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Biosensors for the Detection of *Bacillus anthracis*

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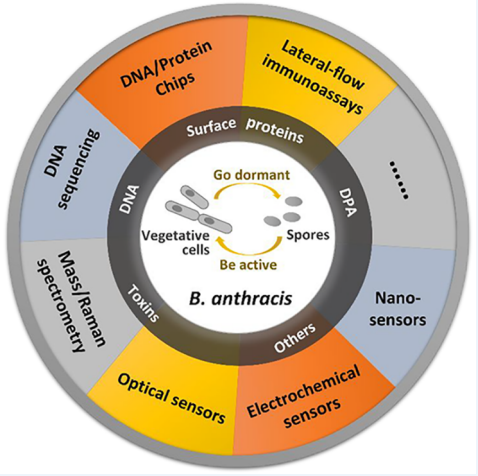
CONSPECTUS: *Bacillus anthracis*, present in two forms of vegetative cells and spores, is a pathogen that infects humans through contact with infected animals or contaminated animal products and is also maliciously used in terrorist acts. Therefore, a rapid and sensitive test for *B. anthracis* is necessary but challenging. The challenge comes from the following aspects: an accurate distinction of *B. anthracis* from other *Bacillus* species due to their high genomic similarity and the horizontal gene transfer between *Bacillus* members; direct detection of the *B. anthracis* spores without damaging them for component extraction to avoid the risk of spore atomization; and the rapid detections of *B. anthracis* in complex samples, such as soil and suspicious powders, without sample pretreatments and expensive large-scale equipment. Although culturing *B. anthracis* from samples is the conventional method for the detection of *B. anthracis*, it is time-consuming and the detection results would not be easy to interpret because many *Bacillus* species share similar phenotypic features such as a lack of motility and hemolysis, resistance to gamma phages, and so on. Intensive and extensive effort has been expended to develop reliable detection technologies, among which biosensors exhibit comprehensive advantages in terms of sensitivity, specificity, and portability. Here, we briefly review the research progress, providing highlights of the latest achievements and our own practice and experience. The contents can be summarized in three aspects: the discovery of detection targets, including genes, toxins, and other components; the creation of molecular recognition elements, such as monoclonal antibodies, single-chain antibody fragments, specific peptides, and aptamers; and the design and construction of biosensing systems by the integration of appropriate molecular recognition elements and transducer devices. These sensor devices have their own characteristics and different principles. For example, the surface plasmon resonance biosensor and quartz crystal microbalance biosensor are very sensitive, while the multiplex PCR-on-a-chip can detect multitargets. Biosensors for direct spore detection are highly recommended because they are not only fast but also avoid contamination from aerosol-containing spores. The introduction of nanotechnology has significantly improved the performance of biosensors. Superparamagnetic nanoparticles and phage-displayed gold nanoparticle ligand peptides have made the results of spore detection visible to the naked eye. Because of space constraints, many advanced biosensors for *B. anthracis* are not described in detail but are cited as references. Although biosensors provide a variety of options for various application scenarios, the challenges have not been fully addressed, which leaves room for the development of more advanced and practical *B. anthracis* detection means.



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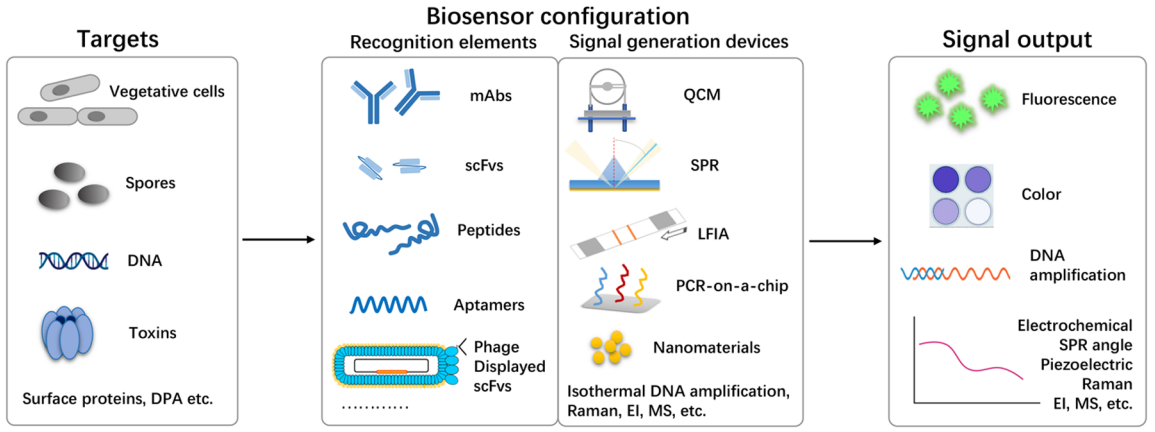

Figure 1. Schematic showing the detection of *B. anthracis*. Abbreviations: DPA, dipicolinic acid; mAbs, monoclonal antibodies; scFvs, single-chain antibody fragments; LFIA, lateral-flow immunoassays; EI, electrical impedance; MS, mass spectrometry; SPR, surface plasmon resonance.

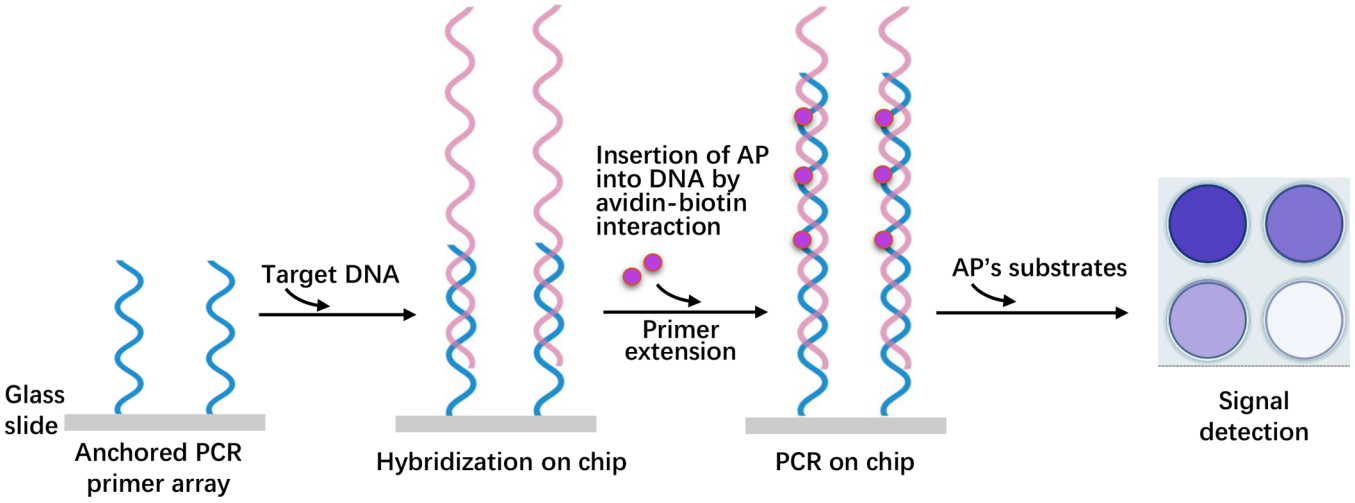

Figure 2. PCR-on-a-chip for the detection of *B. anthracis*. Adapted with permission from ref 15. Copyright (2004) Elsevier B.V.

sensor for the quantitative detection of *B. anthracis* vegetative cells.

- Hao, R.; Wang, D.; Zhang, X. e.; Zuo, G.; Wei, H.; Yang, R.; Zhang, Z.; Cheng, Z.; Guo, Y.; Cui, Z.; Zhou, Y. Rapid detection of *Bacillus anthracis* using monoclonal antibody functionalized QCM sensor. *Biosens. Bioelectron.* **2009**, *24*, 1330−1335.[3] This study developed a QCM-based immunosensor for the detection of *B. anthracis* vegetative cells and spores.

INTRODUCTION

Bacillus anthracis, the causative agent of anthrax, is a Gram-positive, spore-forming bacterium. *B. anthracis* has a high genomic similarity to *B. cereus*, *B. thuringiensis*, and *B. mycoides*.[4] Because of horizontal gene transfer, *B. anthracis*, *B. cereus*, and *B. thuringiensis* have even been suggested to be one species.[5] *B. anthracis* is present in two forms: vegetative cells and spores. The spore state can live for decades and is resistant to high pressure, desiccation, heat, pH, radiation, and other adverse conditions.[6] The virulent *B. anthracis* strain has two plasmids: pXO1 and pXO2. pXO1 carries the genes for toxin synthesis: *pagA* encoding a protective antigen (PA; gene also named *pag*), *cya* encoding an edema factor (EF), and *lef* encoding a lethal factor (LF). pXO2 carries capsular-associated genes *capA*, *capB*, and *capC*. More details regarding the pathogenesis of *B. anthracis* are reviewed by Moayeri et al.[7]

Anthrax is primarily a disease affecting herbivores and is caused by natural infection. Humans usually get anthrax via contact with infected animals or contaminated animal products. It is estimated that 1.8 billion people live within anthrax-suitable areas, including rural areas in Africa, Europe, and Asia.[8] Anthrax suitability is predicted to be increased in northern latitudes with global warming, which will increase the risk of human infection.[9] In addition to natural infection, bioterrorism using *B. anthracis* spores is another serious concern. Because of its high pathogenicity, fatality, and infectivity, *B. anthracis* needs to be tested accurately, rapidly, and sensitively.

The conventional method for the detection of *B. anthracis* and the diagnosis of anthrax is a microbiological test, namely, culturing *B. anthracis* from samples (www.cdc.gov). It requires 12−48 h, and it is not easy to differentiate *B. anthracis* from other *Bacillus* species with similar phenotypic features. Therefore, the microbiological test method has limited applications in the on-site detection of *B. anthracis*.

There are many difficulties involved in the detection of *B. anthracis*, including how to distinguish *B. anthracis* from other *Bacillus* species due to their high genomic similarity, how to detect the spores directly without damaging them for DNA

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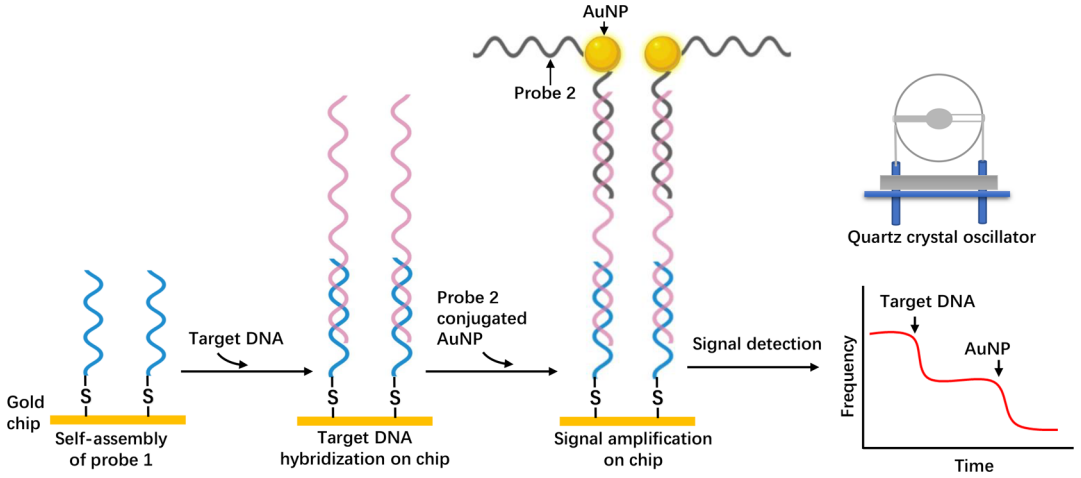

Figure 3. DNA-probe-functionalized QCM biosensor for the detection of *B. anthracis*. AuNP, gold nanoparticles. Adapted with permission from ref 2. Copyright (2011) Elsevier B.V.

extraction and protein purification, which are time-consuming and carry the risk of spore atomization, and how to detect *B. anthracis* in complex environmental samples, such as soil and suspicious powders, without expensive large-scale equipment. Extensive effort has been expended to deal these challenges (Figure 1). Among these practices, biosensors exhibit comprehensive advantages in terms of sensitivity, specificity, and portability. Here, focusing on the relevant research progress, we describe the characteristics and potential application scenarios of various biosensor devices for *B. anthracis* and discuss possible solutions to the problems that still need to be solved.

BIOSENSORS FOR DNA TARGETS OF *B. ANTHRACIS*

Genes involved in the biogenesis of capsules and toxins in the plasmids, pXO1 and pXO2, are usually used as targets for nucleic acid detection in *B. anthracis*. With the development of genomic technology, some specific sequences on chromosomes have been identified as biomarkers, including *BA813*,[10] *RpoB*,[11] and *Gyra*.[12] Chromosome markers are important for the detection of avirulent and attenuated *B. anthracis* strains because these strains often lack pXO1 and pXO2.

There are many nucleic acid amplification-based techniques for the detection of *B. anthracis*, such as the polymerase chain reaction (PCR)[13] and reverse transcriptional PCR (RT-PCR).[14] Using virulence-factor-encoding genes *pagA*, *Ba813*, and *capB* as targets, we established a multiplex PCR-on-a-chip system by combining the use of multiplex PCR, a DNA chip, and and biotin−avidin alkaline phosphatase indicator system (Figure 2).[15] First, the 5′-end anchored primers were fixed on the surface of the mercapto silane-modified chip by chemical cross-linking. Then, the DNA samples were loaded onto the chips and target DNA hybridized with the anchored primers, triggering primer extension. As Biotin-11-dUTPs were admixed in the double-stranded DNA during extension, the amplified DNA products could bind with avidin-labeled alkaline phosphatase (Av-AP) through biotin−avidin interactions. Finally, purple-colored signals were generated after the enzymatic reaction and could be identified with the naked eye. Consequently, *B. anthracis* DNA levels of as low as 1 pg could be detected using this visible gene chip.

Compared with PCR, isothermal amplification can avoid the thermal cycling operation, and thus the detection system can be simplified.[16,17] Our research group developed a loop-mediated isothermal amplification (LAMP) system for the detection of *B. anthracis* spores from both pure culture and various "white powders".[18] Approximately 13 *B. anthracis* strains and 33 non-*B. anthracis* strains were tested. Following the isothermal amplification of *pagA*, *Ba813*, and *capB* at 60−65 °C, this system showed high specificity and sensitivity. The limit of detection (LOD) was 10 spores for the culture and 100 spores for the simulated powder samples. In this system, only a heat blocker is required.

Although gene amplification techniques are highly sensitive, a shortcoming of these methods is the occurrence of false positive results caused by nucleic acid aerosols and other internal factors. The quartz crystal is a type of piezoelectric device, and its surface acoustic wave (SAW) vibrational frequency is related to the mass of its load. Hence, it has been widely used as an excellent quartz crystal microbalance (QCM) and QCM biosensor.[19] To avoid potential false positive results in PCR, we constructed a miniaturized QCM sensor and integrated it with an asymmetric PCR for the quantitative detection of *B. anthracis*.[2] As Figure 3 shows, a thiol-modified DNA probe (probe 1) was immobilized on the surface of the gold chip by self-assembly via the Au−S bond and hybridized with the target single-stranded DNA (*pag* or *BA813*), which was obtained using asymmetric PCR. Because the increase in mass caused a decrease in the resonance frequency of the QCM biosensor, the other DNA probe (probe 2), which is complementary to the targets, was modified with gold nanoparticles and injected onto the chip for signal amplification. This real-time sensing method reached a LOD of 3.5×10^2 CFU/mL with a linear range of 3.5×10^2−3.5×10^7 CFU/mL.

The combined use of nucleic acid amplification technology and different sensing strategies has also been reported. A reverse transcription−PCR−electrospray ionization mass spectrometry system has been proposed to analyze human respiratory samples containing *B. anthracis* and other pathogens, realizing broad pathogen identification.[20] Another example is the surface-enhanced Raman scattering (SERS)-based PCR assay platform in which *pagA* of *B. anthracis* is

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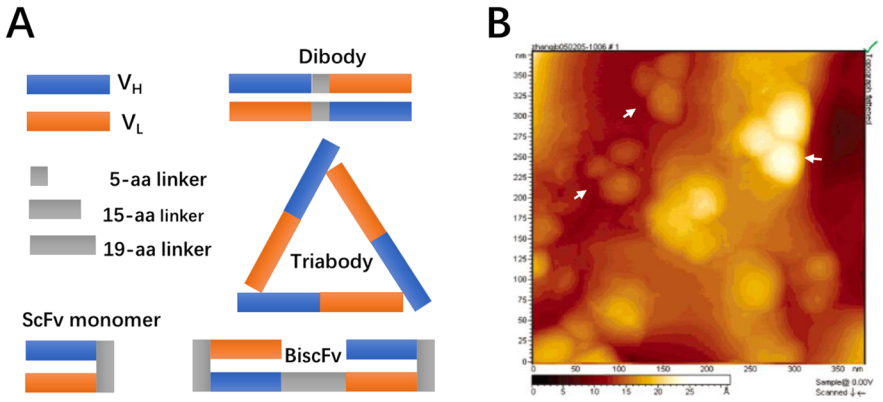

Figure 4. (A) Schematic of ScFvs and its variants. Adapted from ref 26. (B) Image of trimers of scFvs (triabodies) by atomic force microscope. Reproduced with the permission from ref 26. Copyright (2006) American Chemical Society.

detected within a dynamic range of 0.1−1000 pM with a LOD of 960 pM after five amplification cycles.[21]

BIOSENSORS FOR ANTHRAX TOXINS

Anthrax toxins are composed of PA, LF, and EF. PA and LF combine to form lethal toxins, and a mixture of PA and EF forms edema toxins. These toxin components are important targets for the detection of *B. anthracis* as well as the diagnosis of anthrax disease. Compared with nucleic acid amplification, immunoassays based on antigen−antibody recognition show high anti-interference performance. In addition to enzyme-linked immunosorbent assays,[22] various immunosensors have been developed for the detection of toxins. In a recent study, the limits of detection of 1.4 pM for LF and 28 fM for PA have been achieved by the combined use of a sandwich immunoassay and surface-enhanced Raman scattering during the analysis of human serum samples.[23]

In addition to antibodies, aptamers[24] and peptides[25] have been developed for the recognition of PA. Single-chain antibody fragments (scFvs) are engineered antibodies that contain variable regions of the heavy chain (V_H) and light chain (V_L). V_H and V_L are connected by a short, flexible polypeptide. Compared with full-length antibodies, scFvs can be produced in large quantities at low cost by the bacterial expression system and easily fused with other proteins of interest for coexpression and labeling. Using the phage-display technique, we prepared an scFv against *B. anthracis* PA, named 6w10.[26] To further improve the affinity, various variants of this scFv were constructed by shortening or deleting the polypeptide linking V_H with V_L (Figure 4A). Images of trimers of scFvs (triabodies) were acquired by atomic force microscopy (Figure 4B). The recombinant bivalent scFv (biscFv-6w10) showed the highest affinity among the prepared variants, with a kD value of 6.5×10^9 M^{-1}. *E. coli* alkaline phosphatase (EAP) was fused with biscFv-6w10 to amplify the detection signals. Fusion protein EAP-biscFv-6w10, which was thus obtained, could detect as low as 10 pg of PA or 500−1000 vegetative cells in 2 h, and Cy3-labeled biscFV-6W10 had 9-fold higher sensitivity than EAP-biscFv-6w10.

The introduction of nanotechnology has provided enormous opportunities to enhance the performance of biosensors.[27] A polyvalent peptide polymer that was coupled with zinc oxide nanorods allowed for the development of an ultrasensitive fluorescence sensor with a LOD of 15 zM for PA.[28] An electrical graphene aptasensor enabled PA detection with a LOD of 1.2 aM.[29] The combined use of an electrochemical immunosensor with bismuth nanocomposites and cadmium-ion-functionalized titanium phosphates enabled detection even at a volume of as low as 50 pg/mL in 35 min.[30] A gold-nanoparticle-based dual-readout sandwich immunoassay facilitated the colorimetric and scanometric detection of PA without the use of a device with a LOD of 550 fM.[31]

In addition to immunosensors, mass spectrometry (MS) and chromatography with MS have been widely used for the analysis of toxins in recent years. Most of the samples tested using these methods were obtained from patients or infected animal models. The objective is not only limited to the detection of pathogens and the diagnosis of anthrax but also involves examining the kinetics of anthrax toxins and the immune response of the host. In general, most MS-related analysis methods have a LOD range of ng/mL−pg/mL or pM−fM for toxin detection in biological samples, and some of the approaches can reach a LOD of fg/mL.[32] The review by Touriner et al. can be referred to for details.[33]

BIOSENSORS FOR *B. ANTHRACIS* SPORES

Biorecognition Elements

B. anthracis spores are easier to disperse but more difficult to inactivate than vegetative cells, thereby making them a long-term threat to humans and animals. Nucleic acid- and toxin-associated detection methods require disrupting tough spores and extracting DNA or proteins. Therefore, it is important to detect the spores' surface of *B. anthracis* directly and sensitively. However, considering the high genomic similarity between *B. anthracis* and its relatives, the main challenge in the detection of *B. anthracis* spores is the performance of the recognition elements. Several previous studies have attempted to ensure the optimal performance of the recognition element. By screening a phage display peptide library, two peptides were identified to selectively bind to *B. anthracis* spores. The selectivity was enhanced by using these peptides in tandem in simple assays, which were expected to be economical and serve as portable *B. anthracis* spore detectors.[34] In a related phage display screening approach, the most specific spore-binding peptide bound 3.5- to 70-fold better to the spores of *B. anthracis* Sterne than to those of other *Bacillus* species.[35]

Generally, monoclonal antibodies (mAbs) have a higher affinity for their targets than their counterparts (e.g., binding peptides, aptamers, and scFvs). mAbs against exosporium protein BclA[36] and mAbs against the *B. anthracis* cell wall and

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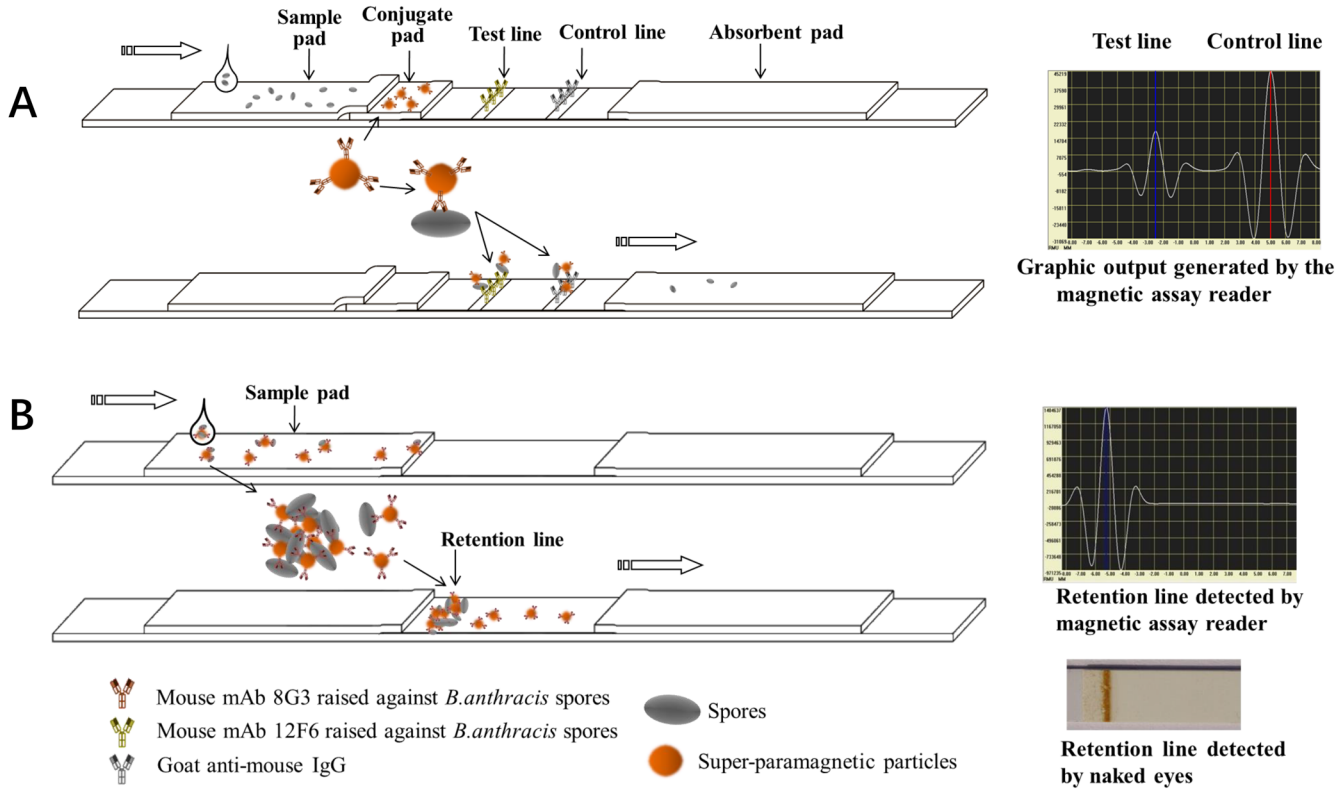

Figure 5. Schematic of superparamagnetic lateral-flow immunoassays (LFIA) for *B. anthracis* spore detection. (A) Classic LFIA. Reproduced with permission from ref 44. Copyright (2013) Elsevier B.V. (B) Road-closure-based LFIA. Reproduced with permission from ref 1. Copyright (2015) Elsevier B.V.

capsule[37] have been reported. Because the adaptive immunity in jawless vertebrates is mediated by clonally diverse lymphocytes that express variable lymphocyte receptors (VLRs, i.e., VLR-A and VLR-B) created by the combinatorial assembly of leucine-rich repeat (LRR) gene segments, a recombinant VLR-B antibody specific for BclA was obtained by the heterologous expression of full-length VLR-B cDNAs derived from lymphocytes of lamprey larvae immunized with the exosporium of *B. anthracis*.[38] These antibodies showed high specificity and sensitivity to *B. anthracis* but solely recognized either vegetative cells or spores.

We used intact *B. anthracis* spores as an immunogen to develop mAbs against the surface components of spores. After several rounds of panning with *B. anthracis* spores (positive) and *B. cereus* spores (negative), several mAbs's with high selectivity and sensitivity were obtained, and the S-layer protein, EA1, was identified as the target antigen.[39] In an extended study, we developed an S-layer-based 2D nanozyme array for the highly sensitive detection of anthrax antibodies by the self-assembly of EA1 *in vitro*.[40] We also found that EA1 existed on the surface of *B. anthracis* spores and vegetative cells, and our mAbs could be used for the species-specific and direct detection of both *B. anthracis* spores and vegetative cells. These mAbs's laid the foundation for the development of several biosensor platforms for the direct detection of *B. anthracis* spores.

SPR Biosensor

Surface plasmon resonance (SPR) biosensors are routinely used to study biomolecular interactions. In the SPR sensor, the SPR angle changes with the refractive index in the immediate vicinity of the surface of the sensor chip, and the refractive index changes with the mass of biomolecules bound to the surface of the sensor chip.[41] Therefore, the affinity of biomolecular interactions can be analyzed in real time by monitoring changes in the SPR angle.

Although SPR has many advantages, such as high sensitivity and easy operation, it is difficult to use it to analyze intact micrometer-sized spores and vegetative cells because large analytes can inhibit the effective penetration of the evanescent field and block the microfluidic channel. Accordingly, we conducted subtractive inhibition assays and detected mAbs not bound to the spores using SPR.[42] We found that this method could detect whole *B. anthracis* spores at a concentration of as low as 10^4 spores/mL (200 μL volume), with no cross reaction with high concentrations of other close *Bacillus* spores. Considering that the 50% lethal dose (LD50) of anthrax inhalation for humans is approximately 3000−50 000 spores,[43] the proposed detection strategy could be used for the detection of *B. anthracis* spores in the field if a portable SPR machine is available.

QCM Biosensor

We constructed a QCM detection system by immobilizing anti-*B. anthracis* mAbs onto the surface of a gold chip.[3] When a voltage was applied across the sensor, the quartz crystal induced a small shear vibration at the oscillating frequency. When the spores were recognized and bound by the immobilized mAbs, the vibration varied with the number of spores bound to the surface layer. As a result, LODs of 10^3 spores/mL for *B. anthracis* spores and 7×10^2 CFU/mL for *B. anthracis* vegetative cells could be achieved within 30 min.

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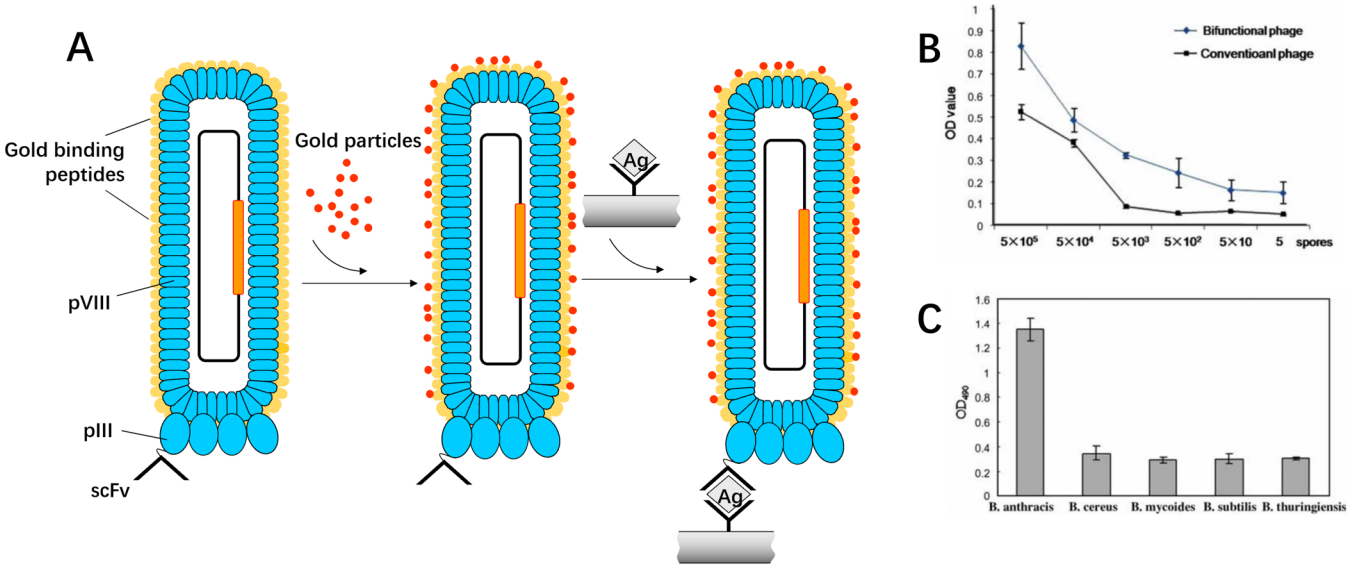

Figure 6. Phage-display-based bifunctional biosensor. (A) Phage-displayed scFvs and many copies of the gold-binding peptide on its surface by fusion expression with pIII and pVIII, allowing *B. anthracis* recognition and signal amplification. (B) Detection sensitivity of a bifunctional phage immunoassay with silver enhancement and phage ELISA for *B. anthracis* spores. (C) Specificity of the bifunctional phage-based immunoassay. Adapted with the permission from ref 45. Copyright (2010) Elsevier B.V.

Compared to the SPR system, the mAb-functionalized QCM sensor has a low LOD and a short detection time.

Superparamagnetic Lateral-Flow Immunoassays (LFIAs)

LFIAs have been widely used for the rapid detection of pathogens, drugs, and other analytes. Superparamagnetic particles have a rapid and sensitive magnetic response, excellent dispersibility, and steady magnetic signals that can be detected by the naked eye as well as with a device. We first developed a LFIA system for the detection of *B. anthracis* spores by labeling *B. anthracis*-specific mAbs with superparamagnetic particles (Figure 5A).[44] The system can detect as few as 400 pure *B. anthracis* spores (4000 spores/mL) in 30 min with a linear range of 4×10^3–4×10^6 spores/mL. For the simulated samples, LODs of 200 spores/mg of milk powder and 130 spores/mg of soil were achieved; interestingly, the large immunocomplex of superparamagnetic nanoparticle-mAb-spores can block the pores of chromatographic strips and form retention lines on the strips. On the basis of the phenomenon of "road closure," we developed a simple LFIA system without test and control lines by manipulating the formation of immunocomplexes and retention lines and realizing the naked-eye and magnetic dual-mode detection of *B. anthracis* spores (Figure 5B).[1] This new approach can detect as few as 500 pure *B. anthracis* spores within 5 min of chromatography or 20 min of total run time when using superparamagnetic nanoparticles of 300 nm diameter. In addition, this LFIA system showed excellent anti-interference performance from white powders, with LODs of 60 spores/g of milk powder, 200 spores/g of starch, and 500 spores/g of baking soda without any sample pretreatment. The road closure LFIA shared a similar detection sensitivity with the former LFIA but had a significantly shortened detection time. In addition, it did not require the preloading of antibodies onto the sample pad; consequently, it had a longer shelf time than the reported LFIA. This low-cost and convenient LFIA can also be used for the detection of many other large analytes.

Phage-Display-Based Biosensor

Phage display technology involves cloning a foreign polypeptide or protein gene into the appropriate position of the phage genome and displaying it on the surface of the phage through fusion expression with the phage coat protein. The displayed polypeptide or protein can maintain a relatively independent spatial structure and biological activity to facilitate the recognition and binding of the target molecule. Considering that the phage display system is easy to produce and engineer at low cost, we used an scFv derived from *B. anthracis*-specific mAbs to construct a phage-display-based bifunctional biosensor for the detection of *B. anthracis* (Figure 6).[45] In this system, the scFv was fused with the protein, pIII, from the bacteriophage for coexpression, and the gold-binding peptide was fused with phage protein pVIII (~2700 copies). The engineered phage can specifically and sensitively recognize *B. anthracis* through the scFv as well as bind to numerous gold nanoparticles with gold-binding peptides for signal amplification and detection with the naked eye.

The phage can be used to display biorecognition molecules and other molecules of interest on its surface; it can also serve as a biorecognition tool.[46] Natural species-specific phages have been reported for the identification of *B. anthracis*.[47] PlyG is a lyase derived from gamma phages that can specifically kill *B. anthracis*.[48] We focused on the interactions of PlyG with *B. anthracis* spores and vegetative cells and identified the key sequences of PlyG for biorecognition.[49] Several groups have combined phages with different sensor platforms to detect *B. anthracis* spores or vegetative cells.[50−52] In the future, a combination of synthetic and structural biology approaches may help to design highly specific biosensing elements by understanding the molecular structure of the natural phages specific to *B. anthracis* spores.

B. anthracis spore simulants and other features of spores such as dipicolinic acid (DPA) were also tested with biosensors.[53−56] Although these sensors were not used to directly detect *B. anthracis* spores, they can be alternative ways to test the new biosensing protocols.

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Table 1. Brief Summary of Biosensing Platforms for the Detection of *B. anthracis*[a]

targets		recognition elements	biosensing platforms	limits of detection	advantages	disadvantages	ref
DNA	*pagA/Ba813/capB*	DNA primers	multiplex PCR-on-a-chip	1 pg DNA	• naked-eye detection • potential method for on-site detection sensitive and low cost	• requires disrupting *B. anthracis* spores or vegetative cells • increases the risk of infection	15
	pagA/Ba813/capB	DNA primers	LAMP	10 spores for culture and 100 spores for powder samples	• only a heat blocker required • potential method for on-site detection • sensitive and low cost	• low anti-interference performance	18
	pagA/Ba813	DNA probes and DNA primers	asymmetric PCR and QCM	3.5×10^2 CFU/mL	• good linear range of 3.5×10^2–3.5×10^7 • CFU/mL potential method for on-site detection		2
	not given	commercial kits	RT-PCR and EI-MS	80.5 ± 36.5 genomes/well	• high sensitivity and specificity • high-throughput detection • multiplex detections of respiratory organisms in BAL fluid specimens		20
	pagA	DNA probes	SERS-PCR	0.1 pM	• enabled DNA detection after only five amplification cycles • wide dynamic range of 0.1−1000 pM		21
toxin-related proteins	LF/PA	antibodies	SERS-immunoassay	1.4 pM for LF and 28 fM for PA	• allow detection in human serum • sensitive	• require disrupting *B. anthracis spores* or vegetative cells • increase the risk of infection	23
	PA	aptamers	SWNT-based nano aptasensor	1 nM	• reusable six times • high anti-interference performance • potential point-of-care diagnosis method		24
	PA	peptides	electrochemical ELISA	170 pg/mL (2.1 pM)	• good thermal stability • sensitive detection in 1% serum		25
	PA	scFvs	protein chip	10 pg PA and 500−1000 vegetative cells	• high specificity • low cost • potential method for on-site detection		26
	PA	peptide polymers	fluorescence nanosensor	15 zM	• ultrasensitive		28
	PA	aptamers	electrical graphene aptasensor	1.2 aM	• ultrasensitive		29
	PA	antibodies	electrochemical immunosensor	50 pg/mL	• short analysis time (35 min) • allow detection in human serum		30
	PA	antibodies	nanosensor	550 fM	• device-free dual readout • allow detection in human serum • sensitive		31
	EF	no	MS	20 fg/mL (225 zM)	• high specificity and sensitivity • allow analysis of human serum/plasma samples • potential early diagnosis method		32
spore surface	not given	peptides	immunoassay	not determined	• direct detection without disrupting spores • high specificity	need to further improve the detection sensitivity	34
	BclA	mAbs	immunoassay	not determined	• direct detection without disrupting spores • high specificity		36
	EA1	mAbs	SPR	10^4 spores/mL (200 μL volume)	• direct detection without disrupting spores		42

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Table 1. continued

targets	recognition elements	biosensing platforms	limits of detection	advantages	disadvantages	ref
				• rapid detection (40 min)		
EA1	mAbs	QCM	10^3 spores/mL	• high specificity		3
EA1	mAbs	superparamagnetic LFIA	4×10^3 spores/mL (100 μL volume) 200 spores/mg of milk powder 130 spores/mg of soil • high specificity and low cost • potential on-site detection method	• direct detection without disrupting spores • rapid detection (30 min) • high anti-interference performance		44
EA1	mAbs	superparamagnetic LFIA	500 pure spores 60 spores/mg of milk powder 200 spores/mg of starch 500 spores/mg of baking soda • high specificity • potential on-site detection method	• direct detection without disrupting spores • rapid detection (20 min) • high anti-interference performance • long shelf time and low cost		1
EA1	scFvs	phage display-based biosensor	5×10^3 spores	• direct detection without disrupting spores • high specificity		45
not given	JRB7 phage	magnetoelastic biosensor	5×10^3 spores/mL	• direct detection without disrupting spores		52

[a]Abbreviations: LAMP, loop-mediated isothermal amplification; QCM, quartz crystal microbalance; EI-MS, electrospray ionization mass spectrometry; BAL, bronchoalveolar lavage; SERS, surface-enhanced Raman scattering; SWNT, single-walled carbon nanotubes; scFvs, single-chain antibodies fragments; MS, mass spectrometry; mAbs, monoclonal antibodies; SPR, surface plasmon resonance; LFIA, lateral-flow immunoassays.

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In summary, although various sensing strategies for *B. anthracis* detection are available, each of them has some advantages and disadvantages (Table 1). No approach can meet all of the requirements of *B. anthracis* detection. Hence, the most appropriate approach needs to be selected on the basis of the test scenario.

CONCLUSIONS

Biosensors are the most suitable tools for field testing *B. anthracis*. Many biosensors have been developed for this purpose. Their performances are comparable to but surpass each other through constantly improving to meet the major requirements of on-site detection: sensitivity, simplicity, and portability. The direct detection of spores is of particular concern against natural infections and biothreats. The current biosensor technology can have an LOD of as low as almost 10^2 spores. Further improvement in the LOD calls for new technology and interference factors in complex samples to be overcome. The comprehensive application of nanobiosensing and artificial intelligence (AI) could be an effective way (i.e, nanobiosensing can have higher sensitivity than the conventional biosensors), and AI can identify the authenticity of signals through machine learning.

Since plasmids carrying the virulence genes of *B. anthracis* may be transferred between *Bacillus* species, exploiting more gene markers on chromosomes is a rigid demand. With the integration and development of multiomics, massive amounts of omics data can be combined with AI technology to overcome the limitations of current technologies. In the future, the competitive detection panels could include a number of targets from various pathogens. On the basis of these findings, gene fingerprints could be designed using high-throughput multiplex-PCR-on-a-chip to sensitively and accurately identify *B. anthracis* as well as the other pathogens.

On the other hand, it has been a trend in the application of gene sequencing technologies, such as next-generation sequencing (NGS) and whole-genome sequencing (WGS), in the identification of infectious pathogens.[57] Miniaturization of the sequencing machines is already under development. A good example is Oxford nanopore technology, which performs DNA or RNA sequencing directly in real time and has commercial portable devices that are available.[58] In principle, it is a perfect integration of electrochemical biosensing and nanobiology. Since the genomic similarity and gene transfer between closely related *Bacillus* species pose considerable challenges, rapid and convenient DNA sequencing platforms would be optimal in the field detection of *B. anthracis*.

Finally, with the development of a mobile network, cloud computing, and flexible electronics, the above-described biosensors will allow the use of smart devices to remotely detect *B. anthracis*, which is of great significance for controlling the dissemination of *B. anthracis*.

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