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Review

Molecular Beacon Nano-Sensors for Probing Living Cancer Cells

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Heterogeneities and oncogenesis essentially result from proteomic disorders orchestrated by changes in DNA and/or cytoplasmic mRNA. These genetic fluctuations, however, cannot be decoded through conventional label-free methods (e.g., patch clamps, electrochemical cellular biosensors, etc.) or morphological characterization. Molecular beacons (MBs) have recently emerged as efficient probes for interrogating biomarkers in live cancer cells. MBs hybridize with their intracellular targets (e.g., mRNAs, DNAs, or proteins), emitting a fluorescent signal that can be quantified and correlated with the expression levels of their targets. In this review we discuss MB probes with different delivery platforms for intracellular probing as well as novel MB designs for detecting a variety of targets in living cancer cells. Finally, we describe current trends in MB-based intracellular biosensors.

Trends

Precisely controlling the dose of MBs delivered into live cells, which allows quantitative analysis at single-cell resolution, is becoming increasingly feasible.

The design of MBs is moving towards increased sensitivity and longer-lasting resistance to nucleases and other enzymes.

The 'specificity' parameter is particularly important for prognostic bio-chips and *in vivo* imaging. MB designs that address single-nucleotide mutations and that can detect multiple targets are becoming popular.

Challenges in Probing Living Cancer Cells

The living cell contains a sophisticated machinery that is responsive to both intracellular and extracellular stimuli. Cellular functions, modulated by nuclear DNA, cytosolic mRNA, and proteins, play a major role in determining cellular proliferation, differentiation, oncogenesis, apoptosis, etc. Decoding genetic information is crucial for gene therapy (e.g., gene editing and adoptive immunotherapy) and cancer diagnosis (e.g., gene mutation and intracellular heterogeneities of cancer stem-like cells) [1–7]. Over the past few decades efforts have been devoted to probing intracellular components (e.g., DNA, mRNA, and proteins). Well-known techniques such as PCR, DNA microarrays, western blotting, and ELISA have demonstrated high resolution for quantitatively analyzing the exact copy number of genes [8–12]. However, these methods are not compatible with live-cell bio-interrogation, and therefore have limited impact in early cancer detection (e.g., for circulating tumor cells) or in precision/personalized medicine. Label-free biosensors are among the commonest tools for monitoring live cells. In particular, advances in the fields of micro-/nanofabrication (top-down) and biomolecular synthesis (bottom-up) have remarkably boosted the sensitivity and specificity of cellular sensors [13]. However, their implementation for gene-based probing remains challenging [14].

MBs have emerged as promising tools for live-cell interrogation with single-molecule resolution [15–17]. MBs are single-stranded (ss) oligonucleotides that form a hairpin structure that confines a fluorophore and quencher in close proximity such that no fluorescence signal is emitted. Once they are delivered into the cytosol, the MBs seek their target molecules (e.g., mRNAs), hybridize (via the complementary binding of the MB on the coding sequence (CDS) of mRNA), and emit a fluorescence signal via the controlled separation of the quencher and fluorophore. Precise titration of MB delivery, for example, can quantify mRNA expression based on fluorescence

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Box 1. Fundamental Working Principle of MBs

Design Principles of MBs

A MB is a hairpin-shaped oligonucleotide probe that is mainly composed of three parts, described below (Figure IA).

Reporter: used to build a signal transduction system. Generally, the reporter consists of a fluorophore located at one end and a quencher dye at the other end of the hairpin oligonucleotide.

Stem: the stem sequence is located at the base of the loop. The stem structure, which acts as a 'lock' to maintain the closed hairpin structure, plays a key role in the design of the MB.

Loop: generally used as the target-binding region. The loop domain consists of a 15–30 nt region of the MB that is complementary to the target sequence.

Working Principle of Molecular Beacons

The hairpin structure, which quenches the fluorophore, is designed to maintain close proximity between the fluorophore and the quencher. After hybridizing with the target sequence (DNA or RNA), the hairpin structure opens up and separates the dye from the quencher (Figure IB). Thus, fluorescence will be emitted based on the separation distance between the dye and quencher. Target mRNAs, for example, can be detected and tracked depending on the fluorescence signal, and the expression level can be directly linked to the fluorescence intensity. If the target sequence is not complementary to the MB probe sequence, hybridization and fluorescence emission do not occur.

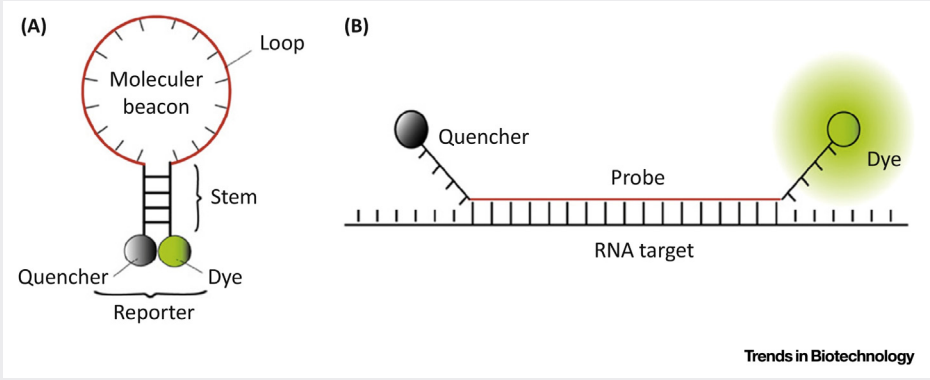

Figure I. (A) Molecular beacon structure. (B) Working principle. Figure reproduced, with permission, from [18].

intensity [18] (Box 1). Different from PCR and other techniques, MB-based bio-interrogation faces two major challenges. First, the cell membrane forms a 'barrier' to 'naked' MBs. Vehicles for transporting MBs across the cell membrane play a crucial role in determining delivery efficiency and diminishing false-positive signals. Second, an appropriate MB is required to achieve high sensitivity, specificity, nuclease resistance, and long-term stability. In recent years much of the research on MBs has centered on these two themes. In this review we give insights into the state of the art of MBs for cancer cell interrogation (*in vivo* and *in vitro*) as well as MB design considerations for various purposes. The purpose of this review is to highlight the importance of developing advanced platforms for cell bio-interrogation and to promote inter-disciplinary collaborations across multiple fields including materials science, engineering, biology, chemistry, and clinical research.

Strategies for MB Delivery into Cells

Successfully transporting MBs across the cell membrane requires methods that yield high delivery efficiencies while preserving cell viability. So far, various approaches have been implemented for intracellular MB delivery, including viral vectors, physical methods (e.g., injection and electroporation), chemical vectors (e.g., reversible membrane permeabilization and

cell-penetrating peptides, CPPs), inorganic nanoparticles (e.g., gold nanoparticles, AuNPs), and other functional nanomaterials (e.g., carbon nanotubes, CNTs). The features and limitations of these strategies are summarized in Table 1.

Viral vectors (e.g., lentivirus or adenovirus) have been employed for MB delivery for decades because of their high infection efficiency [19,20]. Viral methods, however, are known to alter cellular responses and thus have the potential to affect detection outcomes. Recently, attention has shifted to non-viral strategies. Microinjection, for example, is a common physical method for controllably delivering MBs into single cells in a straightforward manner [21]. Cells are typically injected by mechanically disrupting the membrane using a sharp tip (e.g., a glass tip with a diameter of 1–10 μm). This method, however, is mostly limited to relatively large cells. Nano-injection-based approaches have also been explored for MB delivery. Nanowires [22,23] and nanopillars [24,25] are among the most commonly used systems. Shalek *et al.* studied the *Wnt* signaling pathway in live chronic lymphocytic leukemia (CLL) cells by intracellular testing and silencing mRNA expression [22]. Nanopillar-mediated delivery allowed imaging of nuclear deformation *in situ*, in which some important parameters including migration, proliferation, and polarization could be monitored [24] (Figure 1A). In addition to cytoplasmic mRNA, injection-based MB delivery has been used to probe nuclear DNA. Nevertheless, some of the major drawbacks of injection-based methods are low throughput and high cost.

By contrast, electroporation-based strategies have been recognized as simple and effective physical methods for intracellular cargo delivery with high throughput. Commercially available bulk electroporation (BEP) systems can deliver cargo such as MBs to millions of cells by applying high voltages (~1000 V), which typically result in impaired cell viability (20–50%) and random transfection patterns. Alternative approaches, such as microfluidic electroporation (MEP), enable successful transfection at significantly lower voltages. Nevertheless, both BEP and MEP have limited ability to control dosage. Recent advances in nanoscale electroporation (NEP) have enabled precise dosage control with single-cell resolution [26,27]. Boukany *et al.*, for example, used NEP to precisely determine the apoptosis threshold of K562 cells via titrated *Mcl1* siRNA delivery and live-cell monitoring (Figure 1B) [27]. Gallego-Perez *et al.*, by contrast, used a 3D NEP platform to identify aggressive subpopulations of glioma-initiating cells via controlled MB delivery [3]. Nanofountain probe electroporation (NFP-E) has been used to controllably deliver detection probes (e.g., DNA- and RNA-based MBs) into live cells (Figure 1C) [28–30]. Nanostraw-based electroporation has demonstrated benign and efficient intracellular delivery of small molecules, including propidium iodide (PI) dye [31]. The detailed operational principles of frequently used delivery platforms (NEP and NFP) are presented in Box 2.

In addition to physical methods, cargo can also be delivered via chemical permeabilization of the cell membrane. Pore-forming bacterial toxins, such as streptolysin O (SLO), can bind to cholesterol on the cellular membrane to form ring-shaped structures with nanopores (25–30 nm in diameter) which permit cargo translocation across the cell membrane. Pore formation can be reversibly manipulated by adjusting the serum concentrations in SLO solutions. A series of MBs, such as 2-deoxy and 2-*O*-methyl MBs [32], have been successfully delivered into cells for mRNA imaging (e.g., BMP-4 mRNA [33]) and viral RNA (e.g., bovine respiratory syncytial virus [34]) detection using SLO. Moreover, SLO-delivered MBs have been employed to study the mRNA target accessibility with DNA-based MBs and to understand the interplay between the beacon-based probes and BMP-4 mRNA targets [33]. One practical limitation of this method, however, is that delivery efficiency depends on the cell type. SLO protocols must be specifically optimized to increase the affinity for membrane cholesterol in different cells.

The second most popular chemical vectors are CPPs, which have also been used to deliver MBs. The most frequently used peptides include the HIV-1 Tat peptide and its derivatives [35].

Table 1. Currently Available Techniques for MB Delivery[a,b]

Delivery Platform			Typical Platform	Detected Cells	Efficiency[c]	Viability	Throughput	Features	Limitations	Refs
Viral vectors			AAV2	HeLa	High	High	High	High transduction efficiency	Oncogenesis; safety concern; side-effects *in vivo*	[19,20]
Physical vectors	Injection	Microinjection	Micropipettes	PtK2	High	Medium	Low	Direct method; dose control; destination control	Costly equipment; low throughput	[21]
		Nanoinjection	Nanowires; Nanopillars	HeLa, MCF-7, fibroblast	High	High	Low	Direct injection; benign penetration; dose control	Costly equipment; low throughput	[22–25]
	Electroporation	Bulk electroporation	BEP	N/A	N/A	Low	High	Simple; straightforward	Harsh environment; low viability; random transfection	[26,27]
		Microporation	MEP	NIH/3T3	Medium	High	N/A	Benign environment	Random delivery; limited dose control; limited in ability to deliver large cargo/molecules	[26,27]
		Nanoporation	NEP	KG1a, A549	High	High	Low (2D NEP); high (3D NEP)	Instantaneous delivery; benign transfection; dose control	Complicated on-chip cell manipulation	[3,26,27]
			NFP-E	HeLa, HT1080	High	High	Low	Benign environment	Low throughput	[28–30]
Chemical vectors		Reversible permeabilization	SLO	HDF, HeLa	High	High	High	Convenient; less damage to cells; targeted delivery (nucleus)	Limited to certain cell types; long incubation time	[32–34]
		Cell-penetrating peptides	HIV-1 Tat peptide	HeLa, fibroblast	High	N/A	High	Simplicity and low cost; fast delivery	Complex process (covalent conjugation); toxicity/side-effects for *in vivo* applications	[35,36]
		Liposome	Oligofectamine	H460, fibroblast, HEK293T	Medium	High	High	High-throughput; benign carrier	Slow delivery process; MB degradation in endosomes	[37,38]
		Dendrimer	Superfect	SK-Hep1	Medium	High	High	High-throughput; benign carrier		[39]

Table 1. (continued)

Delivery Platform	Typical Platform	Detected Cells	Efficiency[c]	Viability	Throughput	Features	Limitations	Refs
							Slow delivery process; MB degradation in endosomes	
Gold nanoparticles	Nanoflare	HeLa, SKBR3, C166, MCF-7, MCF-10A, HepG2, HL-7702	High	High	High	High quenching efficiency; large quenching distance; enter cells without transfection agents; prevent enzyme degradation	Use limited to breast cancer until now	[42–47]
	hDAuNPs	HeLa, MCF-10, MCF-7/Adr,	High	High	High	Enter cells without transfection agents; resistance to nuclease degradation; enhanced nucleic acid binding	Relatively low detection sensitivity; longer detection cycles	[1,50,51]
Other functional nanomaterials	Carbon nanotubes, graphene nanoribbon, SnO_2, chitosan, quantum dots, PMMA nanoparticles	HeLa, MCF-7, HEK 293, SPC-A1, MMP-2, A549	High	High	High	Efficient delivery; limited side effects; high biocompatibility	Relatively low detection sensitivity; longer detection cycles; exposed to the endosome during endocytosis	[56–61]

[a]Abbreviations: AAV, adeno-associated virus; BEP, bulk electroporation; hDAuNP, hairpin DNA modified gold nanoparticle; MB, molecular beacon; MEP, microporation; N/A, not available; NEP, nanochannel electroporation; NFP-E, nanofountain probe electroporation; Oligofectamine, formulation for transfecting oligonucleotides and siRNA into eukaryotic cells; SLO, streptolysin O; superfect, transfection of eukaryotic cell lines with DNA.
[b]In general, the sizes of the MBs reported in this table were in the range 10–30 nt.
[c]Transfection efficiency: high, >70%; medium, 40–70%; low, <40%.

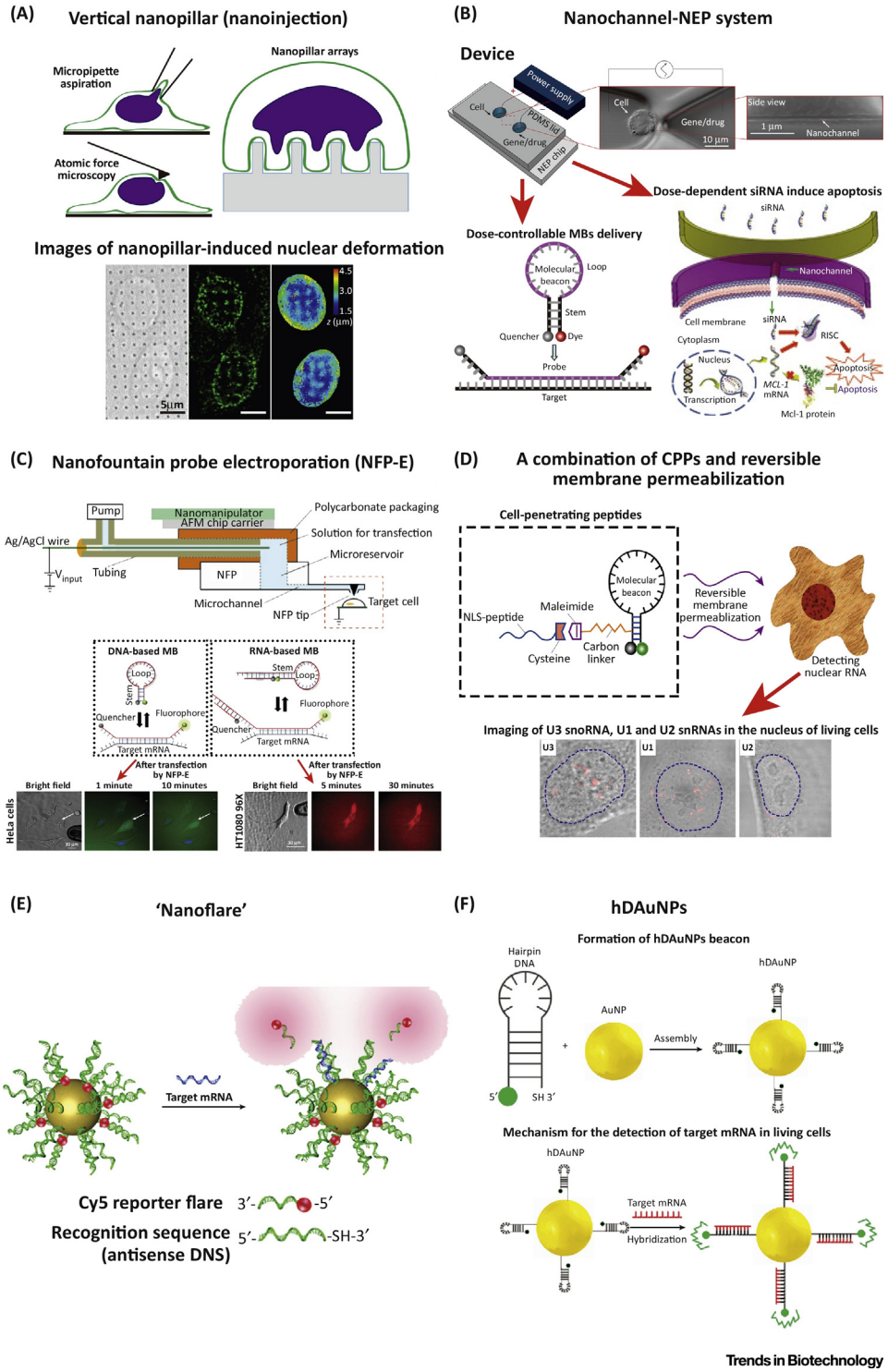

Figure 1. Molecular Beacon (MB) Delivery Strategies into Live Cells. (A) Nanoinjection: illustration of vertical nanopillar-induced nuclear deformation [24]. (B) Illustrations of the nanochannel electroporation (NEP) system for MB and siRNA delivery [26,27]. (C) Nanofountain probe electroporation (NFP-E) for MB delivery at the single-cell level [28,29]. (D) Nuclear delivery of MBs via cell-penetrating peptides (CPPs) and reversible membrane permeabilization [36]. (E) Schematic illustration of the 'nanoflare' concept [47]. (F) Synthesis and operation of hDAuNP (hairpin DNA-functionalized gold nanoparticle)-based MBs [49]. Figures reproduced with permission from the references listed.

Box 2. Operational Principles of Different MB Delivery Platforms

Hairpin DNA-functionalized gold nanoparticles (hDAuNPs): these are composed of a gold nanoparticle (AuNP) covalently bound to thiolated hairpin DNAs with a 5′-end reporter dye. The fluorescence of the 5′-end reporter dye is quenched by its proximity to the AuNP surface, which acts as a quencher. When they hybridize with their targets, hDAuNPs denature the hairpin configuration and subsequently fluoresce.

Nanochannel electroporation (NEP): NEP devices are primarily fabricated through lithographic techniques (e.g., photolithography or soft lithography). In NEP, the cells are first interfaced with individual nanochannels which are fluidically connected with a reservoir system that holds the MBs to be delivered into the cells. A pulsed electric field is then applied across the nanochannel system, and this benignly nanoporates the cell membrane at the cell/nanochannel interface, and electrophoretically delivers MBs into the cytosol in a fast (micro- to milliseconds) and dosage-controlled manner.

Nanofountain probe electroporation (NFP-E): this technique comprises a microfluidic-channel device with a hollow probe tip [i.e., an atomic force microscopy (AFM) tip]. Once the MB solution is loaded into the system, the probe tip is brought into contact with cultured adherent cells, and is then followed by the application of an electric field between the tip and the cell culture substrate. This method causes localized poration and MB translocation into the cytosol.

Nanoflare: the nanoflare platform consists of an AuNP that is densely functionalized with a thiol-modified ssDNA monolayer with complementary sequences to specific targets. Recognition sequences in the ssDNA pre-hybridize to a short DNA competitor containing a fluorescent reporter (i.e., the 'reporter flare'), whose fluorescence is quenched based on its proximity to the AuNP. Upon target hybridization, the reporter flare strand is replaced, thus providing a fluorescent readout.

MBs are covalently linked with the Tat peptide, and the resulting peptide-based delivery system can quickly and efficiently deliver the MBs into live cells for mRNA detection. A combination of the SLO and CPP approaches has been used for probing small nuclear RNA (snRNA) in live cells [36]. A single nuclear localization sequence (NLS) peptide was first conjugated with MBs and then efficiently delivered into the cells (Figure 1D). Nevertheless, the mechanisms underlying CPP-based cargo translocation remain unclear, which limits widespread use of this technology. Although additional chemical methods (e.g., methods based on liposomes [37,38] or dendrimers [39]) have also been developed for delivering cargo (e.g., plasmids [40]), these tend to be limited by additional sub-processes (e.g., diffusion and endocytosis), which leads to slow and inefficient delivery [41].

Inorganic nanoparticles, such as gold nanoparticles (AuNPs), have also been used for MB delivery. So far, 'nanoflare' and hairpin DNA-coated AuNPs (hDAuNPs) are the most common AuNP-based systems for probing intracellular mRNA. The detailed operational principles of these AuNP-based platforms are shown in Box 2. Nanoflares, for example, have been reported to offer high sensitivity, selectivity, and stability (Figure 1E) [42]. This system has been successfully used for *in vivo* probing of breast cancer cells [43–47]. In AuNP (hDAuNP) systems, a gold nanoparticle is covalently linked with a hairpin DNA beacon via a thiol–gold bond [48,49]. hDAuNPs have attracted a great deal of attention owing to their interesting properties, including enhanced nucleic acid binding affinity, resistance to degradation, and a high quench rate (Figure 1F) [1,50,51]. Because of these advantages, many researchers have explored using hDAuNPs to deliver MBs into cancer cells to visualize the intracellular environment. Recently, telomerase-responsive AuNP MBs were developed to monitor intracellular telomerase activity in live cells for cancer theranostics [52]. Endocytosis-mediated translocation of AuNPs, however, results in low efficiencies and stochastic delivery. Efforts have been taken to improve the efficiency with the aid of physical methods, such as ultrasound (i.e., sonoporation) [51,53], electroporation [54], and gene gun [55].

Finally, a wide variety of nanomaterials, such as carbon nanotubes (CNTs) [56], polyethylenimine (PEI)-grafted graphene nanoribbon [57], SnO_2 nanoparticles [58], chitosan nanoparticles [59], quantum dots [60], and polymethyl methacrylate (PMMA) nanoparticles [61], have also been

reported for MB delivery into live cancer cells including HeLa cells (probe: miR-21) [58], lung cancer cells (probe: miR-155) [59], and MDA-MB-231 cells (probe: MMP-2 MBs) [60].

MB Designs for Intracellular Bio-Interrogation of Cancer Cells

Designing MBs to accurately probe biomarkers in cancer cells presents equally challenging opportunities. Conventional assays (e.g., morphological analysis) often lead to low accuracy and uncertainty, and fail to fully document the highly heterogeneous nature of cancer cells. Tumors exhibit specific cellular and microenvironmental characteristics, including unique mRNAs, special tumor biomarkers, a reductive cytosol milieu, and low pH, among others, which provide a host of possibilities for detecting and sensing cancerous cells. MBs have thus gained a great deal of attention recently as powerful tools for thoroughly probing tumor-related biomarkers (Figure 2A). We summarize here the latest advances in MB designs for cancer research.

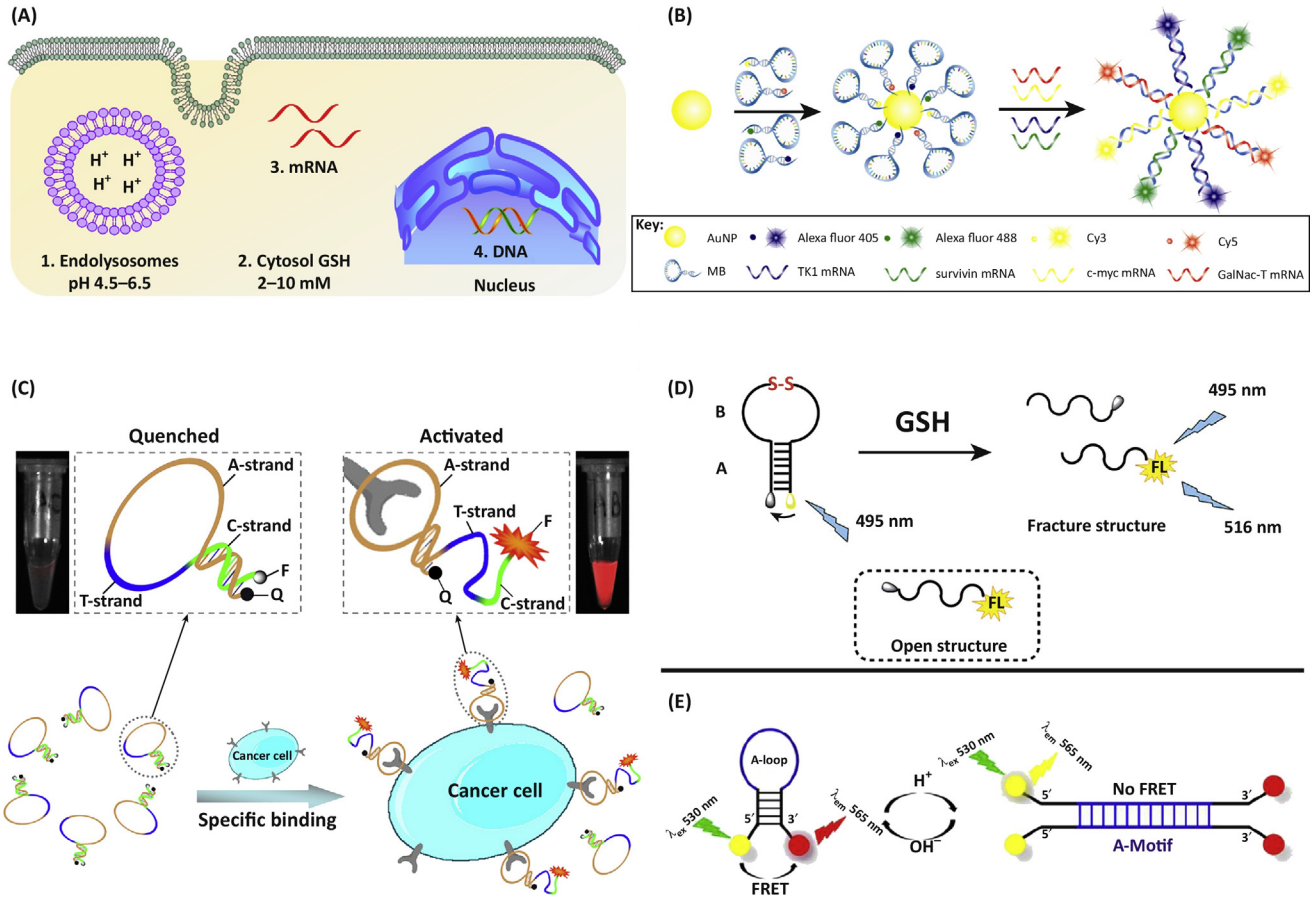

Figure 2. Examples of Cues Detected via MBs. (A) Schematic illustration of potential MB targets in tumor cells. (B) A four-color nanoprobe composed of an AuNP and labeled with Alexa fluor 405, Alexa fluor 488, Cy3, and Cy5 to detect TK1 mRNA, survivin mRNA, c-Myc mRNA, and GalNAc-T mRNA, respectively [64]. (C) A novel strategy for *in vivo* cancer imaging using an activatable aptamer probe based on a conformational change triggered by cell-membrane proteins. The probe consists of three fragments: a cancer-targeted aptamer sequence (A-strand), a poly-T linker (T-strand), and a short DNA sequence (C-strand) complementary to a part of the A-strand, with a fluorophore and a quencher attached at either terminus. In the absence of a target, the probe is hairpin-shaped with quenched fluorescence; upon binding to membrane receptors of the target cancer cell, its conformation is altered, thus resulting in an activated fluorescence signal [73]. (D) A disulfide-decorated MB for the detection of GSH. The disulfide bond is cleaved via a thiol–disulfide exchange in the presence of GSH, which leads to the dissociation of the two fragments and fluorescence enhancement. This system was used to quantify intracellular GSH levels in K562 leukemia cells [82]. (E) A representative pH-sensitive MB sensor labeled with Cy3 and Cy5; upon excitation at 530 nm, the fluorescence intensity ratios of Cy3 versus Cy5 depends on the change of pH from 7 to 3, demonstrating remarkable potency to sense pH within a narrow acidic window [86]. Figures reproduced with permission from the references listed. Abbreviations: AuNP, gold nanoparticle; FRET, fluorescence resonance energy transfer; GSH, glutathione; MB, molecular beacon.

MBs have been primarily designed to target cytosolic mRNAs in cancer cells. Tumor mRNAs can often represent specific markers for therapeutic, diagnostic, and prognostic purposes**.** Recent studies have highlighted the relevance of these probes in early detection efforts. Tang *et al.* developed an effective approach to detect multiple tumor mRNAs [62]. A bimolecular beacon (bi-MB) termed AuNP/bi-MB was self-assembled from gold nanoparticles. This functional hybrid MB could simultaneously target two types of tumor mRNAs, cyclin D1 mRNA and survivin mRNA, in breast cancer cells. This system has been shown to effectively avoid false-positive results and yield more comprehensive and dependable information for the early detection of cancer. The same group recently described the *in vitro* detection of up to three tumor-related mRNAs (c-Myc mRNA, GalNAc-T mRNA, and TK1 mRNA) in different cells. The modified AuNP-MB probes show higher accuracy than the single-marker assays, as well as higher resistance against nucleases, and they have been shown to differentiate tumor cells from normal cells using breast and liver cancer models [63].

To minimize the rate of false positives, Tang *et al.* proposed a four-color nanoprobe that can simultaneously detect up to four types of mRNAs in live cells (Figure 2B) [64]. The nanoprobe consisted of AuNPs and a dense corona of MBs that can discern multiple intracellular mRNA transcripts, which increased the accuracy in cancer cell identification compared to single MBs. In particular, the probe was labeled with Alexa fluor 405, Alexa fluor 488, Cy3, and Cy5 to detect TK1 mRNA, survivin mRNA, c-Myc mRNA, and GalNAc-T mRNA, respectively. Subsequent work has focused on detecting and imaging tumor mRNA *in vivo* [48,50,65–67].

MBs have also been reported to identify DNA mutations with high specificity, sensitivity, and speed [68]. In an early report Yang *et al.* proposed a method to detect the expression levels of multiple genes in both fixed and viable cells using MB-based imaging [69]. Simultaneous delivery of MBs with two different DNA targets (i.e., survivin and cyclin D1) successfully differentiated cancer cells from normal/healthy cells in a breast cancer model. Both cyclin D1 and surviving MBs only generate fluorescent signals upon hybridization with their specific DNA target. Recently, Wu and coworkers reported a primer-integrated label-free multifunctional MB (LMMB) to detect specific DNA sequences via integrating target recognition sequences, polymerization primers, templates, and G-quadruplex structures (also known as G4-DNA, nucleic acids that are rich in guanine and tend to form tetramers via hydrogen bonds) into a single system [70]. Such a system is compatible with target DNA amplification and detection with SYBR green I. Moreover, the LMMB technology can be adapted for amplifying target DNA without an extra DNA probe. The polymerization reaction is thought to generate double-stranded fragments suitable for the intercalation of SYBR green I to detect hybridization between the LMMB and target DNA. By using a G-quadruplex instead of conventional hairpin, the target DNA can induce further conformational changes in the LMMB structure, permitting the detection of single-nucleotide mutations. In recent years, MBs have been reported to detect tumor markers (e.g., p53) [71] and circulating DNA from serum samples in a breast cancer model [72].

MBs can also be designed to detect specific tumor proteins. Aptamer probes have been used to successfully target particular membrane proteins on cancer cells for contrast-enhanced tumor visualization in mice (Figure 2C) [73]. Conformational alterations upon binding to a target protein can result in fluorescence, which is then used to conduct *in vitro* and *in vivo* studies with CCRF-CEM cancer cells (a T cell line associated with human acute lymphoblastic leukemia) and a specific aptamer, sgc8. The activated fluorescence signals show high sensitivity and high specificity for identifying CCRF-CEM cells *in vivo*.

Glutathione (GSH) is an important peptide responsible for modulating a host of biological processes. Tumor cells, in particular, exhibit about a 100- to 1000-fold increase in intracellular GSH levels compared to the extracellular microenvironment [74–76], and this has made the GSH

peptide a potential target of interest in drug development efforts [77–81]. As such, different MB-based approaches have been devised to probe the cytosolic levels of GSH in tumor cells. Guo *et al.* developed a disulfide-bonded MB in which the loop contains ssDNA sequences that are functionalized with a disulfide bond, and the fluorophore–quencher pair is tethered at the hairpin structure (Figure 2D) [82]. Upon targeting GSH, the disulfide bond is cleaved via a thiol–disulfide exchange, which leads to the dissociation of the two fragments and fluorescence enhancement because of its low stability in water. This system was used to quantify intracellular GSH levels in K562 leukemia cells.

Important biological events such as enzyme catalysis and protein conformational changes, among others, are tightly regulated by the intracellular pH. Indeed, changes in intracellular pH can potentially result in cellular dysfunction including tumorigenesis [83]. Tumor cells have particularly acidic intracellular microenvironments, which suggests the possibility of developing pH-sensitive tumor diagnostic and therapeutic MBs-based tools [84,85]. Recently, a MB-based DNA switch was developed as an efficient and reversible pH sensor. Structural transformations between the open (A-motif) and closed (MB) states were successfully achieved within a narrow pH range comparable to the acidic intracellular milieu found in tumors [86]. As shown in Figure 2E, this system was composed of a hairpin-like structure of 24-nucleobases, including 12 consecutive A-bases in the loop part and two stretches of five complementary base pairs labeled with Cy3 and Cy5 fluorescent dyes at the 5′- and 3′-ends, respectively. The hairpin structure was modulated by pH fluctuations. The utility of the MB-based pH sensor was further demonstrated in the analysis and imaging of tumor cells, and this could inspire the development of DNA probes for intracellular pH sensing and drug delivery applications.

Concluding Remarks and Future Perspectives

MBs have positioned themselves as powerful tools for cancer cell bio-interrogation, including enabling the possibility of detecting gene mutation and/or allowing gene editing (see Outstanding Questions). Intercellular heterogeneities, in particular, have been hypothesized to play an important role in cancer relapse because subpopulations of cells (e.g., cancer stem-like cells) have the potential to evade therapy more effectively than other tumor cells, and thus establish new foci of growth locally and/or distally. MB-based systems have therefore been used to identify and analyze such cancer-initiating cellular subpopulations at the single-clone level.

In general, MBs are small molecules (15–30 nt) that facilitate intracellular delivery compared to larger/bulkier cargo (e.g., plasmids) [2,87]. Currently, the primary strategy in the development of MBs for intracellular probing is to design them to hybridize to mRNA in the cytosol. This approach is much easier than designing plasmid probes or probes that hybridize to DNA because such probes must enter the nucleus after long-distance transport across both the cell membrane and the nuclear membrane. In this regard, conventional and/or advanced non-viral methods are well-suited for cytosolic delivery of MBs. However targeted delivery of MBs requires specially modified MBs and/or carriers. For example, MBs could be tethered with target organism-specific ligands such that the probes can be properly deployed to destinations such as the nucleus or mitochondria.

One determining factor for MBs is the loop sequence; in particular, the probe sequence, which is between 15 and 30 nt long, and is generally devoid of secondary structures. Increasing this length would result in improved affinity but also lead to reduced specificity, thus careful choice of probe length and components is necessary to ensure specific target recognition without compromising affinity. Although several MB delivery methods have been developed and tested (e.g., chemical carriers and physical platforms), chemical approaches have been reported to be more prone to MB degradation (e.g., endosomal degradation), which could result in false-positive readings. In addition, external physical stimulation (e.g., electric fields during

Outstanding Questions

How can we avoid 'false' MB signals triggered by the intracellular delivery method, and increase the detection specificity?

How can we evaluate, and ideally diminish, the interference from single-base mutations in intracellular detection?

How exactly can the intracellular trafficking of MBs be evaluated?

How can we conduct clinically relevant research after purifying/isolating cancer cells identified by MBs?

To what extent can MBs be applied in cancer diagnostics and therapy?

electroporation) may also alter the function of the MBs. Multiple studies have therefore used supplementary techniques (e.g., PCR, transcriptome microarray) to verify the reliability of the MB signal [3,26]. Co-delivery of multiple MBs for a single target has also been shown to be an efficient approach to reduce the incidence of false positives [88,89]. Co-delivery of MBs could also enable the development of advanced multiplexed analytic platforms to monitor, in real time, the intracellular levels of multiple targets at the same time. Such targets, however, could potentially be found in different intracellular compartments (e.g., the nucleus vs the cytosol), and this may require the development of novel delivery approaches and/or MB designs that incorporate specific localization sequences compatible with compartmentalized delivery. In addition, further work concerning the trafficking and final fate of MBs within cells is also imperative for the design of MBs.

Finally, for *in vitro* early cancer detection, single-nucleotide mutations remain challenging for MB-based platforms. The ability of MBs to recognize the genetic heterogeneity associated with single-nucleotide mutations is currently limited. In the future, incorporating the PCR technique to add an altered base to the mutated base of the primer may reduce the risk of inducing single-nucleotide mutations and increase the accuracy of MBs in cancer detection. In addition, more studies need to be conducted to better understand the mechanisms underlying the hybridization between MBs and their targets, and any potential downstream influence on cell behavior. Such efforts could play a pivotal role in developing optimized MB designs and/or delivery methods, and these could have a significant impact not only on disease diagnosis and prognosis but also therapeutics for precision medicine.

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