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Molecular Beacon Strategies for Sensing Purpose

Negar Bidar[1], Mohammad Amini[2], Fatemeh Oroojalian[3,4], Behzad Baradaran[2], Seyed Samad Hosseini[1], Mohammad-Ali Shahbazi[5,6,7], Mahmoud Hashemzaei[8], Ahad Mokhtarzadeh[2*], Michael R Hamblin [9,10,11**], Miguel de la Guardia[12***]

[1] Department of Biological Science, Faculty of Basic Science, Higher Education Institute of Rab-Rashid, Tabriz, Iran
[2] Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[3] Department of Advanced Technologies, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran
[4] Natural Products and medicinal plants research center, North Khorasan University of Medical Sciences, Bojnurd, Iran
[5] Drug Research Program, Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki, Helsinki FI-00014, Finland
[6] Department of Pharmaceutical Nanotechnology, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran
[7] Zanjan Pharmaceutical Nanotechnology Research Center (ZPNRC), Zanjan University of Medical Sciences, Zanjan, Iran
[8] Department of Pharmacodynamics and Toxicology, School of Pharmacy, Zabol University of Medical Sciences, Zabol. Iran
[9] Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA, 02114, USA.
[10] Department of Dermatology, Harvard Medical School, Boston, MA, 02115, USA
[11] Laser Research Centre, Faculty of Health Science, University of Johannesburg, Doornfontein 2028, South Africa
[12] Department of Analytical Chemistry, University of Valencia, Dr. Moliner 50, 46100, Burjassot, Valencia, Spain

Corresponding authors:

*mokhtarzadehah@tbzmed.ac.ir (A. Mokhtarzadeh)
Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
** Hamblin.lab@gmail.com (M.R.Hamblin)
Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA, 02114, USA
***miguel.delaguardia@uv.es (M. de la Guardia).
Department of Analytical Chemistry, University of Valencia, Dr. Moliner 50, 46100 Burjassot, Valencia, Spain.

Abstract

The improvement of nucleic acid probes as vital molecular engineering devices will cause a noteworthy contribution to developments in bioimaging, biosensing, and disorders diagnosis. The molecular beacon (MB) which was designed by Tyagi and Kramer in 1996, are loop-stem hairpin-designed oligonucleotides armed with a quencher and a dye (also named reporter groups) at the 3′ or 5′ ends. This construction allows that MBs in the absence of their target complementary molecules do not fluoresce. Through hybridization with their specific targets a spontaneous configuration change on MBs occur and the dye and quencher separate from each other, resulting in emitting the fluorescence. MBs are effective probes for biosensing because of their extraordinary target-specificity, unique structure, inherent fluorescent signal transduction mechanism, low background fluorescence emission, recognition without separation, and favorable thermodynamic properties. In comparison to other probes (such as linear DNA sequences), MBs with the same number of complementary nucleotides matching their target, are multitasking probes. They have advantages of thermodynamic and photostability, flexible ability for conjugation, higher efficient intrinsic signal switching, and ultra-sensitivity. MBs not only are useful for identifying a nucleic acid target but can also be employed for recognition of various non-nucleic acid goals, including heavy metals and cations, enzymes, cells, ATP, etc. Hence, this review highlights the potential of MBs in the improvement of biosensors and their usage in detection of different analytes such as miRNA, mRNA, cocaine, methamphetamine, actin, thrombin, heavy metal and cations and so on.

Keywords: Molecular beacon; Nucleic acid probe; Biosensor; 2′-O-methylated MBs; Peptide nucleic acids; Locked nucleic acids; Aptamer MBs; Catalytic MB

1. Introduction

Nucleic acid probes cause a perfect outline for the molecular engineering of probes for a variety of applications. Among these probes, MBs has been less than thirty years since the introduction of them by Tyagi and co-worker [1]. These molecular probes are formed by a loop and a single stem (Figure 1A)[2].

While inactive in the absence of a specific target, MBs can report the presence of a target during hybridization or a certain detection caused change in configuration (Figure 1B). The benefits of MBs in the ease of their producing, and their appropriateness for constructional modification. Modified MBs such as 2′-O-methylated MBs, peptide nucleic acids (PNAs) based-MBs, locked nucleic acids (LNAs) based-MBs [3], and Aptamer MBs [4], have better stability than regular MBs and able to monitor such as small molecules and proteins at *in vivo* and *in vitro* conditions [5]. Nowadays MBs are broadly applied in the variety of targets such as alleles, messenger-RNA (mRNA) [6], cations [7], heavy metals, genes [8], microRNA (miRNA) [9, 10], viruses such as SARS COV2 RNA [11], and so on. Also, these dual label probes are practical agents for point-of-care diagnostics for infectious disorders and have the ability to progress patient care and antibiotic supervision [12]. This wide spectrum of tenders can be credited to the specific method in which the MBs interact with their targets. The exclusive stem-loop construction and fluorophore/quencher couple permit the detection of target nucleic acids to be reported in real time with excellent sensitivity and selectivity [13]. Important progress in the field of MB-based biosensing has been gained as confirmed by the developing number of tenders: MB is commercially accessible and it has been widely reviewed since the primary review appeared in 2000 [9].

MBs are effective probes for biosensing because of their extraordinary target-specificity, unique structure [14], inherent fluorescent signal transduction mechanism, low background fluorescence emission, and favorable thermodynamic properties [13]. In comparison to other probes (such as linear

DNA sequences), MBs with the same number of complementary nucleotides matching their target, are multitasking probes. They have advantages of thermodynamic [15], photostability owing to the reporter groups, flexible ability for conjugation, higher efficient intrinsic signal switching, ultra-sensitivity [15], and high selectivity [16]. Because of these impressive features, MBs have been successfully employed in a diversity of bioanalytical tenders that involve the recognition of DNA/RNA sequences [13, 17, 18]. These include the monitoring of enzymatic reactions (nucleases [19], ligases, DNA phosphorylation, and methylation) [20], real-time pathway analysis of living cells [18], the study of protein–DNA interactions [20], bioimaging, gene therapy, drug delivery, genotyping, analysis of PCR products, detection of SNPs in cancer diagnosis [16, 20], DNA array development [13], mutation identification, detection of viral infection, and RNA expression [21], etc. MBs also have a variety of beneficial applications in medicine and biology making them attractive to researchers [12-14]. Although MBs have been broadly used in biomedical applications, their role in biosensor development cannot be neglected. Elementary linear switches based on the shift of a fluorophore-tagged reporter chain by have been created. In this conformation, the certain probe was fixed onto a gold nanoparticle (AuNPs) or was interacted with the reporter chain: due to the stabler interaction with the target or the modification in configuration of the aptamer on attaching to the specific target, the reporter chain was deranged and the fluorescence was restored [9].

The goal of this review is to describe the ability of MBs for monitoring biological and non-biological targets, focusing on their engineering methods for this purpose, also, is the presentation of the recent MB methods applied for detection purpose.

2. Type of MBs

Amid various groups of DNA probes, MBs have an exclusive configuration in their unbound state owing to the presence of a self-complementary terminal. According to their applications, MBs can be divided into regular MBs (RMBs) or labeled-MBs, nuclease resistant MBs, aptamer MBs, catalytic MB (CAMB), and label-free MBs. In Table 1, the types of MBs and their properties, applications, advantages, and disadvantages are summarized.

111 Table 1. Examples of different types of MBs for biosensing

Type of MB	Description	Modification	Advantage	Disadvantage	Ref. No.
Based on labeled and unlabeled MBs					
Regular MB (RMB)	Fluorogenic DNA based detection tool composed of a short portion, including a loop and stem construction.	-ISMB (in-stem MB): In this strategy, threoninol nucleotides conjugated to a dye and a quencher are embedded into the stem area of MB as pseudo base pairs -MBs with two fluorophores.	Higher selectivity than linear DNA probes; Photo-stability owing to quencher and dye labels; Non-radioactive labels; Inherent signal transduction mechanism; Flexible conjugation. High quantum efficiency. Unique chemical structures, good biorecognition capacity, chemical simplicity, small size. Good thermal stability, easy synthesis; A good-designed MBs able to produce as high as 200-fold of fluorescence enhancement in optimal circumstances.	The interaction between the RMB and surfaces can make incomplete quenching, limited sensitivity, and low efficiency; difficulty in multi-targets recognition They need to be labeled with two non-native molecules (dye and quencher) that may make them inappropriate for long-term *in vivo* imaging, possible toxicity of labels, difficulties in synthetic routes, the need for purification, and separation procedures	[22-26]
Label free-MB	An MB without fluorophore/ quencher pair. e.g. colorimetry and surface enhanced Raman scattering (SERS)	quadruplex-based MB, Label-free	Cost-effective; High affinity; Noncovalent method; Fast and simple.	Quickly respond to enzymatic reactions *in vivo*.	[18, 27, 28]

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Based on activity and function					
Aptamer-MB (AMBs)	Single-chain RNA or DNA oligonucleotides (usually 25 to 60 nt) which have been generated through systematic evolution of ligands by exponential enrichment method conjugated with MB. (AMBs are similar to RMBs, except that the aptamer sequence replaces the complementary loop sequence between the two self-complementary strands that maintain the fluorophore and quencher pair in close proximity) (Figure 1F)	Aptamer-MB that targets single-stranded DNA-binding protein	Unique chemical features; Good biorecognition system; Chemical simplicity; Small size; Can be simply modified to combine markers or immobilize; It can be formed and utilized in non-physiological conditions. Good thermal stability, easy synthesis, and low cost -could be an alternative to the well-established immunoassay systems.	Many limitations occur in solution, pH, temperature, e.g., aptamers immobilization to the surface, and interactions with the microenvironment affect the aptamer conformation and target-aptamer interactions. These problems mean that interference caused by non-target molecules can occur.	[29-34]
Catalytic MB (CAMB)	DNAzymes are practical nucleic acids that can be isolated by using selection *in vit*ro system termed systematic evolution of ligands by exponential enrichment. To creation a catalytic MB, the DNAzymes acts as the loop section of an MB. The active region can recognize substrates by interaction with short DNA chains at a molecular level. (Figure 1G).	A vital modification is the combination of a 3′-3′ reversed nucleotide at the 3′ end. Phosphorothioate linkages that enhance stability of CAMB.	Positive flexibility that presents its bases to the outside; Ultra-efficient catalysis; Low background fluorescence signal; Low-cost. Easy synthesis, Easy modification can be adapted for the detection of non-nucleic acids targets, containing organic molecules and metal ions such as Pb2+ owing to The DNAzyme section. LNA-DNAzymes have high thermal duplex stability toward complementary RNA	Catalytic DNA has not been detected in nature, and all the molecules have been obtained by *in vitro* selection procedures. The phosphorothioate modification has been associated with toxicity, and it is seldom employed in the DNAzyme platform. -LNA-DNAzymes have been shown to have both biological potency and catalytic action during a single turnover reaction because DNAzymes with a reversed nucleotide at the 3′ terminal is catalytically more effective compared with their LNA-DNAzyme counterparts as a result of more rapid product release.	[35, 36]

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			or DNA, Better solubility because of a configuration similar to nucleic acids, and stability against nucleolytic degradation		
based on stability and resistance to nucleases degradation					
2′-O-Me-MB Adenine-biosensor riboswitch method to measure the regulatory of riboswitch transcriptional action. Nuclease-resistant backbone MB (Figure 1 C, a)	A dual MB (5′-OMB) and (3′OMB) were designed with fluorescein/DABSYL group.	Nuclease resistant with better stability than RMB in the cytoplasm; RNase resistant; Suitable reporters for transcription reactions. could be used as high throughput assays for the detection of new molecules targeting any riboswitch at the messenger RNA (mRNA) level, and with slightly increased affinity for the RNA molecule.	High background because of nonspecific interaction with molecules like proteins; Probe accumulation in the mitochondria and nucleus of cells as well as RNA misfolding, which sometimes may influence the results. (To avoid the nuclear accumulation, 2′-O methylated MBs were associated with a quantum dot (QD) with a dT-biotin linker group at the 3′ end, while the mitochondrial accumulation could be avoided using cyanine dye labels). They decrease the kinetics of the sensing event	[21, 37, 38]
PNA MB PNAs are DNA analogs in which the nucleotide bases are connected to a polypeptide mainstay including repeating N-(2-aminoethyl)-glycine parts (Figure 1C, c), (the interaction of PNA-DNA is shown in Figure 1E).	14- mer PNA immobilized on the Au electrode. 15- mer PNA onto a carbon electrode.	Since they do not contain any phosphate groups, they have an overall neutral charge and are not susceptible to cleavage by nucleases. Unique biochemical and physicochemical features; High stability and resistance to protease degradation. Powerful binding ability at different concentrations of salt, high stability at different pH	Occasional aggregation and reduced solubility limit their usage in vivo Penetration through the cell membrane is difficult. The uptake of PNA-based MBs into cells is difficult can undergo unpredictable changes with slight changes in the sequence, indicating that PNA-MBs may be problematic for widespread use.	[21, 39-41]

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		values Can be used as alternatives to RMBs for the recognition of RNA, DNA, SNPs, viruses, bacteria, etc. PNA-based electrochemical MBs have remarkable features such as rapidity, simplicity, miniaturization, and low-cost, as well as the ability to probe non-deproteinized unpurified DNA samples		
LNA-MB a type of negatively charged nucleic acid. The methylene connection joins the 2′ and the 4′ position of the ribose sugar (Figure 1C, b). The combination of LNA and DNA are depicted in	A stem modified with four stems, alternating LNA/DNA bases (with a ratio 1: 1) This interaction is used to immobilize MB on a glass surface followed by PEG[2].	Reproducibility; Excellent selectivity; Thermostability reflected in efficient target molecule hybridization; Low background signal; Robustness and nuclease-resistance. reproducibility, outstanding base mismatch discrimination ability. can hybridize to either RNA or DNA, show a remarkable increase in melting temperature having good performance when fixed on a solid plane, a better affinity for target sequences, have detection limits in the nanomolar range.	Interactions between the stem with other nucleic acids or between MB-MB and L-DNA can take place. (To solve this limitation, D-DNA is used, which can form steady duplexes with D-DNA. (Figure 1D). This design enhances the MB selectivity and stem stability and increases the Tm).	[21, 24, 39]

112
113 1. Peptide nucleic acid. 2. Poly ethylene glycol.

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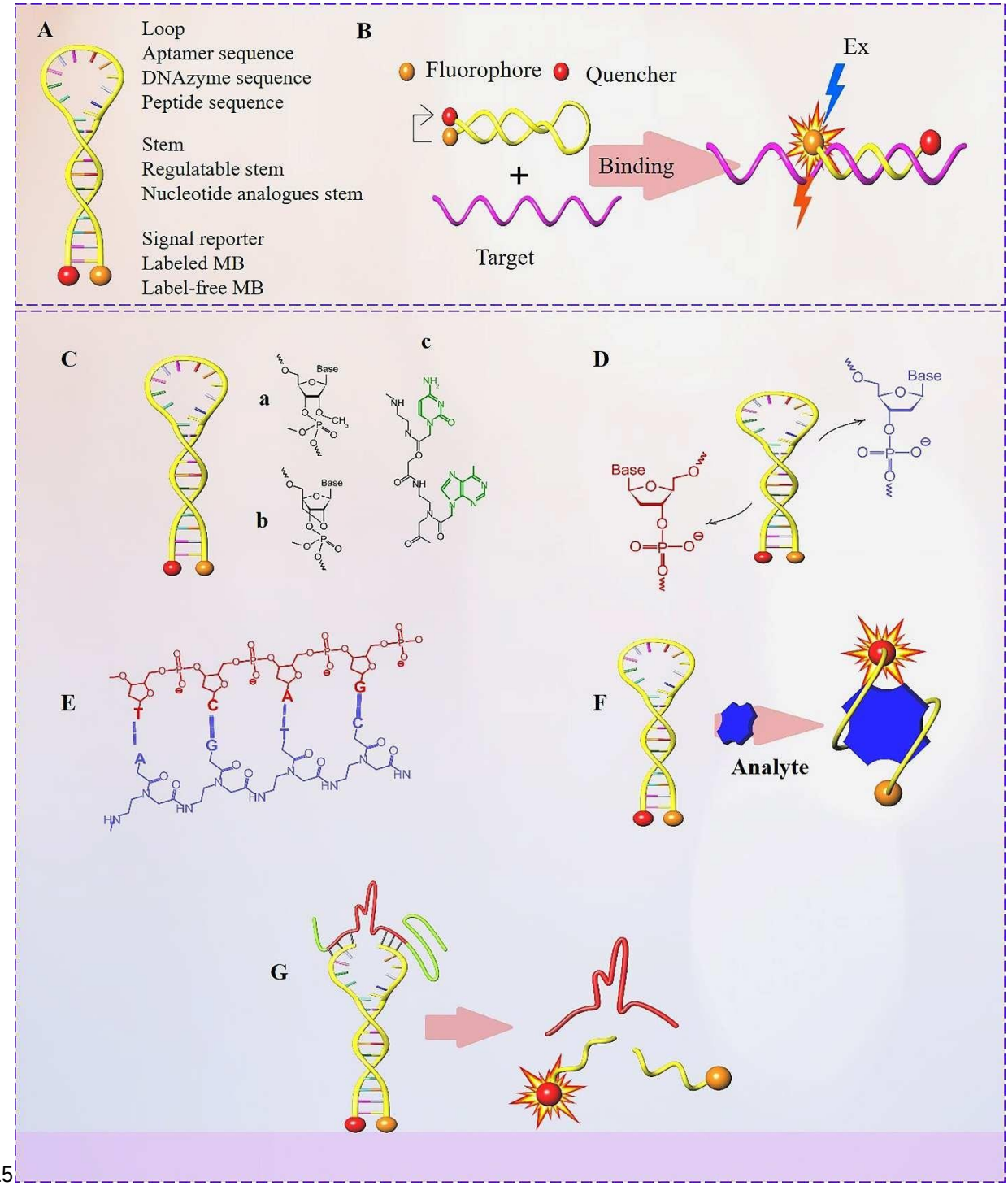
A
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Regulatable stem
Nucleotide analogues stem
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Figure 1. A) The schematic structure of MBs. B) During hybridization with target sequence, the hairpin construction of MB unlocks, which separates the quencher and dye leads to enhanced fluorescence intensity. Redrawn from ref [40]. C, a) Structure of 2′-O-methyl. C, b) LNA, and C, c) PNA. D) The structure of L-DNA. E) The chemical pattern of PNA attached with DNA. F) Schematic illustration of aptamer-MB G) Schematic illustration of CAMB [33].

3. Fluorophore and quencher pairs using in MBs

3.1. Fluorophores

Common examples of frequently used fluorescent dyes in molecular beacons include pyrene [42], cyanines, rhodamine, FAM (carboxyfluorescein), HEX (hexachlorofluorescein), CyTM, (TAMRA), JOE (3, 4′,5′-dichloro-2′,7′-dimethoxy-5(6)-carboxyfluorescein), ROX (carboxy-X-rhodamine), Alexa Fluor 488, Oregon Green 488 [20], EDNAS (5-(2′-aminoethyl) aminonapthalene-1-sulfonic acid), Tet (tetrachloro-6-carboxyfluorescein) [43], nanocrystals (quantum dots, lanthanides [36], and carboxytetramethylrhodamine. These dyes have the appropriate photophysical properties to be used in MBs.

3.2. Quenchers

Molecules that absorb the optical energy, so that it is diverted away from a fluorophore and transformed into heat are termed quenchers. A common example and one of the first compounds employed as a quencher in molecular beacons is Dabsyl (dimethylaminoazobenzenesulfonic acid) [43].

The fluorophore and quencher pair must be well-matched with both wavelength and extinction co-efficient. To optimize this pairing, two important points should be considered when Dabsyl and TAMARA were used in MBs.

I) Dabsyl is a hydrophobic and non-fluorescent molecule that is frequently used as a quencher for many fluorophores. Dabsyl may not be the best quencher, because many of the reporter

couples in common use have fluorescence production at wavelengths longer than 490 nm [20, 43]. Recently, graphene oxide (GO), and AuNPs which all possess a high quenching capacity, have been investigated as alternative quenchers [36, 43].

II) TAMRA is not only used as a quencher in MB probes but also has its own fluorescence emission at 577 nm, which may interfere with the fluorescent signal. Consequently, using a dark quencher without any natural fluorescence is preferable [20]. Traditional MBs with organic dyes and quenchers (e.g., FAM and Dabsyl) have limited sensitivity and deficient quenching [44].

Hence, rationally modified fluorescent MBs, such as quencher-free MBs (e.g., using two fluorophores, without employing a fluorophore and quencher), auto/quenched dimer-MBs, FRET-based MBs [44], label-free MBs, and phosphorescent MBs have all been explored [36]. Recently, approaches to enhance the efficiency of absorption, also to improve the possibility of the interactions between the fluorophore and the quencher with a cooperative quenching result, have involved using several quencher moieties in tandem which has been termed "super quenching" (SQ). This approach increases the Tm (melting temperature) of the MB. The higher Tm can increase the ability of beacon to distinguish between single mismatched sequences and perfectly matched sequences. This approach is useful for monitoring RNA species, which only have low copy numbers within cells (e.g., microRNAs) [21, 36].

4. ON/OFF switching of MBs

Because the target-probe duplex is more stable than the hairpin structure, at a sufficiently high temperature, the target forces the stem to unwind and turns it into a random coil structure. Target probe binding causes a conformational change of MB from the 'OFF' state (closed, dark) to an 'ON' state (open, bright). Then, by disconnecting the reporter from each other, the fluorophore starts to produce the detectable fluorescence signals. Eventually, by raising the temperature

167 further, the target-probe binding is destabilized, and the MB returns to its closed structure, and
168 the fluorescence emission decreases [1, 43, 45]. The melting temperatures of molecular beacons
169 are generally estimated at 60–85°C, while the theoretical Tm depends on the sequence length of
170 their stem regions, the concentration of probe and its target in solution, and the CG content of the
171 stem sequences [1, 20]. Although MBs tend to hybridize spontaneously with the target strand at
172 low temperature, $MgCl_2$ can accelerate the process, due to the strong stabilizing effect of divalent
173 cations like Mg^{2+} on the stem hybridization. For instance, the Tm of a MB with 13 bp stem
174 length is 27 °C in the absence of Mg^{2+}, but it increases to 56°C in solution with Tris-HCl, $MgCl_2$,
175 and pH 7.4 [1, 20]. Conventional MBs possess a fluorophore and a quencher, with 85%-97%
176 quenching efficiency. To improve the quenching, super quencher MB (SQ-MB) were designed
177 with three quenchers assembled together, which could enhance the quenching efficiency to
178 99.7%. Afterward, researchers reported a modern technique to design MBs having a fluorophore
179 molecule at the 5′ termination, known as a molecular groove beacon (MGB). In the absence of
180 the target, MGBs are able to quench the fluorophore like a quencher molecule [46].
181

182 **5. Modifications of MBs for the detection of non-biological targets**

183 **5.1. MBs for the recognition of cations and heavy metals**

184 Heavy metal pollution is a fundamental threat to the environment and health. Water-soluble Hg^{2+}
185 ions are the commonest form of mercury pollution, which can be widely distributed in water,
186 atmosphere, and soil [47]. Chronic exposure to Hg^{2+} ions causes some severe diseases for
187 instance, brain disorder, kidney disease, and many chronic disorders [48, 49]. Hence, the
188 detection of heavy metals in environmental samples and their management and removal is vital
189 for human health and other purposes.

Apart from the specific features of MBs in their loop regions, the stem region has also been used for important purposes. The thymine base has been shown to be an effective ligands to bind Hg^{2+} ions and create T-Hg^{2+}-T bridges [49], leading to a new design of MB to be developed. A short 14 nucleobase sequence with a T-T mismatch in the MB containing a FAM and DABSYL group, led to special interactions between T-T bases in the MB and Hg^{2+} ions occurring, leading to ultrasensitive detection of Hg^{2+} in solution. Similar MBs have been designed for the recognition of biothiols, homocysteine, glutathione, and cysteine [20, 50]. In this modified MB, the fluorescent part is a beneficial approach for the distinguishing the stability coefficients of Hg^{2+}/ligating structure and identification of bridge energies. The open state of the MB in the absence of Hg^{2+} permits the FAM dye to emit signals due to the distance from the quencher. While in the closed state in the existence of Hg^{2+}, this causes the hybridization of the T-T mismatched sequences, and the MB emits almost no fluorescence because the FAM is quenched by Dabsyl [50]. In this modified MB, the fluorescent probe can be used to determine the stability coefficients of the Hg^{2+}/ligating structure and measure the bridge energies (Figure 2A,a) [50]. But this system needed the applying of toxic molecules. To conquer this problem, Wang et al. [51] employed a sensor for Hg^{2+} based on construction-switching DNA. Hg^{2+} ion stimulated the folding of dye labeled DNA chain by T-Hg-T creation, which increased the fluorescence intensity. This biosensor had a LOD of 3.2 nM (Figure 2A, b).

MBs have been used for the detection of other cations like Cu^{2+} and Ag^{+} [52]. In the case of Cu^{2+} detection, graphene oxide (GO) was utilized to increase the selectivity and sensitivity of the MBs toward Cu^{2+} in solution. As shown in Figure 2B, the MB was normally adsorbed on the plate of GO, however, in the presence of Cu^{2+}, and after introducing the H_2O_2, the beacon was cut into small pieces by the ensuing Fenton reaction, leading to fluorescence restoration [53, 54].

213 Although GO has a high specific area, remarkable electrical and optical features, thin nano-
214 sheets, excellent sensitivity, the use of GO can raise the cost of the sensor. Hence, development
215 of the MBs is more practical than using nanoparticles [48, 55].

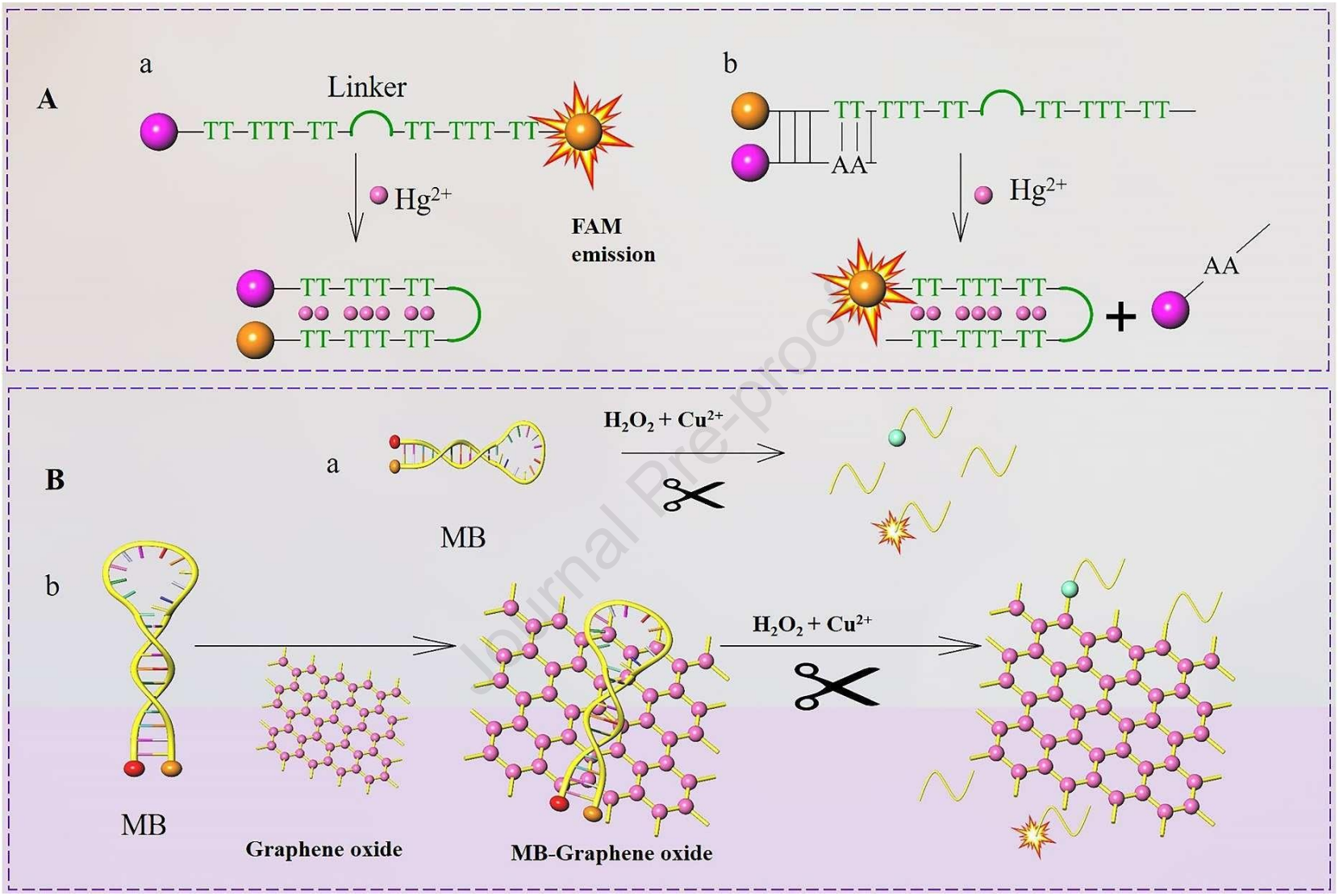

Figure 2. A) Thymine-Hg^{2+}-Thymine sensors which altered with dyes. (a) Diagram illustration of a turn-off sensor. Hg^{2+} ions mediate T-Hg^{2+}-T creation and make hairpin construction to form [50]. (b) Diagram illustration of a turn-on fluorescent Hg^{2+} sensor [51]. B) The principle of MB-GO for Cu^{2+} ion identification [54].

In 2016, a colorimetric Pb^{2+} approach based on MBs and DNAzyme reported by Yun et al. [56]. The substrate chain DNA of DNAzyme could be changed into MB construction with bases of stem section at the both terminations. The MB hybridized with enzyme chain DNA to create DNAzyme, and be triggered after adding Pb^{2+} ions. Then, MB released from the DNAzyme as two produce fragments. The fragments of MB attached to AuNP to effectually stabilize them in contradiction of salt-stimulated aggregation. The enzyme chain DNA was released to catalyze the more reactions. This approach showed high sensitive for Pb^{2+} recognition and a linear range from 0.05 to 5 nM and a LOD of 20 pM [56].

Recently, Xu and co-worker [57] described a DNAzyme/AuNRs approach for the recognition of Pb^{2+} ions using Cy3/ labeled DNA and AuNRs (gold nanorods) as the reporter (Figure 3A). A Pb^{2+} sensitive DNAzyme was used to detect Pb^{2+} ions. In the absence of Pb^{2+} ions, the DNAzyme substrate was firmly bound to the AuNRs providing a strong SERS signal, while in the existence of Pb^{2+} ions, the DNAzyme cleaved the substrate chain into two portions, allowing the Cy3-linked oligonucleotide segment to move away from the AuNR surface and led to a reduction in the SERS signal intensity. The sensitivity of this method for identification of lead ions was among the best of aptamer/based biosensors that have been described. The SERS platform possessed a low LOD (about 0.01 nM), broad linear range (0-100 nM), and remarkable selectivity, and stability. Besides, the DNAzyme is able to be opened and closed several times without losing its activity toward its substrate or its binding capacity [58]. Moreover, the SERS platform could be employed to detect Pb^{2+} ions in biological samples such as human serum samples and water with satisfactory performance.

Another study used a G-quadruplex-based FRET sensor for the recognition of K^+ [57]. The human telomeric DNA (21-mer) was marked with two dye moieties, 6-TAMRA and 6-FAM

243 (**Figure 3C**). In the existence of K^+, the sensor created a firm G-quadruplex with the two dyes in
244 near proximity to let FRET to occur. In another study, a sensitive DNA-aptamer based biosensor
245 was reported by Shi et al. [59] for monitoring K^+ ions. In this strategy, the aptamer was applied
246 as a molecular determination part, and a strand that is complementary to the aptamer section was
247 modified by pyrene at the 5′ and 3′ terminations, to allow the binding K^+ ions to the aptamer. In
248 the existence of K^+ ions, the complementary strand was replaced from the aptamer, which led to
249 excimer fluorescence from the pyrenes since the hairpin configuration of the complementary
250 strand brought them close together. But in the absence of K^+, the pyrenes just gave monomer
251 emission (**Figure 3B**) [59]. This method not only could distinguish K^+ ions with ultra-selectivity
252 in the existence of Ca^{2+}, Na^+, $Mg^{2+,}$ and NH^{4+} ions in biological liquids, but also might be used to
253 determine K^+ levels in living cells and human serum using time-resolved optical measurements
254 [59].
255 Although several scientific reports pay attention to MBs for the recognition of heavy metal ions
256 [7], but most of them were worked on a specific metal ion recognition. Though the recognition of
257 different ions could be gained by applying different MBs approaches in different levels, or by
258 covering the interfering ions, the fractional recognition was achieved. Thus, it is essential to find
259 an operative technique for simultaneously sensing a range number of heavy metal ions with
260 remarkable selectivity [7].
261 In **Table 2**, a comparison between different methods based on MBs for the detection of inorganic
262 cations is provided.

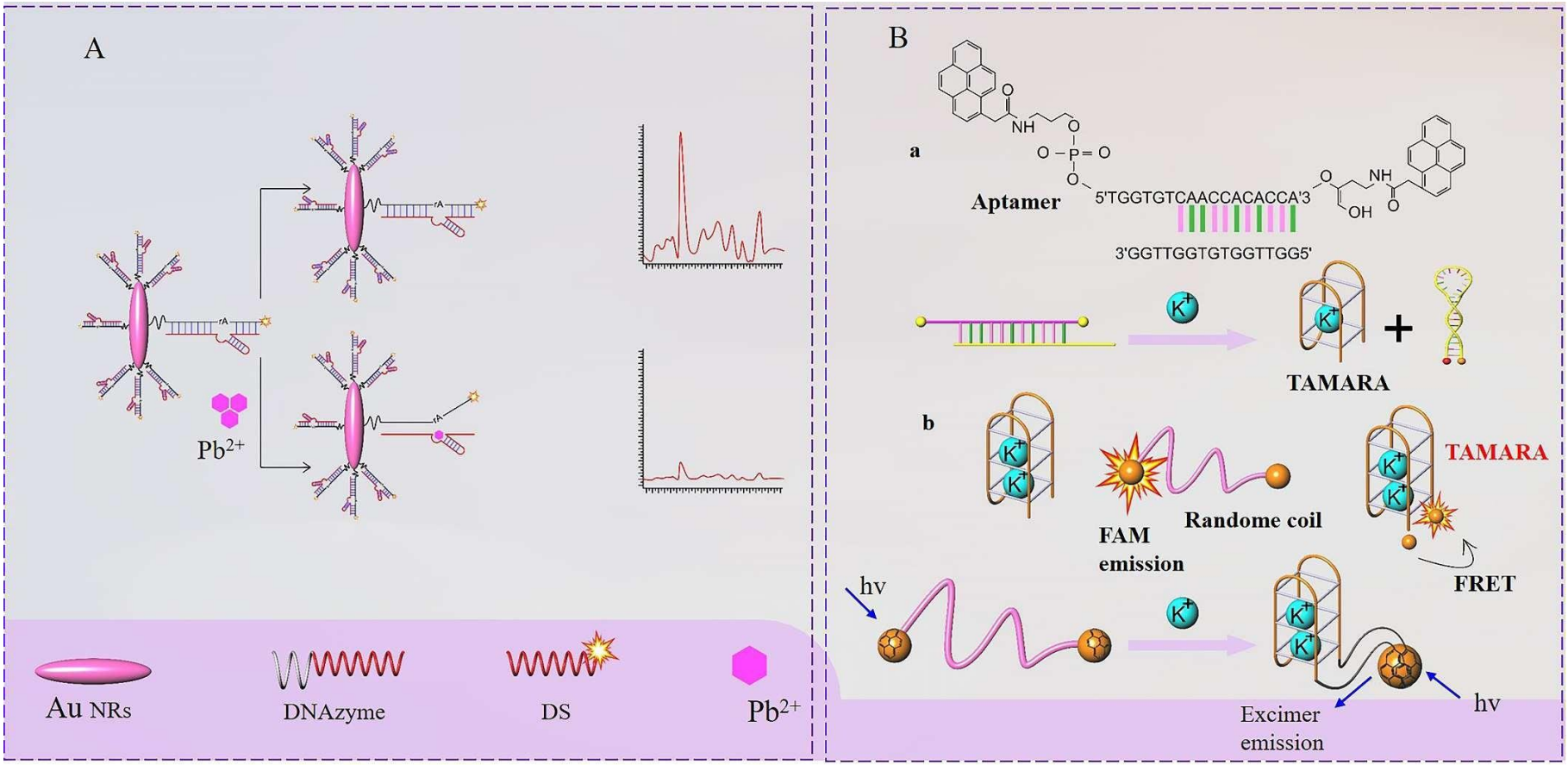

Figure 3. A) The principle of recognition of Pb^{2+} ions via DNAzyme SERS biosensor [57]. B) The general approach for aptamer/DNA sensors, the construction of the pyrene-marked MB, and the predictable hairpin construction creation for creation of the G-quadruplex K^{+} biosensors [59]. C) Structure of a G-quadruplex (a) Representation of a G-quadruplex-based FRET probe (b) Representation of a G-quadruplex probe (c) [57]. Abbreviations: hv, photon energy. G, guanine base.

272
273

Table 2. Different methods based on MBs for the detection of inorganic cations

Method	Target molecule	Type of analysis	LOD	Labels	Mechanism	Ref.
MB-Hg^{2+} with T-T mismatch	Hg^{2+}, homocysteine, cysteine, glutathione	NMR	Hg^{2+} = 19 nM (Hcys = 23 nM)	6-FAM/ DABSYL	Hg^{2+} interacts with the T-T mismatch and can create a T-Hg-T bridge. In the existence of Hcys the competitive ligation of Hg^{2+} by GSH/cy/ and the T-T mismatch occurred, and MB switched to ON state.	[50, 60]
Hg^{2+}-AT-rich-probe-Hoechst compound	Hg^{2+}, homocysteine, cysteine, glutathione	CD (Circular dichroism)	Cys= 0.1 mM Hg^{2+} = 5 nM	Hoechst Dye/ No quencher	The special interaction of Hoechst dye with the Hg^{2+} AT-probe/ hairpin caused a remarkable fluorescence enhancement, that is and sensitive for recognition of Hg^{2+} ions	[61]
MB based on DNAzyme cascade with CAMB	Hg^{2+}	Fluorescent	0.2 nM	FAM/ DABSYL	In the existence of Hg^{2+}, the specific T-Hg^{2+}-T connection induced the sections to generate a Mg^{2+}-dependent DNAzyme, that interacted with a MB to create the CAMB process. The activated DNAzyme could cleave many MB substrates through enzymatic turnover	[45]
MB-T3 MB-T5 MB-T7	Hg^{2+}	Fluorescent	1.9 nM 3.8 nM 44.2 nM respectively	TAMARA/ BHQ2	With T3 mismatches, the MB showed higher sensitivity but a limited dynamic range. By handling the numeral of T-T mismatches to 7, MB showed a broader dynamic range.	[48]
MEF	Hg^{2+}	Fluorescent	1 nM	FAM/ (CH2)3-S-AgNP	The MB beacon was stacked onto the silver nanoparticle surface with thiol-silver chemistry in the existence of Hg^{2+} ion, the hairpin could be opened since Hg^{2+} ions mediate the creation of T-Hg^{2+}-T complex between the non-complementary chain and the hairpin structure leading to fluorescence enhancement.	[62]
graphene-based cu^{2+} ions-MBs	Cu^{2+}	Fluorescent	~50 nM	FAM/ DABSYL	In the OFF-state GO could absorb the MB, after the addition of H_2O_2 and Cu^{2+}, the molecular beacon was cut into small pieces, leading to fluorescence restoration	[54]

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DNAzyme-AuNRs	Pb^{2+}	SERS signal	0.01 nM	Cy3/ labeled DNA designed AuNRs	In the existence of Pb^{2+}, the DNAzyme cut the chain into two portions, releasing a few Cy3/ linked oligonucleotide segments apart from the AuNRs area, the intensity of the SERS signal decreased.	[57]
CAMB-DNAzyme sensor	Pb^{2+}	___	600 pM	FAM/ BHQ	In the existence of Pb^{2+}, the MB was cut into two portions, releasing the label, resulting the increasing of fluorescent signal intensity.	[33]
DNA-aptamer based fluorescent biosensor	K^{+}	Fluorescence spectrum	4×10^{-4} M	Pyrene-Pyrene	In the existence of K^{+}, the complementary chain was replaced from the aptamer, which produced excimer fluorescence of pyrenes since the hairpin configuration of the complementary chain brought the pyrenes close together.	[59]
Pb^{2+} biosensor based on MB and DNAzyme	Pb^{2+}	colorimetric	20 pM	Label-free	DNAzyme could be changed into MB construction with bases of stem section at the both terminations. The MB hybridized with enzyme chain DNA to create DNAzyme, and be triggered after adding Pb2+ ions. Then, MB released from the DNAzyme as two produce fragments. The fragments of MB attached to AuNP to effectually stabilize them in contradiction of salt-stimulated aggregation.	[56]

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5.2. MBs for detection of low-molecular weight organic compounds and protein analytes

Cocaine is a one example of low-molecular weight organic compounds and is the second most consumed drug around the world[63]. In the field of cocaine, a sensitive optical biosensor based on signal amplification by rolling circle amplification (RCA) and an aptamer, with a LOD of 0.48 nM, that was developed by Ma et al. According to this report, the magnetic beads were functionalized by AuNPs on which the anti-cocaine aptamer was immobilized and interacted with a short oligonucleotide chain. In the existence of cocaine, the short oligonucleotide strand (cDNA) was replaced from the aptamer by the cocaine binding. Then, the magnetic beads allowed the short DNA strand to be separated, and it could be used in the RCA process as a primer. The final binding was detected by a fluorescence signal created upon MB hybridization with the final products of RCA, while in the absence of cocaine, there were no RCA products to hybridize with the MB and no fluorescence emission (Figure 4). This system could also be used for the amplified identification of low molecular weight proteins using RCA since there is a wide availability of aptamer molecules. Moreover, cDNA might be attached to AuNPs functionalized magnetic beads for the identification of different analytes [64]. Nevertheless, MB-based RCA techniques suffer from some inherent problems. For instance, DNA hybridization between separate MBs sometimes occurs, and no obvious fluorescence change is detected [35]. RCA is a labor-intensive and time-consuming procedure to create a long-repeat DNA and to produce a circular padlock [65].

In another investigation, a simple, and cost-effective biosensor based on a hemin DNAzyme MB and G/quadruplex was developed for the recognition of methamphetamine (METH) with a LOD about 0.5 nM. The METH concentration in urine specimens measured by the MB. By replacing

some bases in the MBs and selecting different aptamers, this DNAzyme MB probe approach might offer a versatile method for selective and sensitive identification of several analytes [66].

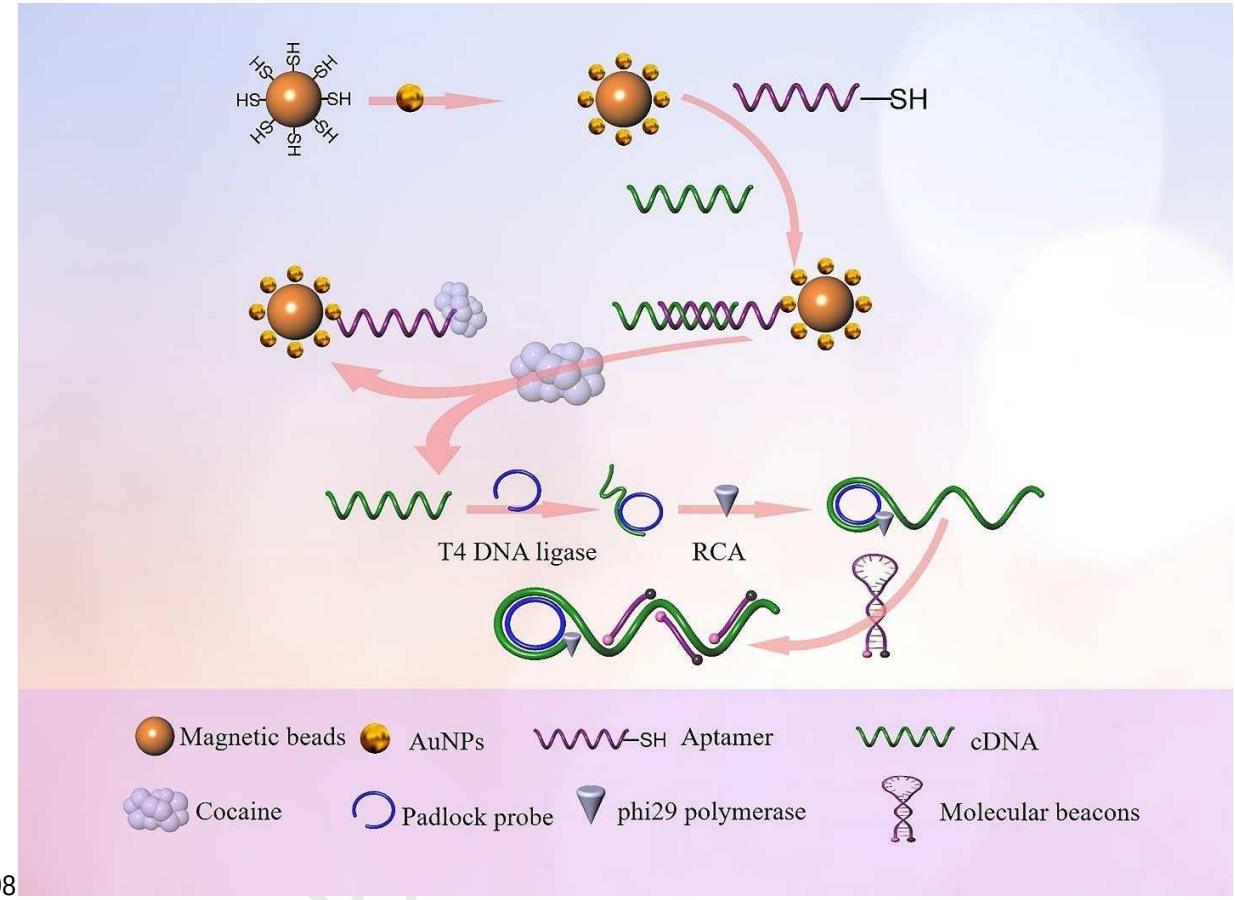

Figure 4. Diagram outline of a sensor for the detection of cocaine [67].

In the field of METH detection, a label-free and cost-effective colorimetric sensor based on the hemin/G-quadruplex DNAzyme MB was designed which had a LOD of 0.5 nM and a linear range from 8 to 500 nM. The probe may suggest a new method for recognition of a varied spectrum of analytes by altering some bases of probe and selecting different aptamers [66].

Gas chromatography and chromatography-mass spectrometry are the traditional instrumental investigations of addictive stimulant drugs such as cocaine and methamphetamine. However, they encounter on complex specimen pretreatment, high-cost tools and accomplished operators

which inhibit their use for onsite recognition. Therefore, portable MBs have been intensively considered as they suggest attractive benefits over old instrumental investigation, e.g. easy operation, cost-effective, and extensive range of analytes. Also, they promise a good ability for the fast recognition of cocaine and methamphetamine [66, 68].

In another gene detection strategy, a simple MB based on the "kissing/hairpin" configuration was developed for the identification of thrombin and β-actin genes. To create a kissing hairpin structure, two corresponding hairpins were mixed equally together. In the existence of the target, the hairpin structure was opened (as shown by kinetic analysis), then the kissing hairpin structure formed a duplex structure leading to fluorescent enhancement. The flexibility, sensitivity, and simplicity of these MBs suggest they could be used for further applications. This platform can also detect small molecules and proteins [69]. Kim et al. employed the electrochemical identification of the thrombin gene using MABs. After thrombin addition, due to a structural change in the MAB, the methylene blue label was released, leading to a decrease in optical absorption. The LOD of this method was 11 nM [36]. Gao et al. [70] developed a method for SERS (surface-enhanced Raman spectroscopy) recognition of thrombin using rhodamine 6G (R6G)-AgNPs as a label by attaching DNAzyme assisted DNA RCA (recycling and rolling circle amplification). In this system, the MB was fixed on a glass plate, and after thrombin-induced switching in the DNA hairpins of probe1, the DNAzyme was released from the caged construction and interacted with the MB in the existence of Zn^{2+} and started the DNA recycling procedure, resulting in the cleavage of MB and the generation of a large amount of primer for the RCA reaction. The RCA products bound to an oligonucleotide with AgNPs used as reporters (Figure 6A) [70].

6. Modification of molecular beacons for detection of biological analytes

6.1. MBs for nucleic acid recognition

Reliable, and convenient recognition of nucleic acid sequences (DNA, RNA) is required in gene therapy, drug screening, and disease diagnosis [35]. Therefore, many investigations using MBs for nucleic acid detection have been performed.

6.1.1. Gene detection

Several bioanalytical platforms have been applied for the recognition of biomarkers, containing flow cytometry and the polymerase chain reaction (PCR) for the recognition of specific genes. The p53 gene is often mutated gene in cancer, resulting in the rapid growth of cancer cells, making it an important tumor biomarker at the protein and nucleic acid level[41, 71]. Li et al. employed a label-free CAMB method for the recognition of p53 DNA. This CAMB was a multi-functional probe that acted as a catalytic DNAzyme, the target recognition sequence, and the primer for the reaction. As depicted in Figure 5, when the MB was assembled into its structure, and after the addition of dNTPs, DNA polymerase and in the existence of p53 gene as a target DNA, interaction of p53 DNA caused a new configuration in the central area to create the DNAzyme-G/quadruplex that could cover the hemin fragment. As a result, an extension from the 3′ terminal occurred. The G-quadruplex construction in the SDA products bound to hemin to form the DNAzyme/aptamer–hemin compound with peroxidase enzyme activity to generate a green color of $ABTS^+$ [72].

In another study, a dual-stem hairpin system was reported for the recognition of the p53. According to this method, in the existence of the p53, a cascade reaction took place, resulting in the nicking of one oligonucleotide probe, which caused aggregation of the G-quadruplexes and a change in the colorimetric signal [35]. Wang and et al. developed a multi-functional rapid

355 detection MB based on reverse SDA and target-induced ICSDP. When p53 DNA hybridized
356 with the MMB, the circular p53-displacement process began and a double chain with a nicking
357 site for Nt. BbvCI was created. Consequently, the dual-chain was cleaved, and a new reaction
358 started, producing many nicked sequences that could hybridize with other MMBs and amplify
359 the signal. ICSDP was used in this sensing method to improve the performance of nucleic acid
360 sequence detection [73].
361 The cancer diagnosis suffers from restriction of deficient accuracy. So, applying MBs as
362 sensitive, fast and adaptive analytical approaches are vital for developing the accuracy of cancer
363 diagnosis but traditional MBs do not meet these necessities. The amplification pathways
364 employing enzymes, DNAzymes and AuNPs, also DNAzyme-G/quadruplex approaches are
365 usually quite practical and are not limited to certain tenders.
366
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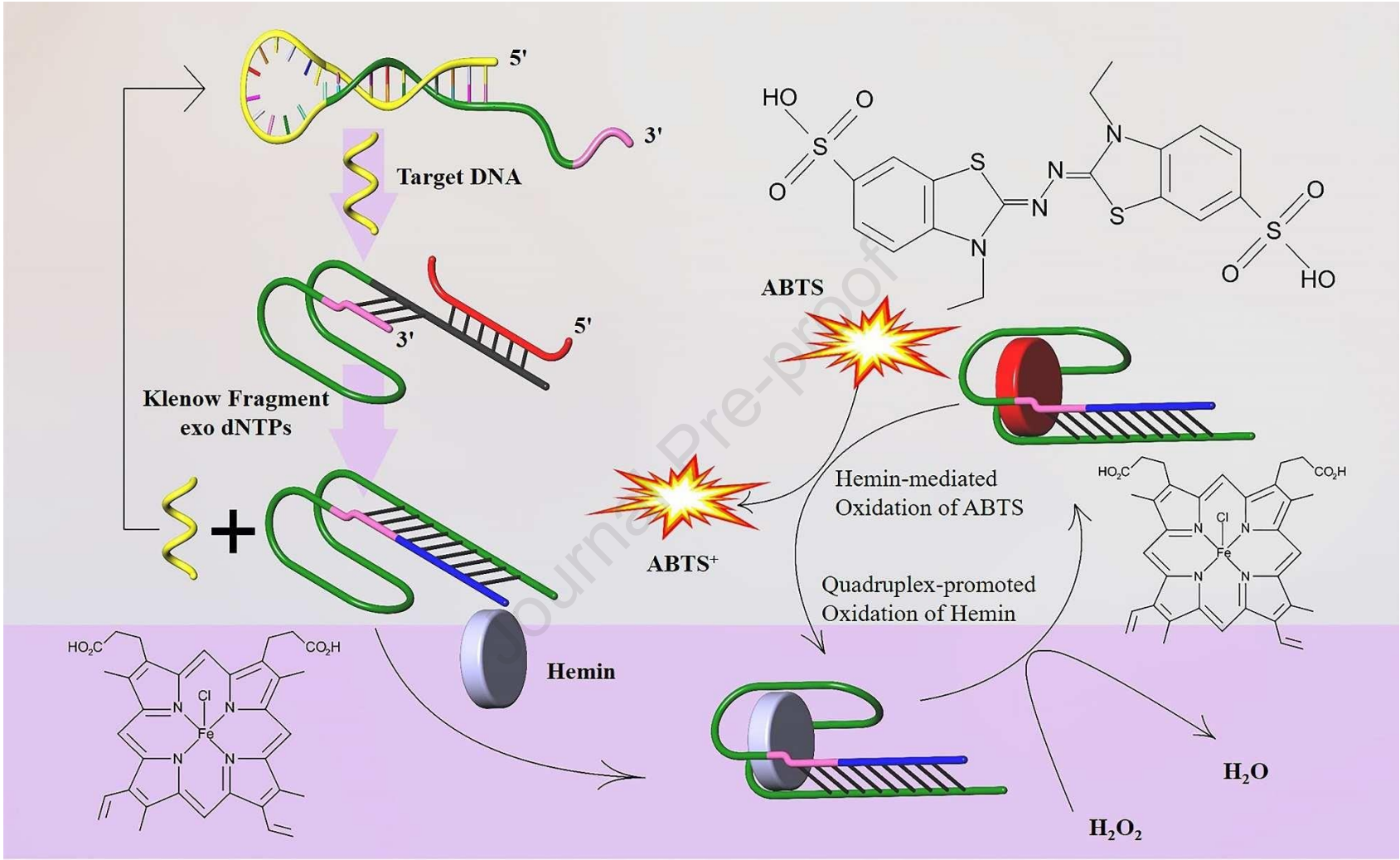

Figure 5. Schematic representation of the DNAzyme/G-quadruplex biosensor [72].

6.1.2. MBs for recognition of SNPs

SNPs make almost 90% of the genetic variations found in humans and are regarded as effective and valuable molecular and genetic markers for clinical diagnosis, biomedical research, disease therapy, and drug development [74]. They can be identified using PCR amplification, but this process is a relatively sophisticated and time-wasting method, which is prone to false-results because of artificial amplification. Therefore, a combination of the PCR method with specific MB design has been employed to develop an improved real-time method to detect base mutations [36]. Research studies have been focused on improved signal transduction methods, including electrochemical, fluorescent, colorimetric, etc.

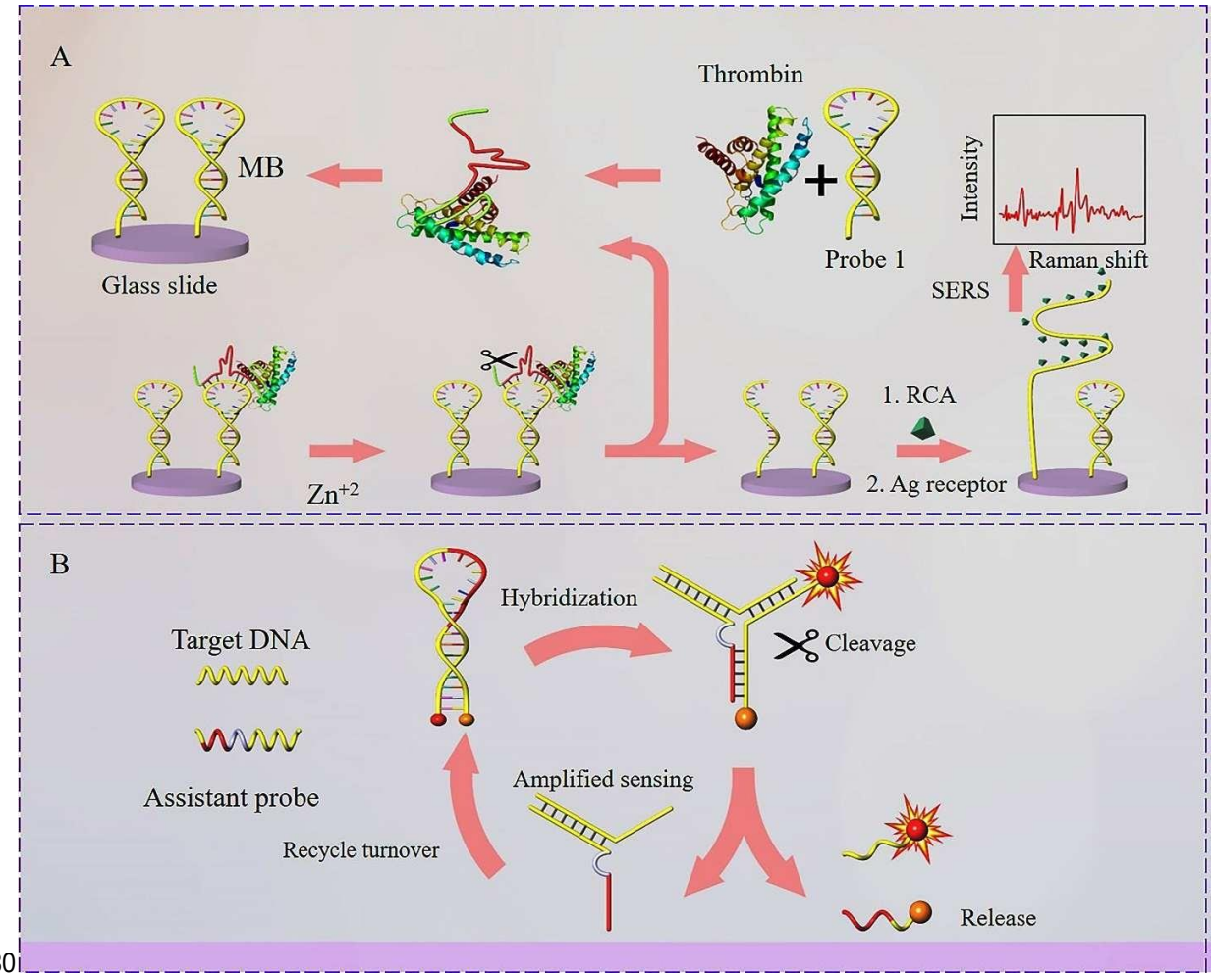

Figure 6. A) Illustration of SERS analyzes for thrombin recognition [70]. B) Illustration of a MB-based connection probe method for SNP recognition [75].

Among these methods, the fluorescence has attracted the most attention due to its low cost, ultra-sensitivity, and rapid response [76]. In this field, an MB-based ‘‘Y’’-designed biosensor with powerful SNP detection capacity was reported. The design of the MB included three sections: a target DNA, an MB probe, and a helper probe. The probe contained the location for the nicking endonuclease enzyme Nt. BbvCI, which bound to the dsDNA (Figure 6B). By changing the helper probes and the target of the MB, the MB could carry out widespread DNA target identification and SNP monitoring [75]. On the other hand, the nicking endonuclease needs a specific location, so it suffers in general tender [77].

Two rapid, sensitive, and simple approaches based on a hairpin structure functionalized LFSB and HO-AuNP biosensor were reported for SNP detection. In the first method, the HO was designed with biotin and a thiol group (at the 3′ and 5′ terminals respectively), which were attached to the AuNPs through self-assembly. After the addition of MUD (11-mercaptoundecanol) as a blocker (to lock the remaining AuNPs as well as to keep the HO probes correctly oriented on the AuNP surface), there was an excellent DNA hybridization. The use of MUD could decrease the nonspecific binding of the AuNP/ HO to streptavidin in the binding site of the LFSB. Because of the long hydrocarbon chain of MUD and the hairpin configuration of HO, the biotin group was protected on the AuNP surface, which made it inactive. Nevertheless, this method required a lengthy process time and had a poor LOD (about 0.5 nM). To overcome these limitations, a dATP blocker was used in the second approach. During the hybridization reaction, the AuNPs bound to the activated biotin group on the LFSB site via interaction with pre-immobilized streptavidin. Finally, the agglomeration of the AuNPs produced a color change

to detect the SNPs. This method provided good selectivity and lower background compared to other MB based SNP identification techniques and could identify as low as 10 pM of well-matched DNA in the presence of one-base-mismatched DNA in 25 minutes. The protocol was cost-effective, rapid, and its simple screening of SNPs might find many applications in diagnosis [78].

Later, Zhang et al. employed a sensitive florescent MB (using FAM and Dabsyl as reporter groups) for detection of SNPs with a LOD of 8 nM and a linearity range of 17.58 nM-1.125μM. This method had some advantages, including excellent reproducibility, ultra-specificity, and sensitivity [76]. Though these MB based SNP identification approaches can conquer some of the shortage of old methods of SNP recognition, these procedures have not yet been used for sensitive, accurate, low-cost, and rapid SNP detection in clinical settings [78].

SNPs which are broadly spread in the genome, have been directly related to human disorders. High quantity approaches are vital for finding the spreading of genetic variations in human and for recognizing the genes in charge of genetic diseases. Among the various methods in detection of SNPs, MBs system has attracted increasing attention. The combination of MBs and AuNPs and other designed of MBs such as HO-AuNP are practical for monitoring SNPs.

6.1.3. MBs for detection of mutations

Mutations in the genome are the cause of many diseases, including K-Ras mutations, which perform a vital function in the early stages of carcinogenesis. Accordingly, one group developed a colorimetric isothermal cascade amplification system based on dual-MB and SDA for the recognition of K-Ras mutation with a LOD of 4 pM. In the existence of K-Ras, a loop area of DHMB was opened, binding to the K-Ras/biosensor duplex in order to start reactions. This led to

the synthesis of dsDNA, including two binding locations for the enzyme while leading to the removal of the hybridized K-Ras. The displaced K-Ras could begin the next reaction sequence. The Nt.BbvCI endonuclease led to the repeated occurrence of strand displacement amplification/nicking reaction in both sections of the resulting dsDNA, producing a G-quadruplex bound DNAzyme that could attach to hemin molecule to achieve ultra-sensitive colorimetric signal. This DHMB could be a new route to analyze mutations occurring in genetic disorders [65].

Furthermore, another rapid, simple, and selective system for DNA identification based on the T7 exonuclease and GO was constructed with a LOD of 0.3 pmol/L. The existence of the target resulted in the creation of dsDNA followed by the digestion of the biosensor by exonuclease, which caused the recovering of target and enhancement of the fluorescence signal. This system did not require extensive modification of the MBs or a time-consuming thermal cycling system [77]. Ting and et al. also reported a nucleic acid identification system based on the immobilization of an adenosine-based MB. In this method, two types of ABMB probes with twelve adenosine bases at 3′ and 5′ terminals were used. By using coralyne (an intercalating molecule for poly-A) which can interact with the adenosine bases, the ABMB took up a hairpin configuration and was immobilized within the microplates by the interaction between streptavidin and biotin. According to this system, the interaction between the ABMB and the target forced a structural change to occur in ABMB. In the presence of the target, 4-MUP was catalyzed by ALP, resulting in a measurable fluorescent emission at 450 nm. This system could be employed to study SNPs as well as identification of single-base mismatch and gene mutations in genetic diseases, such as the fumarylacetoacetate hydrolase gene at room temperature within 1.5 hours [46].

AuNPs are used in nucleic acid detection because of their low toxicity, ultra-chemical stability, plasmonic features, high biocompatibility, and good homogeneity. However, MB assays based on SERS by AuNPs and reporters sometimes suffer from poor Raman enhancement of the reporter group, mainly when the AuNP size is smaller than 15 nm [79].

In another report, a complex QD-AuNP/CdZnSeS-0S1.3-SiO_2.ZnSe1. nanohybrid system was developed as a MB signal producer for sensitive DNA identification. During the interaction between complementary target with MB, the structure of the MB was opened, which induced a LSPR signal from the AuNPs, leading to fluorescence emission [80]. During a study, an electrochemical sensor for recognition of DNA utilizing cascade signal amplification and MB-mediated biotin/streptavidin, CSD (circular strand displacement)/RCA (rolling circle amplification) method was developed [81]. In this method, target interacted with the MB fixed on the Au electrode, which caused the CSD. Additionally, by biotin-streptavidin interaction, the RCA was triggered, allowing many cycles of repeated DNA synthesis for binding a number of biotinylated recognition probes. Song et al. [82] described an electrochemical sensor based on AMB (allosteric molecular beacon) for DNA recognition, which included a streptavidin aptamer locked by its conformation. In the presence of the target, the interaction between the AMB and target DNA made the MB to open and created a SA-aptamer construct, which stimulated the capacity of the AMB to attach to SA. Finally, the probe could conjugate to SA beads, which in turn could fluoresce. The utilize of SA allowed enrichment of the target-bound probe on biocompatible microbeads that enhanced the overall signal intensity. Moreover, this MB not only could be used to selectively determine a varied range of targets, but was also inexpensive to prepare (only requiring a fluorophore) and could recognize various targets in biological

474 specimens. Some reports concerning the use of MBs in detection of DNA-related targets are
475 summarized in table 3.
476
477
478 **6.2. MBs for recognition of messenger RNA in living cells**
479 Messenger RNA (mRNA) is found in some locations within the cytoplasm of cells and is a vital
480 post-transcriptional process [83]. Cancer is a widespread disorder that affects the lives of people
481 global and [84]. Effective treatment for this disease depends on early diagnosis, and detection of
482 specific cancer biomarkers can be utilized to detect cancer and screen its progression and
483 response to treatment [85, 86]. Some mRNAs are presently being employed as biomarkers since
484 the expression levels are related to the progression of the malignancy and the tumor burden [41,
485 87]. It is important to improve techniques for mRNA recognition in living cells. A living cell
486 includes complicated machinery that is varies depending on both extracellular and intracellular
487 stimuli. Proteins, cytosolic mRNA, and nuclear DNA modulate many cellular functions and
488 govern many biological procedures, containing cell differentiation, oncogenesis, apoptosis, and
489 proliferation [88, 89]. To detect and quantify mRNA contents, there are two vital subjects to
490 consider: stability and delivery. In the past, many methods were investigated to deliver MBs
491 through the membrane of the cell, containing the use of cell-penetrating molecules,
492 microinjection, transfection with cationic lipids, and streptolysin O (SLO) [90]. Recently, AuNPs
493 have been applied to deliver MBs into cells[2]. For instance, the intracellular imaging of mRNA
494 utilizing hairpin DNA-coated AuNPs was reported [91]. To demonstrate the value of this
495 approach, h-AuNPs were modified to target the mRNA of the respiratory syncytial virus. Qiao et
496 al. [92], reported an effective method for the identification of several different tumor mRNAs

(Figure 7A). A special bimolecular beacon called AuNP/bi-MB was collected from AuNPs to target two kinds of tumor-specific mRNA (survivin and cyclinD1) in breast cancer cells. This approach does not show false-positives and could be applied for early recognition of tumor. Li and co-workers [93] reported the in vitro multi-color fluorescence detection of three cancer-related mRNAs (TK1, GalNAc-T, c-myc) in different cells. The probe structure has been shown to possess higher resistance against nuclease cleavage, and more precision compared to single-marker probes, with the potential to distinguish tumor cells from normal cells in liver and breast cancer models. In another study, Pan and et al. reported a four-color nanoprobe that was able to distinguish up to four types of mRNA in living cells (Figure 7B) [94]. This nanoprobe contained AuNPs with a thick corona of MBs that could determine several intracellular mRNA transcripts, providing enhanced accuracy for tumor cell recognition compared to single MBs. The MBs were marked with Alexa fluor 488, Alexa fluor 405, Cy5 and Cy3 to detect survivin mRNA, TK1 mRNA, GalNAc-T mRNA, and c-myc mRNA, respectively [94].

Intracellular mRNA monitoring and recognition able to yield appreciated information for adaptive treatment, and new drug discovery. In of the numerous recent live-cell imaging approaches for mRNA, MBs may be the most marvelous since they are not need the complex genetic manipulations of approaches. In this field, some methods such as AuNP/bi-MB and multi-color fluorescence were employed by researchers. AuNP/bi-MB does not show false-positives and could be applied for early recognition of tumor. Multi-color fluorescence detection of mRNAs shown to possess higher resistance against nuclease cleavage.

517 Table 3. MBs for detection of nucleic acids and genes

Method	Target	Category	LOD	Linearity range	Labels	Benefits	Ref.
G-quadruplex MBzymes mediated label-free amplified	P53	Colorimetric	25 fM	25 fM-250 nM	Label-free	Highly effective enhancer for hemin- G-quadruplex DNAzymes complex for biomedical diagnosis and biotechnology.	[72]
DHP-based cascade amplification	P53	Colorimetric	1 fM	1 fM-150 nM	Label-free	Simple structure, cost effective, high stability Colorimetric MBs allow detection of point mutations by the naked eye.	[35]
MB based on R-SDA and ICSDP (MMB)	P53	Fluorescence	1 nM	1 nM-100 nM	FAM/ BHQ-1	Rapid and simple multi-functional MB, high specificity and sensitivity, high stability in human serum, results in 30 min without costly nanomaterials, ICSDP useful for signal amplification.	[73]
CHA-YNEASA circuits	P53/ cancer cell	Fluorescence	0.9 pM	___	FAM/ Dabsyl	Rapid, sensitive, cyto-sensor with hairpin structure, capable of measuring MUC1 positive breast cancer cells, no interference in 10% bovine serum.	[71]
MB-T7 exonuclease-GO	Nucleic acid	Fluorescence	0.3 pmol/L	___	FAM/GO	Does not need modifications of MBs, no time- consuming thermal cycling system	[77]
MB/biotin -Au-NP based DSNAB1 test	Nucleic acid	___	50 pM	0.25-50 nM	Thiol/Biotin	Short experiment time, high sensitivity, no need for high cost sophisticated instruments.	[95]
AuNP-SiO_2-QD-MB	Nucleic acid	Fluorescence	1.4 fM	___	AuNP-SiO_2-QD /BHQ-2	High stability, differentiate between complementary target DNA and single-base mismatch	[80]
MB-mediated CSD[2]-RCA	Nucleic acid	DPV[3]	9×10^{-16} mol L^{-1}	1 fM to 100 pM	SH-(CH2)6/ Biotin	Wide dynamic range, and reproducibility, does not need thermal cycling or complex amplification marks, useful for DNA recognition in clinical diagnosis and pathogen recognition.	[81]

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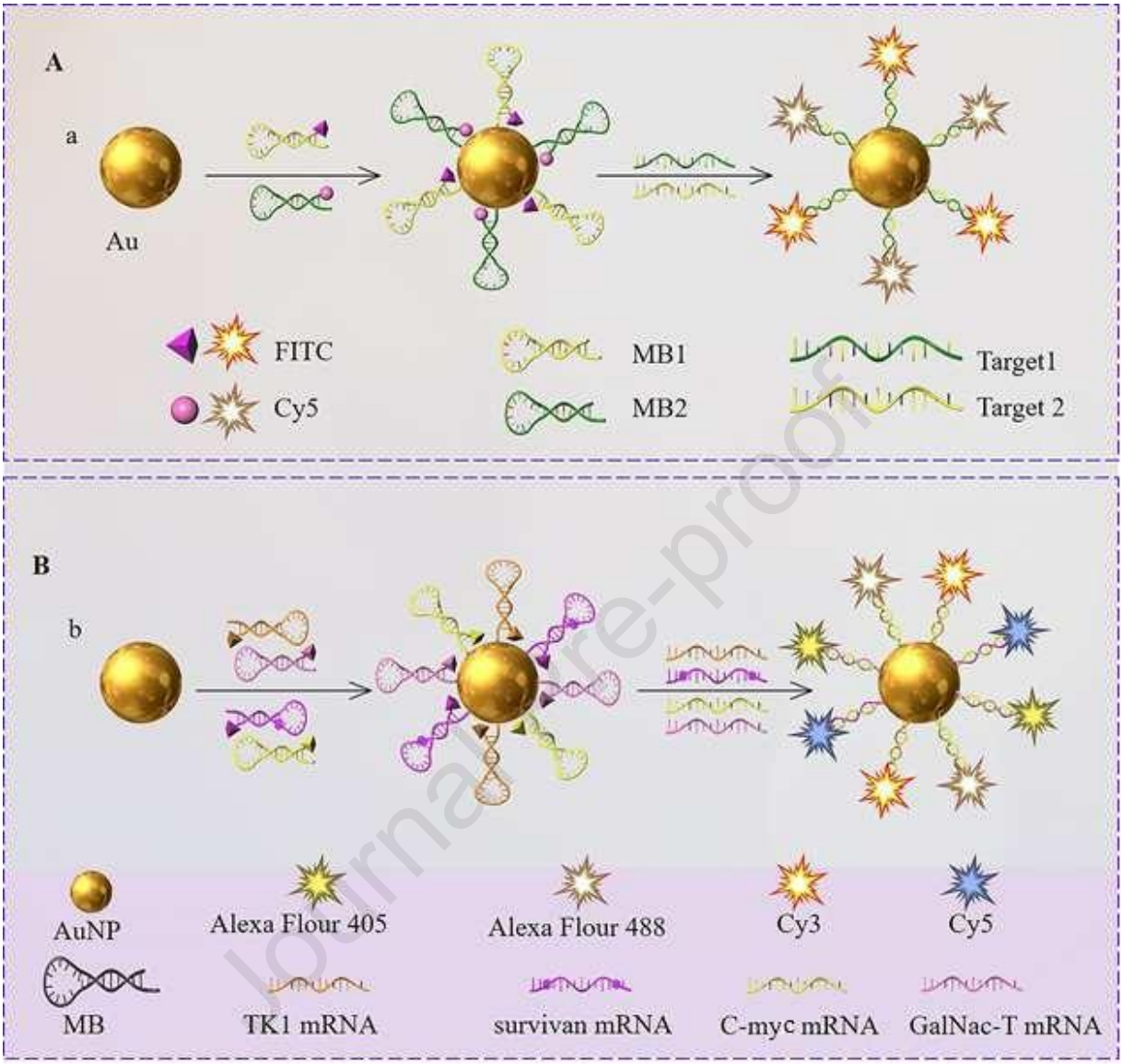
aMB[4]	Nucleic acid	Electrochemical	15pM	___	FAM, TMR, and Cy5	Inexpensive, good sensitivity, selectively detects a range of targets in biological samples	[82]

518

519 1, dry-reagent strip-type nucleic acid biosensor. 2, mediated circular strand displacement. 3, differential pulse voltammetry. 4, allosteric molecular

520 beacon.



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522 **Figure 7**. A) Diagram of the assembly of bi-MB /AuNP [92]. FITC= fluorescein isothiocyanate,
523 Cy5=cyanine 5. B) Diagram illustration of the Four-Color Nanoprobe [94].
524

525 **6.3. MB for the detection of microRNAs (miRNA)**

526 miRNAs which were first described in 1993 have vital roles in regulating many cellular
527 mechanisms, for example, cell differentiation, development, apoptosis, stress responses, and cell
528 proliferation, in both diseased and normal physiological conditions[96-98] .

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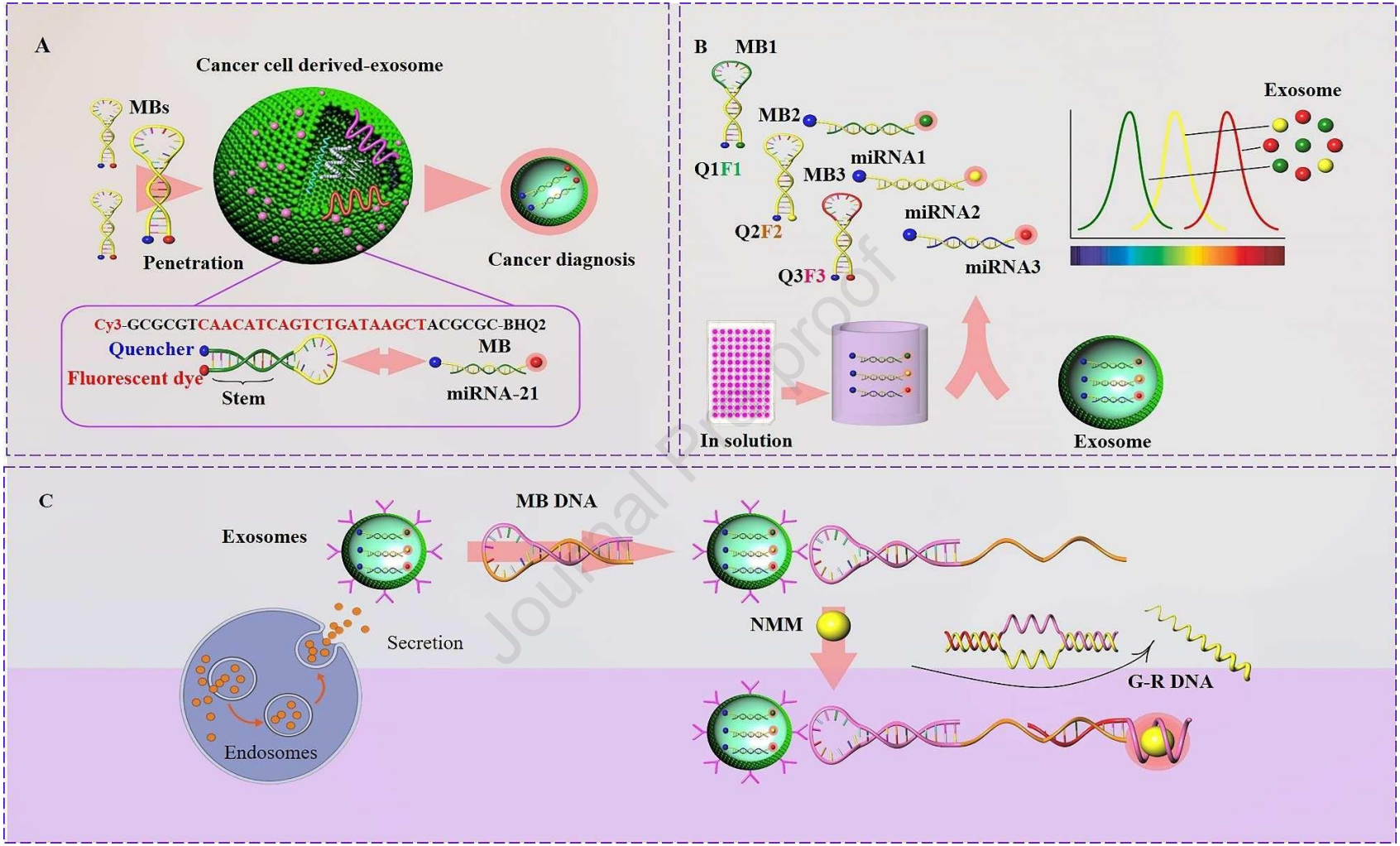
In this field, Lee and et al. employed a MB for the detection of exosomal microRNAs that could be used for the diagnosis of several diseases [99]. Exosomes are tiny extracellular vesicles with 30-100 nm diameter that contain miRNAs derived from their specific cells of origin and can be transported through the circulatory system. In this study, their MB targeted miR-21 expression in the cell line of breast cancer (Figure 8A). They confirmed that the MB could monitor the levels of miR-21 in the tumor-derived exosomes, even in the existence of serum in a fast, simple, and sensitive manner. Later, the same group [100] reported a system for the in situ simultaneous detection of several miRNAs in whole exosomes from MCF-7 breast cancer cells using MBs (Figure 8B). Exosomal miR-27a, miR-21, and miR-375, which all play a significant role in cancer, were selected as the target miRNAs. MBs with different fluorophores (FAM, Cy5) were constructed for each miRNA. This system not only allowed multiplexed recognition of three different miRNAs, but also was high-throughput, highly specific, accurate, and low cost.

Exosomes play significant roles in the cellular invasion, communications, and tumor migration, however, the specific detection of cancer exosomes remains challenging because of their very low concentration in biofluids [101, 102]. An activatable and label-free aptamer probe was considered for the recognition of exosomes in blood [101]. In this report, the exosomes initiated a chain replacement reaction to form a G-quadruplex construction, which led to an enrichment in the fluorescence intensity of N-methyl mesoporphyrin IX owing to bonding between the porphyrin and the G-quadruplex. In the existence of target exosomes, PTK7 (tyrosine-protein kinase-like 7) bound to the sgc8 aptamer and triggered the unfolding of the MB. This initiated the chain displacement reaction to create a G-quadruplex (dark green), resulting in fluorescence signal emission (Figure 8C). The LOD was measured to be about 3.4×10^5 particles/μL. This

552 method had good specificity for the recognition of exosomes derived from different cell lines and
553 was validated in healthy and patient plasma samples [101].
554 miRNAs are small-scale and noncoding transcripts which regarded as targets in the varied ranges
555 of disorders and cancers[103]. Compared to traditional approaches; recently biosensors based on
556 MBs have emphasize on developed sensitivity, specificity, affordability, and reproducibility.
557 These new approaches combine with new materials, such as GO in the MBs have been widely
558 used for miRNA monitoring which improve the simplicity and device manufacturability of MBs
559 [2]. In the future, the research for miRNA study will definitely be improved. Also, noteworthy
560 features such as sample consumption, time-to-result will be vital for the improvement of the new
561 approaches.
562 Some typical methods based on MBs for miRNA detection are summarized in table 4.
563



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565

566 **Figure 8**. A) Design for the recognition of exosomal miR-21 by MB [99]. B) In situ simultaneous recognition of several miRNAs in exosomes by
567 MBs [100]. Q: quencher. F: fluorescent dye. C) Representation diagram of the sensor for quantification of exosomes via label-free strategy [101].
568

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569 Table 4. MBs for the recognition of miRNAs.

Method	Category	Target	Labels	LOD	Advantage	Ref.
RCA[1] coupled with NESA[2]	Fluorescent	miR-155	___	3.9 aM	Specificity, ultra-sensitivity also can be employed in breast cancer diagnosis, Has excellent potential for more applications in primary clinical diagnosis.	[104]
independent DNA mechanism.	Isothermal	miR-122b	FAM/ TAMARA	20 amol	Low-cost, Can detect natural miRNAs among whole RNAs, Can be used for multifaceted logic networks.	[105]
MB based on SDA[3] and CHA[4] with DNAzyme formation.	Colorimetric	different miRs	___	1.7 fM	Satisfactory reproducibility, High sensitivity Can be used to detect different miRNAs by just varying the miRNA-recognition section of the MB	[106]
SiO2/UCNP[5]-MB	Photoluminescence	miR-21 miR-195	COOH/ BHQ3[6]	2 nM	Can be applied for point-of-care analysis and early cancer detection	[107]
Biotinylated LNA[7]-DNA MB-AuNps.	Electrochemical	miR-21	Biotin/ thiol	0.3 pM	Reproducibility, Robust platform for detecting targets in low concentration and short-length miRNA	[108]
Single generic neutravidin probe.	Electrochemical	miR-21 miR-141	biotin-MB-metal NPs	miR-21:0.3 pM miR-141:10 pM	High selectivity due to the electrochemically responsive nano labels, comprising biotin-MB and metal-NPs,	[109]
AuNPs-Dox[8] -MB	Electrochemical	miR-let7d	Label-free	0.17 pM	Good stability, high potential for diagnosis of malignant cancers.	[110]
In situ single step recognition based on MB.	Fluorescent	miR-21	Cy3/ BHQ2	___	Selectivity in mixed exosome preparations and in human serum.	[99]

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miRNA-targeting MBs.	Fluorescent	miR-27a/ miR-21/ miR-375	FAM and Cy5/ ___	___	Potential for high-throughput analysis with high specificity, accuracy and cost-saving.	[100]
CQD[9] based MB.	Fluorescent	miR-21	CQD/ BHQ1	0.3 nM	Can distinguish between microRNA-21 and its single mismatch mutant Useful for early cancer diagnosis.	[111]
Hairpin-mediated quadratic enzymatic amplification.	Fluorescent	miR-21	___	at 37 °C =10 fM at 4 °C= 1 aM	Able to distinguish various miRNA families. Selective and sensitive when used on extracts from PC3 and MCF-7 cell lines.	[112]
Hairpin DNAzymes	Fluorescent	miR-let7	___	50 fmol	May be beneficial in tenders that need direct recognition of DNA, RNA within their normal environment.	[113]
MF[10]-beacon.	Fluorescent	miR-124a	Nanoparticle/ BHQ1	___	Able to screen microRNA 124a expression during P19 cell differentiation, showing an enhanced fluorescence signal with increased levels of miRNA expression	[114]
Imaging multiple miRNAs based MB.	Fluorescent	miR-206/ miR-26a	Texas Red/ BHQ2/ 6-FAM-BHQ1. respectively	___	Suitable for detection of a variety of miRNAs related to cellular processes *in vivo.*	[115]
Dual optical-MB.	Fluorescent	miR-1	Texas Red/ BHQ2	___	Can screen miRNA-1produced during myogenesis *in vivo* and *in vitro*, in animal models related to miRNA-related diseases to get more data on pathogenesis.	[116]
Novel-MB.	Fluorescent	miR-155	Alexa488/ BHQ1	___	Detection of the expression of miR-155 in NSCLC Potentially diagnostic for lung cancer.	[117]
MFAS[12] miRNA MB	Fluorescent	miR-221	MF/BHQ2	___	Simply used to other tumors by altering the sequence of target.	[118]

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Solution-phase detection	Fluorescent	miR-155/ miR-103	6-FAM/ Iowa Black FQ. TEX/Iowa Black RQ respectively	3.4–59.3 nM/ 19.3-79.0 nM respectively	Low detection limit, can be applied for the recognition of solution-phase RNA or DNA also for the recognition of serum miRNAs.	[119]

570
571 1, Rolling circle amplification. 2, Nicking endonuclease signal amplification. 3, Strand displacement amplification. 4, Catalytic hairpin assembly.
572 5, Up-conversion nanoparticle molecular beacons. 6, Black Hole Quencher 3. 7, Locked nucleic acid. 8, Anticancer drugs doxorubicin. 9, Carbon
573 quantum dot. 10, Magnetic fluorescent. 11. Non-small-cell lung cancer. 12. Magnetic fluorescence nanoparticle.
574

6.4. MBs for detection of bacteria

The detection of bacterial DNA sequences is a valuable diagnostic strategy in many kinds of infectious diseases. It is also used in disease prevention and environmental screening[120, 121]. Recently, foodborne illnesses have become a major concern for public health, and can even be a threat to life. Among many pathogenic bacteria, *E. coli. O157:H7* is a damaging species, which can create serious disease and causes death. The ability to handle and control diseases associated with harmful bacteria depends upon the rapid recognition of these pathogens [122].

Several works have been developed for bacterial detection such as Shiga-toxin-creating bacteria by a multiplex PCR method including MBs, and *Chlamydia trachomatis, Neisseria gonorrhoeae*, and *Mycobacterium tuberculosis*, by fluorescence-based PCR analysis [123]. In one study, Jiang et al. [124] designed a sensor based on an allosteric MB (aMB) for the recognition of DNA from *E. coli*. O157:H7. In the absence of target, the aMB created a steady construction, which locked the binding ability of the SA (streptavidin) aptamer. In the presence of target, the MB opened, and the SA was released to bind to biotin-HRP. The horseradish peroxidase (HPR) catalyzed TMB (tetramethylbenzidine) oxidation to produce TMB^{2+} and produced an electrochemical signal. This probe not only had noticeable linear connection between the logarithms of *E. coli*. and presented height of peak, but also had an acceptable regression coefficient (R^2=0.9885). moreover, it showed a good linear of *E. coli*. DNA from 1×10-9g/ml-1×10-6g/ml and could detect the quantitative detection of *E. coli*. O157:H7 DNA [124].

Another pathogenic species is the anaerobic bacillus *Clostridium perfringens* that causes gas gangrene. It expresses chromosomally encoded perfringolysin O and alpha-toxin that causes the pathology in infections [125]. Hence, the detection of *C. perfringens* is significant in analysis. In one study, an electrochemical approach based on MB-SA, and Fe_3O_4/G

599 quadruplex/hemin/AuNPs/ graphene/GCE was developed for recognition of this pathogen [126].
600 In the existence of target DNA, the MB was opened, and the aptamer was activated to inhibit the
601 SA/ Fe_3O_4/alcohol dehydrogenase activity. By a "sandwich" reaction, the hemin/ G-quadruplex
602 was absorbed on the electrode plate, and the electrochemical signal was triggered (Figure 9A).
603 This SPE approach could be an alternative method to recognize *C. perfringens* without the need
604 for bacterial culture or amplification of DNA for point-of-care diagnosis. The combination of the
605 Fe_3O_4 and hemin/G quadruplex permitted the advanced amplification of electrochemical signal.
606 This sensor also presented sufficient stability, good specificity, and reproducibility [126].
607 In 2019 Zhang and co-workers synthesized three MB probes for the recognition of the 16S rRNA
608 genes of *Staphylococcus aureus* [127]. The group performed optimization on some factors such
609 as hybridization buffer components, temperature, and signal/noise ratio. The MB-FISH able to
610 monitor *S. aureus* infection in the blood culture directly. Using MB-FISH, a novel approach was
611 successfully created for the recognition of *S. aureus* and direct detection of pathogenic bacteria
612 in the blood culture using flow cytometry [127].
613 Zheng and et al. constructed a DNA silver nanocluster (DNA-AgNCs) based sensing method
614 based on a magnetic nanoparticle (MNP)-AChE-DNAzyme nanocomposite for the recognition of
615 *E. coli* [128]. In the existence of *E. coli* lysate, the MDA complex, including DNAzyme as the
616 bacterial detection element, AChE as an enzyme to produce a reaction, and MNPs as the
617 separation element, the DNAzyme nucleic acid substrate could be cut into two parts to release
618 AChE. Because of the magnetic separation, the liberated AChE reacted with AgNCs-DNA by
619 hydrolysis of ATCh to create TCh, which could produce the fluorescence signal of the AgNCs-
620 DNA. This DNAzyme platform had good sensitivity with a LOD of 60 $CFU.mL^{-1}$. Besides, the

621 approach could be improved into a potentially versatile organization by only changing
622 corresponding DNAzyme as the item's detection probes. (Figure 9B) [128].
623 A summary of MB-based methods for bacterial detection and their advantages is given in the
624 table 5.

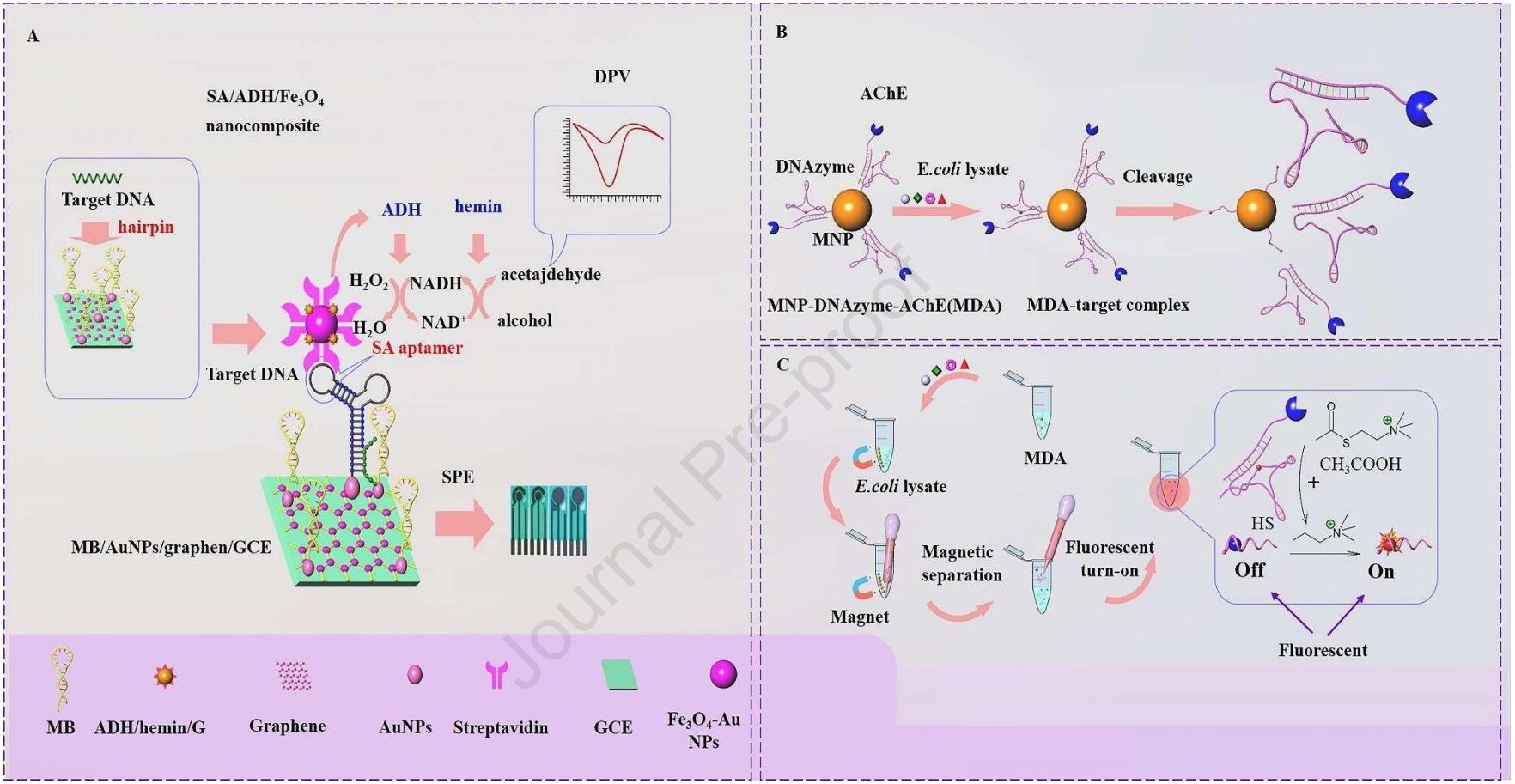

Figure 9. Two different approaches for detection of bacteria using MBs. A) The principle of the aptasensor [126]. B), C) Schematic representation of DNA-AgNCs [128].

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629 Table 5. Methods based on MBs for bacterial detection.

Method	Target	Category	Advantage	Ref.
Genosensor Based on Loop-stem Probe	*Bacillus anthracis*	Electrochemical	Fast, without light optics; sources or high voltage power approach; can be employed several times with excellent analytical parameters.	[129]
DNA-based X sensor	*E. coli 16S rRNA*	Fluorescent	The remarkable specificity of the investigation was confirmed using differentiating *Bacillus subtilis* from *E. coli*.	[130]
aMB	*E. coli O157:H7*	Electrochemical	Noticeable linear connection between the logarithms of *E. coli.* and present height of peak; showed a good linear of *E. coli*. DNA from 1×10^{-9}g/ml-1×10^{-6}g/ml; display an acceptable regression coefficient (R^2=0.9885); can detect the quantitative detection of *E. coli*. O157:H7 DNA.	[124]
MB-SA and hemin/G quadruplex/Fe_3O_4 AuNPs	*C. perfringens*	Electrochemical	High accuracy; hopeful alternative method to distinguish *C. perfringens* without DNA amplification in bacterial culture.	[126]
MB-FISH[1]	16S rRNA gene of *Staphylococcus aureus*	FISH	Creates an approach for identification of *S. aureus* in the blood cultures; beneficial platform for more recognition of pathogenic microorganisms in positive blood cultures via flow cytometry.	[127]
MNP-DNAzyme-AChE complex	*E. coli*	Fluorescence	Can be settled in a useful organization by changing DNAzyme as the item recognition probes.	[128]
Reverse MB (rMB)	*S. aureus and S. epidermidis*	Fluorescence	Can be planned by means of an affinity preconcentrator that able to procedure big input volumes.	[131]
Unnamed	16S rRNA gene of *Francisella tularensis*	Fluorescence	Able to recycle lending to their probable practical tender.	[132]

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Real-time PCR-MB	*B. anthracis*[2] *Y. pestis*[3] *B. mallei* [4] *F. tularensis*[5]	Fluorescence	Flexible analysis that able to be improved to the varied range of spectrofluorometric thermal cyclers.	[133]

630

631 1, Fluorescence in situ hybridization. 2, *Bacillus anthracis. 3, Yersinia Pestis. 3. Burkholderia mallei. 5. Francisella tularensis* .

6.5. MBs for detection of viruses

The prevalence of infectious diseases caused by viruses is increasing throughout the world[134, 135]. Moreover, newly emerged viruses have been detected that can cause serious pandemics [136, 137]. Hence, there is an urgent requirement for powerful novel diagnostic tools, which are capable of detecting pathogenic viruses before they can spread widely to general populations and cause pandemics[138-140]. Accordingly, many studies have investigated the detection of viruses based on MBs. For instance, by using two short RNA strands, Yamamoto et al. used an aptamer-based MB for the recognition of Tat protein of HIV based on fluorescence enhancement in the existence of Tat protein. This method has ability to use in other analytes detections e.g. small molecules and proteins. Furthermore, the MB-aptamer needs half the length of target in comparison with MBs. So, it is possible that an analyte-binding location was added into MBs to change them to signaling beacons [141].

On the other hand, advanced probes that can accurately detect and distinguish several different serotypes of a single virus are required for point-of-care diagnosis. Accordingly, a class of luminescent functionalized ZnSeS/CdSe core/alloy shell QD was created [142]. In this system, the QD-AuNP-nanohybrids were formed by connecting AuNPs and QD. Then, QD-AuNP nanohybrids were attached to the 5′ terminus of the MB that was complementary to Dengue virus (DENV) nucleic acid. The QD-AuNP-MB biosensor had good sensitivity for the detection of several serotypes of dengue virus, with a LOD ranging from 31 to 260 copies per mL. In compared to QD-MB, the AuNP-QD-MB displayed that the localized external plasmon resonance-stimulated signal to the fluorescence intensity of the QD increased the action of the probe. This probe holds good promise for the certain diagnosis of DENV, though the versatile sensor concept is appropriate to any kind of RNA virus [142].

In another study, Adegoke and co-workers reported a CdZnSeS-MB biosensor with good fluorescence under UV irradiation [143]. This detection method was rapid and sensitive and could detect a very low amount (down to 2 copies/mL) of influenza virus H1N1 RNA. It was also specific for the recognition of H1N1 RNA in serum [143].

In 2016 a QD fluorophore with innovative optical properties was designed to detect norovirus (NV) RNA [144]. In this work, the terminal carboxyl groups on the QDs were attached to the amino group at the 5′ terminus of the MB. In the existence of the target, the interaction of the MB with the target RNA increased the distance between the quencher and the QD, leading to the generation of a fluorescent signal. The TGA thiol was coated on the QDs using a ligand-exchange reaction. First, the QDs were functionalized with amino groups and then further reacted to form carboxylate groups, which were then conjugated to the MB using EDC/NHS chemistry. Ultra-sensitive recognition of low concentrations of NV RNA with a LOD of 8.2 copies/mL in serum was gained by applying this probe. The further merits of this probe are improved sensitivity, specificity, and rapidity over conventional probes [144].

In 2018, a MB-based on DNA silver nanoclusters (AgNCs) was constructed by Han and et al [145]. This MB was composed of three parts: a sequence for the creation of AgNCs (C-rich region), a blocking sequence, and a detection sequence for the target DNA (loop region). The essential principle of this sensor was based on the release of the C-rich single-strand DNA (ssDNA) from the blocking sequence induced via binding to the target virus, which then triggered the formation of AgNCs. The fluorescence signal of the DNA-AgNCs could be utilized for the recognition of several target virus genes. By this approach, three infectious disease-connected genes H5N1, H1N, and HIV were detectedwith a LOD of 3.95, 0.12, and 3.53 nM

677 respectively. The probe permits versatile, simultaneous, and specific recognition of diverse target
678 molecules with low cost and easy operation [145].
679 In another report, an electroactive MB-based biosensing approach was developed for the
680 recognition of cauliflower mosaic virus 35s (CaMV 35s) [146]. The tag was attached to the MB
681 using an in-situ modification method. The MB was fixed on the AuE via Au-S bonds. Next, the
682 AbC crown ester was conjugated to 5′-COOH group on the MB. The modified electroactive
683 Cu^{2+}-AbC was located close to the electrode, heading to an increased electrochemical signal.
684 Upon hybridization, the MB construction was opened and transformed into a linear strand,
685 leading to the Cu^{2+}-AbC moving away from the plate, leading to a decreased signal. Therefore,
686 the hybridization event that occurred on the surface of the electrode could be observed (Figure
687 10A). In comparison to traditional approaches for fabricating electroactive MBs, the in-situ
688 assembly method avoided complex procedures such as separation, purification, and
689 homogeneous reaction. This approach was labor-saving, reagent-saving, and much simpler,
690 leading to a LOD equal to 0.060 pM [146].
691 Another electrochemical technique for the recognition of the single-stranded DNA using an
692 oligonucleotide from the hepatitis B virus (HBV) was reported [147]. In this system, the RCA
693 and circular strand displacement (CSD) reactions were triggered by an MB. The MB was fixed
694 on the gold electrode plate using gold-thiol binding. Then, the ssDNA derived from the target
695 DNA of HBV was added, resulting in opening the structure of the MB and hybridization of the
696 primer DNA to the 3′-end of the MB. Consequently, the polymerization of DNA was initiated in
697 the presence of phi29 DNA polymerase and dNTPs, leading to the liberation of the target. Then,
698 the target repeatedly bound to another MB to start the MB-mediated CSD procedure. The 5′-end
699 of the primer anchored the circular RCA onto the electrode surface. In the presence of phi29

DNA polymerase, dNTPs, and T4 DNA ligase, the RCA produced a very long DNA chain with several tandem-repeat sequences. It was observed that methylene blue conjugated to the guanine bases of the DNA and was used as an electrochemical redox probe in the electrochemical detection approach. When the redox probe bound to the long DNA chain, the redox signal of methylene blue was increased. In the absence of target, the MB remained in the closed structure. The RCA did not occur, and the DNA was not produced, and the electrochemical signal of methylene blue was not increased (Figure 10B). Compared to conventional methods with no signal amplification procedure, the redox signal variation was strongly enhanced even at a very low concentration of target DNA. Under optimal experimental conditions, the assay exhibited a high sensitivity with a LOD of 2.6 aM and a linear range from 1.0×10^{-17} M to 7.0×10^{-16} M and outstanding selectivity [147].

Lim and co-workers prepared a peptide-based MB (PEP-MB) for the recognition of H1N1 viruses utilizing a FRET approach [148]. The PEP-MB comprised two complementary sequences in the stem area and a peptide sequence in the core loop area that targeted a conserved sequence in the HA protein of H1N1. The PEP-MB alone created a hairpin structure that displayed fluorescence quenching, but after the detection of the HA1 protein by these molecules, the fluorescence intensity increased. This probe could recognize H1N1 viruses within 5 min and displayed a good fluorescence signal even at low concentrations of the H1N1 virus [148].

In table 6 some important works based on MBs detection for viruses were prepared.

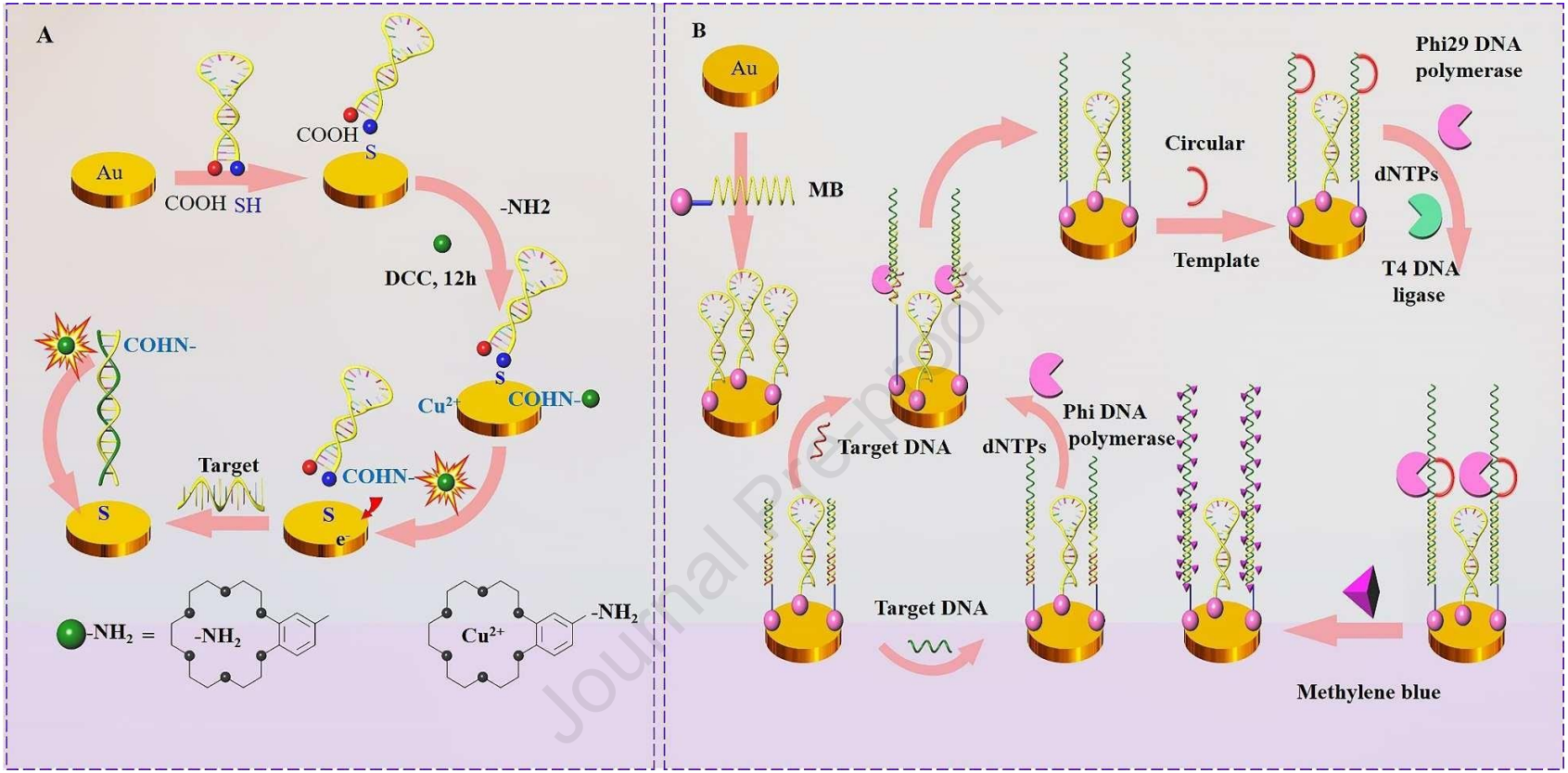

Figure 10. Two different approaches for detection of bacteria using MBs. A) The principle of MB based biosensor using AbC-Cu^{2+} [146]. B) Schematic illustration of the electrochemical biosensor and its tender for detection of HBV DNA [147].

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726

Table 6. MBs for detection of viruses

Method	Target	LOD	Labels	Category	Advantage	Ref.
AuNP–QD[1] -MB	serotypes of DEN[2] virus	Less than 20 copies/mL	NH2/ BHQ2	Photoluminescent	Serotype-specific and high sensitivity,	[142]
NIR[3] alloyed-CdZnSeTeS- MB biosensor	Various Strain of H1N1 virus	4.1 copies/mL	MPA/ BHQ1	Fluorescent	Highly sensitive than conventional tests and fast detection of influenza virus also useful to manage the spread of the disease	[149]
G-rich MB-Ag NCs	HIV, HBV, HTLV-I genes	4.4 nM, 6.8 nM, 8.5 nM, respectively	Label-free	Fluorescent	High selectivity, low-cost also can provide a platform for DNA study, this system has a LOD less than nanomolar as well as differentiate one bases mismatched target.	[150]
SiO-CdZnSeS QD-MB	Norovirus	8.2 copies/ mL in human serum	NH2/ BHQ1	Fluorescent	Specificity and better sensitivity in compared to traditional assay probes.	[144]
MB-based on DNA-AgNCs	HIV, H1N1, and H5N1	3.53 0.12 and 3.95 nM, respectively	Label-free	Fluorescent	Allowing versatile, simultaneous recognition of targets with low cost and easy process.	[145]
MB based biosensor using AbC-Cu^{2+}	CaMV 35s	___	Label-free	Electrochemical	Opens a strategy for the fabrication of electrochemical sensing interface.	[146]
MB mediated CSD and RCA	HBV	2.6 aM	Label-free	Electrochemical	The redox signal variation is amplified at low amount of target.	[147]
QDs-based MB-2′-O-methyl modification	BKPyV [4]	———	QDs, BHQ1	Immunofluorescence	Helps assess the suitability of adopting BKPyV as an indicator organism for water quality.	[36]
CdZnSeTeS-MB	H1N1	1.9 copy/mL	AmMC6-IABkFQ[5]	Fluorescent	Highly sensitive than traditional MB and rapid influenza recognition tests probes.	[149]

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CdZnSeTeS-MB	H1N1 A/California/7/2009	4.1 copy/mL	5AmMC3-DABSYL	Fluorescent	More sensitive than conventional MB and fast influenza detection tests probes, specific and ultrasensitive.	[149]
CdZnSeTeS-MB	H1N1	2.4 copy/mL	5AmMC3-BHQ-1	Fluorescent	Fast influenza detection tests probes, specific and ultrasensitive.	[149]
Peptide-based-MB	HA[6] protein of H1N1	4 copy/mL	Oligo[7] Cy3/ Oligo BHQ2	Fluorescent	Specific, simple, rapid, detect H1N1 within even 5 min, shows strong fluorescence at low viral concentrations	[148]

727

728 1, Quantum dot .2, Dengu. 3, Near-infrared. 4, BK Polyomavirus. 5, Iowa black fluorescence quencher. 6, Hemagglutinin. 7, Oligonucleotide.

7. Conclusion and future perspectives

Since the report of MBs in 1996, several efforts have been done to develop the strategy of MBs and spread their scope of tender, which has made the formation of a big family of effectual MBs. In compared to other probes, MBs have some remarkable features consist of the flexibility of their conjugation, highly effective signal-transduction mechanism, and cost-efficiency which have resulted in the improvement of a wide spectrum of tenders in medicine, biology, and biotechnology. MBs great sensitivity because of the good quenching efficiency is advantageous. Hence, these advantages over other analytical approaches have caused MBs perfect for intracellular investigations of nucleic acids and other molecules sensor improvement. The analysis of gene expression through the native state was can be achieved owing to the great mechanism of MBs. This could be vital since it can animatedly display the cellular responses to outside stimulus, e.g. drug therapy and differential expressions of gene in abnormal and normal tissues. MBs able to accelerate the development of high-throughput analytical approaches with highly kept selectivity to deal with the enormous number of molecules. By applying PCR amplification, MBs have also been broadly applied in diagnosis such as recognition of human pathogens and detection of disease-related to SNPs and alleles. Novel approaches, such as enzymatic amplification [151] and quenching at metal areas [152], have been emerged and employed to MBs design. New quenchers and fluorophores, for example, AuNPs, SQ, conjugated polymers, and pyrene derivatives are too becoming progressively attractive for several tenders. These new approaches cause MBs broadly employed in clinical diagnosis and environment problems, for example, recognition of mRNA, and miRNA, detection of various ions *in vivo* and *in vitro*. The more benefit of MBs is that natural modification of the constructions is fairly straightforward, which permits the usage of flexible synthetic methods.

753 Numerous base modifications have been improved for MBs, for instance, the combination of
754 phosphorthioate derivatives, L-DNA, 2′-OMe, LNA, PNA bases. These modified MBs have
755 further valuable features, including minimized nonspecific protein conjugating, a remarkable
756 hybridization affinity, and resistance to enzymes digestion. In combination with other methods,
757 MBs are applied for molecular computing, enzyme monitoring, and protein evaluates. Recently
758 nucleic acid sensors based on MBs have emphasized on developed affordability, specificity,
759 reproducibility, sensitivity, and analyze simplicity in the company of short hybridization time.
760 In the future, the efforts for allowing molecular study methods based on MBs method will
761 absolutely be advanced. The significance of various molecules detection causes the development
762 of point-of-care study methods extremely important. In these applications, noteworthy features
763 e.g. time-to-result, analyze cost, and sample consumption will be significant for the improvement
764 of the novel approaches. With the improvement of such novel MBs, they will certainly be
765 employed in new drug studies, diagnosis, therapy and so on wider and wider.
766
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Highlights

- Since MB's structure and labelling, they have advantages of thermodynamic, photostability, and good intrinsic signal switching.
- MBs have target-specificity, inherent fluorescent signal production, detection without separation, and non-radioactive labels.
- MBs have been used for sensing in biological and non-biological targets e.g. mutations, heavy metals, bacteria, and viruses e.g. SARS-CoV-2.
- Recently, improved designs of MBs such as LNA and PNA-based MBs have been proposed to improve the hybridization efficiency and nuclease resistance.

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The authors declare no conflict of interests and/or commercial products or companies.