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Review

Constructions, Purifications and Applications of DNA-Antibody Conjugates: A Review

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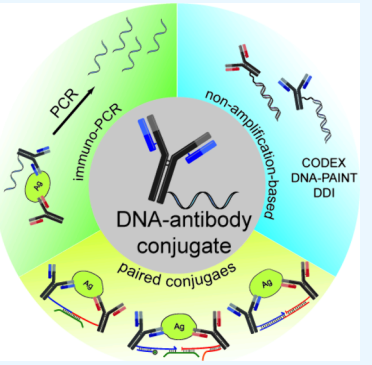
ABSTRACT: A DNA-antibody conjugate is a synthetic molecule that combines the unique functions of both an antibody and DNA. With the increased accessibility of commercialized kits, the procedure for constructing conjugates is simplified and the requirement for chemistry background is reduced. As a result, the difficulty of preparing a DNA-antibody conjugate has been significantly lowered. Therefore, the application of DNA-antibody conjugates has attracted more interest in recent years. The most common application of DNA-antibody conjugates is based on the amplifiable property of DNA through PCR. This includes single-conjugate-based immuno-PCR, paired-conjugates-based proximity ligation assay, and proximity extension assay. These methods achieve highly sensitive or specific detection of target proteins. The conjugated single stranded DNA molecules can also specifically hybridize with another strand containing its complementary sequence. This property can be used to selectively bind fluorophore labeled DNA strands, which plays an important role in tissue imaging and spatial omics. All these factors make DNA-antibody conjugates have a broad range of applications in research, diagnosis, and potentially therapy.



1. INTRODUCTION

Antibody-based immunoassays are important and widely used in life sciences and medical research, clinical diagnostics, drug development and even environmental monitoring.[1] Thanks to the high specificity and variety of antibodies, immunoassays are specific, simple and versatile compared to other assays for detecting or separating certain analytes. Antibodies can be modified with various labels including radioisotopes,[2] fluorescent dyes,[3] chemiluminescent,[4] proteins,[5] and chemical molecules.[6] These modifications combine the unique functionalities of the labels with antibodies, further expanding the capabilities and applications of immunoassays, particularly for quantification purposes.

Cells produce a myriad of biomolecules to carry out various cellular activities. Changes in the amounts, structures, and functions of these biomolecules may be related to pathogenic processes. The molecules, primarily proteins, that can serve as indicators of normal biological processes, pathogenic processes, or responses to an exposure or intervention are known as biomarkers.[7] Accurate and reliable quantification of biomarkers is crucial, as early diagnosis leads to a quicker response to the disease and a higher likelihood of successful treatment. However, biomarker levels can vary greatly and may be low, especially during the early stages of cancer, when changes in biomarker levels can be very subtle and difficult to detect.[8] These challenges highlight the need for the development of sensitive detection methods.

While there are numerous established protein detection techniques, most are only capable of detecting proteins with high abundance or require enrichment before the assay.[9,10] When detecting targets with very low amounts, signal amplification is necessary to increase sensitivity (Table 1).[11,12] For example, enzyme-linked immunosorbent assay (ELISA), a colorimetric or chemiluminescent immunoassay that relies on enzyme labeled antibodies, is a common method for amplifying protein signals through enzymatic reactions. Polymerase chain reaction (PCR), which amplifies nucleic acid molecules, and its derivative methods such as quantitative real-time PCR (qPCR) are ultrasensitive tools for detecting nucleic acids and pathogens.[13] During the COVID-19 pandemic, qPCR was the primary diagnostic method, capable of detecting virus infection even when the patient was asymptomatic.[14] In contrast, PCR is plagued by false positive issues caused by nonspecific amplification, and it cannot differentiate between dead or inactive pathogens, potentially leading to results that do not accurately reflect the disease status.[15] In addition, the level of protein expression may not always correlate strongly

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Table 1. Comparison of Commercial Immunoassays for Human Interleukin-6 (IL-6) Measurement[a]

Methods	Sample volume/μL	Sensitivity (pg/mL)	Measuring range (pg/mL)	Vendors	Catalog numbers
Sandwich ELISA	100	3	3−1000	RayBiotech	ELH-IL6
Sandwich ELISA	100	0.7	3.1−300	R&D Systems	D6050
One-step sandwich ELISA (colorimetric)	50	1.6	7.8−500	Abcam	ab178013
One-step sandwich ELISA (fluorescent)	50	0.4	0.97−2000	Abcam	ab229434
Immuno-PCR	10−25	0.32	0.064−1000	RayBiotech	IQH-IL6
Bead-based Immuno-PCR	5	0.03	0.28−200	RayBiotech	BIQH-IL6
Chemiluminescent Immunoassay	50	7.5	12.5−800	Innovative Research	IHUIL6KTC
NanoBiT bioluminescent Assay	20−80	7.5	18.2−25000	Promega	W6030
Flow Cytometry	25−50	1.6	10−2500	BD Bioscience	558276
Later Flow Immunoassay	100	50	50−10000	Milenia Biotec	MQL6 1
TR-FRET immunoassay	15	11	61−30000	Bioauxilium	KIT-IL6

[a]All the data presented are gathered from the manuals of the respective products, including catalog numbers.

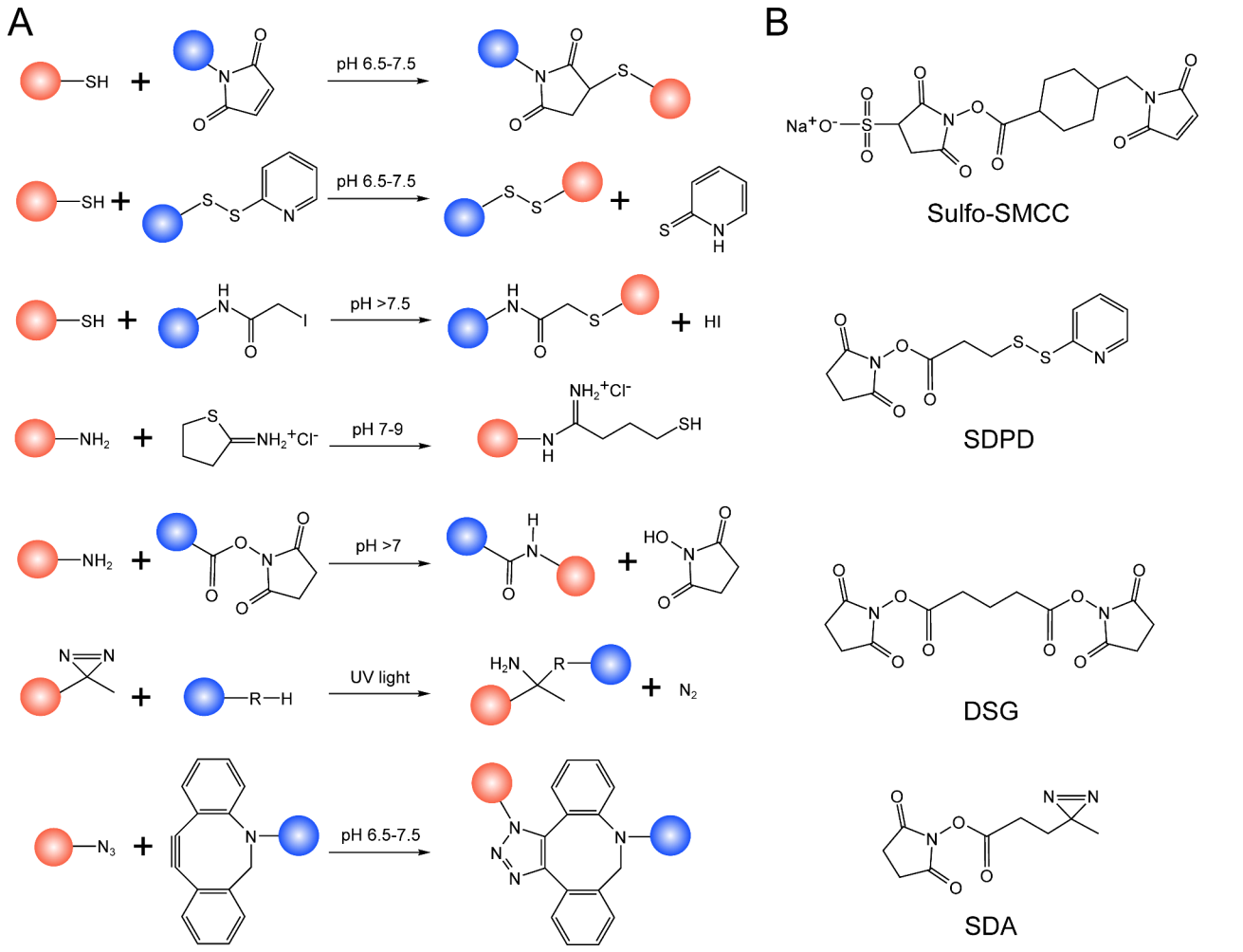

Figure 1. Reaction mechanisms and cross-linkers are used to covalently conjugate antibodies and DNA. (A) The mechanisms involved in conjugation are represented by blue and orange balls, which symbolize the two biomolecules being conjugated. (B) Common cross-linkers include sulfo-SMCC (sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate), SDPD (succinimidyl 3-(2-pyridyldithio)propionate), DSG (disuccinimidyl glutarate), and SDA (succinimidyl 4,4′-azipentanoate)

with transcriptional levels due to factors such as translation regulation, translation efficiency, splicing, post-translation modification, degradation rate, and other biological processes.[16,17] Therefore, directly measuring proteins or other metabolites are still necessary in most cases. However, there is currently no technology available that can directly amplify these non-nucleic acid molecules in each sample. Sano et al. first used a DNA-antibody conjugate to detect proteins and achieved detection for only hundreds of the target molecules.[18] The principle of this experiment, called immuno-PCR, was to conjugate target-specific ligands, including antibodies and aptamers, with nucleic acid probes that can be amplified by PCR so that the signal can be extremely magnified. Based on this idea, a group of ultrasensitive protein detection techniques was developed.[9,18] These methods combine the specific molecule recognition of antibodies with the high amplification

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Table 2. Comparison of Selected Commercial DNA-Antibody Conjugation Kits[a]

Vendors	Abcam	Alphathera	Broadpharm	Cellmosaic	Dynamic biosensers	Raybiotech	Vectorlabs
Catalog number	ab218260	AT1002	bp-50005	CM53405	PF-AB-1	OAC-AMINE	S-9011-1
DNA modification	Amine	Not specified	Amine	Amine	DBCO	Amine	Amine
Optimum oligo length	ss: 10−120 nt ds: ≤80 bp	≤80 bp	ss: 10−120 nt ds: ≤80 bp	≥5 nt	≤150 nt	Not specified	ss: 20−60 nt
Site-specific	No	Fc region	No	No	No	No	No
Residue of antibody	Not specified	Not specified	Lysine	Lysine	Lysine	Lysine	Lysine
Antibody needed/μg	100	25−100	100 or 500	250−1000	50−500	100	100
Antibody activation	Yes	No	Yes	Yes	No	No	Yes
Purification method of activated molecules	Gravity flow size exclusion column	Not required	Ultrafiltration	Ultrafiltration	Spin Desalting Column	Spin Desalting Column	Spin Desalting Column
Purification methods of conjugates	Precipitation	Not provided	Ultrafiltration (optional)	Spin column	Not provided	Not provided	Affinity magnetic beads
Estimated assay ctime[b]	2 h	2 h	3 h	8 h	3 h	1.5 h	11 h
Cost per reaction	High	High	Medium	Medium	Low	Low	High

[a]All the kits require HPLC grade DNA oligo with a modification on either the 5′ or 3′ end. Except for Alphathera, the cost for kits from other vendors does not include oligo synthesis and preparation. Abbreviations: ss, single strand; ds, double strand; nt, nucleotide; bp, base pair. [b]The assay time is estimated based on the minimum incubation time needed for each step. However, most kits suggest incubating for a longer period to ensure the maximum formation of the conjugate.

of DNA templates by PCR and can achieve 100−10000 times higher for target detection compared with traditional ELISA.[19]

Besides simply amplifying the signal, DNA-antibody conjugates have other unique advantages in various applications. To address the issue of cross-activity with antibodies in multiplex protein detection, methods based on paired DNA-antibody conjugates, such as proximity ligation assay, have been developed using a similar principle to immuno-PCR. In these experiments, only targets correctly bound by the two corresponding antibodies can produce a signal, greatly improving specificity. Additionally, the development and easy access to sequencing techniques have significantly increased the throughput for DNA-antibody-conjugates-based assays.

On the other hand, the order of nucleotide bases along a DNA strand contains valuable information. Due to the principle of complementary base pairing, a single stranded DNA molecule can specifically hybridize with another strand containing its complementary sequence. Thus, antibodies conjugated with DNA molecules can also be used to target specific molecules conjugated or containing the complementary sequences.[20−22] This property is important for studying spatial proteomics and visualizing cellular structures, and it has been utilized in many recent applications. Additionally, functional nucleic acids like DNA aptamers can form unique secondary structures and bind to target molecules. Due to their smaller size, aptamers can be easily conjugated to antibodies to form a bispecific detector, potentially serving as an alternative to bispecific antibodies. This type of aptamer-antibody conjugate shows promise for cancer therapy.[23]

In this review, we will first briefly introduce the methods for constructing and purifying DNA-antibody conjugates. Then, we will focus on the application of DNA-antibodies in protein detection and quantification, primarily based on immuno-PCR. Additionally, we will cover some of the latest nonamplification-based applications. These applications are important tools in imaging molecules and spatial omics.

2. CONSTRUCTION AND PURIFICATION OF DNA-ANTIBODY CONJUGATES

DNA-antibody conjugates can be constructed either noncovalently or covalently, depending on the conditions. Covalent conjugation methods involve the same mechanisms as other molecules that conjugate with antibodies (Figure 1. A). Many studies have reported or evaluated these common methods in detail.[24−26] Therefore, we will focus more on the general concept rather than specific approaches.

Noncovalent methods typically require a linker molecule to connect the antibody and the DNA. One common linker molecule used is avidin or streptavidin, as both the antibody and DNA can be easily biotinylated. In some cases, the DNA may be chemically conjugated to streptavidin rather than through biotin. Another option is protein A or protein G, which can specifically bind to the Fc fragment of the antibody, but this method usually still requires the oligo to be covalently conjugated to protein A or protein G in advance.[22] Noncovalent methods are generally easier to perform than covalent methods as the linker molecules can universally bind to the corresponding ligand without any modification. Another advantage of noncovalent methods is the lower number of antibodies required. However, in many cases, noncovalent conjugates are constructed *in situ*, meaning the linker molecules and DNA are added after the antigen−antibody complex formation.[18,27,28] This results in needing more incubation time and wash steps, and the system cannot be used for multiplex detection in these cases. On the other hand, covalent approaches provide a more stable connection between the antibodies and DNA molecules, allowing these conjugates to be used in more conditions. In addition, covalent conjugates are usually prepared in advance, which shortens the downstream experimental procedure and enables multiplex detection as each antibody can be conjugated with different DNA molecules. However, covalent methods have drawbacks. They require a lager input of antibodies, typically 50−100 μg, as the purification step could result in significant loss. To minimize inhibition of the reaction, high-purity DNA such as

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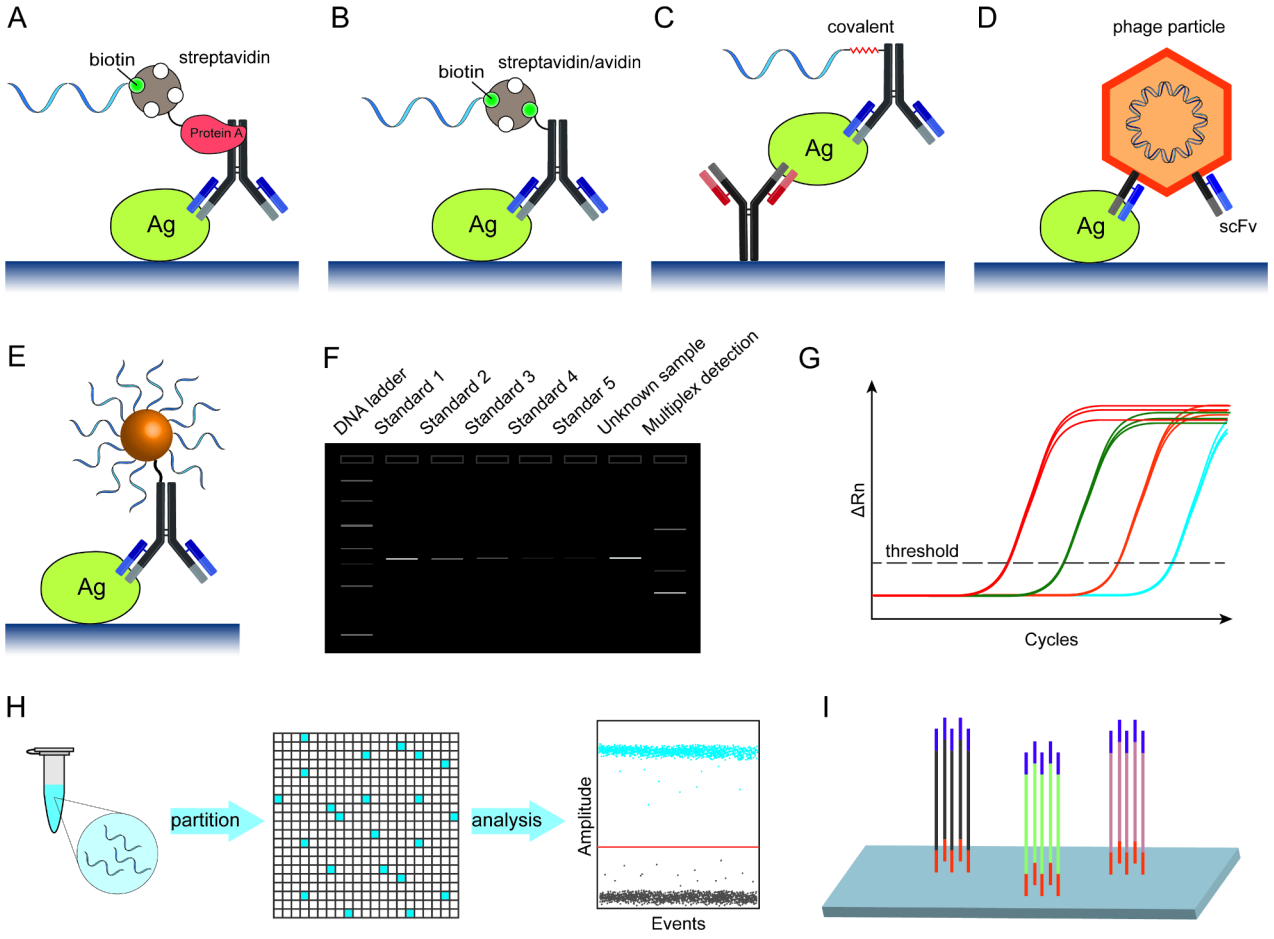

Figure 2. Formats of immuno-PCR and readout methods: (A) The original format of immuno-PCR introduced by Sano et al. through a streptavidin-protein A chimera. (B) The universal immuno-PCR where DNA and antibody were both biotinylated and connected by streptavidin or avidin. (C) DNA-antibody conjugates constructed by covalent methods. (D) Immuno-PCR based phage display technique where svFc is expressed on phage surface. (E) Immuno-PCR made with gold nanoparticles labeled with DNA. (F) Immuno-PCR readout by agarose electrophoresis. Unknown samples can be analyzed by comparing the intensity with standard samples and multiplex detection can be completed by visualizing the amplified DNA bands with different lengths. (G) Immuno-PCR readout by qPCR based on SYBR Green I dye or fluorescent probe. (H) Immuno-PCR readout by dPCR, which analyzes the presence and absence of a fluorescent signal in partitioned PCR reactions. (I) NGS was used to count the number of each barcode for quantifying the target proteins.

HPLC pure is usually required. These requirements significantly increase the cost of making the conjugates, not including the cost of other reagents like cross-linkers (Figure 1. B) or special modifications needed. In addition, covalent methods require researchers to have more knowledge of chemistry and protein engineering, which can be a barrier. While there are now many commercialized DNA-antibody conjugation kits available that simplify the procedure (Table 2), practice and optimization are still necessary since each antibody used is unique, keeping costs high.

Despite these shortcomings, covalent approaches have still attracted a lot of interest in their development and application. In a covalent conjugation reaction, there are two important properties one should consider: conjugation efficiency and degree of conjugation.[24] The percentage of antibodies conjugated with at least one DNA molecule is called conjugation efficiency. Higher conjugation efficiency means a higher yield. Since antibodies are usually expensive, one would like to achieve as high a conjugation efficiency as possible to make full use of the input antibody. The degree of conjugation refers to the number of DNA molecules per antibody.[24] Depending on experimental design, a specific degree of conjugation, such as one DNA molecule per antibody, may be desired. This value can be adjusted using a suitable DNA-antibody ratio for making the conjugate, especially for conjugation reactions that rely on the amine group (lysine) or thiol group (cysteine) of the antibody (Figure 1. A). The average degree of conjugation can be measured using gel electrophoresis, size exclusion chromatography and mass spectrometry. It is important to remember that these reactions randomly occur on the available groups and will always result in a complex mixture of antibodies conjugated with a different number of DNA molecules, even when a low ratio is used.[29] Generally, a higher DNA-antibody ratio can lead to a higher conjugation efficiency and average degree of conjugation. However, a high amount of DNA can cause a strong background in downstream experiments. It is always necessary to conduct preliminary tests to determine the best DNA-antibody ratios based on the selected conjugation method to balance all aspects.

The DNA used is typically in excess, so in most cases, the conjugates need to be purified to remove this excess DNA. Besides, in some instances, the unconjugated antibody that remains can compete with the conjugates to bind the antigen,

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resulting in a lower signal. Therefore, it is important to remove these antibody molecules as well. Various methods can be used for purification, including precipitation, ultrafiltration, dialysis, desalting, and chromatography.[30−33] These methods can be used individually or in combination to achieve the highest purity of conjugates.[34] Since DNA is usually much smaller than the antibody, ultrafiltration, dialysis and desalting can be used to remove these smaller molecules based on their difference in size.[35] These methods are simple and compatible with relatively small amounts of conjugates. Chromatography is commonly used for routine purification of conjugates with three main types: affinity chromatography, size-exclusion chromatography (SEC), and ion exchange chromatography (IEC). Affinity chromatography uses protein A or/and protein G ligands to specifically capture the unconjugated antibody and conjugate, allowing the excess DNA to be washed off. SEC separates molecules based on size differences as they pass through a resin. The conjugates, being the largest molecules, are eluted first, followed by free antibody, and then excess DNA.[27,36] High-resolution columns can separate conjugates with different numbers of DNA attached, making this method useful when specific type of conjugate is needed.[37] IEC is often preferred over the other types of chromatography.[31,38] A high-resolution anion exchange column is typically required for this purification. Due to the strong negative charge of DNA, it binds tightly to the resin and requires a higher salt concentration for elution. In contrast, the antibody has much less charge than DNA under weak alkaline conditions and is eluted with a lower salt level. After optimization, unreacted antibody, conjugates and excess DNA can be eluted sequentially with increasing salinity. Antibodies with different numbers of DNA attached are also eluted in order to increase DNA numbers. While chromatography can provide excellent purity, there is often a significant loss of conjugates. Additionally, the collected fractions usually need to be concentrated and buffer exchanged into a specific storage buffer, further reducing the recovery. The expensive equipment and trained personnel required for chromatography can limit its use. To address these issues, spin column versions of chromatography have been developed allowing for simpler use through centrifugation. While purity may not be as high as with traditional columns, spin columns can still meet certain requirements.

3. EVOLUTION OF IMMUNO-PCR

Immuno-PCR was originally introduced by Sano et al. in 1992.[18] In their experiments, bovine serum albumin (BSA) was used as antigen and immobilized on a plate. After incubating with a detection antibody, a biotinylated linear plasmid DNA molecule was connected to the antibody through a streptavidin-protein A chimera (Figure 2.A). The plate was then subjected to PCR and the yield of PCR product was used for quantifying antigens. Using this method, they claimed that they could detect as low as 580 antigen molecules, making the limit of detection (LOD) 5 orders of magnitude lower than conventional ELISA. However, streptavidin-protein A chimera, the key component, brought several limitations to the assay. For example, protein A only has a strong affinity to immunoglobulins of certain species, limiting its application with other antibodies. But most importantly, it was found that only direct ELISA could be used since the Fc of the capture antibody used in sandwich ELISA could also be bound by protein A, leading to higher background and lower specificity.[28]

In 1993, Ruzicka et al. and Zhou et al. respectively improved immuno-PCR by discarding the hard-to-get streptavidin-protein A chimera and instead using free avidin, streptavidin, and biotinylated antibodies (Figure 2. B).[28,39] These molecules were commercially available or could be easily prepared. This modification made the assay no longer restricted by the sources or (sub)classes of the antibodies. Therefore, Zhou et al. called this new assay “universal immuno-PCR”. However, since both streptavidin and avidin are homotetramers, the formation of DNA-antibody conjugates through these methods may not be homogeneous and could have a batch effect for each experiment.[40]

The covalently conjugated antibody (Figure 2. C) was first introduced in immuno-PCR in 1995 by Hendrickson et al..[27] In contrast to the noncovalent conjugation made *in situ*, these conjugates constructed by the heterobifunctional cross-linker sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC, Figure 1. B) were prepared and purified in advance. This shortened the assay procedure greatly and made the sandwich format possible. The most significant improvement in this study is that direct conjugation allows for the detection of various targets simultaneously, which is not common in immunoassays. At that time, the signal readout still relied on agarose gel electrophoresis and staining for the PCR products. By labeling the antibodies with DNA of different lengths, the PCR product can be easily separated in electrophoresis and analyzed separately (Figure 2. F).[18,27] Theoretically, any DNA sequences can be used as the reporter, providing potentially unlimited probes to use. The same research group also explored the differences between ssDNA and dsDNA, different primers, but they did not find a noticeable effect on the assay detection limit.[34]

One factor that may restrict the extension of the detection limit is the DNA detection methods.[18,27] Initially, ethidium bromide was the most used dye for DNA gel staining, capable of detecting a band at the nanogram level. While alternative dyes can achieve higher sensitivity, measurements after the entire PCR cycle can only show the concentration of the original template within a certain range, as all samples made with the same master mix saturate at the same level.[41,42] Compared to agarose gel electrophoresis, ELISA-based methods for detecting the PCR product increase sensitivity but are even more time-consuming and laborious.[40] By adding ethidium bromide to the PCR system, which binds to the PCR product after each cycle, Higuchi et al. enabled the monitoring of the PCR process.[43] This laid the foundation for qPCR (Figure 2. G), but this technique was not introduced into immuno-PCR to replace the agarose gel electrophoresis for detecting the amplification of labeled DNA until 2000.[44] qPCR can detect the signal directly during the PCR procedure, eliminating problems caused by cross-contamination while increasing sensitivity and automation of analysis. However, due to channel limitations, smaller panels must be designed when measuring multiple targets. In such cases, agarose gel may still be a powerful tool. An example of this is a photocleavable DNA barcode-antibody conjugate system for cell protein identification designed by Agasti et al.[45] When the DNA-antibody conjugates bind to cell receptors, the DNA barcodes are released by light irradiation. After PCR amplification, the barcodes are identified using gel electrophoresis for multiplexed detection of protein biomarkers from single cells. In

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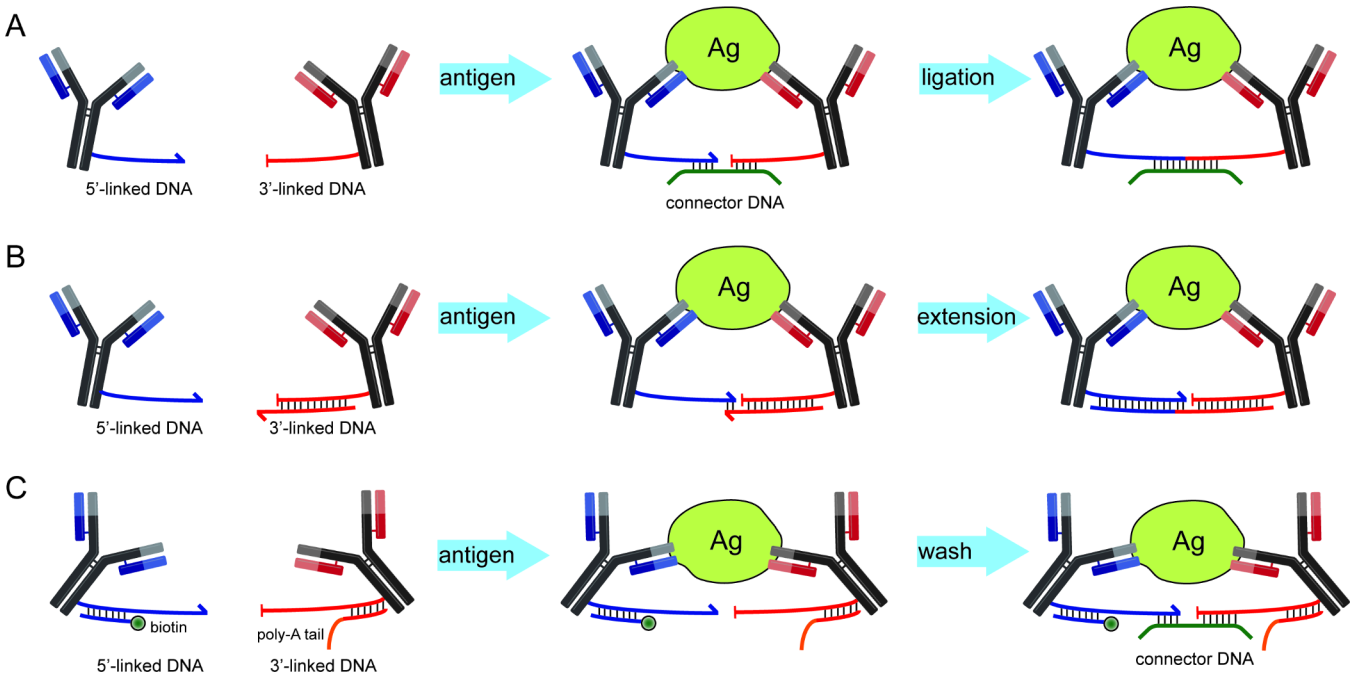

Figure 3. Scheme of amplification-based detection using paired DNA-antibody conjugates. Only when the two probes bind the same antigen are the DNA molecules brought into proximity so that the final amplification template is formed. The signal can be detected by qPCR or NGS. (A) PLA: the final amplification template is produced by ligase. (B) PEA: the final amplification is produced by DNA polymerase. (C) NULISA: after the probes bind antigen, oligo dT magnetic beads are used to capture the immunocomplex and excess probe containing poly-A. After washing, captured molecules are released from oligo dT beads and recaptured by streptavidin beads. Only the immunocomplex containing both probes can be captured, reducing background significantly.

contrast, by utilizing DNA barcodes, it offers a superior solution for multiplexing, as the vast diversity of DNA barcode sequences and sizes allows for virtually limitless identification (Figure 2F).

Although the basic form of immuno-PCR is already mature and in use today, there have been numerous studies aimed at improving it further. One area of exploration focuses on the DNA component, specifically the different forms of DNA molecules used to create DNA-antibody conjugates. These modifications are generally aimed at overcoming challenges in preparing antibody-DNA conjugates and enhancing the signal, ultimately improving sensitivity. By utilizing the phage display (Figure 2. D) technique, antibody fragments such as single chain variable fragments (scFv) can be expressed on the surface of phage particles and still bind specifically to the corresponding antigen.[46,47] The recombinant phage is then used as the detection antibody, with its genomic DNA acting as the reporter DNA and amplified for detection. However, scFv has a lower binding affinity than its parent antibody, which limits the application of this method. Gold nanoparticles have also been used as cross-linkers for the detection antibody and DNA molecules.[48,49] This technique allows each gold nanoparticle to be conjugated with more DNA molecules than traditional methods (Figure 2. E), increasing the sensitivity by enlarging the "degree of conjugation". Improvements have also been made in readout techniques. Digital PCR (dPCR, Figure 2. H) is an approach that detects the absolute amount of target nucleic acids, offering higher sensitivity than traditional qPCR. dPCR eliminates the reliance on amplification efficiency for result readout, providing better reproducibility and tolerance to PCR inhibitors.[50−53] Next-generation sequencing (NGS, Figure 2. I) has become more affordable and is now a routine research tool. This technology transfers the traditional signal detection by fluorescent of qPCR to counting the fragments with specific sequences called barcodes, allowing for the simultaneous detection of thousands of targets in a single run. NGS has advantages over qPCR in terms of throughput, as it is not limited by the number of channels and dyes.[54,55] In terms of solid support for the assay, microspheres, particularly magnetic beads, have been introduced for coating antigens or capture antibodies.[56−58] These particles have a larger surface area than traditional microtiter plates, enabling the coating or capture of more molecules even at low physical concentrations.[57] Magnetic beads simplify washing and reduce incubation time as they can be easily suspended by constant agitation and separated using a magnet. The use of beads also improves specificity and multiplexing capabilities.[59,60] However, not all particles are compatible with PCR, requiring the release of DNA molecules from the immunocomplex before PCR.[56,61,62] Loss of beads during washing is also a concern, as fluctuations in bead numbers among wells can significantly affect the final results.[57] Careful operation and quantification of beads for calibration are essential to address these issues.

The most common application of immuno-PCR is the ultrasensitive detection of certain biomarkers whose concentrations may be low in samples.[63,64] In recent years, by combining various techniques such as microfluidic technique, immuno-PCR may also be used to detect a wider range of targets. One example is cellular indexing of transcriptomes and epitopes by sequencing (CITE-Seq),[65] which can simultaneously detect the proteomics and transcriptomics at a single-cell level. The DNA molecules conjugated to the antibodies are carefully designed to contain a barcode for identification, a poly(dA) mimicking mRNA and other elements required for PCR. Cells stained with barcoded antibodies are individually partitioned into a droplet with a magnetic bead whose surface

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is coated with oligonucleotides containing poly(dT) for capturing mRNA and the DNA-conjugated antibody. After cell lysis, the released mRNA and antibody are purified by the beads for library construction, followed by NGS. Due to differences in lengths, libraries constructed from mRNA and antibodies can be easily separated. As a result, single cell multiomics data can be collected. CITE-seq has been proven to be as accurate as flow cytometry, which is the gold standard for enumerating cell subsets by quantifying different cell surface markers.[65] In addition, immuno-PCR has been applied in the diagnosis of bacterial infections, offering significant advantages in detecting *Mycobacterium tuberculosis* and other pathogenic microbes.[19,66−68]

4. AMPLIFICATION BASED DETECTION USING PAIRED DNA-ANTIBODY CONJUGATES

Ideally, the negative controls should not generate any signal, but this is almost impossible in practice. The sensitivity of qPCR is good enough to detect as low as several copies. Therefore, any nonspecific binding of the conjugates, remaining excess DNA, or contamination will lead to background in the assay. On the other hand, the cross activity of the antibodies, especially under high multiplex conditions, can lead to a lot of false positive signals and restrict the number of targets to be detected in a single reaction.[69] One of the improvements to solve these problems is proximity ligation assay (PLA, Figure 3. A). In this assay, the target protein is recognized by two binding reagents, called probes, attached with DNA molecules. Originally protein binding DNA aptamers were used as the binding reagents, but antibodies are also compatible and quickly adopted.[30,70] The coincidence of probes binding to the target brings the two DNA molecules into proximity and can be joined by ligase with the help of a connector DNA. The newly formed ligation product is then amplified and quantified to measure the number of analytes. As the probe concentrations are low enough to minimize the formation of ligation product without the target protein, the background is significantly reduced, and the specificity increased. Unlike conventional immuno-PCR which requires a solid phase, the whole PLA reaction happens in the liquid phase without requiring a solid support, therefore eliminating the need for extensive washes. In addition, this difference also can reduce the volume required for reaction and sample to 5 μL and 1 μL, respectively.[30] However, a solid phase may still be helpful when the samples contain ligase or polymerase inhibitors, or when the analyte concentration is low and concentration is needed.[71]

To ensure the successful PLA, the DNA oligo used in the assay must be carefully designed to avoid any secondary structure that may prevent correct hybridization with the connector.[30] Additionally, it is important to ensure there is a hydroxyl group and phosphate group on the 3′ and 5′ end, respectively, so that the ligation reaction can occur, as synthesized DNA oligos may lack them by default setting.[72] Even though, components of biological fluid samples could interfere with the assay and reduce the recovery, especially DNA ligase.[73,74] Proximity extension assay (PEA, Figure 3. B) is derived from PLA, where the two probes are joined by DNA polymerase to create the final template for amplification, helping to mitigate this issue.[73] Enzymes are required for both PLA and PEA, which necessitate attention to storage and higher costs. One potential improvement is using hybridization chain reaction (HCR), which does not rely on enzymes for amplification.[73,75]

PLA and PEA have been used extensively in research to quantify cytokines, growth factors, and pathogens.[76−78] Like immuno-PCR, the antibody-dependent PLA approach is now being utilized in commercially available preconjugated kits or services offered by some companies. One of the main providers of these kits is Sigma-Aldrich which acquired the DuoLink PLA kits from Olink Bioscience in 2015. Since the acquisition, Olink Bioscience has shifted its focus to PEA and has successfully achieved proteomic-scale protein detection with a streamlined and automated platform.[79]

The performance of PLA is strongly correlated to the affinity of the two probes used. Therefore, PLA can be used to estimate the affinities of interactions between molecules such as protein−protein interactions (PPI).[30,80] Proteins rarely function in isolation; instead, they assemble into complexes and networks that oversee essential cellular functions, ranging from signal transduction to gene expression and metabolic pathways. PPIs within cells are critical as they support nearly all biological processes. Understanding PPIs provides insights into the mechanisms that regulate these processes, offering vital information to decode cellular signaling cascades and pathways. Over the past decade, numerous methods have been developed to detect cellular PPIs, with many relying heavily on gene constructs.[81] However, these methods cannot be applied to clinical samples. For such samples, PLA offers a novel approach for investigating PPIs even within cells. For example, Söderberg et al. developed an immuno-based rolling-circle amplification assay (RCA) to directly detect endogenous PPIs between Myc and Max oncogenic transcription factors after being stimulated by interferon-γ signaling and low molecular-weight inhibitors.[82] In this method, when DNA-antibody conjugates bind to two interacting proteins, the DNA tails guide connector DNA to form circular DNA strands. These DNA circles then serve as templates for localized rolling-circle amplification. The repeated sequences are labeled with fluorescent complementary DNA sequences, which enable visualizing and amplifying the signal in cells. The superiority of immuno-based RCA exhibits several advantages. It realizes the goal of detecting PPIs within cell pathways and this method can be applied to various samples, including clinical samples. Furthermore, RCA enhances signal amplification by increasing the number of fluorophores per detected protein interaction, allowing single events to be clearly visualized by microscopy as bright fluorescent spots or analyzed by flow cytometry, while minimizing the impact of nonspecifically bound fluorescent probes.[83] This technique has also been successfully used to detect the protein post-translational modifications *in situ*.[84]

Nucleic acid linked immuno-sandwich assay (NULISA, Figure 3. C) is one of the latest forms of immunoassay based on PLA.[85] What makes it unique is the reduction of background though two separations of the immunocomplex by magnetic beads. In this assay, each antibody is conjugated with a partially hybridized dsDNA. The long strands of the dsDNA on each antibody are mainly used for PLA, but the short strands, containing either a poly-A tail or biotin, are responsible for binding the magnetic beads so that the immunocomplex will not be removed with other unbound molecules during wash. Compared to the traditional PLA method, NULISA claims a 10,000-fold background reduction and the LOD at attomolar level. Although this technique has only been publicly available for a short time, it has already been

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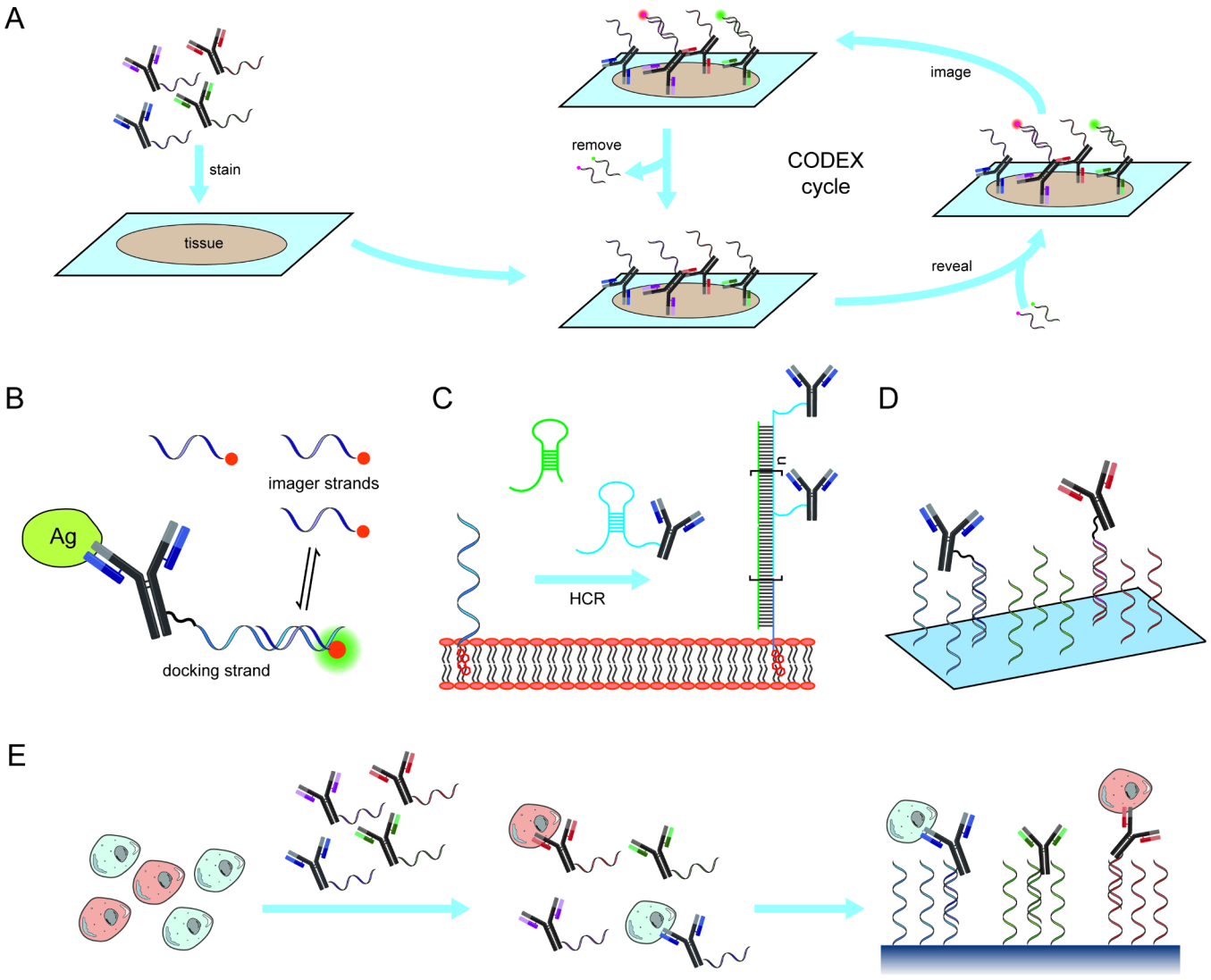

Figure 4. Nonamplification-based applications of DNA-antibody conjugates. (A) CODEX: Tissue samples are stained with DNA conjugated antibodies. To visualize the sample, a subset of complementary oligonucleotides with a fluorescent label is applied to the samples. After imaging, the reporter oligonucleotides are dissociated and removed. The cycle is repeated until all antibodies used have been revealed and imaged. (B) DNA-PAINT: After staining the sample with docking strand conjugated antibodies, fluorescently labeled imager strands are added to the sample. The transient binding of the imager strand generates a signal, which can be imaged. (C) Cholesterol modified DNA is embedded in the cell membrane and extended by HCR, allowing multiple DNA-antibody conjugates to be displayed. The displayed antibodies on the cell surface dramatically increase the ability to recognize other cells expressing the corresponding antigen. (D) DDI: DNA-antibody conjugates can be incubated with a DNA array containing the complementary strand of the conjugated DNA so that the antibodies can be immobilized through hybridization. (E) DEAL for cell sorting: Cell surface markers are recognized by DNA-antibody conjugates. The cell resuspension is then incubated with a DNA array containing the complementary strand of the conjugated DNA. The cells with different markers are separated.

used in some disease research and could be potent in the future.[86,87]

5. NONAMPLIFICATION-BASED APPLICATIONS OF DNA-ANTIBODY CONJUGATES

Cell membrane proteins and endogenous proteins are associated with a variety of biological functions, including cell differentiation, cell signaling, and nutrient transportation. Therefore, these proteins are highly appealing as targets for biological, diagnostic and therapeutic applications. Currently, antibodies are the top choice for targeting proteins due to their exceptional specificity and wide commercial availability for numerous targets. DNA nanotechnology's precision has introduced a robust method for adding unique, programmable labels to antibodies, enabling the detection of cell proteins through various fluorescence-based or PCR-based techniques. For example, Goltsev et al. developed a novel technology for multiplexed tissue imaging called Co-detection by indexing (CODEX, Figure 4. A).[88] In this approach, multiple DNA-antibody conjugates were utilized to bind numerous cellular proteins simultaneously within a single tissue section. Each antibody was labeled with a unique DNA barcode, facilitating its identification through hybridization with complementary DNA sequences labeled with fluorophores. Following visualization, these fluorophore-labeled sequences were subsequently removed using chemical treatments. This iterative process enables the visualization of a vast number of proteins in a single sample. CODEX facilitates single-step antibody staining and cyclic detection of fluorophore-labeled DNA probes for up to 60 markers in a single tissue section, reducing overall

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Table 3. Selected Clinical Applications of Antibody-DNA Conjugation in Recent Years

Method/platform	Disease/application	Main finding/conclusion	Year [ref]
PEA	Aging	Plasma proteomics can be used to measure biological aging and involved in age-related diseases.	2024[103]
Immuno-PCR	Alzheimer's disease	Immunomagnetic exosomal PCR can be used to quantify the levels of exosomal biomarkers related to Alzheimer's disease.	2024[104]
NULISA	CAR T-cell therapy	New proteins and functional pathways associated with CAR T-cell-induced toxicity were identified.	2024[105]
Catalytic hairpin self-assembly	Laryngeal carcinoma	CYFRA21−1 expression level can be used for early diagnosis of laryngeal carcinoma.	2023[106]
Immuno-PCR	Tuberculosis	Immuno-PCR can be used for diagnosis of extrapulmonary tuberculosis.	2023[107]
dPCR	Cancer diagnosis	Multisubpopulation extracellular vesicles counting through surface proteins profiling is a way to diagnose breast cancer.	2023[108]
DNA hybridization	Drug delivery	DNA conjugated drugs can be delivered through DNA conjugated antibody	2020[109]
Immunostaining	Cancer imaging	DNA-dot conjugated antibody is a toxic-free, swift and efficiency fluorescent imaging tool for cancer cell detection.	2020[110]

procedure time and overcoming spectral overlap limitations.[89] CODEX and similar techniques including immunostaining with signal amplification by exchange reaction (immuno-SABER), DNA-Exchange Image (DEI), and CosMx Protein Assay (Nanostring) are highly multiplexed immunostaining methods enabling *in situ* imaging of a given sample and are applied in spatial mono-omics and multiomics studies.[90−93]

Observing cellular structures is crucial for understanding the spatial organization and functional dynamics of cellular components. It is essential for deciphering how cells operate and communicate. Super-resolution imaging allows for the visualization of cellular structures on a nanoscale level. DNA point accumulation in nanoscale topology (DNA-PAINT, Figure 4. B) technology is a sophisticated super-resolution imaging approach that utilizes temporary interactions between short, matching DNA sequences to produce highly resolution images. Jungmann et al. pioneered the use of DNA-PAINT for imaging cellular structures, including microtubules, mitochondria, Golgi and peroxisomes, based on DNA-antibody conjugates.[94] In this method, antibodies specific to target proteins are conjugated with short DNA sequences, known as docking strands. Fluorescently labeled complementary DNA strands, called imager strands, transiently bind to these docking strands, producing a fluorescence signal that can be detected and localized with nanometer precision. By cycling the binding and unbinding of imager strands, DNA-PAINT generates high-resolution images, allowing researchers to observe the spatial organization within their native cellular environments with exceptional specificity and detail.[95,96]

Cell-cell interactions are essential for coordinating biological processes and maintaining tissue integrity in multicellular organisms. They enable communication between cells, which is crucial for functions such as immune therapy.[97] Antibodies can specifically recognize targets on cell surfaces, while DNA can be modified with lipid residues to enable display on cell membranes through lipid insertion. Combining these components to create DNA-antibody conjugates offers a promising approach to enhance cell−cell interactions (Figure 4. C). For example, Wang et al. displayed multiple antibodies on the cell surface for enhanced cell recognition.[98] The initiator DNA sequence was modified with cholesterol. After being inserted into the natural killer cell membrane, it triggered the formation of polyvalent antibody-DNA polymer through the hybridization of two DNA-antibody conjugates. The displayed antibody on the cell surface dramatically increased the ability of natural killer cells to recognize and kill cancer cells. This method is flexible and versatile as it is independent of cell types and antibody types.

As mentioned earlier, the DNA conjugated to antibodies can be used to dock molecules containing complementary sequences. There are several applications that take advantage of this property. One example is the fabrication of antibody arrays, which are robust tools for detecting proteins with small sample requirements, high sensitivity and high throughput.[99] However, printing antibody arrays is usually problematic because each antibody used must be individually stored to avoid cross contamination. Besides, antibody arrays suffer from loss in protein structure and function regardless of how they are printed.[100] In contrast, DNA arrays do not have such stability issues and have higher efficiency in immobilization. Niemeyer et al. explored the possibility of using DNA-antibody conjugates to transform a DNA array into a protein array through DNA-directed immobilization (DDI, Figure 4. D).[101] This process can be completed under mild conditions and in a short amount of time, maximizing the activity of antibodies. Due to the specificity of DNA hybridization, this technique allows for the immobilization of multiple antibodies simultaneously, which can be stored in a mix.[33] In addition, other advantages of DNA arrays such as smaller spot size and higher spot density may also be transferred to antibody arrays. DNA-antibody conjugates can also be utilized for cell sorting. Cell sorting allows researchers to isolate and study specific cell populations based on their characteristics, enabling detailed analysis of cell function, behavior, and interactions in biological research and clinical applications. Therefore, a simple and rapid platform for cell sorting is necessary. Bailey et al. developed DNA-encoded antibody libraries (DEAL, Figure 4. E) for sorting cells. DNA-antibody conjugates were mixed with a cell mixture, allowing specific cells to be labeled with these conjugates.[102] The DNA arrays were modified with complementary DNA (cDNA) corresponding to the conjugates, enabling the capture of cells when introduced into the cell-DNA-antibody conjugate complex on the spotted DNA array.

6. CONCLUSION AND PERSPECTIVES

A DNA-antibody conjugate combines the unique functions of both antibodies and DNA in a single molecule, allowing for a wide range of applications. These applications range from ultrasensitive protein measurements based on PCR to the spatial detection of biomolecules. In recent years, DNA-antibody conjugates have also been widely used for clinical diagnosis and therapy (Table 3). The versatility of immunoassays opens up the possibility for further expansion of DNA-

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antibody conjugate applications in the future. Various DNA-antibody conjugation kits are now available, providing researchers with multiple options for constructing DNA-antibody conjugates. Additionally, many companies offer services for creating purified DNA-antibody conjugates, making it easier for researchers to obtain them. The accessibility of related techniques such as NGS, dPCR, and super-resolution microscopy is increasing, leading to a higher demand for the use of DNA-antibody conjugates. Overall, DNA-antibody conjugates will continue to play a crucial role in biological studies, diagnostics, and therapies.

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Notes

The authors declare the following competing financial interest(s): We certify that some authors (Tao Wang, Xuelin Wang, Peng Zhang, Na Li, Shuhong Luo, Ruo-Pan Huang) are employees of Raybiotech and have a financial stake in the company. All other authors have no conflicts of interest.

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