

Rapid and Specific Detection of *Bacillus cereus* Using Phage Protein-Based Lateral Flow Assays

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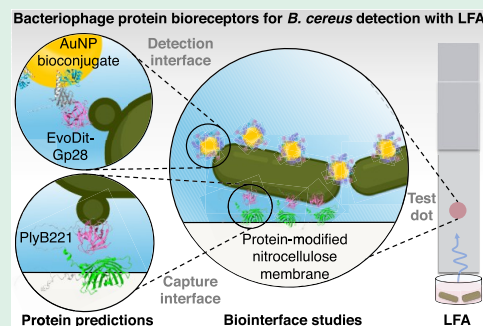
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ABSTRACT: Rapid and precise diagnostic techniques are essential for identifying foodborne pathogens, including *Bacillus cereus* (*B. cereus*), which poses significant challenges to food safety. Traditional detection methods are limited by long incubation times and high costs. In this context, gold nanoparticle (AuNP)-based lateral flow assays (LFAs) are emerging as valuable tools for rapid screening. However, the use of antibodies in LFAs faces challenges, including complex production processes, ethical concerns, or variability. Here, we address these challenges by proposing an innovative approach using bacteriophage-derived proteins for pathogen detection on LFAs. We used the engineered endolysin cell-wall-binding domain (CBD) and distal tail proteins (Dit) from bacteriophages that specifically target *B. cereus*. The protein-binding properties, essential for the formation of efficient capture and detection biointerfaces in LFAs, were extensively characterized from the microstructural to the LFA device level. Machine-learning models leverage knowledge of the protein sequence to predict advantageous protein orientations on the nitrocellulose membrane and AuNPs. The study of the biointerface binding quantified the degree of attachment of AuNPs to bacteria, providing, for the first time, a microscopic model of the number of AuNPs binding to bacteria. It highlighted the binding of up to one hundred 40 nm AuNPs per bacterium in conditions mimicking LFAs. Eventually, phage proteins were demonstrated as efficient bioreceptors in a straightforward LFA prototype combining the two proteins, providing a rapid colorimetric response within 15 min upon the detection of 10^5 *B. cereus* cells. Recombinantly produced phage binding proteins present an opportunity to generate a customizable library of proteins with precise binding capabilities, offering a cost-effective and ethical alternative to antibodies. This study enhances our understanding of phage protein biointerfaces, laying the groundwork for their utilization as efficient bioreceptors in LFAs and rapid point-of-care diagnostic assays, thus potentially strengthening public health measures.

KEYWORDS: *Bacillus cereus*, bacteriophage, bioreceptor, lateral flow assay (LFA), gold nanoparticle (AuNP), AlphaFold



1. INTRODUCTION

The detection and identification of bacterial pathogens in food and the environment have become of primary importance for health and ecosystem safety reasons.¹ The *Bacillus cereus* group, also referred to as *B. cereus sensu lato* (*s.l.*), includes several species known for their pathogenic potential including the causative agent of anthrax: *Bacillus anthracis* (*B. anthracis*).² Also, some *B. cereus* strains are commonly recognized as food-poisoning agents representing a serious threat for food safety as their toxins have been some of the most frequently reported causes of food-poisoning outbreaks due to bacterial toxins in 2022 at the EU level, according to the European Food Safety Authority (EFSA).³ Conventional, standardized detection methods for *B. cereus* in food and environmental samples, such as agar plate-based counting (ISO 7932:2004), require long incubation periods (up to 72 h for the whole detection process) and costly laboratory settings.⁴ Since more than two decades, the emergence of nanobiotechnologies, molecular chemistry processes, and functional nanomaterials have

enabled the development of rapid point-of-care diagnostic biosensors to address these drawbacks, including lateral flow assays (LFAs).^{5,6} LFAs work by loading a liquid sample (about 100 μ L) onto a strip composed of cellulosic pads and a nitrocellulose (NC) membrane. The sample migrates through the strip via capillary action. Detection in LFAs can occur in two formats, competitive and sandwich assays, with the latter being the most commonly reported for whole-cell bacterial detection. In the sandwich format, detection is achieved through the formation of an immunoassay sandwich at the designated test line (TL), involving two distinct biointerfaces. First, the capture biointerface is created by coating the NC

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membrane with specific bioreceptors, which enables the immobilization of bacterial cells at the TL. Second, the detection biointerface confirms the presence of bacteria at the TL when nanoparticles coated with specific bioreceptors bind to the bacterial cells. This interaction causes the TL to become visible to the naked eye, and it appears red when gold nanoparticles (AuNPs) are used. To date, antibodies (Abs) remain the gold standard bioreceptors in LFAs. For instance, Cox et al. reported an antibody-based LFA for rapid point-of-need *B. anthracis* detection with a limit of detection (LOD) of about 2.5×10^4 CFU/mL.⁷

Despite the ubiquitous applicability of antibodies as bioreceptors in LFAs, they present some limitations due to their production process, which still primarily depends on animal host infection and bleeding. They can suffer from variability (batch-to-batch), low specificity and stability, and relatively high prices due to the dependency and high pressure on the suppliers, especially highlighted during COVID-19 pandemics.⁸ Antibody generation using animal immunization raises scientific and ethical concerns.⁹ Also, generating, selecting, and mass-producing antibodies requires 16–24 months, which is much longer than the critical initial period when an outbreak is emerging. Therefore, there is an urgent need to find alternatives to antibodies when it comes to the development of new in vitro diagnostic tests.

Bacteriophages, or phages, are viruses that exclusively replicate within bacteria and are equipped with highly specific mechanisms to recognize, adsorb, infect, and eliminate their bacterial hosts during their life cycle. Their inherent stability across a wide range of pH values and temperatures renders them well-suited for applications in biotherapeutics¹⁰ and bacterial capture and detection.^{11–13} While whole phages have been used as bioreceptors in biosensor applications,¹⁴ their efficiency is hampered by their frequent incorrect orientation when immobilized on sensor surfaces, still posing a significant technical barrier to their widespread use in biosensor applications.¹⁵ Phages employ different proteins, enabling them to specifically bind components of the bacterial cell envelope. Among them, receptor-binding proteins and other accessory proteins such as evolved distal (EvoDit) facilitate phage adsorption onto the cell surface in the initial recognition step. Endolysins, through their cell-wall-binding domain (CBD), are involved in binding to the peptidoglycan, leading to the fatal lysis of the bacterial host following phage replication.¹⁶ The characterization of phage binding proteins has revealed their high affinity for bacterial cells and potential to display a broader host activity spectrum than their parental phages, which often target specific subsets of bacterial species.¹⁷ Furthermore, progress in cloning and gene expression techniques have enhanced the efficiency of producing these proteins as recombinant entities, for instance, by the *E. coli* expression system.¹⁸ Consequently, phage proteins hold promise for the detection of critical bacterial pathogens. They have been tested in various biosensor designs due to their suitable sizes and engineering adaptability from their genetic sequences,^{19,20} such as immobilized bioreceptors in the enzyme-linked immunosorbent assay (ELISA),²¹ on NC membranes²² and gold electrodes²³ for bacterial detection by electrochemical impedance spectroscopy, on AuNPs for surface plasmon resonance (SPR)-based detection,²⁴ and for magnetic separation for preconcentration purposes²⁵ or combined with bioluminescence detection²⁶ and real-time polymerase chain reaction (PCR) detection.²⁷ Although these

detection methods offer advantageous detection limits, they often require cumbersome and expensive equipment and complex operational procedures.

To address these limitations, Kong et al. showcased the use of a single phage binding protein, a modified CBD of LysB4 endolysin from *B. cereus* B4 phage, for developing capture and detection biointerfaces of a prototype lateral flow device that showed superior results compared to the antibody-based LFA, without any significant cross-reactivity.²⁸ Shin et al. exploited the same strategy to develop a multistep LFA device for the multiplex detection of foodborne pathogens, including the detection of *B. cereus* cells in approximately 20 min with an LOD of 10^5 CFU/mL.²⁹ Despite achieving functionality of the lateral flow assays, the single phage protein strategy developed by these authors leads to a scenario where both capture and detection biointerfaces target the same bacterial surface receptors. Therefore, the LFAs still require a two-step operation and necessitate absorbent pad replacement in a vertical arrangement, hindering the user-friendliness of the new devices compared with conventional LFAs and limiting their potential application. Additionally, the evaluation of the CBD-formed biointerfaces (e.g., protein orientation) was limited and predominantly reliant on theoretical assumptions. Given the pivotal role of bioreceptor immobilization on the NC membrane and AuNPs within LFAs,³⁰ a comprehensive characterization of the binding properties of bioreceptors is crucial.⁸ With the aim of exploring credible alternative bioreceptors to antibodies for bacterial detection in LFAs, these studies highlight the strong potential of using phage-binding proteins. However, they also underscore the need for further research to fully substitute antibody-based LFA functionalities with phage protein-based LFAs. This particularly emphasizes the importance of understanding the protein interaction dynamics at the biointerface level for effective integration into LFAs.

This study addresses these challenges by employing, for the first time, two phage binding proteins as specific bioreceptors for the capture and detection of *B. cereus* strains in LFA formats. We selected two recently identified phage binding proteins, i.e., the CBD of an endolysin (PlyB221)¹⁷ and an evolved distal tail (EvoDit-Gp28),³¹ encoded respectively by Deep-Blue³² and Deep-Purple³³ phages infecting the *B. cereus* group. These proteins were chosen to provide a broader binding spectrum compared with their parental phages while maintaining high specificity for the *B. cereus* group. Our approach aims to provide a deeper characterization of phage protein biointerfaces for biosensing, surpassing previous studies.^{25,28} We conducted a comprehensive assessment of their binding properties at the microstructural, biointerface, and LFA device levels.

At the microstructural level, computational analyses were performed to predict the molecular structures and binding orientations of PlyB221 and EvoDit-Gp28 on NC and AuNP surfaces, respectively. At the biointerface level, protocols were developed for the optimal implementation of the capture and detection biointerfaces, confirming their functionality and specificity through detailed characterizations. Specifically, we quantified the binding functionality and specificity of the protein–AuNP conjugates toward *B. cereus* whole cells by scanning electron microscopy (SEM), providing valuable insights into the adsorption capacity of nanoparticles on bacterial cells, which is crucial for determining the LFA performance. Finally, we developed a prototype AuNP-based

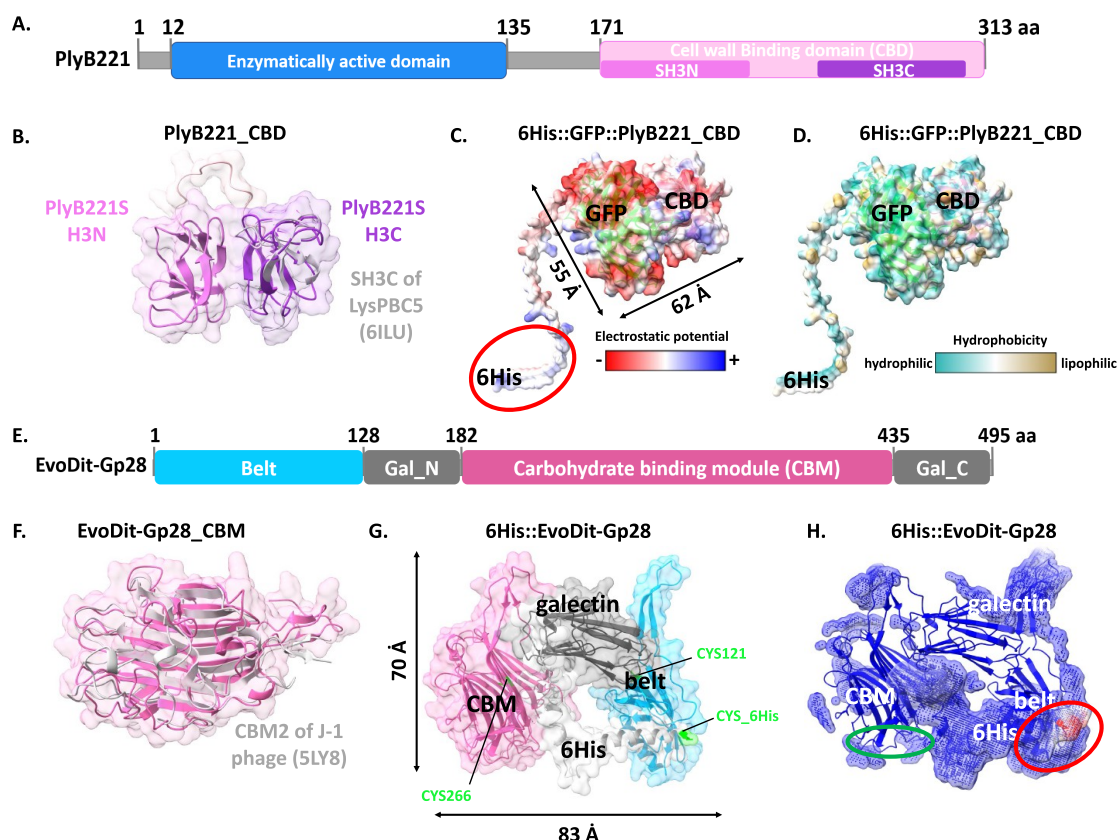


Figure 1. In silico analysis of PlyB221 and EvoDit-Gp28. (A) PlyB221 two-domain organization. The enzymatically active domain is responsible for the bacterial cell lysis, while the CBD (made of two SH3 domains) is responsible for the adsorption on the cell surface. (B) Ribbon and transparent surface representation of the predicted structure of the PlyB221 domain with its SH3_N (pink) and SH3_C (violet) subdomains. SH3_C is superposed with its closest structural homologue identified by Dali server, the SH3_C of LysPBC5 endolysin from *B. cereus* phage PBC5, whose crystal structure is available in the PDB server (gray, 6ILU). (C) Electrostatic surface potential distribution of the CBD of PlyB221 fused to an N-terminal GFP and a hexa-histidine tag used for its purification (6His::GFP::PlyB221_CBD). This protein, including the histidine tag, is used as the capture bioreceptor in our study. The red circle highlights the region potentially binding to the NC membrane. (D) Color representation of the hydrophobicity of 6His::GFP::PlyB221_CBD. (E) EvoDit-Gp28 had a three-domain organization. (F) Ribbon and transparent surface representation of the predicted structure of EvoDit-Gp28_CBM (pink) and superposition with its closest structural homologue, EvoDit_CBM2 of *Lactobacillus* phage J-1 (gray, 5LY8). (G) Ribbon and transparent surface representations of the Gp28 protein fused to an N-terminal hexa-histidine tag (6His::EvoDit-Gp28) with cysteine residues highlighted in red. This protein is used as the detection bioreceptor in our study. (H) Prediction of the preferred bound surface of 6His::EvoDit-Gp28 on the AuNP taking both the affinity scale and the surface accessibility into account using the blue to red scale (red highlights the most preferred bound surface).⁴¹ The accessible area of the protein calculation with NACCESS stand-alone program is depicted by surface representation.^{42,43} The red circle highlights the part of the protein predicted to bind to the AuNP that is distant from the part of the protein involved in binding to bacteria (green circle).

lateral flow device to demonstrate the applicability of our novel design using distinct phage binding proteins as bioreceptors for the specific detection of *B. cereus* strains, maintaining the user-friendly format of conventional LFAs.

This work enhances our understanding of recombinantly produced phage proteins and promotes their use in rapid diagnostics to substitute for antibody-based methods. This supports the transition to more ethical, versatile, and cost-effective diagnostic devices.

2. RESULTS AND DISCUSSION

2.1. Prediction of Phage Protein Molecular Structures and Binding Orientations. PlyB221 is the endolysin encoded by the *B. cereus* phage Deep-Blue (family *Hereleviridae*). Its peptidoglycan-degrading activity, involved in phage-mediated lysis of the bacterial host at the end of the phage replication cycle, has been confirmed and characterized in a previous work.¹⁷ PlyB221 was shown to display a broad activity spectrum, targeting all of the tested strains of the *B.*

cereus group and being restricted to *Bacillus* spp. PlyB221 turned out to be active in the pH range of 6–9 and to withstand up to 200 mM NaCl without loss of activity. The binding activity of its CBD, the domain of the protein responsible for its specificity, was also confirmed through a cell wall decoration assay, fusing a green fluorescent protein (GFP)-tagged CBD adsorbed onto the bacteria. In this work, the bioreceptor used as the LFA capture biointerface is the CBD of PlyB221 fused to an N-terminal GFP and a hexa-histidine tag used for purification (named PlyB221 hereafter; molecular weight (MW): 20.5 kDa; isoelectric point (pI): 5.79).¹⁷

In recent years, the microstructural characterization and engineering of such affinity proteins have been largely facilitated by the emergence of accurate bioinformatics tools including AlphaFold2, the DeepMind machine-learning protein structure prediction program that has largely revolutionized structural biology.^{34,35} Leveraging these tools holds promise not only for deepening our understanding of

bioreceptor characteristics but also for enabling the precise engineering of biointerfaces, thereby driving forward the development of LFAs. Therefore, *in silico* analyses were conducted to further characterize PlyB221, including the protein structure prediction using AlphaFold2. This confirmed that PlyB221_CBD consists of two tandem SH3 domains, which are similar to the tandem SH3 domains of LysPBC5, the endolysin of *B. cereus* phage PBC5 (Figure 1A,B). The CBD binds to the peptidoglycan (PG) at the bacterial cell surface via the tandem SH3 domains in a bidentate manner and confers the host specificity to the endolysin.³⁶

Capture bioreceptors are mainly immobilized through adsorption on the NC membrane. While the initial electrostatic interactions between the NC and protein bioreceptors play a significant role in the adsorption dynamics, hydrophobic forces, van der Waals forces, and the surrounding buffer environment also contribute to the overall long-term stability and immobilization on the NC membrane surface.³⁷ These forces can affect protein orientation and binding stability, which vary depending on the pH, protein isoelectric point, buffer composition, and NC membrane surface properties.³⁰

Figure 1C highlights the positive charges of the PlyB221 hexa-histidine purification tag that are likely to interact with the strong dipole of the nitrate group of the NC membrane (electrostatic affinity). Indeed, the purification tag contains approximately twice more positive residues compared to the GFP and approximately three times more positive residues compared to the CBD. Notably, at the neutral pH used during LFA operation, the side chains of arginine and lysine are predicted to be positively charged. Figure 1D shows a uniform distribution of hydrophobic and hydrophilic residues between domains, which is therefore not likely to play a role in the preferential binding of the protein to the NC (ca. 85% of the residues are hydrophobic or hydrophilic residues in each domain). All in one, our analysis strongly suggests an attachment of PlyB221 to the NC membrane through its purification tag, primarily driven by electrostatic interactions between the positive residues of the purification tag and the negatively charged membrane. In addition, the strong negative charges on the fused GFP could be responsible for electrostatic repulsion within the protein, potentially promoting the deployment of its different domains, which increases their accessibility. The GFP therefore most likely plays the role of a spacer between the purification tag and CBD, allowing reduced steric hindrance of the CBD for binding to bacterial cells.

The *B. cereus* siphophage Deep-Purple encodes an EvoDit protein termed EvoDit-Gp28, which was shown to bind to numerous *B. cereus* strains.³¹ EvoDits form central hexameric hubs in the phage tails and display at least one carbohydrate binding module (CBM) per monomer that binds to the bacteria and likely plays an accessory role in the adsorption process (i.e., recognition of the bacterial host at the onset of the phage life cycle). In this work, the AuNP labels were conjugated with the Gp28 protein fused to an N-terminal hexa-histidine tag.³¹ Further bioinformatic analyses of EvoDit-Gp28 confirmed the presence of a CBM, comprising long stretches and folds, which likely bind to the bacterial cell surface. Besides the CBM fold, EvoDit-Gp28 displays galectin and belt structural domains. The predicted structure of the EvoDit-Gp28 CBM fold is very similar to the CBM2 fold of *Lactobacillus casei* phage J-1 (Figure 1E,F).³⁸

The orientation adopted by proteins on AuNP surfaces determines their activity and interactions with bacteria or other

living organisms. The passive adsorption of proteins to AuNPs is a three-step noncovalent process based on a combination of separate but dependent phenomena: (a) long-range electrostatic interactions between the negatively charged nanoparticles and the positively charged sites on the protein; (b) hydrophobic attraction between the protein and the metal surface, reorganizing the protein layer; (c) a thiol–gold dative binding between the metal and protein cysteine residues definitively anchors the protein on the AuNP, as it was shown that cysteine has the highest affinity toward AuNPs and can irreversibly oxidize on the AuNP surface.^{39,40} Charged and hydrophobic residues are quite dispersed in EvoDit-Gp28 (data not shown), and the protein harbors three cysteine residues (Figure 1G). Recently, Xu et al. developed a model enabling high-throughput predictions of protein behavior on AuNPs considering the affinity scale and surface accessibility of both residues.⁴¹ This algorithm was applied to EvoDit-Gp28, which predicted an attachment of the protein to the AuNPs by the Cys residue in the region preceding EvoDit-Gp28 (containing, among others, the purification tag). This residue is located away from the bacteria binding surface of the CBM, therefore leaving its access sterically unaffected (Figure 1H). The attachment of EvoDit-Gp28 onto AuNPs via this surface cysteine residue guarantees an optimal orientation of the bioreceptor on the AuNP surface, with the CBM radially exposed toward the exterior of the surface. This configuration maximizes the probability of binding to bacterial cells.

These results suggest that engineered PlyB221 and EvoDit-Gp28 exhibit favorable orientations for bacterial cell capture and detection when immobilized on NC membranes and AuNPs, respectively.

2.2. PlyB221 Coating on NC Membranes as the Specific Capture Biointerface. NC membranes were coated with PlyB221 proteins through a process of adsorption influenced by electrostatic, hydrophobic, and hydrogen-bonding interactions and were observed by fluorescence microscopy due to the GFP-fused tag. Confocal laser scanning microscopy (CLSM) experiments were performed to explore the in-depth functionalization of the NC membranes with PlyB221 solution at 1 mg/mL concentration. Figure 2A shows the characteristic porous microstructure of the CN95 NC membrane revealed by PlyB221. To assess the distribution of the immobilized proteins within the membrane, the distribution of the fluorescence intensity emitted was investigated by CLSM in Z-stack mode.⁴⁴ Fluorescence intensity was observed in each stack slice after thorough washing. While this suggests a sufficient immobilization and distribution of PlyB221 to form an adequate biointerface, it should be noted that NC membranes typically used in LFAs may incorporate proprietary additives from various manufacturers that can further affect the protein binding and orientation.⁴⁵ Despite the fluorescence intensity not being identical on the upper and lower sides of the NC membrane, the coating was considered complete, validating the homogeneous functionalization of the membrane with such a protein concentration. These results are corroborated by previously published results on the analysis of NC membrane sections coated with fluorescent antibodies.⁴⁶

In order to validate the preservation of PlyB221 binding functionalities after immobilization, CLSM was performed to visualize the capture of *B. thuringiensis* (GBJ002) whole cells within NC membranes. NC membranes modified with PlyB221 proteins were incubated in *B. cereus* suspensions containing 10⁷ CFU/mL cells expressing the cyan fluorescent

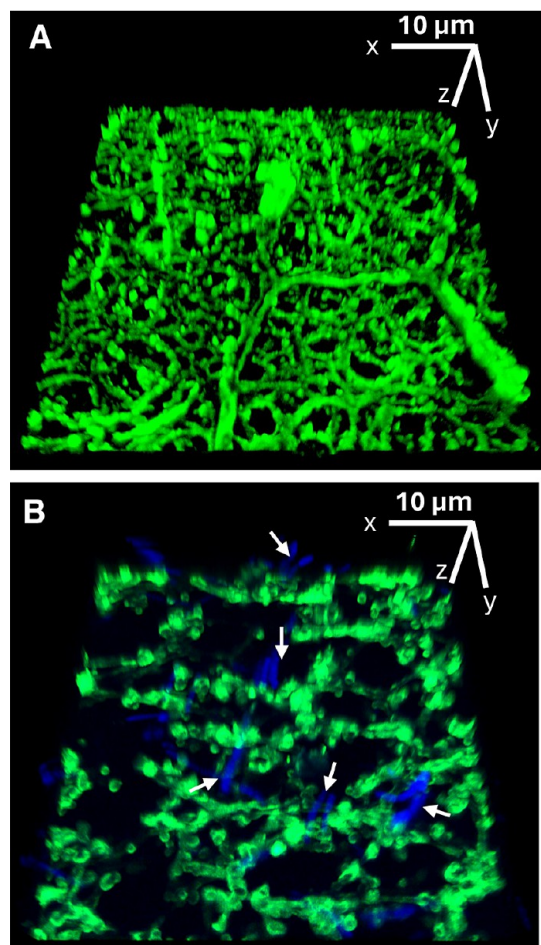


Figure 2. Assessment of PlyB221 on the NC membrane as the capture biointerface. (A) PlyB221-functionalized NC membrane (green) without bacterial cells (negative control). (B) Functionalized NC membrane in the presence of captured *B. cereus* whole cells in pores (blue), indicated by white arrows, built from Z-stack CLSM images over a 15 μm height.

protein (CFP), washed twice with phosphate-buffered saline (PBS), and then observed by CLSM, with laser settings such as to capture emissions from both the GFP-fused PlyB221 and CFP-fused retained bacterial cells. The visualization of *B. cereus* cells immobilized in the pores of the modified NC membrane was performed by 3D CLSM imaging (Figure 2B). Bacterial cell capture was confirmed down to 10 μm within the NC membrane (Figure S1A). SEM images of modified NC membranes incubated with bacteria provided a comparative visualization, confirming the CLSM results (Figure S1B). Time-series images recorded over 60 s via CLSM assessed the dynamics of bacterial cell capture on the NC membrane. Figure S2 shows bacterial attachment occurring within a minimum duration of 30 s. Although slight movements of bacteria around fixed points were observed, most bacteria remained immobilized against the membrane wall, indicating a strong binding affinity between the capture biointerface and the bacteria trapped within the NC pores.

Overall, our findings suggest a high binding affinity, providing robust immobilization of *B. cereus* cells on the biointerface, thus highlighting the potential of PlyB221 as a capture bioreceptor in LFA schemes.

2.3. Preparation of AuNP-EvoDit-Gp28 Conjugates.

The appropriate morphology (shape, size, and size distribution) and optical properties of AuNPs are essential for achieving the sensitivity and reproducibility of colorimetric LFAs. Based on previous studies on LFA detection of whole-cell bacteria,⁴⁷ 40 nm round-shaped AuNPs were selected as colorimetric labels to build the detection biointerface. Transmission electron microscopy (TEM) images of the bare AuNPs confirmed their homogeneous and highly spherical shapes (Figure 3A). Moreover, TEM image analysis shows an average diameter of 42.5 ± 3.5 nm (Figure S3A). Dynamic light scattering (DLS) experiments were also conducted on the bare AuNPs, confirming an average hydrodynamic radius of about 18 nm (Figure 3B). The slightly larger size observed in TEM may result from variations in the image analysis methods. The DLS measurement is considered more accurate as it aligns with the supplier data. The visible spectrum of the 40 nm AuNPs shows a sharp absorbance peak around 522 nm due to the SPR effect of the AuNPs, indicating that they emit a deep red color as expected (Figure 3C).

Careful optimization of the conjugation of EvoDit-Gp28 to AuNPs is imperative to regulating the loading density and orientation of the immobilized proteins. This optimization is essential to maintain the properties of AuNPs and the binding functionality of EvoDit-Gp28 toward *B. cereus* cells, crucial factors influencing the analytical performance of LFAs. The pH is recognized for its role in modulating the net charge of proteins through protonation/deprotonation of amino acids. Consequently, this alteration affects the electrostatic interactions between positively charged regions on the proteins and the negatively charged AuNPs, which is attributed to their citrate coverage layer, essential for achieving stable suspension.⁴⁸ This modulation of the net charge significantly influences protein immobilization processes.⁴⁹ The optimization of the AuNP conjugation with EvoDit-Gp28 binding proteins was performed via a gold aggregation test (Figure S4). Eight protein concentrations and three pH conditions were screened to identify the most stable conjugates requiring the lowest protein concentration. The optimal stability was achieved at pH 8 with the EvoDit-Gp28 concentration set at 80 $\mu\text{g/mL}$. TEM, DLS, and UV-vis spectroscopy measurements were performed on AuNPs after conjugation with EvoDit-Gp28 to evaluate the changes in the size and stability of the nanoparticles. TEM images revealed blurred corona (~ 10 nm thickness) around the AuNPs, which has already been reported as successive protein layers adsorbed on the AuNPs (Figure 3D).⁵⁰ The TEM images also showed the repetitive occurrence of small aggregates (2–10) of AuNPs, suggesting small aggregation during the conjugation process. This was supported by DLS experiments showing a slightly larger mean hydrodynamic radius for the conjugated AuNPs (ca. 42 nm, biggest peak) compared with the bare AuNPs, which can be attributed to a protein coating deposited on the surface of the nanoparticles and associated with a broader range of small aggregate sizes (Figure 3B). The peak observed at around 3–4 nm is most likely due to residues, impurities, or protein aggregation in the solution. This is a common issue, sometimes termed as the solvent peak. The 400 nm peak can also be attributed to the presence of small aggregates of AuNPs after conjugation, confirming the TEM results and indicative of the complex interactions that can occur during the passive conjugation process. Similar aggregation phenomena have previously been documented for 40 nm AuNPs when utilizing

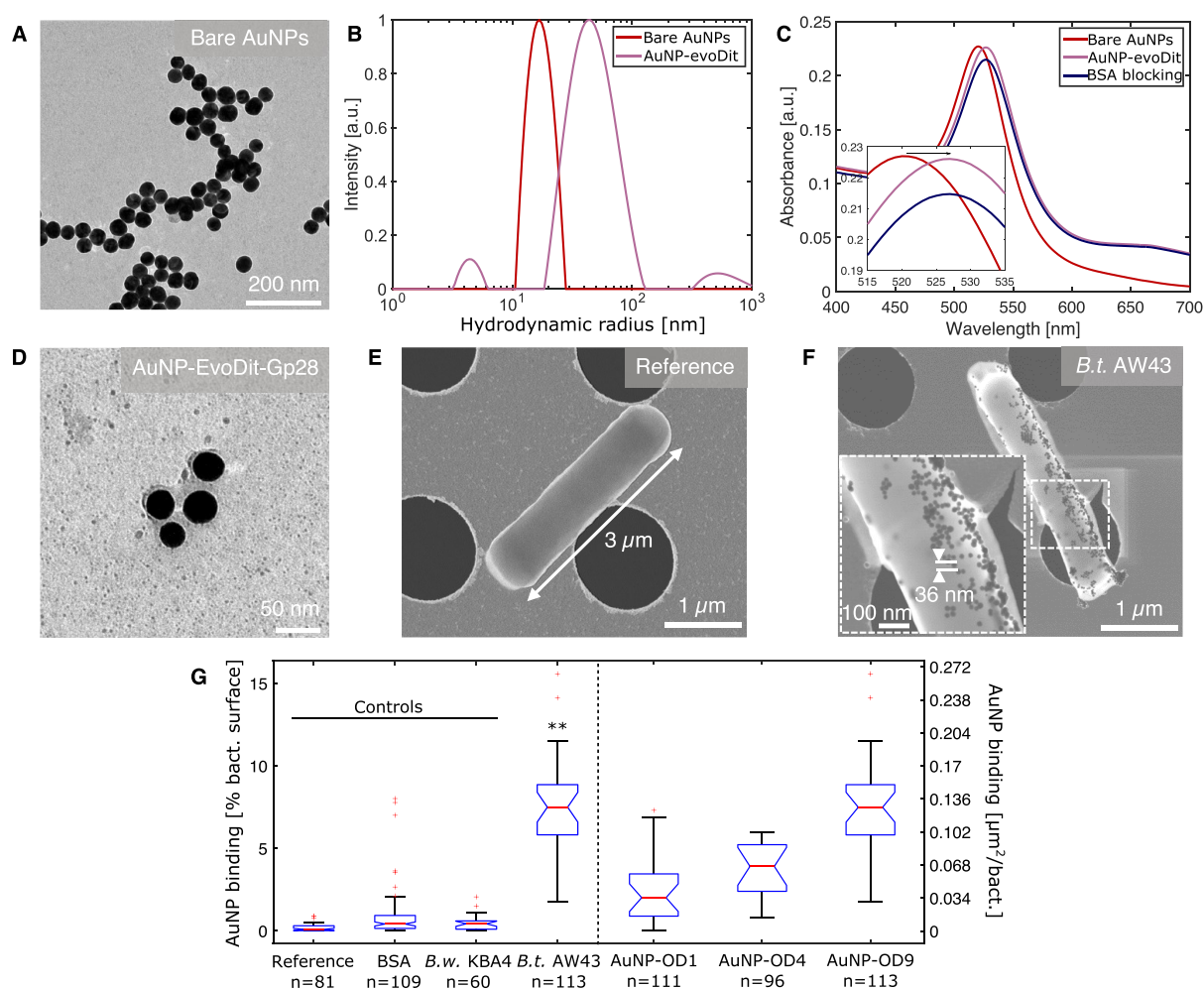


Figure 3. Characterization of the AuNP-EvoDit-Gp28 detection biointerface. (A) TEM image of bare AuNPs. (B) Size distribution by intensity of the hydrodynamic radii of bare and conjugated AuNPs measured by DLS. (C) Visible spectra of bare AuNPs, AuNPs conjugated with EvoDit-Gp28, and AuNPs blocked after conjugation. The slight shift of the absorbance peak from 522 to 528 nm reflects the immobilization of the proteins onto the AuNP surface (inset). (D) TEM image of 40 nm AuNPs conjugated with EvoDit-Gp28. (E) STEM reference image of *B. cereus* cells without AuNPs, exhibiting a rod shape and sizes varying from 1 to 3 μm. (F) STEM images of *Bacillus thuringiensis* AW43 cells labeled with AuNP-EvoDit-Gp28 conjugates (black spheres) after incubation and centrifuge washings. (G) (Left) Quantification of the AuNP labeling on bacterial cells indicates a significant decrease in binding events under control conditions compared with the sensitive strain *B. thuringiensis* AW43 (BSA: bacterial cells subjected to incubation with AuNPs functionalized exclusively with BSA, i.e., negative control). This observation implies the binding functionality of AuNP-EvoDit-Gp28 conjugates to *B. cereus* cells characterized by strong adsorption and specificity. Two independent experiments were conducted, showing similar differences. Data shown are means of *n* bacterial cells. Significant shifts (***p* < 0.001) are indicated. (Right) Quantification of the AuNP labeling of *B. cereus* cells for different concentrations of AuNPs (OD 1, 4, and 9). Quantitative results are presented in Table 1 (OD: optical density; data from two independent experiments).

alternative bioreceptors (i.e., antibodies).⁴⁷ The visible spectrum of AuNPs-EvoDit-Gp28 reveals a peak at 528 nm, indicating a peak shift of 6 nm compared to the bare AuNP solution (Figure 3C). This shift reflects the binding of the proteins onto the AuNP surface.⁵¹ To prevent nonspecific adsorption of the AuNP conjugates in the NC membrane, the modified nanoparticles were subsequently blocked with a bovine serum albumin (BSA) layer. The adsorption of BSA onto the AuNP surface was characterized by a slight decrease of the UV absorbance peak (Figure 3C). The stability of the AuNP conjugates blocked with BSA was assessed over 1 week by UV-vis spectroscopy. The absorbance peaks remained unchanged (Figure S3B), confirming the good stability of the conjugate particles.

2.4. Cell Binding of AuNP-EvoDit-Gp28 as a Specific Detection Biointerface. Prior to LFA testing, we compre-

hensively assessed and optimized the detection biointerface by validating the ability of AuNP conjugates to specifically bind to *B. cereus* host cells, which is pivotal to their suitability in LFAs.

Conjugate AuNPs were incubated with bacterial suspensions to investigate the interaction between EvoDit-Gp28 immobilized on AuNPs and bacteria in aqueous solutions. Bacterial binding, or labeling, was subsequently examined through the acquisition of STEM micrographs. To enhance the contrast between the cell membrane and AuNPs, fixation of the bacteria–AuNP complexes with glutaraldehyde solution was necessary. Unlabeled bacteria exhibited the expected rod shape and dimensions after glutaraldehyde fixation (Figure 3E). When incubated with AuNP conjugates, the STEM images of bacterial cells clearly show an inhomogeneous covering of the AuNPs on the bacterial surfaces (Figure 3F). In the absence of glutaraldehyde treatment, image interpretation is hindered by

Table 1. Quantification of AuNP Binding to *B. cereus* Cells for Different Concentrations of AuNP Conjugates

AuNPD OD ^a	AuNP number ^b [#/sample]	bact. cover. ^c [% bact. surf.]	bact. cover. [$\mu\text{m}^2/\text{bact.}$]	numb. AuNP [# /bact.]	AuNP bound [%]
1	3.15×10^9	2.54 ± 2.18	0.044 ± 0.04	35 ± 32	≈ 1.7
4	1.4×10^{10}	3.92 ± 1.67	0.066 ± 0.03	53 ± 25	≈ 0.4
9	3.15×10^{10}	7.82 ± 3.05	0.13 ± 0.072	103 ± 58	≈ 0.2

^aOD (absorbance at 520 nm). ^bConsidering 35 μL of 40 nm AuNP conjugates incubated with 150 μL of 10^7 CFU/mL overnight *B. cereus* suspension. ^cBact. cover.: Bacteria surface coverage.

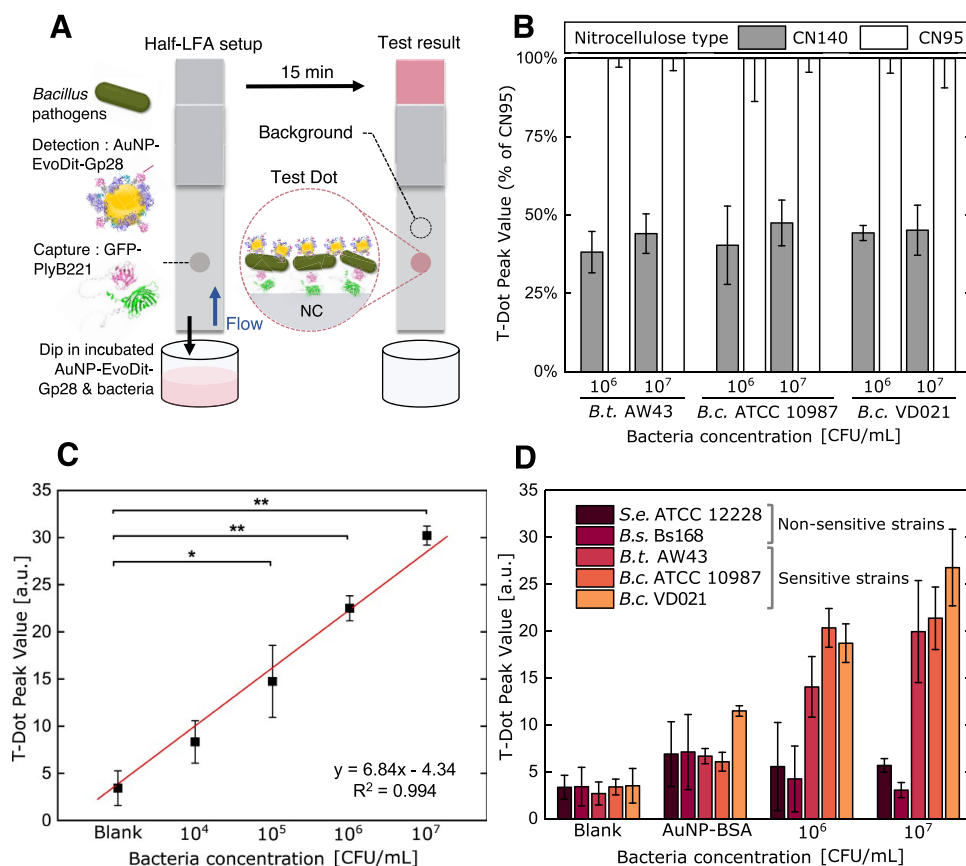


Figure 4. Half-stick testing for phage protein binding assessment in the LFA format. (A) Schematic representation of the test dot based on half-stick LFA. (B) Half-stick testing with CN140 and CN95 NC membranes reveals that CN95 is more appropriate to whole-cell bacteria flow for the three tested *B. cereus* s.l. strains (*B. thuringiensis* AW43 and *B. cereus* ATCC 10987 and VD021), most probably due to its larger pores (8 and 12 μm for CN140 and CN95, respectively). (C) Sensitivity study of the half-stick LFA toward VD021 strain, suggesting an optical LOD of about 10^5 CFU/mL. Significant results (* $p \leq 0.05$, ** $p \leq 0.01$) are indicated. (D) The detection of 10^6 and 10^7 CFU/mL suspensions of the three *B. cereus* sensitive strains with respect to blanks (no bacteria in solution) confirmed the broad applicability of the phage proteins in the LFA format. The specificity of the sensor was validated with bacterial species nonsensitive to the phage proteins (*Staphylococcus epidermidis*, S.e.; *Bacillus subtilis*, B.s.). AuNPs coated solely with BSA (AuNP-BSA) were tested as the negative control to evaluate nonspecific adsorptions. All of the conditions were tested with triplicate half-sticks ($n = 3$). Experiments (B)–(D) were performed independently with different batches of half-sticks.

the limited contrast between AuNPs and bacteria, and the morphological integrity of bacteria is substantially compromised (data not shown). Given that the bacteria labeled with AuNPs underwent washing before the fixation process, these findings indicate a robust affinity between the AuNP-EvoDit-Gp28 conjugates and the *B. cereus* surface.

The specificity of AuNP labeling on *B. cereus* cells was evaluated by quantitatively comparing the extent of AuNP binding with bacterial surfaces among different *B. cereus* s.l. strains. Image analyses were conducted on the high-contrast STEM micrographs to quantitatively measure the surface coverage of bacterial cells by AuNPs. First, 10^7 CFU/mL *Bacillus thuringiensis* AW43 cells, sensitive to EvoDit-Gp28,³¹ were incubated with AuNP conjugates at OD 9 to serve as the reference. Here, the OD of the AuNP solution refers to the

absorbance value at 520 nm. Absorbance values are directly related to the number of AuNPs in the solution.⁵² STEM images of the strain AW43 showed significant binding between AuNP-EvoDit-Gp28 conjugates and the cell surface (Figure S5). The adhesion efficiency is validated through statistical analysis, revealing ca. $7.85 \pm 3.05\%$ coverage of the bacterial surface with AuNP conjugates, equivalent to $0.13 \pm 0.07 \mu\text{m}^2$ surface area covered by the AuNP conjugate (Figure 3G), demonstrating significant variability among bacteria. To prove the specificity of the labeling, AuNP conjugates were then incubated with *Bacillus weihenstephanensis* KBA4, insensitive to EvoDit-Gp28.³¹ The binding events exhibited a significant reduction compared with the sensitive bacteria (Figure S5), with adhesion efficiencies of $0.49 \pm 0.47\%$ bacterial surface coverage, or $9.1 \times 10^{-3} \pm 1 \times 10^{-2} \mu\text{m}^2/\text{bacterium}$ (Figure

3G), thereby confirming the specificity of the conjugates. To investigate the extent of nonspecific adsorption of AuNPs on *B. cereus* s.l., strain AW43 cells were incubated with AuNPs coated solely with BSA, which lacks specific interaction with bacterial cell surface receptors (negative control). Again, binding events of BSA-capped AuNPs were significantly low ($1.14 \pm 1.69\%$ bacterial surface coverage) as compared to AuNP-EvoDit-Gp28 conjugates. The notable variability observed (evidenced by several outliers in Figure 3G) and the presence of relatively large AuNP-BSA covered areas on the bacterial surface (Figure S5) may be attributed to the formation of medium- to large-sized aggregates resulting from uncontrolled BSA coating. These aggregates could facilitate nonspecific adsorption onto bacterial cells due to their size. A reference condition lacking any AuNP conjugates was included (Figure 3E). The analysis algorithm applied to STEM images reported minimal AuNP coverage of the bacterial surface ($0.19 \pm 0.23\%$), primarily attributable to measurement errors arising from variations in bacterial shapes, occasionally incorporating dark areas from outside the bacterial surface. Finally, the significance of the specificity tests was confirmed using a two-sample *t*-test on the percentage of the bacterial surface covered by AuNPs, establishing that the mean AuNP binding coverage for the AW43 strain significantly differed from that of the control conditions (p -value $< 10^{-4}$ for each).

Bacteria possess numerous specific receptors on their cell surface, facilitating the binding of phage proteins and enabling the capture of numerous nanoparticles. To optimize the concentration of AuNP conjugates required for the adequate coverage of bacteria and consistent detection of bacterial cells in LFAs, sensitive *B. cereus* cells were further incubated with varying concentrations of AuNPs (OD 1, 4, and 9), and STEM image analyses were conducted. Different levels of adsorbed AuNPs were observed on the surfaces of bacterial cells (Figure S6), as reported in Figure 3G and detailed in Table 1 (refer to the Supporting Information for computation details). The number of AuNPs bound per bacterial cell clearly increases with higher AuNP conjugate concentrations, despite high variability between cells. The greater the accumulation of AuNP conjugates on the bacterial surface, the higher the expected sensitivity of the lateral flow test, as the brightness of the TL depends on the amount of AuNPs accumulated at the TL.⁵³ As indicated in Table 1, AuNP conjugates at OD 9 appear to be the most suitable for achieving a significant coverage of the bacterial surface.

Considering the well-established phenomenon wherein the grafting of biomolecules onto other surfaces can alter their intrinsic structure and properties,⁵⁴ it was crucial to assess the impact of the grafting of the phage protein used in this study on their binding performance and specificity. Taken together, our findings indicate favorable binding properties of the AuNP conjugates, suggesting suitable immobilization orientation of EvoDit-Gp28 on the AuNPs and demonstrating the retention of its specificity. The quantitative analysis of the AuNPs bound to individual *B. cereus* cells enabled us to establish a model describing the binding of conjugated AuNPs to host cells. Our findings suggest the necessity of adding AuNP conjugates in excess in the LFA design to maintain a relatively consistent ratio of adsorbed AuNPs to bacteria in each detection experiment.

2.5. Proof-of-Concept of *Bacillus* Whole-Cell Detection in the LFA Format. To assess the robustness of phage protein biointerfaces under LFA settings, specifically, the flow

within a NC membrane, we employed a half-stick assay format, serving as a prototype LFA (Figure 4A). Herein, PlyB221 and EvoDit-Gp28 phage binding proteins were used for the capture and detection of *B. cereus* whole cells, respectively. As recommended by methodological studies of LFA development,⁸ we opted for dotted half-sticks over full LFAs due to their quick preparation, versatility in protocol optimization, and reduced reagent requirements. Indeed, while producing recombinant phage proteins is easier and more cost-effective compared to animal-derived antibodies in a university lab, creating large volumes of capture bioreceptors at required concentrations (1 mg/mL) is still challenging. Thus, we used a half-stick format and test dots to minimize material usage and facilitate prototype development.

As studied by Bergua et al., due to their large size, the proper flow of whole-cell bacteria is challenging and must be optimized by selecting the appropriate NC membrane.⁴⁷ The size of *B. cereus* cells, about $0.5\text{--}1.0\text{ }\mu\text{m} \times 2\text{--}5\text{ }\mu\text{m}$, and their tendency to form aggregates justify the selection of two NC membranes with large pore sizes (8 and $12\text{ }\mu\text{m}$ for CN140 and CN95, respectively) since clogging and retention to the membrane should be avoided to enable the flow of large bacterial cells. To determine the most suitable type of NC membrane, we evaluated the performance of a half-stick assay to various *B. cereus* strains sensitive to the phage binding proteins, including *B. thuringiensis* (AW43) and two *B. cereus* strains (ATCC 10987 and VD021). Test dots (T-Dots) were prepared by depositing PlyB221 onto CN140 and CN95 NC membranes. The quantification of AuNP-labeled bacterial cell capture was accomplished by assessing the intensity of the red coloration observed on the T-Dot (T-Dot intensity). Figure 4B depicts the T-Dot intensity values for the CN140 and CN95 NC membranes, depicted on a relative scale compared to the T-Dot intensity value of the CN95 membrane. Specifically, the CN95 membrane demonstrated the highest sensitivity for detecting bacterial suspensions of 10^6 and 10^7 CFU/mL for the three tested *B. cereus* strains. The CN140 membranes exhibited a persistent red coloration at the base of the half-stick, indicating partial pore clogging, likely caused by bacterial cells. Overall, numerous strips displayed partial pore clogging at the base when testing a bacterial concentration of 10^7 CFU/mL. Pore clogging in LFA pads caused by whole-cell bacteria is a commonly documented issue, a consideration that must be addressed in the complete LFA format. Various strategies exist to facilitate the optimal flow of bacteria and AuNPs between the pads, such as ensuring sufficient overlap between the conjugate pad and the NC membrane, or replacing cellulosic sample pads with glass fiber, which provides increased porosity and reduced nonspecific interactions.⁴⁷

The half-stick assays demonstrate the ability to optically quantify bacterial suspensions down to 10^5 CFU/mL within 15 min with significant statistical confidence (Figure 4C). The blank signal corresponds to the T-Dot intensity signal for half-sticks dipped in a solution devoid of bacteria. The difference in the signal between the 10^4 CFU/mL sample and the blank sample was not statistically significant at the 95% confidence level. Linear fitting ($R^2 > 0.99$) reveals a mathematical detection limit (3σ) of 9.37×10^4 CFU/mL and suggests good sensitivity with a linear range from 10^5 to 10^7 CFU/mL.

The successful detection of the three sensitive strains proves the broad-range applicability (versatility) of our phage protein LFA toward *B. cereus* cells (Figure 4D). Despite the variability observed among the strains and the slightly lower T-Dot

Table 2. Performance Comparison of Conventional Laboratory-Based Methods with AuNP-Based LFAs for the Detection of *B. cereus*

method	LOD [CFU/mL]	time	costs ^a	complexity	ref
culture	1	>48 h	medium	low	4
ELISA	10 ² – 10 ⁶	>2 h	medium	medium	4
PCR	1	>1 h	very high	very high	57
MALDI-TOF	10 ⁵	20 min ^b	very high	very high	4
LFA (Ab ^c)	2 × 10 ⁴	20 min	low	low (2 h with amplification)	7
LFA (Bact. CBD ^d)	10 ⁴	17 min	low	low (2-steps: AP replac. ^e)	28,29
LFA (Bact. CBD, Dit ^f)	9 × 10 ⁴	15 min	low	low (1-step)	this work

^aInclude equipment costs. ^bAfter sample preparation (requires culture). ^cBioreceptors: antibodies. ^dBioreceptors: bacteriophage CBD. ^eAbsorbent pad (AP) replacement required during the assay. ^fBioreceptors: bacteriophage CBD & EvoDit.

intensity for AW43, statistical analysis revealed a significant difference between the three tested *B. cereus* strains and the blanks (p -value ≤ 0.05 , Figure S7), while highlighting the nonsignificant variation among the three strains ($p > 0.1$).

Finally, to assess the specificity of the half-stick detection, we selected different bacterial species that are not targeted by the proteins used in our experiments: *Bacillus subtilis* Bs168, another member of the Bacilli, and *Staphylococcus epidermidis* ATCC 12228 from another genus. The binding at the T-Dot for these species was strongly reduced, resulting in a decrease in signal intensity by 70–80%, which greatly decreases the risk of false-positive results in the testing. However, the signal was not consistently as low as the blank (negative control), indicating the occurrence of nonspecific interactions between the AuNP conjugate, the control strains, and the PlyB221 capture bioreceptors. To investigate nonspecific interactions in the LFA format, we also assessed the detection of each strain with BSA-coated AuNPs (negative control). The signal intensities were decreased by more than 50% compared to the detection of the sensitive *B. cereus* s.l. strains, as BSA is insensitive to bacteria. Nonetheless, the residual signal indicates ongoing nonspecific adsorption, especially for strain VD021, corroborating the findings in Figure 3G which demonstrates the occasional but significant nonspecific adsorption of BSA-coated AuNPs on bacterial surfaces.

It is important to note that using phage proteins instead of whole phages enhances the selectivity of the assay. While PlyB221 shows sensitivity to all tested *B. cereus* strains, EvoDit-Gp28 binds to 17 out of 22 tested strains of *B. cereus*, demonstrating a considerable binding spectrum, surpassing that of the whole phage.¹⁶ Thus, incorporating multiple proteins in our design allows for the detection of a broader range of *B. cereus* strains compared with the use of whole phages.

We can attempt to correlate the LOD determined from our sensor sensitivity study with the earlier quantification of the binding of AuNP to *B. cereus* cells. We found that approximately 100 AuNPs were observed to bind to each bacterial cell under conditions of excess AuNP conjugates. Considering that the accumulation of around 10⁶–10⁷ AuNPs at the TL is typically required for an LFA to produce a visible colorimetric signal without amplification,⁵⁵ the minimal number of AuNP required to produce a sufficient signal at the TL in our system could be delivered by a sample of approximately 10⁵ CFU/mL. This number correlates well with the LOD identified in our sensitivity study. The LODs reported in the literature for such systems are commonly around 10⁵ CFU/mL,⁴⁷ which corroborates the results of our sensitivity study. However, we emphasize that this correlation

should be approached with caution, as each bacterial strain (and potentially each individual cell) expresses its binding receptors differently. Additionally, the derivation of an LOD in LFA systems is not solely dependent on the number of AuNPs bound to the bacterial cell but is also influenced by factors such as the flow rate, the amount of capture bioreceptor immobilized on the membrane, the wettability of the membrane, and the affinity of complex formation.

As this study aimed to establish a proof-of-concept for bacterial detection using multiple phage proteins within an LFA prototype, other optimizations of the assay were not included. For instance, the weak residual nonspecific adsorption observed in the strips could be reduced by membrane blocking with BSA.⁵¹ In addition, future work will be essential to evaluate the sensor performances in complex biological samples containing different bacterial species and varying ratios, as this will be critical to assessing the selectivity and robustness of the sensor under real-world conditions. Spiked sample testing will be an important next step in advancing our phage protein LFA toward practical applications in food safety.

Leveraging the two characterized phagebinding proteins allowed us to overcome the operational complexity observed in previous attempts to incorporate phage proteins in LFAs, which employed the same phage protein for both capture and detection.^{28,29} To the best of our knowledge, our device is the first instance of an LFA combining two phage proteins targeting the same bacterial strains, enabling a user-friendly operation while leveraging all of the inherent benefits of phage proteins. Our LFA design addresses the issues of long incubation times (over 48h) and relatively high costs (typically a few tens of dollars depending on the country) associated with traditional culture methods for bacterial detection (Table 2). Our assay achieves a detection time of 15 min, and the production costs of LFAs are generally around a few dollars per test at most, with a sale price per strip between 2 and 5 times the production cost depending on the application, making it a cost-effective alternative to laboratory-based methods.⁵⁶

Finally, the effective incorporation of our phage binding protein as new bioreceptors in LFAs requires ensuring that the bioreceptor exhibits sufficient characteristics of stability, strong binding affinity, specificity, and fast association kinetics with the target cell surface receptors.^{8,45} While the binding ability and specificity of the biointerfaces have been predicted and experimentally demonstrated through this work, the stability of phage binding proteins has been established by previous studies.¹⁷ While not systematically reported, the protein association kinetics, expressed by its association constant K_D , have been reported for a few phage endolysin CBDs,^{13,58,59}

indicating that CBDs exhibit a strong binding affinity toward bacterial hosts. K_D values span from the picomolar to the nanomolar range (Figure S8), which are typical values reported for antigen–antibody systems, underscoring the potential of phage proteins as viable alternatives to antibodies in LFAs. However, to date, no K_D values for distal proteins such as EvoDit-Gp28 have been documented. Access to crucial information such as K_D is particularly important for scientists involved in identifying suitable LFA bioreceptors, as previously highlighted.⁸ Therefore, additional characterization of bacteriophage binding proteins could facilitate their informed implementation in immunoassays such as LFAs.

3. CONCLUSIONS

This study aimed to demonstrate the potential of recombinant affinity phage proteins as capture and detection biointerfaces in LFAs for whole-cell pathogen detection. To this end, the phage protein binding properties were thoroughly characterized and demonstrated at the molecular, biointerface, and LFA levels.

Recent machine-learning algorithms were used to predict the microstructure and physicochemical properties of PlyB221 and EvoDit-Gp28, two recently derived phage proteins targeting *B. cereus*. These detailed predictions provided valuable insights into the potential interactions and preferred anchorage of these proteins on the NC membrane and AuNPs used for bacterial capture and detection in LFAs. Notably, predictions suggested a favorable orientation for EvoDit-Gp28 binding on AuNPs at the cysteine residue level, which may influence its performance. While our findings enhance the understanding of the microstructural aspects of phage proteins, the implications for their application as bioreceptors in LFAs should be interpreted cautiously, particularly regarding the preservation of their binding performance. Subsequently, we developed, assessed, and validated the capture and detection biointerfaces, incorporating respectively PlyB221 onto NC membranes and EvoDit-Gp28 onto AuNPs. The biointerfaces exhibited specificity and robustness in capturing and detecting whole *B. cereus* cells in model experiments. Through these experiments, we investigated the labeling of *B. cereus* surfaces with AuNPs and proposed a model that correlates the quantity of conjugate AuNPs used in LFA with the coverage of bacterial cells. If applied with caution, this model could provide insights for designing AuNP-based in vitro diagnostic applications, including colorimetric immunoassays for detecting foodborne pathogens.⁶⁰ The binding performances of the new phage protein biointerfaces were finally demonstrated in an LFA format by detecting strains of the *B. cereus* group with high specificity. Unlike previous attempts to incorporate phage protein biointerfaces in LFAs reported in the literature, our approach leverages two distinct phage proteins to preserve the user-friendly nature of LFAs. This renders our strategy appropriate for point-of-care applications with minimal sample preparation and operational steps, which is the primary advantage of LFAs. In particular, the use of a phage distal tail protein as an LFA bioreceptor has not been previously demonstrated.

Phage affinity proteins represent an underexploited class of affinity proteins with promising potential for point-of-care diagnostics. Their recombinant expression in bacterial cells renders them as simpler and more sustainable alternatives to the animal-based production of antibodies traditionally employed in lateral-flow-based diagnostics.⁶¹ Moreover, these affinity proteins can be engineered and fully customized based

on their genetic sequences, allowing for the optimization of various binding properties, including the recognition of specific epitopes of interest in the pathogen or attachment to specific assay substrates. With the increasing number of phage proteins discovered, the combination of these advantageous characteristics paves the way for the establishment of a library of proteins with well-controlled specificities to rapidly address diagnostic testing needs in emergency situations. This work expands our understanding of phage protein biointerfaces for LFAs and paper-based diagnostics, establishing a foundation for the application of phage binding proteins as highly effective bioreceptors in LFAs or other point-of-care diagnostic assays.

4. EXPERIMENTAL SECTION

4.1. Bioinformatics and Structure Prediction. Proteins Deep-Blue PlyB221 and Deep-Purple EvoDit-Gp28 were retrieved from the NCBI database: YP_009285532.1 and YP_009833669.1, respectively. Structure predictions of tail proteins were made using a ColabFold notebook v1.5.5 on February 2024 (available at <https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb> #scrollTo = kObIAo-xetgx). The predicted structures were then submitted to the Dali server⁶² to identify the closest structural homologues in the PDB database. Structure analysis and representation were generated with ChimeraX.⁶³

4.2. Materials. PBS 10 mM, sodium chloride solution (1 M NaCl), BSA, Tween-20, and sucrose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized (DI) water was produced in our facilities (conductivity $\sigma = 6.6 \times 10^{-6}$ S/m). Commercial 40 nm diameter AuNPs were purchased from Nano-Flow (Liège, Belgium). The AuNPs exhibit a negative charge due to their citrate coverage. Indeed, the manufacturing process of AuNPs yields nanoparticles coated with citrate. This layer is essential for achieving a stable suspension. Lateral flow strips made of NC membranes on a polyester backing (UniSart Lateral Flow CN95 and CN140 Backed, 12 and 8 μ m nominal pore size, respectively) were purchased from Sartorius (Göttingen, Germany). Lysogeny broth (LB), cellulose membrane (CFSP001700), and glass fiber were purchased from Merck Millipore (Billerica, MA, USA). Supporting adhesive cards were purchased from Kenosha (Amstelveen, The Netherlands). TEM grids (carbon film 300 MESH Copper grids CF300-CU) were purchased from Electron Microscopy Sciences. Lateral flow strips were cut using a guillotine strip cutter (ref ZQ2002, Shanghai Kinbio Tech. Co. Ltd., China). Lateral flow strip T-Dot intensity measurements were carried out by using a scanner (HP Scanjet G4010). SEM images were acquired using a JEOL 7600F. CLSM images were captured with a Zeiss LSM 710 confocal microscope. Bacteria were cultivated at 37 °C in an incubator (Memmert GmbH, Germany). Concentrations of bacterial suspensions were adjusted by analyzing the OD₆₀₀ value. Visible spectra of AuNPs were acquired using a wavelength sweep from 400 to 700 nm, with a step size of 1 nm (spectrophotometer Ultraspec8000PC, Life Sciences). DLS experiments were performed on a CGS-3 Compact Goniometer System (ALV, Germany) equipped with a LSE-5003 light scattering electronics and multiple tau digital correlator.

4.3. Bacterial Strains and Growth Conditions. *B. thuringiensis* AW43⁶⁴ and *B. weihenstephanensis* KBA4⁶⁵ were used for the STEM experiments; *B. cereus* strains ATCC 10987 and VD021,⁶⁶ *Bacillus subtilis* Bs168,⁶⁷ and *Staphylococcus epidermidis* ATCC 12228, were used for LFA experiments. The bacteria were plated on LB agar plate and grown overnight (O/N) at 30 °C. Next, an individual colony was used to inoculate 5 mL of LB, and the suspension was incubated at 30 °C with agitation (120 rpm). Then, the culture was washed twice with an appropriate buffer (water or diluted PBS) to remove residual LB (centrifugation at 10,000g for 5 min at room temperature (RT)). The pellet was finally resuspended in the appropriate buffer yielding a bacterial suspension of ca. 10⁷ CFU/mL. The CFU/mL values were obtained by performing standard plate counting experiments. *B. thuringiensis* (GBJ002(pSW4::CFP))⁶⁸ expressing the CFP was used

for the CLSM imaging experiments. The growth parameters were the same as described above with the only exceptions of antibiotic use being kanamycin (50 $\mu\text{g/mL}$), due to a resistance gene to this antibiotic on the plasmid, and acid nalidixic (25 $\mu\text{g/mL}$), since GBJ002 displays innate resistance to this antibiotic.

4.4. Phage Protein Expression and Purification. A detailed description of the expression and purification of the CBD of PlyB221 endolysin, encoded by phage Deep-Blue, and fused to a GFP for fluorescence assays, has been previously published by Leprince et al.¹⁷ The CBD was fused to a GFP for fluorescence assays. The protein concentration was adjusted to 1 mg/mL.

Similarly, a detailed description of the expression and purification of EvoDit-Gp28 is available in the study of Leprince et al.³¹ The protein concentration was adjusted to 100 $\mu\text{g/mL}$.

The protein expression was evaluated by the Bradford method, and the purity of the protein was assessed by SDS-PAGE (Biorad, Hercules, CA, USA). The protein was supplemented with 5% glycerol and stored at $-20\text{ }^{\circ}\text{C}$. The protein was finally dialyzed (Tube-O-DIALYZERMedi, 8 kDa, Biosciences) O/N at $4\text{ }^{\circ}\text{C}$ with agitation against PBS before use.

4.5. Preparation and Characterization of the PlyB221 Capture Biointerface on the NC Membrane. The PlyB221 capture biointerface on NC membranes was prepared following a previously reported protocol.²² Briefly, a 1 mg/mL solution of purified PlyB221 in PBS buffer was drop cast onto NC membranes. Membranes were then dried in an oven for 60 min at $37\text{ }^{\circ}\text{C}$, followed by desiccation for 30 min at RT to fix the protein to the membrane. Membranes were then washed twice for 2 min with DI water to remove protein molecules in excess. The effective binding of specific bacteria was assessed by incubating a droplet of 10^7 CFU/mL *B. thuringiensis* (GBJ002) suspension on the membranes, followed by a washing step in PBS buffer. CLSM imaging was immediately performed on a membrane with and without bacterial capture. Simultaneous observation of proteins expressing GFP and bacterial strains expressing CFP was achieved by combining CFP filters (observation window 454–494 nm) and GFP filters (observation window 510–574 nm). 3D fluorescent images were built from Z-stack CLSM images over a 15 μm height within the NC membrane.

4.6. Preparation of AuNP-EvoDit-Gp28 as a Detection Biointerface. To determine the optimal coupling conditions of EvoDit-Gp28 onto AuNPs, a gold aggregation test was performed according to a previously published procedure.⁵¹ In summary, 150 μL of AuNPs is incubated in a 96-well microplate with 10 μL of the EvoDit-Gp28 protein at different initial concentrations under three different pH conditions (7, 8, and 9), in a duplicate test (see Figure S4a–c). After 20 min of incubation at 600 rpm, 20 μL of 10% (wt/vol) NaCl solution was added to the suspension, inducing the aggregation of nanoparticles that are not optimally covered by phage protein. Quantitative analysis of the UV–vis spectra (absorbance peak intensity, peak position, and spectra shape) was performed to determine the degree of aggregation, thus identifying the optimal pH and the minimum amount of EvoDit-Gp28 protein required to reach sufficient coverage of the nanoparticles to keep them well dispersed in solution (see Figure S4d–i).

To prepare the optimized AuNP-EvoDit-Gp28 conjugate used in this work, the AuNP solution pH was first adjusted to pH 8. Then, 100 μL of EvoDit-Gp28 at an initial concentration 100 $\mu\text{g/mL}$ was added to 1.5 mL of AuNP suspension at OD 0.4 to reach a final protein concentration of 6.25 $\mu\text{g/mL}$. It was followed by an incubation step (Thermoshaker for microtubes, Eppendorf) at 650 rpm (RT) for 20 min. Afterward, 100 μL of a solution of 1 mg/mL BSA in Milli-Q water was added to the suspension, and then an additional incubation step at 650 rpm (RT) for 20 min was performed. The resulting AuNP-EvoDit-Gp28 conjugate was centrifuged at $21,913 \times g$ for 20 min at $4\text{ }^{\circ}\text{C}$, and the supernatant was discarded to remove free phage proteins that may interfere within the LFA. The pellet was resuspended in 500 or 125 μL of conjugate pad buffer (10 mM PBS containing 5% (w/v) sucrose, 1% (w/v) BSA, and 0.5% (v/v) Tween-20) to reach a final conjugate OD of about 1 or 4. To prepare the conjugate at OD 9, 10 μL of EvoDit-Gp28 at an

initial concentration 1 mg/mL was incubated with 250 μL of AuNP suspension at OD 8 to reach a final protein concentration of about 38.5 $\mu\text{g/mL}$. The pellet was resuspended in 225 μL of conjugate pad buffer to reach the final conjugate OD of about 9. Conjugate AuNPs were cooled and carefully stored at $4\text{ }^{\circ}\text{C}$ for further use.

4.7. *B. cereus* Cell Binding by AuNP-EvoDit-Gp28. In order to mimic similar amounts of AuNP conjugate and sample volume used in the application, e.g., in a 5 mm wide LFA, we incubated 150 μL of fixed O/N bacterial suspensions with 35 μL of the AuNP conjugate. The concentration of the bacterial suspension was fixed at the O/N bacterial suspensions, i.e., 10^7 CFU/mL, to study the binding between the AuNP conjugate and bacteria cells for the extreme case where bacteria would be the most numerous in the sample. The choice of the AuNP ODs used for this experiment was based on previous observations and published studies. For instance, the use of AuNP conjugates at OD 4 (at 520 nm) was reported by Anfossi et al.⁶⁹ for toxin detection and by Bergua et al.⁴⁷ for pathogen detection using 40 nm AuNP-based LFAs. Therefore, we selected OD 9 for the control experiments (before dilution in the sample suspensions), and OD 1, 4, and 9 (at 520 nm, before dilution in the sample suspensions) as a range to optimize the bacterial surface coverage.

The experiment consists of labeling bacterial cells with the AuNP conjugate, followed by the reticulation (fixation) of the complex cell–AuNP conjugate to ease the SEM imaging. After incubation and before the fixation process, the first centrifugation is conducted to wash the sample, i.e., removal of the supernatant containing unbound AuNP conjugates ($10,000 \times g$ for 5 min at RT). To fix the complexes, the pellets were resuspended in a 5% glutaraldehyde solution in PBS. Samples were left for 30 min at RT to allow the fixation of the bacterial cells through the crosslinking of the bacterial cell walls and intracellular structures.⁷⁰ Dehydration of the sample was performed by successively immersing the pellets for 10 min in DI supplemented with an increasing amount of ethanol (10–30–50–70–90% ethanol). The immersion solution was removed by centrifuging the suspension at $10,000 \times g$ for 10 min (RT).

Quantitative SEM image analysis was performed with ImageJ software (NIH). For each image, a region of interest containing the bacteria labeled with the AuNP conjugate was selected. Subsequently, the image was inverted and converted to black and white by using a threshold to best identify the surface of the bacteria covered by the nanoparticles. An algorithm was then applied to calculate the surface area covered by the nanoparticles.

4.8. Half-Stick LFA Experiments. NC membranes were fixed onto laminated cards. A cellulose absorbent pad was assembled on the laminated card, overlapping 2 mm with the NC membrane. To provide enough bed volume and prevent a quick decrease in the bacterial cell flow, two consecutive pieces of absorbent pads were assembled in a row at the end of the strip. The card was then cut into 5 mm wide lateral flow strips with a guillotine strip cutter. The test area on the NC membrane was formed by manually placing a drop of 0.3 μL of PlyB221 solution at 1 mg/mL onto LFA strips to form test dots (T-Dots) and then drying for 1 h at $37\text{ }^{\circ}\text{C}$. The strips were stored in aluminum pouches with desiccants at RT to preserve the biological activity of the PlyB221 proteins.

The detection protocol consisted of incubating 150 μL of the bacterial suspensions of interest (diluted in PBS for the calibration curve) with 35 μL of AuNP-EvoDit-Gp28 in a 96-well plate for 5 min to allow the binding of AuNP conjugates to the bacterial cells. Then, a half-stick dotted strip was immersed vertically in the well containing the suspension. After 15 min, the T-Dot color intensity was stable and the half-stick was scanned with a scanner to determine the T-Dot intensity. The T-Dot area was quantified using ImageJ software (NIH). To this end, a circular region of interest was selected containing the T-Dot. The region was then inverted, converted into a 32-bit image, and its average gray scale intensity was measured. Another rectangular region of interest was selected from the background, and the image analysis process was repeated to measure the background intensity. The background intensity value was subtracted from the T-Dot intensity value to obtain the normalized T-Dot intensity used to compare the different LFA detection

conditions. The calibration curve was obtained by linear fitting of the data using Origin software (Origin(Pro), Version 2024. OriginLab Corporation, Northampton, MA, USA).

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsabm.4c00965>.

Additional data about the preparation and characterisations of the PlyB221 capture biointerface (CLSM images, time-lapse series) and EvoDit-Gp28 detection biointerface (TEM size histogram, stability study, aggregation test); TEM images for AuNP labeling experiments, computation details about AuNP labeling, the half-stick specificity testing of different *B. cereus* strains, and a review of affinity constants of phage-binding proteins (PDF)

Capture dynamics of bacterial cells within PlyB221-functionalized NC membranes (AVI)

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Notes

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■ REFERENCES

- (1) Ikuta, K. S.; et al. Global mortality associated with 33 bacterial pathogens in 2019: a systematic analysis for the Global Burden of Disease Study 2019. *Lancet* **2022**, *400*, 2221–2248.
- (2) Ehling-Schulz, M.; Lereclus, D.; Koehler, T. M. The *Bacillus cereus* Group: *Bacillus* Species with Pathogenic Potential. *Microbiol. Spectr.* **2019**, *7*, 18.
- (3) Authority (EFSA), E. F. S.. European Centre for Disease Prevention and Control (ECDC), The European Union One Health 2022 Zoonoses Report. *EFSA J.* **2023**, *21*, No. e8442.
- (4) Zourob, M.; Elwary, S.; Turner, A. P. F. *Principles of bacterial detection: biosensors, recognition receptors, and microsystems*; Springer: New York, 2008.
- (5) Younes, N.; Yassine, H. M.; Kourentzi, K.; Tang, P.; Litvinov, D.; Willson, R. C.; Abu-Raddad, L. J.; Nasrallah, G. K. A review of rapid food safety testing: using lateral flow assay platform to detect foodborne pathogens. *Crit. Rev. Food Sci. Nutr.* **2023**, *64*, 9910–9932.
- (6) Hua, Z.; Yu, T.; Liu, D.; Xianyu, Y. Recent advances in gold nanoparticles-based biosensors for food safety detection. *Biosens. Bioelectron.* **2021**, *179*, No. 113076.
- (7) Cox, C. R.; Jensen, K. R.; Mondesire, R. R.; Voorhees, K. J. Rapid detection of *Bacillus anthracis* by γ phage amplification and lateral flow immunochromatography. *J. Microbiol. Methods* **2015**, *118*, 51–56.
- (8) Hu, L.; Calucho, E.; Fuentes-Chust, C.; Parolo, C.; Idili, A.; Álvarez Diduk, R.; Rivas, L.; Merkoçi, A. Selection and characterisation of bioreceptors to develop nanoparticle-based lateral-flow immunoassays in the context of the SARS-CoV-2 outbreak. *Lab Chip* **2022**, *22*, 2938–2943.
- (9) Gray, A.; Bradbury, A. R. M.; Knappik, A.; Plückthun, A.; Borrebaeck, C. A. K.; Dübel, S. Animal-free alternatives and the antibody iceberg. *Nat. Biotechnol.* **2020**, *38*, 1234–1239.
- (10) Mu, A.; Lawley, T. D. Bacteriophage discovery to advance biotechnology and biotherapeutics. *Nat. Rev. Microbiol.* **2023**, *21*, 279.
- (11) Paczesny, J.; Richter, U.; Holyst, R. Recent Progress in the Detection of Bacteria Using Bacteriophages: A Review. *Viruses* **2020**, *12*, 845.
- (12) Richter, U.; Janczuk-Richter, M.; Niedziółka-Jönsson, J.; Paczesny, J.; Holyst, R. Recent advances in bacteriophage-based methods for bacteria detection. *Drug Discov Today* **2018**, *23*, 448–455.
- (13) Schmelcher, M.; Loessner, M. J. Application of bacteriophages for detection of foodborne pathogens. *Bacteriophage* **2014**, *4*, No. e28137.
- (14) Abdelhamied, N.; Abdelrahman, F.; El-Shibiny, A.; Hassan, R. Y. A. Bacteriophage-based nano-biosensors for the fast impedimetric determination of pathogens in food samples. *Sci. Rep.* **2023**, *13*, 3498.
- (15) Templier, V.; Roux, A.; Roupiez, Y.; Livache, T. Ligands for label-free detection of whole bacteria on biosensors: A review. *Trends Anal. Chem.* **2016**, *79*, 71–79.
- (16) Leprince, A.; Mahillon, J. Phage Adsorption to Gram-Positive Bacteria. *Viruses* **2023**, *15*, 196.
- (17) Leprince, A.; Nuytten, M.; Gillis, A.; Mahillon, J. Characterization of PlyB221 and PlyP32, Two Novel Endolysins Encoded by Phages Preying on the *Bacillus cereus* Group. *Viruses* **2020**, *12*, 1052.
- (18) Makino, T.; Skretas, G.; Georgiou, G. Strain engineering for improved expression of recombinant proteins in bacteria. *Microb. Cell Fact.* **2011**, *10*, 32.
- (19) Schmelcher, M.; Loessner, M. J. Bacteriophage endolysins: applications for food safety. *Curr. Opin. Biotechnol.* **2016**, *37*, 76–87.

- (20) Filik, K.; Szermer-Olearnik, B.; Oleksy, S.; Brykała, J.; Brzozowska, E. Bacteriophage Tail Proteins as a Tool for Bacterial Pathogen Recognition—A Literature Review. *Antibiotics* **2022**, *11*, 555.
- (21) Ning, Y.; Teng, T.; Wu, X.; Wang, M.; Jiao, X.; Qiao, J. Development of an enzyme-linked phage receptor-binding protein assay (ELPRA) based on a novel biorecognition molecule- receptor-binding protein Gp130 of *Pseudomonas aeruginosa* bacteriophage Henu5. *Enzyme Microb. Technol.* **2024**, *177*, No. 110442.
- (22) Le Brun, G.; Hauwaert, M.; Leprince, A.; Glinel, K.; Mahillon, J.; Raskin, J.-P. Electrical Characterization of Cellulose-Based Membranes towards Pathogen Detection in Water. *Biosensors* **2021**, *11*, 57.
- (23) Tolba, M.; Ahmed, M. U.; Tlili, C.; Eichenseher, F.; Loessner, M. J.; Zourob, M. A bacteriophage endolysin-based electrochemical impedance biosensor for the rapid detection of *Listeria* cells. *Analyst* **2012**, *137*, 5749.
- (24) Singh, A.; Arya, S. K.; Glass, N.; Hanifi-Moghaddam, P.; Naidoo, R.; Szymanski, C. M.; Tanha, J.; Evoy, S. Bacteriophage tailspike proteins as molecular probes for sensitive and selective bacterial detection. *Biosens Bioelectron* **2010**, *26*, 131–138.
- (25) Wang, L.; Lin, H.; Zhang, J.; Wang, J. Phage long tail fiber protein-immobilized magnetic nanoparticles for rapid and ultra-sensitive detection of *Salmonella*. *Talanta* **2022**, *248*, No. 123627.
- (26) Kretzer, J.; Schmelcher, M.; Loessner, M. Ultrasensitive and Fast Diagnostics of Viable *Listeria* Cells by CBD Magnetic Separation Combined with A511::luxAB Detection. *Viruses* **2018**, *10*, 626.
- (27) Poshtiban, S.; Javed, M. A.; Arutyunov, D.; Singh, A.; Banting, G.; Szymanski, C. M.; Evoy, S. Phage receptor binding protein-based magnetic enrichment method as an aid for real time PCR detection of foodborne bacteria. *Analyst* **2013**, *138*, 5619.
- (28) Kong, M.; Shin, J. H.; Heu, S.; Park, J.-K.; Ryu, S. Lateral flow assay-based bacterial detection using engineered cell wall binding domains of a phage endolysin. *Biosens Bioelectron* **2017**, *96*, 173–177.
- (29) Shin, J. H.; Hong, J.; Go, H.; Park, J.; Kong, M.; Ryu, S.; Kim, K.-P.; Roh, E.; Park, J.-K. Multiplexed Detection of Foodborne Pathogens from Contaminated Lettuces Using a Handheld Multistep Lateral Flow Assay Device. *J. Agric. Food Chem.* **2018**, *66*, 290–297.
- (30) Low, S.; Shaimi, R.; Thandaithabany, Y.; Lim, J.; Ahmad, A.; Ismail, A. Electrophoretic interactions between nitrocellulose membranes and proteins: Biointerface analysis and protein adhesion properties. *Colloids Surf. B Biointerfaces* **2013**, *110*, 248–253.
- (31) Leprince, A.; Nuytten, M.; Mahillon, J. Viral Proteins Involved in the Adsorption Process of Deep-Purple, a Siphovirus Infecting Members of the *Bacillus cereus* Group. *Appl. Environ. Microbiol.* **2022**, *88*, No. e02478-21.
- (32) Hock, L.; Gillis, A.; Mahillon, J. Complete Genome Sequence of Bacteriophage Deep-Blue Infecting Emetic *Bacillus cereus*. *Genome Announc* **2016**, *4*, , Publisher: American Society for Microbiology.
- (33) Hock, L.; Gillis, A.; Mahillon, J. Complete genome sequence of bacteriophage Deep-Purple, a novel member of the family Siphoviridae infecting *Bacillus cereus*. *Arch. Virol.* **2018**, *163*, 2555–2559.
- (34) Jumper, J.; et al. Highly accurate protein structure prediction with AlphaFold. *Nature* **2021**, *596*, 583–589.
- (35) Goulet, A.; Cambillau, C. Present Impact of AlphaFold2 Revolution on Structural Biology, and an Illustration With the Structure Prediction of the Bacteriophage J-1 Host Adhesion Device. *Front. Mol. Biosci.* **2022**, *9*, No. 907452.
- (36) Lee, K. O.; Kong, M.; Kim, I.; Bai, J.; Cha, S.; Kim, B.; Ryu, K.-S.; Ryu, S.; Suh, J.-Y. Structural Basis for Cell-Wall Recognition by Bacteriophage PBC5 Endolysin. *Structure* **2019**, *27*, 1355.e4–1365.e4.
- (37) Fridley, G. E.; Holstein, C. A.; Oza, S. B.; Yager, P. The evolution of nitrocellulose as a material for bioassays. *MRS Bull.* **2013**, *38*, 326–330.
- (38) Dieterle, M.-E.; Spinelli, S.; Sadovskaya, I.; Piuri, M.; Cambillau, C. Evolved distal tail carbohydrate binding modules of *Lactobacillus* phage J-1: a novel type of anti-receptor widespread among lactic acid bacteria phages: Phage J-1 evolved Dlt CBM2 is a receptor binding protein. *Mol. Microbiol.* **2017**, *104*, 608–620.
- (39) Thobhani, S.; Attree, S.; Boyd, R.; Kumarwami, N.; Noble, J.; Szymanski, M.; Porter, R. A. Bioconjugation and characterisation of gold colloid-labelled proteins. *J. Immunol Methods* **2010**, *356*, 60–69.
- (40) Wang, A.; Vangala, K.; Vo, T.; Zhang, D.; Fitzkee, N. C. A Three-Step Model for Protein–Gold Nanoparticle Adsorption. *J. Phys. Chem. C* **2014**, *118*, 8134–8142.
- (41) Xu, J. X.; Alom, M. S.; Yadav, R.; Fitzkee, N. C. Predicting protein function and orientation on a gold nanoparticle surface using a residue-based affinity scale. *Nat. Commun.* **2022**, *13*, 7313.
- (42) Hubbard, S.; Thornton, J. 'NACCESS', department of biochemistry and molecular biology; University College: London, 1993.
- (43) Ding, J.; Arnold, E. In *International Tables for Crystallography*, 1st ed.; Fuess, H.; Hahn, T.; Wondratschek, H.; Müller, U.; Shmueli, U.; Prince, E.; Authier, A.; Kopský, V.; Litvin, D. B.; Rossmann, M. G.; Arnold, E.; Hall, S.; McMahon, B.; Rossmann, M. G.; Arnold, E., Eds.; International Tables for Crystallography; International Union of Crystallography: Chester, England, 2006; Vol. F, pp 685–694.
- (44) Mujawar, L. H.; Maan, A. A.; Khan, M. K. I.; Norde, W.; van Amerongen, A. Distribution of Biomolecules in Porous Nitrocellulose Membrane Pads Using Confocal Laser Scanning Microscopy and High-Speed Cameras. *Anal. Chem.* **2013**, *85*, 3723–3729.
- (45) Wong, R.; Tse, H. Eds. *Lateral Flow Immunoassay*; Humana Press: Totowa, NJ, 2009.
- (46) Le Brun, G.; Raskin, J.-P. Material and manufacturing process selection for electronics eco-design: Case study on paper-based water quality sensors. *Procedia CIRP* **2020**, *90*, 344–349.
- (47) Bergua, J. F.; Hu, L.; Fuentes-Chust, C.; Álvarez Diduk, R.; Hassan, A. H. A.; Parolo, C.; Merkoçi, A. Lateral flow device for water fecal pollution assessment: from troubleshooting of its microfluidics using bioluminescence to colorimetric monitoring of generic *Escherichia coli*. *Lab Chip* **2021**, *21*, 2417–2426.
- (48) Park, J.-W.; Shumaker-Parry, J. S. Structural Study of Citrate Layers on Gold Nanoparticles: Role of Intermolecular Interactions in Stabilizing Nanoparticles. *J. Am. Chem. Soc.* **2014**, *136*, 1907–1921.
- (49) Ruiz, G.; Tripathi, K.; Okyem, S.; Driskell, J. D. pH Impacts the Orientation of Antibody Adsorbed onto Gold Nanoparticles. *Bioconjugate Chem.* **2019**, *30*, 1182–1191.
- (50) Robson, T.; Shah, D. S. H.; Solovyova, A. S.; Lakey, J. H. Modular Protein Engineering Approach to the Functionalization of Gold Nanoparticles for Use in Clinical Diagnostics. *ACS Appl. Nano Mater.* **2018**, *1*, 3590–3599.
- (51) Parolo, C.; Sena-Torralba, A.; Bergua, J. F.; Calucho, E.; Fuentes-Chust, C.; Hu, L.; Rivas, L.; Álvarez Diduk, R.; Nguyen, E. P.; Cinti, S.; Quesada-González, D.; Merkoçi, A. Tutorial: design and fabrication of nanoparticle-based lateral-flow immunoassays. *Nat. Protoc.* **2020**, *15*, 3788–3816.
- (52) Haiss, W.; Thanh, N. T. K.; Aveyard, J.; Fernig, D. G. Determination of Size and Concentration of Gold Nanoparticles from UV-Vis Spectra. *Anal. Chem.* **2007**, *79*, 4215–4221.
- (53) Khlebtsov, B. N.; Tumskiy, R. S.; Burov, A. M.; Pylaev, T. E.; Khlebtsov, N. G. Quantifying the Numbers of Gold Nanoparticles in the Test Zone of Lateral Flow Immunoassay Strips. *ACS Appl. Nano Mater.* **2019**, *2*, 5020–5028.
- (54) de Puig, H.; Bosch, I.; Gehrke, L.; Hamad-Schifferli, K. Challenges of the Nano–Bio Interface in Lateral Flow and Dipstick Immunoassays. *Trends Biotechnol.* **2017**, *35*, 1169–1180.
- (55) Gupta, R.; Gupta, P.; Wang, S.; Melnykov, A.; Jiang, Q.; Seth, A.; Wang, Z.; Morrissey, J. J.; George, I.; Gandra, S.; Sinha, P.; Storch, G. A.; Parikh, B. A.; Genin, G. M.; Singamaneni, S. Ultrasensitive lateral-flow assays via plasmonically active antibody-conjugated fluorescent nanoparticles. *Nat. Biomed. Eng.* **2023**, *7*, 1556–1570.
- (56) Yetisen, A. K.; Akram, M. S.; Lowe, C. R. Paper-based microfluidic point-of-care diagnostic devices. *Lab Chip* **2013**, *13*, 2210.

- (57) Zhao, X.; Bhat, A.; O'Connor, C.; Curtin, J.; Singh, B.; Tian, F. Review of Detection Limits for Various Techniques for Bacterial Detection in Food Samples. *Nanomaterials* **2024**, *14*, 855.
- (58) Schmelcher, M.; Tchang, V. S.; Loessner, M. J. Domain shuffling and module engineering of *Listeria* phage endolysins for enhanced lytic activity and binding affinity: Engineering of *Listeria* phage endolysins. *Microb Biotechnol* **2011**, *4*, 651–662.
- (59) Kong, M.; Sim, J.; Kang, T.; Nguyen, H. H.; Park, H. K.; Chung, B. H.; Ryu, S. A novel and highly specific phage endolysin cell wall binding domain for detection of *Bacillus cereus*. *Eur. Biophys J.* **2015**, *44*, 437–446.
- (60) Park, S.-H.; You, Y. Gold Nanoparticle-Based Colorimetric Biosensing for Foodborne Pathogen Detection. *Foods* **2024**, *13*, 95.
- (61) Holstein, C. A.; Griffin, M.; Hong, J.; Sampson, P. D. Statistical Method for Determining and Comparing Limits of Detection of Bioassays. *Anal. Chem.* **2015**, *87*, 9795–9801.
- (62) Holm, L.; Laakso, L. M. Dali server update. *Nucleic Acids Res.* **2016**, *44*, W351–355.
- (63) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Meng, E. C.; Couch, G. S.; Croll, T. I.; Morris, J. H.; Ferrin, T. E. UCSF ChimeraX: Structure visualization for researchers, educators, and developers. *Protein Sci.* **2021**, *30*, 70–82.
- (64) Wilcks, A.; Jayaswal, N.; Lereclus, D.; Andrup, L. Characterization of plasmid pAW63, a second self-transmissible plasmid in *Bacillus thuringiensis* subsp. *kurstaki* HD73. *Microbiology* **1998**, *144* (Pt 5), 1263–1270.
- (65) Lapidus, A.; et al. Extending the *Bacillus cereus* group genomics to putative food-borne pathogens of different toxicity. *Chem. Biol. Interact* **2008**, *171*, 236–249.
- (66) Van Der Auwera, G. A.; Feldgarden, M.; Kolter, R.; Mahillon, J. Whole-Genome Sequences of 94 Environmental Isolates of *Bacillus cereus* *Sensu Lato*. *Genome Announce.* **2013**, *1*, No. e00380-13.
- (67) Barbe, V.; Cruveiller, S.; Kunst, F.; Lenoble, P.; Meurice, G.; Sekowska, A.; Vallenet, D.; Wang, T.; Moszer, I.; Médigue, C.; Danchin, A. From a consortium sequence to a unified sequence: the *Bacillus subtilis* 168 reference genome a decade later. *Microbiology (Reading)* **2009**, *155*, 1758–1775.
- (68) Sastalla, I.; Chim, K.; Cheung, G. Y. C.; Pomerantsev, A. P.; Leppla, S. H. Codon-Optimized Fluorescent Proteins Designed for Expression in Low-GC Gram-Positive Bacteria. *Appl. Environ. Microbiol.* **2009**, *75*, 2099–2110.
- (69) Anfossi, L.; Di Nardo, F.; Giovannoli, C.; Passini, C.; Baggiani, C. Increased sensitivity of lateral flow immunoassay for ochratoxin A through silver enhancement. *Anal Bioanal Chem.* **2013**, *405*, 9859–9867.
- (70) Araujo, J. C.; Téran, F. C.; Oliveira, R. A.; Nour, E. A. A.; Montenegro, M. A. P.; Campos, J. R.; Vazoller, R. F. Comparison of hexamethyldisilazane and critical point drying treatments for SEM analysis of anaerobic biofilms and granular sludge. *J. Electron Microscop.* **2003**, *52*, 429–433.