Supporting Information (SI)

Naked-eye Detection of *Legionella pneumophila* Using Smart Fluorogenic Polymers Prepared as Hydrophilic Films, Coatings, and Electrospun Nanofibers

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S1. Materials, instrumentation and general methods.

Materials

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: *N*-vinyl-2-pyrrolidone (VP) (Acros Organic, 99%), 4-aminostyrene (TCI, 98%), methylmethacrylate (MMA) (Aldrich, 99%), ethylene glycol dimethacrylate (E) (Aldrich, 98%), hydrochloric acid (VWR, 37%), acetone (VWR, 99%), acetonitrile (VWR, 99.9%), dimethylsulfoxide (VWR, 99%), dimethylformamide (Supelco, 99.9%), diethyl ether (VWR-Prolabo, 99.9%), glutaraldehyde (Thermo Scientific, 25% aq. Soln.) glycerol (Aldrich, +99.5%), sodium dodecyl sulphate (SDS) (Sigma-Aldrich, 95%), bromophenol blue (Alfa Aesar), dithiothreitol (DTT) (TCI, 98%), glycine (Alfa Aesar, 99%), 40 % acrylamide/bis solution, 37.5:1 (BioRad, 99%), ammonium persulphate (BioRad), tetramethylethylenediamine (TEMED) (BioRad), Multicolour protein ladder, broad range, Spectra™ (Thermo Fisher Scientific), Coomassie Brilliant Blue R-250 (Thermo Scientific), acetic acid glacial (Merck, ≥99.7%), methanol (VWR-Prolabo, 99.9%), Legionella buffered charcoal yeast extract (BCYE) Agar Base (Liofilchem), Legionella BCYE Growth Supplement (Liofilchem), ACES (VWR, ≥99%), yeast extract (Sigma Aldrich), sodium hydroxide (VWR, 99%), 2-oxoglutaric acid (TCI, >99%), L-Cysteine HCL (VWR-polabo, 100%), iron nitrate (VWR, 100%), Trizma (Aldrich, 99.5%), sodium chloride (VWR, 98%), magnesium chloride hexahydrate (Fisher Scientific, >99%), bacto™ tryptone (Gibco), soy peptone (VWR), agar (WVR), K_2HPO_4 (VWR, 99.7%), D(+)-glucose (Merck, 100%), beef extract (Sigma-Aldrich), peptone (Millipore), fluorescein (Fluka, ≥99%), resorcinol (Alfa Aesar, 99%), filter paper in reams (Filter Lab, 73 g/m^2). Fluorogenic peptide substrate: Fluorescein isothiocyanate-6-Aminohexanoic acid-phenylalanine-phenylalanine-lysine-Dabcyl (FITC-Ahx-FFK-Dabcyl) was synthesized by GenScript company (N-Terminus: FITC-Ahx, 97.0%, additional information in Figures S1 and S2, SI-Section S2). Azo-bis-isobutyronitrile (AIBN, Aldrich, 99%) was recrystallised twice from methanol.

Instrumentation and general methods

The material's thermal properties were determined using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). DSC analysis was conducted with a TA Instruments Q200 DSC analyser at a heating rate of 20 ºC/min, using 10-15 mg of the sample under a nitrogen atmosphere. TGA analysis was performed with a TA Instruments Q50 TGA analyser at a heating rate of 10 ºC/min, utilising 10-15 mg of the sample under both synthetic air and nitrogen atmospheres.

Infrared spectra (FTIR) were recorded using a Jasco FT/IR-4200 infrared spectrometer equipped with an ATR-PRO410-S single reflection accessory.

Water swelling percentage (WSP) measurements were performed in triplicate and calculated using the following equation.

$$WSP = \left[\frac{g_{swelled\ material} - g_{dried\ material}}{g_{dried\ material}}\right] \times 100 \qquad \text{(eq. 1)}$$

The powder X-ray diffraction (PXRD) patterns were obtained using a Bruker D8 Discover (Davinci design) diffractometer operating at 40 kV with Cu(Kα) radiation. The scan step time was 2 seconds, and each spectrum was acquired from 5° to 70° with a step size of 0.05° (2θ).

Inductively coupled plasma mass spectrometry (ICP-MS) measurements were recorded using an Agilent 7500 ICP-MS spectrometer.

Raman spectra were acquired using a confocal AFM-Raman system, model Alpha300R—Alpha300A AFM from WITec. A laser radiation of 532 nm was employed, with magnifications set at 100×. All spectra were collected at room temperature.

Samples for PXRD and ICP-MS analyses were prepared by milling the polymeric materials using a ball mill machine, model MM400, from Retsch. The equipment operated at 25 Hz for 5 min, ensuring thorough homogenisation of the samples. Additionally, the capsules were pre-immersed in liquid nitrogen for 20 min before milling, facilitating efficient grinding and minimising potential thermal effects.

Gel images were obtained using the Gel DocTM EZ Imager (BioRad). The Synergy HT microplate reader (BioTek®) was used to measure fluorescence.

Electrospinning experiments were performed using a Spinbox Electrospinner System (Fluidnatek) and a rotating collector. The polymer was dissolved in DMF (15 % in weight) using a 20 mL vial and left in an orbital stirrer overnight.

Bacterial strains and inoculum preparation

In this study, *Legionella pneumophila* CECT 7109, *Staphylococcus aureus* CECT 239, *Klebsiella pneumoniae* CECT 8453, *Salmonella enterica* CECT 915, *Escherichia coli* O157:H7 CECT 4972 and *Pseudomonas aeruginosa* CECT 109 were used. *L. pneumophila* CECT 7109 was grown on BCYE Agar Base (Liofilchem) supplemented with Legionella BCYE Growth Supplement at 37 °C for 7 days. *S. aureus* CECT 239, *K. pneumoniae* CECT 8453 and *S. enterica* CECT 915 were grown on tryptocasein soy agar (TSA; tryptone 15g/L, soy peptone 5 g/L, sodium chloride 5 g/L, adjust pH to 7.2, agar 15 g/L) at 37 °C for 18 h. *E. coli* O157:H7 CECT 4972 and *P. aeruginosa* CECT 109 were grown on nutrient agar I (beef extract 5 g/L, peptone 10 g/L, sodium chloride 5 g/L, adjust pH to 7.2, agar 15 g/L) and nutrient agar II (beef extract 1 g/L, yeast extract 2 g/L, peptone 5 g/L, sodium chloride 5 g/L, adjust pH to 7.2, agar 15 g/L) respectively, for 18 h at 37 °C.

One colony of each bacteria was inoculated into BYE liquid medium (ACES 10 g/L, yeast extract 10 g/L, potassium hydroxide 2.2 g/L, α-ketoglutarate 1 g/L, L-cysteine HCL 0.4 g/L, iron nitrate 135 µg/mL, adjust pH to 6.8-6.9 [42]) for *L. pneumophila*; tryptone soy broth (TSB; tryptone 17 g/L, soy peptone 3 g/L, sodium chloride 5 g/L, glucose 2.5 g/L, K_2HPO_4 2.5 g/L, adjust pH to 7.2) for *S. aureus, K. pneumoniae* and *S. enterica*; nutrient broth I (beef extract 5 g/L, peptone 10 g/L, sodium chloride 5 g/L, adjust pH to 7.2) for *E. coli* and nutrient broth II (Beef extract 1 g/L, yeast extract 2 g/L, peptone 5 g/L, sodium chloride 5 g/L, adjust pH to 7.2) for *P. aeruginosa* and growth at 225 rpm for *L. pneumophila* or 180 rpm for the rest of bacteria at 37 °C overnight to attain a viable cell concentration ranging from 1×10^8 to 3×10^8 CFU/mL.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

30 µL of protein extracts were denatured using a loading buffer (50 mM Tris-HCl pH 6.8, 1% SDS, 0.02% bromophenol blue, and 10% glycerol) with DTT 1 mM for 5 min at 95 ºC. Then, the samples were ready to load in the electrophoresis gel. Electrophoretic detection of proteins was performed using 10% denaturing polyacrylamide gels (10% acrylamide/bis solution, 37.5:1, 0.375 M buffer Tris-HCl pH 8.8, 0.01% SDS, 5% glycerol, 0.005% APS, TEMED 10% APS volume). The electrophoresis was carried out using Tris-HCl 25 mM pH 8.3, glycine 192 mM, and 0.1% SDS as cathode buffer and Tris-HCl 25 mM pH 8.3, glycine 192 mM as anode buffer in the BioRad Mini Protein Electrophoresis Cell System for 60 minutes at 100 V and once the samples had entered the separating gel, run for 90 minutes at 200 V. In addition, Spectra™ Multicolor Broad Range Protein Ladder (Thermo Fischer) was used as a weight molecular pattern. After the electrophoresis, the gel was submerged into a staining solution of 0.25% Coomassie Blue R-250 in 40% methanol and 10% acetic acid to stain the proteins. The gel was initially destained with an aqueous solution containing 50% methanol and 10% acetic acid for 1 hour, followed by treatment with an aqueous solution containing 5% methanol and 7% acetic acid overnight. Gel images were obtained using Gel Doc™ EZ Imager (BioRad).

S2. Electrospun nanofibers characterisation.

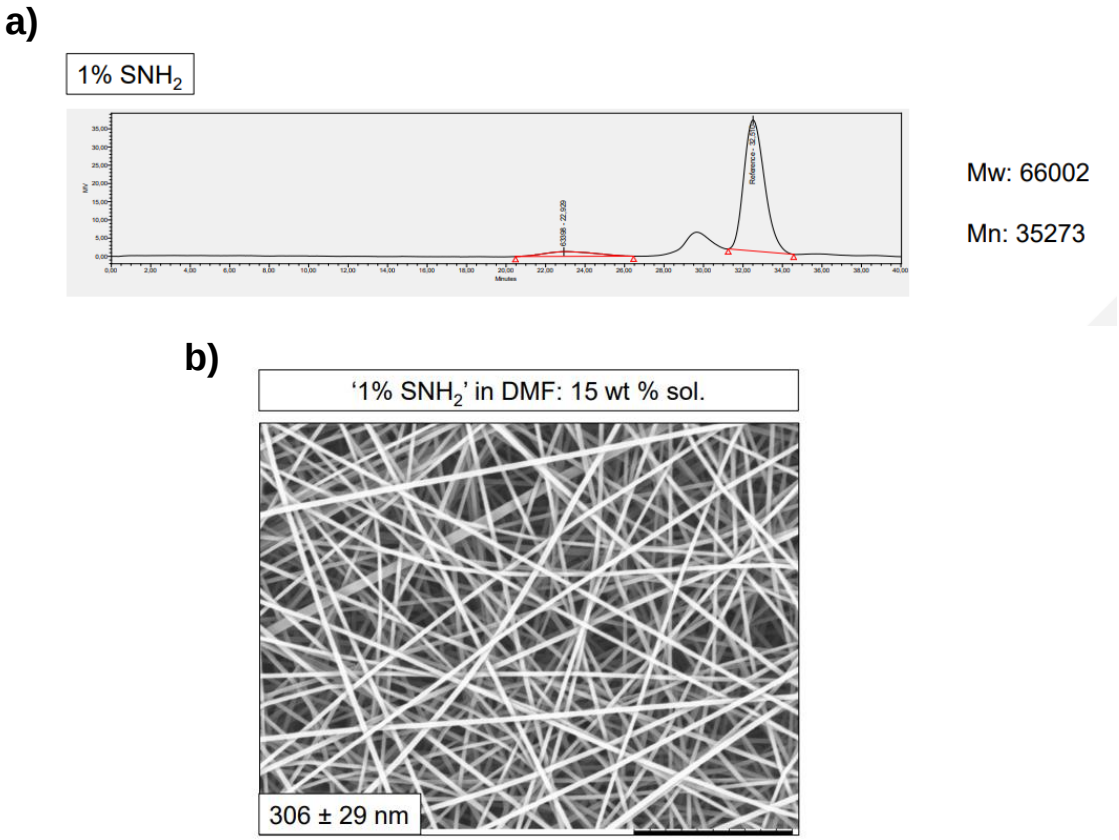

Figure S1. (a) Gel Permeation Chromatography (GPC) spectrum of the copolymers used for the electrospinning experiments. The samples were dissolved in THF at a concentration of 5 mg/mL and filtered (polyamide 45 µm) before injection into the GPC, which consisted of a pump (Shimadzu LC-20AD), three columns (Styragel HR2, HR4 and HR6), and a refractive index detector (Waters 2410). Chromatograms were obtained at 35 °C. THF was used as continuous phase at flow rate of 1 mL/min. The obtained molecular weights were related to polystyrene standards. **(b)** SEM micrographs of the electrospun polymer nanofibers (average nanofiber diameter was 306 ± 29 nm). Scanning Electron Microscope (SEM), Hitachi TM 3030, was used to characterise the morphology of the nanofibers obtained by electrospinning. 3 photos were taken at different places of the sample with x6000 magnification. ImageJ™ open-source software (National Institute of Health) was used to measure the mean average fibre diameter. Fifty measurements were done from the three separate images.

S3. Characterisation of the fluorogenic peptide substrate FITC-Ahx-FFK-Dabcyl.

a)

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CERTIFICATE OF ANALYSIS

Product Name	peptide
Order ID	U1519IA240_1
Lot No.	U1519IA240-1/PE9591
Sequence	{PHE}{PHE}{LYS(DABCYL)}
Modification	N - Terminal: FITC-Ahx
Length	3AA
Storage	-20°C
Recommended Solvent*	Dimethyl sulfoxide(Analytical grade)
Comments	TFA salt

Test Items	Specifications	Results
Molecular Weight	Theoretical MW: 1194.38	Consistent
HPLC purity	≥90.0%	97.0%
Appearance	Red lyophilized powder	Conforms
Gross Weight	80-100 mg	100.0mg

*Note: Above recommended solvents for reference only. If there is any request for detailed dissolution conditions,we suggest you choose our 'Peptide Solubility Test Service'.

Caution:
For laboratory or further manufacturing use only. Not intended for household use. If you have any questions about the Certificate of Analysis, please contact our customer service representative at 1-877-436-7274 (Toll-Free), or 1-732-885-9188.

Certified by: Yi Gao Date:02/23/2023

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b)

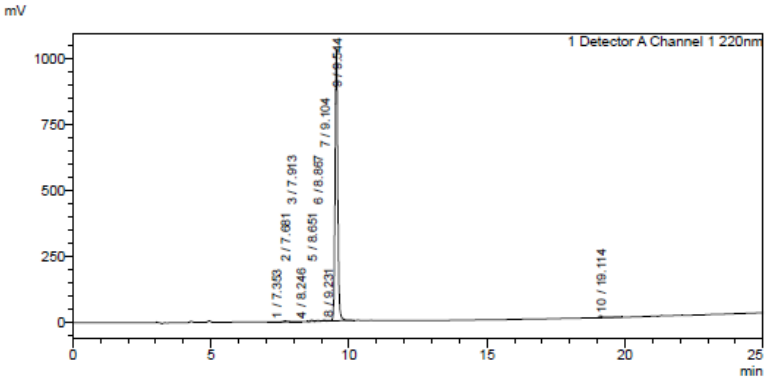
Sample Name :peptide
Sample ID :U1519IA240-1
Time Processed :13:27:29
Month-Day-Year Processed :02/21/2023

Pump A : 0.065% trifluoroacetic in 100% water (v/v)
Pump B : 0.05% trifluoroacetic in 100% acetonitrile (v/v)
Total Flow:1 ml/min
Wavelength:220 nm
<<LC Time Program>>

Time	Module	Command	Value
0.01	Pumps	B.Conc	35
25.00	Pumps	B.Conc	95
25.01	Pumps	B.Conc	95
32.00	Pumps	B.Conc	95
32.01	Pumps	B.Conc	5
35.00	Pumps	B.Conc	5
35.01	Controller	Stop	

<<Column Performance>>
<Detector A>
Column :Inertsil ODS-SP 4.6 x 250 mm
Equipment: GR11010440

<Chromatogram>



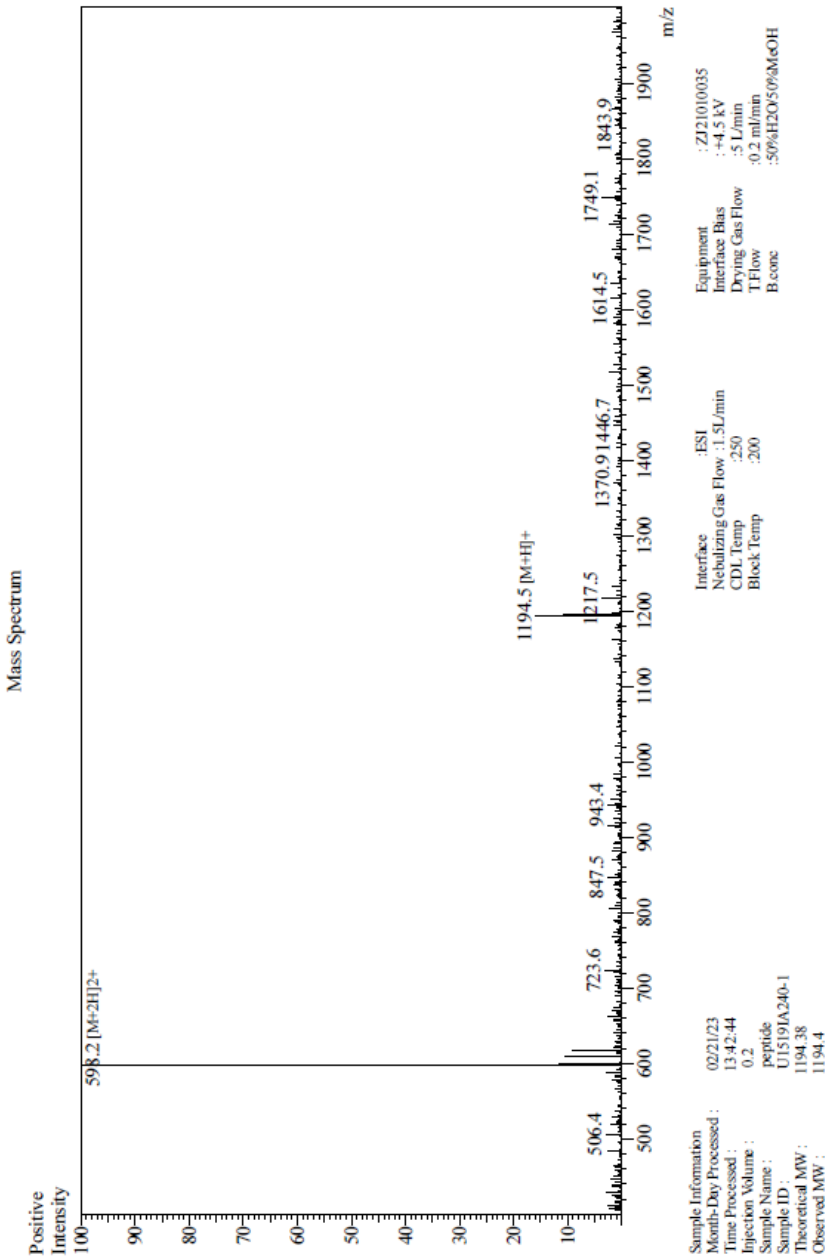
<Peak Table>

Detector A Channel 1 220nm

Peak#	Ret. Time	Area	Height	Area%
1	7.353	5718	370	0.080
2	7.681	27096	4155	0.380
3	7.913	1459	245	0.020
4	8.246	1223	141	0.017
5	8.651	34069	5438	0.478
6	8.867	34304	3663	0.482
7	9.104	30316	3969	0.426
8	9.231	12747	2394	0.179
9	9.544	6910334	1028800	97.021
10	19.114	65213	7278	0.916
Total		7122479	1056452	100.000

Figure S2. **(a)** Certificate of analysis and **(b)** HPLC of fluorogenic peptide substrate FITC-Ahx-FFK-Dabcyl.

a)



b)

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Peptide Qualitative Solubility Test Report

Name	peptide
Order ID	U1519IA240-1

Solvent	Results
ultrapure water	Insoluble
1X DPBS * (pH 7.1±0.1)	Insoluble
DMSO	Soluble

DPBS Dulbecco's Phosphate Buffered Saline, containing Potassium Chloride(KCl),Potassium Phosphate monobasic (KH_2PO_4),Sodium Chloride (NaCl)and Sodium Phosphate dibasic (Na_2HPO_4-$7H_2O$);

Comments:

1. The solubility of peptides is largely determined by the polarity of the peptides. Acidic proteins are dissolved in alkaline solutions, basic proteins can be dissolved in acidic solutions, and hydrophobic and neutral polypeptides containing a large number of uncharged polar amino acid residues or hydrophobic amino acids can be dissolved in a small amount of organic solvents first. Then dilute with water. Peptides with higher hydrophobicity are recommended to be dissolved in pure DMSO.
2. Freely soluble: the solvent is added to the sample, the sample dissolves immediately, and the solution is clear and transparent. Soluble: the solvent is added to the sample, the sample dissolves after shaking or sonication , and the solution is clear and transparent. Insoluble: The solvent is added to the sample, the solution is cloudy or flocculent by shaking or sonication. Note: The dissolved concentration of the sample is about 1mg/ml.
3. Peptides containing Cysteine (C), Methionine (M) or Tryptophan (W) are sensitive to oxidation by DMSO. We advise that peptides dissolved in DMSO be used immediately or stored at -20°C (or preferably -80°C)prior to use. Usually, we recommend that the peptides be used in time after dissolving. If the solution peptides need to be stored, it is recommended to store them in small samples to avoid repeated freezing and thawing.
4. When the peptide is insoluble in the solvent of your choice, please refer to the table above for other suggested solvents.
5. Please note that distinct dissolution behaviors may happen between small amounts and large amounts of gross peptide in the same solvent. Generally, larger amounts of peptide take longer to dissolve.
6. The test results are for reference only, and the user needs to choose a suitable solvent according to the experimental needs.

Tested by: Ying Lu
02-21-2023

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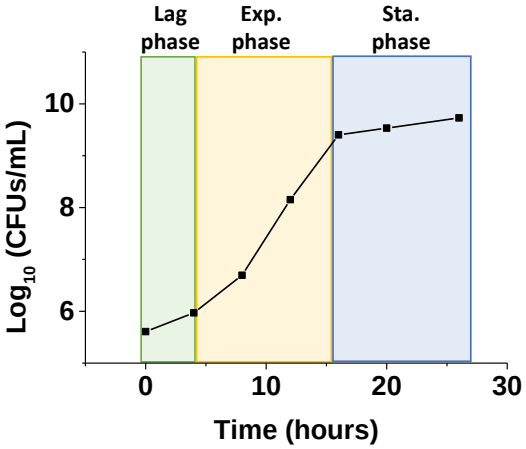
Figure S3. **(a)** Mass spectrum and **(b)** solubility test of fluorogenic peptide substrate FITC-Ahx-FFK-Dabcyl.

S4. Growth curves and extracellular protein extracts characterisation of *L. pneumophila*.

a)



b)

Time (h)	O.D 600	CFUs/mL	Log10 (CFUs/mL)	Growth phase
0	0.04	4.05E+05	5.61	Lag pase
4	0.15	9.30E+05	5.97	Lag pase
8	0.3	4.95E+06	6.69	Exponential pase
12	0.5	1.42E+08	8.15	Exponential pase
16	1.5	2.53E+09	9.40	Early stationary pase
20	2.3	3.40E+09	9.53	Stationary pase
26	3.4	5.35E+09	9.73	Stationary phase

Figure S4. Growth data of *L. pneumophila* strain. (a) Growth curve of *L. pneumophila* at 37 ˚C and 225 rpm expressed as Log_{10} of CFUs/mL. **(b)** *L. pneumophila* culture growth data; time of growth, optical density measured at 600 nm, the corresponding CFUs/mL and the growth phase are indicated. Lag, exponential (Exp.), and stationary (Sta.) phases are resalted in green, yellow and blue, respectively.

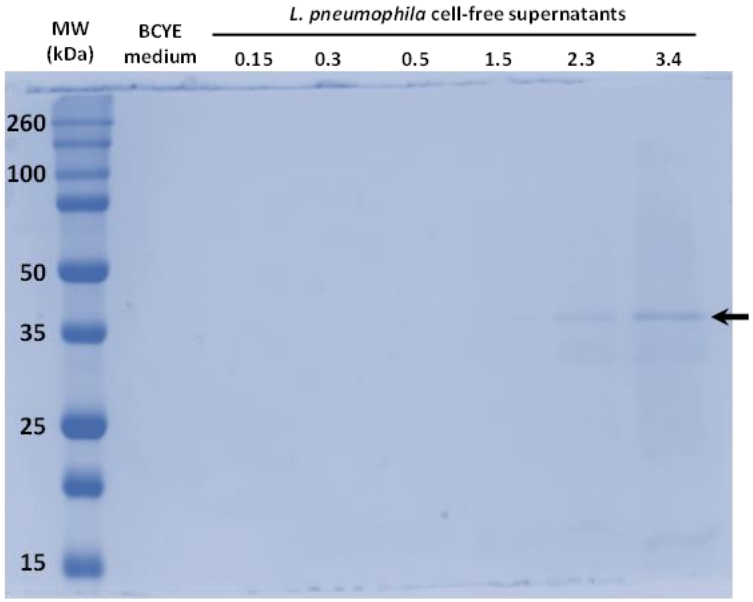


Figure S5. Analyses of Legionella extracellular protein extracts. SDS-PAGE of secreted proteins from *L. pneumophila* to the extracellular media at different stages of growth. The black arrow indicates the major secretory protein band. Spectra™ Multicolor Broad Range Protein Ladder (Thermo Fischer) was used as a weight molecular pattern.

S5. Detection limits of smart materials for the detection of *L. pneumoniae.*

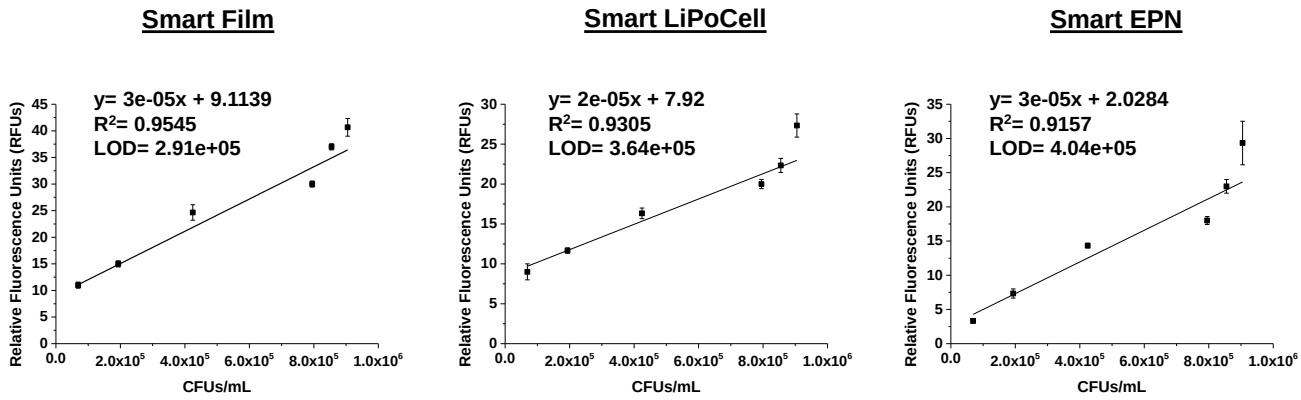


Figure S6. Calibration curves and detection limits of smart materials for *L. pneumoniae.*

S6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

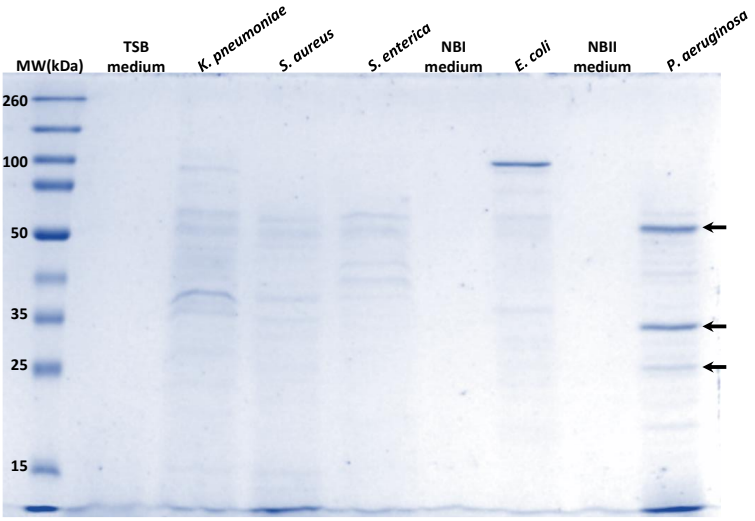
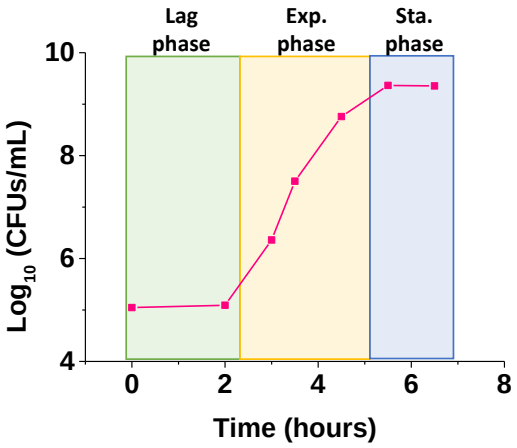


Figure S7. Analyses of extracellular protein extracts from *K. pneumoniae*, *S. aureus*, *S. enterica*, *E. coli*, and P. aeruginosa at the stationary growth phase. Black arrows indicate the most prominent bands found in the P. aeruginosa cell-free supernatant. TBS, NBI, and NBII mediums are the control mediums without proteins for each microorganism, depending on their culture conditions. Spectra™ Multicolor Broad Range Protein Ladder (Thermo Fischer) was used as a weight molecular pattern.

S7. Growth curves and extracellular protein extracts characterisation of *P. aeruginosa*.

a)



b)

Time (hours)	O.D 600	CFUs/mL	Log 10 (CFUs/mL)	Growth phase
0	0.04	1.12E+05	5.05	Lag phase
2	0.15	1.24E+05	5.09	Lag phase
3	0.3	2.30E+06	6.36	Exponential phase
3.5	0.5	3.19E+07	7.50	Exponential phase
4.5	1.5	5.74E+08	8.76	Exponential phase
5.5	2.3	2.31E+09	9.36	Stationary phase
6.5	3.4	2.26E+09	9.35	Stationary phase

Figure S8. Growth data of *P. aeruginosa* strain. (a) Growth curve of *P. aeruginosa* at 37 ˚C and 225 rpm expressed as Log10 of CFUs/mL. **(b)** *P. aeruginosa* culture growth data; time of growth, optical density measured at 600 nm, the corresponding CFUs/mL and the growth phase are indicated. Lag, exponential (Exp.), and stationary (Sta.) phases are resalted in green, yellow and blue, respectively.

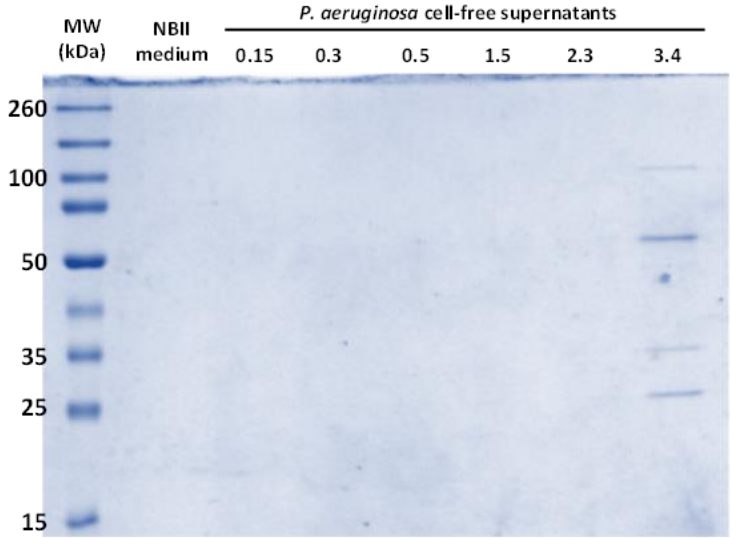


Figure S9. Analyses of *P. aeruginosa* extracellular protein extracts. SDS-PAGE of secreted proteins from *P. aeruginosa* to the extracellular media at different stages of growth. Spectra™ Multicolor Broad Range Protein Ladder (Thermo Fischer) was used as a weight molecular pattern.