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Devising novel near-infrared aggregation-induced-emission luminogen labeling for point-of-care diagnosis of *Mycobacterium tuberculosis*

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SUMMARY Detecting and appropriately diagnosing a *Mycobacterium tuberculosis* infection remains technologically difficult because the pathogen commonly hides in macrophages in a dormant state. Described here is novel near-infrared aggregation-induced-emission luminogen (AIEgen) labeling developed by the current authors' laboratory for point-of-care (POC) diagnosis of an *M. tuberculosis* infection. The selectivity of AIEgen labeling, the labeling of intracellular *M. tuberculosis* by AIEgen, and the labeling of *M. tuberculosis* in sputum samples by AIEgen, along with its accuracy, sensitivity, and specificity, were preliminarily evaluated. Results indicated that this near-infrared AIEgen labeling had satisfactory selectivity and it labeled intracellular *M. tuberculosis* and *M. tuberculosis* in sputum samples. It had a satisfactory accuracy (95.7%), sensitivity (95.5%), and specificity (100%) for diagnosis of an *M. tuberculosis* infection in sputum samples. The current results indicated that near-infrared AIEgen labeling might be a promising novel diagnostic tool for POC diagnosis of *M. tuberculosis* infection, though further rigorous verification of these findings is required.

Keywords *Mycobacterium tuberculosis*, near-infrared aggregation-induced emission luminogen, point-of-care diagnosis, metabolic labeling, high-throughput screening

Tuberculosis (TB) is a common infectious disease caused by infection with *Mycobacterium tuberculosis* that may spread *via* aerosol and involve various organs (*1*). More than 10 million cases are confirmed per year. Other than COVID-19, TB is the second leading cause of death due to infectious disease (*2,3*). TB remains a major public health concern, particularly in developing countries, and indeed it cannot be ignored or underestimated. Earlier appropriate diagnosis and timely anti-TB therapy may result in better clinical outcomes. However, many conventional diagnostic methods, such as biomedical imaging, bacteriological diagnosis, molecular diagnosis, and immunologic testing, suffer from several methodological flaws, resulting in a low specificity, low sensitivity, and demanding conditions for pathogen detection (*4-6*). The main technological difficulty lies in the fact that *M. tuberculosis* might be shielded by host macrophages, so latent and dormant intracellular *M. tuberculosis* is extremely difficult to detect or eradicate (*7,8*). An ideal method of diagnosing TB in clinical practice should have several characteristics: *i*) satisfactory sensitivity and specificity; *ii*) rapid *M. tuberculosis* detection achieved with minimal sample processing; and *iii*) a high sensitivity so that it can be used to screen patients with TB from patients with a suspected *M. tuberculosis* infection (*9,10*). Since conventional methods are far from satisfactory, development of rapid, sensitive, and accurate tools for diagnosis of intracellular *M. tuberculosis* is an urgent task for global TB researchers.

Unlike other bacteria, *M. tuberculosis* has a special cellular structure. The cytoderm of *M. tuberculosis*

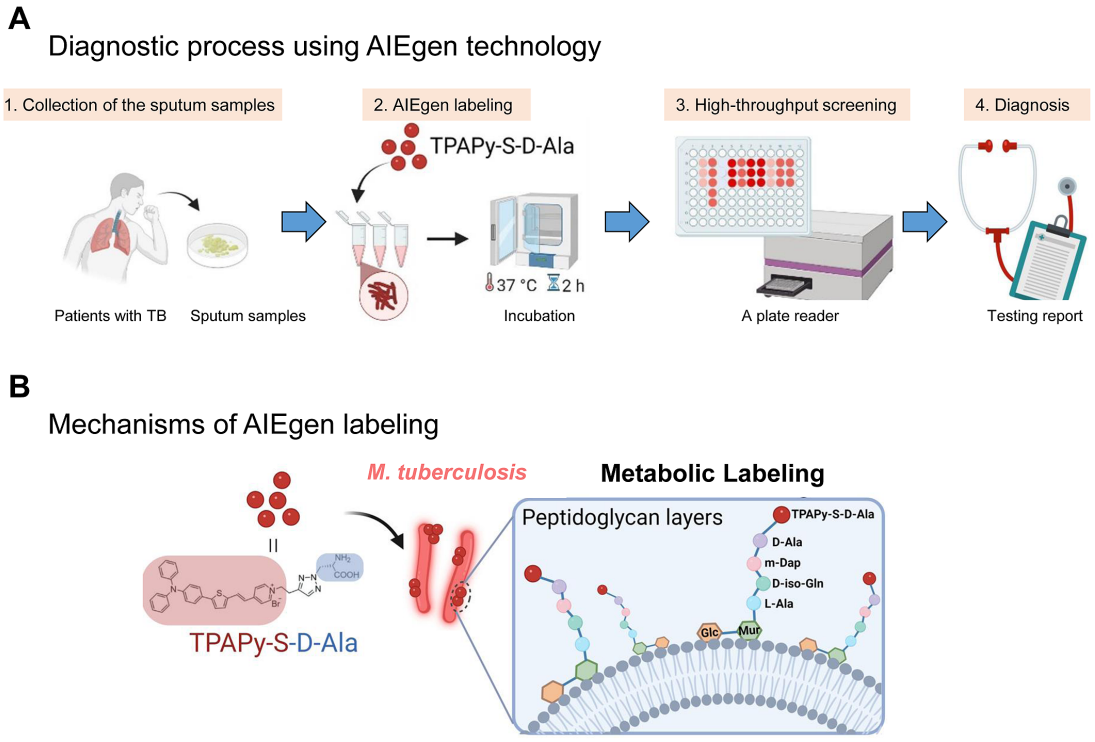

Figure 1. Introduction of a newly developed AIEgen for POC diagnosis of a *Mycobacterium tuberculosis* infection. (A) Process for diagnosis of an *M. tuberculosis* infection using TPAPy-S-D-Ala, an AIEgen with near-infrared fluorescence developed by the current authors' laboratory. Sputum samples were collected and labeled with TPAPy-S-D-Ala at 37°C for 2 h. Then, a plate reader was used to conduct high-throughput tuberculosis screening. **(B)** Underlying mechanisms of TPAPy-S-D-Ala in the metabolic labeling of *M. tuberculosis*. TPAPy-S-D-Ala molecules are integrated into the peptidoglycan layer of the cytoderm and "turn on" the fluorescence of *M. tuberculosis* after incubation for 2 h.

is reported to consist of a cytoplasmic lipid bilayer, peptidoglycan (PG) layer, arabinogalactan layer, mycomembrane, and capsule that contribute to building a solid barrier that facilitates the survival and virulence of *M. tuberculosis* (*11*). However, the complicated structure of the *M. tuberculosis* cytoderm enables the exploration of novel targets for detecting *M. tuberculosis*, such as targets in the PG layer. Specific pentapeptides (L-Ala-D-iso-Gln-m-DAP-D-Ala-D-Ala) are crossed-linked with N-acetylmuramic acid (MurNAc) in the PG layer *via* L, D-transpeptidases catalysis (*12*). Studies have reported that D-Ala-linked fluorescent probes have marked potential to label the PG layer of the *M. tuberculosis* cytoderm by serving as an external D-Ala substrate, enabling visualization of *M. tuberculosis* replication, thereby distinguishing living *M. tuberculosis* from dead cells (*13,14*). However, many of the commercially available fluorophores have a low emission efficiency and low sensitivity in an aggregate state due to the phenomenon of aggregation-caused quenching (ACQ) (*15*). Accordingly, aggregation-induced emission luminogens (AIEgens), which are molecules that are highly emissive in an aggregate state (limited intramolecular motion), had to be developed to address this problem (*16,17*). Thus, development of a metabolic-labeling AIEgen is a potential good solution for accurate point-of-care (POC) diagnosis of an *M. tuberculosis* infection. A previous study by the current authors described an *M. tuberculosis* cytoderm-labeling AIEgen for rapid detection and intracellular ablation of *M. tuberculosis* that had better detection sensitivity compared to conventional acid-fast staining (*17*). AIEgen with near-infrared fluorescence was urgently needed in order to better penetrate so as to facilitate *in vivo* labeling of *M. tuberculosis* in subjects (*18*). Thus, the current authors developed a near-infrared AIEgen (TPAPy-S-D-Ala) for metabolic labeling of PG to enable high-throughput diagnosis of *M. tuberculosis* (Figure 1). The AIE functional motif (TPApy-S) and the metabolic motif (D-Ala, with PG-layer-specific metabolic labeling) are linked using click chemistry to yield TPAPy-S-D-Ala. The entire diagnostic process involving TPAPy-S-D-Ala is shown in Figure 1A and it includes collection of the sputum sample, obtaining of TPAPy-S-D-Ala, incubation of samples with TPAPy-S-D-Ala, high-throughput screening, and finally readout of the diagnostic results (Figure 1A). The mechanisms of incubation are clear but extremely important (Figure 1B), namely, the external D-Ala molecules of the AIEgen are efficiently integrated into PG and are solidly aggregated on the cytoplasmic membrane, resulting "turning on" fluorescence so that the *M. tuberculosis* cells can be labeled. The fluorescence intensity of labeled *M. tuberculosis* can be quantified with a microplate reader to enable the diagnosis of TB (Figure 1).

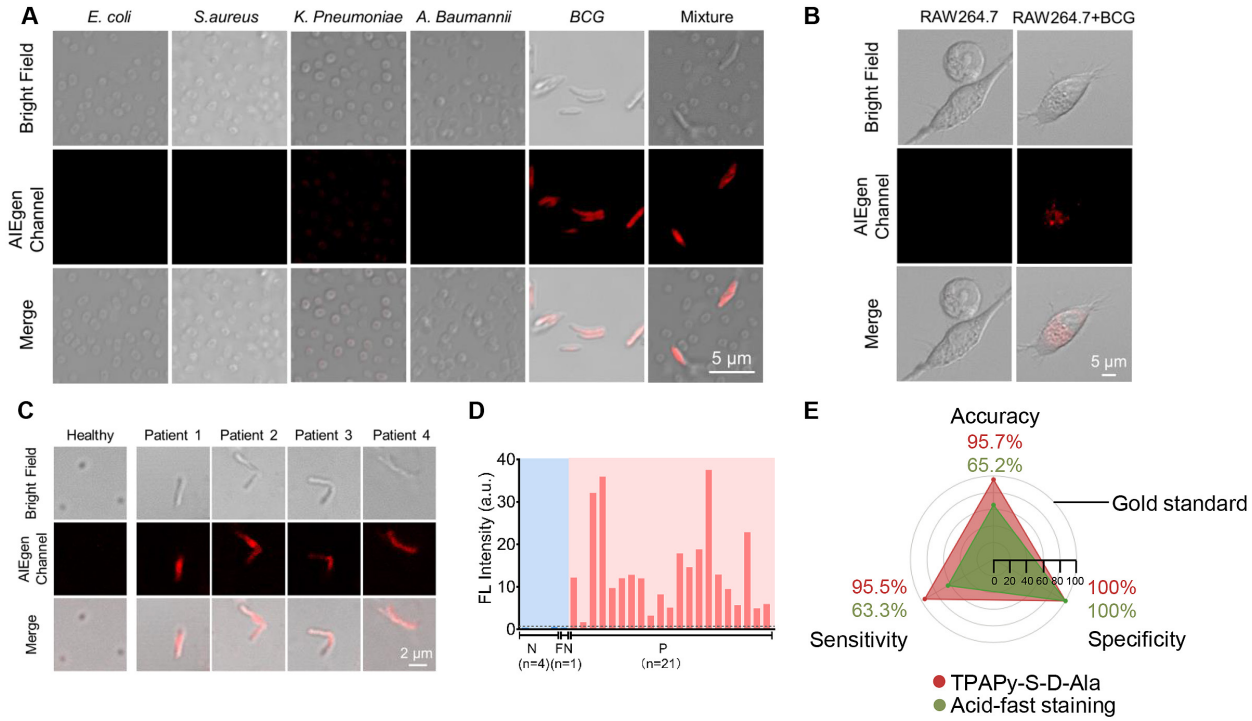

Figure 2. Verification of the labeling of *M. tuberculosis* by AIEgen. (A) Verification of the selective labeling of *M. tuberculosis* by TPAPy-S-D-Ala. Obviously, only samples infected with *M. tuberculosis* (BCG and a mixed infection) were labeled. Microbes were labeled in red by AIEgen. **(B)** Verification of the intracellular effects of TPAPy-S-D-Ala on *M. tuberculosis*. Only cells infected with *M. tuberculosis* were labeled. Microbes were labeled in red by AIEgen. **(C)** Verification of the labeling of *M. tuberculosis* in sputum samples by TPAPy-S-D-Ala. Only *M. tuberculosis* in sputum samples from infected patients was labeled. Microbes were labeled in red by AIEgen. **(D)** Outcomes of *M. tuberculosis* identification by TPAPy-S-D-Ala labeling. A total of 26 sputum samples (22 samples from patients infected with *M. tuberculosis* and 4 samples from healthy controls) were observed. N means negative, FN means false negative, and P means positive. **(E)** Evaluation of the accuracy, sensitivity, and specificity of TPAPy-S-D-Ala labeling in comparison to conventional acid-fast staining of sputum samples.

The metabolic labeling efficiency of TPAPy-S-D-Ala was preliminarily verified in terms of the following aspects: *i*) Selectivity of TPAPy-S-D-Ala The specificity with which TPAPy-S-D-Ala identified *M. tuberculosis* was investigated *via* clinical digestion and incubation with various bacteria. The species used included *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and Bacillus Calmette-Guerin (BCG, a model for *M. tuberculosis*). As shown in Figure 2A, only the panels that included *M. tuberculosis* (BCG and a mixed infection) were found to be labeled by TPAPy-S-D-Ala, indicating that TPAPy-S-D-Ala was highly selective at labeling an *M. tuberculosis* infection. *ii*) Evaluation of the labeling efficiency for intracellular bacteria TPAPy-S-D-Ala was co-incubated with RAW264.7 cells infected with BCG and intact RAW264.7 cells (control) for 2 h. The results were observed using a laser scanning confocal microscope (Zeiss 700, Germany; excitation, 488 nm). Results indicated that the fluorescence intensity of intracellular BCG labeled by TPAPy-S-D-Ala was markedly greater than that of the control (Figure 2B). These results indicated that the AIEgen was effective at labeling intracellular *M. tuberculosis*, so it might be able to serve as a promising tool for diagnosis of latent TB (Figure 2A, B). *iii*) Evaluation of the labeling of *M. tuberculosis* in sputum samples by AIEgen Sputum samples from *M. tuberculosis*-positive patients and healthy controls were collected and exposed to TPAPy-S-D-Ala. As shown in Figure 2C, *M. tuberculosis*-positive sputum samples exhibited a clear "turning on" of fluorescence after TPAPy-S-D-Ala labeling, whereas no fluorescence was observed in *M. tuberculosis*-negative sputum samples from the healthy controls (Figure 2C). The fluorescence intensity of the *M. tuberculosis*-positive sputum samples was significantly greater than that of the sputum samples from the healthy controls (Figure 2D). *iv*) Evaluation of the accuracy, sensitivity, and specificity of the AIEgen A comprehensive gold standard was established to definitively diagnose TB by comprehensively considering clinical manifestations, radiological findings, and laboratory results from a given patient. Conventional acid-fast staining was performed in parallel. Based on the index of fluorescence intensity, TPAPy-S-D-Ala labeling had an accuracy of 95.7%, a sensitivity of 95.5%, and a specificity of 100% in diagnosing an *M. tuberculosis* infection in sputum samples (vs. an accuracy of 65.2%, a sensitivity of 63.3%, and a specificity of 100% for acid-fast staining) (Figure 2E). Hence, the selective labeling of intracellular *M. tuberculosis* and *M.*

tuberculosis in sputum samples by this near-infrared AIEgen (TPAPy-S-D-Ala), along with its accuracy, sensitivity, and specificity, were preliminarily verified. This near-infrared AIEgen was therefore considered to have great potential for use in the clinical diagnosis of TB due to its rapid, sensitive, and precise nature. However, well-designed randomized controlled trials (RCTs) with a large sample are needed to obtain robust evidence for clinical use of this AIEgen, and these RCTs are now being planned.

In conclusion, a near-infrared AIEgen (TPAPy-S-D-Ala) was developed for POC diagnosis of an *M. tuberculosis* infection. TPAPy-S-D-Ala can efficiently aggregate in the PG layer of the bacterial cytoderm by acting as an exogenous substrate of D-amino acid during PG biosynthesis, and this may facilitate the rapid and sensitive detection of living *M. tuberculosis* in macrophages. Preliminary data indicated that this near-infrared AIEgen selectively labels intracellular *M. tuberculosis* and *M. tuberculosis* in sputum samples, with satisfactory accuracy, sensitivity, and specificity in comparison to conventional acid-fast staining. Hence, TPAPy-S-D-Ala is a promising diagnostic tool for rapid and accurate detection of *M. tuberculosis* and it might be a good tool to mass screen for an *M. tuberculosis* infection, particularly in people in developing countries. The efficiency and safety of TPAPy-S-D-Ala should be further verified in large-scale RCTs before this labeling is widely used in clinical practice.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

Ethical issues: This study was approved and supervised by the Ethics Committee of the Third People's Hospital of Shenzhen (approval number 2021-030). The study protocol was explained to all of the participants, who then provided written informed consent to participation in this study.

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