JACS Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

pubs.acs.org/jacsau
Perspective

Nanobiosensors Enable High-Efficiency Detection of Tuberculosis Nucleic Acid

Published as part of JACS Au special issue "DNA Nanotechnology for Optoelectronics and Biomedicine".

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Cite This: *JACS Au* 2025, 5, 536−549
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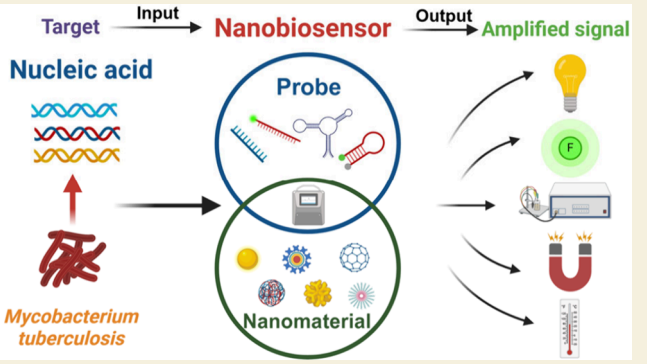
ABSTRACT: Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*), with a complex pathogenesis that poses a long-term threat to human health globally. Early and accurate diagnosis of TB provides a critical window for timely and effective treatment. The development of nucleic acid testing (NAT) based on polymerase chain reaction (PCR) has greatly improved the diagnostic efficiency of TB. However, balancing detection accuracy, efficiency, and cost in TB NAT remains challenging. Functionalized nanomaterials-based nanobiosensors have demonstrated exceptional performance in detecting TB nucleic acid by integrating their unique physicochemical properties with diverse biological probes that exploit Mtb characteristics to effectively amplify biological signals. Compared to traditional NAT, nanobiosensors simplify nucleic acid detection, improve accuracy, and reduce reliance on external conditions, thereby contributing to more immediate and accurate TB diagnosis. In this perspective, we provide a comprehensive summary and discussion on current strategies for detecting *Mtb* biomarkers using nucleic acid along with novel solutions for TB diagnosis. Additionally, we explore the advantages and challenges associated with applying nanotechnology to the clinical management of TB, particularly point-of-care testing (POCT).



KEYWORDS: *Nanobiosensors, tuberculosis, nucleic acid, diagnosis, DNA nanotechnology*

1. INTRODUCTION

Tuberculosis (TB) is a complex public health issue that affects people around the world and has once again become the leading cause of death among infectious diseases. It is a respiratory infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*) and is primarily transmitted through the air. It is estimated that over 10 million new TB cases occur globally each year, with 10.8 million cases reported in 2023. However, only 8.2 million of these cases were diagnosed, leaving approximately 25% of patients without a definitive diagnosis or unrecorded.[1,2] The primary methods for detecting *Mtb* in clinical laboratories, such as acid-fast bacilli (AFB) smear microscopy, *Mtb* culture, *Mtb* nucleic acid testing (NAT), specific immune-based diagnosis and *Mtb* antigen detection,[3] play a crucial role in the prevention and treatment of TB. Despite their importance, these methods still face numerous challenges in accurately diagnosing TB. First, a low specimen positivity rate poses a significant challenge. *Mtb* grows in clusters and distributes unevenly within lesions, leading to difficultly predict the likelihood of obtaining pathogens with each sample taken. Furthermore, nontuberculous mycobacteria (NTM) share similarities with *Mtb* in smear staining morphology and antigenic characteristics, which can easily lead to misdiagnosis. Thus, the pass rate for sputum specimens is often unreliable, and they can easily be contaminated by saliva. Second, highly contagious and pathogenic *Mtb* necessitates a higher biosafe level in clinical laboratories, preventing many local hospitals from implementing detection technologies such as *Mtb* culture and smear microscopy.[4,5] They have traditionally been considered the gold standards for obtaining direct pathogenic evidence, culture requires more than 3 weeks of testing time, making it challenging to provide timely information for clinical treatment. Smear microscopy often struggles to yield effective positive results due to difficulties in ensuring specimen quality and a low positive rate.

Currently, NAT has become an essential method for TB disease.[6] The clinical detection of TB nucleic acid markers

Received: December 10, 2024
Revised: February 2, 2025
Accepted: February 4, 2025
Published: February 12, 2025

primarily relies on nucleic acid amplification tests (NAATs).[7−9] These techniques based on the principle of complementary base pairing ensure the specificity in detection. By employing the central dogma of molecular biology, NAT enables extremely small amounts of target signals to multiply exponentially, thereby enhancing sensitivity.[10] The WHO has recommended NAATs as a replacement for traditional microscopy, as they can simultaneously assist in TB diagnosis and drug resistance identification. Polymerase chain reaction (PCR), as the most common amplification strategy, exhibits excellent sensitivity. Currently, multiple advanced NAATs are employed for *Mtb* nucleic acid detection like Xpert MTB/RIF assay.[11−13] The Xpert MTB/RIF technology permits the simultaneous detection of pathogens and drug resistance by amplifying and detecting the *Mtb*-specific region of the rifampicin resistance-related gene *rpoB*. In Xpert MTB/RIF, the amplification method design employs a separate ink cartridge, three specific primers, and five molecular probes, effectively preventing contamination interference and augmenting the specificity (98%) and sensitivity (86%) of *Mtb* detection.[14−16] Despite its impressive detection capabilities, Xpert technology exhibits suboptimal sensitivity in extrapulmonary TB detection. Furthermore, its need for expensive instruments and fully equipped laboratories limits its availability in many primary hospitals. Both PCR and Xpert MTB/RIF rely on complex temperature control systems and long turnaround time which limit their effectiveness for rapid clinical diagnosis.[14,17] Conversely, loop-mediated isothermal amplification (LAMP) technology can be executed under constant temperature conditions and does not necessitate complex instrumentation or laboratories. LAMP technology has 4 specific primers designed for 6 regions of the target gene, and under the action of strand replacement DNA polymerase (*Bst* DNA polymerase), isothermal amplification can be performed in the temperature range of 60−65 °C.[18] Accordingly, it can function as a portable and instantaneous detection method. However, LAMP's sensitivity is lower than that of Xpert, implying it may overlook certain cases.[19] In addition, two isothermal amplification technologies, recombinase polymerase amplification (RPA) and rolling circle amplification (RCA), have also been applied to TB NAT. RPA uses the protein−DNA complex formed by the binding of recombinase and primers, and searches for homologous sequences in double-stranded DNA to mediate the formation of a chain exchange reaction and initiate DNA synthesis, and exponentially amplify the target area on the template. RPA can obtain detectable levels of amplification products within 10 min at 37 °C, realizing rapid detection of target nucleic acids. At present, there have been studies using nucleic acids generated by RPA for lateral flow detection, which has good specificity (93.3%) and a low detection limit (5 fg/μL), but poor sensitivity (53.2%).[20−22] RCA uses circular DNA as a template and generates single-stranded DNA under the catalysis of polymerase through a short DNA primer. This single-stranded DNA contains hundreds or thousands of repeated template complementary fragments. Chen et al. applied RCA to the mutation detection of TB resistance gene *rpoB*, with a sensitivity and specificity of 96.6% and 89.5%, respectively.[23,24] Although current NAATs have shown impressive results, further optimization is needed to balance their detection performance with portability, cost, and accessibility.[3]

Nanobiosensors, nanomaterials-based biosensors, are widely used in TB diagnosis and are a key application of nanotechnology over the past decades.[25] A biosensor usually consists of two parts: the biorecognition part, which binds and enriches target substances to generate biological signals, and the signal transduction part, which converts biological signals into other forms of signals, such as optical or electrical signals. Nanomaterials have exceptional optical, electrical, and mechanical properties that make them highly effective in amplifying and converting biomolecular signals within biosensors.[26] The high specific surface area of nanomaterials allows for greater loading densities of sensor recognition components, such as antibodies, enzymes, and aptamers. Nanomaterials also have high surface activity, including enzymatic activity, which facilitates various chemical reactions.[27−29] In addition, nanobiosensors exhibit quantum size effects and quantum tunneling effects, which further enhance their signal amplification capabilities compared to general sensors, enabling highly sensitive biomolecular detection.[30−36] The quantum size effect refers to the phenomenon where, as the size of a particle decreases to a certain threshold, the electron energy levels near the Fermi level transition from quasi-continuous to discrete. The quantum tunneling effect describes the scenario in which a microscopic particle, despite having energy lower than a high-energy barrier, can probabilistically penetrate the barrier and access the area beyond it. In quantum sensing, both of these effects are harnessed to create highly sensitive sensors capable of detecting specific nanoparticles or tunneling currents. Compared to the clinical detection of TB nucleic acids, nanobiosensors offer several advantages. First, most nanobiosensors operate effectively at room temperature and can achieve rapid nucleic acid detection in under 1 h, making them more cost-effective with shorter turnaround times. Additionally, nanobiosensors leverage the efficient signal conversion properties of nanomaterials to directly provide high-sensitivity detection of the target. This simplification of reaction steps reduces the risk of misdiagnosis and missed diagnoses. Moreover, nanobiosensors are more user-friendly, converting biological signals into various output modes. They also require less specialized experience and qualifications from detection personnel, enhancing their overall versatility. Nanobiosensor has advanced point-of-care testing (POCT) in medical diagnostics. POCT aims to develop simple, rapid, low-cost, and efficient disease detection technologies, making it easier to diagnose diseases in remote and developing areas. This is particularly important for TB diagnosis and screening. Many TB patients reside in economically underdeveloped or remote regions, such as mountainous and pastoral areas, where hospitals often lack the necessary resources to equip advanced laboratories, such as molecular diagnostic laboratories, or to utilize complex instruments. In contrast, POCT employs portable instruments and straightforward operating procedures to enable rapid TB detection. This approach facilitates quick and cost-effective screening of potential patients in high-risk areas, aiding in patient triage and further diagnosis and treatment. Moreover, the low environmental requirements of POCT allow clinicians to conduct tests directly in patient wards, minimizing time loss and the risk of sample contamination associated with sample transport. This capability is particularly beneficial for doctors who need to adjust treatment strategies promptly in urgent situations. Nanobiosensors, known for their high sensitivity and user-friendliness, align well with the principles of point-of-care testing. By integrating nanobiosensors with advanced technologies such as mechanical engineering, biochip technology, microfluidics, and smart devices, numerous point-of-care detection strategies with potential clinical applications

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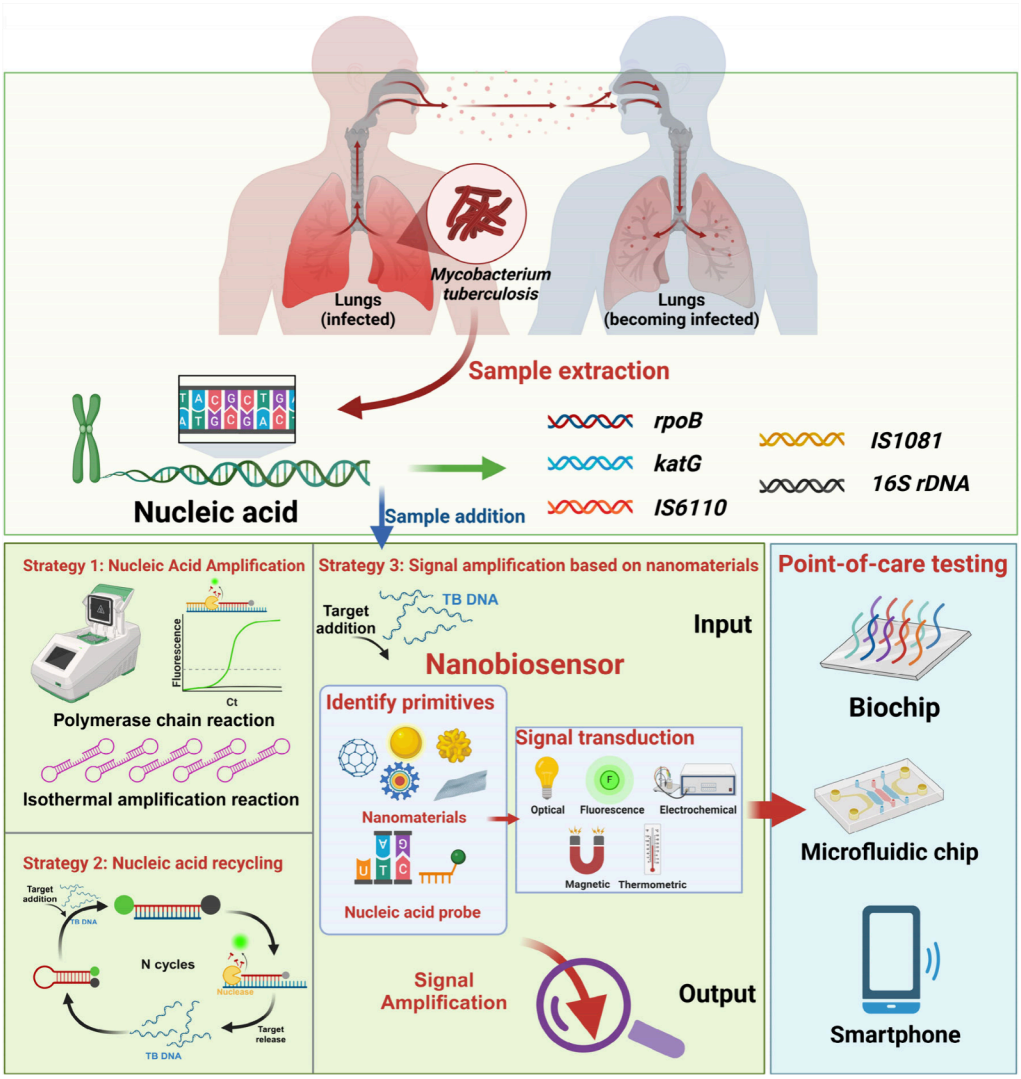

Figure 1. Nanotechnology constructs TB NAT strategies and nanobiosensors for TB POCT. Created with BioRender.com.

have been developed, thereby advancing medical diagnostics.[37−39]

Improving the sensitivity of clinical sample detection remains a major challenge in the development of NAT. This challenge involves two aspects: (1) Capturing and hybridizing minimal amounts of target nucleic acids with nucleic acid probes. (2) Converting and identifying the signal released after the probe binds to the target nucleic acid. Zuo et al. summarized four current strategies to enhance NAT's sensitivity: nucleic acid amplification, nucleic acid recycling, signal amplification, and nucleic acid sequencing.[40−42] These four strategies cover the design of most nucleic acid biosensors. In addition to sequencing, the other three strategies can be flexibly connected to build a variety of rapid nucleic acid detection devices. Among them, nanotechnology is mainly used to achieve effective signal conversion and amplification. In fact, strategies based on nucleic acid amplification, nucleic acid recycling, and nanotechnology signal amplification complement each other and jointly improve the sensitivity of NAT. These three strategies focus on different aspects. Amplification can exponentially increase the amount of low-abundance target nucleic acids. Nucleic acid recycling aims to release signals multiple times from the same nucleic acid. Nanotechnology focuses on enhancing the performance of biosensors, including the identification of target nucleic acids and signal conversion and amplification. The first two strategies amplify the signal of the nucleic acid itself, while the third strategy amplifies the existing nucleic acid signal using other signals. Therefore, these three strategies are responsible for different stages of NAT and can be combined in various ways to achieve an overall improvement in detection sensitivity. Nanobiosensors provide many effective solutions to solve the current TB diagnostic problems and achieve highly efficient detection of nucleic acid biomarkers.[25,43,44] Researchers are leveraging different strategies to complement current clinical approaches. They developed nanobiosensors based on different transduction principles to detect *Mtb* nucleic acids with high sensitivity.[3,30] In this perspective, we summarize the strategies for TB nucleic acid detection using nanotechnology (Figure 1). We analyze potential solutions that integrate nanotechnology theory to address the challenges faced in TB clinical management. Furthermore, we explore the clinical application potential of these research findings. By integrating nanotechnology into TB NAT, we can not only introduce new ideas and designs but also foster innovation and development of traditional clinical techniques which shows the value of interdisciplinary research.

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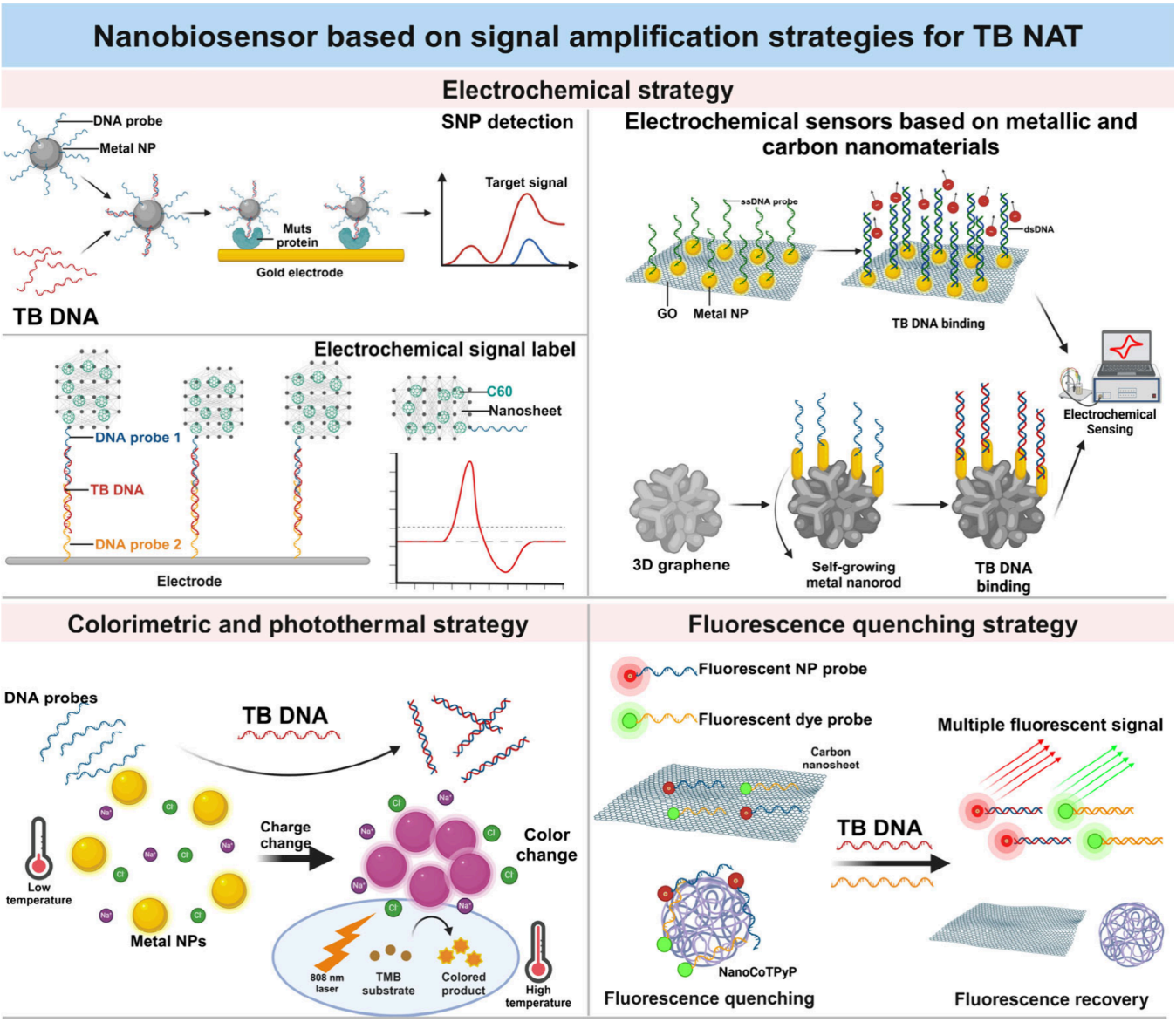

Figure 2. Nanobiosensors based on signal amplification strategy for TB NAT. Created with BioRender.com.

2. APPLICATION OF NANOTECHNOLOGY IN SAMPLE PREPARATION FOR TB DETECTION

The low concentration and uneven distribution of *Mtb* in body fluids, including sputum, cerebrospinal fluid, and urine, make the positive detection rate highly dependent on sample quality. Additionally, obtaining sputum specimens can be challenging for children, elderly patients, and individuals with HIV. The concentration of bacteria in urine and cerebrospinal fluid specimens is very low, and current detection methods often lack the sensitivity needed to differentiate them. Consequently, incorporating additional concentration or purification steps can enhance the concentration of *Mtb* in the sample and effectively enrich various markers, thus reaching the detection limit of existing technologies. Since the implementation of new technologies typically requires extensive research, development, and verification—along with significant costs—many clinical laboratories prefer to use optimized sample pretreatment methods. Paris et al. screened a copper complex active dye, RB221, which can effectively identify the *Mtb*-specific antigen LAM in samples, facilitating the enrichment of the pathogen.[45]

Similarly, some *Mtb* affinity materials can adsorb and enrich free bacteria. Antibodies and aptamers serve as more suitable recognition elements that bind to *Mtb* with greater specificity. Nanoparticles, due to their high specific surface area, can carry more recognition elements. Magnetic particles are easier to manipulate and are the most widely used materials for enrichment and purification. Similar to immunoprecipitation, these antibody- or aptamer-functionalized nanoparticles can adsorb *Mtb* and separate them from other liquid components for further elution and identification. The eluted and purified samples can be used for marker detection, microscopy, and imaging. Currently, magnetic bead-based purification methods are widely employed to enrich various nucleic acid substances and proteins. A feasible strategy involves using multiple magnetic beads that can bind to different sites to jointly adsorb and enrich target bacteria. Xue et al. utilized Fc-fused mannose-binding lectin (MBL)-coated Fe_3O_4 (Fc-MBL@Fe_3O_4) and human IgG-coated Fe_3O_4 (IgG@Fe_3O_4) to create comagnetic beads for the identification and enrichment of both G^+ and G^- bacteria.[46] Mannose also exists on the surface of *Mtb* and can be

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JACS Au 2025, 5, 536−549

recognized and bound by MBL, suggesting that it can theoretically be enriched using this comagnetic bead system. In addition to the aforementioned marker enrichment method, membrane-based filtration is another simple and effective approach. These membranes feature specific-sized pores that can remove other components in the sample while retaining *Mtb*. By properly digesting and lysing the specimen, interference from larger substances and cells can be minimized. The *Mtb* filtered on the membrane can then be eluted and enriched for further detection and analysis.[47]

3. NANOBIOSENSORS BASED ON SIGNAL AMPLIFICATION STRATEGY

Signal amplification strategies are the primary focus of nanotechnology applied to NAT. Nanomaterials' unique properties, such as conductivity, luminescence, enzyme activity, and magnetism, are utilized to convert and amplify trace amounts of nucleic acid signals into other detectable signals like fluorescence, visible light, current, or temperature.[48] Consequently, the use of nanotechnology to construct *Mtb* nucleic acid detection biosensors has become a prominent area of research in TB diagnostic methods. This part delves into various strategies for developing *Mtb* nucleic acid sensors using nanotechnology (Figure 2).

3.1. Electrochemical Biosensors for TB NAT

Electrochemical (EC) biosensors play a significant role in applying nanotechnology to TB NAT. These biosensors detect biological signals by identifying target biological substances as electrons or ions. Through integration design, EC biosensors can easily be transformed into POCT for instant clinical diagnosis.[37,49] In terms of NAT, since the nucleic acid itself does not generate electron transfer in the sensor, affinity recognition, using nucleic acid probes, is needed to generate electrical signals. Electroactive labels, particularly nanomaterials, are key to this type of nucleic acid EC biosensors. Nanomaterials enhance the performance of EC biosensors due to their higher specific surface area, conductivity, and stability. The high specific surface area of nanomaterials enables them to load more nucleic acid probes, capture more target nucleic acids, and generate stronger signals. Additionally, the higher specific surface area provides more reaction space for various electroactive substances. The combination of a high loading rate and high conductivity enhances the charge transfer capability of the sensor, resulting in improved detection sensitivity.[41,50] The primary nanomaterials used for the electrochemical detection of *Mtb* nucleic acids are gold nanoparticles (AuNPs), carbon-based nanomaterials (e.g., graphene), and polypyrrole materials. Chen et al. utilized AuNPs to design an EC sensor for detecting the *Mtb IS6110* sequence. In this system, when the target DNA, specific probes, and AuNPs are present simultaneously, the hybridized DNA strands can be adsorbed to the AuNPs at an extremely high density. Moreover, a specially modified electrode incorporating self-assembled monolayers (SAMs) and MutS protein was designed to specifically adsorb AuNPs that bind to target nucleic acids, generating electrical signals. The MutS protein recognizes base mismatches and can bind to DNA double strands, ensuring high selectivity of detection through specific probes. The system leverages the slight miscibility of butanol and water to further concentrate all detection components, amplifying the nucleic acid signal for more sensitive detection. This nucleic acid EC biosensor has a limit of detection (LOD) as low as 1.8×10^{-11} μM and has successfully detected *Mtb* single nucleotide polymorphisms (SNPs) in synovial fluid.[51] Many researches were conducted on combining AuNPs with graphene materials to create efficient tuberculosis DNA detectors.[52] The electrode is constructed using the good film-forming ability of graphene materials and the abundance of group-bonded AuNPs. AuNPs play a key role in loading capture probes effectively and converting biological signals into electrical signals after nucleic acid hybridization. By further reducing GO and removing the oxidized functional groups, reduced graphene oxide (RGO) can be synthesized. RGO also exhibits good conductivity and has more defects, resulting in better electrocatalytic activity and more electroactive sites. Mogha et al. developed a sensing electrode by fixing AuNPs on RGO nanoribbons (RGONRs). They covalently modified single-stranded DNA (ssDNA) onto AuNPs to enable specific recognition of *Mtb* DNA. When ssDNA and the target nucleic acid bind to the electrode, the negatively charged DNA phosphate backbone in the electrolyte repels $[Fe(CN)_6]^{3-/4-}$ in the solution, leading to a decrease in electron mobility. As the concentration of target nucleic acid fragments increases, the rate of electron transfer continues to decrease. The LOD for this detection method can be as low as 1×10^{-9} μM.[53,54] In addition to constructing solid-phase electrodes, the combination of graphene materials and AuNPs can also be directly utilized as electrical signal labels to achieve signal conversion and amplification. Bai et al. combined fullerene (C_{60}), nitrogen-doped graphene nanosheets (NGS), and AuNPs to create a nanocomposite (Au-nano-C_{60}/NGS) that serves as an electrical signal label for signal amplification. This nanocomposite exhibits excellent electrical conductivity and redox activity. After the target nucleic acid is recognized and captured by an additional solid-phase probe, the biological signal is converted into an electrical signal through binding to Au-nano-C60/NGS. The LOD of this sensor is 3×10^{-9} μM, and the linear range is $1 \times 10^{-8} \sim 1 \times 10^{-1}$ μM.[55] Constructing three-dimensional porous graphene materials represents a potential breakthrough in improving the specific surface area and electrocatalytic activity. Perumal et al. anchored AuNPs on 3D graphene, instead of sheet-like graphene materials, to fabricate an electrochemical sensor for detecting the *Mtb IS6110* gene. The unique spatial structure of 3D graphene enables the self-assembly of AuNPs into gold nanorods *in situ*, resulting in a large active surface area and high charge transfer performance. The porous spatial structure of this nanocomposite, combined with the characteristics of the nanogold material itself, allows for the covalent adsorption of more DNA probes and exhibits strong electrocatalytic activity, thus enabling highly sensitive nucleic acid detection. The LOD of this detector is 1×10^{-8} μM, and the detection linear range is $1 \times 10^{-8} \sim 1 \times 10^{-1}$ μM.[56] Polypyrrole (PPy) is a conductive polymer (CP) widely used in electrochemical biosensors. It has electrical properties comparable to inorganic semiconductors and metals, making it an excellent electron transfer medium for biosensors.[57,58] PPy offers advantages such as low cost, good biocompatibility, high conductivity, and stability. Thin films of PPy can be synthesized through simple electrochemical or chemical methods. Scientists have explored various modifications of PPy to develop sensors for *Mtb* DNA detection. Haddaoui et al. created a film by combining PPy with magnetic Fe_3O_4 nanoparticles and functionalizing it with polyamidoamine (PAMAM) dendrimers. Naphthoquinone (spaNQ), a redox center, was covalently bound to PAMAM along with DNA probes to identify target nucleic acids and generate charge transfer. This sensor successfully detected the *rpoB* gene and aided in determining

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JACS Au 2025, 5, 536−549

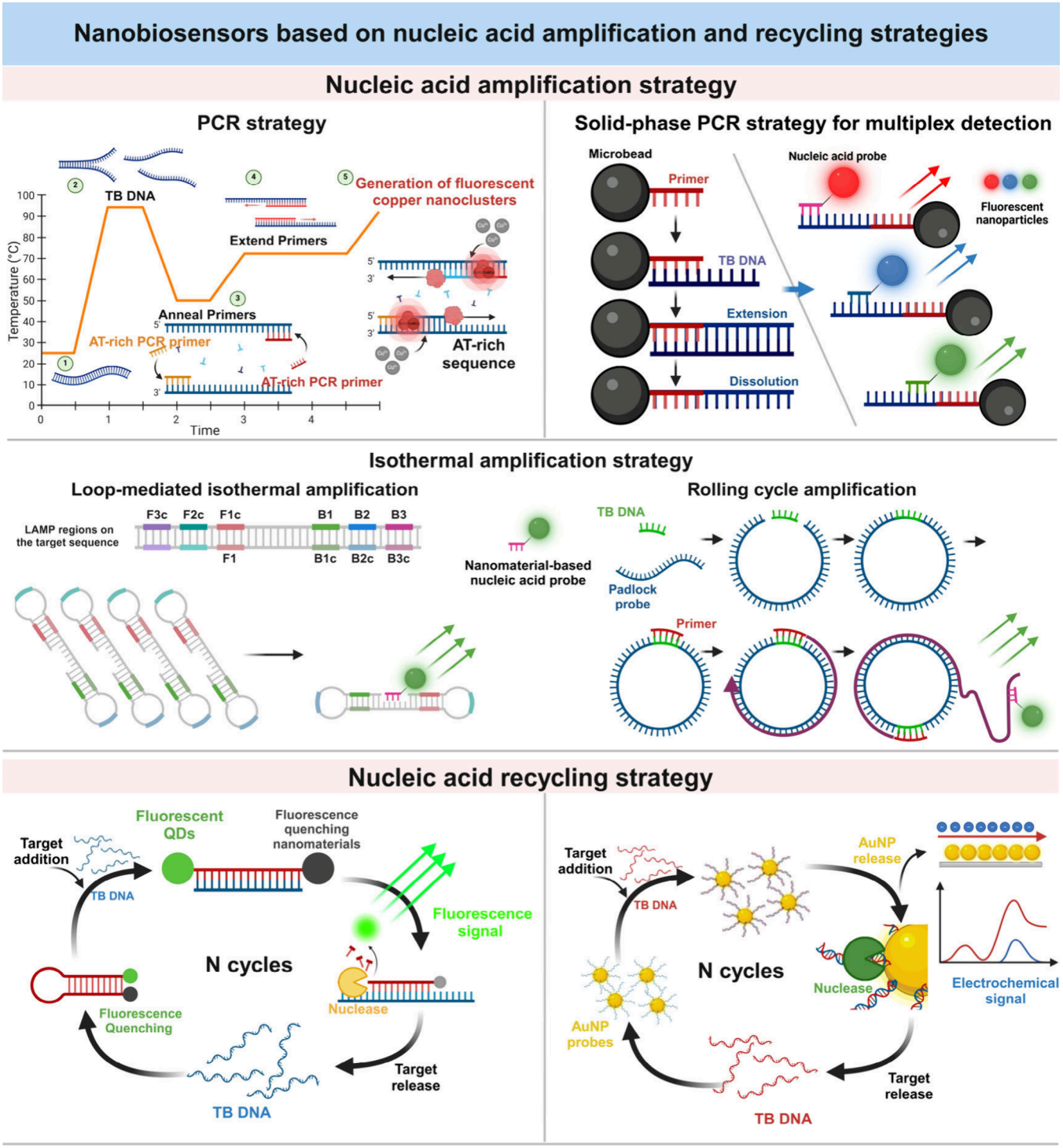

Figure 3. Nanobiosensors based on nucleic acid amplification and recycling strategy for TB NAT. Created with BioRender.com.

the presence of rifampicin resistance, with a LOD of 1×10^{-9} μM.[59] Similarly, Khoder et al. used polypyrrole nanowires (nw-PPy) with a high specific surface area. They did not employ additional nanomaterials to modify PPy and used ferrocene groups as redox reporter groups. This sensor also demonstrated utility in *rpoB* gene detection.[60−62]

3.2. Colorimetric and Photothermal Nanobiosensors for TB NAT

In addition to being an excellent conductive material for constructing EC biosensors, AuNPs possesses special properties that can also be utilized in the design of other types of sensors. One example is the ability of AuNPs to transition from a dispersed state to an aggregated state when the DNA probe hybridizes with the target nucleic acid in the system, resulting in a change in charge. This change in the properties of AuNPs, such as color and photothermal effects, can be used to design colorimetric and photothermal sensors for NAT.[63] An example of this is the work by Tsai et al., who constructed a *Mtb* nucleic acid colorimetric sensor. In this sensor, the binding of the ssDNA probe to the target double-stranded DNA (*IS6110* gene)

https://doi.org/10.1021/jacsau.4c01206
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changes the surface charge density of the AuNPs, causing them to aggregate. This aggregation process leads to a red shift in the plasmon resonance, causing the color of the suspension to change from wine-red to blue, which can be detected using a spectrophotometer. This colorimetric sensor has a LOD of 4.8×10^{-7} μM and a turnaround time of 60 min.[64] Another approach is the use of the photothermal effect of AuNPs aggregation to construct a *Mtb* nucleic acid photothermal sensor. Zhou et al. achieved this by irradiating dispersed and aggregated AuNPs with an 808 nm laser, resulting in different temperature changes that can distinguish the presence or absence of the target nucleic acid. This analytical method is simple, requiring only a thermometer for detection, but it exhibits high sensitivity with a LOD of 2.8×10^{-4} μM and a turnaround time of 40 min.[65] The team also utilized the chemical catalytic activity of AuNPs to design another photothermal sensor for the detection of Mtb DNA. By forming a sandwich structure of a capture probe, target nucleic acid, and AuNPs-labeled detection probe, the AuNPs catalyze the oxidation reaction of 3,3′,5,5′-tetramethylbenzidine (TMB) and generate a temperature signal under 808 nm laser irradiation. This sensor also requires only a thermometer to determine the results and is portable and low-cost. It has a LOD of 3.9×10^{-2} μM and a turnaround time of 2 h.[66]

3.3. Nanobiosensors Based on Fluorescence Quenching Mechanism for TB NAT

Biosensors that utilize the fluorescence quenching mechanism of nanomaterials have been widely employed for detecting markers of various diseases. This approach involves synthesizing selective fluorescent probes and choosing appropriate fluorescence quenching materials for different detection purposes. It is a straightforward, convenient, and cost-effective strategy.[67] The detection process typically involves the following steps: (1) the nanomaterial absorbs and suppresses fluorescence of the probe, and (2) the probe binds to the target, detaches from the nanomaterial, and restores fluorescence.[68,69] Some studies have explored the application of this type of fluorescence quenching sensor for *Mtb* nucleic acid detection. Liang et al. utilized CdTe QDs-labeled dsDNA as a fluorescent probe and a new two-dimensional metal−organic framework (Cu-TCPP) as a fluorescence quenching material to create an *Mtb* DNA sensor for detecting the *IS6110* gene. In the absence of target nucleic acid, the fluorescent probe binds to Cu-TCPP through $\pi-\pi$ stacking interactions, resulting in quenched fluorescence via fluorescence resonance energy transfer (FRET). When the fluorescent probe hybridizes with the target nucleic acid, forming a DNA double strand, the probe dissociates from Cu-TCPP, leading to restored fluorescence. The selective quenching ability of Cu-TCPP for nucleic acid probes effectively reduces the "false positive" effect caused by background interference. The LOD of this fluorescence quenching sensor is 3.5×10^{-5} μM, with a detection linear range of $5 \times 10^{-5} \sim 1 \times 10^{-3}$ μM, and the detection process can be completed within 50 min.[70] Fluorescence quenching sensors can also be employed for multiplex detection of *Mtb* nucleic acids by designing different fluorescent probes. Chen et al. designed and employed two fluorescent probes simultaneously to detect the *rpoB* gene (associated with rifampicin resistance) and the *katG* gene (associated with isoniazid resistance). In this sensor, nano-CoTPyP serves as the fluorescence quenching material, while two different fluorescent QDs act as fluorescent labels. The fluorescent probes bind to nanoCoTPyP through various interactions, including electrostatic interactions, $\pi-\pi$ stacking, and hydrogen bonds, resulting in fluorescence quenching via FRET and photoinduced electron transfer (PET). The specific detection process is similar to the aforementioned study. The results of this multiple test provide comprehensive information for determining *Mtb* drug resistance. Based on the different outcomes, drug resistance can be classified as nonresistant, only isoniazid-resistant, or multidrug-resistant. In turn, this facilitates prompt identification of drug resistance and aids clinical decision-making. The LODs of this sensor are 2.4×10^{-5} μM (*rpoB*) and 2.0×10^{-5} μM (*katG*).[71]

4. NANOBIOSENSORS BASED ON NUCLEIC ACID AMPLIFICATION STRATEGY

Amplification-based strategy has proven to be effective in enhancing the sensitivity of NAT and has gained widespread use in the clinical diagnosis of TB. This approach mimics the DNA replication process in cells, allowing for the exponential amplification of low-abundance target nucleic acids *in vitro*, thereby increasing the nucleic acid signal.[72−74] Nucleic acid amplification strategies and nanomaterial-based signal amplification strategies work in tandem, as the amplified nucleic acids require additional signal tags to enable labeling and output in the form of common signals. Currently, fluorescent biosensors using the fluorescence properties of nanomaterials are extensively utilized in NAT based on amplification strategies to amplify nucleic acid signals through fluorescence (Figure 3). For traditional PCR products, the amplified DNA can serve as a template to generate fluorescent copper nanoparticles (CuNPs) *in situ*, effectively converting nucleic acid signals into fluorescent signals. Tsai et al. introduced primers containing polycytosine AT-TA sequences during the conventional PCR process, allowing the amplified product to template the formation of fluorescent CuNPs and subsequently produce a fluorescence signal. This process eliminates the need for conventional PCR purification and electrophoresis, resulting in time and labor savings. Fluorescent CuNPs generated *in situ* using DNA as a template possess a large Stokes shift, effectively avoiding background interference. This strategy was employed for the genetic detection of *Mtb IS6110*, achieving a LOD of 1.2×10^{-7} μM, a detection linear range of $2.4 \times 10^{-7} \sim 2.4 \times 10^{-6}$ μM, and a postamplification fluorescence signal reading time of less than 3 min.[75] To enable the simultaneous detection of multiple hotspot locations in the rifampicin resistance-determining region (RRDR) of the *rpoB* gene, Ibrahim et al. developed a solid-phase polymerase chain reaction (SPPCR) and utilized oligonucleotide-functionalized CdSe/CdS nanorods (CdSe/CdS NRs) as multicolor fluorescent probes. The SPPCR reaction immobilizes the amplification primers on microbeads, ensuring high specificity and facilitating further nucleic acid signal detection. However, the amplification efficiency of SPPCR is lower than that of conventional PCR, necessitating a more powerful signal amplification strategy to ensure detection sensitivity. The strong fluorescence of CdSe/CdS NRs, along with the design of multisite detection, compensates for the limitations of SPPCR. By adjusting the ratio of raw materials during the synthesis process, CdSe/CdS NRs emitting green, yellow, and red high-brightness fluorescence can be obtained. Nucleic acid probes possessing different targeting properties can correspond to different fluorescence colors, thereby providing more accurate and comprehensive information for nucleic acid detection and drug resistance analysis. The LOD of this multisite detection fluorescent nucleic acid sensor is 1×10^{-7} μM, and the turnaround time, including the amplification step, is 2.5 h.[76] In

https://doi.org/10.1021/jacsau.4c01206
JACS Au 2025, 5, 536−549

addition to fluorescent nanomaterials, magnetic nanomaterials can also be used for the detection of nucleic acid amplification products. Tian and his colleagues devised a nick-enhanced roller cycle amplification (NickRCA) strategy and employed the optomagnetic effect of magnetic nanoparticle (MNP) dimers for the detection of the amplification products. RCA utilizes a short primer to generate long single-stranded DNA containing numerous repeated template complementary fragments.[77] In this study, two nucleic acid probes targeting different sites of the amplification product were employed for the modification of MNP, resulting in the formation of MNP dimers through hybridization, thereby generating optical and magnetic signals. This optomagnetic sensor was successfully applied to *rpoB* gene detection, achieving a LOD of 1.5×10^{-8} μM and a total detection time of 100 min.[78]

5. NANOBIOSENSORS BASED ON NUCLEIC ACID RECYCLING STRATEGY

The strategy based on nucleic acid recycling is an effective approach to enhance the sensitivity of NAT. In this strategy, the same target nucleic acid binds repeatedly to the signal tag without increasing its copy number, effectively recycling the signal for output. This strategy transforms the original 1-to-1 signal recognition output into a 1-to-n format, allowing for efficient signal accumulation.[40,79,80] Nucleic acid recycling strategies can also complement nanotechnology-based signal amplification techniques, as demonstrated in *Mtb* nucleic acid detection (Figure 3). Currently, the nucleic acid recycling strategies used in *Mtb* DNA detection primarily involve enzyme assistance. These enzymes, typically nucleases, recognize and cleave the probe-target nucleic acid complex, releasing the target nucleic acid. This release process promotes signal conversion in the sensor and facilitates binding of the target nucleic acid to more probes, triggering the recognition-signal output process in a cycle, ultimately achieving signal amplification. Chen et al. developed a nick nuclease-assisted nucleic acid recycling technology for electrochemical detection of the *IS6110* gene. They designed a capture probe fixed on a C_{60} electrode and an auxiliary probe to collect the target nucleic acid and promote complex cleavage by nick nuclease, thereby regenerating the nucleic acid. This process generates a large number of cleaved solid-phase capture probes for further electrical signal conversion. The sensor utilizes a CNTs-PAN nanohybrid material consisting of clustered carbon nanotubes (CNTs) doped with polyaniline (PAN) as an electrical signal label, working in collaboration with C_{60} to achieve efficient conversion and amplification of electrochemical signals. The sensor has a LOD of 3.3×10^{-10} μM and a linear detection range of $1 \times 10^{-9} \sim 1\times 10^{-3}$ μM.[81] Zhang et al. developed a multichannel series piezoelectric quartz crystal (MSPQC) sensor using an exonuclease III (Exo III)-assisted nucleic acid recycling strategy and autocatalytic growth of AuNPs. In this system, capture probe-modified AuNPs hybridize with the target nucleic acid, and Exo III selectively cleaves the capture probe, releasing the target nucleic acid. The released intact target nucleic acid then hybridizes with the capture probe on the next AuNPs, initiating the next round of probe digestion until all capture probes are digested and the material surface is exposed. The exposed AuNPs undergo autocatalytic growth in glucose and $HAuCl_4$ solutions, increasing in volume and releasing piezoelectric signals. This piezoelectric sensor detects *Mtb*-specific 16S rDNA fragments with a LOD of 20 CFU mL^{-1} and completes the detection within 3 h.[82] The same team designed another Exo III-assisted nucleic acid recycling strategy and conducted a similar test. They designed a hairpin-shaped double-stranded DNA probe with a protruding 3′ end, including a recognition fragment (section I probe) and a connecting nucleic acid fragment (section II probe). After the target nucleic acid binds to the probe, Exo III cleaves it, releasing the II segment probe and the complete target nucleic acid. The released nucleic acid fragments enter the next digestion cycle and generate more segment II probes. The II-section probe immobilizes oligonucleotide-functionalized AuNPs on the electrode and undergoes autocatalytic growth in $HAuCl_4$ and nicotinamide adenine dinucleotide (NADH) solutions, releasing piezoelectric signals. This sensor has a LOD of 30 CFU mL^{-1} and can detect 16S rDNA fragments in 3 h.[83] Hu et al. designed a fluorescent nucleic acid sensor that combines RPA, multicomponent nuclease (MNAzyme)-assisted nucleic acid recycling, and QD nanobeacon (QD-NB) fluorescence detection. RPA is a constant-temperature amplification technology that uses recombinase to assist DNA melting and can complete the amplification of target nucleic acids within 20 min. The target nucleic acid combines with the QD-NB fluorescent probe to form a complex, which is then cleaved by MNAzyme in the presence of Mg^{2+}. After cleavage, the intact target nucleic acid is released for the next cycle. The quenching group in the QD-NB fluorescent probe is cleaved, resulting in strong green fluorescence that can be observed by the naked eye under UV light. This fluorescent nucleic acid sensor with multiple coordinated mechanisms was successfully applied to the highly sensitive detection of *Mtb IS1081* gene, with a LOD of 3.3×10^{-12} μM.[84]

6. NANOTECHNOLOGY AND TB POCT

Driven by recent advances in analytical chemistry and biomedicine, disease diagnostic technology is progressing toward high throughput, high sensitivity, and high selectivity. However, to improve global disease diagnosis, attention must be paid to POCT in the current research and development of disease diagnostic technology.[85] This is especially crucial for TB diagnosis. Although current *Mtb* detection technologies have greatly improved detection performance, they often rely on central laboratories with complex infrastructure. These laboratories require ample space for assembly line instruments and equipment, as well as a stable supply of electricity, water, and suitable temperature and humidity conditions. While these laboratories offer efficient and accurate processing of large numbers of specimens, they are expensive, geographically distant from patients, require longer specimen transportation times, and necessitate many specialized technical personnel. This is clearly impractical for TB diagnosis in remote areas, which are often the worst affected by *Mtb* infection.[3] POCT for *Mtb* fills these gaps, enabling wider TB screening, reducing the spread of *Mtb*, and achieving true point-of-care testing for patients. In a review, Drancourt et al. defined POCT as a user-friendly, rapid, low-cost testing technology that can be performed at the point of patient care without requiring sophisticated laboratory infrastructure or expertise.[86,87] Furthermore, POCT must ensure adequate detection performance, including sensitivity and specificity. TB screening often requires highly sensitive detection methods. Nanobiosensors have facilitated the development of *Mtb* POCT by enabling the use of portable devices and generating simple electrical or optical signals. Compared to traditional detection methods that use biological enzymes or antibodies, nanomaterials have signifi-

https://doi.org/10.1021/jacsau.4c01206
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cantly reduced the cost of detection. These nanobiosensors do not require complex preprocessing steps and have shorter response times, thereby reducing the overall detection turnaround time. Importantly, these detection technologies also offer high sensitivity and specificity. Therefore, nanobiosensors are an excellent strategy for constructing *Mtb* POCT. The emergence of new technologies and nanotechnology has led to the development of more practical *Mtb* POCT, including biochips, microfluidics, and smartphones.

By densely distributing biomonitoring probes on the chip surface using integrated technology, it is possible to collect a large amount of biological information in a small size for disease diagnosis. This biochip is compact and easy to use, making it highly portable and suitable for mass production to improve the rate of disease diagnosis. Currently, some conventional detection methods, such as immunoassays, have been combined with nanotechnology to construct biochips.[88] The application of microfluidic technology in biochips further advances the development of *Mtb* POCT.[89] Microfluidic technology is a precise liquid manipulation technology that combines microelectromechanical system technology, nanotechnology, and material science. In the field of biomedicine, microfluidic chips built with microfluidic technology can achieve high integration of the entire detection process, including sampling, preprocessing, sensor detection, and signal output, which is referred to as "lab-on-a-chip technology".[90] By combining well-designed flow paths, reagent freeze-drying processes, and nanobiosensors, microfluidic biochips can automate all detection steps and ensure highly sensitive detection of small-volume samples. Overall, microfluidic biochips are highly miniaturized, automated, and integrated POCT systems that can truly achieve "sample in, result out".[91−93] However, traditional microfluidic technology has limitations, such as complex channel design, expensive materials, and unstable external pumps and valves. These drawbacks restrict the clinical application of microfluidic chips. In recent years, paper-based microfluidic technology has emerged as a solution to these limitations. Paper-based microfluidics can create fluidic channels by drawing hydrophobic barrier patterns or shaping/cutting the paper. These materials can be mass-produced using specific printing technologies. Compared to traditional microfluidic technology, paper-based microfluidics offers advantages such as flexible and simple graphic design, cost-effectiveness, the ability to manipulate fluids without external power, and the capability to store reagents. This makes it suitable for building POCT devices.[94] In the field of *Mtb* detection, some studies have combined AuNPs with paper-based microfluidic technology to develop *Mtb* POCT. These studies have utilized different characteristics of nanomaterials to achieve various forms of signal detection. For example, Tsai et al. constructed a paper-based microfluidic sensor by incorporating *Mtb* nucleic acid capture probes and AuNPs into cellulose. By simply dropping a small amount of clinical specimen on this paper-based sensor, a color change can be observed within 1 h, which can be recognized by a smartphone application. The use of paper as the main material makes this sensor easy to produce, store, and transport, making it cost-effective and suitable for remote areas.[64] Another study by Zhou et al. integrated oligonucleotide-functionalized AuNPs onto a low-cost paper/polymer hybrid device to develop a paper-based microfluidic chip for *Mtb* nucleic acid detection. When a target nucleic acid fragment binds to a probe on the chip, the resulting temperature change can be directly detected using a conventional thermometer. The accessibility of commercial lasers has made this low-cost paper-based microfluidic chip more widely available.[66] The specific mechanisms of these two studies have been detailed previously, and their attempts to integrate nanobiosensors onto paper provide valuable insights for future *Mtb* POCT studies.

It is worth noting that many current studies on biosensors are exploring the use of smartphones as an auxiliary signal recognition tool, which has become a new trend in the development of POCT. There are several advantages to utilizing smartphones in this context. First, smartphones possess advanced computing power and graphics analysis capabilities due to the rapid development of electronic information technology. By combining smartphone apps with artificial intelligence and machine learning, more sophisticated identification and analysis of medical test results can be achieved. Additionally, smartphones have high-coverage network signals and high-speed network connections, enabling them to function effectively in complex environments and facilitate real-time signal transmission. Moreover, smartphones are highly portable and user-friendly, making them easily accessible and usable for professional examiners, clinicians, and patients alike. Furthermore, smartphones have gained extensive popularity worldwide, with approximately one-third of the global population owning smartphones and over 80% of adults in some developed countries and regions being equipped with smartphones.[95] In summary, the integration of smartphones in disease diagnosis presents a new opportunity for the advancement of POCT. For instance, Franco et al. utilized a smartphone as a colorimetric analysis tool for capturing and analyzing images of a modified immunoassay of gold nanostars (AuNSt). This sensor leveraged the peroxidase-like activity of AuNSt to generate a color reaction that could be detected by mobile phones. With appropriate calibration, the performance of mobile phone detection was found to be comparable to that of dedicated colorimetric sensors. The LOD of this detection strategy was determined to be 7.2×10^3 CFU mL^{-1}, with a turnaround time of 3 h.[96] In addition to providing colorimetric results for visible light, smartphones can also analyze fluorescent signals by connecting to portable nucleic acid amplification devices. Chen et al. developed a 3D-printed ultraviolet light-emitting diode (UV-LED) device that can easily detect small sample amounts of amplified products in capillaries by measuring fluorescent signals. These signals can be imported into smartphones for image analysis and detection.[97] The functional expansion of smartphones includes the optimization of imaging capabilities, enhancements in information processing functions, robust Internet connectivity, and various methods for remote communication. This integration enhances their potential roles in detection, result analysis, output, and clinical guidance. Zheng et al. utilized machine learning and artificial intelligence to enhance the analysis algorithm and workflow for TB test results, enabling the integration and analysis of patient information, nucleic acid test results, and antigen test results. They also incorporated this system into a portable testing device connected to a smartphone for result analysis and reading, facilitating instant detection of *Mtb* in more remote hospitals. This technology combines nanotechnology, POCT, and machine learning to significantly improve the efficiency of traditional immunoassays. In this system, smartphones not only record and analyze test results but also serve as data storage and conduct intelligent algorithm analysis for result output. The researchers employed this POCT for TB diagnosis in HIV-infected children, identifying 58 positive cases out of 78 cases of

https://doi.org/10.1021/jacsau.4c01206
JACS Au 2025, 5, 536−549

tuberculosis, detecting 48 positive cases out of 66 missed cases, and successfully distinguishing latent tuberculosis infection.[98] Currently, mobile phones are primarily employed for graphics or color analysis and data calculation in *Mtb* detection sensors, thereby replacing bulky colorimetric tools. However, smartphones and other smart mobile devices, such as smartwatches, have recently exhibited enhanced functionalities, offering a more comprehensive range of options for POCT design in the future. This unprecedented popularity and portability will undoubtedly usher in significant changes in *Mtb* POCT and even extend to a wider spectrum of disease diagnosis.

7. CONCLUSIONS AND FUTURE OUTLOOK

TB remains a major public health problem and has once again become the leading cause of death from infectious diseases, surpassing HIV. The unique *in vivo* infection cycle and immune evasion abilities of *Mtb* make the clinical diagnosis of TB extremely challenging. Many medical institutions still rely on outdated testing techniques to confirm TB infection. For over a century, acid-fast staining of sputum smears has been the preferred method for detecting *Mtb* in clinical laboratories, despite being invented in 1882.[17,99] Despite technological advancements and the discovery of *Mtb*'s pathogenic mechanism, we still lack a definitive solution to combat the increasingly severe TB epidemic. Currently, multiple technologies are used to collaboratively diagnose TB, including sputum smear staining, *Mtb* culture, *Mtb* genetic testing, IGRAs, and drug susceptibility analysis. However, these diagnostic methods impose a high economic, environmental, and personnel burden on clinical laboratories, particularly at the grassroots level. Centralized laboratories also face challenges in maintaining rapid turnaround times for TB diagnosis.[100] In this perspective, we discuss the applications of nanobiosensors in TB NAT. These research findings aim to address the challenging issues in clinical TB management using interdisciplinary approaches. Nanotechnology plays a crucial role in improving the sensitivity of TB nucleic acid detection by enabling efficient signal conversion and amplification. Nanobiosensors convert *Mtb* biological signals into various types of signals that can be easily detected by simple instruments. These signals can be communicated to operators through simple operations, facilitating timely diagnosis of TB. The advancement of engineering technology has led to the development of portable or wearable nanobiosensor devices, while biochips and microfluidic technologies enable the detection of trace samples. The abundance of nanobiosensor options provides significant potential for improving TB diagnosis in both clinical laboratory settings and point-of-care diagnosis.

However, it must be acknowledged that the current state of clinical translation of nanobiosensors is not optimiztic, with much work still in the preclinical research stage. Overcoming this situation requires addressing several challenges. First, there are cost-effectiveness concerns surrounding the clinical applications of nanobiosensors.[101] Currently, there are very few approved nanomaterials for clinical use, and most of them are expensive, making them unaffordable for many developing countries and regions. Second, the complex synthesis steps lead to batch-to-batch variations in nanomaterials, causing instability in subsequent detection or treatment effects. As a result, many potential nanobiosensors remain confined to the pages of scientific documents. Therefore, policies and financial support that encourage the clinical translation of nanobiosensors are necessary. Simplifying and standardizing the production steps of nanomaterials will facilitate large-scale production of nanobiosensors.[43,102,103] Additionally, clinical settings present more complex and diverse challenges compared to laboratories with highly optimized conditions. In some nanobiosensor research, artificially prepared solutions are used to evaluate the sensor's performance. However, these solutions do not accurately represent actual clinical samples. Laboratory physicians or technicians often encounter problems like small sample sizes, impurity contamination, and issues related to sample storage and transportation. Blood samples, for example, may be affected by jaundice, hemolysis, coagulation, and other factors that can impact test results. Sputum samples can be influenced by the patient's age, disease status, and technical issues, leading to small sample sizes, as well as contamination from saliva and oral bacteria. Therefore, the ability to handle complex clinical sample-related problems is crucial for nanobiosensors in clinical testing.[104] Consequently, extensive verification experiments using many clinical sample pools are necessary before the clinical application of nanobiosensors to ensure sufficient reliability and repeatability. Lastly, nanobiosensors with high detection efficiency typically utilize highly reactive nanomaterials capable of detecting low-abundance biomarkers through mild chemical transformation processes. However, the high reactivity of these nanomaterials often results in low stability; for instance, some nanomaterials are prone to oxidation during use. Consequently, achieving a balance between detection efficiency and stability is essential when selecting nanomaterials for nanobiosensors. In addition to the intrinsic stability of the nanomaterials, appropriate storage conditions are vital for maintaining the stability of nanobiosensors. Factors such as temperature, humidity, pH, oxygen concentration, and light exposure can influence the physical and chemical properties of the materials, potentially leading to decreased activity and even misdiagnosis. Long-term storage of certain water-soluble nanomaterials may cause precipitation or a reduction in the effectiveness of active substances, resulting in decreased concentration and inconsistent quality, which could lead to unstable test results. Moreover, if proper aseptic handling is not adhered to during preparation and storage, biological sources of nanomaterials, such as proteins, can easily become contaminated, leading to detection failures. Therefore, stringent storage conditions must be established for each nanomaterial, considering factors such as time and environmental requirements. Additionally, hydrophilic modification and the use of solvents can enhance the performance of nanomaterials and reduce their dependence on specific storage environments. However, it is crucial to strictly control the concentration of solvents (such as DMSO), as these solvents may damage some organic components in the system, such as causing the quenching of fluorescent groups.[105]

Overall, the application of nanobiosensors in the detection of TB nucleic acid presents both opportunities and challenges. Although interdisciplinary research in nanotechnology is rapidly progressing worldwide, the clinical translation of these technologies continues to encounter numerous obstacles. Nonetheless, given the limited options available to clinicians for TB diagnosis, it is worthwhile to develop supportive policies and invest significant resources in relevant research. Every effort made will contribute to the realization of a "TB-free" world.

https://doi.org/10.1021/jacsau.4c01206
JACS Au 2025, 5, 536−549

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (32371439, 82003262); Science and Technology Program of Sichuan Province (2024NSFSC1922, 2022YFS0095); Zigong Academy of Medical Sciences 2022 (ZGYKY22KF003); Sichuan University Postdoctoral Interdisciplinary Innovation Fund; Post-Doctor Research Project, West China Hospital, Sichuan University (2021HXBH009).

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