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(54) Title: DETECTION OF BACTERIA

(57) Abstract: A method for bacteria detection and the assessment of the antimicrobial susceptibility profile uses a bacterial viability assay based on combining a highly efficient affinity bacteria enrichment/ separation with an electrochemical redox interrogation measurement. The method comprises: the affinity enrichment/ separation of bacteria from a liquid sample by using ligands-modified paramagnetic or superparamagnetic particles; the subsequent introduction of the bacteria isolate together with a solution containing a metabolic redox marker into a container for incubation; transferring the solution containing the metabolic redox marker and bacteria complexes to an electrochemical cell containing electrodes with magnets outside the reservoir so as to form a bacteria isolate (super)paramagnetic agglutinate on or close to the detection electrode, and the electrochemical detection of the change of the oxidation state of the metabolic redox marker inside the bacteria isolate (super)paramagnetic agglutinate as a direct measure of bacterial metabolic activity. Antimicrobial susceptibility testing (AST) is performed by adding an antibiotic to the sample at any stage, such as before the affinity enrichment/ separation.

DETECTION OF BACTERIA

Background to the Invention

[0001] This invention relates to a method for the electrochemical detection of viable bacteria in a sample.

[0002] Bacterial detection and the determination of the antimicrobial susceptibility pattern represent a cornerstone of clinical microbiology. Bacterial detection methods can be grouped into genotypic techniques that profile an organism's genetic material (primarily its DNA) and phenotypic techniques that profile an organism's metabolic attributes. The genotypic methods include techniques such as real-time polymerase chain reaction (PCR), fluorescence *in situ* hybridization (FISH) and 16S ribosomal RNA gene sequencing, which are independent of the physiological state of the organism and which are not influenced by the composition of the growth medium or by the organism's growth phase.^{9, 21, 24} However, the stability and the reproducibility of these methods can barely reach the requirements for routine clinical diagnosis. Phenotypic techniques provide understanding of the physiological and functional activities of the organism at the protein level. The predominant proteomic phenotypic technologies include Matrix Assisted Laser Desorption Ionisation-Time of flight-Mass Spectrometry (MALDI-TOF-MS), ElectroSpray Ionisation-Mass Spectrometry (ESI-MS), Surface Enhanced Laser Desorption Ionisation (SELDI) and Sodium DodecylSulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).⁹ Indeed, the main drawback of all these technologies is the culture-based approach, meaning that a lengthy culture enrichment is still necessary prior to the bacteria detection.

[0003] Antimicrobial susceptibility testing (AST) of bacterial pathogens for the determination of the antimicrobial susceptibility profile is considered as the gold standard in hospitals and microbiology laboratories, since the antimicrobial resistance (AMR) is a major threat to the public health worldwide. It is estimated that around 700,000 deaths occur annually due to the AMR.^{21, 22, 24-26} In hospitals, AST is conducted using phenotypic antimicrobial drug culture approaches like the broth (or agar)

microdilution method, disk diffusion method, gradient tests, agar dilution and breakpoint tests, which are based on the continuous exposure of a bacterial isolate to a set of antimicrobials followed by the optical detection of the growth.²⁴ Standard AST requires 1 - 3 days in order to allow the bacteria to multiply to detectable levels (culture enrichment). After bacteria pre-culture (hours or days), additional 12 - 24 hours are required to perform the susceptibility tests.²⁵ Until the AST results are received, the patients are usually treated with broad-spectrum antibiotics to cover the most probable pathogens. This procedure can result in incorrect initial therapy leading potentially to further infection and increased mortality.

[0004] Other techniques have been developed for the detection of the antimicrobial resistance including nucleic acid staining²⁹, nanoliter optical sensing of stochastic confinement¹³, motion detector using atomic force microscopy¹⁷, mass monitoring of bacterial growth using cantilevers¹² and surface plasmon resonance⁵. Molecular diagnostic assays that target DNA and genetic mutations associated with drug resistance provide faster diagnostics but require pre-knowledge with respect to the genetic basis of the antibiotic resistance for a target pathogen. Polymerase chain reaction (PCR)-based assays have complex nucleic acid amplification steps, and false positive and negative results may be obtained due to the amplification of complex matrices and enzymatic inhibition from different blood and urine components.⁹ Almost all of these techniques require bulky and complex equipment.

[0005] Bacterial viability assays have been employed for decades to provide a simple, reliable and cost-effective detection of metabolically-active cells, either by direct¹⁴ or indirect measurements of bacterial metabolites. Indirect methods include the monitoring of oxygen consumption in the bacteria medium or monitoring the oxidation state of redox markers (e.g. ferricyanide^{6, 11, 19}, resazurin², tetrazolium salts^{2, 20, 23, 27}). For the latter approach, bacteria are incubated together with the redox marker in physiological solution. If bacterial cells are metabolically active their metabolites create a chemically reducing environment that will reduce the oxidation state of the redox marker. This change can be followed using spectroscopic methods (UV-Vis

absorbance or fluorescence) or by electrochemical methods (voltammetry). Recently, viability assays have been adapted for antimicrobial susceptibility testing, where an antibiotic is added to the medium containing bacteria and the redox marker. In such cases, the resistant bacteria continue to multiply and metabolize the redox marker, i.e. reducing the oxidation state of the redox marker, while susceptible bacteria do not.²

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[0006] The sensitivity of the bacterial viability assays depends on the requirement of a sufficiently detectable quantity of the reduced form of the redox marker, which is directly related to the bacteria concentration and the reaction time of bacteria with the redox marker. For example, bacterial viability assays performed in microtiter plate formats (consuming microliter volumes) can require up to 8 h for the detection of low bacteria concentration, i.e. few cells mL⁻¹.

[0007] Indeed, different strategies have been followed to improve the sensitivity of bacterial viability assays. One strategy is based on increasing the local concentration of bacteria by using microfluidic devices in which bacteria are confined in microliter or nanoliter volumes.^{2, 3, 8, 16, 30} Despite this, the required time for analysis remains long (i.e. several hours). Very sensitive optical techniques for microfluidic devices operate with samples divided into nanoliter droplets inside a microfluidic channel where the signal is gathered sequentially from each droplet with a high-powered fluorescence microscope.^{3, 7, 15} Plenty of the microfluidic devices detect either the presence of bacteria or the antimicrobial susceptibility profile.^{2, 15, 28, 31}

[0008] Another strategy to improve the sensitivity of bacteria viability assays is to employ bacteria enrichment/separation and culture-independent immunological methods. These methods have been successfully used to improve the sensitivity for bacteria detection using MALDI-TOF-MS and fluorescence spectroscopy. For instance, Fe₃O₄ nanoparticle-graphene composite nanosheets decorated with chitosan were used to capture *Pseudomonas aeruginosa* and *Staphylococcus aureus* and perform the bacteria detection using fluorescence spectroscopy or MALDI-TOF-MS analysis.¹ Commercial anti-*Salmonella* Dynabeads® (Lake Success, USA) were used to

selectively isolate *Salmonella choleraesuis* for MALDI-TOF-MS analysis, with the limit of detection (LOD) being 107 cells mL⁻¹ in water and 109 cells mL⁻¹ in human urine or chicken blood.¹⁸ Janus magnetic mesoporous silica nanoparticles coated with anti-*E. coli* antibodies were used for foodborne bacterial enrichment and fluorescent identification of *E. coli* that can be combined with MALDI-TOF-MS.⁴

Summary of the Invention

[0009] The present invention relates to a method based on an electrochemical bacterial viability assay with a highly efficient affinity bacteria enrichment/separation in form of a bacteria isolate (super)paramagnetic agglutinate on or close to the detection electrode.

[0010] The invention provides a method according to claim 1. The method provides a significant reduction of bacteria culturing time and very sensitive detection of the oxidation state of the metabolic redox marker, which is directly linked to the number of viable bacteria in the sample. The method can be applied for total or species-specific bacteria detection and bacteria quantification (bacterial counts). If an antibiotic is added to the sample at any stage, such as before bacteria affinity enrichment/separation, the present method can be used to perform antimicrobial susceptibility testing (AST). Optional features of the invention are set out in the dependent claims.

Brief Description of the Drawings

[0011] The invention will now be described in more detail, by way of example only, with reference to the accompanying drawings, in which:

[0012] **Fig. 1a** schematically shows a method for the electrochemical viability assay based on a highly efficient affinity enrichment/separation of bacteria from a liquid sample;

[0013] **Fig. 1b** schematically shows details of steps in the method of **Fig. 1a** preceding the bacteria isolate (super)paramagnetic agglutinate formation;

[0014] **Fig. 2a** shows a typical differential pulse voltammogram illustrating the electrochemical detection of Gram-negative bacteria (*E. coli*);

[0015] **Fig. 2b** shows a typical differential pulse voltammogram illustrating the electrochemical detection of Gram-positive bacteria (*B. subtilis*);

[0016] **Fig. 2c** illustrates the detection of *E. coli* by using ferricinium methanol as the redox marker;

[0017] **Fig. 2d** shows a typical linear sweep voltammogram illustrating the detection of *E. coli* by using a microelectrode and ferricinium methanol as the redox marker;

[0018] **Fig. 3a** shows the bacterial count curve for *E. coli* (strain DH5a) obtained by using a fluorometric bacteria viability assay;

[0019] **Fig. 3b** shows the bacterial count curve for *E. coli* obtained using the method presented in **Fig. 1**;

Detailed Description of Particular Embodiments

[0020] **Figs. 1a and 1b** schematically illustrate the principle of the present invention. The method incorporates a highly efficient affinity enrichment/separation of bacteria from a liquid sample by using ligands-modified paramagnetic or superparamagnetic particles. Bacteria isolate-ligands-modified (super)paramagnetic particles are then incubated with a solution containing the metabolic redox marker in its oxidized form (RM_{OX}) prior to the transfer into the reservoir containing a detection electrode with a magnet positioned outside the reservoir, but close enough to the electrode, to accumulate the (super)paramagnetic particles and form a bacteria isolate (super)paramagnetic agglutinate on or close to the detection electrode (**Fig. 1b**). Bacteria metabolites present in the agglutinate react with the metabolic redox marker with higher oxidation state (i.e. in oxidized form) RM_{OX} and reduce them to a lower

oxidation state (i.e. reduced form) RM_{RED} . The RM_{OX} molecules or ions that were not reduced by the bacteria are then electrochemically reduced at the detection electrode using a competitive mode: the more viable bacteria, the more RM_{RED} is produced and the less RM_{OX} is electrochemically detected. The present invention can be employed for both qualitative and quantitative bacteria analysis. The method can be used for the detection of total viable bacteria when non-specific ligands are used or for the detection of certain bacteria species when bacteria species-specific ligands are used. Furthermore, if an antibiotic is added to the sample at any stage, preferably before bacteria affinity enrichment/separation, the electrochemical read-out can be used for assessing the antimicrobial susceptibility profile: susceptible bacteria killed by the antibiotic do not reduce RM_{OX} , while resistant bacteria reduce RM_{OX} .

[0021] **Figs. 2a-c** shows exemplary differential pulse voltammograms (DPV) illustrating the detection of Gram-negative (*E. coli*, 5×10^8 cells mL^{-1} , redox markers resazurin and ferricinium methanol, **Fig. 2a** and **Fig. 2c**, respectively) and Gram-positive bacteria (*B. subtilis*, 5×10^8 cells mL^{-1} , redox marker resazurin, **Fig. 2b**) by using the method presented in **Fig. 1**. DPV parameters for resazurin were the following: potential range 0.0 V to -0.6 V vs. Ag/AgCl quasi-reference electrode, potential step 0.01 V, potential pulse 0.1 V, pulse time 0.05 s and the scan rate 0.02 V/s. DPV parameters for ferricinium methanol were the following: potential range 0.4 V to 0 V vs. Ag/AgCl quasi-reference electrode, potential step 0.01 V, potential pulse 0.1 V, pulse time 0.05 s and the scan rate 0.02 V/s. Carbon nanotubes were used to form the working electrode, Ag/AgCl served as a quasi-reference electrode and a second carbon layer served as a counter electrode. The supporting electrolyte was Luria-Bertani medium. **Fig. 2d** shows a linear sweep voltammogram (LSV) illustrating the detection of *E. coli* (5×10^8 cells mL^{-1}), recorded with a platinum disk microelectrode, 25 μm in diameter, surrounded by an insulating glass sheath in a ferricinium methanol - ferrocene methanol mixture (ratio 4:1). The LSV parameters were the following: potential range 0.5 V to -0.2 V vs. Ag-QRE, potential step 0.005 V and potential scan rate 0.01 V/s, Ag wire as quasi-reference electrode and a platinum wire as a counter electrode.

[0022] Fig. 3 shows the results of quantitative bacteria analysis (bacterial counts) for *E. coli* obtained by using the fluorometric bacteria viability assay (Fig. 3a) and by using the present invention (Fig. 3b). The concentration range of *E. coli* was: a) 5×10^8 , b) 5×10^7 , c) 5×10^6 , d) 5×10^5 , e) 5×10^4 , f) 5×10^3 , g) 5×10^2 , h) 50, i) 5 and j) 0 cells mL^{-1} . Both methods used the same redox marker (i.e. resazurin) and the readout was conducted every 30 min until 8 h.

[0023] Table 1 shows the antimicrobial susceptibility testing (AST) report for multiresistant strain of *E. coli* DH5a (concentration 5×10^5 cells mL^{-1}) obtained by using the standard automatic phenotypic system based on the broth dilution method (bioMérieux Vitek 2 system) and by using the method presented in Fig. 1. Nine different antibiotics were tested in the following concentrations: 0 (control), 1, 2, 4, 8, 16, 32 and 64 $\mu\text{g mL}^{-1}$. The resistance phenotype was confirmed and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, version 2017. MIC - presents minimum inhibitory concentration, the lowest antibiotic concentration that can inhibit the growth of bacteria; R - resistant bacteria, S - susceptible bacteria.

[0024] Table 2 shows the antimicrobial susceptibility testing (AST) report for the blood sample containing the multi-resistant *E. coli* strain DH5a, obtained by using the method presented in Fig. 1. Nine different antibiotics were used in the following concentrations: 0 (control), 1, 2, 4, 8, 16, 32 and 64 $\mu\text{g mL}^{-1}$. The resistance phenotype was confirmed and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, version 2017. MIC - presents minimum inhibitory concentration, the lowest antibiotic concentration that can inhibit the growth of bacteria; R - resistant bacteria, S - susceptible bacteria.

Results

[0025] Fig. 2 shows that the present invention can be employed for qualitative bacteria analysis when using bacteria species-specific ligands. Paramagnetic particles coated with anti-*E. coli* antibodies (Abs) and anti-*B. subtilis* Abs were employed for the

specific capturing of *E. coli* and *B. subtilis* and the representative differential pulse voltammograms (DPVs) obtained at macroelectrodes by using resazurin as the redox marker are presented in **Fig. 2a** and **Fig. 2b**, respectively. The DPV obtained for the detection of *E. coli* at macroelectrodes by using ferricinium methanol as the redox marker is presented in **Fig. 2c**. A linear sweep voltammogram (LSV) at microelectrodes for the detection of *E. coli* by using ferricinium methanol as the metabolic redox marker is presented in **Fig. 2d**. The shapes of all of the voltammograms clearly indicate the differences between the blank samples and the samples containing specific bacteria. When the targeted bacteria species were present in the sample and captured by the species-specific ligand, it reduced a certain amount of RM_{OX} so that a lower amount of RM_{OX} was detected by the electrochemical reduction at the detection electrode, leading therefore to the decrease in the reduction/peak current (DPV) or reduction plateau current (LSV). Furthermore, due to the small finite volume of the redox marker solution in the electrochemical cell, coulometric measurements rather than amperometric measurements can be carried out.

[0026] **Fig. 3** shows that the present invention can be employed for quantitative bacteria analysis (bacterial counts). The presented method was compared with the standard commercial fluorometric bacteria viability assay (alamarBlue™ Cell Viability, ThermoFischer) for quantitative analysis of *E. coli* (strain DH5a). The obtained results indicate that the present method allows the detection of 5 millions of bacteria per mL within 1 hour (**Fig. 3b**) rather than 4 hours that are required for the standard fluorometric method (**Fig. 3a**).

[0027] **Table 1** shows that the present invention can be employed for the assessment of the antimicrobial susceptibility from pure bacteria cultures. The method was compared with a standard phenotypic system used in hospitals worldwide (bioMérieux Vitek 2 system) for antimicrobial susceptibility testing of the same multi-resistant *E. coli* strain DH5a. Due to the bacteria enrichment strategy and the sensitive

electrochemical detection, the presented method provides the antimicrobial susceptibility testing report in 2.5 h instead of >9 h, as required for the Vitek 2 system.

Table 1

Antibiotic	bioMérieux Vitek 2		Present method	
	MIC ($\mu\text{g mL}^{-1}$)	Interpretation	MIC ($\mu\text{g mL}^{-1}$)	Interpretation
Ampicillin	≥ 32	R	32	R
Cefuroxime	≥ 64	R	64	R
Cefpodoxime	≥ 8	R	16	R
Ceftriaxone	≥ 64	R	64	R
Gentamicin	≥ 16	R	16	R
Ciprofloxacin	≥ 4	R	8	R
Tetracyclin	≤ 1	S	1	S
Nitrofurantoin	≤ 16	S	8	S
Fosfomycin	≤ 16	S	8	S

[0028] Table 2 shows that the present invention can be employed for the assessment of the antimicrobial susceptibility directly from blood samples. Thanks to the highly efficient affinity enrichment/separation of bacteria from the blood samples, it was possible to start the antimicrobial susceptibility testing after only 6 h of blood culturing. Normally, the antimicrobial susceptibility testing is started when the blood bottles turn positive, which usually requires days of blood culturing. Furthermore, the antimicrobial susceptibility testing report was provided in 2.5 h, with an overall time from the blood sampling to the AST result being only 8.5 h.

Table 2

Antibiotic	Present method	
	MIC ($\mu\text{g mL}^{-1}$)	Interpretation
Ampicillin	32	R
Cefuroxime	64	R
Cefpodoxime	16	R
Ceftriaxone	64	R
Gentamicin	16	R
Ciprofloxacin	16	R
Tetracyclin	2	S
Nitrofurantoin	8	S
Fosfomycin	16	S

Advantages of the present invention

[0029] Bacterial viability assays have been employed for decades for bacteria detection based on the direct or indirect measurement of bacteria metabolites. For the indirect method, bacteria are incubated in solution together with a metabolic redox marker and if bacterial cells are metabolically active their metabolites create a reducing environment that will cause a change in the oxidation state of the redox marker that can be followed by spectroscopic or electrochemical methods. The principle is simple but challenging, because of limitations on achieving the necessary sensitivity due to the requirement of lengthy culture-based enrichment procedures. By using the present invention, bacterial cells are first captured from the sample by ligands-modified paramagnetic or superparamagnetic particles and by using an external

magnet are then transferred into a liquid medium containing the redox marker in low concentrations. Due to the highly efficient bacteria enrichment/separation step, long bacteria culturing times are reduced or even no longer necessary, depending on the bacteria concentration in the sample. Furthermore, the captured bacteria (super)paramagnetic particles are then introduced into the reservoir containing the electrodes and the magnets positioned so as to localize the bacterial metabolites on or close to the detection electrode's surface and to provide a very sensitive detection of the redox marker as a direct measure of bacterial viability. Accordingly, the present invention can be used for total or species-specific bacteria detection and bacteria quantification (bacterial counts). Moreover, if an antibiotic is added in the sample medium at any stage, such as before bacteria affinity enrichment/separation, the invention can be used for the detection of antimicrobial resistance. Furthermore, the electrochemical detection can operate in dark and turbid samples where spectroscopic detection methods are hindered.

Examples

Example 1 - Bacteria detection using the electrochemical viability assay with highly efficient affinity enrichment/separation

[0030] *Escherichia coli* (*E. coli*) strain DH5a and *Bacillus subtilis* (*B. subtilis*) strain 168 were grown as pre-cultures in Luria-Bertani medium at 37 °C for 6 h with continuous shaking at 200 rpm. 100 µL of each pre-culture was added into 3 mL of Luria-Bertani medium and incubated overnight at 37 °C with continuous shaking at 200 rpm. The obtained fresh cultures were afterwards used as stock solution for further experiments. Concentrations of bacterial cells in Luria-Bertani medium were determined by measuring the optical density at 600 nm (OD_{600nm}). 1.0 OD_{600nm} corresponds to $\sim 8 \times 10^8$ cells mL^{-1} .

[0031] In order to demonstrate the feasibility of the present method for bacteria detection, two model solutions were prepared: the first solution containing 5×10^8 5×10^8 cells mL^{-1} of *E. coli* in Luria-Bertani medium and the second solution containing

5×10^8 cells mL^{-1} of *B. subtilis* in Luria-Bertani medium. Protein A/G-coated superparamagnetic beads (MBs, Thermo Scientific) were modified with anti-*E. coli* (2 mg mL^{-1} , polyclonal, IgG isotype, Abcam) and anti-*B. subtilis* antibodies (4 mg mL^{-1} , polyclonal, IgG isotype, Abcam) and washed with phosphate buffered saline containing 0.05% (V/V) of Tween 20. The mixture was incubated for 1 h at room temperature with continuous shaking and transferred into 1 mL of the model solutions containing *E. coli* or *B. subtilis*. After incubation with continuous shaking for 30 min at 37°C , the superparamagnetic beads with captured bacterial cells were collected using a magnetic separation stand.

[0032] The collected superparamagnetic complexes were then incubated with 25 μL of Luria-Bertani medium containing redox marker (1 mM resazurin, 2 mM ferricinium methanol or a 2 mM mixture of ferricinium methanol and ferrocene methanol (e.g. in a ratio of 4:1)) at 37°C for 25 min. After incubation, the mixture was transferred into a reservoir of a microchip. The microchip was composed of eight reservoirs, each containing an inkjet-printed carbon nanotube working electrode (WE), Ag/AgCl quasi-reference electrode (QRE) and a second carbon layer as a counter electrode (CE). The microchip was placed in a holder containing eight integrated magnets that are located below the working electrode of each reservoir, separated by the microchip plastic support. In this way, the bacteria cells captured by the superparamagnetic beads were concentrated closely to the working electrode surface. The electrochemical read-out was conducted by employing differential pulse voltammetry (DPV) at carbon nanotube (CNT) macroelectrodes and linear sweep voltammetry (LSV) at platinum microelectrodes. DPV parameters for resazurin were as follows: potential range 0.0 V to -0.6 V vs. Ag/AgCl quasi-reference electrode, potential step 0.01 V, potential pulse 0.1 V, pulse time 0.05 s and the scan rate 0.02 V/s. DPV parameters for ferricinium methanol were as follows: potential range 0.4 V to 0 V vs. Ag/AgCl quasi-reference electrode, potential step 0.01 V, potential pulse 0.1 V, pulse time 0.05 s and the scan rate 0.02 V/s. LSV parameters for ferricinium methanol were as follows: potential range 0.5 V to -0.2 V vs. a Ag quasi-reference electrode, potential step 0.005 V and potential scan rate 0.01 V/s. The recorded differential pulse voltammograms

for the samples with *E. coli* and *B. subtilis* obtained by using resazurin as the redox marker are presented in **Fig. 2a** and **Fig. 2b**, respectively; the differential pulse voltammogram for the sample with *E. coli* obtained by using ferricinium methanol as the redox marker is presented in **Fig. 2c**; the linear sweep voltammogram for a sample with *E. coli* obtained by using an 4:1 ferricinium methanol:ferrocene methanol mixture as the redox marker system is presented in **Fig. 2d**. The plots clearly indicate the differences between the blank samples and the samples containing specific bacteria species.

Example 2 - Bacterial counts - quantitative analysis of microbes using the electrochemical viability assay with highly efficient affinity enrichment/separation

[0033] Bacterial counts were conducted using the standard fluorometric method and the present method. The fluorometric method was adopted from the protocol of alamarBlue™ Cell Viability kit by ThermoFischer. *E. coli* strain DH5a was cultured as described in *Example 1*. The bacteria were incubated with 1 mM of resazurin at 37°C. The cell concentrations were set as 5×10^8 , 5×10^7 , 5×10^6 , 5×10^5 , 5×10^4 , 5×10^3 , 5×10^2 , 50, 5 and 0 cells mL⁻¹, obtained by 10-fold serial dilution of the *E. coli* stock solution. The fluorescence emission at 590 nm of each solution was measured after every 30 min of incubation, which was carried out continuously for 8 h. The fluorescence measurement was conducted with a PerkinElmer LS 55 fluorescence spectrometer. The instrumental parameters were set as excitation wavelength at 540 nm, emission wavelength window from 555 nm to 700 nm, excitation slit 4.4 nm, emission slit 4.4 nm and low gain. Bacterial counts obtained by the fluorometric method are presented in **Fig. 3a**.

[0034] Bacteria counts were conducted also by using the present method based on bacteria species-specific enrichment/separation. As described in *Example 1*, protein A/G-superparamagnetic beads were coated with anti-*E. coli* antibodies and transferred into 1 mL of Luria-Bertani medium containing different concentrations of *E. coli* (5×10^8 , 5×10^7 , 5×10^6 , 5×10^5 , 5×10^4 , 5×10^3 , 5×10^2 , 50, 5 and 0 cells mL⁻¹), obtained

by 10-fold serial dilution of the stock solution. After incubation with continuous shaking for 30 min at 37°C, the superparamagnetic beads with captured *E. coli* were collected using the magnetic stand.

[0035] The collected complexes for each bacteria concentration were incubated with 25 μ L of Luria-Bertani medium containing the redox marker (1 mM resazurin) at 37°C for 25 min. After incubation, the mixture was transferred into a reservoir of a microchip where the bacteria cells captured by superparamagnetic beads were concentrated closely to the working electrode surface (as described in *Example 1*). The electrochemical read-out was conducted every 30 min for up to 8 h by employing differential pulse voltammetry (DPV). Obtained bacterial counts show that the present method allows the detection of 5 millions of bacteria per mL within 1 h rather than 4 h that are required for the fluorometric method, as shown in **Fig. 3b**.

Example 3 - Antimicrobial susceptibility testing (AST) of pure bacteria cultures by using the electrochemical viability assay with highly efficient affinity enrichment/separation

[0036] The multi-resistant *E. coli* strain DH5a was provided by the Hospital of Valais, Sion, Switzerland. For each bacteria sample, bacterial cells were harvested from the growth medium by centrifugation at 7,500 g for 3 min, then washed two times with deionized water and suspended in deionized water (18.2 M Ω cm) to reach a concentration of $\sim 5 \times 10^5$ cells mL⁻¹ in Luria-Bertani medium. A detailed antimicrobial susceptibility profile of the *E. coli* strain was obtained by using the bioMérieux VITEK 2 automated AST system in Hôpital du Valais, Sion, Switzerland (**Table 1**).

[0037] Antimicrobial susceptibility testing of the same *E. coli* strain was conducted using the present method. Briefly, in each Eppendorf tube containing 1 mL of 5×10^5 cells mL⁻¹ a certain concentration of an antibiotic was added at 37°C and incubated with continuous shaking for 1 h. Nine different antibiotics were used for the test: ampicillin, cefuroxime, cefpodoxime, ceftriaxone, gentamicin, ciprofloxacin, tetracycline, nitrofurantoin and fosfomycin (Sigma-Aldrich). Based on the broth dilution method, the antibiotic concentrations in different tubes were: 0 (control), 1, 2,

4, 8, 16, 32 and 64 $\mu\text{g mL}^{-1}$. After the first incubation, superparamagnetic beads (10 mg mL^{-1}) conjugated with the anti-*E. coli* antibodies were added into each tube to capture viable *E. coli* cells and incubated under continuous shaking at 37°C for 30 min. Afterwards, the superparamagnetic beads with captured viable *E. coli* cells were collected and the supernatant was discarded. The collected complex was incubated with 25 μL of the redox marker solution (200 μM resazurin in Luria-Bertani medium) at 37°C for 25 min and then introduced into the reservoir of the microchip (as described in *Example 1*). In order to obtain the antimicrobial susceptibility profile, the electrochemical read-out was conducted by employing differential pulse voltammetry (DPV). The DPV signal obtained from a measurement with the antibiotics was compared with that without the antibiotic (the control). If the former is the same as the latter, bacteria were defined as resistant against that concentration of the antibiotic. Otherwise, the bacteria were defined as susceptible to that concentration of the antibiotic. The resistance phenotype was confirmed and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, version 2017. A detailed antimicrobial susceptibility testing report is shown in **Table 1**. The present method provides the AST results in 2.5 h instead of > 9 h required for the standard phenotypic system Vitek 2.

Example 4 - Antimicrobial susceptibility testing (AST) for blood stream infections by using the electrochemical viability assay with highly efficient affinity enrichment/separation

[0038] The bacteria concentration in adult patients' blood with bloodstream infections is typically 1 - 100 CFU (colony forming unit) per mL. To simulate real diagnosis, 6 mL of whole blood collected from a patient was spiked with the multi-drug resistant *E. coli* cells (100 cells mL^{-1}), injected into a BacT/Alert® FA Plus aerobic blood culturing bottle (containing 30 mL of broth) and cultured at 37°C with continuous shaking. After 6 h of incubation, 1 mL of the culture liquid was firstly centrifuged at 1200 rpm for 5 min to remove red blood cells; then at 13,000 rpm for 3 min to precipitate bacteria cells. The precipitated cells were re-suspended in 1 mL of Luria-

Bertani medium and captured with the superparamagnetic beads conjugated with anti-*E. coli* antibodies. The captured bacteria complex was incubated at 37°C for 60 min with 24 μL of Luria-Bertani medium containing one antibiotic for each measurement. Nine different antibiotics were tested: ampicillin, cefuroxime, cefpodoxime, ceftriaxone, gentamicin, ciprofloxacin, tetracycline, nitrofurantoin and fosfomycin (Sigma-Aldrich). Based on the broth dilution method, the antibiotic concentrations were: 0 (control), 1, 2, 4, 8, 16, 32 and 64 $\mu\text{g mL}^{-1}$. After the incubation, 1 μL of the redox marker (5 mM resazurin) was added and the mixture was incubated for 30 min to allow the reduction of redox marker by living bacteria. The mixture was introduced into the reservoir of the inkjet-printed microchip (as described in the *Example 1*). The antimicrobial susceptibility profile was obtained as explained in the *Example 3*. With the present method it was possible to start AST after only 6 h of blood culturing. Afterwards, the AST results are obtained in 2.5 h with the overall time from the blood sample to the AST result being only 8.5 h (**Table 2**).

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Claims

1. A method for the electrochemical detection of viable bacteria in a sample, the method comprising:

- affinity enrichment/separation of bacteria from a liquid sample using ligands modified paramagnetic or superparamagnetic particles, to form bacteria isolate-(super)paramagnetic complexes;
- placing the complexes together with a metabolic redox marker in solution into a container for incubation;
- subsequently transferring the solution containing the metabolic redox marker and complexes to an electrochemical cell having a detection electrode with an external magnet close to the detection electrode, such that the complexes form a bacteria isolate (super)paramagnetic agglutinate on or close to the detection electrode; and
- using an electrochemical read-out procedure to detect a change in oxidation state of the metabolic redox marker as a direct response to the metabolic activity of viable bacteria.

2. A method according to claim 1, wherein, in order to identify viable bacteria in the sample, the ligands modified paramagnetic or superparamagnetic particles include bacteria species-specific ligands.

3. A method according to claim 2, wherein the bacteria species-specific ligands contain affinity labels antibodies, single-stranded DNA aptamers or bacteriophages.

4. A method according to claim 1, wherein, in order to quantify the presence of viable bacteria in the sample in terms of end-point or kinetic assays, the ligands-modified

paramagnetic or superparamagnetic particles include bacteria species non-specific ligands showing affinity to bacterial cells.

5. A method according to claim 4, wherein the non-specific ligands comprise lectins, mammalian immunoglobulins, antimicrobial peptides, carbohydrate-based polymers or biomimetic macromolecular constructs.

6. A method according to any preceding claim, wherein the electrochemical read-out procedure comprises an amperometric method, the detection electrode being used to measure a current, in association with a reference and/or counter electrode.

7. A method according to claim 6, wherein the electrochemical read-out procedure comprises steady-state voltammetry, linear sweep voltammetry or differential pulse voltammetry.

8. A method according to claim 6 or 7, wherein the detection electrode is one of: a screen-printed graphite or carbon electrode, an inkjet-printed carbon nanotube electrode, carbon, gold or platinum microelectrode, for example made by photolithography.

9. A method according to any one of claims 1 to 5, wherein the electrochemical read-out procedure comprises a coulometric method.

10. A method according to any one of claims 1 to 5, wherein the electrochemical read-out procedure is a potentiometric measurement for the redox marker oxidation state, or for the associated pH variation.

11. A method according to claim **10**, wherein the detection electrode is a potentiometric redox sensor or a pH glass electrode.

12. A method according to any preceding claim, wherein the sample is one of: blood, serum, plasma, urine, sputum, saliva, or interstitial fluid; in pure, diluted or any other processed liquid form.

13. A method according to any one of claims **1** to **11**, wherein the sample is selected from a laboratory prepared bacteria culture, an environmental sample, an industrial sample, a food sample or a beverage sample.

14. A method according to any preceding claim, wherein the metabolic redox marker is a water-soluble substrate for a mitochondrial or cytoplasm enzyme produced by viable bacteria.

15. A method according to claim **14**, wherein the metabolic redox marker is one of: a water soluble oxidised metallocene such as ferricinium methanol, an organometallic complex such as osmium tris-bipyridine, a redox dye such as a tetrazolium salt, resazurin, methylene blue or formazan.

16. A method according to any preceding claim, wherein one or more antibiotics are introduced into the sample, which contains bacteria, and wherein the change in oxidation state of the metabolic redox marker indicates if bacteria are resistant or susceptible to the one or more antibiotics.

17. A method according to claim **16**, wherein the one or more antibiotics are in solid form, liquid form or solubilized and introduced to the sample before the affinity enrichment/separation.

18. The method of claim **17**, wherein the one or more antibiotics are coated on paper, nitrocellulose, a hydrogel, or a polymer.

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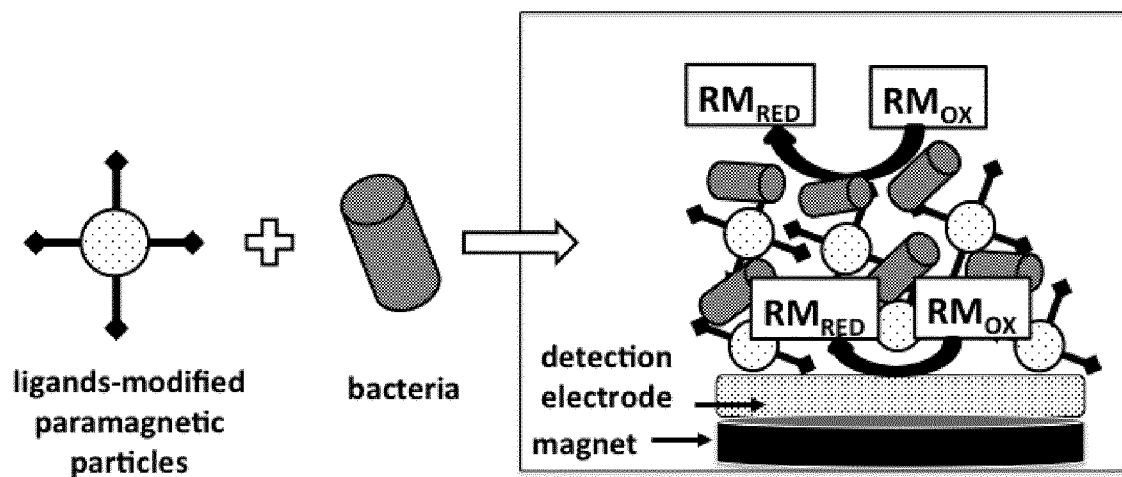


Fig. 1a

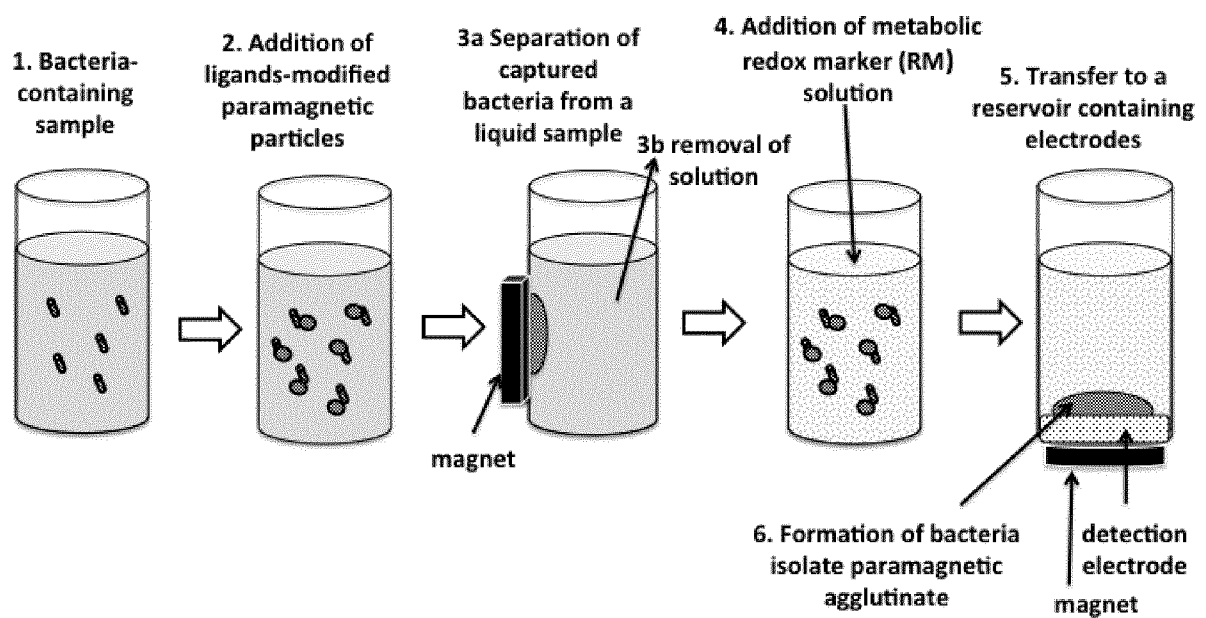


Fig. 1b

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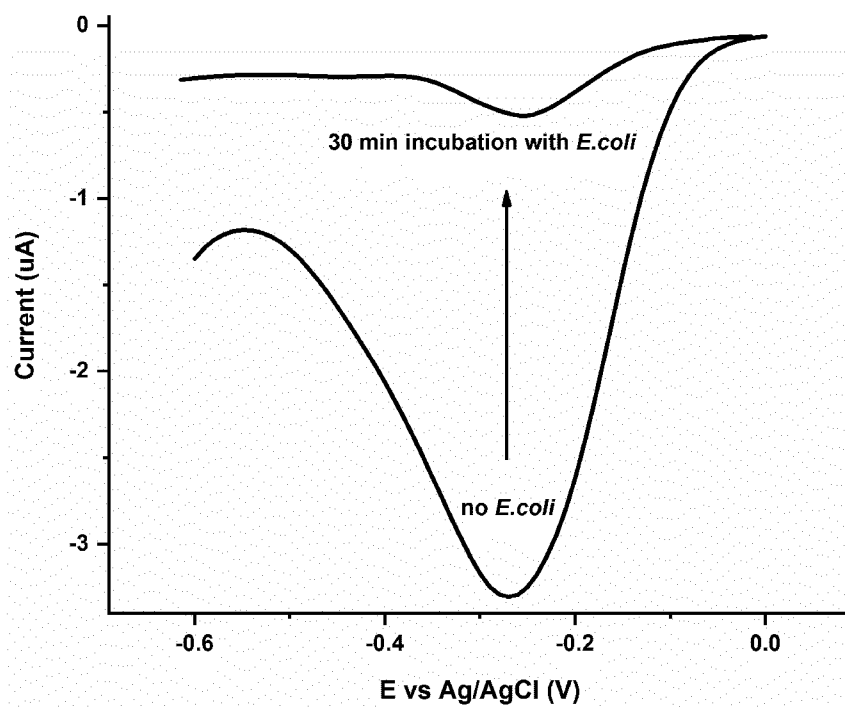


Fig. 2a

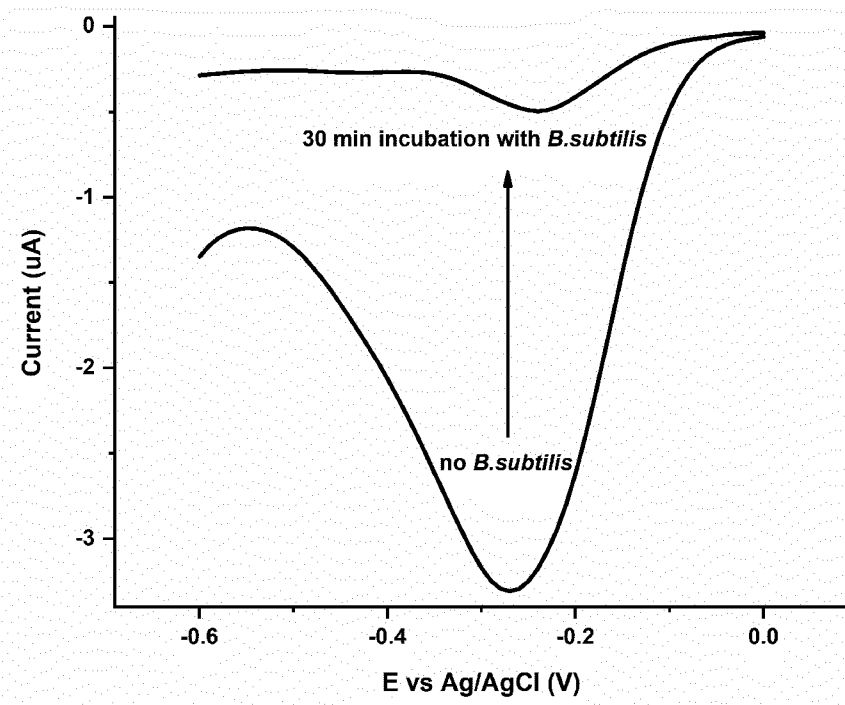


Fig. 2b

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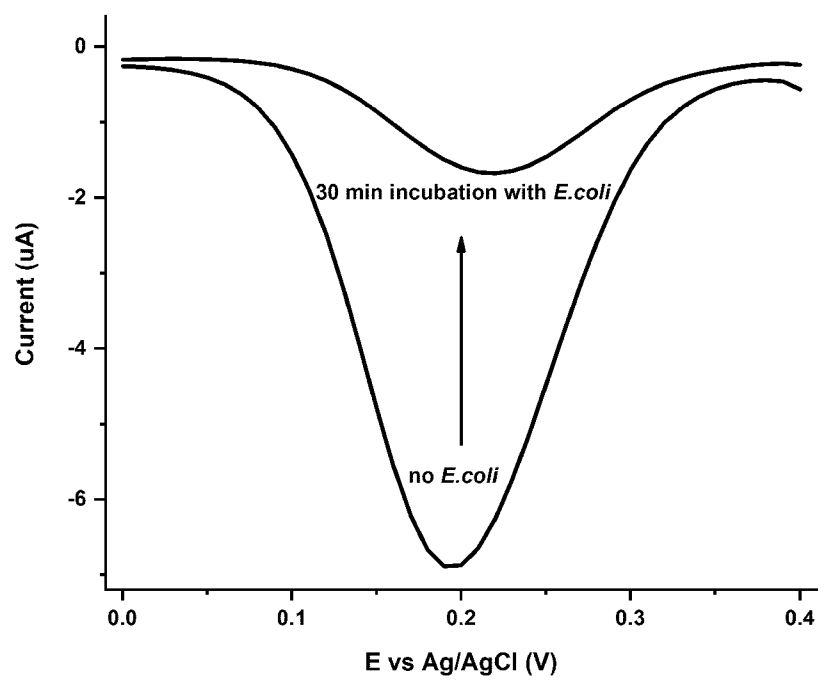


Fig. 2c

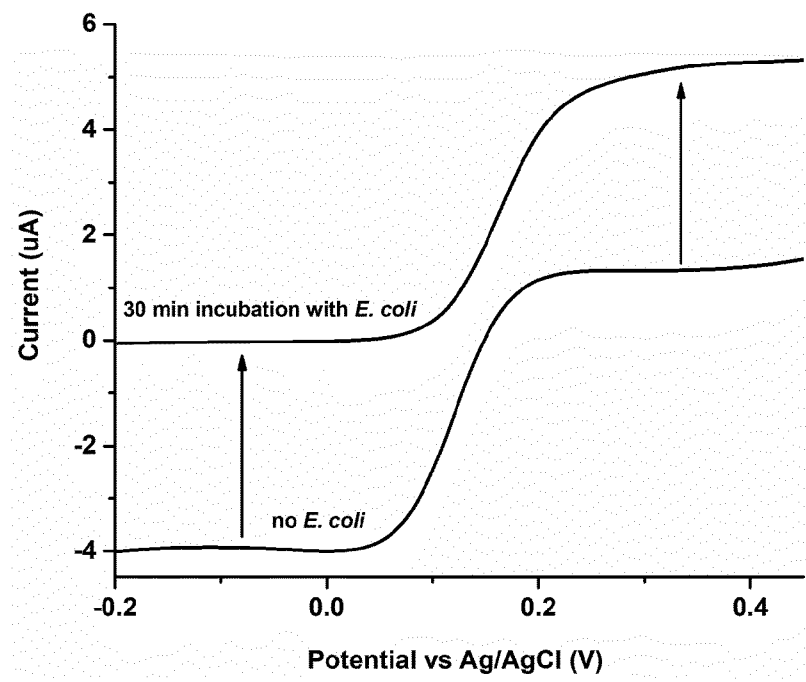


Fig. 2d

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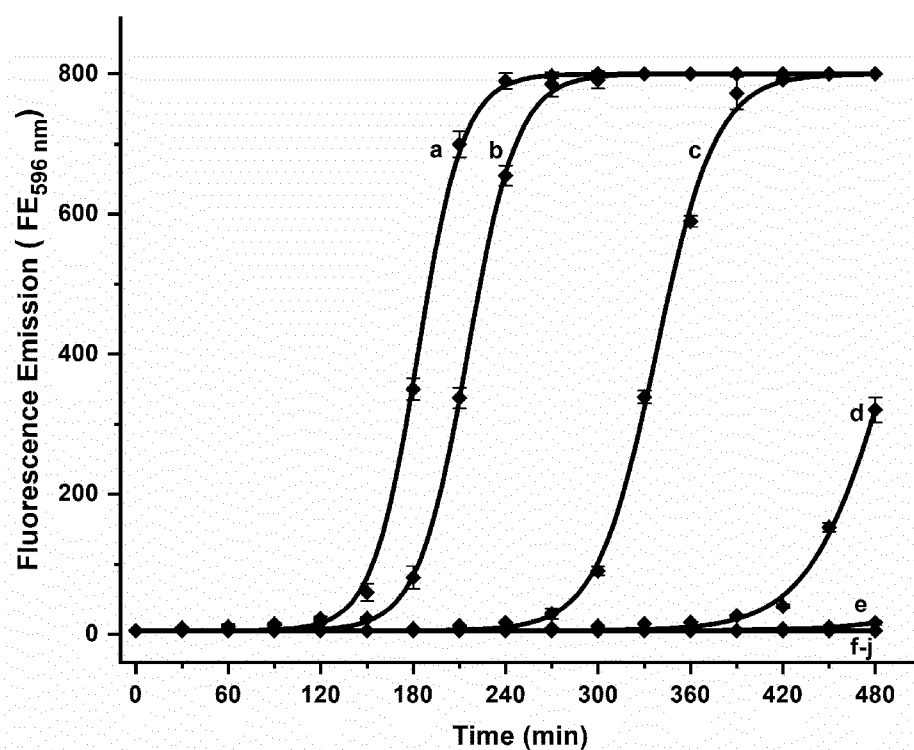


Fig. 3a

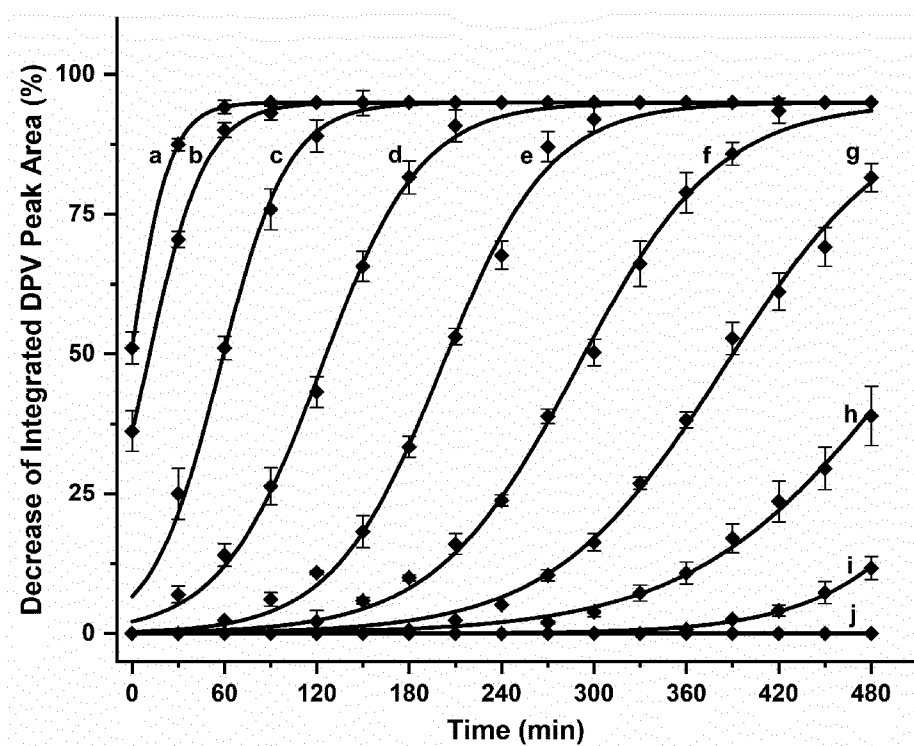


Fig. 3b

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/059905A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/04 C12Q1/18
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FIDEL G PEREZ ET AL: "Immunomagnetic Separation with Mediated Flow Injection Analysis Amperometric Detection of Viable Escherichia coli 0157", ANALYTICAL CHEMISTRY, vol. 70, no. 11, 24 April 1998 (1998-04-24), pages 2380-2386, XP055587689,	1-7,9-18
Y	abstract page 2381 - page 2382; figure 1 ----- -/--	14,15



Further documents are listed in the continuation of Box C.



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Date of the actual completion of the international search

8 July 2019

Date of mailing of the international search report

16/07/2019

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Lunter, Pim

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/059905

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LUKAS NEJDL ET AL: "Remote-controlled robotic platform ORPHEUS as a new tool for detection of bacteria in the environment : Microfluidics and Miniaturization", ELECTROPHORESIS, vol. 35, no. 16, 13 March 2014 (2014-03-13), pages 2333-2345, XP055603212, ISSN: 0173-0835, DOI: 10.1002/elps.201300576	1,4-13, 16-18
Y	abstract page 2334 - page 2335; figure 1	14,15
X	ISMAIL H BOYACI ET AL: "Amperometric determination of live Escherichia coli using antibody-coated paramagnetic beads", ANALYTICAL AND BIOANALYTICAL CHEMISTRY, SPRINGER, BERLIN, DE, vol. 382, no. 5, 29 June 2005 (2005-06-29), pages 1234-1241, XP019327458, ISSN: 1618-2650, DOI: 10.1007/S00216-005-3263-8	1-11, 16-18
Y	abstract	14,15
Y	NGA-CHI YIP ET AL: "Real-time electrocatalytic sensing of cellular respiration", BIOSENSORS AND BIOELECTRONICS, vol. 57, 11 February 2014 (2014-02-11), pages 303-309, XP055156142, ISSN: 0956-5663, DOI: 10.1016/j.bios.2014.01.059	14,15
Y	abstract	
Y	JUSTIN D. BESANT ET AL: "Rapid electrochemical phenotypic profiling of antibiotic-resistant bacteria", LAB ON A CHIP, vol. 15, 13 May 2015 (2015-05-13), pages 2799-2807, XP055407704, ISSN: 1473-0197, DOI: 10.1039/C5LC00375J	14,15
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