ACSNANO

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REVIEW

DNAzyme-Based Biosensors: Immobilization Strategies, Applications, and Future Prospective

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Cite This: *ACS Nano* 2021, 15, 13943−13969

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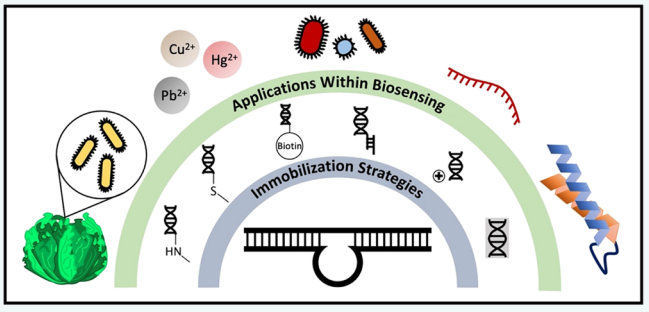
ABSTRACT: Since their discovery almost three decades ago, DNAzymes have been used extensively in biosensing. Depending on the type of DNAzyme being used, these functional oligonucleotides can act as molecular recognition elements within biosensors, offering high specificity to their target analyte, or as reporters capable of transducing a detectable signal. Several parameters need to be considered when designing a DNAzyme-based biosensor. In particular, given that many of these biosensors immobilize DNAzymes onto a sensing surface, selecting an appropriate immobilization strategy is vital. Suboptimal immobilization can result in both DNAzyme detachment and poor accessibility toward the target, leading to low sensing accuracy and sensitivity. Various approaches have been employed for DNAzyme immobilization within biosensors, ranging from amine and thiol-based covalent attachment to non-covalent strategies involving biotin−streptavidin interactions, DNA hybridization, electrostatic interactions, and physical entrapment. While the properties of each strategy inform its applicability within a proposed sensor, the selection of an appropriate strategy is largely dependent on the desired application. This is especially true given the diverse use of DNAzyme-based biosensors for the detection of pathogens, metal ions, and clinical biomarkers. In an effort to make the development of such sensors easier to navigate, this paper provides a comprehensive review of existing immobilization strategies, with a focus on their respective advantages, drawbacks, and optimal conditions for use. Next, common applications of existing DNAzyme-based biosensors are discussed. Last, emerging and future trends in the development of DNAzyme-based biosensors are discussed, and gaps in existing research worthy of exploration are identified.



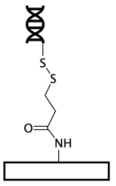
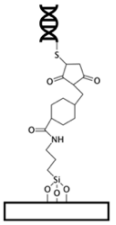
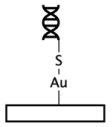
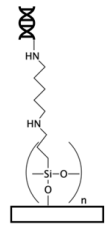
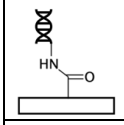
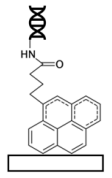
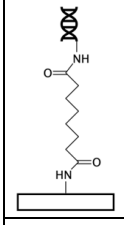
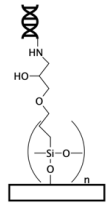
KEYWORDS: *DNAzyme, biosensor, sensing, immobilization, metal ion detection, pathogen detection, biomarker detection, cancer detection, food monitoring*

Deoxyribozymes (DNAzymes) have continuously intrigued the scientific community due to their potential as biosensors for various clinical and environmental applications.[1] As the name implies, these functional oligonucleotides have enzymatic properties that allow them to mediate various chemical reactions including DNA and RNA cleavage, ligation, and phosphorylation.[2] The activity of some DNAzymes is dependent on the presence of specific molecules, such as metal ions and proteins.[3] Because of this property, these DNAzymes can be used to engineer biosensors capable of detecting such molecules. Many DNAzymes have been shown to have the capability to distinguish between similar stimuli—such as different strains of bacteria, which allows for the development of targeted biosensors with high specificity.[4,5]

DNAzyme-based biosensors offer various advantages over traditional detection strategies. Enzyme-linked immunosorbent assays (ELISAs) are one of the most common procedures used for the detection of target analytes. While a valuable tool, ELISA performance is heavily impacted by environmental conditions, and the assays exhibit poor long-term stability due to the sensitive nature of their expensive protein components.[6] ELISAs also offer limited sensing capabilities, preventing the

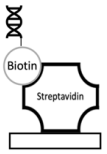
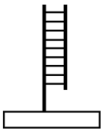
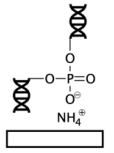
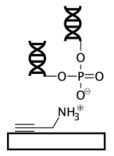
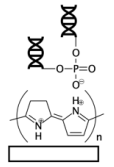
Received: May 21, 2021
Published: September 15, 2021

Table 1. Comprehensive Overview of Existing DNAzyme Immobilization Techniques for Biosensing

Immobilization Strategy	Surface Functionalization	DNAzyme Functionalization	Detection Targets	Transduction Methods	Considerations	Schematic	Ref
Thiol-mediated	N-succinimidyl 3-(2-pyridyldithio) propionate-treated	Thiolated	Lead ions	Fluorescence	*Advantages* 1. Robust covalent immobilization 2. Easy implementation 3. Crosslinker not needed if using Au 4. Inherent quenching properties if using Au *Disadvantages* 1. Increased cost 2. Limited to certain transduction methods		40
	Sulfosuccinimidyl-4-(N-maleimidomethyl) -cyclohexane-1-carboxylate		Uranyl ions	Electrochemical			28
	Gold (*via* electro-deposition or treatment with cysteamine, Fe_3O_4 or chromium)		Lead, copper, nickel, uranyl ions	Fluorescence, electrochemical			12,16,17,25,34,37,38
			Avian influenza virus	Electrochemical			
			L-histidine				
Aldehyde-mediated	Aldehyde (hydroxyl + APTES + glutaraldehyde or pre-purchased)	Amine	Lead	IR spectroscopy	*Advantages* 1. Robust covalent immobilization 2. Wide selection of available chemistries 3. Compatibility with all transduction methods 4. Well established immobilization technique with extensive supporting literature 5. Limited cost *Disadvantages* 1. Introduction of a crosslinker		41,45–48
			H_2O_2	Colorimetric			
			Thrombin				
Ester-mediated	NHS-ester (COOH + EDC/NHS)		Lead	Electrochemical			49–51
			Prostate specific antigen	Colorimetric			
	1-pyrenebutanoic acid succinimidyl ester		Silver, mercury, copper ions	Electrochemical			52,53
	Bis(sulfosuccinimidyl) suberate		Glucose	Colorimetric			54
Epoxy-mediated	Epoxy (GPTMS or pre-purchased)		Lead	Electrochemical, pH			24,55,56
			Food pathogen detection	Fluorescence			

https://doi.org/10.1021/acsnano.1c04327
ACS Nano 2021, 15, 13943−13969

Table 1. continued

Immobilization Strategy	Surface Functionalization	DNAzyme Functionalization	Detection Targets	Transduction Methods	Considerations	Schematic	Ref
Biotin-streptavidin interaction	Streptavidin (*via* spotting, bonding with AuNPs, EDC-NHS mediation, or pre-purchased)	Biotinylated	Lead	Fluorescence, electrochemical, colorimetric	*Advantages* 1. Very stable interaction 2. Compatibility with all transduction methods *Disadvantages* 1. Need to label sensing entities with protein		13,26,63–67,167
			Tetracycline	Colorimetric			
			Bacteria (*Helicobacter pylori*, *Vibrio anguillarum*)	Fluorescence, colorimetric			
DNA hybridization	Coated with DNA complimentary to DNAzyme	N/A	Copper ions	Fluorescence, electrochemical	*Advantages* 1. Provides mediation of sensing cascade 2. Compatibility with all transduction methods *Disadvantages* 3. Requires very specific sensor design		39,70–72
			Uranyl ions	Colorimetric			
			Lysozymes				
Electrostatic interactions	Ammonium	N/A	Lead, mercury ions	EIS	*Advantages* 1. High efficacy with reduced graphene oxide *Disadvantages* 1. Relatively weak interactions 2. Limited to electrochemical systems 3. Existing work with DNAzymes is limited		18
	Plasma-polymerized propargylamine		DNA fragments				19
	Plasma-polymerized polypyrrole		Lysozymes, *Salmonella*	Electrochemical			20,76
Physical entrapment	Polysaccharide-polynucleotide coacervate microdroplets	N/A	Ultraviolet irradiation	Fluorescence	*Advantages* 1. Unmodified DNAzymes can be used 2. Pullulan provides strong long-term stability 3. Increased DNAzyme activity upon sol-gel matrix optimization *Disadvantages* 1. Not compatible with many existing platforms 2. Requires significant sensor-specific optimization 3. Stability of non-pullulan approaches are unknown 4. Limited studies		14,26,78–80,168
	Polydopamine		H_2O_2	Electrochemical			
	Pullulan		*Klebsiella pneumoniae*, *Helicobacter pylori*	Fluorescence, colorimetric			
	Sol-gel-derived matrices		Metal ions mixture (multiplex detection)	Fluorescence			

detection of target analytes present at concentrations below 10^{-12} M. DNAzyme-based sensors offer greater stability and higher detection sensitivity at a lower cost.[6] Alternatively, when a nucleic acid target can be used for the detection of a particular entity, polymerase chain reaction (PCR) is often employed due to its strong detection capabilities. However, while significant progress has been made in the development of on-site PCR technologies, such systems remain quite complex and require a wide range of expensive components.[7] Additionally, when working with complex clinical samples, PCR platforms needs to be optimized extensively based on the nature of the sample, making their widespread use as a

https://doi.org/10.1021/acsnano.1c04327
ACS Nano 2021, 15, 13943−13969

detection platform quite cumbersome.[8] Contrarily, DNAzyme-based biosensing platforms can be easily modified for varying applications through the incorporation of the desired target-specific DNAzyme.

The process of identifying DNAzymes for specific applications is well-documented within literature. In essence, it comprises an *in vitro* selection process involving a large DNA pool consisting of as many as 10^{16} distinct DNA strands.[2] These strands undergo function-based selection steps that ultimately identify the strands that engage in enzymatic activity in response to the desired target—a process commonly referred to as systemic evolution of ligands by exponential enrichment (SELEX), which is also commonly used for the identification of other functional oligonucleotides such as aptamers.[2,9] While SELEX has historically been a very laborsome process, recent advancements have presented methods that make the procedure more efficient. Such advancements have been detailed at length elsewhere.[10,11] Once a DNAzyme sequence has been identified, large quantities can be easily produced at a low cost, given that DNA synthesis has become very inexpensive. This makes DNAzyme-based biosensors ideal for commercial use.

The subsequent process of taking an identified DNAzyme and developing a functional biosensor is much more ambiguous. That being said, many DNAzyme-based biosensors immobilize DNAzymes onto an underlying substrate, given that surface immobilization has been shown to improve detection sensitivity through a reduction in background noise.[12] This reduction is driven by the ability to wash away unreacted signaling entities, which is otherwise not possible within a solution-based sensor. Surface immobilization has also resulted in the development of reusable sensors.[12] For such surface-immobilized DNAzyme-based biosensors, various considerations—such as the nature of the underlying substrate and the signal transduction method—need to be made to optimize performance. Common substrates include various polymers,[13−15] gold,[12,16,17] and graphene,[18−20] each of which possess distinct properties that make them suitable for particular sensing systems. These properties include cost, potential for mass production, the degree of background noise, and the chemical groups present for surface functionalization. This last factor is key in the surface immobilization of a DNAzyme. If a surface-based sensor is prone to DNAzyme detachment, its accuracy and stability will be impaired. Thus, the selection of an appropriate immobilization strategy is critical in the development of a relevant biosensor.

DNAzyme-based biosensors have been used for widespread applications, given their strong detection capabilities and functionality within diverse environments. Namely, these biosensors have been used extensively for the detection of food contaminants,[21−24] pathogens,[25−27] metal ions,[13,15,28] and various clinically relevant biomarkers.[29−31] Compared to traditional detection methods, DNAzyme-based biosensors have shown robust accuracy, high detection sensitivity, and resilience to non-specific entities that can often hinder sensing cascades.[32,33]

With the high degree of research activity in the field of DNAzyme-based detection, a plethora of immobilization methods have emerged. These strategies have characteristics that make them ideal for varying detection platforms. Accordingly, there has been extensive growth in the target applications of these biosensors. Herein, we provide a comprehensive review of existing immobilization methods, their respective advantages and disadvantages, as well as detection platforms for which they are most appropriate. Subsequently, we detail common applications of DNAzyme-based biosensors, with a focus on recent developments. Finally, emerging trends and future directions are discussed in relation to both DNAzyme immobilization strategies and target applications of DNAzyme-based biosensors. Discussions in this Review are informed by all three DNAzyme classes—RNA-cleaving DNAzymes, DNA-cleaving DNAzymes, and peroxidase-mimicking DNAzymes—to provide a comprehensive understanding of the field.

DNAzyme IMMOBILIZATION STRATEGIES FOR BIOSENSING

A wide range of covalent and non-covalent immobilization strategies have been implemented in the functionalization of sensing substrates with DNAzymes. While some strategies—such as DNA hybridization and DNA-mediated electrostatic interactions—are specific to oligonucleotides, many DNAzyme immobilization strategies are non-specific to DNA entities. Rather, they are used for the immobilization of various biomolecules. However, the ways in which these strategies are selected and implemented revolve around fundamental design considerations specific to DNAzyme-based sensors. Such considerations include the nature of the target entity, the properties of the underlying substrate, and the desired signal transduction method. All such considerations are discussed at length for individual immobilization methods, in the context of recent studies involving DNAzyme-based biosensors. A detailed summary of DNAzyme immobilization methods is additionally provided in Table 1.

Covalent Immobilization. *Thiol-Based Attachment.* Thiol-based attachment has presented itself as a premier strategy for DNAzyme immobilization. The resulting attachment is both robust, given its covalent nature, and easy to implement, as its constituent parts are often already involved in the sensing pathway. Specifically, platforms using gold can use this metal as a binding site for thiolated DNAzymes, yielding Au−S bonds.[12] The strong binding affinity associated with thiol-based immobilization has been shown to increase biosensor shelf life and has made certain sensing devices reusable.[12,34] This is especially true for DNAzyme-based biosensors, given that these functional oligonucleotides are capable of maintaining functionality in a wide range of conditions.[35,36]

Using thiol-based immobilization, significant improvements in detection limits have been achieved. In particular, Swearingen *et al.* found that the detection limit for lead ions (Pb^{2+}) can be reduced from 10 nM to 1 nM through immobilization onto an Au substrate, due to a reduction in background signals.[12] Their sensor involved the immobilization of a quencher-containing RNA-cleaving DNAzyme onto Au films, which would subsequently hybridize with a fluorophore-containing substrate strand. While initial fluorescence was minimal, a substantial increase was observed upon Pb^{2+}-induced cleavage of the substrate strand due to separation between the fluorophore and quencher.[12] Following a sensing cycle, replenishing the attached DNAzymes with uncleaved substrate strands was all that was needed to prepare the sensor for subsequent samples, as the immobilized DNAzyme was unaffected by the sensing cascade.[12] Electrochemical detection has also been achieved on an Au substrate by Yang *et al.*, who immobilized thiolated nickel (Ni^{2+})-

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ACS Nano 2021, 15, 13943−13969

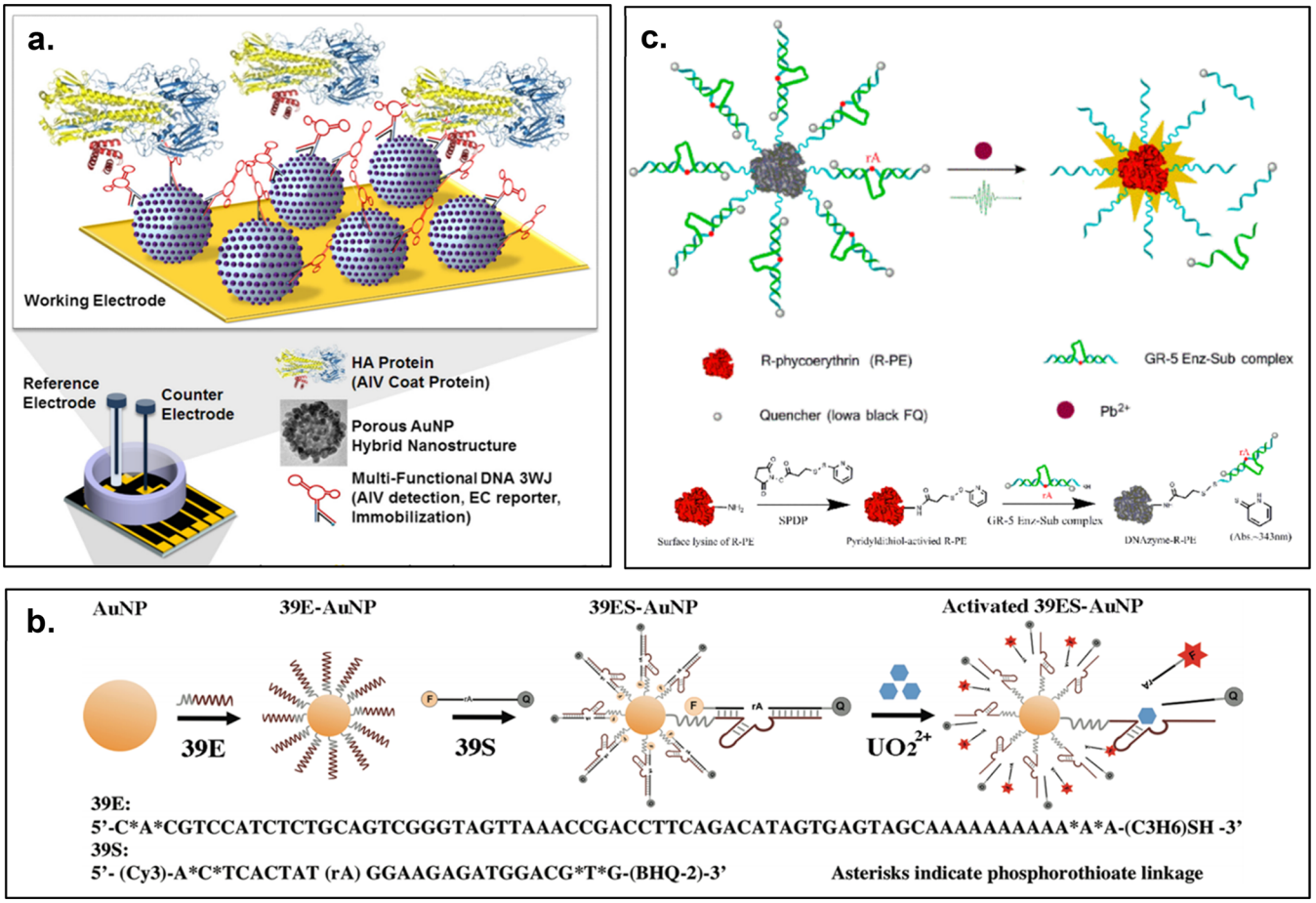

Figure 1. Immobilization of DNAzymes onto biosensing components using thiol-based attachment. (a) Hemagglutinin-responsive DNAzymes immobilized onto porous AuNPs, which maximize available surface area for binding. Reprinted with permission from ref 25. Copyright 2019 Elsevier. (b) Immobilization of thiolated DNAzymes hybridized with fluorophore-quencher construct onto AuNPs for uranyl ions detection. Reprinted with permission from ref 16. Copyright 2013 American Chemical Society. (c) Pb^{2+}-responsive DNAzymes hybridized with a quencher-containing fragment immobilized onto R-phycoerythrin using *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) as a cross-linking agent. Reprinted with permission under a Creative Commons CC-BY license from ref 40. Copyright 2019 Multidisciplinary Digital Publishing Institute.

responsive RNA-cleaving DNAzymes onto an Au electrode. The DNAzymes were hybridized with substrate strands coupled with cadmium selenide quantum dots. Upon Ni^{2+}-induced cleavage, the dislocation of the quantum dots from the electrode surface was detectable through differential pulse anodic stripping voltammetry with a limit of detection of 6.67 nM.[37] Importantly, sensors aiming to immobilize biomolecules onto Au substrates are washed with mercapto-hexanol following incubation with the DNAzyme to disrupt any Au−N non-specific interactions that may have been formed between Au substrates and oligonucleotides.[34,38] Such a treatment ensures a monolayer of Au−S bonded DNAzymes across the sensing surface.[34]

A wide range of three-dimensional form factors have been used when incorporating Au within DNAzyme-based biosensors. Specifically, Au nanoclusters (AuNCs),[34] nanoparticles (AuNPs),[16,17,25,35] and nanoflowers (AuNFs)[39] have all been used to maximize the surface area available for thiol-based attachment of biosensing components. Compared to planar sensing surfaces, these substrates can result in an improved limit of detection. Under this principle, Hu *et al.* coated glass carbon electrodes with AuNCs and subsequently used Au−S bonds to immobilize thiolated copper (Cu^{2+})-responsive DNA-cleaving DNAzymes.[34] The DNAzymes cleaved a complementary substrate strand when exposed to Cu^{2+}, resulting in a change in the interfacial electron-transfer resistance on the surface of the electrode, which was detectable for Cu^{2+} concentrations as low as 0.07 nM.[34]

Zhang *et al.* developed a Pb^{2+} biosensor by coating magnetic AuNPs with Pb^{2+}-responsive RNA-cleaving DNAzymes, hybridized with a substrate strand. Upon exposure to Pb^{2+}, the substrate strand was cleaved, and the magnetic properties of the NPs were used to separate the particles from the cleaved oligonucleotide fragments.[17] These fragments were then subjected to a DNA extension reaction and subsequently exposed to *N,N′*-bis (2-(trimethylammonium iodide) propylene) perylene-3,4,9,10-tetracarboxyldiimide (PDA^+), to which the negatively charged DNA strands could then electrostatically interact. Upon transferring this solution to an electrode, its prior attachment interactions with DNA prohibited PDA^+ from binding to the electrode. This led to a detectable electrochemical shift for concentrations above 30 pM.[17] In another recent work, Lee *et al.* used porous AuNPs to further maximize the available surface area on these particles.[25] Using thiol-based immobilization, these particles were coated with a peroxidase-mimicking DNAzyme specific to a hemagglutinin

https://doi.org/10.1021/acsnano.1c04327
ACS Nano 2021, 15, 13943−13969

protein associated with avian influenza virus, as depicted in Figure 1a. This sensor achieved a limit of detection in the 1 pM range. Interestingly, thiol-based immobilization has also been used to deliver DNAzymes into cells for biosensing. Wu *et al.* coated AuNPs with RNA-cleaving DNAzymes responsive to uranyl ions to detect the presence of these carcinogenic entities within live cells, as shown in Figure 1b.[16] The thiol-based attachment showed both stability in serum and resistance to the cellular environment. Cleavage within a fluorophore−quencher substrate strand allowed for uranyl ion quantification through fluorescence.

Ultimately, the primary advantage of this immobilization strategy is that no cross-linker needs to be introduced into the sensing platform. For many of the sensors outlined here, Au entities play an integral role in the sensing cascade, while providing the additional benefit of immobilization. Even when Au is solely implemented for immobilization, it proves to be easier to implement compared to chemical cross-linkers, which require substantial optimization. For fluorescence-based biosensors, AuNPs provide the added benefit of acting as a quencher, providing lower background fluorescence than organic quenchers.[40] That being said, Au integration into a biosensor can increase cost substantially. To this end, thiol-based immobilization has been induced through the use of *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) as a cross-linking agent. Wu *et al.* coated R-phycoerythrin—a naturally fluorescent protein isolated from red algae—with Pb^{2+}-responsive, thiol-modified RNA-cleaving DNAzymes hybridized to substrate strands containing a quencher, as shown in Figure 1c.[40] SPDP bonded the thiol group of the DNAzyme and the amine group of R-phycoerythrin, resulting in a disulfide and an amide bond, respectively. Sulfosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (Sulfo-SMCC) has also been used for thiol-based DNAzyme immobilization. Wen *et al.* used this cross-linker to immobilize DNAzymes onto aminated mesoporous silica nanoparticles, as its maleimide groups were able to bind to thiolated DNAzymes through thioether linkages.[28]

Amine-Based Attachment. One of the most common DNAzyme immobilization strategies involves the attachment of aminated DNAzymes to functionalized surfaces. The covalent nature of this approach ensures robust immobilization. A variety of cross-linkers for amine attachment have been explored in the context of DNAzyme-based biosensing, typically containing an aldehyde, ester or epoxy group that can react with amine modified oligonucleotides.

Surface functionalization with aldehyde groups can be induced through a range of different pathways. For macroscale surface treatments, induction of hydroxyl groups *via* oxygen plasma or piranha incubation and subsequent treatment with aminopropyltriethoxysilane (APTES) provides an excellent platform for glutaraldehyde immobilization.[41−44] Specifically, the amine group present on APTES can react with an aldehyde group on glutaraldehyde in a condensation reaction resulting in the formation of an imine group known as a Schiff base. The second aldehyde group on glutaraldehyde can then react with aminated oligonucleotides, resulting in immobilization. Wang *et al.* recently used this approach to immobilize a peroxidase mimicking DNAzyme-containing construct for the chemiluminescent detection of thrombin on glass.[41] For biosensors using heavily hydroxylated substrates, APTES deposition can readily take place on the substrate without any prior treatment. Notably, nanoporous alumina has been directly coated with APTES and then glutaraldehyde to immobilize Pb^{2+}-responsive RNA-cleaving DNAzymes, leading to a biosensor that achieved a limit of detection of 12 nM using interferometric reflectance spectroscopy.[45] In the case of a substrate that is rich in amine groups, glutaraldehyde has also been successfully added directly as the sole cross-linking agent for DNAzyme immobilization. Specifically, Chen *et al.* immobilized a peroxidase mimicking DNAzyme onto eggshell membranes for chemiluminescent detection of hydrogen peroxide.[46] Other studies have relied on pre-purchased reagents such as aldehyde-terminated agarose microbeads and aldehyde-coated glass slides for DNAzyme-based detection of heme and Pb^{2+}, respectively.[47,48]

Alternatively, surface functionalization with esters provides another means by which aminated DNAzymes can be immobilized. Most prominently, this pathway involves inducing carboxyl groups onto the surface and subsequent treatment with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). The resultant NHS ester can then covalently bind with primary amine groups to form amide linkages. Such a strategy has been applied for DNAzyme immobilization in both electrochemical and chemiluminescent detection platforms for Pb^{2+} detection.[49,50] Ye *et al.* demonstrated the applicability of this strategy by treating carboxylated Fe_3O_4 magnetic spheres with EDC-NHS to immobilize aminated DNAzymes.[51] The resultant electrochemical biosensor was capable of detecting prostate-specific antigen to a limit of detection of 0.3 fg/mL.

DNAzyme immobilization onto single-walled carbon nanotubes (SWNTs) has been an area of active research given the improvement in detection sensitivity when using these entities for biosensing.[52] In this case, 1-pyrenebutanoic acid succinimidyl ester (PBASE) is used, given that its pyrene ring can form $\pi-\pi$ interactions with the SWNT bulk material.[52] Simultaneously, its ester group can be used to immobilize aminated DNAzymes. This strategy has been used for silver, mercury, and copper ion DNAzyme-based detection thus far.[52,53] Bis(sulfosuccinimidyl)suberate (BS3) has also been used as a cross-linker for DNAzyme immobilization—specifically to glucose oxidase.[54] BS3 contains two NHS esters, which are separated by an eight-carbon chain, making it ideal for linkage between two aminated entities.

Epoxy-based immobilization typically involves using (3-glycidyloxypropyl)trimethoxysilane as a cross-linker between a hydroxylated surface and the aminated DNAzyme. Such an approach has been used for immobilization onto hydroxylated magnetic beads for the detection of Pb^{2+} at picomolar and nanomolar concentrations through electrochemical and pH detection systems, respectively.[55,56] Pre-treated epoxy surfaces have also been used for the immobilization of DNAzymes for pathogen detection in food samples. By immobilizing bacterium-specific RNA-cleaving fluorescent DNAzymes on flexible substrates and attaching them onto food wraps, Yousefi *et al.* were able to create a platform that provided strong fluorescence signals in response to pathogenic contamination.[24]

Amine-based attachment is one of the most versatile immobilization methods, given its compatibility with all common signal transduction methods. The wide range of cross-linkers available means that sensing entities do not need to undergo significant modifications for attachment to any given substrate. The primary drawback of this strategy is in that it requires the introduction of an otherwise unnecessary

https://doi.org/10.1021/acsnano.1c04327
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reagent. Given the ultrasensitive nature of DNAzyme biosensors, any additional entity has the potential to interfere with the sensing cascade, resulting in reduced detection performance.

Non-covalent Immobilization. *Biotin-Based Interactions.* Avidin, NeutrAvidin, and streptavidin are three biotin-binding proteins that are often paired alongside biotin to immobilize biomolecules onto surfaces. With association constants in the range of 10^{15} M^{-1}, the interactions between biotin and these three proteins represent some of the strongest non-covalent interactions present in nature.[57] Of the three, avidin exhibits the highest degree of non-specific absorption. This is attributed to its glycosylated nature, which results in an overall positive charge on the protein, resulting in interactions with the various negatively charged entities present in sensing environments.[57] Streptavidin and NeutrAvidin both lack this glycoprotein portion, making them more suitable for biosensing applications. NeutrAvidin in particular exhibits the most neutral isoelectric point, resulting in the lowest degree of non-specific absorption.[58] Nonetheless, all three proteins are widely used to immobilize biomolecules. Biotin-based immobilization allows for easy implementation and compatibility within all common signal transduction methods. Additionally, the mild nature of this immobilization strategy makes it ideal for immobilization onto delicate substrates that are prone to damage.[13] Implementation is simple, given that DNAzymes can be modified with a biotin molecule at either the 3′ or 5′ end of the functional oligonucleotide. That biotin can then bind to binding proteins immobilized on a surface.

The use of avidin within biosensing platforms is limited due to the aforementioned prominence of non-specific adhesion. Nevertheless, its ease of use has led to its incorporation within numerous sensing platforms. Specifically, avidin graphite–epoxy composites represent one of the most common avidin-containing substrates used for biomolecule immobilization. These composites can be prepared using dry chemistry strategies, which result in the facile incorporation of avidin onto the target substrate.[59] Such composites have been most notably used for the immobilization of biotinylated Cu^{2+}-responsive DNAzymes.[60] Additionally, avidin–biotin interactions were used by Song *et al.* to aid in the immobilization of biotinylated DNAzymes onto mesoporous silica nanoparticles.[61] An avidin–DNAzyme complex was formed between two biotinylated enzymatic DNAzyme strands and one avidin protein. The formation of this complex resulted in the entrapment of fluorescein within the nanoparticles, giving the binding interaction a secondary function. Upon DNAzyme-induced cleavage of the substrate strand, the biotinylated enzymatic strand was displaced resulting in the release of fluorescein, but its avidin interactions were maintained due to their high affinity.[61]

While NeutrAvidin presents itself as an excellent alternative to avidin, its use within DNAzyme-based platforms is scarce. NeutrAvidin-coated substrates have been used to immobilize DNA probes, which validate their compatibility with oligonucleotides.[62] Its minimal use may be due to the limited commercial availability of diverse NeutrAvidin-coated substrates and bioconjugates. The abundance of streptavidin-coated substrates and bioconjugates has resulted in a significant volume of literature making use of this biotin-binding protein.

Streptavidin is easily and extensively physically absorbed by various substrates—such as those composed of poly(methyl methacrylate) (PMMA) or nitrocellulose.[13,63] For sensing platforms that require particle-based immobilization, streptavidin-coated magnetic and agarose beads are both readily available and have been successfully used in the development of DNAzyme-based biosensors.[26,64] For materials that cannot readily absorb streptavidin, numerous methods of streptavidin functionalization have been developed. Such approaches are instrumental within electrochemical detection systems, as the sensitivity and stability of such sensors rely heavily on the underlying substrate. In particular, substrates composed of two-dimensional nanomaterials have gained significant attention due to their beneficial physical and chemical properties. In a recent study by Ji *et al.*, tin dioxide-functionalized reduced graphene oxide, a two-dimensional nanomaterial with high electron mobility and surface area, was functionalized with APTES and AuNPs. Streptavidin was then able to bind to the AuNPs *via* amino–Au interactions.[65]

Likewise, nanotubes have also garnered significant interest for biosensing applications. Their electrical conductance changes considerably in response to variations in electrostatic charge and the absorption of molecular entities onto their surface, which makes them ideal for detecting ongoing reactions with high sensitivity. Yim *et al.* reported a strategy for immobilizing DNAzymes onto nanotubes using biotin–streptavidin interactions.[66] In their work, multi-walled carbon nanotubes were acid-treated to induce carboxyl groups on the surface and then functionalized with streptavidin following EDC-NHS-mediated carboxyl activation.[66] Streptavidin was thus covalently attached onto the surface and used to immobilize biotinylated DNAzymes. Notably, the Michaelis–Menten kinetics of this system indicated that immobilization onto the nanotube–streptavidin structure did not hinder enzymatic activity in any way, further supporting the use of such constructs for future studies.[66]

Given streptavidin's four binding sites, biotin–streptavidin is also compatible with complex systems that require binding between multiple molecular entities. This property has been particularly advantageous within biosensors that work *via* an enzyme-linked aptamer assay. Gong *et al.* recently developed such a biosensor for tetracycline (TC), in which a surface was coated with TC conjugated with bovine serum albumin (BSA) and subsequently exposed to a biotinylated peroxidase-mimicking DNAzyme, a biotinylated aptamer specific to TC and free TC, which competed with immobilized TC for binding to the aptamer.[67] Streptavidin used two of its binding sites to act as a cross-linker between the two biotinylated molecules. Considering that the aptamer and DNAzyme are the target detector and signaling reaction catalyzer respectively within this system, this linkage was instrumental for the sensor's functionality.

Overall, biotin-based systems are highly effective and versatile for DNAzyme immobilization. However, given that avidin, NeutrAvidin, and streptavidin are proteins, their structure and binding affinity may be affected by storage under non-refrigerated conditions. Assays performed in solutions that lead to protein denaturation or in the presence of proteases also stand to deteriorate biotin-based immobilization. Alongside these constraints, this approach requires sensing entities to be labeled with biotin and/or biotin-binding proteins—labels that provide no benefit to the sensing platform outside of immobilization. An attempt to incorporate immobilization entities into other aspects of the sensing system to improve performance has been a key contributor to the

https://doi.org/10.1021/acsnano.1c04327
ACS Nano 2021, 15, 13943–13969

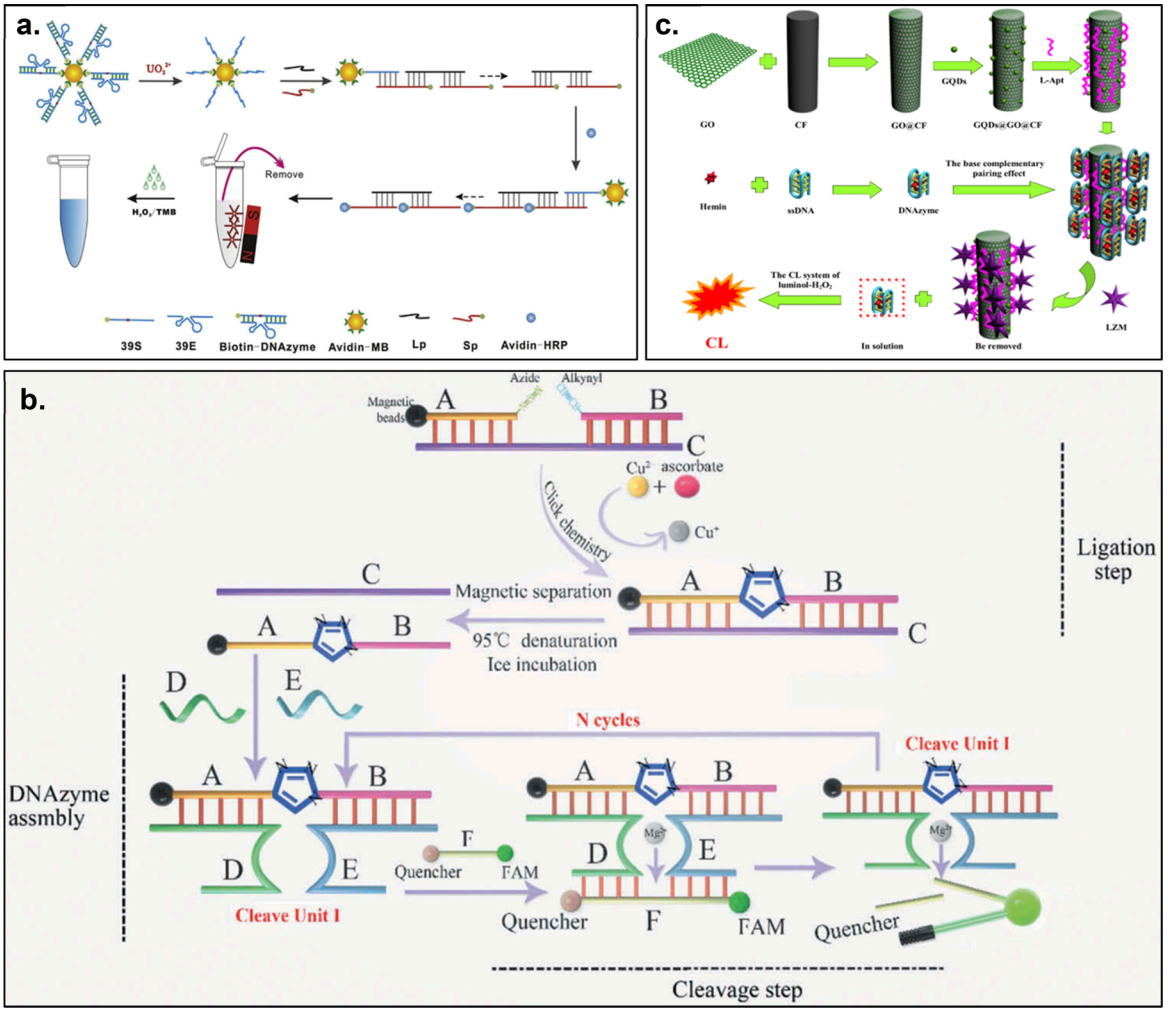

Figure 2. DNA hybridization-mediated immobilization of DNAzymes for sensing applications. (a) Uranyl ion-responsive DNAzymes hybridized to a substrate strand for immobilization onto magnetic beads. Reprinted with permission from ref 70. Copyright 2017 Elsevier. (b) Hybridization-based click chemistry for the detection of copper ions through fluorescence. Reprinted with permission from ref 71. Copyright 2020 Taylor & Francis. (c) Hybridization of hemin/G-quadruplex DNAzymes with lysozyme aptamers for immobilization onto carbon fiber composites. Reprinted with permission from ref 72. Copyright 2019 Elsevier.

preference for other immobilization strategies within biosensors.

DNA Hybridization. Studies have used the hybridization capabilities of complementary DNA sequences to immobilize DNAzymes onto target substrates for decades.[68,69] This method overcomes the need to introduce cross-linking reagents, which may interfere with the sensing cascade, resulting in reduced detection sensitivity and accuracy. The basic premise for this method is the hybridization of a DNAzyme to a complementary substrate strand already immobilized on a given entity. Such a system allows for a greater degree of creativity within the sensing cascade as the pathway through which detection occurs following cleavage can be altered in a multitude of ways to maximize detection sensitivity. In fact, three different strategies that use post-cleavage products for detection have been developed.[39,70−−72] Following target-induced activation of surface-immobilized DNAzymes, sensing has been performed by (1) detecting the presence of the cleavage product; (2) probing the presence of the residually attached DNAzyme fragment left behind on the surface following cleavage; or (3) subsequently adding an oligonucleotide that may initiate a sensing cascade depending on whether cleavage has occurred.

Xu *et al.* developed a Cu^{2+} sensor by coating a metal−organic framework possessing inherent peroxidase-like activity with substrate strands capable of hybridizing to a Cu^{2+}-responsive DNA-cleaving DNAzyme.[39] Upon exposure to Cu^{2+}, the substrate strand was cleaved by the DNAzyme, resulting in detachment between the two strands. The portion of the substrate strand attached to the organic framework was then free to bind to a complementary DNA strand present on an Au-modified electrode. The peroxidase-like activity of the now immobilized organic framework onto the Au-modified electrode could then output an electrochemical signal when engaging in a catalytic reaction with 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2. The signal amplification provided by this platform allowed for a limit of detection of 0.457 nM.[39]

Zhang *et al.* developed a colorimetric sensor for the detection of uranyl ions under a similar premise, depicted in Figure 2a.[70] The well-known uranyl ion-responsive RNA-cleaving DNAzyme was hybridized with a substrate strand already attached to magnetic beads. Upon exposure, the DNAzymes cleaved the substrate strand and were mobilized off

https://doi.org/10.1021/acsnano.1c04327
ACS Nano 2021, 15, 13943−13969

the surface of the beads. The portion of the substrate strand residually attached to the bead then underwent a hybridization chain reaction with other oligonucleotides present in the system to create a long strand capable of capturing horseradish peroxidase (HRP). The captured HRP could then catalyze the oxidation of TMB in the presence of H_2O_2.[70]

Hybridization has specifically yielded biosensors with high sensitivity *via* the fragmentation of DNAzymes for signal amplification. Wu *et al.* used this principle as described in Figure 2b,[71] to design a sensing platform capable of detecting Cu^{2+} ions at concentrations as low as 2 nM. In this study a Mg^{2+}-responsive DNAzyme was fragmented into four segments, named A, B, D, and E. Fragment A was bonded to a magnetic bead and attached to Fragment B *via* an azide−alkyne click chemistry reaction mediated by Cu^{2+}. This reaction was supported by a complementary assistant strand named Fragment C. Upon separation from Fragment C, Fragments A and B hybridized to Fragments D and E, respectively, resulting in the formation of a functional Mg^{2+}-responsive RNA-cleaving DNAzyme. When exposed to Mg^{2+}, this construct would effectively cleave a fluorophore−quencher DNA construct, leading to fluorescence output. Given that one Mg^{2+} DNAzyme can cleave multiple fluorophore−quencher constructs, the signal from one Cu^{2+} ion in the first step can induce substantial cleavage, thus allowing for a very low limit of detection.[71] In the absence of Cu^{2+}, Fragments A and B would be unable to bind, preventing the reaction from progressing.

Target-specific DNA aptamers can be used to immobilize highly catalytic DNAzymes onto surfaces, which creates a simple system for target-dependent release of the DNAzyme from the surface to the solution.[72] In the absence of the target, the aptamer hybridizes with the DNAzyme keeping it on the surface, but in the presence of the target, the aptamer preferentially binds the target and releases the DNAzyme. After separation of the liquid and solid phases, the presence of the DNAzyme in the solution can be probed by adding a reporter substrate and monitoring signal generation. This approach was explored recently by Sun *et al.*, when they hybridized a peroxidase-mimicking DNAzyme to lysozyme-binding DNA aptamers immobilized onto carbon fiber composites for the detection of lysozymes[72]—a marker for mucosal immune competence,[73] atherosclerosis,[74] and certain categories of leukemia.[75] Their sensing system is shown in Figure 2c. Since lysozymes bind with high specificity to lysozyme aptamers, they would kick the DNAzymes off the aptamers. The released DNAzymes then coordinated with H_2O_2 to mediate the oxidation of luminol present in the system, giving rise to chemiluminescence.[72] This approach is especially useful for the detection of targets for which no active DNAzymes have been identified.

What these studies collectively demonstrate is the multidimensional nature of this strategy. Its advantages go beyond simple immobilization, as it also provides a means by which the sensing pathway can be mediated, and by which signals can be amplified. The primary drawback of this approach is in its need for specificity, in that it cannot be implemented into any given platform as easily as a cross-linking reagent. Whereas cross-linkers can be applied universally through the incorporation of appropriate chemical modifications, hybridization requires the use of oligonucleotides, which are specific to a given sensing system. Thus, it is not easily adaptable into existing technologies. That being said, its use alongside electrochemical, chemiluminescent, fluorescent, and colorimetric detection platforms shows its versatility within different environments.

Electrostatic Interactions. Aside from hybridization, electrostatic interactions reliant on the charge and hydrophobicity of sensing components have been employed for immobilization. For these systems, reduced graphene oxide (rGO) functions as an excellent base material, especially within electrochemical detection platforms due to its high electrochemical activity.[18] Single-stranded DNA can be spontaneously absorbed onto such GO substrates through $\pi-\pi$ stacking interactions.[18] While promising, these interactions typically do not retain sufficient DNA for biosensing applications. As a result, various surface modification techniques have been developed to increase DNA retention.

Inducing amino groups onto the sensing surface is one strategy reported in literature for improved DNA retention. Under this approach, positively charged ammonium groups on the surface can interact with the negatively charged phosphate backbone of oligonucleotides. Wang *et al.* used this approach to immobilize Pb^{2+}- and Hg^{2+}-responsive RNA-cleaving DNAzymes.[18] Nitrogen plasma enhanced chemical vapor deposition was used to introduce amino groups onto a rGO-coated gold electrode. Raman spectroscopy confirmed that nitrogen groups were uniformly distributed across the rGO surface and that the chemical structure of rGO was not significantly altered by plasma treatment. DNAzymes were prehybridized with single-stranded DNA probes that would more readily interact with the amine groups on the substrate. Using electrochemical impedance spectroscopy (EIS), the resulting multiplex detection system achieved ultrasensitive limits of detection of 7.8 pM and 5.4 pM for Pb^{2+} and Hg^{2+}, respectively.[18]

A structural strategy that has been used for increased DNA retention involves using three-dimensional (3D) rGO, as opposed to the conventional layered form. This results in an increased surface area-to-volume ratio and improved in-plane conductivity in the context of electrochemical detection.[20] While 3D-rGO has not yet been applied to the immobilization of DNAzymes, Wang *et al.* used this approach to increase the retention of aptamers for lysozyme detection.[20] This study paired 3D-rGO with plasma-polymerized polypyrrole (PPy), which has been used extensively to improve DNA absorption for biosensing applications. PPy is a positively charged conductive polymer that can form electrostatic interactions with negatively charged DNA entities.[20] The primary hindrance in its use is its poor electrochemical properties, which leads to a significant deterioration in the performance of resultant electrochemical biosensors. To circumvent this issue, nanostructured PPy has been introduced as a means by which the impact of the polymer can be reduced. In the context of the aforementioned study, 3D-rGO provided a substrate for the formation of such a nanoscale layer of PPy. Nanoporous silicon has also been used as a scaffold for PPy for the same purpose.[76] As an alternative to PPy, plasma-polymerized propargylamine (PpPG) has garnered interest for use in biosensing due to its improved electrochemical properties.[19] He *et al.* paired this polymer with graphene to create nanofilms to be used in sensing platforms for DNA targets. By further inducing amino groups on the surface, they achieved a limit of detection of 1.84 nM *via* EIS.[19]

Outside of electrochemical detection, Hui *et al.* used electrostatic interactions to immobilize fluorescently labeled

https://doi.org/10.1021/acsnano.1c04327
ACS Nano 2021, 15, 13943−13969

Table 2. Overview of Existing Literature Involving the Use of DNAzymes for Environmental Detection, with Studies Segregated into Subsections Pertaining to Food Contaminant, Pathogen, and Metal Ion Detection

detection method	DNAzyme name	immobilization strategy	target	sample type	LOD	ref
		Food Contaminant Detection				
electrochemical	G-quadruplex-hemin DNAzyme	aptamer-mediated binding	*Escherichia coli*	buffer and milk samples	8 CFU/mL	84
fluorescence	*E. coli*-specific RNA-cleaving DNAzyme	hybridization	*E. coli* O157:H7	drinking water and apple juice	1.57 CFU/mL	85
fluorescence	*E. coli*-specific DNAzyme	biotin−streptavidin	*E. coli*	buffer, tap water, and milk	60 CFU/mL	87
fluorescence	*E. coli*-specific RNA-cleaving DNAzyme	$\pi-\pi$ stacking interactions	*E. coli*	buffer	10^4 CFU/mL	98
fluorescence	DAh1T1	physical entrapment	*Aeromonas hydrophila*	buffer, drinking water, and milk	36 CFU/mL	88
fluorescence	*E. coli*-specific RNA-cleaving DNAzyme	epoxy-mediated	*E. coli*	buffer, apple juice, and meat	10^3 CFU/mL	24
colorimetric	G-quadruplex-hemin DNAzyme	hybridization	*Vibrio parahemolyticus*	buffer and salmon	10 CFU/mL	91
		Pathogen Detection				
electrochemical	HRP-mimicking DNAzyme	thiol−gold	avian influenza virus	chicken serum	undisclosed	25
colorimetric	G-quadruplex-hemin DNAzyme	thiol−silver	hepatitis B virus	human serum samples	0.2 nM	169
fluorescence	RNA-cleaving fluorogenic DNAzyme	non-covalent functionalization; $\pi-\pi$ stacking interactions	*E. coli*	blood samples and *E. coli* K12 culture	10^5 CFU/mL	98
electrochemical	Hemin/G-quadruplex HRP-mimicking DNAzyme	thiol−gold	hepatitis B virus surface antigen	serum samples	0.19 pg/mL	27
fluorescence	RNA-cleaving fluorogenic DNAzyme	not specified	*E. coli* − lysozyme	drinking water, milk, apple juice	100 cells/mL	170
colorimetric, smartphone readout	RNA-cleaving DNAzyme	hybridization	*E. coli* K12	juice, milk, or complex samples	10^3 CFU/mL	171
fluorescence	DNAzyme-based fluorescent paper sensor	covalent	*Klebsiella pneumoniae*	buffer and bacterial isolates	10^5 CFU/mL	78
fluorescence	RFD-CD1	not specified	*Clostridioides difficile*	buffer and crude extracellular mixture	not specified	95
electrochemical	VAE-1 and VAE-2 RNA-cleaving DNAzymes	biotin−streptavidin	*Vibrio anguillarum*	water samples and fish tissue	4000 CFU/mL	64
colorimetric	RNA-cleaving DNAzyme	biotin−streptavidin	*Helicobacter pylori*	human stool samples	10^4 CFU/mL	26
fluorescence	cis (hairpin) and trans DNAzyme	amide−agar media	*E. coli*	buffer and wastewater treatment plant	10^4-10^5 cells/mL	172
		Metal Ion Detection				
plasmonic (uv−vis spectroscopy)	15−12 DNAzyme	thiol−gold	lead ions	tap water	8.0 nM	108
electrochemical	custom Pb^{2+} DNAzyme	polyadenine binding to AuNPs; thiol−gold; hybridization	lead ions	tap water	96 pM	173
colorimetric	GR-5 DNAzyme	thiol−gold; biotin−streptavidin	lead ions	soil	0.05 nM	107
electrochemical	8−17 DNAzyme	hybridization	lead ions	lake and tap water	0.07 pM	174
surface plasmon resonance	GR-5 DNAzyme	thiol−gold; hybridization	lead ions	hepes and nacl buffer	80 pM	175
colorimetric (interferometric reflectance spectroscopy)	K^+-stabilized hemin G-quadruplex; Pb^{2+}-stabilized quadruplex	aldehyde-mediated	lead ions	buffer and seawater	12 nM	45
fluorescence	Pb^{2+} DNAzyme	$\pi-\pi$ stacking and non-covalent hydrophobic interactions	lead ions	tap and lake water	6.7 pM	176
electrochemical	8−17 DNAzyme	biotin−streptavidin	lead ions	tap, lake, and pool water	38 fg/mL	65
electrochemical	8−17 DNAzyme	NHS-ester	lead ions	tap and river water	17.4 fM	50
colorimetric	GR-5 DNAzyme	biotin−streptavidin	lead ions	soil	0.05 nM	107
electrochemiluminescence	Hemin/G-quadruplex DNAzyme	NHS-ester	lead ions	river water	0.98 fM	177
electrochemical	8−17 DNAzyme	thiol−gold	lead ions	drinking water, serum	0.29 pM	178
fluorescence	Mg^{2+}-dependent DNAzyme	thiol−gold	mercury ions	herbs	30 pM	115
digital drop PCR	GR-5 DNAzyme	biotin−streptavidin	lead ions	lake water	500 pM	179
colorimetric	Cu^{2+}-dependent DNAzyme	biotin−streptavidin	copper ions	bottled, lake, and domestic sewage water	8 nM	106
fluorescence	GR-5 DNAzyme	$\pi-\pi$ stacking; hydrogen bonding	lead and magnesium ions	tap and lake water	96 pM (Pb^{2+}); 356 pM (Hg^{2+})	114
electrochemical	UO_2^{2+}-specific DNAzyme	thiol−gold	uranyl ions	river water	20 pM	180

https://doi.org/10.1021/acsnano.1c04327
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Table 2. continued

detection method	DNAzyme name	immobilization strategy	target	sample type	LOD	ref
		Metal Ion Detection				
electrochemical	Pb^{2+}-specific DNAzyme	thiol−gold	lead and magnesium ions	tap and lake water	0.034 pM	181
Raman scattering	Pb^{2+}-specific DNAzyme	thiol−gold	lead ions	lake, tap, and industrial water	8.9 pM	182

Table 3. Overview of Existing Literature on the Use of DNAzymes for Clinical Applications, with Studies Segregated into Sub-Sections Pertaining to Nucleic Acid, Protein, and Cancer Detection

detection method	DNAzyme name	immobilization strategy	target	sample type	LOD	ref
		Nucleic Acid Detection				
colorimetric	hemin/G-quadruplex DNAzyme	physical entrapment	Y human amelogenin fragment	buffer	45.7 ng	29
electrochemical	trivalent DNAzymes	thiol−gold	microRNA let-7a	dilute human serum	0.46 fM	122
electrochemiluminescent	hemin/G-quadruplex DNAzyme	NHS-ester	miRNA-155	cell lysate	0.3 fM	183
		Protein Detection				
chemiluminescence	hemin/G-quadruplex DNAzyme	hybridization	lysozyme	human urine	1.25×10^{-11} g/L	72
electrochemical	hemin/G-quadruplex DNAzyme	metal−thiol interactions	cardiac troponin I	PBS spiked with human serum	7.5 pg/mL	128
electrochemical	Pb^{2+}-dependent DNAzyme	thiol−gold	prostate-specific antigen	clinical serum samples	0.1 pg/mL	184
electrochemical	Pb^{2+}-dependent DNAzyme	thiol−gold	thrombin	PBS and dilute serum	1.8 pM (in PBS)	130
electrochemical	hemin/G-quadruplex DNAzyme	hybridization	thrombin	spiked serum	0.3 fM	131
chemiluminescence	hemin/G-quadruplex DNAzyme	hybridization	thrombin	spiked serum	6.3×10^{-15} mol/L	132
fluorescence	8−17 DNAzyme	thiol−gold	thrombin	serum samples	4.5 pM	185
colorimetric	8−17 DNAzyme	hybridization	platelet-derived growth factor BB	buffer	0.11 fM	186
electrochemical	hemin/G-quadruplex DNAzyme	thiol−gold	prion protein	diluted serum samples	0.38 pg/mL	118
electrochemical	Pb^{2+}-dependent DNAzyme	thiol−gold	alpha-fetoprotein	clinical serum samples	0.8 pg/mL	187
photoelectrochemical	hemin/G-quadruplex DNAzyme	thiol−gold	alpha-fetoprotein	diluted serum samples	14.7 pg/mL	117
fluorescence	3D DNA motor	thiol−gold	telomerase	living cells of MCF7, A549, and HeLa	46 cells/ml	145
		Cancer Detection				
colorimetric	hemin/G-quadruplex DNAzyme	aldehyde-based	telomerase	telomerase activity in HeLa cells	0.5 HeLa/μL	146
fluorescence	DZM-21 and DZM 205 − Na^{2+}DNAzymes	thiol−gold	miRNA-21; miRNA-205	A549 and H520 cells	97 fM; 137 fM	150
fluorescence	Cu^{2+}-DNAzyme-sgc8c and Mg^{2+}-DNAzyme-TD05 Cy5	thiol−gold	CTC	whole blood sample	10^2−10^3 cells/mL	136
electrochemical	Pd−Pt nanocage−HRP−cDNA/hemin/G-quadruplex hybrid nanoprobes	hybridization/thiol−gold	CTC	HepG2 tumor cells	5 cell/mL	140

aptamers onto a rGO-coated nitrocellulose membrane.[77] Introduction of the target led to aptamer dissociation and a resultant fluorescence signal. Further, the released aptamer then acted as a primer for rolling circle amplification, resulting in the formation of multiple peroxidase-mimicking DNAzymes capable of providing colorimetric output in the presence of H_2O_2 and TMB.

While a viable immobilization method, electrostatic interaction-based immobilization has thus far been largely limited to electrochemical systems, with limited studies exploring its use alongside other transduction methods. As such, its use is restricted and its stability is questionable given its non-covalent nature. Additionally, while a proven method for the immobilization of DNA entities, its use with DNAzymes is limited. The displacement of immobilized DNAzymes by other molecules is one potential challenge with using electrostatic interactions for immobilization. In particular, complex samples may possess molecules with the ability to bind to the sensing surface through non-specific electrostatic interactions, reducing the concentration of DNAzymes on the surface. Nonetheless, under appropriate circumstances, this approach presents itself as an ideal candidate for further exploration.

Physical Entrapment. While largely unexplored, physical entrapment of DNAzymes has also been reported as a means of immobilization. In particular, Liu *et al.* developed a fluorescence biosensor for the detection of ultraviolet irradiation.[14] Polysaccharide polynucleotide coacervate micro-

https://doi.org/10.1021/acsnano.1c04327
ACS Nano 2021, 15, 13943−13969

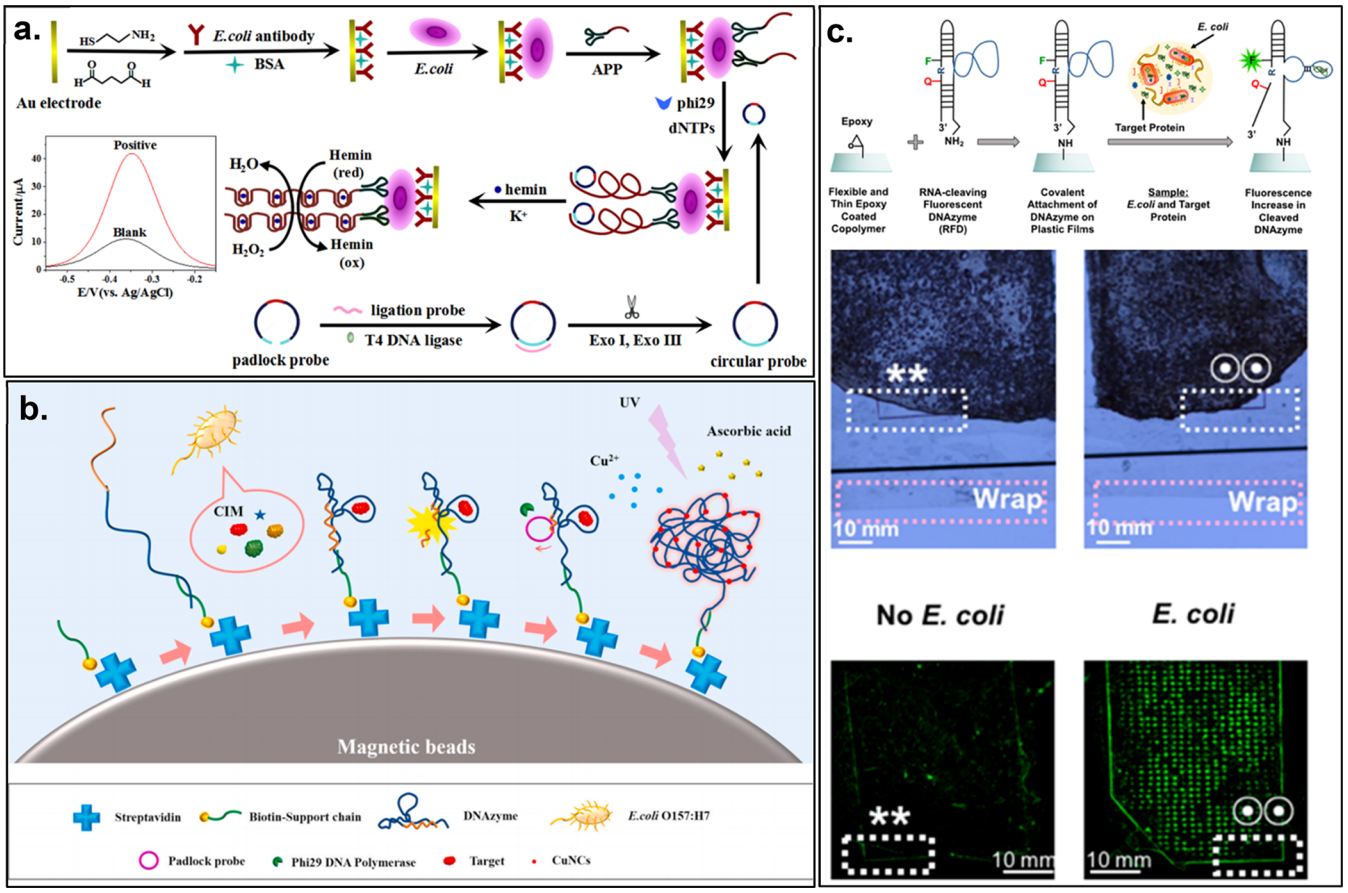

Figure 3. DNAzyme-based sensors for the detection of *Escherichia coli*. (a) Antibody-mediated electrochemical sensing system for *E. coli* using rolling circle amplification for ultrasensitive limit of detection. Reprinted with permission from ref 84. Copyright 2016 Elsevier. (b) Ultrasensitive fluorescence detection system for *E. coli* O157:H7 involving rolling circle amplification. Reprinted with permission from ref 85. Copyright 2020 Elsevier. (c) RNA-cleaving fluorescent DNAzyme sensor immobilized on flexible patch for on-package, real-time detection of *E. coli*. Associated images show sensor performance when in contact with spiked food samples. Reprinted with permission from ref 24. Copyright 2018 American Chemical Society.

droplets (PCMs) consisting of diethylaminoethyldextran chloride (DEAE-dextran) and double-stranded DNA were fabricated and homogeneously immobilized within a hydrogel. PCMs located at the input site of the hydrogel were loaded with titanium dioxide and silver NPs, which reduce dioxygen into H_2O_2 in response to ultraviolet light. H_2O_2 molecules then migrated to the output site of the hydrogel, where the microdroplets were loaded with Amplex Red and peroxidase-mimicking DNAzymes. Peroxidation of H_2O_2 concurrently oxidized Amplex Red into resorufin, which emits a detectable fluorescent signal.[14] Other studies that have developed fluorescence biosensors using physical entrapment for DNAzyme immobilization have relied on pullulan and various sol−gel matrices. While pullulan entrapment has not been explored extensively, studies have found that it provides entrapped DNAzymes with a protective environment that ensures maintenance of its catalytic properties. Pullulan entrapment has been used most notable for a fluorescence biosensor for *Klebsiella pneumoniae*—a multi-drug-resistant pathogen.[78] Similarly, sol−gel strategies have also been minimally applied within DNAzyme-based biosensing platforms. The use of this approach has been reviewed elsewhere.[79]

In relation to electrochemical sensing, Gao *et al.* developed a biosensor for H_2O_2 detection that relied on the physical absorption of peroxidase-mimicking DNAzymes onto the surface of an electrode and trapped these entities using polydopamine. By oxidizing dopamine diluted in phosphate buffered silane, polydopamine was formed, resulting in the immobilization of the DNAzymes onto the electrode's surface. The resultant biosensor exhibited a limit of detection of 2.2 μM.[80]

Ultimately, the nature of physical entrapment makes it difficult to implement in most sensing platforms, and its stability over time will be a challenge. The lack of existing literature using this strategy makes it difficult to characterize its effectiveness for DNAzyme immobilization.

DNAzyme APPLICATIONS IN BIOSENSING

This section provides a review of the most common immobilized DNAzyme sensing applications for efficient analyte detection. Applications were selected to highlight the potential for DNAzymes in the context of their immobilization strategies. As detailed earlier and given the variety of molecular targets, DNAzymes can be applied to a range of systems, including food safety, environmental monitoring, and clinical diagnostics. As such, this analysis will focus mainly on active research in these areas within the past 5 years. Reviews discussing earlier studies can be found in literature.[81,82] Specifically, contaminant detection in food and biological

https://doi.org/10.1021/acsnano.1c04327
ACS Nano 2021, 15, 13943−13969

settings, as well as heavy metal detection in a variety of settings, will be explored. Recent work in developing DNAzymes for biomarker analysis in the context of clinical diagnostics, with a focus on protein and nucleic acid sensing, will then be detailed. Finally, we conclude with a summary of applications in cancer research, which has seen the most progress in recent years. Further, existing literature on food contaminant, pathogen, and metal ion detection are summarized in Table 2, while the detection of clinical and cancer biomarkers is summarized in Table 3.

Detection of Contaminants within Food. *Escherichia coli.* Foodborne illnesses have a pronounced impact on human health worldwide, with hundreds of millions being affected annually.[83] Bacterial contaminants are largely to blame, meaning that developing strategies for rapid detection is paramount. *Escherichia coli* presents itself as one of the most common food contaminants, making it a focus in ongoing research. Recent studies have developed DNAzyme-based biosensors for *E. coli* detection that communicate its presence through electrochemical, fluorescence, and colorimetric means.

Guo *et al.* developed an electrochemical DNAzyme sensor for the detection of *E. coli* that used rolling circle amplification to achieve high sensitivity (Figure 3a).[84] Following antibody-mediated immobilization of *E. coli* onto a gold electrode, a primer-modified *E. coli*-specific aptamer was bonded to the bacterial cells. A plasmid containing a region complementary to the primer on the aptamer was then introduced. This plasmid also encoded sequences complementary to that of peroxidase-mimicking DNAzymes. The transcribed DNAzymes then became functional once complexed with hemin. The DNAzyme-mediated reduction of subsequently added H_2O_2 gave rise to an electrochemical signal. The developed biosensor exhibited an ultrasensitive limit of detection of 8 CFU/mL, which presents strong real-world applicability when paired with its rapid test time.

In an effort to achieve such ultrasensitive detection limits within a fluorescence platform, Zhou *et al.* also used rolling circle amplification in the development of an *E. coli* biosensor, shown in Figure 3b.[85] *E. coli*-responsive RNA-cleaving DNAzymes were immobilized onto magnetic beads. Upon exposure to a target protein present in the crude intracellular mixture of this bacterial species, cleavage of RNA within the DNAzyme sequence freed a segment of DNA that acted as a primer for rolling circle amplification. The amplified sequence was T-rich, which resulted in the formation of copper nanoclusters in the presence of sodium ascorbate and copper sulfate. These nanoclusters have inherent fluorescence that enabled quantitative detection. This sensor showed an ultrasensitive limit of detection of 1.57 CFU/mL and effectiveness within contaminated water and apple juice samples. That being said, the stability of this biosensor was not assessed within this study and remains the primary challenge in its incorporation with food products. Nonetheless, both the aforementioned studies demonstrate the potential advantages rolling circle amplification can provide. With a recent study uncovering that such amplification reactions proceed better on surface-immobilized systems compared to in-solution systems, further use of this strategy within immobilized DNAzyme biosensors is warranted for the development of alternatives to equipment-dependent lab analysis of food samples.[86] These systems, however, still fail to address the need for real-time food monitoring. Widespread monitoring largely requires a greater focus on sensor stability—which the aforementioned studies did not evaluate. Contrarily, other studies have focused on developing simpler platforms that hold more potential for such widespread use.

Recognizing that package-integrated biosensors are the key to developing tangible solutions to real-time food contamination, Yousefi *et al.* used RNA-cleaving fluorescent DNAzymes for the detection of *E. coli* within food products, as depicted in Figure 3c.[24] *E. coli* responsive DNAzymes were covalently immobilized onto flexible polymeric surfaces that could be implemented onto food wraps. Upon bacterial exposure, cleavage within the fluorophore-quencher construct would lead to separation between the two components, resulting in a detectable fluorescence signal. This sensor showed the ability to detect the presence of *E. coli* within meat samples to a limit as low as 10^3 CFU/mL. While the sensitivity of this biosensor is lower than other existing platforms, the real-world applications of this system are particularly intriguing, given that its form factor as a patch on food packaging allows for individualized product screening in real time without the need to open food samples. Tests on the developed biosensor involving variations in pH showed sustained performance irrespective of such an environmental change. However, variations in DNAzyme activity in response to temperature and fluorophore photobleaching are obstacles that need to be addressed to ensure reliable detection.

Circumventing stability issues associated with the use of traditional fluorophores, entities with inherent fluorescence properties have shown applicability within DNAzyme-based detection platforms, thus presenting an alternative. Namely, Zheng *et al.* used silver nanoclusters, which have controllable fluorescence properties and offer strong stability, fluorescence intensity, and biocompatibility.[87] This study hybridized *E. coli*-responsive RNA-cleaving DNAzymes with a substrate strand containing acetylcholinesterase and immobilized this construct onto magnetic beads. Upon target exposure, cleavage and subsequent release of acetylcholinesterase into solution allowed for its separation from the magnetic beads. Released acetylcholinesterase then mediated the hydrolysis of acetylthiocholine into thiocholine, the latter of which can enhance the fluorescence of DNA-templated silver nanoclusters added to the solution. While effective, this solution-based nature of the platform complicates its potential for on-package applications, and the cost of such a system is unclear. Nonetheless, this study provides the foundation for further work involving the incorporation of silver nanoclusters within DNAzyme-based sensors for the detection of various pathogenic food contaminants.

Other Bacterial Contaminants. The development of biosensors targeting pathogens other than *E. coli* is a very active area of research. Specifically, sensors relying on fluorescence-based detection represent a growing body of work. For example, Ma *et al.* developed a fluorescence-based biosensor for *Aeromonas hydrophila*—a highly pathogenic bacteria that is often present within food products.[88] The platform relied on a conventional RNA-cleaving fluorescent DNAzyme system in which target-induced cleavage of a fluorophore-quencher substrate by an *A. hydrophila*-responsive DNAzyme resulted in separation between the two entities, giving rise to a detectable fluorescence signal. Interestingly, the sensor provided a positive signal within 10 min of target exposure when tested with contaminated milk and water samples, heavily supporting its potential for real-world applicability.

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ACS Nano 2021, 15, 13943−13969

While undoubtedly a viable tool, fluorescence-based detection systems are limited by the need for imaging tools for food monitoring. Thus, sensors utilizing colorimetric signals for signal transduction largely occupy this space, as they allow for equipment-free monitoring at all stages of the food product pipeline. Colorimetric detection platforms for food pathogen detection largely rely on peroxidase-mimicking DNAzymes, which interact with hemin to yield defined optical changes. This design has been used in the development of sensors targeting a range of bacteria. Mondal *et al.* focused on the highly pathogenic enterotoxin B-harboring *Staphylococcus aureus*, developing a DNAzyme-based colorimetric sensor for its detection within food samples.[22] Upon antibody-based immobilization and lysing of bacterial cells, the resulting media was exposed to forward and reverse primers specific to the enterotoxin B gene. The forward primer was modified with a sequence complementary to the peroxidase-mimicking DNAzyme. Following PCR amplification, exposure to TMB and hemin in the presence of H_2O_2 gave rise to a colorimetric change. The sensor demonstrated a limit of detection of 10^2 CFU/mL within spiked milk samples. Li *et al.* used a similar strategy for the detection of *Salmonella*, by integrating the sequence of a peroxidase-mimicking DNAzyme onto PCR primers.[23] While the primer initially formed a hairpin structure, DNA extension led to opening of the hairpin, allowing for interactions with hemin and TMB and subsequent colorimetric change. Lastly, Liu *et al.* used a collection of four target-specific primers that resulted in the formation of peroxidase-mimicking DNAzymes for the detection of *Listeria monocytogenes*.[89] This foodborne pathogen is known to give rise to listeriosis—a life-threatening condition associated with septicemia and meningitis. This colorimetric sensor gave rise to an optical limit of detection of 47.5 CFU per reaction. While all three of these sensors present viable tools with strong applicability, the abundance of reagents in these systems make them unfeasible for package-integrated sensing, rather positioning their use as rapid, low cost alternatives to the laboratory testing of collected food samples.

In an attempt to achieve ultrasensitive colorimetric detection, Liu *et al.* used a split peroxidase-mimicking DNAzyme design for the detection of *Cronobacter sakazakii*—a foodborne pathogen that results in neurological or developmental disorders in 94% of infected patients.[90] In the absence of *C. sakazakii*, an aptamer specific to the bacterium combined the two halves of the split DNAzyme through complementary sequences present on the DNAzyme strands, giving rise to a functional DNAzyme. A colorimetric change was resultantly observed following the addition of hemin, H_2O_2, and 2,20-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid. When the target was present, the aptamer preferentially bonded with the bacterium, meaning that the DNAzyme remained split. Thus, no colorimetric change was observed. This split system demonstrated a limit of detection of 1.2 CFU/mL. An aptamer-peroxidase-mimicking DNAzyme-pairing has also been used for the detection of *Vibrio parahaemolyticus*—a pathogen found in seafood.[91] A *V. parahaemolyticus*-specific aptamer was used to induce competition between the bacterial target and DNAzymes labeled with a sequence complementary to the aptamer. Preferential binding of the target resulted in the release of the DNAzyme, leading to a colorimetric signal with a limit of detection of 10 CFU/mL. The ultrasensitive detection limits of aptamer−DNAzyme paired systems indicate strong applicability within food contamination testing. The lack of aptamers available for specific bacterial targets limits the widespread use of this system, but with aptamer selection representing a rapidly growing field of work, such systems can be expected to grow in popularity.

Finally, while a resultant biosensor is yet to be developed, Rothenbroker *et al.* recently identified and characterized an RNA-cleaving DNAzyme specific to *Legionella pneumophila*—a lethal pathogen often responsible for outbreaks in water supplies.[92] The study demonstrated a limit of detection as low as 10 CFU/mL through denaturing polyacrylamide gel electrophoresis.

Chemical Contaminants. Alongside pathogenic contamination of food, there is growing concern regarding chemical entities, present both naturally and due to human activities. Developing effective means for their detection is vital, given that many of these chemicals are known toxins, often with carcinogenic effects. Aflatoxins are of particular interest given their common presence within food samples. Naturally produced, these toxins are metabolites of *Aspergillus* fungi.[21] Aflatoxin B (AfB) is especially carcinogenic, making it a focus in research. Wang *et al.* and Setlem *et al.* both detailed strategies that involved using peroxidase-mimicking DNAzymes for the colorimetric detection of AfB.[21,93] While Wang *et al.* used a split DNAzyme design and competitive aptamer binding for the detection of the target, Setlem used a closed loop of blocking linker to inhibit DNAzyme binding to an AfB aptamer. In this case, AfB binding to the aptamer led to the loop opening and subsequent DNAzyme attachment for colorimetric detection. Both sensors provided ultrasensitive limits of detection of 0.02 ng/mL and 22.6 parts per billion, respectively.

Similar to AfB, Ochratoxin A (OTA) is another metabolite of *Aspergillus* with significant toxic effects. Yu *et al.* used an exonuclease-mediated method for the detection of OTA, centered upon the merging of an OTA aptamer and a peroxidase-mimicking DNAzyme sequence into one strand.[94] In the presence of OTA, its binding to the designed strand prevented exonuclease activity, allowing for subsequent hemin binding and colorimetric detection. In its absence, the strand was digested by the exonuclease, preventing any signal.

Outside of natural metabolites, human activity-related contaminants have been explored in recent years, especially with growing concerns surrounding antibiotic resistance. Tetracycline—a widely used antibiotic—has been found to be present within food products as a result of misuse.[67] Gong *et al.* developed a DNAzyme-based sensor for its detection and achieved a colorimetric limit of detection of 8.1×10^{-2} ng/mL in buffer.[67] Effectiveness within contaminated milk samples was also demonstrated.

Pathogen Detection in Biological Samples. In addition to detection in food samples, and notwithstanding the recent COVID-19 pandemic, rapid, high-throughput, and sensitive detection of infectious pathogens in biological samples is critical to identify, diagnose, and isolate potential outbreaks. With the diversity of immobilization techniques previously described, it stands that the application of DNAzymes can thus be tailored to settings wherein traditional pathogenic detection tactics, such as selective enrichment, plating and polymerase chain reaction, may not be feasible. As well, in clinical settings where rapid results are often critical, such as in the diagnosis of acute sepsis, DNAzymes could provide both a rapid and precise alternative for routine detection. Within clinical

https://doi.org/10.1021/acsnano.1c04327
ACS Nano 2021, 15, 13943−13969

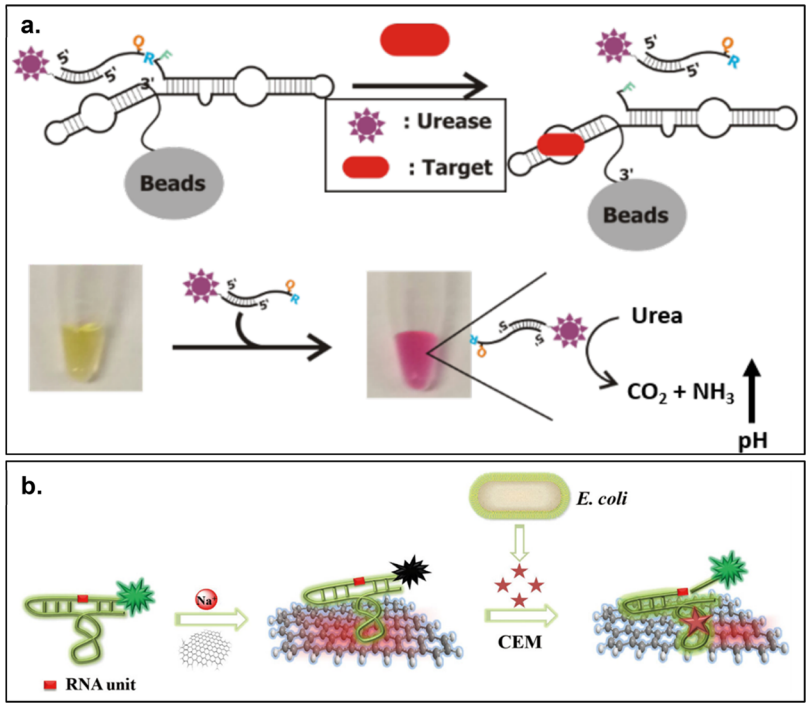

Figure 4. Pathogen detection using immobilized DNAzymes. (a) Colorimetric detection of *H. pylori* through the immobilization and release of urease upon DNAzyme-induced cleavage. Reprinted with permission from ref 26. Copyright 2019 John Wiley and Sons. (b) Fluorescence-based detection of *E. coli* using RNA-cleaving DNAzymes immobilized on graphene through $\pi-\pi$ stacking interactions. Reprinted with permission from ref 98. Copyright 2018 Springer Nature.

detection, a variety of DNAzyme-based strategies have been proposed to overcome these challenges. In fact, DNAzymes have been employed to detect different strains of pathogenic bacteria, including *Klebsiella pneumoniae*,[78] *Clostridium difficile*,[95] and *Helicobacter pylori*.[26] In relation to *H. pylori* specifically, Ali *et al.* developed a colorimetric detection system for the pathogen (Figure 4a) and transferred the sensor onto a paper platform, providing a strong case for the use of such sensors as point-of-care systems. More recently peroxidase-mimicking DNAzymes, have been proposed as a potential mechanism for the detection of SARS-CoV-2.[96] It is certain that accurate sensing of pathogenic bacteria and viruses in clinical settings poses a particular challenge in complex samples, such as blood, saliva, and urine, which are rife with other potentially cross-reactive biomolecules and other contaminants.[97]

While *in vitro* testing with purified samples has been met with early success, successful translation into clinical care—particularly for point-of-care detection—requires innovative strategies that can provide highly specific detection. In this regard, the robust nature of DNAzymes, coupled with effective and stable immobilization approaches, makes them particularly suitable for addressing these needs.

With the aim of detecting the avian influenza virus (H5N1), Lee *et al.* developed a sensor based on the application of a multifunctional DNA 3-way junction structure, consisting of a target recognition site, a peroxidase-mimicking DNAzyme, and a thiol immobilization tether.[25] This DNA 3-way junction was then conjugated onto porous gold nanoparticles and layered onto Au electrodes *via* the thiol group. Detection of hemagglutinin protein, which makes up the viral envelope of avian influenza, was accomplished through binding and cyclic voltammetry measurements, with results demonstrating detection down to 1 pM. In another approach, Liu *et al.* applied a non-covalent immobilization technique to detect *E. coli*, as depicted in Figure 4b.[98] To simplify fabrication steps and avoid the need for lengthy and costly functionalization steps, immobilization of an *E. coil*-responsive RNA-cleaving DNAzyme was achieved using $\pi-\pi$ stacking interactions onto graphene. Before the introduction of *E. coli* into the system, a fluorophore-laden DNAzyme was non-covalently adsorbed onto the surface of the graphene, which quenched the signal of the fluorescent unit. However, when *E. coli* was introduced, directed cleavage occurred, which subsequently released the fluorophore from the graphene, resulting in fluorescence output. A limit of detection of 10^5 CFU/mL was achieved using this system, with demonstrated stability in blood indicating its potential for clinical translation. In addition to these approaches, an overview of recent studies investigating DNAzyme-based pathogen detection and their respective immobilization techniques is presented in Table 2.

Heavy Metal Detection within Water and the Environment. Heavy metals detection makes up a large body of DNAzyme-based biosensing research, due to the abundance of metal ion-responsive DNAzymes.[99] Equally, research demonstrating the link between heavy metals and cancer,[100] and corresponding environmental regulations,[101] indicates a need for rapid and sensitive detection mechanisms. Given that detection of metal ions using DNAzymes has recently been reviewed extensively in the literature,[99,102−104] we focus this overview on immobilization strategies, innovative designs, and applications for detection in environmental samples. Further, while many metal-ion-responsive DNAzymes are now available for biosensing studies, we will focus on three of the most studied analytes: copper, lead, and mercury ions. A

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ACS Nano 2021, 15, 13943−13969

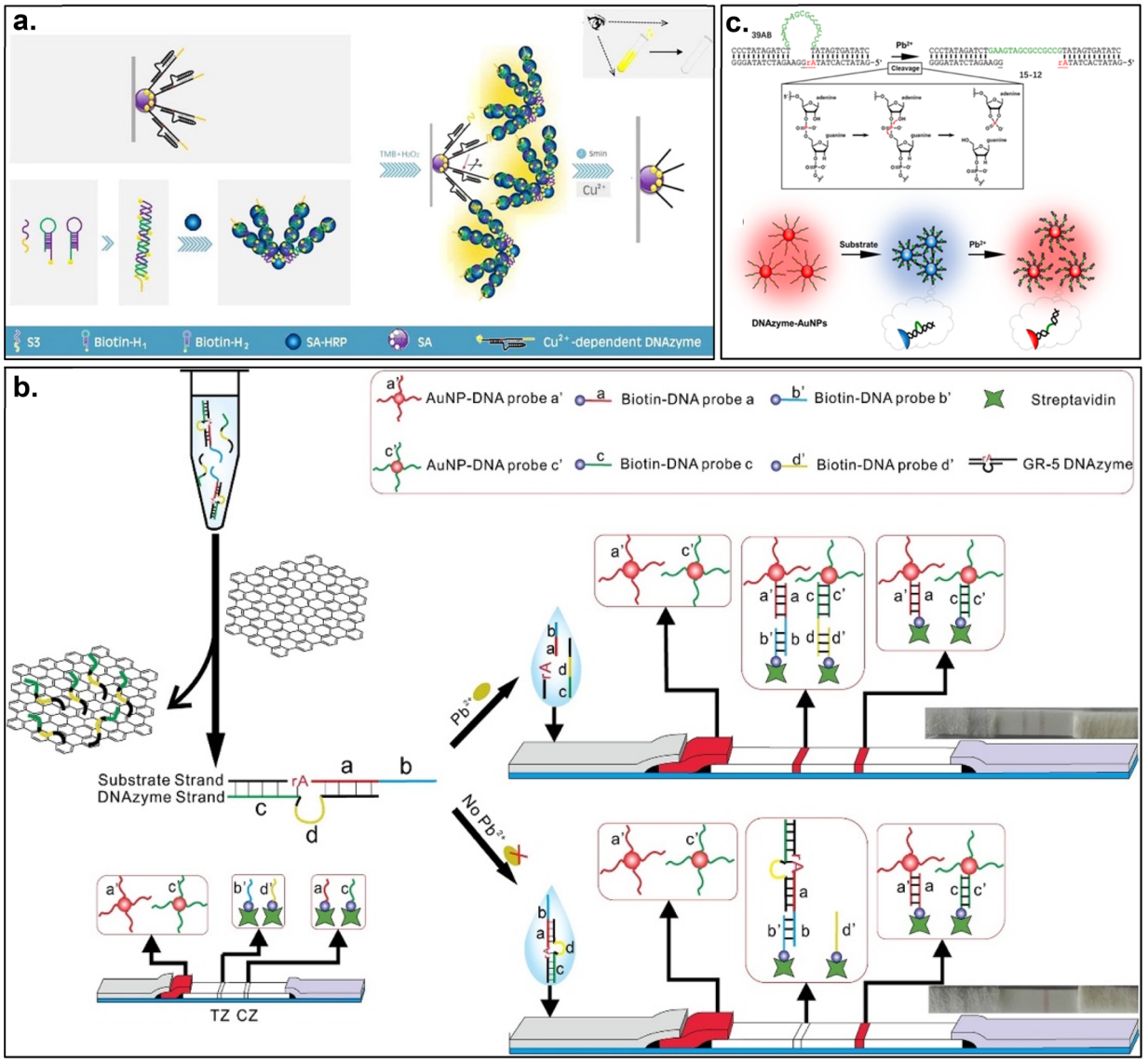

Figure 5. Metal ion detection using DNAzyme-based biosensing platforms. (a) A hybridization chain reaction-based horseradish peroxidase concatemer sensing system for Cu^{2+} detection. Reprinted with permission under a Creative Commons CC-BY license from ref 106. Copyright 2017 Springer Nature. (b) A nanoparticle-based lateral flow test strip for Pb^{2+} detection. Reprinted with permission from ref 107. Copyright 2018 Elsevier. (c) Gold nanoparticles for plasmonic detection of Pb^{2+} ions. Reprinted with permission from ref 108. Copyright 2020 American Chemical Society.

comprehensive list of metal detection approaches—largely in environmental settings—is presented in Table 2.

Copper. While copper is an essential nutrient with established daily recommended intake levels, acute and chronic copper exposure has resulted in changes in liver function, gastrointestinal symptoms, nausea, and even death.[105] Thus, copper(II) ion (Cu^{2+}) detection is important to test for potential water contamination. While traditional approaches to Cu^{2+} detection, such as inductively coupled plasma mass spectrometry and atomic absorption spectroscopy, offer low detection limits and high specificity, both techniques require specialized equipment and trained personnel. The lack of portability and high cost are the driving factors for innovative approaches for rapid and on-site high-throughput sensing and accurate monitoring. With this in mind, the application and versatility of DNAzymes to detect heavy metal ions, such as Cu^{2+}, has great potential to address these issues. For example, Zu *et al.* developed a "turn-off" sensing system based on the immobilization of a Cu^{2+}-dependent DNA-cleaving DNAzyme by biotin−streptavidin conjugation onto a polystyrene 96-well plate.[106] For detection, streptavidin−HRP was introduced, resulting in hybridization chain reactions with designer DNA hairpins and an initiator, which then complexed with the immobilized DNAzyme. Without Cu^{2+} the entire complex would remain in the well yielding a characteristic colorimetric reaction, while upon addition of Cu^{2+}, the complex was cleaved and washed away, as shown in Figure 5a.

In another approach, presented in Figure 2b, Wu *et al.* presented a Cu^{2+} sensing strategy developed around the concept of combining click chemistry with fluorescence.[71] Briefly, immobilization of a DNAzyme subunit was achieved using EDC to link −COOH magnetic beads with an NH_2-oligomer. This was followed by the hybridization of specific sequences to form a double-stranded oligomer complex. These sequences were tailored in such a way that, in the presence of Cu^{2+} and sodium ascorbate, a cycloaddition reaction resulted

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ACS Nano 2021, 15, 13943−13969

in the formation of a triazole ring ligated complex. Upon temperature cycling, the two strands were separated, and then Mg^{2+}-reactive RNA-cleaving DNAzyme assembly was initiated using the triazole ring strand. Detection was carried out in the presence of Mg^{2+} by the formed DNAzyme, which catalyzed the cleavage of a reporter substrate molecule tethered to a fluorescent probe. In this way, fluorescence intensity could be related to the presence of Cu^{2+}, with a detection limit of 2 nM.

Lead. Lead pollution is one of the most widespread and concerning forms of environmental contamination, with severe effects demonstrated on multiple organ systems.[109] Most sources of lead poisoning originate from petroleum, batteries, and other chemical industries, commonly leaching into waterways and soil to subsequently be introduced into the food chain. While many robust Pb^{2+} detection approaches have been in use for decades, most techniques still suffer from a lack of portability and sensitivity for rapid detection. The application of DNAzymes offers high specificity, with the potential for ultra-sensitive detection given that Pb^{2+} can serve as a powerful co-factor for enzymatic catalysis.

While DNAzyme/Pb^{2+} specificity itself is well established,[110] as with all DNAzyme platforms, the critical components for robust detection is the selection of a signal amplifier, immobilization strategy, and sample handling platform. With this in mind, Wang *et al.* demonstrated the application of GR-5 (a Pb^{2+}-dependent RNA-cleaving DNAzyme), coupled with a lateral flow test system and graphene oxide as a mechanism to remove un-hybridized DNA from the test solution.[107] As shown in Figure 5b, the lateral flow text strip was prepared by immobilizing two gold nanoparticle-DNA probes with specificity toward cleaved and un-cleaved DNA strands. Ultimately, in the presence of Pb^{2+} and successful hybridization to the appropriate gold nanoparticle−DNA probes, the test strip would yield colorimetric detection in both the test and control strips, with reported Pb^{2+} recognition down to 0.05 nM. While this scheme employs a graphene oxide-based pretreatment step with the need for a centrifuge, through the development of strategic sorting, DNAzyme-based lateral flow detection platforms could be a particular area of expansion given that they typically require little in the way of instrumentation and trained labor and can be conducted on-site.

In another approach, Diao *et al.* developed a highly sensitive DNAzyme-based mechanism for Pb^{2+} detection using a "turn-on" strategy, as depicted in Figure 5c.[108] Briefly, the system consisted of a thiolated Pb^{2+}-dependent DNAzyme bonded to gold nanoparticles, which were subjected to hybridization of the substrate strand. Thus, when hybridization occurred, base stacking caused the nanoparticles to aggregate, which in turn decreased their localized surface plasmon resonance (LSPR). However, when Pb^{2+} was introduced into the system, the substrate was cleaved, disassembling the stacking and increasing LSPR. Overall, these two systems demonstrate the potential for Pb^{2+} detection using DNAzyme-centered strategies with flexibility in terms of design and system optimization.

Mercury. Mercuric ions (Hg^{2+}) are another environmentally pervasive heavy metal contaminant, with well-characterized toxicity at both the acute and chronic exposure levels.[111] While Hg^{2+} detection *via* DNAzymes is notably less common than Pb^{2+}, several nanomaterial, chemical, and hydrogen-bonding techniques are described in the literature,[112] with approaches ranging from peroxidase-mimicking DNAzymes[113] to strategies coordinating with graphene oxide[114] and incorporation into hydrogel systems.[15] The system developed by Yun *et al.* is particularly notable given that the strategy is based on an RNA-cleaving DNAzyme motor.[115] Specifically, Hg^{2+} sensing was achieved by integrating thiolated DNA probes onto the surface of AuNPs. With this tethered system, quenching of the fluorophore was achieved, while the introduction of Hg^{2+} resulted in cleavage and fluorescent detection down to 30 pM.

Detection of Clinically Relevant Biomarkers. Applications in the field of medical diagnostics and monitoring constitute a major area of DNAzyme research and advancement.[102,116] The potential for miniaturization in point-of-care devices, rapid results, and high sensitivity and target selectivity enable the translation of technologies from the bench to the bedside. While nearly any illness or disease state with defined biomarkers, be they nucleic acids, proteins, or other small molecules, has the potential for DNAzyme-based diagnostics, this section presents recent advances in protein-based biomolecule detection, followed by a discussion of DNA and microRNA detection, with a brief overview of cardiovascular marker sensing. Applications specifically in cancer detection are presented subsequently. Generally, two types of DNAzymes are widely applied in biomarker detection: RNA-cleaving DNAzymes, which are primarily used as the recognition elements, and peroxidase-mimicking DNAzymes, which function exclusively as reporter molecules because of their ability to coordinate with hemin to oxidize particular substrates, yielding a colorimetric or chemiluminescent signal in the presence of H_2O_2.

Protein Detection. As detailed in Table 3, several protein-based biomarkers have been investigated for detection *via* DNAzymes, including alpha-fetoprotein,[117] lysozyme,[72] and prions.[118] In most of these systems, peroxidase-mimicking DNAzymes are exploited in an analogous method to how HRP is utilized as an enzyme label in ELISAs. In other words, peroxidase-mimicking DNAzymes confer catalytic conversion of a particular substrate, such as TMB, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), or luminol in the presence of a metal ion (Pb^{2+}, NH_4^+, K^+) and H_2O_2, which then generates a signal for detection and subsequent quantification.[119]

By and large, these peroxidase-mimicking DNAzymes exhibit lower activity compared to their protein counterparts.[120] However, these oligonucleotides offer several advantages that justify their use in such applications. Specifically, the structural stability of these enzymes in a wide range of chemical and thermal environments is enhanced compared to that of protein-based enzymes. The ease of manufacturing through chemical synthesis and polymerase chain reaction is generally preferred over more complicated protein synthesis techniques—a particular advantage in biosensing applications that could involve several complex fabrication steps.

A system developed by Chen *et al.* for the detection of thrombin is a prime example of the peroxidase-mimicking DNAzyme approach.[121] Briefly, Au-coated octahedral Cu_2O nanocrystals were functionalized with aminated thrombin binding aptamers (TBAs). These nanocrystals functioned primarily as signal amplifiers. A gold-coated electrode was similarly functionalized with TBAs. When present, thrombin acted as a binding agent between the nanocrystals and the electrode *via* interactions with TBA. Interestingly, thrombin binding triggered a conformational change within TBA, resulting in the adaptation of a G-quadruplex structure.

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ACS Nano 2021, 15, 13943−13969

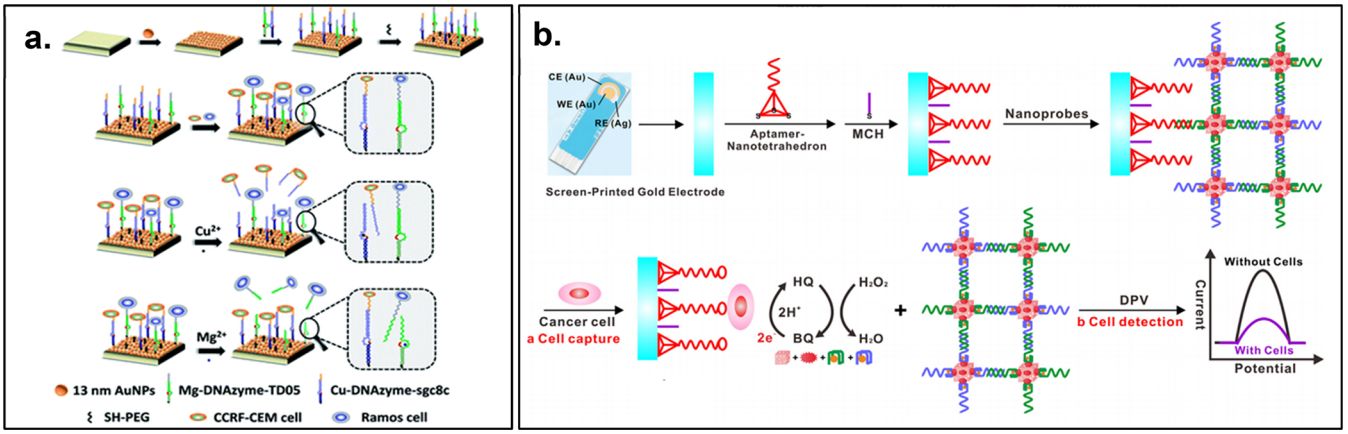

Figure 6. Detection of circulating tumor cells using immobilized DNAzymes. (a) DNAzyme-based platform for the capture and release of circulating tumor cells. Reprinted with permission under a Creative Commons Attribution 3.0 unported license from ref 136. Copyright 2020 Royal Society of Chemistry. (b) Target cancer cells compete with nanocages to bind to nanotetrahedron-aptamer probes, triggering release from screen-printed gold electrodes. Reprinted with permission from ref 140. Copyright 2018 Elsevier.

Subsequent attachment of hemin gave rise to a peroxidase-mimicking DNAzyme, which, in this case, was used for the electrochemical detection of the target to an ultrasensitive limit of detection of 23 fM.

Nucleic Acid Detection. Detection of nucleic acids is also widely applicable to various fields: environment, agricultural, forensic, and, as detailed in this section, markers for clinical diagnostics. In this regard, recent drives toward personalized medicine and pharmacogenetics mean that rapid detection of, for example, genetic variants could result in rapid assessment of a patient's potential response to a particular treatment based on their genotype. While next-generation sequencing has made significant strides toward this reality, costs, time, and labor associated with even the simplest of sequencing procedures make such activities challenging to execute in many clinical settings. DNAzymes are thus poised to offer a rapid alternative with high specificity when incorporated into point-of-care devices.

For example, Azuake-Hualde *et al.* demonstrated the application of a paper-based device for the detection of single-stranded DNA using physical entrapment within wax-printed circles.[29] Specifically, they demonstrated DNAzyme-based detection of a synthetic fragment of the Y amelogenin gene—a model for the detection of genetic variations. The system achieved precise colorimetric detection, showcased through its ability to distinguish between the target and a control that only differed by six base pairs. Interestingly, this methodology allowed for a visual detection limit of 143 ng. With a mobile phone camera, the limit of detection was reduced to 37 ng. The ultrasensitive capabilities of this sensing platform indicate great potential for use within real-world enabled sensors.

In another approach, a fluorescence-based system was devised for microRNA detection with potential for the detection of single nucleotide polymorphisms in clinical and biomedical research.[122] In this work, miRNA let-7, a potential marker for stroke,[123] cardiomyopathy,[124] traumatic brain injury,[125] and various cancers,[126,127] was used as an analyte in a system that combined a peroxidase-mimicking DNAzyme with an RNA-cleaving DNAzyme. In this method, thiolated DNA probes, with a recognition site for a Mg^{2+}-dependent RNA-cleaving DNAzyme and a region capable of complexing with K^+ and hemin to form the peroxidase-mimicking DNAzyme, were immobilized onto a Au-coated electrode. In the presence of the target miRNA, a suite of non-immobilized hairpin structures would assemble to produce the RNA-cleaving DNAzyme, which could then cleave the immobilized probe at the recognition site, thus releasing a portion of the probe to form the peroxidase-mimicking DNAzyme required for electrochemical sensing. The limit of detection was 0.46 fM in pure buffer and 10 pM in diluted human serum.

Cardiovascular Biomarker Detection. Cardiovascular biomarkers similarly play a significant role in diagnostics and patient monitoring in both acute and chronic vascular diseases. One such marker, cardiac troponin I, validated as a marker for myocardial tissue damage, has been studied extensively with a variety of immobilization strategies.[30,128,129] For example, an oligonucleotide aptamer-based sensor was developed based on a sandwich-type assay, wherein a DNA nanotetrahedron structure with a cardiac troponin aptamer serving as the capture molecule, was thiolated to a gold electrode.[128] This was coupled with the application of multifunctional nanoparticles complexed with a peroxidase-mimicking DNAzyme and HRP. This electrochemical sensing system demonstrated a limit of detection of 7.5 pg/mL, which was on the order of clinically relevant values for damaged myocardium. Another vascular-related biomarker is thrombin, a serine protease that engages in various activities, typically pro-inflammatory, and is used clinically to diagnose thrombosis and other clot-related disorders. The application of peroxidase-mimicking DNAzymes in biosensing systems has also shown significant potential in the pursuit of ultrasensitive and rapid thrombin detection.[121,130−133] Recently, a magnetic composite based on the application of a Pb^{2+}-dependent RNA-cleaving DNAzyme showed detection of thrombin at 1.8 pM.[130] This system was based on the immobilization of a DNA substrate onto Fe_3O_4@Au nanoparticles. The substrate contained a cleavage site-specific to Pb^{2+}-dependent RNA-cleaving DNAzymes, while also exhibiting complementarity to a thrombin-specific aptamer. In the absence of thrombin, the aptamer would be bonded to the immobilized substrate strand, preventing DNAzyme-mediated cleavage. However, in the presence of thrombin, the aptamer would preferentially bind to thrombin, leaving the immobilized substrate exposed, allowing for its cleavage. The subsequent addition of methylene blue—which binds to DNA molecules—quantified the presence of

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ACS Nano 2021, 15, 13943−13969

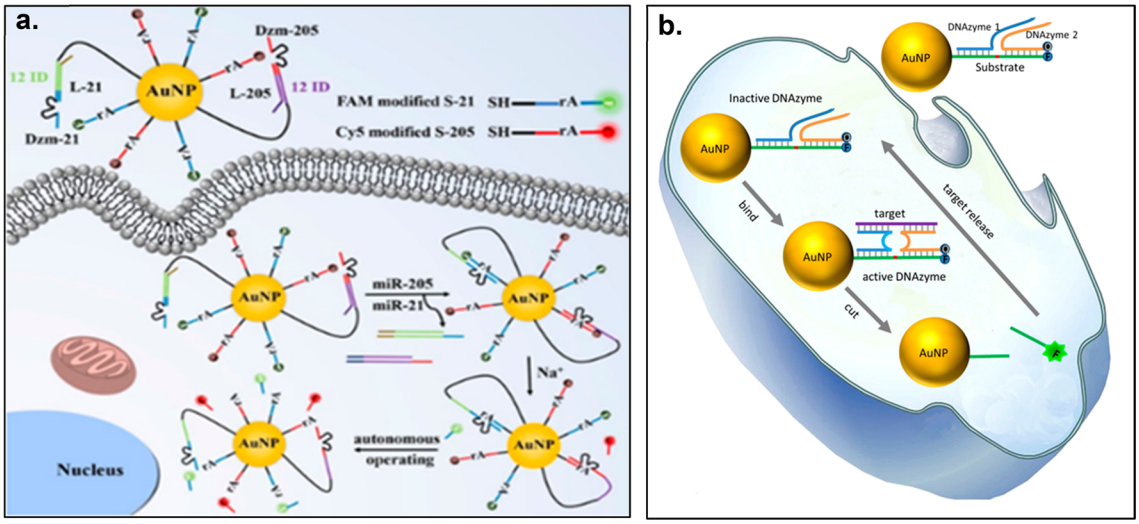

Figure 7. MicroRNA detection using immobilized DNAzyme-based platforms. (a) A platform of miRNA-initiated and intracellular sodium fueled DNAzyme probes to differentiate lung cancer cell subtypes. Reprinted with permissions from ref 150. Copyright 2020 American Chemical Society. (b) DNAzymes conjugated to gold nanoparticles for intracellular miRNA detection. Reprinted with permissions from ref 152. Copyright 2017 American Chemical Society.

thrombin, given that the amount of methylene blue directly correlated to the length of DNA present.

Detection of Cancer Markers. To successfully improve cancer survival rates, better diagnostic tools capable of early stage detection are necessary. Traditional approaches for cancer detection—such as tumor biopsies—are limiting, and their effectiveness is influenced by tumor size and location. Alternative approaches such as liquid biopsies may provide a solution.[134] To this end, DNAzymes have been proposed as valuable tools to analyze tumor biomarkers in liquid biopsies for the detection of clinically relevant targets, including exosomes,[135] circulating tumor cells (CTCs),[136] circulating tumor DNA,[137] and microRNA (miRNA).[138] For the purpose of this review, we focus on three target categories most widely studied in DNAzyme systems: CTCs, intracellular markers, and miRNA. A list of DNAzyme-based cancer detection strategies is presented in Table 3 to complement this discussion.

Circulating Tumor Cell Detection. CTCs are shed from primary or metastatic tumors and circulate in the blood.[139] They carry proteins, DNA, and RNA associated with the tumor of origin and can be used as biomarkers in liquid biopsies. However, given that their concentrations typically remain low relative to other cells, there is a pressing need for the development of technologies capable of highly specific capture and detection of CTCs. DNAzyme-based platforms can be engineered with various isolation strategies to overcome these limitations.

Although not yet incorporated into a detection strategy, Zhang *et al.* designed a platform to capture and selectively release multiple CTCs using DNAzymes, as shown in Figure 6a.[136] This design utilized two aptamers coupled with Mg^{2+}- and Cu^{2+}-dependent RNA-cleaving DNAzymes to select for two distinct cell types (CCRF-CEM and Ramos). The thiolated DNAzymes were immobilized onto a substrate covered with AuNPs. Upon capture, cellular release was initiated *via* the introduction of Mg^{2+} or Cu^{2+} for selective CTC enrichment. Results using blood samples from lung cancer patients indicated that the platform could be adapted for clinical use. Sun *et al.* also developed a CTC capture and release platform, with their sensor capable of detecting HepG2 cells associated with liver cancer (Figure 6b).[140] This method relied on the immobilization of DNA nanotetrahedron structures containing HepG2-specific aptamers onto the surface of screen-printed gold electrodes *via* thiol-based chemistry. Palladium−platinum nanocage-based structures incorporating HRP and peroxidase-mimicking DNAzymes were then hybridized onto the nanotetrahedrons. When present, target cancer cells outcompeted the nanocage structures for nanotetrahedron binding. This led to their release from the surface, allowing them to engage in an oxidation reaction that transduced signals *via* differential pulse voltammetry. The system demonstrated a detection limit of 5 cells/mL. Cellular release was also demonstrated by breaking Au−S bonds through the application of negative potential, with minor losses in cellular viability.

Telomerase Detection. Cancer cells exhibit increased telomerase activity relative to healthy cells,[141] which has led to the development of platforms that use this property for diagnostic purposes.[142,143] While there are many approaches to telomerase detection, DNAzyme-based strategies provide avenues for both ultrasensitive and *in situ* detection.[144] For example, Xu *et al.* developed a sensor capable of imaging telomerase activity through a DNAzyme motor consisting of gold nanoparticles functionalized with thiolated Mn^{2+}-dependent, RNA-cleaving DNAzymes.[145] Using a locking strand method, fluorescence signals were quenched owing to their proximity to gold. However, in the presence of telomerase, DNAzyme activity was initiated, yielding cleavage of the fluorescent tag, which could be detected *via* confocal imaging. This sensor was successfully translated to use within live cells. Notably, this approach utilized the inherent function of telomerase, incorporating a telomerase substrate (TS) onto the gold surface to yield a functional DNAzyme in the presence of telomerase.

Wang *et al.* similarly exploited the innate function of telomerase using magnetic beads with a TS.[146] An amine-aldehyde reaction achieved immobilization of TS onto the magnetic beads. In the presence of telomerase, the strategic addition of TTAGGG resulted in G-rich sequences that could

https://doi.org/10.1021/acsnano.1c04327
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form peroxidase-mimicking DNAzymes in the presence of K^+ and hemin. Telomerase activity in HeLa cells could be detected down to a concentration of 0.5 cells/μL using a UV−vis spectrometer and 1 cell/μL with the naked eye. As evidenced by these strategies, the nature of DNAzymes paired with telomerase activity are suited for the screening of cancerous cells.

MicroRNA Detection. Given recent developments in the use of miRNAs as clinical biomarkers, it follows that methods for their detection, particularly in cancer, are needed.[147] An investigation of several DNAzyme-based approaches for miRNA-driven cancer detection has recently been conducted.[148] For an in-depth analysis of DNAzyme approaches for miRNA detection overall, the reader is directed to a recent review by Mahdiannasser *et al.*[149] However, we highlight a few cancer-based applications herein.

Chen *et al.* developed a system for the intracellular detection of miRNA-21 and miRNA-205 in A549 and H520 lung cancer cells.[150] As depicted in Figure 7a, their system was based on a DNAzyme motor, which consisted of AuNPs coated with both Na^{2+}-dependent RNA-cleaving DNAzymes hybridized to locking strands preventing their activity and fluororphore-conjugated substrate strands specific to each DNAzyme. In the presence of target miRNA, the locking strands preferentially hybridized to the target strand, allowing the DNAzymes to interact with their corresponding substrate strands. Cleavage of the substrate strands would then result in the release of the fluorophores off the AuNPs. The dependence of DNAzyme activity on a Na^{2+} cofactor is of particular note given the ubiquity of Na^{2+} in cells, which enabled the system to progress *in vitro* without any additional components. In addition, miRNA-21 is tied to many other cancers, including gastric, prostate, breast, and neuroblastoma, further expanding applications beyond lung cancer.[151]

In another approach to miRNA-21 detection, Wu *et al.* engineered a thiolated split-DNAzyme probe, which was conjugated to AuNPs (Figure 7b).[152] This split probe formed active RNA-cleaving DNAzymes in the presence of the target miRNA, which resulted in cleavage from the AuNP and activation of the fluorophore. Without the target miRNA, the DNAzyme would remain in an inactive form, leaving the fluorophore in a quenched state. Results showed promising evidence for accurate miRNA imaging in live cells, with applications in fundamental research and cancer diagnostics.

FUTURE DIRECTIONS

This Review aimed to provide an overview of current immobilization techniques and biosensing applications of DNAzymes, with an emphasis on recent developments in the detection of food contamination, pathogens, metal ions, and clinical biomarkers. Given the magnitude of research in this space, rapid advancements can be anticipated in the sensitivity, specificity, and feasibility of DNAzyme-based biosensors for real-world applications. These developments can be expected to coincide with improvements in functionalization and immobilization strategies. Given the advantages of DNAzymes, including stability across a variety of environmental conditions and ease of synthesis, particularly compared to protein enzymes, we anticipate that the diversity and translational capabilities of these technologies will continue into the future.[119]

While many of the discussed immobilization strategies have been well established for quite some time, recent studies have aimed to compare their effectiveness in immobilizing DNA under various conditions.[153] Such studies will continue to better our understanding of the compatibility of different strategies for particular sensing systems. Contrarily, strategies such as physical entrapment and graphene-mediated electrostatic interactions have gained traction only recently. Given the advantages these methods present compared to more traditional immobilization strategies, further research into their use can be anticipated. Further, many of the discussed strategies have only been applied in the detection of a handful of targets. With a growing body of literature supporting their viability in diverse detection environments, more widespread application of the discussed strategies is expected. Considerations moving forward should include surface stability, the shelf life of immobilized substrates, compatibility within target environments (*e.g.*, water, serum or food), and the minimization of non-specific binding, which is a challenge in achieving the very low limits of detection needed within many applications. Ultimately, the performance of DNAzyme-based biosensors depends on immobilization, activity after immobilization, and adequate transduction of signals, which supports the need for advances in robust and stable immobilization techniques. In addition, fabrication costs should be considered to allow for potential implementation within large-scale applications.

Recent developments involving the structural modification of DNAzymes into a three-dimensional form can be expected to continue. These DNA structures provide numerous benefits, including the ability to physically adsorb onto substrates such as paper without the need for any chemical modifications, resistance to nuclease activity, high signal-to-noise ratios, and suppression of non-specific protein adsorption.[154] In terms of modifications to the sensing surface, lubricant infusion has garnered interest as a scalable modification that can improve biosensor performance through the omniphobicity it induces on sensing surfaces.[155−157] In particular, lubricant infused surfaces have demonstrated excellent blood repellency, making them ideal for use within clinical biosensors seeking to detect biomarkers within blood samples.[158−164] Furthermore, through the attachment of capture ligands onto such lubricated surfaces, selective cell adhesion has been achieved.[159,163,165] This presents a promising avenue toward cell-specific intracellular detection of biomarkers with increased precision. Hierarchical structuring has also been identified as a means through which omniphobicity can be induced and used for improvements in biosensing, presenting itself as another possible surface modification for future works.[166] The incorporation of such surface modifications stands to provide sensors with improved accuracy and lower limits of detection.

Perhaps one of the most intriguing applications for DNAzyme-based biosensors is in the detection of food contaminants. Given the global implications of foodborne illnesses and food waste, there is a defined need for the development of in-package sensors capable of providing easy-to-detect signals of contamination and spoilage without the need to open packaging. By relying on such dynamic indicators of safety and freshness, societal reliance on generic expiry dates may be eliminated. Fluorescence and colorimetric transduction platforms are of particular interest given the ease with which signals could be detected along the food production pipeline.

Similarly, regarding pathogen, metal ion, and biomarker detection in both environmental and clinical settings, the application of DNAzymes might be able to address the selectivity and sensitivity needed to advance point-of-care and

https://doi.org/10.1021/acsnano.1c04327
ACS Nano 2021, 15, 13943−13969

on-site testing. Within the context of reducing lengthy waiting periods and labor costs associated with most clinical and heavy metal testing, DNAzyme-integrated sensors may challenge traditional approaches, offering an alternative that is both time- and cost-effective. With that said, careful consideration regarding the efficacy, reproducibility, and scale-up, particularly at the immobilization level, will be critical to achieving this reality. While the robust nature of covalent linkages is in widespread use, during initial design and conception, non-covalent as well as physical entrapments should be considered to consolidate fabrication steps and potentially decrease costs.

In a similar vein, DNAzyme-based platforms may overcome the hurdle of low biomarker concentrations that characterize early-stage cancer. Additionally, the potential to multiplex various targets—such as CTCs and miRNAs—could further assist in clinical characterization and staging. Combined with improved specificity and sensitivity, the versatility to integrate such DNAzyme-based strategies into point-of-care devices may lead to these platforms leading the next generation of clinical tests in cancer detection.

DNAzymes also have the potential to play a substantial role in increasing our chances of success in our fight against infectious diseases. The COVID-19 pandemic has highlighted the need to have methods that enable rapid, simple, and equipment-free detection of infectious agents, such as SARS-CoV-2. We expect soon to see the identification of DNAzymes that are able to detect and report the presence of specific proteins in viruses surfaces (such as the spike protein), as well as the identification of DNAzymes that can be activated by the presence of specific genomic sequences, such as the presence of the RNA sequence that encodes for the production of the spike protein in SARS-CoV-2. This second approach would avoid the need to use RT-PCR for nucleic acid-based detection of the virus. As highlighted in this review, DNAzymes are tools that are ideally suited to serve as the backbone for highly specific and simple tests, including paper-based sensors.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

S.K. is supported by a Vanier Canada Graduate Scholarship awarded by the Natural Sciences and Engineering Research Council. K.D. is supported by the North Carolina A&T State University KL2 Scholar Award from the National Center for Advancing Translational Sciences, National Institutes of Health, through Grant KL2TR002490 and start-up funds from the Joint School of Nanoscience and Nanoengineering, North Carolina A&T State University. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. T.F.D. is supported by NSERC Discovery and CRD grants, Ontario Early Researcher Award grant, and McMaster start-up funds.

VOCABULARY

Hemin/G-quadruplex, a peroxidase-mimicking DNAzyme often used as a reporting entity within DNAzyme-based sensing systems; **signal transduction**, the portion of the sensing cascade responsible for converting target-induced nanoscale events into detectable signals; **limit of detection**, the lowest concentration at which a target analyte can be reliably detected; **hybridization chain reaction**, an enzyme-free amplification approach for improved detection sensitivity that occurs in either the presence or the absence of the desired target analyte; **rolling circle amplification**, a DNA amplification strategy that involves the use of a short primer, circular DNA template, and DNA polymerase to create repeated copies of a desired sequence; in the context of DNAzyme-based sensors, this strategy is often used to improve the limit of detection through the formation of large quantities of signal transduction-related entities.

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https://doi.org/10.1021/acsnano.1c04327
ACS Nano 2021, 15, 13943−13969