorphin-7 (BCM-7) peptide. Aptamers (Seq.7 and Seq.U5) selected in previous work for BCM-7 as well as their variable regions and selected truncated segments of variable regions were evaluated for recognition of BCM-7. Aptamers or biotinylated aptamers or amino group attached to aptamer and BCM-7 or biotinylated BCM-7 or BCM-7-peroxidase were used in different assay geometry using streptavidin-coated gold nanoparticle or streptavidin-coated magnetic beads or streptavidin-coated 96-well plate or nitrocellulose & nylon membranes. In different assay formats, biotinylated full-length aptamers (Seq.7 and Seq.U5) as well as biotinylated variable region of aptamer Seq.U5 (Seq.U5v) recognized BCM-7. However, truncated aptamers from Seq.U5v failed to recognize BCM-7. Assay formats using aptamer-BCM-7 interaction can be adapted for studying aptamer-target recognition. Seq.7 and Seq.U5 are promising aptamers for developing protocols in different assay geometry for measuring BCM-7 in the urine sample.

Keywords: Aptamer, β-casomorphin-7, truncated aptamer, gold nanoparticle, magnetic bead

INTRODUCTION

Casein is a major protein in milk and accounts for 80% of total protein. Casein itself is not a single protein but comprises of three subtypes referred to as α_S-casein, β-casein, and κ-casein. There are two genetic variants of β-casein, namely β-casein A1 and β-casein A2. These two proteins are 209 amino acids long but differ from each other at single amino acid at the 67th position. At this position, A1 has histidine while proline is present in the A2 protein.[1,2] The presence of histidine in β-casein A1 on digestion produces seven amino acid long peptide, referred to as β-casomorphin-7 (BCM-7).[3-6] It is believed that BCM-7 has some role in aggravating autism, schizophrenia, and other disorders.[7-9] A high concentration of BCM-7 has been reported in autistic patients' urine and serum samples.[10-13] Another report suggests that diabetes mellitus type 1 (DM1) and heart-related issues are associated with A1 milk consumption.[14] Human trial in China has also indicated A1 milk association with physiological discomfort.[15] The harmful effect of A1 milk is still not

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URN:NBN:sciencein.nanomed.2023.v3.554

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conclusively proved and more human clinical studies covering a large population are needed.[16] Methods for the detection of BCM-7 in digested milk, blood, and urine are developed and these methods involve ELISA,[13] MS[12,17] and HPLC techniques.[12,17] MS and HPLC are expensive equipment where ELISA method depends on specific antibodies. Alternate methods for the measurement of BCM-7 in low amounts in urine samples of autistic patients are required. Ligands against target molecules are very useful for developing methods for the detection and estimation of the analyte. Aptamers have emerged as a new class of ligand molecules.

Aptamers are single-strand (ss) DNA, RNA or peptide molecules that have an affinity toward targeted analytes. They are usually less than 80 nucleotides long and selected from a random aptamer library through a repetitive process referred as 'selection of ligand by exponential enrichment' (SELEX). Each aptamer in the library comprises the constant region (usually 18 nucleotides long) at both terminal ends and the variable region (usually 36 nucleotides long) in the middle. The variable region contributes approximately 50% of total nucleotides. The randomness in nucleotide sequence allows the selection of aptamers from trillion of random sequences present in aptamer library. Tracer moieties such as biotin, peroxidase, and linkers comprising of a few base or methylene residues are added at terminal ends of aptamer for their use in assay protocols for analytes. The modification of aptamer may affect its binding ability for the target molecule. Thus prior to their use in any assay format, it is desirable to evaluate the retention of binding ability of aptamers. Here, we have reported recognition of β-casomorphin-7 by modified BCM-7 specific aptamers named Seq.7 and Seq.U5 that were identified in an earlier study[18]. Different assay formats involving aptamer-target and avidin-biotin interactions on the surface of streptavidin-coated gold nanoparticle (ST-GNP), streptavidin-coated magnetic beads (ST-magnetic beads), streptavidin-coated 96 well plate (ST-96 wells), nitrocellulose membrane (NC membrane), and nylon membrane were performed. Further, recognition of BCM-7 by aptamer has been assessed in terms of either horseradish peroxidase activity or stability of gold nanoparticles (GNP). Developed protocols can be adapted for evaluating any aptamer-target recognition.

MATERIALS AND METHODS

Biotin-labelled aptamers 'Seq.7' and 'Seq.U5' comprising of 18 nucleotides long constant region at terminal ends and 36 nucleotides long middle variable region identified in the earlier study[18], biotin-labelled variable region of Seq.U5 (Seq. U5v), truncated variable region from Seq. U5v (Seq.U5$_{VT1}$, Seq.U5$_{VT2}$, Seq.U5$_{VT3}$, Seq.U5$_{VT4}$, Seq.U5$_{VT5}$ & Seq.U5$_{VT6}$) and 5′amino group tagged Seq.U5 (aptamers sequences are given in Table-1) were purchased from Integrated DNA Technologies (IDT), Belgium. Dynabeads MyOne Streptavidin T1 magnetic beads (ST-beads, code-65601) were obtained from Invitrogen, USA. BCM-7 (YPFPGPI) peptide, BCM-7-cysteine conjugated to horseradish peroxidase (HRP-CYPFPGPI or BCM-7-HRP) and biotin linked at the N-terminal end of BCM-7 (biotin-BCM-7) were purchased from Biolinkk India Pvt. Ltd., India. Streptavidin immobilized ELISA plate (ST-96 wells plate, code-786-745) was procured from G-biosciences, USA. Streptavidin conjugated gold nanoparticle (ST-GNP, code-AC-40-04-05) was procured from Cytodiagnostics, Canada. Tetramethylene benzidine (TMB, code-T0440 & T0565) and positively charged Nylon 66 membrane (code-11209272001) were obtained from Sigma-Aldrich, Germany. 3,3′-diaminobenzidine (DAB, code-SFE51090) and streptavidin-conjugated horseradish peroxidase enzyme (ST-HRP, code-N100) were purchased from GeNei, India, and Thermo Fisher, USA respectively. Nitrocellulose membrane (code- SCNJ8102XXXX101) was purchased from Advanced Microdevices, India. Other chemicals such as streptavidin, BSA, and PBS were purchased from Hi-Media, India.

Recognition of target by aptamer was assessed on the surface of ST-GNP, ST-magnetic beads, ST-96 well plate, nitrocellulose (NC), and nylon membranes. The target molecule was either BCM-7 or biotin-conjugated to BCM-7 (biotin-BCM-7) or horseradish peroxidase-conjugated to BCM-7 (BCM-7-HRP) while aptamers were as listed in Table-1 or biotin-conjugated aptamers or amino conjugated aptamer. All incubations were carried out at room temperature.

Assessment of recognition of biotin-BCM-7 by biotin-Seq.7 or biotin-Seq.U5 on the surface of ST-GNP

Recognition of biotin-BCM-7 by biotin-Seq.7 or biotin-Seq.U5 on the surface of ST-GNP was studied in 1.5 ml microcentrifuge polypropylene tubes (Figure 1). Stock ST-GNPs

Table-1: Nomenclature and sequence of aptamers, their variable regions, and truncated variable regions used in the detection of BCM-7

Aptamer name	(Sequence written in 5'-3'direction) (Constant region)	(Random region)	(Constant region)
Seq.7	ATACGGGAGCCAACACCA	**CAAGTTGACTTGGAGGAGGGTCTAACTAGTTCGAGG**	AGAGCAGGTGTGACGGAT
Seq.7$_C$	ATCCGTCACACCTGCTCT	**CCTCGAACTAGTTAGACCCTCCTCCAAGTCAACTTG**	TGGTGTTGGCTCCCGTAT
Seq.U5	ATCCGTCACACCTGCTCT	**ATACACATTGTGTTTACTCCCAGTTTTTTAGACTTA**	TGGTGTTGGCTCCCGTAT
Seq.U5$_V$		**ATACACATTGTGTTTACTCCCAGTTTTTTAGACTTA**	
Seq.U5$_{VT1}$		**ATACACATTGTGTTTACT**	
Seq.U5$_{VT2}$		**CCCAGTTTTTTAGACTTA**	
Seq.U5$_{VT3}$		**ATACACATT**	
Seq.U5$_{VT4}$		**GTGTTTACT**	
Seq.U5$_{VT5}$		**CCCAGTTTT**	
Seq.U5$_{VT6}$		**TTAGACTTA**	

- Seq.7$_C$, complementary sequence to Seq.7
- Seq.U5$_V$, variable region of aptamer Seq.U5
- Seq.U5$_{VT1-6}$, truncated sequences from Seq.U5v
- The variable region is written in bold (red color) alphabets while other alphabets indicate constant regions of aptamer

suspension (0.15 mg/ml) was seven times diluted with PBS and 30 µl of diluted ST-GNPs were distributed to each of the five polypropylene tubes numbered as tube No.1, tube No.2, tube No.3, tube No.4, and tube No.5. Tube No.1 and tube No. 2 were used for studying the recognition of biotin-BCM-7 by biotin-Seq.U5 while tube No.3, tube No.4, and tube No.5 were marked for recognition by biotin-Seq. 7.

After the addition of 30 µl of diluted ST-GNPs to tube No.1 and tube No. 2, 10 µl of 1 µg/ml of biotin-BCM-7 was added to tube No.2. After 1h incubation, 25 µl of 1 mg/ml of biotin was added to tube No.1 and tube No.2 for blocking the remaining sites on ST-GNPs. Excess biotin was removed by centrifugation (12000g, 5 min). Tubes were further treated with 50 µl of 2% BSA for 2h for blocking protein binding sites. After centrifugation, 10 µl of 1.0 µM of biotin-Seq. U5 was added to tube No.2. and contents were further incubated for 1h. Unbound biotin-Seq. U5 was removed by washing ST-GNPs with PBS three times. Then, 50 µl ST-HRP (1/16000 times diluted in PBS) was added and the contents were incubated for 30 min. Subsequently, tubes were washed four times with PBS to remove unbound ST-peroxidase. Then, 10 µl of TMB (code-T0440) mixed in 40 µl of PBS were added. After 20 min, tubes were photographed.

For studying recognition of biotin-BCM-7 by biotin-Seq.7, 10 µl of 1.0 µM of biotin-Seq.7 were added in tube No. 4 and tube No. 5. After 1h incubation, ST-GNPs were washed with PBS and then resuspended in 30 µl PBS. Subsequently, ST-GNPs in tube No.3, tube No.4, and tube No.5 were blocked similar to the method described for tube No. 1 and tube No. 2. Also, 10 µl of 1.0 µg/ml of BCM-7 were added to tube No. 5. After 1h, ST-GNPs were washed and resuspended in 50 µl PBS. Tubes containing ST-GNPs were photographed after 5.0 min. These ST-GNPs were further evaluated for their affinity towards Seq.7c and for this purpose, nitrocellulose membrane (NC; pore size 0.45 µm) was spotted with 10 µl of 1.0 mg/ ml of streptavidin at two spots (Spot No. 2 and Spot No. 3). 10 µl of 1.0 µM of biotin-Seq.7_C was later added on these two spots. Spot No.1, marked as control where streptavidin and biotin- Seq.7_C were not spotted. After 1 h of incubation with biotin- Seq.7_C, NC was blocked by 5% of BSA (overnight, 4°C). Subsequently, 10 µl ST-GNPs suspension from tube No. 3, tube No.4, and tube No. 5 were layered over Spot No. 1, Spot No. 2, and Spot No. 3 respectively on the NC membrane. After 45 min, the membrane was washed with PBS to remove unbound ST-GNP and immediately photographed.

Assessment of recognition of target by aptamer on the surface of ST- magnetic beads

Recognition of biotin-BCM-7 by biotin-Seq.7 or biotin-Seq.U5:

ST- magnetic beads (5.0 µl) were added to each of the three 1.5 ml microcentrifuge tubes (tube No. 1, tube No. 2, and tube No. 3),(Figure2). Beads were washed with binding buffer (binding buffer composition- 5.0 mM Tris-HCl, 0.5 mM EDTA, and 1.0 M NaCl, pH- 7.5). Subsequently, 200 µl of 1.0 µM biotin-Seq.U5 and biotin-Seq.7 (prepared in binding buffer) were added to tube No. 2 and tube No.3 respectively. After 45 mins of incubation, tubes containing ST-magnetic beads were blocked by employing the method described for blocking of ST-GNP earlier. Then, the beads were washed with PBS. Subsequently, a 100 µl human urine sample spiked with 50 ng/ml biotin-BCM-7 was added to tube No. 2 and tube No.3 while an un-spiked urine sample was added to tube No. 1. After further 45 mins. of incubation, beads present in three tubes were washed with PBS. These tubes were further treated with ST-HRP and TMB similar to the method described for ST-GNP earlier. Developed color in tubes was photographed.

Recognition of BCM-7-HRP and Biotin-BCM-7 by Biotin-Seq.$U5_V$:

In each of the four microcentrifuge tubes numbered as tube No.1, tube No.2, tube No.3, and No.4, 5.0 µl of washed ST-beads (refer to section 2.2.1) were added (Figure 3). Then, 200 µl of 1.0 µM biotin-Seq.U5v (suspended in binding buffer) was added to tube No. 2 and tube No.4. After 45 min., ST-magnetic beads were blocked as per the method described for blocking ST-GNP (refer to section 2.1). After washing of beads with PBS, 100 µl urine sample spiked with 50 ng/ml BCM-7-HRP was added to tube No. 1 and tube No. 2, while biotin-BCM-7 at the same concentration was added to tube No. 3 and tube No. 4. After 45 min of incubation, beads were washed with PBS. Beads in tube No. 3 and tube No. 4 were further incubated with ST-peroxidase as per the method described in section 1. After washing, TMB substrate was added in all tubes for color development and was photographed after 20 min incubation.

Assessment of competitive binding of BCM-7-HRP and BCM-7 for biotin-Seq.$U5_V$:

In each of three microcentrifuge tubes numbered tube No. 1, tube No.2, and tube No.3 (Figure4), 5.0 µl ST-beads were added and washed as described in section 2.2.1. Two hundred microlitre of 1.0 µM biotin-Seq.U5v (prepared in binding buffer) was added to tube No. 2 and tube No.3. ST-beads were then blocked as per the method described in section 1 and then washed with PBS. Further, 100 µl of urine spiked with 50 ng/ml BCM-7-HRP were added to tube No.1 & tube No.3 while in tube No. 2, 50 ng/ml each of BCM-7-HRP and BCM-7 spiked in urine was poured. After incubation for 45 min. beads were washed with PBS. Then, TMB substrate was added (refer to section 2.1) and tubes were photographed immediately and after 15 min incubation.

Assessment of recognition of target by aptamer on the surface of ST-96 wells

Recognition of biotin-BCM-7 by biotin-Seq.7 or biotin-Seq.U5:

Recognition of biotin-BCM-7 by biotin-Seq.7 or biotin-Seq.U5 was studied in ST-96 wells plate. 100 µl of biotin-BCM-7 (1.0 µg/ml) diluted with 150 µl of PBS was added to each of six wells numbered as well No.1, well No.2, well No.3, well No.4, well No.5, and well No.6 (Figure 5). After 2h incubation at room temperature, wells were washed with PBS and then blocked by the addition of 250 µl of biotin (1 mg/ml) in each well. After 45 min., wells were washed with PBS and then were further blocked which required the addition of 250 µl of 2% BSA (prepared in PBS) and incubation for 4 h at room temperature. Wells were then washed with PBS. Well No.1, well No.2, and

well No.3 were used for studying the recognition of biotin-BCM-7 by biotin-Seq.U5 while wells numbered as well No.4, well No.5, and well No.6 were used for biotin-Seq.7. Accordingly, 20 µl (1.0 µM) of Biotin-Seq.U5 was added in well No.1, well No.2, well No.3, while biotin-Seq. 7 (20 µl, 1.0 µM) was added in well No.4, well No.5, and well No.6. Further, 80 µl water was added to well No. 1 and well No.4; 80 µl urine spiked with BCM-7 (600 ng/ml) was added to well No. 3 and No. 6 and 80 µl urine un-spiked urine was added to well No. 2 and well No. 5. After 1h incubation, wells were washed with PBS. Treatment of these wells with ST-HRP and color development with TMB were carried out using the method described earlier. Developed color in wells was photographed after 15 min incubation.

Recognition of BCM-7-HRP by truncated sequences from aptamer 'Seq.$U5_V$':

In different wells of the ST-96-well plate, 250 µl of 1.0 µM of each of the biotin labelled Seq.$U5_V$, aptamer and its truncated sequences viz. Seq.$U5_{VT1}$, Seq.$U5_{VT2}$, Seq.$U5_{VT3}$, Seq.$U5_{VT4}$, Seq.$U5_{VT5}$, and Seq.$U5_{VT6}$ (prepared in PBS) were added. After 2 h incubation, wells were blocked as per the method described in section 2.3.1. After washing of wells with PBS, 100 µl of urine spiked with 50 ng/ml BCM-7-HRP was added to each well. After 45 min, wells were washed with PBS and then TMB was added as described in section 2.3.1.

Assessment of recognition of target by aptamer on the surface of NC membrane

Recognition of BCM-7-HRP & biotin-BCM-7 by biotin-Seq.7 or biotin-Seq.U5:

Streptavidin (1.0 µl, 1.0 mg/ml) was spotted at Spots No. 1, 2, 3, 4, 5 and 6 on nitrocellulose membrane (Figure 6). After drying of spots, 10 µl of 1.0 µM of biotin-Seq. U5 was layered over Spots No. 1, 2, and 3 while biotin-Seq.7 was placed over Spots No. 4, 5, and 6. After 45 min, the membrane was blocked by its immersion in 5% BSA overnight at 4°C and then washed with PBS. All subsequent steps for membrane treatment and color development were carried out under diminished light. Then,10 µl of urine spiked with 1.0 µl of BCM-7-HRP (1.0 µg/ml) was layered over Spots No. 1 and 4 while water spiked with the same concentration of BCM-7-HRP was placed over Spots No. 2 and 5. At Spots No. 3 and 6, besides urine spiked with BCM-7-HRP, BCM-7 (1.0 µl of 1.0 µg/ml) was additionally layered. After 45 min, the membrane was washed with PBS. Then, 5.0 µl, DAB solution (prepared as per manufacture's protocol) was placed over each spot. After 15 min., the membrane was photographed.

Effect of different concentrations of BCM-7-HRP on interaction with biotin-Seq.U5:

Effect of different concentration of BCM-7-HRP (0.25 µg/ml, 0.50 µg/ml, 1.0 µg/ml & 1.5 µg/ml) on interaction between biotin-Seq.U5 and BCM-7-HRP on blot intensity was similarly studied on NC membrane as described in section 2.4.1 (Figure 7).

Recognition of BCM-7-HRP by biotin-Seq.U5 or amino-Seq.U5:

Recognition of BCM-7-HRP by amino-Seq.U5 or biotin-Seq.U5 was studied on a separate piece of NC membranes (Figure 8). Spot No.1 was kept untreated and marked as a control. 10 µl of 1.0 µM of amino-Seq. U5 was layered at Spot No.2. Then, membranes having Spot No.1 and Spot No.2 were exposed to 254 nm UV radiation for 10 min.

On the second membrane, streptavidin (1.0 µl, 1.0 mg/ml) was spotted at Spot No. 3 and Spot No. 4. Spot No.3 was marked as a control. Subsequently, 10 µl of 1.0 µM biotin-Seq.U5 was layered over Spot No. 4.

Membranes having spots were then blocked and washed with PBS using the method described earlier. Then, 10 µl of urine sample spiked with 1.0 µl of 1.0 µg/ml of BCM-7-HRP was layered on all spots. After 45 min incubation, membranes were washed with PBS followed by the addition of DAB on these spots using the method described earlier.

Recognition of biotin-BCM-7 by amino-Seq.U5v in a competitive manner:

Competitive binding of BCM-7 and biotin-BCM-7 for amino-Seq.U5v was assessed on NC membrane whereon 5 µl of 1.0 µM of amino-Seq.U5v was spotted on Spots No. 2 and 3 (Figure 9). Then, the membrane was exposed to UV light, blocked, and washed using the method described earlier. At Spot No.1 and Spot No.3, 10.0 µl of urine spiked with 1.0 µl of 2.0 µg/ml of biotin-BCM-7 (prepared in PBS) was loaded while at Spot No. 2, 10.0 µl of urine sample was spiked with 1.0 µl of 2.0 µg/ml of each of BCM-7 & biotin-BCM-7 was placed. After 45 min. of incubation, the membrane was washed with PBS. Further, 10 µl ST-GNP (0.15 mg/ml) diluted with 40 µl of PBS were placed over each spot. After 20 min, the membrane was quickly washed three times with PBS for removing unbound GNPs, and then bound GNPs were photographed.

Assessment of recognition of BCM-7-HRP by amino-Seq.U5v on the surface of Nylon membrane

Recognition of BCM-7-HRP by amino-Seq.$U5_V$ was assessed on a nylon membrane (Figure 10). 5.0 µl of 1.0 µM of amino-Seq.U5v was spotted on Spot No. 2, and Spot No. 3 of the membrane. For negative control (Spot No.1), amino-Seq.U5v was not loaded. The nylon membrane was exposed to UV light (254 nm) for 3.0 min for fixing of the aptamer. Then, the membrane was blocked by immersing in 5% SDS for 12 h at RT. After washing the membrane with PBS, 10.0 µl of urine sample spiked with 1.0 µl of 2.0 µg/ml of BCM-7-HRP were layered over Spot No. 1 and Spot No.3 while at Spot No. 2, 10.0 µl of urine spiked with 1.0 µl of 2.0 µg/ml of each of BCM-7 & BCM-7-HRP was placed. After 45 min. of incubation, the membrane was washed with PBS. Then, 5.0 µl of TMB (code T0565) was spotted at all spots under diminished light. After color formation, blots were photographed.

Ethical clearance for the use of human urine

The first author provided his urine sample in a voluntary manner and informed consent was given to the institutional ethical committee of the National Dairy Research Institute.

RESULT

Ligand binds to the target molecule and the binding can be carried out on several surfaces including GNP, magnetic bead, 96-well plate, membrane, electrode, and quartz. Binding of ligand or target to these surfaces as well as for studying the interaction between ligand and target invariably require chemical

modification. We have evaluated whether modified aptamer and target molecules still retain affinity for each other and for this purpose aptamer selected against BCM-7 in earlier work were used. Results obtained on the surface of GNP, magnetic beads, 96-well plate, and membranes are delineated below.

Recognition on the surface of streptavidin-coated gold nanoparticle:

Interaction between streptavidin and biotin is strong and is also quickly established. Experiments were performed in microcentrifuge tubes containing ST-GNP. Either biotin-Seq.7 (Figure 1, tubes No. 4 and 5) or biotin-BCM-7 (Figure 1, tube No. 2) was allowed to interact first with streptavidin-coated on the surface of gold nanoparticles. Interaction between biotin-Seq.7 and BCM-7 (tube No. 3, 4, and 5) was evaluated in terms of stability of GNP. ST-GNP (tube No. 3), as well as ST-GNP, coated with biotin-Seq.7 (tube No.4) remain red in colour which is indicative of the stable nature of gold nanoparticles under incubation condition. When BCM-7 was added to ST-GNP coated with biotin-Seq.7 (tube No.5), such gold nanoparticles

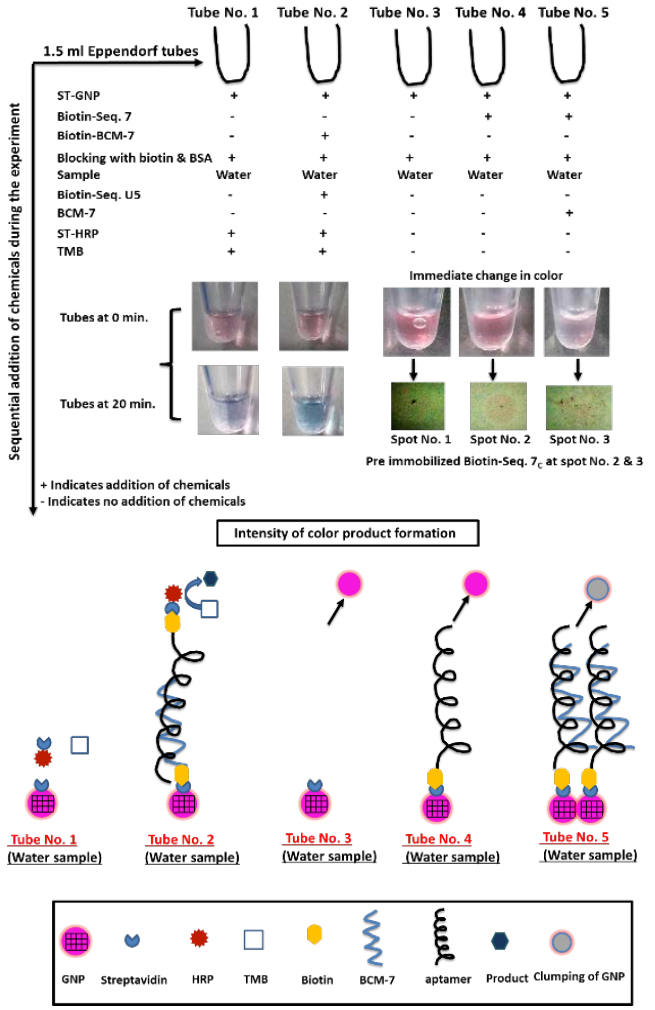

Figure 1. Evaluation of recognition of biotin-BCM-7 by biotin-Seq.7 or biotin-Seq.U5 on the surface of streptavidin-coated gold nanoparticle. Biotin-BCM-7 and biotin-Seq.7 was immobilized on ST-GNP in tube No. 2 and tube No. 4 & 5 respectively. Biotin-Seq.U5 was added in tube No.2 while BCM-7 was added to tube No. 5. ST-peroxidase was further added to tube No. 1 & tube No. 2 and color was developed after the addition of TMB. Color change in tube No.5 refers to the instability of gold nanoparticles on the binding of BCM-7 to Seq.7; Spot No.1, Spot No.2, and Spot No.3 were generated on the transfer of an aliquot from tube No.4, tube No.5, and tube No.6 respectively on nitrocellulose membrane pre-treated with (Spot No.2 and Spot No.3) or without (Spot No.1) sequence 'Seq.7C'. (a). Sequential addition of different chemicals and color change in tubes. (b). Diagrammatic representation of interaction in each tube.

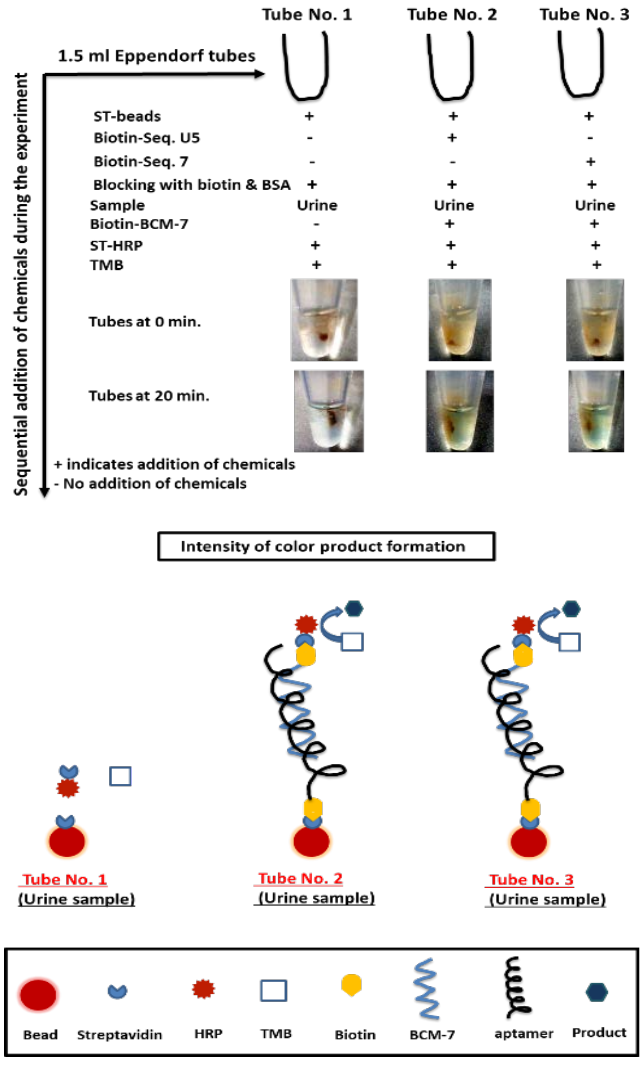

Figure 2. Evaluation of recognition of biotin-BCM-7 by biotin-Seq.7 or biotin-Seq.U5 on the surface of streptavidin-coated magnetic beads. Biotin-Seq.U5 and biotin-Seq.7 were immobilized on ST-beads in tube No.2 and tube No. 3 respectively and then biotin-BCM-7, spiked in urine was added. ST-peroxidase was further added in all tubes and color was developed after the addition of TMB. (a). Sequential addition of different chemicals and color change in tubes. (b). Diagrammatic representation of interaction in each tube.

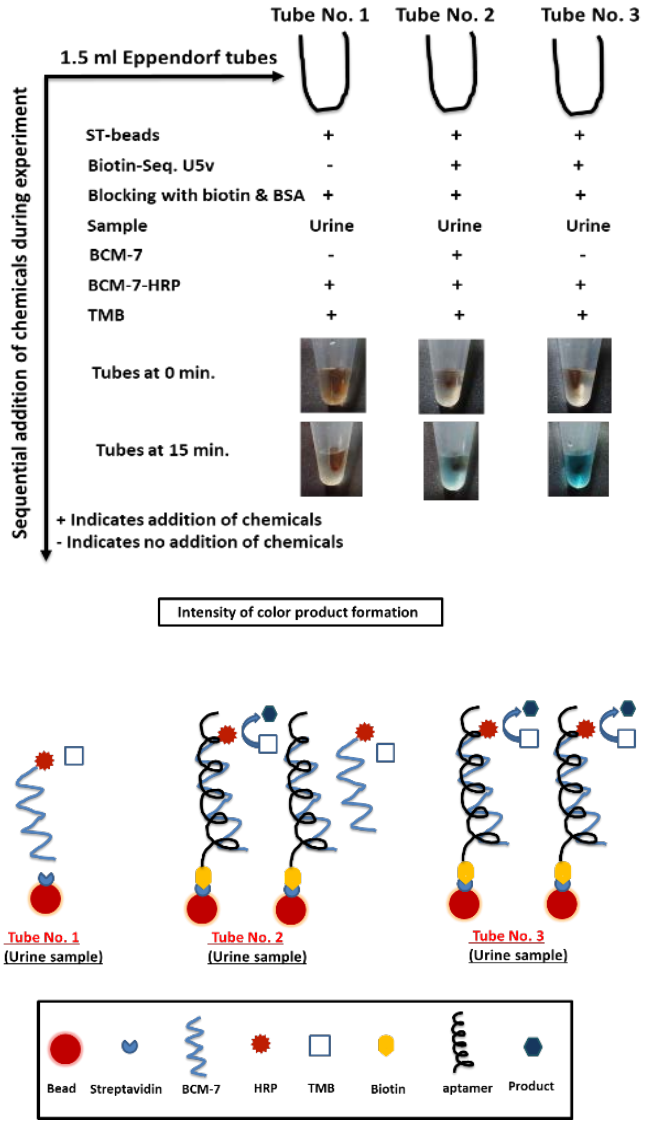

Figure 3. Evaluation of recognition of BCM-7-HRP & biotin-BCM-7 by biotin-Seq.U5$_V$ on the surface of streptavidin-coated magnetic beads Biotin-Seq.U5$_V$ was immobilized on ST-beads in tube No. 2 and tube No. 4. BCM-7-HRP, spiked in urine and was added in tube No. 1 and tube No. 2 while biotin-BCM-7 (spiked in urine) was added in tube No. 3 and tube No. 4. Also, ST-peroxidase was added to tube No. 3 and tube No. 4. Later, in all tubes, TMB was added for color development. (a). Sequential addition of different chemicals and color change in tubes. (b). Diagrammatic representation of interaction in each tube.

lose stability and result in colour change from red to purple (tube No.5). This shows that BCM-7 interacted with aptamer Seq.7.

Lower stability of aptamer coated gold nanoparticles in presence of target molecules has been observed by other workers [18, 20, 22]. When contents of tubes No. 4, 5, and 6 were placed over nitrocellulose membrane at indicated spot numbers pre-immobilized with (spot No. 2 and 3) or without (spot No. 1) biotin-conjugated with complementary sequence to Seq.7 or 'Seq. 7c', contents from tube No. 4 (see spot No. 2) in comparison to tube No. 5 (see spot No. 3) firmly adhered to the membrane. This observation is on the expected line and indicated Seq.7 complex with BCM-7 binds poorly to its complementary sequence.

A slightly different approach was used for assessing the interaction between biotin-BCM-7 and biotin-Seq. U5 (tubes No. 1 and 2). Bound biotin-Seq.U5 was evaluated in terms of peroxidase activity which required an additional step of incubation with the streptavidin-peroxidase conjugate. The relatively more intense blue colour as indicative of peroxidase activity in tube No.2 in comparison to the control tube (tube No.1) indicates interaction between biotin-BCM-7 and biotin-Seq.U5.

Recognition on the surface of streptavidin-coated magnetic beads:

Magnetic beads can be easily separated under a magnetic field and this property enables its preferred use in protocols requiring the separation of micro materials from the liquid phase. Ligands can be attached to magnetic beads using different chemistries. Recognition of biotin-BCM-7 by biotin-Seq.7 or biotin-Seq.U5 on the surface of streptavidin-coated magnetic beads was evaluated and this interaction is reflected in terms of peroxidase activity (Figure 2). Relatively more intense blue colour in tube No. 2 and tube No. 3 in comparison to tube No. 1 suggests that aptamer Seq.7 as well as aptamer Seq.U5 interacted with BCM-7.

A similar protocol was also used for the evaluation of the interaction between Seq.U5$_V$ (variable region of full-length sequence U5) and BCM-7-peroxidase or biotin-BCM-7. Both BCM-7-peroxidase (Figure 3, tube No. 2) and biotin-BCM-7 (Figure3, tube No. 4) interacted with aptamer Seq.U5$_V$. The blue color in tube No. 2 is far more intense in comparison to tube No.1(negative control) and this indicates that BCM-7-peroxidase interacts well enough as visualized from the color intensity. The use of BCM-7-peroxidase can be preferred over biotin-BCM-7 as it avoids additional step of incubation of beads with biotin-HRP (Figure 3).

Competition between BCM-7 and BCM-7-HRP for binding to biotin-Seq.U5$_V$ was also evaluated on ST-beads. The blue colour intensity in presence of BCM-7 (Figure 4, tube No. 2) was lower in comparison to in its absence (Figure 4, tube No. 3). This implies that BCM-7 competed with BCM-7-HRP for binding to biotin-Seq.U5$_V$ and the design can be used for measuring BCM-7 in the urine sample.

Recognition on the surface of streptavidin-coated 96 well plate

Ninety-six well plate enables simultaneous analysis of many samples and analysis of samples using low volumes of expensive reagents. Streptavidin-coated 96-well plates are commercially available for binding of biotinylated ligand molecules. Two aptamers Seq.U5$_V$ and Seq.7 were evaluated for their binding to BCM-7 and biotin-BCM-7 in 96 well formats (Figure 5). Since, BCM-7 is to be measured in urine sample, effect of urine *vis a vis* water on interaction was also assessed. There was no apparent adverse effect of urine sample in recognition of biotin-BCM-7 by either Seq.U5$_V$ (see well No.1 and No.2) or Seq.7 (see well No.4

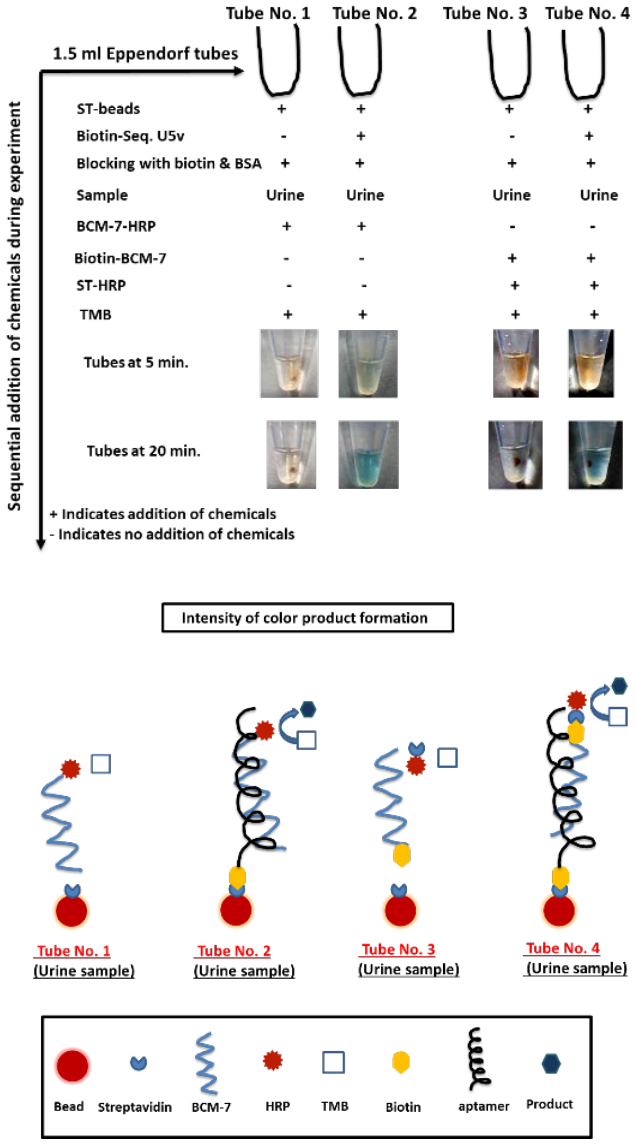

Figure 4. Evaluation of competitive binding of BCM-7-HRP and BCM-7 for biotin-Seq.U5v on the surface of streptavidin-coated magnetic beads. Biotin-Seq. U5v was immobilized on ST-beads (tube No. 2 and tube No. 3). BCM-7-HRP alone (tube No.1 and tube No. 3) or in combination with BCM-7 (Tube No. 2), spiked in urine and was added. After the addition of TMB, photographs of tubes were taken immediately and after 15 min. interval. (a). Sequential addition of different chemicals and color change in tubes. (b). Diagrammatic representation of interaction in each tube.

and No.5) measured in terms of peroxidase activity. Also, competitive binding between BCM-7 and biotin-BCM-7 for biotin- Seq.U5v (Well No.3) or biotin- Seq.7 (Well No.6) was evaluated. Reduction in color intensity in well No.3 *vis a vis* well No.1 or well No.2 indicates that BCM-7 and biotin-BCM-7 competed for biotin- Seq.U5v. Similar results were obtained with biotin- Seq.7 (see well No. 4, 5 and 6). It has been also noted that fragments of Seq.U5v failed to recognize BCM-7 (data not shown).

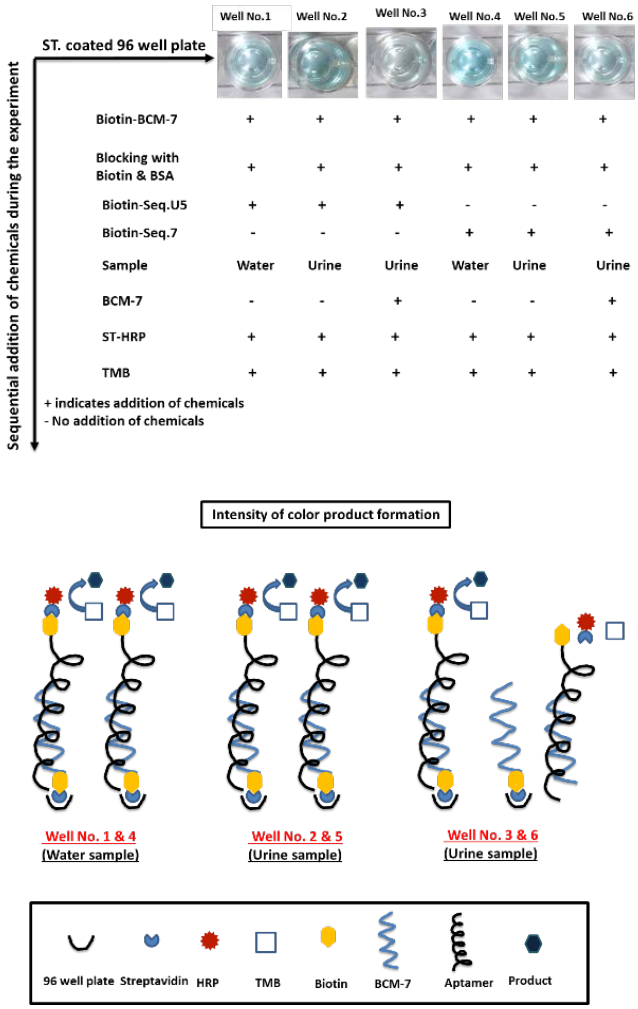

Figure 5. Evaluation of recognition of biotin-BCM-7 by biotin-Seq.7 or biotin-Seq.U5 on the surface of streptavidin-coated 96 well ELISA plate. Biotin-BCM-7 was immobilized on ST-96 wells. Biotin-Seq.U5 was added in well Nos. 1, 2, and 3 while Biotin-Seq. 7 was placed in well Nos. 4, 5, and 6 respectively. Also, BCM-7 was added in wells No. 3 and 6. Later, ST-peroxidase was added to all wells, and color was developed after the addition of TMB. (a). Sequential addition of different chemicals and color change in wells. (b). Diagrammatic representation of interaction in each well.

Recognition on the surface of nitrocellulose and nylon membrane

Nitrocellulose or nylon membranes are used in dot blot assay where presence or absence or increase in color intensity or decrease in color intensity in generated blot can be interpreted for recognition of target molecule by ligand which includes aptamer. On nitrocellulose membrane precoated with streptavidin, competitive binding between BCM-7 and BCM-7-HRP for biotin-Seq.7 (spot No.3) or biotin-Seq.U5 (spot No.6) was noted (Figure 6) and this resulted in lower colour color intensity at spot No.3 *vis a vis* spot No. 1 or spot No.2 as well as at spot No.6 *vis a vis* spot No. 4 or spot No. 5. Almost similar blot intensities at spot No.1 and spot No.2 indicated that urine did not interfere in

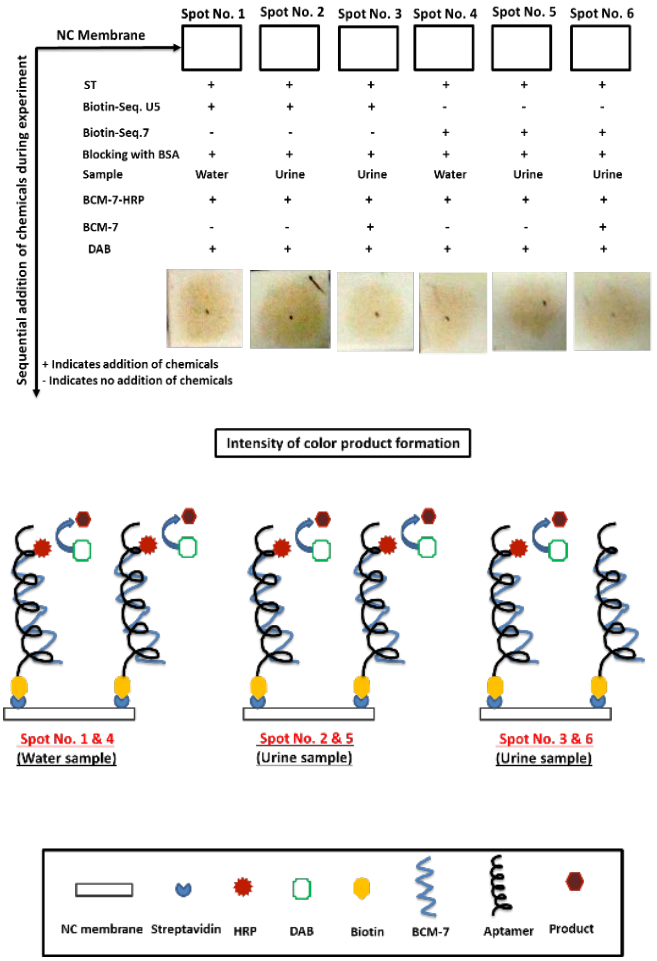

Figure 6. Evaluation of recognition of BCM-7-HRP by biotin-Seq.7 or biotin-Seq.U5 on the surface of nitrocellulose membrane. Streptavidin was spotted on the NC membrane. Biotin-Seq.U5 was immobilized on Spots No. 1, 2 & 3 while Biotin-Seq. 7 was placed on Spots No. 4, 5, and 6 respectively. Subsequently, BCM-7-HRP, spiked in either water (Spot No. 1 & 4) or urine (Spots No. 2, 3, 5 & 6) was placed. Also, BCM-7, spiked in urine was added to Spots No. 3 & 6. After washing of NC membrane, DAB was added to all spots for color development. (a). Sequential addition of different chemicals and development of dot-blot on NC membrane. (b). Diagrammatic representation of interaction on NC membrane.

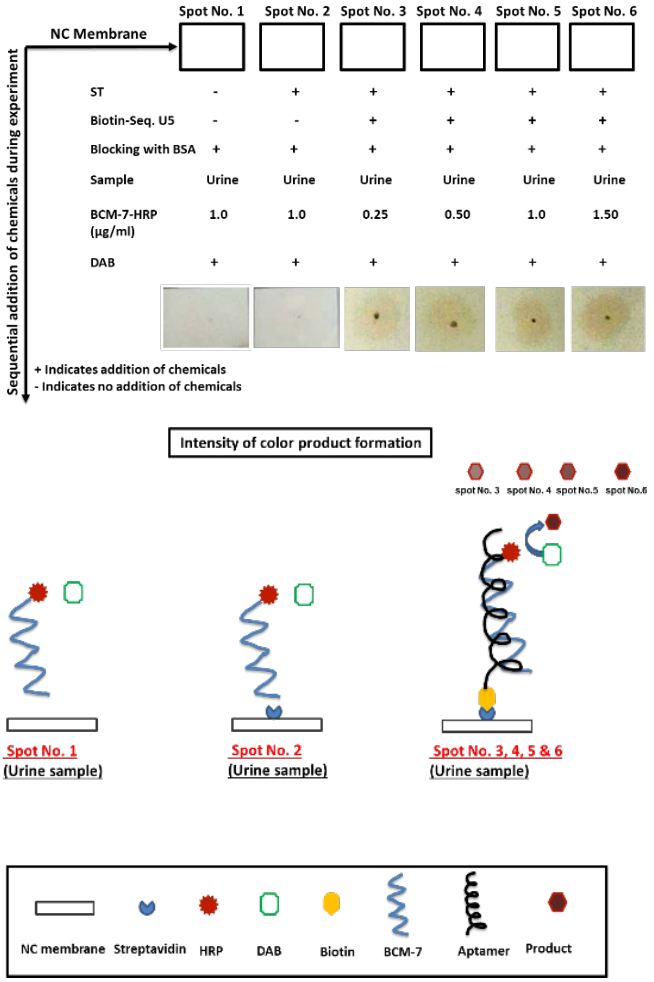

Figure 7. Dependence of intensity of color in dot blot assay on the concentration of BCM-7-HRP involving interaction between biotin-Seq.U5 and BCM-7-HRP on the surface of nitrocellulose membrane After sequential immobilization of streptavidin and biotin-Seq.U5, the membrane was incubated with different concentrations of BCM-7-HRP. Blots were developed after exposing the membrane with DAB. (a). Sequential addition of different chemicals and development of dot-blot. (b). Diagrammatic representation of interaction on the membrane.

blot assay. A similar result was obtained in the comparison of spot No.4 and spot No.5.

Further, on precoated nitrocellulose membrane, the dose-response of BCM-7-HRP for recognizing aptamer Seq.U5 was studied by using its different concentrations (0.25 to 1.50 µg/ml). There was a progressive increase in blot intensity with an increase in BCM-7-HRP concentration (Figure 7; spot No. 3, No.4, spot No.5 and spot No.6). The result suggests that BCM-7-HRP interacted with Seq.U5 on nitrocellulose membrane and a blot assay for BCM-7 can be made.

Besides immobilization of biotin-Seq.U5 on ST-coated nitrocellulose membrane, immobilization of amino-Seq.U5 on the uncoated membrane was attempted by introducing an additional step of UV-irradiation (Figure 8). On this immobilized aptamer, BCM-7-HRP interacted and interaction was measured in terms of peroxidase activity using DAB as substrate. Higher color intensity at spot No.2 in comparison to spot No.1 indicates that amino-Seq.U5 can also be immobilized by employing UV-irradiation. The result is similar to biotin-Seq.U5 immobilized on ST-membrane as indicated by higher blot intensity at spot No.4 in comparison to spot No. 3.

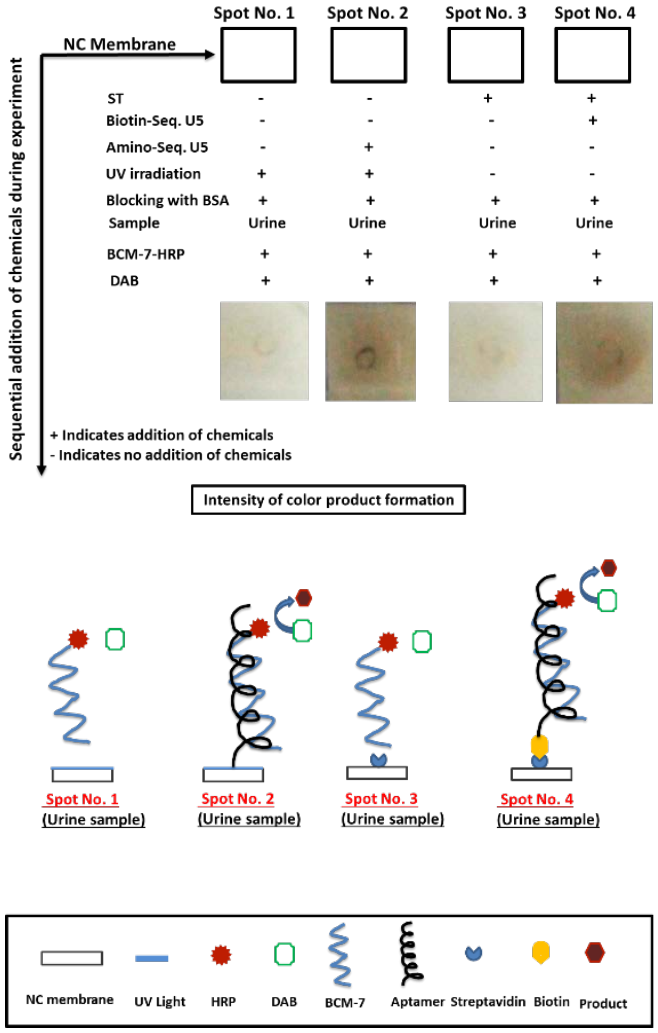

Figure 8. Evaluation of recognition of BCM-7-HRP by biotin-Seq.U5 or amino-Seq.U5 on the surface of nitrocellulose membrane. Streptavidin was spotted at Spot No.3 and Spot No.4. Biotin-Seq.U5 was immobilized on Spot No. 4. Amino-Seq.U5 was spotted on Spot No. 2 and immobilized by UV irradiation. BCM-7-HRP, spiked in urine was layered over, all spots. Blots were developed after incubation of the membrane with DAB. (a). Sequential addition of different chemicals and development of dot-blot. (b). Diagrammatic representation of interaction on the membrane.

Usually, blots are visualized by incubating the membrane with an appropriate substrate of peroxidase. We attempted a different approach by using ST-GNP. For this purpose, amino-Seq.U5 was immobilized on a nitrocellulose membrane using UV irradiation (Figure 9). Biotin-BCM-7 and BCM-7 competed for Seq.U5 as the relatively low color intensity at spot No.2 in comparison to spot No.3 was noted. Results indicate gold nanoparticles can also be used for the visualization of blots.

Besides nitrocellulose membrane, nylon membrane was also assessed for study interaction between aptamer Seq.U5$_V$ and BCM-7, and for this purpose amino-Seq.U5$_V$ was immobilized by employing UV-irradiation. BCM-7-HRP and BCM-7 competed for Seq.U5$_V$ (Figure 10). Relatively low intensity of blot at spot No. 2 in comparison to spot No. 3 indicates BCM-7 and Seq.U5$_V$ interacted on the surface of nylon membrane. Nylon membrane is thus also suitable for such work.

Discussion

Aptamer-target recognition is central to the use of aptamer in the development of sensors. After the selection of aptamers through SELEX, the dissociation constant of the aptamer-target complex is determined for prioritizing selected aptamers in

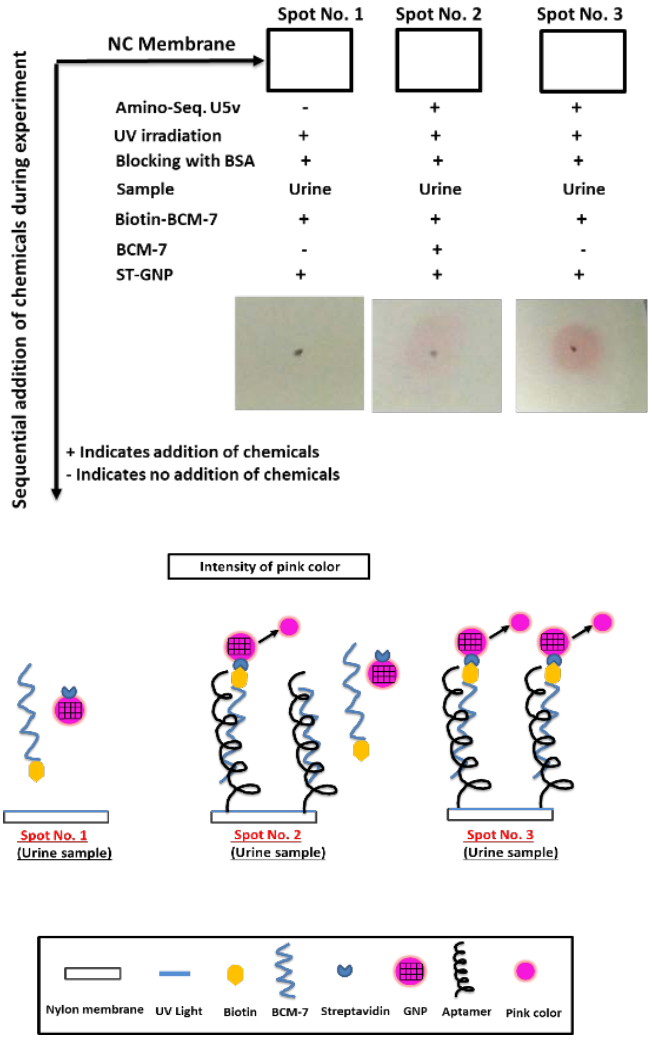

Figure 9 Evaluation of competitive binding of BCM-7-HRP and BCM-7 for amino-Seq.U5$_V$ on the surface of nitrocellulose membrane involving streptavidin-conjugated gold nanoparticle. Amino-U5$_V$ was spotted at Spot No. 2 and Spot No. 3 and immobilized by UV irradiation. Biotin-BCM-7 alone (Spot No.1 and Spot No.3) or in combination with BCM-7 (Spot No. 2) spiked in urine was layered. Later, ST-GNP was layered over, all spots. The photograph was taken after washing of membrane. (a). Sequential addition of different chemicals and development of pink color on the membrane in presence of ST-GNP. (b). Diagrammatic representation of interaction on the membrane.

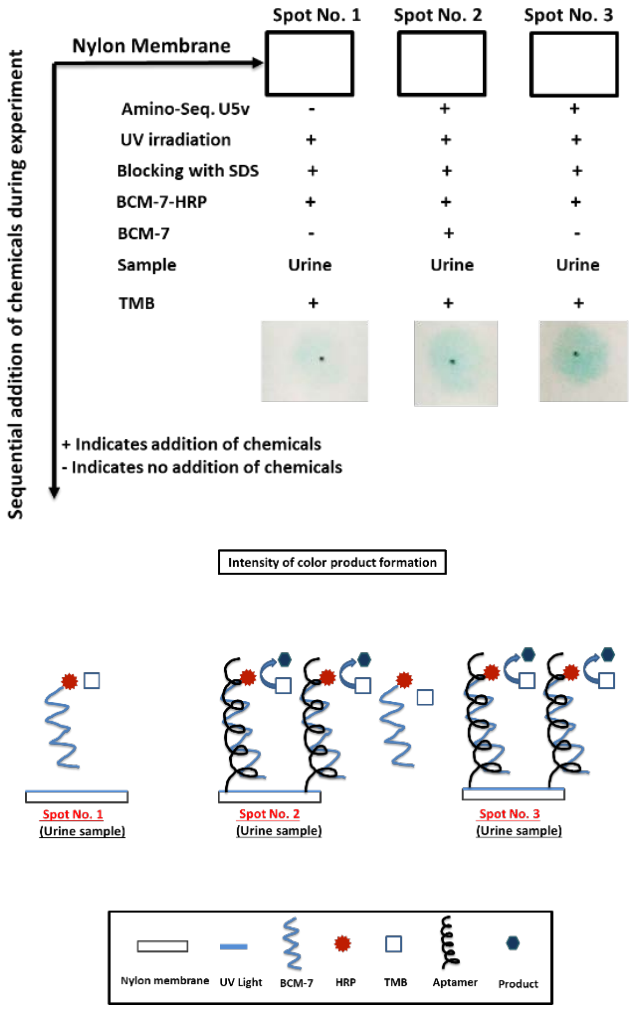

Figure 10. Evaluation of competitive binding of BCM-7-HRP and BCM-7 for amino-Seq.U5v on the surface of nylon membrane. Amino-U5v was spotted at Spot No. 2 and Spot No. 3 and immobilized by UV irradiation. BCM-7-HRP alone (Spot No.1 and Spot No. 3) or in combination with BCM-7 (Spot No. 2), spiked in urine was layered. After incubation and washing, blots were developed after the addition of TMB at all spots. Later, the photograph was taken of all spots. (a). Sequential addition of different chemicals and development of dot-blot. (b). Diagrammatic representation of interaction on the membrane.

sensor application. Coating of aptamer on GNP surface and differential salt stability of the coated nanoparticles in the presence and absence of target resulting in its color change are easy steps in the quick screening of aptamers. This approach has been used by several researchers.[18-22] Coating protocols for aptamer on gold nanoparticles are available and color change can be seen even by the naked eye. This enables the use of this approach across the laboratories. Aptamer or thiolate-conjugated aptamer can be effortlessly conjugated to GNP. In the current work, biotinylated aptamer was conjugated to ST-GNP. However, the sensitivity of gold nanoparticle-based detection system is not sufficient to detect target molecules at low concentrations, especially with toxic molecules. Development of a sensitive detection method invariably requires conjugation of aptamer with biotin or conjugation of the target with biotin or HRP, and an inert surface coated with ST. The present investigation has evaluated modified aptamers (biotinylated, amino-linked) identified in earlier work (Seq.U5 and Seq.7) for BCM-7 that can recognize BCM-7, biotin-BCM-7 or BCM-7-HRP on different surfaces. These two aptamers are 72 nucleotides long comprising 38 nucleotides long variable middle region and 18 nucleotides long constant region at both terminals ends. It appears that biotin-modified aptamers Seq. U5 and Seq. 7 retain recognition for BCM-7 (Figure 1, Figure 2, Figure 5, and Figure 6). Larger is the length of aptamer, minimum is the chance of alteration in recognition potential of aptamer on modification. We have also checked modified aptamers lacking constant regions (Seq. U5v) which have just 50 % length of their parent aptamers and noted that even 38 nucleotides long variable regions can recognize BCM-7 (Figure 3, Figure 4, Figure 9 and Figure 10). There are several successful truncated aptamers.[23-32] and majority of them are 21 to 33 nucleotide long.[23-31] Truncated aptamers in most cases result in reduced Kd values and improved LOD.[23-31] Although 72 nts long truncated aptamer for ofloxacin has shown less sensitivity as compared to the parent sequence,[33, 34] further truncation of variable region (9 and 18 nucleotides long) of Seq.U5v and subsequent modification with biotin fail to recognize BCM-7 (unpublished). Thus, there is a limit to which aptamer length can be truncated without seriously compromising recognition. The optimum length of aptamer may differ from aptamer to aptamer.[35]

The present investigation also does suggest that aptamer-target recognition can be achieved on the surface of gold nanoparticles (Figure 1), magnetic beads (Figure 2, Figure 3 & Figure 4), 96-well ELISA plate (Figure 5), nitrocellulose membrane (Figure 6, Figure 7, Figure 8 & Figure 9), and nylon membrane (Figure 10) and such design can be adopted for any other aptamer.

Conclusion

Interaction between aptamers for BCM-7 as well as between modified aptamers and modified target (BCM-7) can be studied on different surfaces. Variable regions (38 nts long) from full-length aptamers (72 nts long) for BCM-7 identified in earlier work are also able to recognize BCM-7. Interaction between aptamer and target has been established in a competitive format on different surfaces. BCM-7-HRP conjugate retains BCM-7 structure assessable for binding to aptamer and therefore, assay formats for BCM-7 should preferably use BCM-7-HRP in competitive design employing aptamers Seq.U5 and Seq.7 identified earlier.

Conflict of Interest

Authors declare no conflict of interest of any kind – academic or financial for the publication of this piece of work.

Acknowledgments

Indian Council Medical Research (ICMR) provided the fellowship & funding. National Dairy Research Institute

(Deemed University under Indian Council of Agricultural Research, ICAR) gave the laboratory facilities.

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