International Journal Of

Recent Scientific Research

ISSN: 0976-3031

Volume: 7(4) April -2016

STUDY OF rs699 SNP OF HYPERTENSIVE PATIENTS WITH GOLD SURFACE IMMOBILIZED MOLECULAR BEACON BIOSENSOR

Verma N., Kaur N and Singh A K

THE OFFICIAL PUBLICATION OF
INTERNATIONAL JOURNAL OF RECENT SCIENTIFIC RESEARCH (IJRSR)
http://www.recentscientific.com/ recentscientific@gmail.com

ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 4, pp. 10276-10281, April, 2016

International Journal of Recent Scientific Research

Research Article

STUDY OF rs699 SNP OF HYPERTENSIVE PATIENTS WITH GOLD SURFACE IMMOBILIZED MOLECULAR BEACON BIOSENSOR

***Verma N., Kaur N and Singh A K**

Biosensor Technology Lab, Department of Biotechnology Punjabi University Patiala, India 147002

ARTICLE INFO

Article History:

Received 15th January, 2016
Received in revised form 21st February, 2016
Accepted 06th March, 2016
Published online 28th April, 2016

Keywords:

Molecular Beacon, hypertension, Biosensor, SNP, immobilization etc.

ABSTRACT

DNA hairpins are sensitive probes for oligonucleotides identification that discriminate between two sequences over single base pair change. When these hairpins are immobilized onto surface this not only increases their sensitivity but also provides new path in development of microarray based biosensor. Current work includes molecular beacon based biosensor development for hypertensive SNP rs699 recognition. Hybridization efficiency was found to be higher in rs699 mutation complement probe than in non-compliment one. The developed biosensor has response time of 5 min and optimum working temperature of 35ºC. Immobilization of oligonucleotide was cross confirmed by electrochemical analysis of surface via electrochemical Impedance, Cyclic Voltammetry and Differential Pulse Voltammetry. Further biosensor could be applied to clinical hypertensive and normotensive patients, to ensure maximum efficacy of drug and minimal adverse effect.

INTRODUCTION

Single nucleotide polymorphism (SNP) exhibits largest source of diversity in our genome SNPs can be distinguished from other variations by more than 1% frequency in the human population when one of three nucleotides is replaced by a single nucleotide. There are about 10~30 million SNPs in human genome with an average SNP of 100~300 bases. NCBI dbSNP Build 129 is a database where human SNPs are publicly available and their number exceeds more than 5 million. A SNP in a protein coding sequences induces amino acid change thus resulting in functional changes in the protein. Some of these variations are directly linked to human diseases.

The main aim of pharmacogenomics is to develop rational means to optimize drug therapy, with respect to genotype of patient, to ensure maximum efficacy of drug and minimal adverse effects. Genetic screening of SNPs (Single Nucleotide Polymorphism) provides important information for disease diagnosis and drug therapy.

Hypertension is a clinical condition that affects a large proportion (25-30%) of the adult population and is a one of major risk factor for cardiovascular and renal diseases. Heritable factors account for 20% to 60% variations in human (Gould and Jeunemaitre, 1997). Thus many genes involved in blood pressure regulation have been screened till date and are candidates of hypertension. According to the World Health Statistics 2012 report, India has low rates of hypertension compared to world figures. In India, 23.10 per cent men and 22.60 per cent women above 25 years suffer from hypertension than the global average of 29.20 in men and 24.80 in women respectively.

Hypertension is a complex multifactorial and polygenic trait where casual genes contribute between 30% to 50% variation in blood pressure among individuals (Tong *et al*, 2004). Interaction of these genetic determinants with environmental factors such as change in dietary salt habits results in final disease phenotype. One of major challenge includes genetic dissection of human essential hypertension. Among candidate gene study angiotensinogen (AGT) emerges as strong contender (Tyagi and Kumar, 1996; Tsourkas *et al*, 2003; Drake and Tan, 2004; Kolpaschchikov, 2012). This AGT gene forms a part of Renin-Angiotensinogen-Aldosterone System (RAAS) that regulates the blood pressure (BP) and cardiovascular (CV) hemodynamics via angiotensin II, a potent vasoconstrictor and electrolyte balance (Figure 1) (Elton *et al.* 2010).

The Michaelis constant of enzymatic reaction between AGT and renin is nearly close to plasma concentration of AGT

Corresponding author: **Verma N*
Biosensor Technology Lab, Department of Biotechnology Punjabi University Patiala, India 147002

(Gould and Green, 1971). Thus rise in plasma AGT levels can lead to parallel increase in angiotensin II formation that may result in hypertension. Hypertensive patients have higher levels of AGT in blood then the normotensives. Also AGT gene is expressed at higher levels in kidney, brain, adrenal, placenta and vascular walls that are directly involved in blood regulation (Kumar *et al*, 2005). The rs699 is angiotensin AGT SNP that encodes that functional hypertension. This is also known as M235T or Met235Thr. It is occasionally known as C4072T. C allele encodes for threonine and this variant protein contribute to higher plasma angiotensin levels and thus higher blood pressure leading to hypertension disorders.

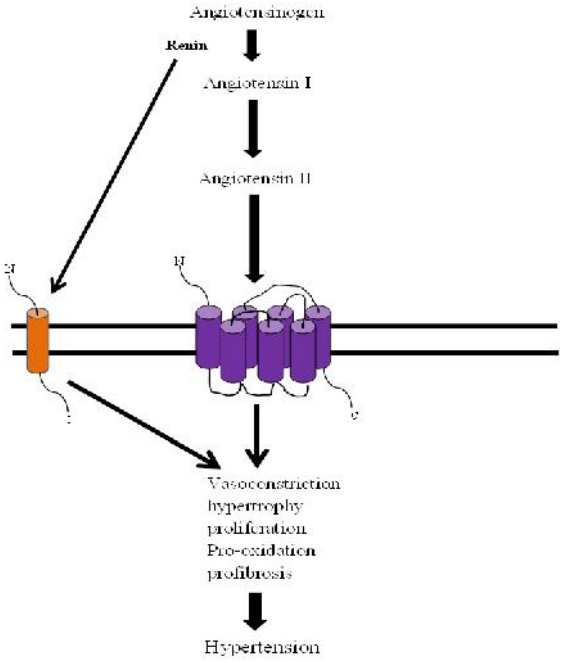


Figure 1 effect of *agt* gene on hypertension (Elton *et al*., 2010).

One of potent method for detection of SNPs is molecular beacons. Molecular beacons consist of DNA hairpins that are functionalized at one terminus with a fluorophore and at the other with a quencher. In the absence of their complement strand, these exist in a closed conformation where fluorescence is quenched. Hybridization occurs when complementary oligonucleotides are introduced that open up the hairpin and allows for fluorescence. DNA hairpins exhibit more stability, better selectivity, and higher specificity than other assays performed using single-stranded DNA. High specificity of DNA hairpins can be demonstrated by their ability to discriminate between single base-pair mismatch. Over a wide range of temperatures only perfectly matched probe- target hybrid force opens the stem hybrid. At substantially lower temperatures, probe-target mismatch are formed. Hence a wide range of temperatures exists in which perfectly complementary probe-target hybrids fluoresce while the mismatched ones don't.

The use of molecular beacons as biosensors is expanding rapidly (Fang *et al*, 1999; Wang *et al*, 2002; Drake and Tan, 2004; Chen *et al*, 2011; Epstein *et al*, 2003; Stobiecka and Chalupa, 2015). MBs are immobilized onto solid surface with high efficiency and must be optimized for use at liquid-solid interface. For immobilization of MBs onto surface either biotin-avidin interaction or thiol-gold linkage and amide bond could be employed. Different surfaces such as glass (Fang *et al*, 1999), gold (Du *et al*, 2003; Du *et al*, 2005), polyacrylamide and agarose (Wang *et al*, 2005) have been used. All these surfaces not only provide efficient but also stable immobilization for response, sensitivity and reproducibility of MB based biosensors and biochips.

Background fluorescence generation in case of biosensor is big hurdle. Inculcation of space between the MB and the surface is one possibility (Li *et al*, 2001; Horejsh *et al*, 2005; Fan *et al*, 2003). Poly T linker introduced as spacer improved little sensitivity. M.B attached to surface of functionalized hydrophilic gel film of agarose, polyacrylamide also showed lower background fluorescence, higher sensitivity, faster response and better selectivity (Fang *et al*, 1999; Wang *et al*, 2005). However MB immobilization onto gold surface not only lowered the background fluorescence but also enhanced hybridization with target (Du *et al*, 2003; Du *et al*, 2005). Apart from fluorescence analysis enzymatic signal detection and electrochemical electron transfer have been developed. The reporter is enzyme or electrochemical agent linked to free end of MB. When MB hybridizes to target nucleic acid signal is released by reporter (Conformation of reporter changes) (Bockisch *et al*, 2005; Graf and Krämer, 2006; Steemers *et al*, 2000).

Beads functionalized with MB have been used for multiplexed analyte detection. Here MB's are immobilized onto microspheres that were entrapped with in array of wells that was etched upon optical fiber (Steemers *et al*, 2000). This array had a fast response time led to accurate analysis of multiple genetic mutations. Flow cytometery is another high throughput technique that could be combined with MB coated microsphere arrays to detect nucleic acids. Based on size and color coding signals from these are differentiated. Such assays that are simple, fast and accurate help in genetic analysis and genotyping for disease diagnosis and therapy.

Molecular beacons can be used in conjunction with amplification of target DNA and simultaneously detection of amplified products in real time PCR. One of the most advantages is that it is not necessary to isolate the probe-target hybrids as non-hybridized molecular beacons don't fluoresce. We hereby report Molecular Beacon (MB) based biosensor for SNP analysis of rs699 variation in human hypertensive and normotensive patients. MBs were pre-synthesized against wild and mutant gene.

MATERIALS AND METHODS

Chemicals

Silicon wafer with gold coated was procured from IIT Delhi, India. The oligonucleotides Sequence of Molecular Beacons, respective target, non-target and negative sequence are shown in Table 1. All oligonucleotides were purchased from Regeneration Technologies, Chandigarh, India. Molecular Beacons (MBs) were functionalized at 5'end with thiolh group and 3'end with Tetramethylrhodamine (TMR). All other chemicals used were of molecular biology grade, without further purification. Ultrapure water used in preparation of buffers and rinsing solution had resistivity of 18.2 MΩ as

produced by WaterPro water purification system (Labconco Corporation, Kansas City, MO). The buffered Saline used was 20mM Cacodylic Acid, 0.5 M NaCl and 0.5mM EDTA, pH=7.

Table 1 Molecular Beacon Probes and their Targets

Serial No.	Sequence Designation	Oligonucleotide Sequence
1.	Molecular Beacon Sequence 1 (MB1) SNP	5'-C-6-thiol- TGGAAGACTGTGTCCACACTGGCTCCCGTCAGGGAGCAGCCAGTCTTCCA-3' Amino-C7-TMR-3'
2	Molecular Beacon Sequence 2 (MB2) wild type	5'-C-6-thiol- TGGAAGACTGTGTCCACACTGGCTCCCATCAGGGAGCAGCCAGTCTTCCA-3' Amino-C7-TMR-3'
3	Target Sequence 1(TS1)	5'-TGGAAGACTGGCTGCTCCCTGACGGGAGCCAGTGTGGACA-3'
4	Target Sequence 2 (TS2)	5'-TGGAAGACTGGCTGCTCCCTGATGGGAGCCAGTGTGGACA-3'
5	Negative Sequence (NS)	5'-GATCGACTGATACTAGTATCAGCTATGATACTGACTAGTC-3

METHODS

Preparation of self-assembled oligonucleotides on Gold surface

Gold coated silicon wafers were cleaned with piranha solution (4:1 concentrated H_2SO_4/ 30% H_2O_2) overnight at room temperature and then rinsed with ultrapure water. The self-assembly process of hairpin oligonucleotides on gold surface was done by pouring the mixture of hairpin oligonucleotides and 3-mercaptopropanol. After two hours the modified chip was thoroughly rinsed with hot water (90ºC or higher) to remove any unbound oligonucleotides. Rinsing the gold surface after self-assembly is an important step; otherwise, some probe molecules will attach to the gold surface causing a large background signal, Next, the gold chip carrying the mixed monolayers was immersed in buffered saline for hairpin formation. Hybridization with target to the hairpin probes on the gold was performed at room temperature under the same conditions.

Optimization of immobilization parameter

Volume ratio of oligonucleotides and 3-mercaptopropanol was optimized by using oligonucleotides (85.3 µM) and 3-mercaptopropanol (85.3 µM) i.e in (1:9, 3:7, 5:5) ratio with final volume to 10µl.

Characterization of modified gold surface by electrochemical methods

Electrochemical Impedance, Cyclic Voltammetry (CV), Differential Pulse Voltammetry (DPV) experiments were performed using electrochemical workstation (CH Instruments, USA) model 660 electrochemical analyzer with three electrodes system. The experiments were carried out in 10ml conventional cell with gold chip (3mm x 3mm) attached with copper wire was used as working electrode, Ag/AgCl (3M KaCl) as reference electrode and platinum wire as counter electrode. CV and DPV were recorded in 0.1 M PBS (pH 7.0). Whereas, impedance was recorded at the frequency range from 10^{-1} to 10^{5} Hz in a bias potential of -0.25 V versus Ag/AgCl, amplitude 5mV in 0.1M HEPES buffer.

Determination of Fluorescence

Target oligonucleotides (243.82µM) were added on molecular beacon immobilized gold surface for hybridization in hybridization buffer that provides 3M NaCl and 300mM Sodium Citrate). The working principle of the biosensor is shown in Figure 2.

Hybridization time and temperature were analyzed using Mini Opticon, BioRad, USA, set with a 435±5 nm excitation filter and a 556±5 nm emission filter. Response time was optimized by taking fluorescence intensity at different time intervals (5min to 60 min).

After optimizing time, temperature was also optimized ranging from 25 to 75 ºC. The process of fluoresce measurement of both molecular beacon (SNP and wild type) are represented in **scheme 1**.

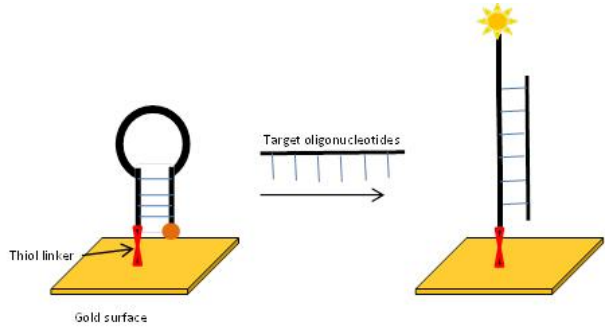
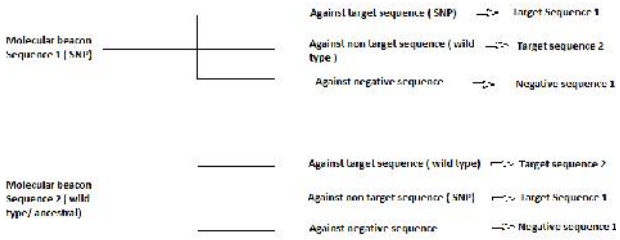


Figure 2 Hybridization of molecular beacon to target sequence



Scheme 1 It demonstrates the experimental proceedings designed for molecular beacon sequence 1 and 2 against the target sequence of human AGT gene (both SNP/ ancestral type gene sequence).

RESULT AND DISCUSSION

Optimization of Molecular Beacon and 3-MPA ratio

The molecular beacon and 3-mercaptopropanol ratio was studied by using different combinations by v/v like 1:9, 5:5, and 3:7 for total 10 µl volume immobilized onto gold surface. The optimum ratio was found to be 5:5 as shown in **figure 3**. The equal ratio of MBs and 3-Mercaptopropanol produced best results so further all studies were carried out by using this ratio.

Optimization of response time and temperature for hybridization of target oligonucleotides

Hybridization of target oligonucleotides, non-target and negative control oligonucleotides sequences with immobilized MBs onto gold surface was studied at different temperature range from 25 to 75 ºC. The optimum temperature was found to give maximum response at 35°C (**Figure 4**). Further all the experiments were run at 35 ºC. Response time of the biosensor was also optimized by taking fluorescence intensity at different

time intervals from 5 to 60 min. The optimum response time was found to be 5 min (**Table 2**).

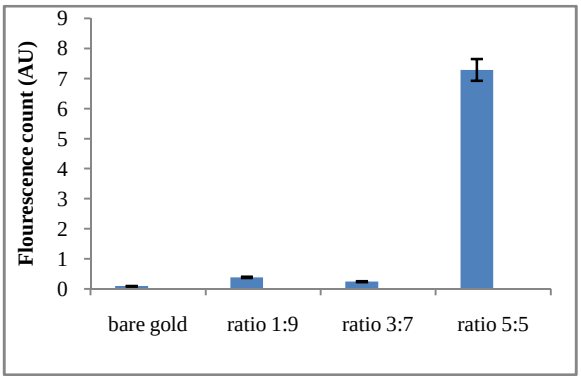


Figure 3 The molecular beacon to 3-mercaptopropanol ratio used for immobilization on gold surface

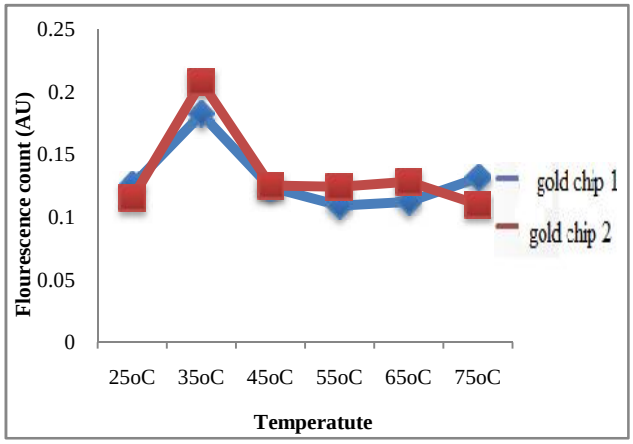


Figure 4 Representing fluorescence at 5 min interval on different temperature range

Table 2 Fluorescence measurement of dry chips treated with buffer saline at 35 ºC

Gold chips	Buffer Saline incubation [M.B + Hairpin formation]	5 min	10 min	15 min	20 min	25 min	30 min	60 min
1	0.132	**0.182**	0.17375	0.16925	0.1675	0.166	0.1632	0.1552
2	0.1175	**0.2075**	0.19075	0.1815	0.1767	0.173	0.169	0.159

Characterization of immobilized MB and hybridization with target by electrochemical methods

The characterization of immobilized molecular beacon on gold surface and binding with target oligonucleotides was studied by electrochemical impedance methods and we found increase in impedance because of increase in resistance (**Figure 5**). Immobilization of molecular beacons with 3-mercaptopropanol was further confirmed by CV and DPV. There was enhancement in current due to polarization of electro active species present (**Figure 6**).

Fluorescence study of hybridization of Molecular beacon with target sequences

As shown in **figure 7**, there is 3 fold increase in fluorescence with target sequence and there is no or negligible fluorescence observed in non-target and negative sequences. It predicts that the fluorescence enhancement has led to hybridization of the target sequence to immobilized molecular beacon for rs699.

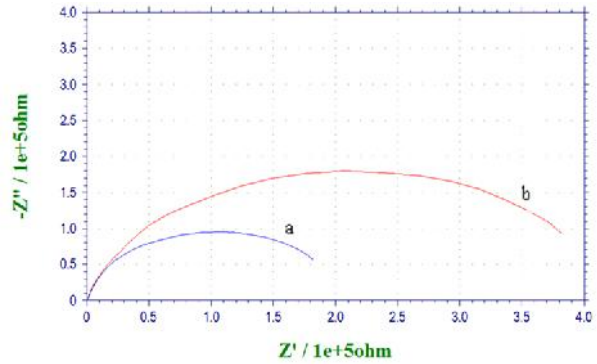


Figure 5 electrochemical Impedance spectra of gold surface with immobilized MBs (a) and after hybridization with targets (b).

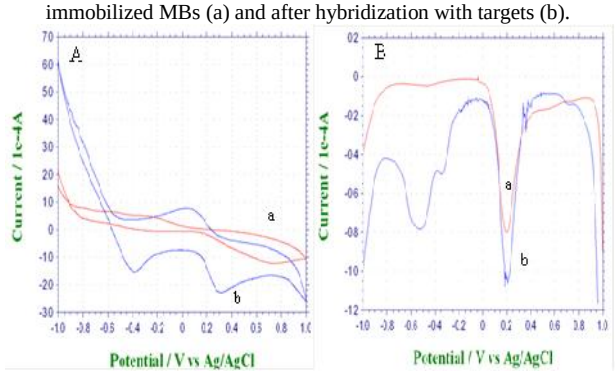


Figure 6 (A) cyclic voltammetry response of bare gold electrode (a) and electrode immobilized with molecular beacon (b), (B) DPV response of the bare (a) and electrode modified with MB (b).

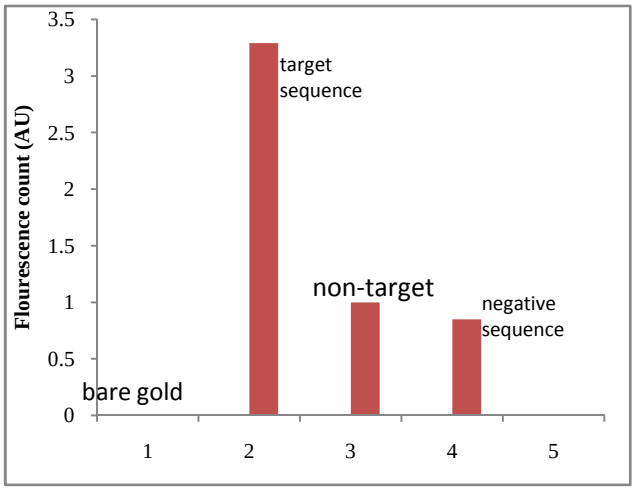


Figure 7 the fluorescence enhancement in immobilized molecular beacon for rs699 hybridizes to the target sequence, non-target and negative sequence.

DISCUSSION

According to Tsourkas *et al.* (2003) probe selection is important since it is critical for determining hybridization efficiency and thus chip performance. Under similar hybridization conditions the conformation of a molecular beacon will be determined by the competition between the hairpin configuration and the duplex. Since the free energy of duplex formation is much more favorable than that of hairpin formation, the duplex form, which results in restoration of fluorescence. Most molecular beacon studied shows that a remarkable balance between selectivity and sensitivity is attained with 15-25 loop nucleotides together with a 5-7 base

pair stem (Tan *et al.* 2000). In our studies we used 50 nucleotides probe (30 loop nucleotides with 10 base pair stem) that provides better surface and balanced. For molecular beacon assays on a solid surface, the specific/nonspecific signal ratios obtained are in the range of only 2-6 (Liu and Tan, 1999; Frutos *et al.* 2002; Dodge *et al.* 2004). By comparison, our surface immobilized molecular beacons can distinguish a G/C mismatch of its 28th nucleotide position. SNP nucleotide of target oligonucleotides binds to the central position (28th) of the loop of molecular beacon and 3 fold enhancement of fluorescence are obtained.

CONCLUSIONS

Adequate temperature was found to be 35 ºC for molecular beacon and target DNA hybridization. Highest fluorescence was observed at 5 min after introduction of target molecule [FRET break] to gold chip with immobilized molecular beacon. The optimum volume ratio of molecular beacon and 3-mercaptopropanol was found to be 5:5 in 10 µl sample volume. 3 fold fluorescence enhancements were observed in case of target and molecular beacon hybridization as compared to negligible fluorescence in case of non-target and negative sequence. Hence developed molecular beacon based biosensor can be successfully employed for clinical samples analysis to check *agt* gene based hypertension, which can be helpful in pharmacogenomics of the patient.

Acknowledgment

The authors are thankful to DBT-Punjabi University Patiala Interdisciplinary Programme in Life Sciences for Advance Research and Education for providing the partial financial assistance in carrying out the study (File No.: BT/PR4548/INF/22/146/2012).

References

Bockisch B, Grunwald T, Spillner E, Bredehorst R (2005) Immobilized stem-loop structured probes as conformational switches for enzymatic detection of microbial 16S rRNA. Nucleic Acids Res, 33(11):101.

Chen A K, Rhee W J and Ba O G (2011) Delivery of molecular beacons for live-cell imaging and analysis of RNA. Methods Mol Biol, 714: 159-74.

Dodge A, Turcatti G, Lawrence I, Rooij N F and Verpoorte E (2004) a Microfluidic Platform Using Molecular Beacon-Based Temperature Calibration for Thermal Dehybridization of Surface-Bound DNA. Anal Chem, 76: 1778-1787.

Drake T J., Tan W (2004) Molecular beacon DNA probes and their bioanalytical applications Appl Spectrosc, 58(9): 269A-280A.

Du H, Strohsahl C M, Camera J, Miller B L and Krauss T D (2005) Sensitivity and specificity of metal surface-Immobilized “molecular beacons”, Biosensor. J Am Chem Soc , 127(21): 7932-7940.

Du H, Disney M D, Miller B L and Krauss T D (2003) Hybridization-based unquenching of DNA hairpins on au surfaces: prototypical "molecular beacon" biosensors. J. Am. Chem. Soc, 125 (14):4012-4013.

Elton T S, Sansom S E and Martin M M (2010) Cardiovascular Disease, Single Nucleotide Polymorphisms; and the Renin Angiotensin System: Is There a MicroRNA Connection?. Int J Hypertens, Article ID 281692,doi:10.4061/2010/281692.

Epstein J R, Leung A P, Lee K H and Walt D R (2003) High-density, microsphere-based fiber optic DNA microarrays. Biosens Bioelectron, 541-546.

Fan C, Plaxco K W and Heeger A J (2003) Electrochemical interrogation of conformational changes as a reagentless method for the sequence-specific detection of DNA. Proc Natl Acad Sci, 100(16): 9134-9137.

Fang X H, Liu X J, Schuster S and Tan W H (1999) Designing a novel molecular beacon for surface-immobilized DNA hybridization studies. J. Am. Chem. Soc, 121: 2921–2221.

Frutos A G, Pal S, Quesada M, Lahiri J (2002) Method for detection of single-base mismatches using bimolecular beacons. J Am Chem Soc, 124: 2396-2397

Gould A B and Green D (1971) Kinetics of the human renin and human substrate reaction. Cardiovasc Res, 5:86–89.

Gould A B and Jeunemaitre X (1997). Molecular genetics of human hypertension: role of angiotensinogen. Endocr Rev, 18:662-667.

Graf N and Krämer R F (2006) Enzymatic amplification in a bioinspired, autonomous signal cascade. Chem Commun (Camb), 42: 4375-4376.

Horejsh D, Martini F, Poccia F, Ippolito G, Di Caro A, Capobianchi M R (2005) A molecular beacon, bead-based assay for the detection of nucleic acids by flow cytometry. Nucleic Acids Res, 33(2): 13.

Kolpaschchikov D M (2012) An elegant biosensor molecular beacon probe, challenges and recent solutions. Scientifica, doi: 10.6064/2012/928783.

Kumar A (2005) Angiotensinogen gene polymorphism and hypetension. In: contemporary cardiology: Cardiovascular genomics (Eds. Raizada M K et al.) Humana Press Inc. Totowa, NJ, pp.19-40.

Li J, Tan W, Wang K, Xiao D, Yang X, He X and Tang Z (2001) Ultrasensitive optical DNA biosensor based on surface immobilization of molecular beacon by a bridge structure. Anal Sci, 10: 1149-1153.

Liu X and Tan W (1999) Fiber-optic evanescent wave DNA biosensor based on novel molecular beacons. Anal Chem, 71: 5054-5059.

Steemers F J, Ferguson J A, Walt D R (2000) Screening unlabeled DNA targets with randomly ordered fiber-optic gene arrays. Nat Biotechnol, 18(1): 91-94.

Stobiecka M and Chalupa A (2015) Biosensors based on molecular beacons. Chemical Papers, 69 (1): 62–76.

Tan W, Fang X, Li J, Liu X (2000) Molecular Beacons: A Novel DNA Probe for Nucleic Acid and Protein Studies. Chem Eut J, 6: 1107-1111.

Tong W., Wang K., Drake T (2004) Molecular beacons. Current Opinion in Chemical Biology, 8 547–553.

Tsourkas A., Behlke M A , Rose S D and Bao G (2003) Hybridization kinetics and thermodynamics of molecular beacons. Nucleic Acids Res., 31(4):1319-1330.

Tyagi S and Kramer F R (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol, 14(3): 303-308,

Wang H, Li J, Liu H, Liu Q, Mei Q, Wang Y, Zhu J, He N and Lu Z (2002) Label-free hybridization detection of

a single nucleotide mismatch by immobilization of molecular beacons on an agarose film. Nucleic Acids Res. 30(12):e61.

Wang L, Yang C J, Medley C D, Benner S A and Tan W (2005) Locked nucleic acid molecular beacons. J Am Chem Soc, 127(45): 15664-15665.

****

How to cite this article:

Verma N., Kaur N and Singh A K.2016, Study of Rs699 SNP of Hypertensive Patients with Gold Surface Immobilized Molecular Beacon Biosensor. *Int J Recent Sci Res.* 7(4), pp. 10276-10281.

TSSN 0976-3031