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Simple and rapid peptide nanoprobe biosensor for the detection of *Legionellaceae*

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This study demonstrates the development of a sensitive, specific, and quantitative peptide-based nanoprobe prototype assay for the detection of *Legionellaceae* in a simple way and in a short time. In this work, proteases present in the culture supernatants of *Legionella* spp. were used as a biomarker. Fluorogenic peptide substrates, specific to *Legionella* strains culture supernatant proteases, were identified. Peptidases produced a significant increase in the fluorescence intensity following the cleavage of the dipeptide fluorogenic substrates. The specific substrates were identified and coupled with carboxyl-terminated nano-magnetic particles (NMPs). On the other hand, the C-terminal was conjugated with the cysteine residue to covalently integrate with a gold sensing platform *via* the Au–S linkage. Four different sensors were fabricated from the four specific substrates, which were treated with the protesase of six different species of *Legionella*. In the presence of specific protease, the peptide sequence is digested and the magnetic nanobeads moved out of the gold surface, resulting in the apparence of gold color. One of the nanoprobes sensitivity detects as low as 60 CFU mL^{-1} of *Legionella anisa*, *Legionella micdadei*, and *Fluoribacter dumoffii*. The cross-reactivity of the sensors was tested using other closely associated bacterial species and no significant cross-reactivity of the sensors was found. It is envisaged that this assay could be useful for screening purposes or might be supportive for the fast and easy detection of *Legionella* protease activity for water monitoring purposes.

1. Introduction

Legionella pneumophila is a pleomorphic Gram-negative facultative intracellular bacterial parasite co-evolved within freshwater protozoa (amoeba).[1,2] In July 1976, *L. pneumophila* was recognized to be pathogenic to humans, following an outbreak of acute pneumonia at the American Legion convention in Philadelphia, USA.[3] This pathogen was reported to contaminate warm engineered water systems[4,5] and to be associated with the occurrence of Legionnaires' disease (LD), which is a pneumonic devastating[6,7] life-threatening infection with a fatality rate of more than 50%, particularly in individuals with compromised health conditions.[2] *L. pneumophila* was found to proliferate and persist within biofilms. Thus, it possesses the ability to resist various purification systems in aquatic media. Accordingly, the inhalation of *L. pneumophila*-contaminated aerosols occurring in, for example, showers, hot tubs, spas, faucets, dental lines, air conditioning systems, and cooling towers, may cause human infections by attacking the alveolar mucosa.[8]

In 1978, Winn *et al.* proposed the contribution of *L. pneumophila* products to the pathogenesis of LD.[9,10] Then,

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Dreyfus *et al.* purified and characterized an extracellular metalloprotease, also called tissue-destructive protease or major secretory protein.[11] This extracellular protease exhibited hemolytic, cytotoxic, and dermal ulcerative activities,[12–14] which is considered as one of the virulent factors of this organism.

In practice, LD diagnosis is based on the culture methodology, which can detect all *Legionella* spp. and serogroups specifically. However, this assay is time-consuming, as it takes more than three days to obtain the results and fails to detect viable but non-culturable (VBND) *Legionella* cells in freshwater.[15–17] Another medically approved test is based on the detection of *Legionella* antigen in urine using ELISA.[15] This test possesses moderate sensitivity and specificity in comparison with the culture and PCR methods, but the *Legionella* antigen is typically detectable in urine in the initial 2 to 3 days after the onset of clinical symptoms.[15] In addition, test results may remain positive for a year upon treatment and the ELISA test is unable to detect pathogenic *Legionella* species other than the *L. pneumophilia* serogroup 1.[6,17] In clinical practice, real-time polymerase chain reaction (rtPCR) is an ultra-sensitive assay for LD diagnosis. This method is quantitative, rapid with a short turnaround time, and sensitive in comparison with to the culturing technique. The PCR method is based on the amplification of conserved regions of rRNA sequences; however, these regions are not specific and, hence, can be applied for the detection of any *Legionella* subspecies.[17] Moreover, this technique is unable to differentiate between viable and dead cells (except with PMA or EMA). Also, this method requires trained personnel.[15,17] Alternative methods such as direct fluorescent antibody staining and immunohistochemistry are highly specific, but with procedural difficulties.[18] Other valuable tools for *Legionella* detection include matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. This method is simple, reproducible, and inexpensive but cannot identify *Legionella* strains at the serogroup level.[19]

In accordance, the development of an easy and fast, in-field, colorimetric, rapid, sensitive, and highly specific test would be an excellent tool for screening purposes to support laborious detection methodologies. In this work, we present a fluorescence-based, portable, and low-cost colorimetric assay for the detection of *L. pneumophila* extracellular proteases as a biomarker.

At first, various fluorescence resonance energy transfer (FRET) peptides were selected from a FRET peptide library.[20] Subsequently positive substrates were conjugated to nano-magnetic particles (NMPs) terminated with a thiol group to covalently integrate with a gold sensing platform *via* the Au–S linkage, offering a colorimetric sensor. Ideally, this could be useful for application in water monitoring purposes.

2. Materials and methods

2.1. Chemicals and reagents

Turbobeads™ carboxyl-terminated nanomagnetic particles (NMPs) (50 nm diameter) were purchased from Turbobeads (Zurich, Switzerland). *N*-Hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC), pH stripes, potassium phosphate, sodium chloride, bovine serum albumin (BSA), ethylenediaminetetraactic acid (EDTA), sodium azide, and Tris buffer were purchased from Sigma-Aldrich (Dorset, UK). Self-adhesive magnetic sheets A4 were purchased from a polarity magnets company (Wickford, UK). Brain heart infusion broth (BHI), blood agar, and buffered charcoal yeast extract (BCYE) agar were purchased from SDA Oxoid, Ltd (Basingstoke, UK). Sterile filters 0.22 μm were purchased from Millipore (UK). The wash/storage buffer (10 mM Tris base, 0.15 M sodium chloride, 0.1% (w/v) bovine serum albumin, 1 mM ethylenediaminetetraacetic acid, 0.1% sodium azide, pH 7.5), and the coupling buffer (10 mM potassium phosphate, 0.15 M sodium chloride, pH 5.5) were prepared from chemicals of analytical grade. FRET-based dipeptide library was purchased from PepScan Presto B.V. (Lelystad, The Netherlands).

The peptide library consists of 115 protease substrates with two amino acids. The substrates were designed using (a) L-amino acid; (b) C-terminal L-amino acid and N-terminal D-amino acid; (c) a combination of L- and D-amino acids. L-Amino acids are denoted in uppercase letters, whereas D-amino acids are denoted in lowercase letters.[20] Fluorogenic substrates were synthesized with a purity of approximately >90%. Substrate identity was confirmed by mass spectrometry. All substrates were C-terminally flanked with fluorescein isothiocyanate (FITC); fluorescent probe and N-terminally flanked with Dabcyl (KDbc), a lysin-coupled quencher. The peptides sequences used for the paper based sensing platfrom was purchased from Pepmic Co. (Suzhou, China). The peptide sequence was conjugated with cystein residue-aminohexanoic acid (Ahx) at the C-terminus of the substrate to covalently attached to the gold sensing platform, and elongated with the aminohexanoic acid (Ahx) spacer at the N-terminal.

2.2. Bacterial culture

Legionella pneumophila (*L. pneumophila*) ATCC 33155, *L. pneumophila* ATCC 33152, *Legionella micdadei* (*L. micdadei*) ATCC 33218, *Legionella anisa* (*L. anisa*) ATCC 35292 and *Fluoribacter dumoffii* (*F. dumoffii*), ATCC 33279 were purchased from ATCC (Manassas, VA, USA). These organisms were cultured in buffered charcoal yeast extract agar at 37 °C, 5% CO_2 for 48–72 h. Other pathogens such as *Campylobacter* ATCC 33560, *Escherichia coli* (*E. coli*) O157:H7, and *Salmonella enterica* (*S. enterica*) ATCC 13312 were purchased from ATCC (Manassas, VA, USA) and cultured in BHI. *Campylobacter* was cultured at 45 °C for 24 h under microaerophilic conditions, whereas *E. coli* and *S. enterica* were incubated at 37 °C for 18–24 h.

2.3. Preparation of *Legionella* spp. culture supernatant proteases

Legionella pneumophila (*L. pneumophila*) ATCC 33155, *L. pneumophila* ATCC 33152, *Legionella Tatlockia micdadei* (*T.L. micdadei*) ATCC 33218, *Legionella anisa* (*L. anisa*) ATCC 35292, and *Fluoribacter dumoffii* (*F. dumoffii*) ATCC 33279 were purchased in the lyophilized form from ATCC (Manassas, VA, USA). All the strains were resuspended with BHI broth, then cul-

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tured on buffered activated charcoal and yeast extract agar incubated at 37 °C, 5% CO_2, and humidity for 48 –72 h. Afterwards, the strains were harvested from the plate in liquid BHI tubes. Following appropriate bacterial culture, bacterial cell count was estimated by McFarland standards and the used cell concentration was diluted to 6×10^8 CFU mL^{-1} for all bacterial strains. Subsequently, each bacterial culture was pelleted by centrifugation at 3000*g* for 10 min and the suspension was filtered using a 0.5 micrometer filter to obtain the crude protease solution with specific proteolytic activity proportional to the bacterial concentration in the range of 6×10^1 to 6×10^8 CFU mL^{-1}. All the bacterial growth media were pushed from Saudi Prepared Media Laboratory Company Limited (Riyadh, Saudi Arabia).

2.4. Substrate detection using the FRET assay

A high throughput screening method for the selection of a specific peptide substrate was performed in a clear bottom black 96-well-plates (Corning, Lowell, USA) using a FRET-peptide library,[20,21] as shown in Fig. 1A. All the peptide substrates were labeled with FITC fluorophore at the C-terminal and KDbc quencher at the N-terminal. The proteolytic activity of the different organisms was monitored using 0.5 μL of each substrate (800 μM) in a 1 : 1 filtered PBC supernatant and PBS buffer (100 μL). Culture media was used as the negative control. The change in the fluorescence intensity was monitored every minute for 2 h at 37 °C using an excitation wavelength of 485 and an emission wavelength of 535 nm. The relative fluorescence unit (RFU) of each sample was calculated by subtracting the negative control values of the culture media. Proteolytic activity was defined in RFU per minute. The test was performed in triplicate.

2.5. Preparation of peptide–NMPs

Carboxylated NMPs suspension (15 mg mL^{-1}) was dispersed by sonication and activated by washing three times with the coupling buffer (10 mM potassium phosphate, 0.15 M sodium chloride, pH 5.5).[22–26] Next, one mL of NMPs suspension (15 mg mL^{-1} in coupling buffer) was mixed with the peptide solution (1 mg of peptide substrate dissolved in 1 mL of DMSO), freshly prepared coupling agent EDC (0.57 mg mL^{-1}), and NHS (2 mg mL^{-1}).[22–26] The mixture was shaken on a rotary shaker overnight at 4 °C. Next, peptide–NMPs conjugates were collected using a magnetic separator and the supernatant was aspirated to remove uncoupled peptides. Finally, collected peptide–NMPs conjugates were washed three times with washing buffer (10 mM Tris base, 0.15 M NaCl, 0.1% (w/v) BSA, 1 mM ethylenediaminetetraacetic acid, 0.1% sodium azide, pH 7.5) and stored at 4 °C in a storage buffer.[22–27]

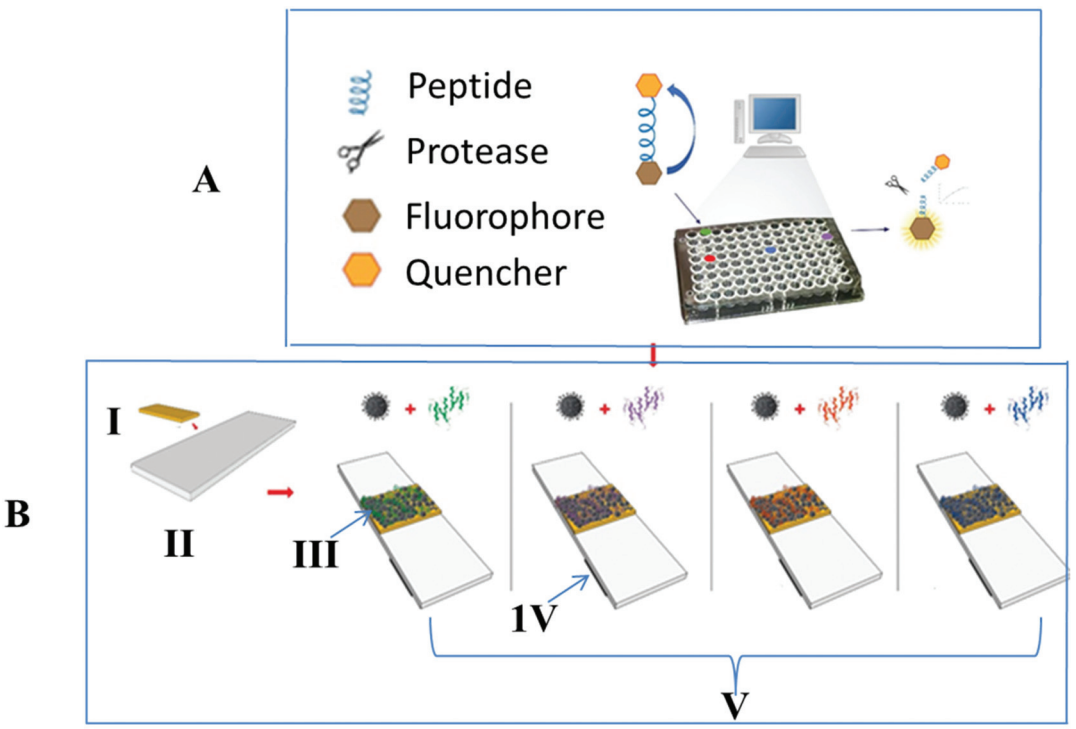

Fig. 1 Schematic overview of *Legionella* spp. detection using the peptide–MNPs probe. A Screening for peptide substrates susceptible to cleavage by *Legionella* proteases by a highthroughput method using the FRET-based dipeptide library. All peptide substrates were labeled with the FITC fluorophore at the C-terminal and KDbc quencher at the N-terminal. The proteolytic activity of different *Legionella* strains was monitored. B Colorimetric biosensors for the detection of *Legionella* strains using different peptide–NMPs conjugate probes. (I) Gold plated self-adhesive tape. (II) Solid physical support. (III) Carboxy-terminated NMPs functionalized with certain peptide substrates were immobilized over a gold sensing platform to form the sensing assay platform. (IV) Round self-adhesive magnet (1 mm in diameter) was fixed at the sensor platform backside. (V) Ready to use peptide–NMPs probe sensing platform.

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2.6. Preparation of the *L. pneumophila* sensing platform

Adhesive sheet purchased from Whatman (London, UK) was coated with a thin layer of gold, which was sputtered at the School of Engineering at King Abdullah University of Science and Technology (KAUST). As shown in Fig. 1BI, the self-adhesive sheet was shaped in a specific size (1–2 mm width) and fitted over a flexible water-resistant strip (Fig. 1BII), which served as the physical support for the sensor. 10 μL of the peptide-conjugated MNP was added over the engineered gold surface and allowed to stand at room temperature until dry (Fig. 1BIII). Afterward, an external magnet (12.5 mm × 12.5 mm × 5 mm, Polarity Magnets Company, Wickford, UK) with a field strength of 3360 gauss was passed over the platform (at 10 mm distance) to remove the unbound peptide–NMPs conjugate. The color change from gold to black following peptide–NMP immobilization over the gold surface indicated the formation of a colorimetric sensor platform (Fig. 1BIII). Then, a circular self-adhesive magnet (1 mm in diameter, prepared by punching an adhesive magnet sheet (purchased from Polarity Magnets Company, Wickford, UK)) was fixed on the sensor platform backside (Fig. 1BIV) and beneath (2–3 mm) the sensing platform. This magnet will assist the collection of the cleaved peptide–NMPs-fragments. Finally, a ready-to-use peptide–NMPs probe was developed (Fig. 1BV).

3. Results and discussion

A simple paper-based protease assay was used for the detection of *Legionella* bacteria. First, we screened a large number of different peptides and selected four common peptide sequences that are specific to *Legionella* species. These selected sequences were further used for the quantitative detection of *Legionella* using the total protease from the culture supernatant. To date, several methods have been developed to detect *Legionella* bacteria. However, these assays have several drawbacks including technical difficulties, lack of sensitivity, and the possibility of false-positive results. Nanotechnology implemented biosensors have guided the development of a cost-effective and time-saving tool and can be applied on-site for the detection of pathogens.[22,23,25,26] Recently, electrochemical impedance spectroscopy-based immunosensor (EIS), a bioaffinity group sensor, has been developed to detect *Legionella* spp. in cooling towers (Table 1). This sensor successfully detected *Legionella* spp. within hours; however, the conductivity of the assay media may affect the measurements and the sensor specificity was not validated.[28] Other optical-based biosensors (Table 1) detected different concentrations of RNA from *L. pneumophila* with high spatial resolution and higher bit depth;[29] however, these sensors reported high detection limits.[29]

The majority of Legionnaires cases are caused by invasive and envasive opportunistic *L. pneumophila*. This pathogen utilizes proteases as the virulence factors to invade alveolar mucosa and subsequently cause human infection, following the inhalation of contaminated warm engineered water systems.[4,5] Remarkably, *Legionella*-mediated proteolysis may be considered valuable biomarkers for detecting the presence or absence of *Legionella* strains in hotels, hospitals, and/or other public areas for regular water monitoring and conse-

Table 1 Sensitivity comparison between commonly used methods for the detection of *Legionella* spp. and the developed biosensor

Detection techniques	LOD	Sample media (matrix)	Positivity rate	Assay time	Ref.
Culture	1 CFU mL^{-1} (for 1 L sample)	Water-BCYE agar, BPAV charcoal media	0.4%	Days	15, 17 and 33
BinaxNOW@UAT			0.8%	Hours	15, 17 and 34
PCR	10^2–10^3 CFU mL^{-1}	Water	2.7%	Hours	15, 17 and 35
Microelectrode array biosensor	10^5–10^8 CFU mL^{-1}	Water		Hours	28
Magnetic-nanoparticle conjugated peptide probe	Up to 6×10^1 CFU mL^{-1}	Water		Minutes	Current study

Table 2 FRET-based cleavage efficiency of the dipeptides by 6×10^8 CFU mL^{-1} culture supernatant of the various *Legionella* strains; *L. pneumophila* ATCC 33155, *T. micdadei* ATCC 33218, *L. anisa* ATCC 35292, *F. dumoffii* ATCC 33279, *L. pneumophila* ATCC 33152, and *F. bozemanae* ATCC 33217

Substrate	33155	33218	35292	33279	33152	33217
FITC-Ahx-L-L-K(Dabcyl)	100.00	36.67	22.80	68.13	72.20	—
FITC-Ahx-F-F-K(Dabcyl)	95.90	100.00	5.40	69.36	67.90	—
FITC-Ahx-W-W-K(Dabcyl)	52.20	19.05	28.10	100.00	100.00	12.3
FITC-Ahx-Y-Y-K(Dabcyl)	85.60	65.20	100.00	60.50	58.70	7.6
FITC-Ahx-M-M-K(Dabcyl)	7.20	0.04	4.79	32.68	33.12	6.7
FITC-Ahx-A-A-K(Dabcyl)	7.60	0.50	6.50	48.02	48.70	−3.4
FITC-Ahx-Y-y-K(Dabcyl)	2.70	5.50	—	10.86	10.90	6.4
FITC-Ahx-t-t-K(Dabcyl)	0.26	0.69	1.08	4.50	4.45	−2.9
FITC-Ahx-y-y-K(Dabcyl)	—	—	—	—	0.30	—

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quent infection prevention measures.[22,23,25,26] In this work, we report the development of a simple and rapid peptide–MNPs based biosensor for the specific detection of *Legionella* spp. proteolytic activity.

3.1. Screening FRET peptide library

The proteolytic activity of different *Legionella* strains was determined by screening 115 fluorogenic dipeptide substrates.[20] Specific fluorogenic dipeptide substrates' cleavage was identified using bacterial culture supernatant by monitoring the change in the fluorescence intensity for six different *Legionella* strains. The maximum change in the fluorescence intensity was considered as 100 percent and the relative percentage change compared to the maximum was calculated. Peptidase released from all *Legionella* strains except the bozemanae strain produced a significant increase in the fluoresce intensity following the cleavage of the dimeric amino acids (L–L), (F–F), (W–W), and (Y–Y) FRET substrates. The proteolytic activity of

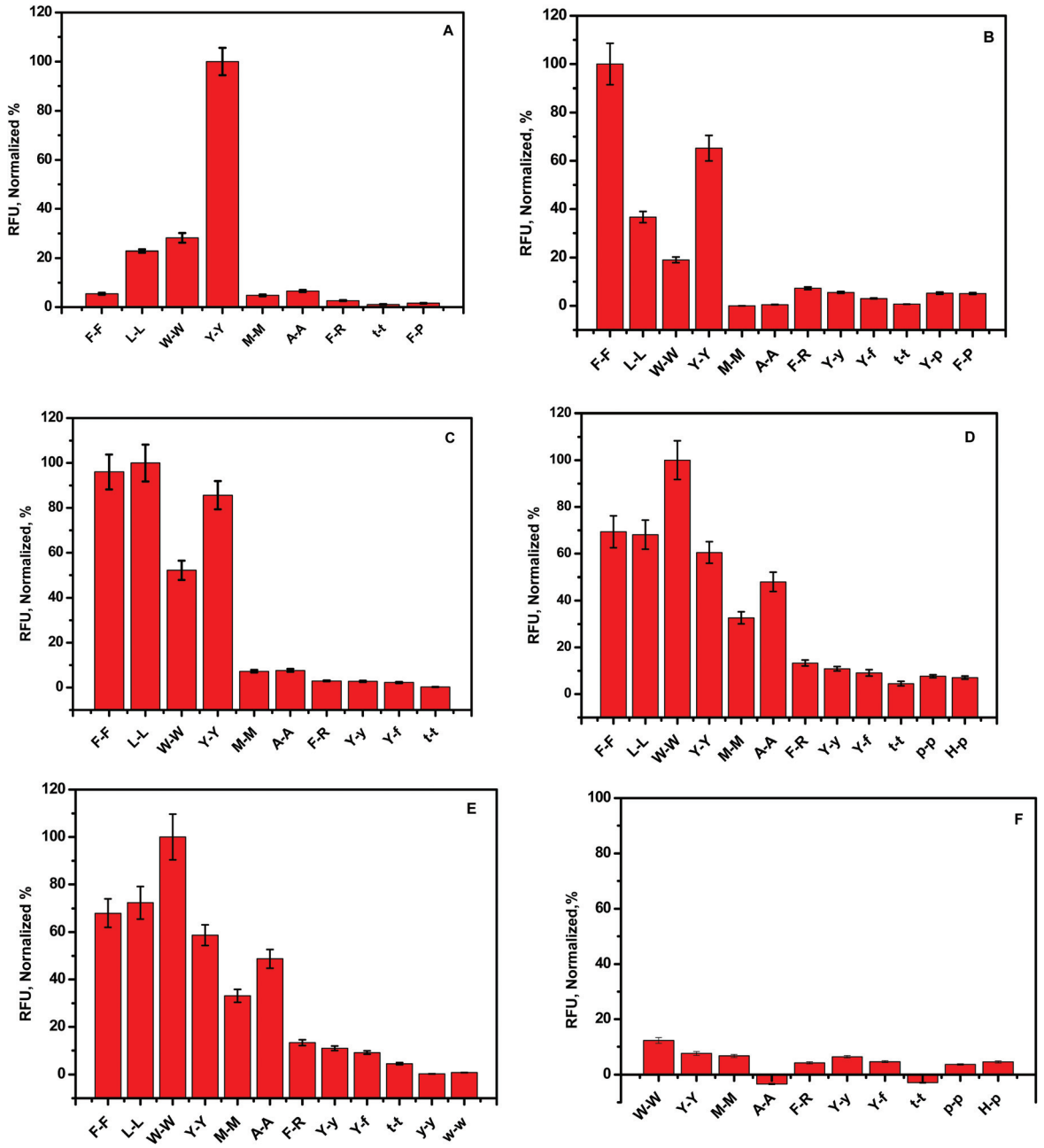

Fig. 2 Change in the fluorescence intensities of different fluorogenic peptide substrates in the presence of different *Legionella* strains culture supernatant after 30 minutes (*L. anisa* ATCC 35292 (A), *L. micdadei* ATCC 33218 (B), *L. pneumophila* ATCC 33155 (C), *F. dumoffii* ATCC 33279 (D), *L. pneumophila* ATCC 33152 (E), and *F. bozemanae* ATCC 33217 (F). All *Legionella* strains specifically cleaved L-aromatic amino acids dipeptide and LL dipeptide. No cleavage was observed with D-amino acid containing substrates.

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Legionella strains was found to specifically target L-amino acids containing dipeptides (Table 2). Notably, proteolysis was observed in the substrates containing aromatic L-amino acids (tyrosine, tryptophan, and phenylalanine) except for leucine-leucine (FITC-Ahx-LL-K(Dabcyl). On the other hand, no hydrolysis was observed in dipeptide substrates having both a D- and L-amino acid. The proteolytic activity of *Legionella* strains was stereo-specific. For example, no change in the fluorescence intensity could be detected when the y–y dipeptide substrate was examined (Table 2, the substrates having Y–Y and y–y, Fig. 2E) compared to the enantiomeric Y–Y substrate (58.7–100% fluorescence intensity change with different *Legionella* strain). Notably, *L. pneumophila* ATCC 33155 exerted efficient proteolytic activity against F–F, L–L, and Y–Y, and moderate activity against W–W (Fig. 2C), whereas the W–W substrate was cleaved efficiently by *L. pneumophila* ATCC 33152 (Fig. 2E). Moreover, *L. anisa* ATCC 35292 specifically digested Y–Y peptide, while no significant increase in the fluorescence intensity was observed with other substrates (Fig. 2A). F–F dipeptide was cleaved effectively by *L. micdadei* ATCC 33218 and moderate cleavage was detected in the Y–Y peptide. On the other hand, no significant proteolysis of L–L and W–W dipeptides was observed (Table 2 and Fig. 2B). However, in contrast to the other five species, *F. bozemanae* 33217 supernatant had no significant effect on the cleavage of any FRET substrates, which may be explained by the difference in the phenotypic characterization as well as the differences in the DNA G–C content and DNA hybridization data of *F. dumoffii* ATCC 33279 from another *Legionella* strains.[30] Furthermore, *Legionella* strain-specific cleavage of the FRET substrates were

Table 3 FRET assay for F–F, L–L, W–W, and Y–Y substrates in the presence of bacterial culture supernatants

Bacteria	F–F	L–L	W–W	Y–Y
L. anisa ATCC 35292	+	++	++	++++
T. micdadei ATCC 33218	++++	++	+	+++
L. pneumophilia ATCC 33155	++++	++++	++	+++
L. pneumophilia ATCC 33152	+++	+++	++++	++
F. dumoffii ATCC 33279	+++	+++	++++	++
E. coli O157:H7	—	—	—	—
S. enterica ATCC 13312	—	—	—	—
Campylobacter Jejuni ATCC 33560	—	—	—	—
F. bozemanae 33217	—	—	—	—

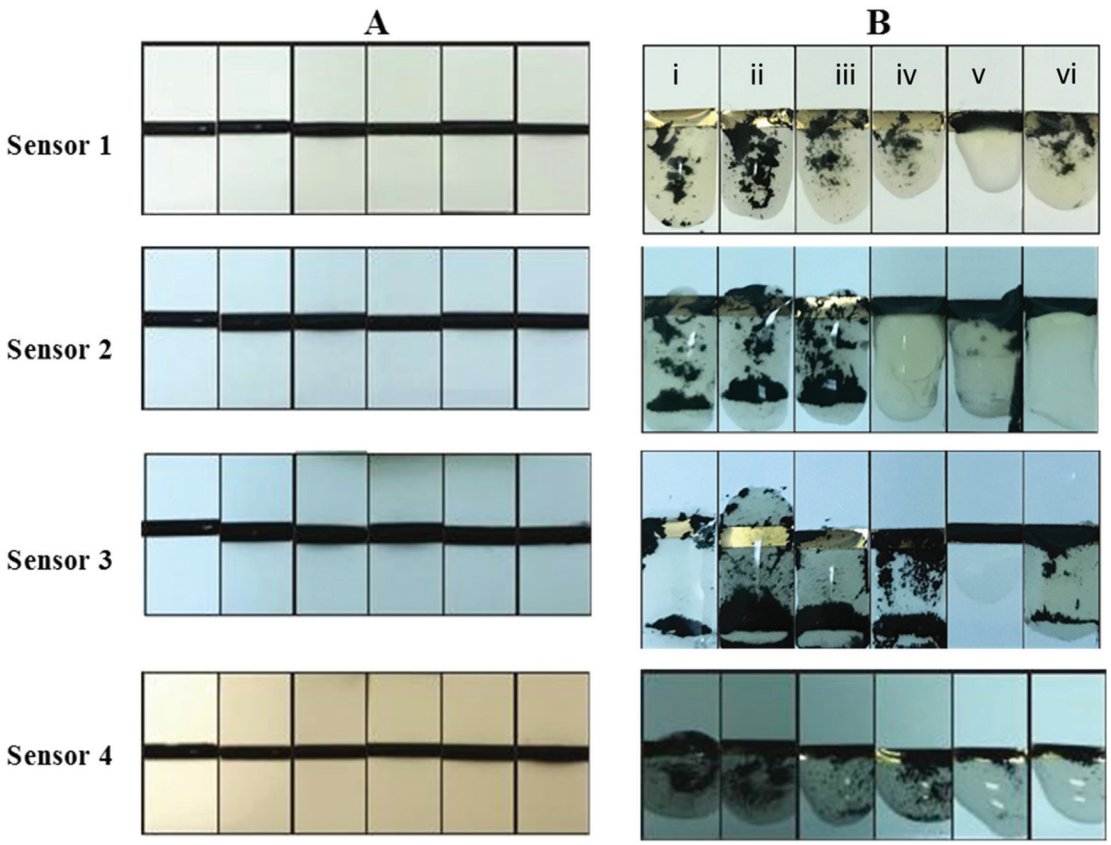

Fig. 3 Colorimetric detection of *Legionella* strains proteolytic activity using four peptide sensor nanoprobe. (A) Biosensor platform functionalized with black color peptide–NMPs conjugates. (Sensor 1) Ahx-YY-Ahx-Cys substrate covalently bound to MNPs. (Sensor 2) Ahx-WW-Ahx-Cys substrate covalently bound to MNPs. (Sensor 3) Ahx-FF-Ahx-Cys substrate covalently bound to MNPs. (Sensor 4) Ahx-LL-Ahx-Cys substrate covalently bound to MNPs. (B) Biosensor platform under the effect of (i) *L. pneumophila* ATCC 33155, (ii) *T. micdadei* ATCC 33218, (iii) *L. anisa* ATCC 35292, (iv) *F. dumoffii* ATCC 33279, (v) *L. pneumophila* ATCC 33152, and (vi) *F. bozemanae* ATCC 33217 culture supernatant (6×10^8 CFU mL^{-1}).

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compared with the associated bacteria such as *E. coli*, *S. Enterica*, and *Campylobacter jejuni* (Table 3).

3.2. Biosensor structuring

Legionella protease-specific dipeptides were identified from the above FRET assay. These dipeptides were used for the paper-based protease assay. Biosensor peptide probes were prepared using L-amino acid dipeptide substrates elongated with an amino hexanoic acid Ahx-residue spacer on either terminus of the peptide (NH_2-Ahx-Y-Y-Ahx-Cys, NH_2-Ahx-W-W-Ahx-Cys, NH_2-Ahx-F-F-Ahx-Cys, NH_2-Ahx-L-L-Ahx-Cys) for easy accesability of the peptide bond in the dipeptide by the *Legionella* protease. The amine group of the substrates was coupled with carboxylic acid-functionlized NMPs to produce black colored peptide–NMPs conjugates. When the MNP-conjugated peptide was pipeted out on the golded surface, the thiol group (–SH) in the cysteine residue at the C-terminus of the substrate forms an irreversible covalent anchor with the golden platform to fabricate a colorimetric sensor.

3.3. Biosensor testing

This method aimed to visually detect the proteolytic activity of *Legionella* based on the use of specific peptide substrates by the naked eye, sandwiched between NMPs and gold platforms *via* a solid physical support. In the absence of *Legionella* protease supernatent, the gold surface is completely covered with the magnetic beads and it appears black (Fig. 3A). When the *Legionella* strains (*L. pneumophila* ATCC 33155, *L. micdadei* ATCC 33218, *L. anisa* ATCC 35292, *F. dumoffii* ATCC 33279, *L. pneumophila* ATCC 33152, and *F. bozemanae* ATCC 33217 culture supernatants (prepared from a 6×10^8 CFU mL^{-1} culture)) were dripped over the fabricated peptide sensor nanoprobes, the protease in the culture supernatant digest the peptide linkage between the amino acids in the dipeptide and break the peptide bond. The proteolytic activity of the *Legionella* strains was visualized by the dissociation of the peptide–NMPs segments from the sensor surface and the consequent restoration of the sensor golden color for the positive read-out result (Fig. 3B). The cleaved peptide–NMPs segments were trapped by a paper magnet fixed under the paper support. The fabricated sensors was Ahx-Y-Y-Ahx-Cys, NH_2-Ahx-W-W-Ahx-Cys, and NH_2-Ahx-F-F-Ahx-Cys substrates were efficiently cleaved by three *Legionella* species ((i) *L. pneumophila* ATCC 33155, (ii) *L. micdadei* ATCC 33218, and (iii) *L. anisa* ATCC 35292). Sensor 1 (NH_2-Ahx-Y-Y-Ahx-Cys) is effectively digested by (iv) *F. dumoffii* ATCC 33279 in addition to these three species. *F. bozemanae* ATCC 33217 (vi) supernatant shows significantly less digestion on all the four sensors. However, *L. pneumophila* ATCC 33152 (v) did not cleave any of the peptides effectively compared to the other five species of *Legionella*. Compared to sensors 1, 2, and 3, sensor 4 did not effectively digest all the six species of *Legionella*. The overall results of the four sensors response to six different species interestingly show that the sensors fabricated from the dipeptide with L-amino acids having aromatic side-chain were efficiently cleaved by all the *Legionella* species family (Fig. 3B, sensor 1, sensor 2, and sensor 3). On the other hand, sensor 4 fabricated from aliphatic amino acid (NH_2-Ahx-L-L-Ahx-Cys) did not show a significant effect by the proteases from six different species of *Legionella* family (Fig. 3B, sensor 4). The results are in correlation with the results obtained from FRET, where the dipeptide with the aromatic side chain has high fluorescence in the presence of *Legionella* species (Fig. 2). It is clear that the L-amino acid dipeptide substrate with aromatic side chains are specifically cleaved by the *Legionella* family of bacteria.

3.4. Quantification measurement

To further explore the sensitivity of the designed sensors, target biosensing methods were implemented for the quantitative detection of *L. pneumophila* ATCC 33155, *L. micdadei*

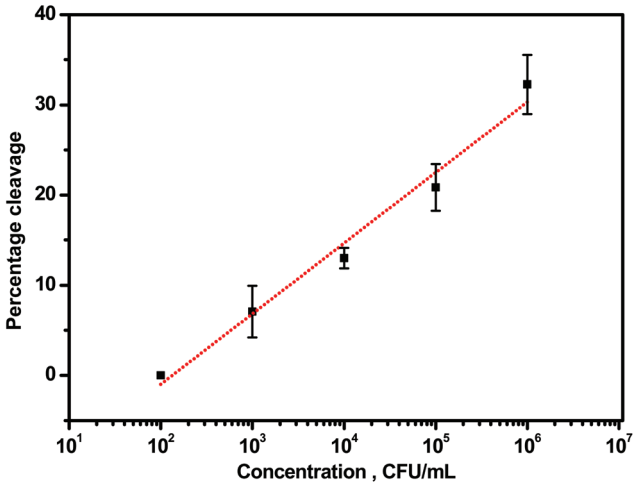

Fig. 4 The percentage cleavage of the MNP-conjugated peptides at variable concentrations of *L. pneumophila* ATCC 33152. All data show mean ± STD of triplicate measurements.

Table 4 Lower detection concentrations (6×10^x CFU mL^{-1}) of *L. pneumophila* ATCC 33155, *T. micdadei* ATCC 33218, *L. anisa* ATCC 35292, *F. dumoffii* ATCC 33279, *L. pneumophila* ATCC 33152 of the colorimetric biosensors

	Bacteria	Sensor 1 (Ahx-YY-AHX-C)	Sensor 2 (Ahx-WW-AHX-C)	Sensor 3 (Ahx-FF-AHX-C)	Sensor 4 (Ahx-LL-AHX-C)
A	*L.. anisa* ATCC 35292	10^1	10^5	10^6	10^3
	T. micdadei ATCC 33218	10^1	10^7	10^1	10^5
	L. pneumophilia ATCC 33155	10^4	10^7	10^3	10^1
	F. dumoffii ATCC 33279	10	10^7	10^2	10^2
	L. pneumophilia ATCC 33152	10^7	10^1	10^1	10^1

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ATCC 33218, *L. anisa* ATCC 35292, *F. dumoffii* ATCC 33279, and *L. pneumophila* ATCC 33152. Each strain was tested in various densities (Bacterial concentration (CFU mL^{-1})). The activity was examined using all the four peptide nanoprobes, namely, sensor 1 (Ahx-YY-Ahx-Cys), sensor 2 (Ahx-WW-Ahx-Cys), sensor 3 (Ahx-FF-Ahx-Cys), and sensor 4 (Ahx-LL-Ahx-Cys). The newly developed biosensors successfully detected the five different *Legionella* strains. The limit of detection (LOD) of *Legionella anisa* ATCC 35292 was 60 CFU mL^{-1} with sensor 1, followed by 6×10^3 and 6×10^5 CFU mL^{-1} with sensor 4 and sensor 2, respectively. The LODs of *L. micdadei* ATCC 33218 with sensor 1 and sensor 3 were 6×10^1 CFU mL^{-1}. However, sensors 4 and 2 show high LODs of 6×10^5 and 6×10^7 CFU mL^{-1}, respectively (Table 4). For clearer presentation, the calibration curve for *L. pneumophilia* ATCC 33155 sensor, as an example, was represented as the percentage cleavage *vs.* log concentration. The data acquisition and analysis included a computer with a data acquisition ImageJ program (developed at the National Institute of Health).[23–25] The calibration curve is depicted in Fig. 4. The LOD was calculated from the equation $\mathrm{LOD} = \frac{3.3\mathrm{SD}}{s}$, where SD is the standard deviation of the signal of blank samples in the absence of target protease and s is the slope obtained from the linear calibration curve.

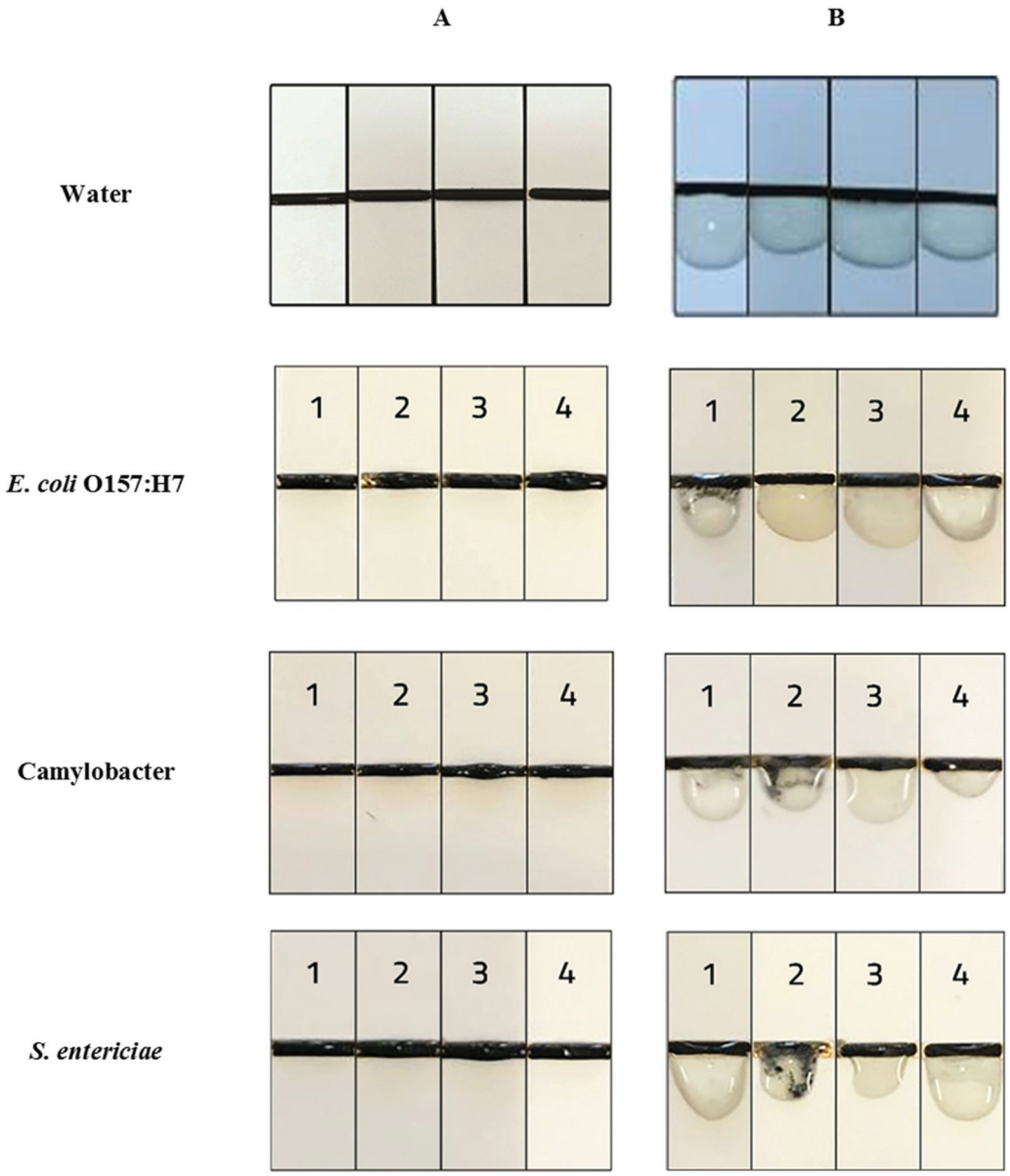

Fig. 5 Specificity of the *Legionella* colorimetric peptide nanoprobs biosensor. (A) Biosensor platform functionalized with black color peptide–NMPs conjugates. (Sensor 1) Ahx-YY-Ahx-Cys substrate covalently bound to MNPs. (Sensor 2) Ahx-WW-Ahx-Cys substrate covalently bound to MNPs, (Sensor 3) Ahx-FF-Ahx-Cys substrate covalently bound to MNPs, (Sensor 4) Ahx-LL-Ahx-Cys substrate covalently bound to MNPs. (B) *Legionella* nanoprobes (Sensor 1, Sensor 2, Sensor 3, and Sensor 4) under the effect of *E. coli* O157:H7, *Campylobacter*, and *S. entericia*.

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Notably, sensor 1 (Ahx-YY-AHX-Cys) was able to detect three *Legionella* strains (*L. anisa* ATCC 35292, *L. micdadei* ATCC 33218, and *F. dumoffii* ATCC 33279) with high sensitivity (60 CFU mL^{-1}) and sensor 4 (Ahx-LL-AHX-Cys) detected both *L. pneumophilia* strains with comparable sensitivity (60 CFU mL^{-1}).

This difference in the *Legionella* strains proteolytic cleavage patterns is in agreement with that reported by Lei *et.al*., where different *Legionella* strains had variable digestion activity on the tested synthetic peptide substrates. This may be correlated to the structural, functional, and genetic differences between the *Legionella* strains despite some observed similarities.[28]

This biosensor is simple in term of the detection reporter (showing positive readout visually), speedy in comparison with other detection methods that require minimum 2 h for PCR and few days for culturing (Table 1), sensitive with a lower limit of detection (LOD) of 60 CFU mL^{-1} for *L. anisa*, *L. micdadei*, and *F. dumoffii* using the Ahx-YY-Ahx-Cys nanoporobe, and *L. pneumophilia* using the Ahx-LL-Ahx-Cys nanoprobe (Table 4). The LOD was based on the visual evaluation of the lowest *Legionella* protease concentration incapable of restoring the biosensing platform's golden color following the cleavage of peptide–NMPs fragments. Visual detection was done side by side with the control so that any reader with normal visual capability could ascertain the LOD. A minimum of three random respondents confirmed the color change (black to yellow).

Moreover, this biosensor has the ability to specifically discriminate *Legionella* from other pathogens and so could be considered as a powerful field diagnostic tool (Fig. 5). A summary of some commonly applied assays for the detection of *Legionella* strains such as LOD, sample media (matrix), positivity rate, and assay time are presented in Table 1. To this end, the reported methods do not fully satisfy the optimal detection performance criteria for field applications and require further simplification for point-of-care testing.

3.5. Biosensor specificity and cross-reactivity

Sensor stability was validated by testing the water matrix.[31,32] As seen in Fig. 3, no reaction with water samples was detected. The sensor specificity was also evaluated using proteases extracted from another common organisms. In this study, *E. coli*, *Campylobacter*, and *S. enterica* culture supernatant proteases were used to check possible cross-reactivity (Fig. 5) against sensors prepared with each of the specific substrates. Interestingly, no significant cleavage was observed by the other bacterial culture supernatants, thus strengthening the sensors specificity. In contrast, the tested *Legionella* spp. showed significant color change. The sensor with the Ahx-WW-Ahx-Cys substrate was decolored slightly by the addition of *Campylobacter* and *Salmonella enterica* proteases, indicating that there is an insignificant cross-reactivity by these species. However, the other species have no effect on the sensors. Thus, the developed biosensors are a promising detection tool for *Legionella* spp. because of their low cost, specificity, and rapidity.

4. Conclusions

Herein, we have selected *Legionella* specific dipeptide fluorogenic substrates by FRET methods. The selected dipeptides were employed for the fabrication of the paper-based biosensors. The new calorimetric biosensors were able to detect the *Legionella* species, *Legionella anisa*, *Legionella micdadei*, and *Fluoribacter dumoffii* as low as 60 CFU mL^{-1} using the Ahx-YY-AHX-Cys nanoporobe, and detected *L. pneumophilia* using the Ahx-LL-AHX-Cys nanoprobe with comparable sensitivity within minutes and with high specificity. The cross-reactivity test confirms that there is no interference from the other closely related bacterial species on the sensor. This portable disposable platform confers several advantages for use onsite in hotels, hospitals, and other public areas for regular water monitoring. It allows fast and proper water treatment without the need for sample processing.

Conflicts of interest

All authors declare no conflict of interest.

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