Analytical Biochemistry 505 (2016) 18–25

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Bacteriophage immobilized graphene electrodes for impedimetric sensing of bacteria (*Staphylococcus arlettae*)

Neha Bhardwaj [a, b], Sanjeev K. Bhardwaj [a, b], Jyotsana Mehta [a, b], Girish C. Mohanta [a, b], Akash Deep [a, b, *]

[a] Central Scientific Instruments Organisation (CSIR–CSIO), Chandigarh 160030, India
[b] Academy of Scientific and Innovative Research, CSIR–CSIO, Chandigarh 160030, India

ARTICLE INFO

Article history:
Received 20 March 2016
Received in revised form
11 April 2016
Accepted 14 April 2016
Available online 22 April 2016

Keywords:
Bacteriophage
Graphene
Impedimetric sensor
Bacteria
S. arlettae

ABSTRACT

Bacteriophages are a class of viruses that specifically infect and replicate within a bacterium. They possess inherent affinity and specificity to the particular bacterial cells. This property of bacteriophages makes them an attractive biorecognition element in the field of biosensor development. In this work, we report the use of an immobilized bacteriophage for the development of a highly sensitive electrochemical sensor for *Staphylococcus arlettae*, bacteria from the pathogenic family of coagulase-negative staphylococci (CNS). The specific bacteriophages were covalently immobilized on the screen-printed graphene electrodes. Thus, the fabricated bacteriophage biosensor displayed quantitative response for the target bacteria (*S. arlettae*) for a broad detection range (2.0–2.0 × 10^6 cfu). A fast response time (2 min), low limit of detection (2 cfu), specificity, and stability over a prolonged period (3 months) are some of the important highlights of the proposed sensor. The practical utility of the developed sensor has been demonstrated by the analysis of *S. arlettae* in spiked water and apple juice samples.

The *Staphylococcus* genus consists of approximately 40 known species that are subdivided into coagulase-positive and coagulase-negative groups based on their ability to produce coagulase enzyme [1,2]. A majority of these species fall under the coagulase-negative staphylococci (CNS)[1] group. Considered as saprophytic or rarely pathogenic, the CNS group species were initially dismissed as culture contaminations. Lately, several of the CNS species have been discerned as potentially pathogenic bacteria. They have been linked with bacteremia-related infections in implanted medical devices such as intravenous catheters, prosthetic heart valves, and orthopedic implants [2–5]. The treatment of many of such infections has become complicated over the years as the bacteria develop antibiotic resistance [6–8]. Conventional methods for the identification of the CNS species are primarily based on microbiological and biochemical analysis. Although they are highly accurate, the above detection procedures require long analysis durations and do not necessarily provide a point-of-care diagnostic solution [9]. Enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and polymerase chain reaction (PCR) are some other popularly used techniques for the quantitative detection of bacteria [10,11].

Biosensors have been widely considered as an attractive detection platform for the qualitative and quantitative analysis of bacteria due to a low cost of the device, simple handling, and high sensitivity [12–21]. Among the several classes of biosensors, electrochemical biosensors are predominantly popular due to their convenient upscaling with microfabrication technologies [22]. The use of electrochemical impedance spectroscopy (EIS) in the development of label-free electrochemical biosensors is particularly advantageous because this technique can record even the smallest changes at the solution–electrode interface, which helps to achieve high detection sensitivities with fast response times

* Corresponding author. Central Scientific Instruments Organisation (CSIR–CSIO), Chandigarh 160030, India. Fax: +91 172 2657082.
E-mail addresses: dr.akashdeep@csio.res.in, dr.akashdeep@gmail.com (A. Deep).

[1] *Abbreviations used:* CNS, coagulase-negative staphylococci; EIS, electrochemical impedance spectroscopy; LOD, limit of detection; SPE, screen-printed graphene electrode; EDC, 1-(3-dimethylaminopropyl)ethylcarbodiimide hydrochloride; BSA, bovine serum albumin; NaCl, sodium chloride; KCl, potassium chloride; Na_2HPO_4, disodium hydrogen phosphate; KH_2PO_4, potassium dihydrogen phosphate; SCD, soya casein digest; PBS, phosphate-buffered saline; FE-SEM, field emission scanning electron microscopy; AFM, atomic force microscopy; FTIR, Fourier transform infrared spectroscopy; UV–Vis, ultraviolet–visible; CV, cyclic voltammetry; R_{ct}, charge transfer resistance.

http://dx.doi.org/10.1016/j.ab.2016.04.008
0003-2697/

[23,24]. EIS-based bacterial sensors offer significantly faster analyses (e.g., 30 min or less) compared with the microbial growth-based conventional biosensing techniques [25–30].

The important biological recognition elements used in the development of bacterial biosensors include enzymes/enzymatic reaction [31–33], antimicrobial peptides [34], aptamers [35], antibodies [36,37], whole cells [36], and bacteriophages [38,39]. The use of antibodies is very effective in terms of high specificity and affinity for the target bacterial cell, but their production requires laborious and time-consuming steps. Moreover, the biological activity and specificity of the anti-bacteria antibodies tend to be influenced by the varying environmental conditions and nature of samples [30,40,41]. Bacteriophages represent an important class of biorecognition elements. They are obligate parasites that infect and hijack the cellular machinery of a host bacterium for their own growth and multiplication. Bacteriophages use their tail proteins to recognize the host bacterium with target specificity to the strain level. The above properties of bacteriophages can be extremely useful for the development of highly specific bacterial biosensors with added advantages of differentiation between the viable and dead cells, no requirement of sample pre-processing, self-signal amplification, and a favorable cost of device production [38,42,43]. Furthermore, the sensor stability in variable environmental conditions (e.g., change in pH or temperature) becomes a very important aspect of bacteriophage-based devices [38,44]. In some recent examples, Shabani and coworkers used the bacteriophage immobilized carbon electrodes for the detection of *Escherichia coli* [27,29]. These methods were based on the bacterial lysis process and offered a limit of detection (LOD) of 10^3 to 10^4 cfu ml^{-1}. It may be pointed out here that the electrochemical monitoring of the bacterial lysis process is suitable only for bacteria having short lifecycles (e.g., 20 min in the case of *E. coli*). The technique may be time-consuming for other bacteria that need a longer duration for their growth [27,28].

In the current study, we demonstrate for the first time the application of a bacteriophage biosensor for highly sensitive and selective impedimetric detection of *Staphylococcus arlettae*. The chosen bacterium belongs to the CNS species and was first isolated from the skin and nares of poultry and goats [45]. Recently, pathogenic strains of *S. arlettae* have been reported in the blood of cardiovascular patients with virulence genes [46,47]. The detection of *S. arlettae* and its strains is also useful for the monitoring of certain environmental processes such as the biodegradation of industrial textile azo dyes [48] and the reduction of chromium (VI) [49]. The biosensor proposed here was assembled by immobilizing a target-specific bacteriophage on screen-printed graphene electrodes (SPEs). We chose screen printed graphene electrodes because they are readily available in the market and can be conveniently integrated with modern portable biosensing instrumentation as a disposable sensor chip. Moreover, it is easy to reproduce the results, with these electrode having precise dimensions and material coverage. As a transducer material, graphene offers high electron mobility and surface area along with the possibility of convenient functionalization with −COOH groups, which is important for achieving a covalent linkage between the electrode surface and the bacteriophage head.

A simple monitoring of the electrochemical properties during the bacteriophage–bacteria binding event may be highlighted as an important feature of this work over the previously reported bacteriophage biosensors where the analyte detection was based on the lysis of bacteria. As already mentioned, the lysis-based sensing methods may be time-consuming, particularly for bacteria requiring prolonged growth periods. The proposed biosensor is environmentally stable and offers a fast response time, high sensitivity, and analyte specificity. The technique can also be extended for the rapid detection of several other pathogens.

Materials and methods

Materials

1-(3-Dimethylaminopropyl)ethylcarbodiimide hydrochloride (EDC), hydrochloric acid, bovine serum albumin (BSA), sodium chloride (NaCl), potassium chloride (KCl), disodium hydrogen phosphate (Na_2HPO_4), and potassium dihydrogen phosphate (KH_2PO_4) were purchased from Sigma–Aldrich/Merck. Soya casein digest (SCD) medium (HiMedia Laboratories) was used for propagation of the bacteria and enrichment of the bacteriophage. Phosphate-buffered saline (PBS, pH 7.4) was prepared by mixing 0.31 M NaCl, 0.002 M KCl, 0.01 M Na_2HPO_4, and 0.001 M KH_2PO_4. Prior to their use, the SCD medium, PBS buffer, and distilled water were sterilized by autoclaving at 121 °C. The screen-printed graphene electrodes (DropSens) were purchased from Metrohm India. The total dimensions of the electrode were 3.4 × 1.0 × 0.05 cm (length × width × height).

Bacteriophage preparation

Highly specific lytic bacteriophages against *S. arlettae* were used in this study. The isolation and purification of the bacteriophage was carried out according to an earlier report [50]. Before immobilizing the bacteriophage on electrode surface, the titer was amplified using the following enrichment procedure. SCD broth (50 ml) containing 2 mM $CaCl_2$ was taken in a conical flask. It followed the addition of 5 ml of bacteriophage solution and 2 ml of overnight grown *S. arlettae* culture. The above SCD broth was left for an overnight incubation (100 rpm, 37 °C). The resulting enriched phase was then centrifuged (8000 rpm, 10 min) to separate out the bacterial debris. The supernatant was then filtered through a 0.22-μm Millipore filter paper to remove any residual bacteria. Thus, enriched bacteriophage solution was determined for the titer concentration using the double agar overlay method. Briefly, 500 μl of the diluted bacteriophage sample (10^1 to 10^6 × the enriched sample) was added to a 300-μl overnight grown bacterial culture. The different infection mixtures were mixed with sterile molten top agar and then quickly poured over the SCD agar plates. The plates were then left for an overnight incubation at 37 °C. A plate with a suitable number of colonies (10^6 × sample) was analyzed, and the titer of the bacteriophage in the stock and other diluted samples was estimated using the following equation [51]:

$$\text{Plaque Forming Unit(pfu/mL)} = \frac{\text{no. of plaques} \times 1000 \times \text{reciprocal of dilution factor}}{\text{volume of bacteriophage sample}(\mu\text{L})} \quad (1)$$

Host specificity of the isolated bacteriophage toward *S. arlettae* was investigated by the standard spot test method. This important

desired property of the isolated bacteriophage was tested with respect to *Staphylococcus aureus* (gram-positive, coagulase-positive bacteria), *E. coli* (gram-negative bacteria), and *Staphylococcus lentus* (gram-positive, coagulase-negative member). Briefly, a loopful of sample bacteria strains (i.e., *S. arlettae, S. aureus* 96, *E. coli* 614, *and S. lentus* 2292) was inoculated on different zones of an SCD agar plate. Then 10 μl of the bacteriophage sample was dispensed at the center of each test zone. This plate was incubated at 37 °C for 24 h before visual analysis.

Electrode functionalization and bacteriophage immobilization

As a first step, the working electrode area of the SPEs was electrochemically oxidized to generate carboxyl (−COOH) groups. The counter and reference electrodes were masked before the electrochemical reaction. The SPE with the exposed working electrode area was suspended in a three-electrode cell using Pt and Ag/AgCl electrodes as separate counter and reference electrodes, respectively (see Fig. S1 in online supplementary material). A solution of 0.1 M HCl was used as the electrolyte. Electrochemical oxidation of graphene surfaces of the SPEs was carried out in chronoamperometry mode at a fixed potential of +1.0 V (reaction time: 180 s; equipment model: Autolab PGSTAT 302; acquisition software: Nova 1.6). The above-treated SPEs were then left in contact with a solution of 0.1 M EDC in 0.12 N HCl for 10 min in order to activate their surface −COOH functional groups. These electrodes were then incubated with the enriched bacteriophage solution for 2 h ($T = 37$ °C) to process the covalent immobilization of the bacteriophage. As the activated electrode was incubated with bacteriophage, the formation of amide bond took place via the linking between the −COOH groups (of the electrode surface) and $-NH_2$ groups (present on the bacteriophage's head). Thus, the SPE could be modified with the immobilized bacteriophage in an oriented manner, that is, with their head bound to the electrode and the tail free to bind the target bacteria. Finally, the counter and reference electrode areas were unmasked, and the bacteriophage immobilized SPEs were washed with PBS buffer, dried under vacuum, and stored at 4 °C.

Characterization of bacteriophage immobilized SPE

Field emission scanning electron microscopy (FE-SEM; Hitachi S4300 SE/N), atomic force microscopy (AFM; XE-NSOM), and Fourier transform infrared spectroscopy (FTIR; Nicolet iS10) were used to confirm the immobilization of bacteriophage on SPEs. Before FE-SEM and AFM measurements, the sample electrodes were washed several times with PBS buffer and then dried under room temperature conditions. Ultraviolet−visible (UV−Vis) absorption spectroscopic investigations were carried out with a Varian Cary 5000 system.

Electrochemical detection of bacteria

Cyclic voltammetry (CV) studies were carried out in a standard cell. Before the analysis, the electrodes were first incubated with 10 μl of the aqueous bacterial solution. A solution of 0.1 M PBS was used as the electrolyte. The potential was scanned from −1.0 to +1.0 V at a scan rate of 100 mV s^{-1}. The negative control experiments (with respect to nonspecific *S. aureus* and *E. coli*) were also performed in a similar fashion.

EIS was used for the quantitative analysis of *S. arlettae*. Aliquots (10 μl) of a stock bacterial solution (2×10^8 cfu ml^{-1}, concentration standardized with standard colony counting method) and its serial dilutions (10^2, 10^4, and 10^6) were incubated with the bacteriophage immobilized SPE (37 °C). The EIS parameters were then recorded in the presence of $Fe^{3+/2+}$ redox probe [5 mM $K_3Fe(CN)_6$ + $K_4Fe(CN)_6 \cdot 3H_2O$, prepared in 0.1 M PBS]. The frequency range was selected as 0.1 Hz−1 MHz while keeping a fixed amplitude of 5 mV. The effective number of bacterial colonies (cfu) analyzed with the bacteriophage sensor was calculated as follows:

$$\text{Effective no. of bacterial colonies} = \frac{\text{Bacterial concentration(in cfu/mL)}}{100} \quad (2)$$

The LOD has been calculated from the calibration curve according to the formula LOD = 3σ, where σ is the standard deviation at $C = 0$.

Analysis of real samples

A demonstration of the practical utility of the bacteriophage sensor was carried out by the analysis of *S. arlettae* in spiked samples of river water and apple juice. In a typical preparation method, the samples were spiked with an unknown concentration of the bacteria. The samples were then filtered with a 0.45-μm hydrophilic filter paper. The bacteria separated on the filter paper were resuspended in a similar volume of PBS buffer.

The validation of the data was done with the standard colony counting method. The samples with spiked bacteria were diluted in different fractions (1×, 10^2×, 10^3×, and 10^4×) and then cultured to form the colonies. A plate with an easily countable number of colonies (10^4×; see Fig. S2 in supplementary material) was analyzed to determine the bacterial concentration in all of the prepared samples.

In a separate experiment, the analysis of *S. arlettae* (known concentration of 2×10^2 cfu) in real samples was also studied in the co-presence of *S. aureus* 96, *E. coli* 614, and *S. lentus* 2292 (known elevated concentration of 2×10^6 cfu each). The sensor's output characteristics were recorded in terms of charge transfer resistance (R_{ct}).

Results and discussion

Determination of bacteriophage titer

The titer of the stock bacteriophage solution (isolated against *S. arlettae*) was assessed before using it for the preparation of bacteriophage biosensor. The plates of different sample dilutions are shown in Fig. S3 of the supplementary material. The appearance of clear zones (plaques) indicated the presence of lytic phage. The plate with bacteriophage dilution of 10^6 showed the presence of approximately 50 plaques. Thus, the titer of the stock bacteriophage solution was determined as 1×10^8 pfu ml^{-1} (Eq. (1)). This level of the enriched bacteriophage concentration is suitable enough for using it in the preparation of a bacteriophage biosensor [29]. The studies on the host specificity of the bacteriophage have indicated its exclusive selectivity for *S. arlettae* (Fig. S4). The experimental studies proved that the isolated bacteriophage caused the lysis of *S. arlettae* (target bacteria used in the study), whereas other test zones of *S. aureus* 96, *E. coli* 614, and *S. lentus* 2292 remained unaffected.

Characterization of bacteriophage immobilized SPE

FE-SEM images of a bare SPE and the bacteriophage immobilized SPE are shown in Fig. 1. A comparison of the blank SPE and

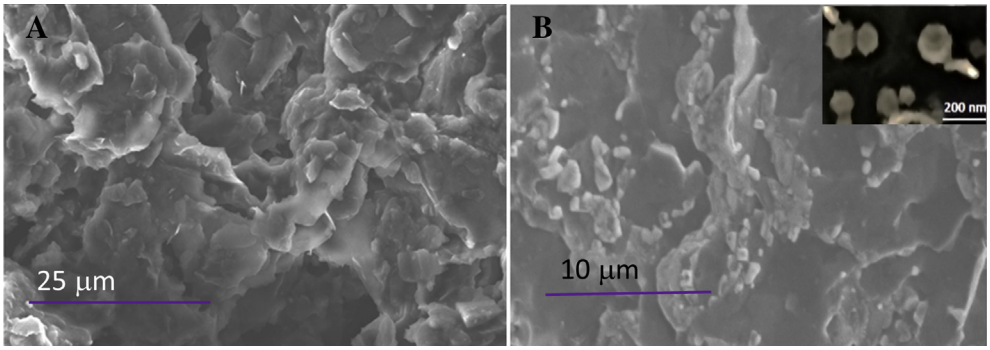

Fig.1. FE-SEM images of blank SPE (A) and bacteriophage immobilized SPE (B).

bacteriophage–SPE suggested that the proposed method successfully modified the SPE with bacteriophage particles. A zoomed image area indicated the presence of individual bacteriophage-like structures characterized by a typical length of 200–300 nm. An AFM scanning analysis of the bacteriophage immobilized SPE is shown in Fig. 2A (larger area scan) and Fig. 2B (higher resolution). This study also suggested a successful bacteriophage immobilization. The line (indicated by red lines) and region profile (indicated by green region) analyses in Fig. 2A and B verified a homogeneous distribution of the bacteriophage particles over the SPE. The surface roughness of the biosensor's surface was limited to ±10 nm. High-resolution AFM scanning (Fig. 2B) indicated the presence of some individual bacteriophage structures with a typical length of approximately 200 nm, which agreed well with the FE-SEM data.

The immobilization of bacteriophage on SPE was also verified with reflectance mode UV–Vis and FTIR studies. The absorption spectrum of the bacteriophage sample was characterized by a shallow maximum at 269 nm (Fig. 3A), in accordance with the available literature [52]. The absorption spectrum of the bacteriophage immobilized SPE also contained the characteristic information of the native bacteriophage, thereby again suggesting the desired modification of the bare SPE. A comparison between the FTIR spectra of the bare SPE and the bacteriophage–SPE clearly highlighted a successful assembly of the bacteriophage particles on SPEs (Fig. 3B). The bacteriophage–SPE sample showed characteristic peaks at 3280 cm^{-1} (broad signal of proteins) and 1650 cm^{-1} (amide I absorption). Small peaks at 1400 and 1025 cm^{-1} can be assigned to the carbohydrates and $-PO_2$ symmetric stretching, respectively.

Evaluation of biosensor's specificity and response time with CV studies

Cyclic voltammograms of the bare SPE and bacteriophage–SPE are presented in Fig. 4. A low degree of current flow in the case of the bare SPE may be attributed to the surface roughness. CV of the bacteriophage–SPE was characterized by an increased flow of current. It has been reported in the literature that the bacteriophages may lower the redox potential of surrounding medium [53], which explains better charge flow characteristics of the bacteriophage modified SPE. As a control experiment, the CV response of the bacteriophage–SPE was first checked with respect to the two

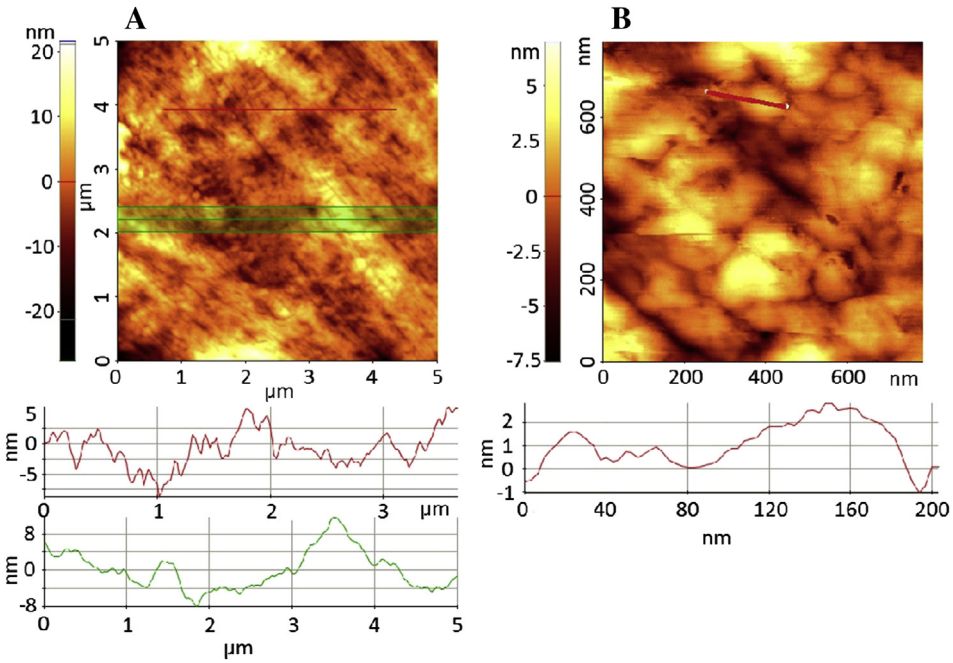

Fig.2. AFM images and line analyses of bacteriophage immobilized SPE: (A) scan of larger sample area; (B) scan of smaller area to map the presence of individual bacteriophage.

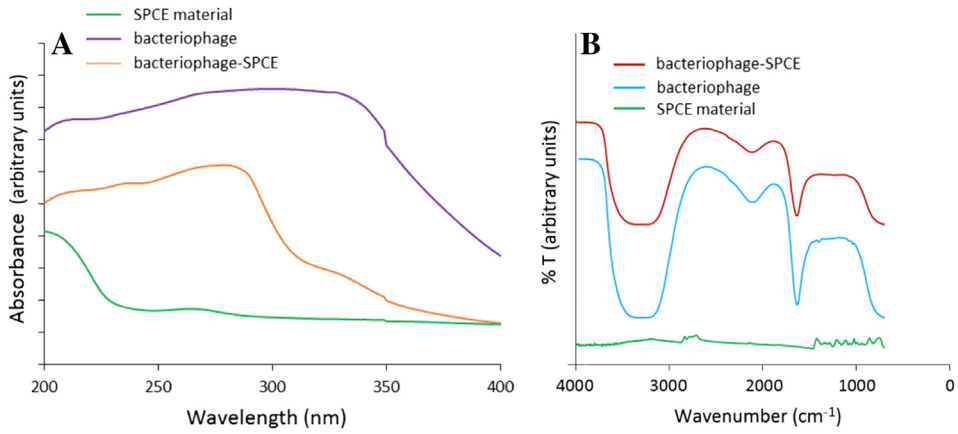

Fig.3. UV–Vis (A) and FTIR (B) spectra of SPE, bacteriophage, and bacteriophage–SPE.

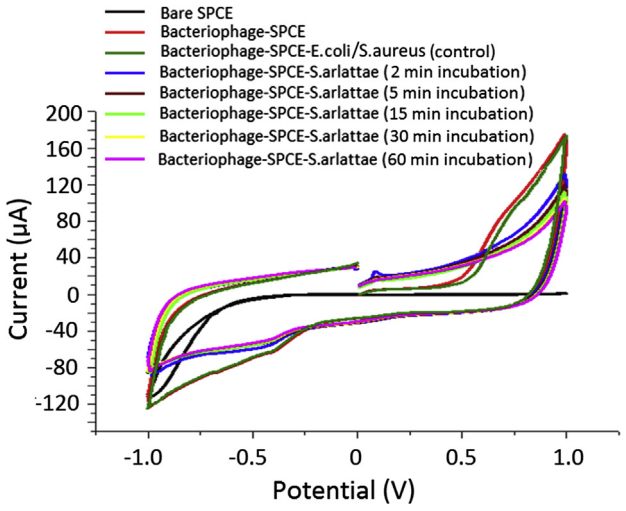

Fig.4. Cyclic voltammograms of bare SPE, bacteriophage–SPE, and bacteriophage–SPE toward *E. coli* and *S. aureus* (nonspecific bacteria) along with CV characteristics of bacteriophage–SPE toward *S. arlettae* for different incubation times. [*S. arlettae*] = 2×10^4 cfu ml^{-1}; [*E. coli*] = 2×10^4 cfu ml^{-1}; [*S. aureus*] = 2×10^4 cfu ml^{-1}.

nonspecific bacteria samples, namely *S. aureus* and *E. coli.* The CV curves in these studies were similar to the baseline curve, thereby proving that the developed biosensor does not respond to the nonspecific bacteria.

For recording the CV response of the bacteriophage–SPE sensor for the target analyte *S. arlettae,* different electrodes were incubated with a fixed concentration of the bacteria (2×10^8 cfu ml^{-1} or 2×10^6 cfu) for different time periods (2–30 min, 37 °C). The biosensor electrode responded to the target bacteria, as seen by the decrease in the current flow. An incubation time of only 2 min was sufficient to obtain the desired stability in the signal. The results were similar for longer incubation times.

EIS detection of S. arlettae

The EIS technique was used for the quantitative detection of *S. arlettae.* The electrochemical impedance data are generally expressed in the form of a Nyquist plot in which the imaginary impedance component (Z'', out-of-phase) is plotted against the real impedance component (Z', in-phase) at different excitation frequencies. We used Randle's equivalent electrical circuit to model the data and estimate the values of charge transfer resistance (Fig. 5).

The Nyquist plots for the SPE, bacteriophage–SPE, and bacteria incubated bacteriophage–SPE (bacteria–bacteriophage–SPE) are shown in Fig. 5A. The effective impedance (expressed in terms of R_{ct}) of the bacteriophage–SPEs did not change in the presence of a nonspecific bacteria *E. coli* but showed increasing values with the increasing concentration of *S. arlettae.* The expending diameters of semicircles and the increase in impedance values can be attributed to the hindered diffusion of the redox probe molecules toward the electrode surface. The above behavior can be related to the buildup of the insulating bacteria–bacteriophage layer. The computed values of R_{ct} were plotted against the corresponding numbers of the bacterial colonies detected (Fig. 5B). A linear increase was observed in the values of R_{ct} with respect to the increasing bacteria concentration. The LOD from the proposed bacteriophage biosensor was estimated as 2 cfu, which is an excellent achievement in comparison with the earlier works [28–30,54,55].

Because the bacterial concentration may also influence the mobility of charge carriers toward the sensor's surface, we investigated the sensor's response time towards 2×10^6, 2×10^4, 2×10^2, and 2 cfu of *S. arlettae.* The results of these measurements are shown in Fig. S5 of the supplementary material. In these cases, the response was measured in term of R_{ct} values. Here again, an incubation time of 2 min was found to be sufficient to achieve a stable response of the bacteriophage–SPE biosensor.

Reproducibility and stability of biosensor

The reproducibility and regeneration of the biosensor was investigated by repetitive analysis of 10^2 cfu *S. arlettae* solution. After each test, the electrode was washed with PBS buffer. The results of five numbers of such analysis cycles are presented in Fig. S6 of the supplementary material. We observed insignificant changes (coefficient of variation of 2.5%) in the measured values of R_{ct}. The stability of the bacteriophage–SPE was investigated by recording its CV scans over a period of time. The values of current (at an applied voltage of 0.75 V) were measured at regular intervals over a period of 120 min. The sensor's response did not change during the course of the above experiment (Fig. S7), thereby suggesting its environmental stability. The long-term stability of the electrode was also tested by recording its CV response over a period of 3 months. The sensor output was nearly stable (±5%) even after such a long storage time (Fig. S8). Therefore, it can be highlighted that

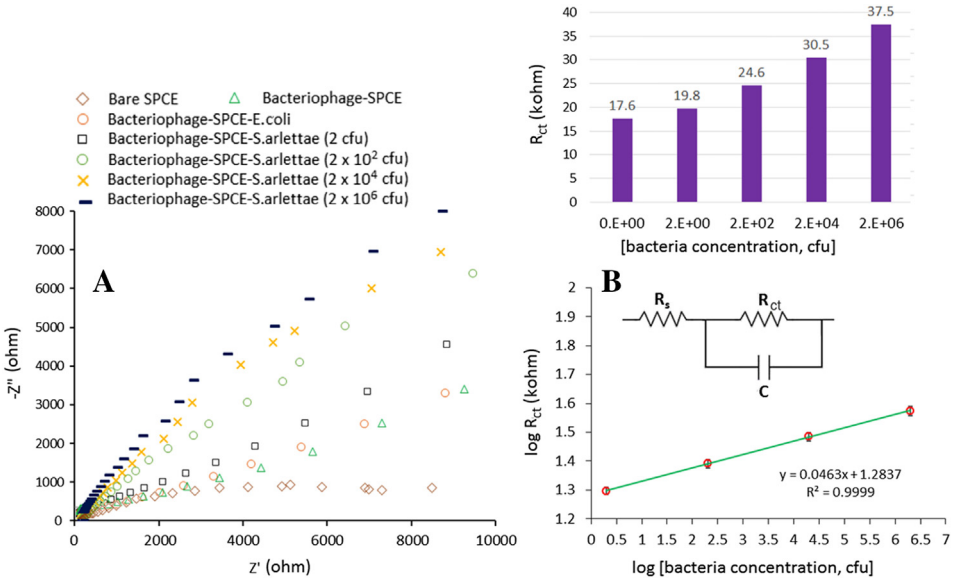

Fig.5. (A) Nyquist plots of bare SPE and bacteriophage–SPE along with the data when bacteriophage–SPE was separately exposed to *E. coli* and different concentrations of *S. arlettae*. (B) Variation in the values of charge transfer resistance with respect to the number of bacterial colonies analyzed (2, 2×10^2, 2×10^4, or 2×10^6 cfu *S. arlettae*).

the proposed sensor system is reasonably stable for long-term use. The intra-assay reproducibility of the developed sensor was also assessed. For this, the response of five different bacteriophage–SPEs was studied with respect to a fixed bacterial concentration. All five electrodes were prepared using the same batch of the electrode material under identical experimental conditions. The observed response was highly reproducible (Fig. S9).

Analysis of S. arlettae in spiked river water and apple juice samples

Fig. 6 and Table 1 show the results of the analysis of *S. arlettae* in the spiked river water and apple juice samples. The Nyquist plots and the corresponding R_{ct} values are shown in Fig. 6. The estimated analyte concentrations were in close agreement with the analysis data obtained from the conventional colony counting method (Table 1). The analysis data of the samples spiked with *S. arlettae* along with *S. aureus, E. coli,* and *S. lentus* are given in Table 2. No significant change was observed in the R_{ct} values even when the samples contained other possible interfering bacteria. This result further emphasized the practical utility of the proposed bacteriophage biosensor.

Conclusions

The current work has demonstrated the application of a bacteriophage biosensor for the electrochemical detection of *S. arlettae.* A robust immobilization of the bacteriophage on SPE and quick response time suggest the practical utility of the sensor. The CV studies provided useful information on the sensor's response time and specificity. These studies also revealed some useful electrochemical characteristics of the biosensor. The usefulness of the EIS plots is reflected in the quantitative analysis experiments. The bacteriophage–SPE biosensor offers the detection of a very low bacterial concentration. The demonstrated technique may be an attractive approach to develop rapid, simple, affordable, and reliable biosensors for pathogen detection in food samples. Because the method directly records signals for a bacteria–bacteriophage binding event, the response is much faster than the previously reported bacteria–lysis techniques. The

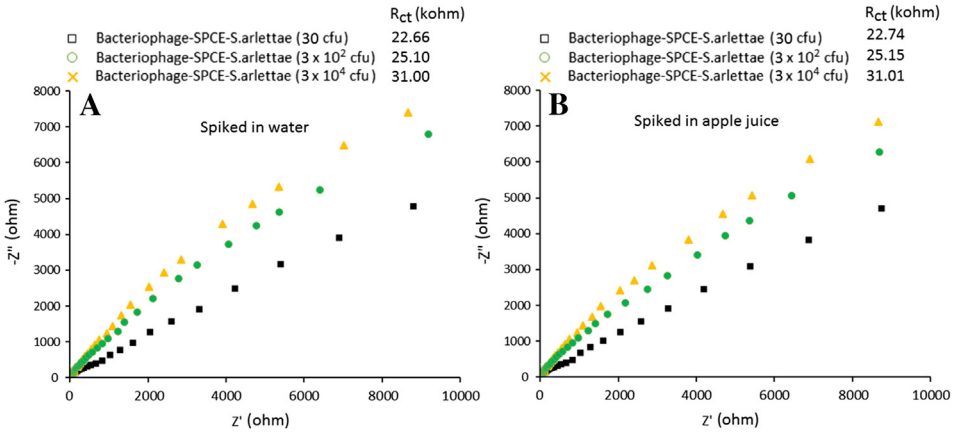

Fig.6. Nyquist plots and the corresponding values of R_{ct} during the analysis of different spiked concentrations of *S. arlettae* in river water (A) and apple juice (B) samples.

Table 1
Analysis of *S. arlettae* in spiked samples of river water and apple juice using bacteriophage–SPE sensor along with the results of validation studies with colony counting method.

Spiked concentration of *S. arlettae*		Response[a] of bacteriophage–SPE sensor and number of corresponding colonies detected	Data from colony counting technique[b]
River water sample	Unknown cfu/ml	R_{ct} = 31.00 KΩ Bacterial colonies detected = 30,675 cfu Bacterial concentration in solution[c] = 3.06×10^6 cfu ml^{-1}	Concentration = 3×10^6 cfu ml^{-1}
	Unknown (10^2 × dilution)	R_{ct} = 25.10 KΩ Bacterial colonies detected = 330 cfu Bacterial concentration in solution[c] = 3.3×10^4 cfu ml^{-1}	Concentration = 3×10^4 cfu ml^{-1}
	Unknown (10^3 × dilution)	R_{ct} = 22.66 KΩ Bacterial colonies detected = 35 cfu Bacterial concentration in solution[c] = 3.5×10^3 cfu ml^{-1}	Concentration = 3×10^3 cfu ml^{-1}
	Unknown (10^4 × dilution)	R_{ct} = 20.05 KΩ Bacterial colonies detected = 2.5 cfu Bacterial concentration in solution[c] = 2.5×10^2 cfu ml^{-1}	Concentration = 3×10^2 cfu ml^{-1}
Apple juice sample	Unknown cfu/ml	R_{ct} = 31.01 KΩ Bacterial colonies detected = 30,810 cfu Bacterial concentration in solution[c] = 3.08×10^6 cfu ml^{-1}	Concentration = 3×10^6 cfu ml^{-1}
	Unknown (10^2 × dilution)	R_{ct} = 25.15 KΩ Bacterial colonies detected = 334 cfu Bacterial concentration in solution[c] = 3.34×10^4 cfu ml^{-1}	Concentration = 3×10^4 cfu ml^{-1}
	Unknown (10^3 × dilution)	R_{ct} = 22.74 KΩ Bacterial colonies detected = 38 cfu Bacterial concentration in solution[c] = 3.8×10^3 cfu ml^{-1}	Concentration = 3×10^3 cfu ml^{-1}
	Unknown (10^4 × dilution)	R_{ct} = 20.15 KΩ Bacterial colonies detected = 2.8 cfu Bacterial concentration in solution[c] = 2.8×10^2 cfu ml^{-1}	Concentration = 3×10^2 cfu ml^{-1}

[a] The response of the sensor in terms of R_{ct} values was converted to the concentration values with regression equation $y = 0.0463x + 1.2837$.
[b] A stock solution of unknown bacterial concentration was synthetically prepared. Its serial dilutions were analyzed with the bacteriophage–SPE sensor. The validation of the results was done with the colony counting method.
[c] Bacterial concentration in the corresponding solution was estimated according to Eq. (2).

Table 2
Analysis of *S. arlettae* in spiked samples of river water and apple juice in the co-presence of *S. aureus, E. coli,* and *S. lentus*.

Spiked concentration of bacteria		Response of bacteriophage–SPE sensor	Variation in sensor's response[a]
River water sample	2×10^2 cfu *S. arlettae*	R_{ct} = 24.63 KΩ	
	2×10^2 cfu *S. arlettae* + 2×10^6 cfu each of *S. aureus, E. coli,* and *S. lentus*	R_{ct} = 24.59 KΩ	±0.05 KΩ
Apple juice sample	2×10^2 cfu *S. arlettae*	R_{ct} = 24.65 KΩ	
	2×10^2 cfu *S. arlettae* + 2×10^6 cfu each of *S. aureus, E. coli,* and *S. lentus*	R_{ct} = 24.58 KΩ	±0.07 KΩ

[a] With respect to the analysis in pure *S. arlettae* spiked sample only (based on the average of three determinations).

practical utility of the proposed bacteriophage biosensor was demonstrated by the analysis of *S. arlettae* in spiked river water and apple juice samples.

Acknowledgments

The current research work was funded through a grant from the Council of Scientific and Industrial Research (CSIR), India (OMEGA/PSC0202/2.2.5). One of the authors (Jyotsana Mehta) also thanks the University Grant Commission, India, for her research fellowship. We are grateful to the director of CSIR–CSIO (Central Scientific Instruments Organisation), Chandigarh, India.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2016.04.008.

References

[1] J. Huebner, D.A. Goldmann, Coagulase-negative staphylococci: Role as pathogens, Annu. Rev. Med. 50 (1999) 223–236.
[2] F. Layer, B. Ghebremedhin, K.-A. Moder, W. König, B. König, Comparative study using various methods for identification of *Staphylococcus* species in clinical specimens, J. Clin. Microbiol. 44 (2006) 2824–2830.
[3] G. Choi, M.P. van den Borne, C.E. Visser, M.J. Kersten, A.P. Kater, Invasive infections with a coagulase-negative staphylococcus in an immunocompromised patient: Case report and review of the literature, Ann. Hematol. 87 (2008) 771–772.
[4] K.L. Rogers, P.D. Fey, M.E. Rupp, Coagulase-negative staphylococcal infections, Infect. Dis. Clin. North Am. 23 (2009) 73–98.
[5] E. Tornero, E. García-Oltra, S. García-Ramiro, J.C. Martínez-Pastor, J. Bosch, C. Climent, L. Morata, P. Camacho, J. Mensa, A. Soriano, Prosthetic joint infections due to *Staphylococcus aureus* and coagulase-negative staphylococci, Int. J. Artif. Organs 35 (2012) 884–892.
[6] J.F. John, A.M. Harvin, History and evolution of antibiotic resistance in coagulase-negative staphylococci: Susceptibility profiles of new anti-staphylococcal agents, Ther. Clin. Risk Manag. 3 (2007) 1143–1152.
[7] L. May, E.Y. Klein, R.E. Rothman, R. Laxminarayan, Trends in antibiotic resistance in coagulase-negative staphylococci in the United States, 1999 to 2012, Antimicrob. Agents Chemother. 58 (2014) 1404–1409.
[8] I. Raad, A. Alrahwan, K. Rolston, *Staphylococcus epidermidis*: emerging resistance and need for alternative agents, Clin. Infect. Dis. 26 (1998) 1182–1187.
[9] P.-E. Fournier, G. Dubourg, D. Raoult, Clinical detection and characterization of bacterial pathogens in the genomics era, Genome Med. 6 (2014), http://dx.doi.org/10.1186/s13073-014-0114-2.
[10] S.D. Gan, K.R. Patel, Enzyme immunoassay and enzyme-linked immunosorbent assay, J. Invest. Dermatol. 133 (2013) 1–3.
[11] M.A. Jensen, J.A. Webster, N. Straus, Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms, Appl. Environ. Microbiol. 59 (1993) 945–952.

[12] R.L. Bunde, E.J. Jarvi, J.J. Rosentreter, Piezoelectric quartz crystal biosensors, Talanta 46 (1998) 1223–1236.
[13] X. Fan, I.M. White, S.I. Shopova, H. Zhu, J.D. Suter, Y. Sun, Sensitive optical biosensors for unlabeled targets: a review, Anal. Chim. Acta 620 (2008) 8–26.
[14] R. Monošík, M. Streďanský, E. Šturdík, Biosensors—classification, characterization, and new trends, Acta Chim. Slovaca 5 (2012) 109–120.
[15] N.J. Ronkainen, H.B. Halsall, W.R. Heineman, Electrochemical biosensors, Chem. Soc. Rev. 39 (2010) 1747–1763.
[16] M.L. Sin, K.E. Mach, P.K. Wong, J.C. Liao, Advances and challenges in biosensor-based diagnosis of infectious diseases, Expert Rev. Mol. Diagn 14 (2014) 225–244.
[17] M. Strianese, M. Staiano, G. Ruggiero, T. Labella, C. Pellecchia, S. D'Auria, Fluorescence-based biosensors, in: W.M. Bujalowski (Ed.), Methods in Molecular Biology, Spectroscopic Methods of Analysis, 875, Humana Press, Totowa, NJ, 2012, pp. 193–216.
[18] A.P. Turner, Biosensors: sense and sensibility, Chem. Soc. Rev. 42 (2013) 3184–3196.
[19] D. Grieshaber, R. MacKenzie, J. Voeroes, E. Reimhult, Electrochemical biosensors—sensor principles and architectures, Sensors 8 (2008) 1400–1458.
[20] J. Wang, Electrochemical biosensors: towards point-of-care cancer diagnostics, Biosens. Bioelectron. 21 (2006) 1887–1892.
[21] E. Wijaya, C. Lenaerts, S. Maricot, J. Hastanin, S. Habraken, J.-P. Vilcot, R. Boukherroub, S. Szunerits, Surface plasmon resonance-based biosensors: from the development of different SPR structures to novel surface functionalization strategies, Curr. Opin. Solid State Mater. Sci. 15 (2011) 208–224.
[22] M. Zhou, S. Dong, Bioelectrochemical interface engineering: toward the fabrication of electrochemical biosensors, biofuel cells, and self-powered logic biosensors, Acc. Chem. Res. 44 (2011) 1232–1243.
[23] L. Cunci, M.M. Vargas, R. Cunci, R. Gomez-Moreno, I. Perez, A. Baerga-Ortiz, C.I. Gonzalez, C.R. Cabrera, Real-time detection of telomerase activity in cancer cells using a label-free electrochemical impedimetric biosensing microchip, RSC Adv. 4 (2014) 52357–52365.
[24] J.-G. Guan, Y.-Q. Miao, Q.-J. Zhang, Impedimetric biosensors, J. Biosci. Bioeng. 97 (2004) 219–226.
[25] P.B. Lillehoj, C.W. Kaplan, J. He, W. Shi, C.-M. Ho, Rapid, electrical impedance detection of bacterial pathogens using immobilized antimicrobial peptides, J. Lab. Autom. 19 (2014) 42–49.
[26] Y. Wang, Z. Ye, Y. Ying, New trends in impedimetric biosensors for the detection of foodborne pathogenic bacteria, Sensors 12 (2012) 3449–3471.
[27] A. Shabani, C.A. Marquette, R. Mandeville, M.F. Lawrence, Magnetically-assisted impedimetric detection of bacteria using phage-modified carbon microarrays, Talanta 116 (2013) 1047–1053.
[28] A. Shabani, C.A. Marquette, R. Mandeville, M.F. Lawrence, Carbon microarrays for the direct impedimetric detection of *Bacillus anthracis* using gamma phages as probes, Analyst 138 (2013) 1434–1440.
[29] A. Shabani, M. Zourob, B. Allain, C.A. Marquette, M.F. Lawrence, R. Mandeville, Bacteriophage-modified microarrays for the direct impedimetric detection of bacteria, Anal. Chem. 80 (2008) 9475–9482.
[30] M. Tolba, M.U. Ahmed, C. Tlili, F. Eichenseher, M.J. Loessner, M. Zourob, A bacteriophage endolysin-based electrochemical impedance biosensor for the rapid detection of *Listeria* cells, Analyst 137 (2012) 5749–5756.
[31] L.-H. Guillemot, M. Vrignaud, P.R. Marcoux, C. Rivron, T.-H. Tran-Thi, Facile and fast detection of bacteria via the detection of exogenous volatile metabolites released by enzymatic hydrolysis, Phys. Chem. Chem. Phys. 15 (2013) 15840–15844.
[32] O.R. Miranda, X. Li, L. Garcia-Gonzalez, Z.-J. Zhu, B. Yan, U.H. Bunz, V.M. Rotello, Colorimetric bacteria sensing using a supramolecular enzyme–nanoparticle biosensor, J. Am. Chem. Soc. 133 (2011) 9650–9653.
[33] E. Tait, J.D. Perry, S.P. Stanforth, J.R. Dean, Bacteria detection based on the evolution of enzyme-generated volatile organic compounds: determination of *Listeria monocytogenes* in milk samples, Anal. Chim. Acta 848 (2014) 80–87.
[34] N.V. Kulagina, M.E. Lassman, F.S. Ligler, C.R. Taitt, Antimicrobial peptides for detection of bacteria in biosensor assays, Anal. Chem. 77 (2005) 6504–6508.
[35] Y.-X. Wang, Z.-Z. Ye, C.-Y. Si, Y.-B. Ying, Application of aptamer based biosensors for detection of pathogenic microorganisms, Chin. J. Anal. Chem. 40 (2012) 634–642.
[36] A. Ahmed, J.V. Rushworth, N.A. Hirst, P.A. Millner, Biosensors for whole-cell bacterial detection, Clin. Microbiol. Rev. 27 (2014) 631–646.
[37] P.D. Skottrup, M. Nicolaisen, A.F. Justesen, Towards on-site pathogen detection using antibody-based sensors, Biosens. Bioelectron. 24 (2008) 339–348.
[38] M. Schmelcher, M.J. Loessner, Application of bacteriophages for detection of foodborne pathogens, Bacteriophage 4 (2014) e28137.
[39] R. Van der Merwe, P. van Helden, R. Warren, S. Sampson, N.G. van Pittius, Phage-based detection of bacterial pathogens, Analyst 139 (2014) 2617–2626.
[40] B. Byrne, E. Stack, N. Gilmartin, R. O'Kennedy, Antibody-based sensors: principles, problems, and potential for detection of pathogens and associated toxins, Sensors 9 (2009) 4407–4445.
[41] S.L. Keasey, K.E. Schmid, M.S. Lee, J. Meegan, P. Tomas, M. Minto, A.P. Tikhonov, B. Schweitzer, R.G. Ulrich, Extensive antibody cross-reactivity among infectious gram-negative bacteria revealed by proteome microarray analysis, Mol. Cell. Proteomics 8 (2009) 924–935.
[42] E. Kutter, A. Sulakvelidze, Bacteriophages: Biology and Application, CRC Press, Boca Raton, FL, 2004, pp. 381–436.
[43] A. Singh, S. Poshtiban, S. Evoy, Recent advances in bacteriophage based biosensors for food-borne pathogen detection, Sensors 13 (2013) 1763–1786.
[44] V. Verma, K. Harjai, S. Chhibber, Characterization of a T7-like lytic bacteriophage of *Klebsiella pneumoniae* B5055: a potential therapeutic agent, Curr. Microbiol. 59 (2009) 274–281.
[45] K. Schleifer, R. Kilpper-Bälz, L. Devriese, *Staphylococcus arlettae* sp. nov., *S. equorum* sp. nov., and *S. kloosii* sp. nov.: three new coagulase-negative, novobiocin-resistant species from animals, Syst. Appl. Microbiol. 5 (1984) 501–509.
[46] V. Dinakaran, L. John, A. Rathinavel, P. Gunasekaran, J. Rajendhran, Prevalence of bacteria in the circulation of cardiovascular disease patients, Madurai, India, Heart Lung Circ. 21 (2012) 281–283.
[47] V. Dinakaran, M. Shankar, S. Jayashree, A. Rathinavel, P. Gunasekaran, J. Rajendhran, Genome sequence of *Staphylococcus arlettae* strain CVD059, isolated from the blood of a cardiovascular disease patient, J. Bacteriol. 194 (2012) 6615–6616.
[48] F. Elisangela, Z. Andrea, D.G. Fabio, R. de Menezes Cristiano, D.L. Regina, C.-P. Artur, Biodegradation of textile azo dyes by a facultative *Staphylococcus arlettae* strain VN-11 using a sequential microaerophilic/aerobic process, Int. Biodeterior. Biodegrad. 63 (2009) 280–288.
[49] S. Sagar, A. Dwivedi, S. Yadav, M. Tripathi, S.D. Kaistha, Hexavalent chromium reduction and plant growth promotion by *Staphylococcus arlettae* strain Cr11, Chemosphere 86 (2012) 847–852.
[50] N. Bhardwaj, S. Bhardwaj, P. Devi, S. Dahiya, M.L. Singla, C. Ghanshyam, M. Prasad, Phage immobilized antibacterial silica nanoplatform: application against bacterial infections, Adv. Anim. Vet. Sci. 3 (1) (2015), http://dx.doi.org/10.14737/journal.aavs/2015/3.1s.1.9.
[51] A.M. Kropinski, A. Mazzocco, T.E. Waddell, E. Lingohr, R.P. Johnson, Enumeration of bacteriophages by double agar overlay plaque assay, in: M.R.J. Clokie, A.M. Kropinski (Eds.), Bacteriophages: Methods and Protocols, Isolation, Characterization, and Interactions, 1, Humana Press, Totowa, NJ, 2009, pp. 69–76.
[52] V. Nanduri, I.B. Sorokulova, A.M. Samoylov, A.L. Simonian, V.A. Petrenko, V. Vodyanoy, Phage as a molecular recognition element in biosensors immobilized by physical adsorption, Biosens. Bioelectron. 22 (2007) 986–992.
[53] A. Shabani, M. Zourob, B. Allain, R. Mandeville, Phage-based method for the detection of bacteria, U.S. patent 20090068638 A1 (2009).
[54] M. Mejri, H. Baccar, E. Baldrich, F.J. Del Campo, S. Helali, T. Ktari, A. Simonian, M. Aouni, A. Abdelghani, Impedance biosensing using phages for bacteria detection: generation of dual signals as the clue for in-chip assay confirmation, Biosens. Bioelectron. 26 (2010) 1261–1267.
[55] C. Tlili, E. Sokullu, M. Safavieh, M. Tolba, M.U. Ahmed, M. Zourob, Bacteria screening, viability, and confirmation assays using bacteriophage–impedimetric/loop-mediated isothermal amplification dual-response biosensors, Anal. Chem. 85 (2013) 4893–4901.