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Investigation Of Oriented Immobilization Of Bacteriophage For Fabrication Of Free Label Biosensor

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Abstract

One of the dangerous human pathogens is *Escherichia coli* (*E. coli*). It causes life-threatening difficulties such as bloody diarrhea and renal failure. The study and preparation of *E. coli* biosensors are very important and required for diagnostic laboratories. Therefore, we studied oriented immobilized *E. coli* bacteriophage for label-free biosensor of *E. coli* bacteria detection and oriented immobilized bacteriophage was investigated in many methods such as FESEM, DLS, UV-Vis spectroscopy, and electrochemistry. For oriented immobilization of phage on the electrode, it was necessary to optimize the concentration of biotin. So, the biotin concentration was optimized by plaque-forming a unit, which was 8.5 mg/ml biotin. The stages of bacteriophage immobilization on the electrode were studied with FESEM, DLS, and CVs. Also, the effect of pH changes on bacteriophage/Au electrodes were studied in a wide range and pH=7 was the candidate. *E. coli* bacteria were measured by LCR meter in the capacitance method. It detected *E. coli* in the wide concentration range from 4×10^2 to 1.4×10^4 N/mL. We prepared label-free, inexpensive, cheap, rapid, and renewable E. coli biosensors by immobilizing oriented bacteriophage on modified gold bacteriophage biosensors. It seems that this system has the ability to be used as a platform for other pathogens.

Keywords: biosensors, bacteriophage, capacitance

Introduction

Microorganisms (viruses and bacteria) are generally found as infectious agents in the environment, food, marine and estuarine waters, soil, and the intestinal tracts of humans and animals. Some bacteria such as *Campylobacter*, *Salmonella*, *Listeria monocytogenes*, *E. coli*, *Staphylococcus aureus*, and *Bacillus cereus* are the most important food-borne pathogen bacteria, which are caused major food-borne illnesses [1]. Hence, it is really important to develop a simple and cost-reasonable method for detecting the food-borne pathogenic bacteria in food, water, and air as its critical role in public health. Culturing microorganisms are gold-standard microbiological methods for traditional biochemical pathogenic exams, but they are costly and time-consuming [2]. It needs to pre-enrichment of the bacteria and then culturing them on selective media. In especial, the major problems of current standard technologies are their enrichment steps and time-consuming up to 7–10 days, resulting in many problems in food pathogens, clinical diagnosis and environmental monitoring. Of course, several pathogenic detection methods were developed as a faster technique using current standard techniques: PCR, microarrays and ELISA. However, they are inefficient to analyze large sample volume without pre-enrichment and have high costs [3]. And also, most of the conventional methods involve complex instrumentation and may not be used on-site. Thus, developing biosensors for detecting pathogens as a fast and specific technique is growing. A biosensor can be defined as a compact analytical device or unit incorporating a biological or biologically-derived sensitive element integrated or associated with a physiochemical transducer. Moreover, biosensors are miniaturized laboratories that perform hundreds or thousands of simultaneous biochemical or serological reactions. So, the design of biosensors can promote detection and diagnostics aim. Bio-probes are the most important components of biosensors because responsible to bind and identify the analytes targets. Usually, the most common bio-probes for bacteria detection are antibodies, which are time-consuming, complicated and expensive to produce. Bacteriophage can be a good candidate as a bio-probe [4]. Compared to antibodies, bacteriophages are more selective towards their host organism, resistant in wide range of pH values (3–14) and resist high temperature (90–97 ^{0}C). Also, bacteriophages can be produced in large quantities easily and cheaply. They are safe to use since they do not infect humans [5]. These factors have made bacteriophage a good bio-recognition tool for the detection of foodborne bacterial pathogens. So, bacteriophage-based

biosensors offer several benefits for rapid bacterial detection. Although, many biosensors were reported based on bacteriophage bio-probe for *E coli* bacteria detection in various methods [6, 7, 8, 9]. But their detection methods such as; SPR, bioluminescence and fluorescent bioassay are very expensive and some of them haven't appropriate reports about analytical parameters such as linear range, detection limit, sensitivity, selectivity and etc. In this work, we designed a rapid and cost-effective biosensor constructed by oriented bacteriophage on the modified gold template for *E. coli* bacteria detection based on LCR meter in the capacitance method. The layout of the biosensor is patterned as a two electrodes configuration. These templates will be made by immobilization of oriented bacteriophage on modified gold template via streptavidin-biotin interaction. Consequently, the *E. coli* trapping by oriented bacteriophage on biosensor reported by capacitance method. It should be mentioned that this biosensor was designed for *E. coli* bacteria detection as a model through its importance and spread, and it can be developed for other pathogens, too.

Apparatus and procedure

The morphology of the prepared bacteriophage/Au electrode was achieved by means of a field emission scanning electron microscope (FE-SEM) (Hitachi S-4160, UK). All of the capacitance evaluations were done by an LCR meter (GPS 3131B, Benchtop LCR Meter, Germany). Cyclic voltammetry (CVs) analysis was accomplished via an Autolab potentiostat/galvanostat (model 302N, Eco Chemie, Ultecht, Netherlands). The capacitance in this system was evaluated through two similar Au electrodes in 2 mm diameter (presented in Fig. 1) as a capacitor.

Reagents

Linker biotin-NHS with 341.38 MW and streptavidin from Sigma, United States of America, LB Broth culture LB media culture (Broth & Solid) from Merck Germany, Polyethylene glycols (PEG) 6000 from Merck, Germany, 1-Ethyl-3-(3-dimethylamino propyl) carbodiimide (EDAC) with 155.24 MW from Sigma, United States of America, 2-(N-morpholino) ethane sulfonic acid (MES) buffer with 951.24 MW Sigma, United States of America, Dimethyl sulfoxide (DMSO), Dihydrogen phosphate (KH2PO4), dipotassium hydrogen phosphate (K2HPO4), hydrochloric acid (HCl) and hydrogen peroxide (H_2O_2) were obtained from Merck, Germany. A phosphate buffer solution (0.1 M, pH=7) was applied as a supporting electrolyte. Ultrapure H_2O by a Millipore-Milli Q (18 MΩ cm, Barnstead, Dubuque, USA) system was used for all solution preparations. All the reagents were used as received, without any purification, and all experiments were done at room temperature (25 0C). *E. coli* bacteria and bacteriophage were gotten from the Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran. It should be noted that, in this study, we used serotypes of *Escherichia coli O157:H7* bacteria.

Experimental section

Preparation of bacteriophage/streptavidin/Au electrode

There were 3 steps for preparing bacteriophage/streptavidin/Au electrode. In the first step, biotinylated bacteriophage prepared as follows. NHS-Biotin dissolved in Dimethyl sulfoxide (DMSO) instantly in 22 mg/mL concentration. NHS-Biotin solution was added to the total volume of the bacteriophage 10%, stirred gently, and incubated at room temperature for 4 hours. In the second step, streptavidin was immobilized on 11-MUA modified Au electrode; in the following order. Firstly, 3 µL of 11-MUA (5% W/V) in ethanol solution was dropped on the Au electrode. In the other vessel, 0.7 mg of streptavidin was dispersed in MES buffer (1.5 mL, 50 mM, pH= 6.1). Then, N-Hydroxysuccinimide (NHS) (0.5 mL, 50 mg/mL) which was prepared in MES was added to the above suspension. While stirring the suspension severely, 0.3 mL of fresh EDAC solution (10 mg/mL prepared in MES buffer) was combined quickly, and the consequence mixture was stirred at room temperature for 45 minutes repeatedly. After that, 20 µL of activated streptavidin was immediately dropped on the Au electrode and set the electrode was stable for 2 hours, to react slowly. Then, the streptavidin/Au electrode was washed with MES buffer three times for removing the excess EDAC, NHS. In the third step, biotinylated bacteriophage was immobilized on a streptavidin/Au electrode. For this purpose, 300 µL biotinylated bacteriophage solutions were capped on streptavidin/Au electrode and kept away from light overnight at room temperature, then stabilized bacteriophage on streptavidin/Au electrode washed with phosphate-buffered solution (PBS) three times. In this stage oriented bacteriophage was attached to the Au electrode surface.

Procedure for *E. coli* detection on bacteriophage/streptavidin/Au electrode

The quantification of *E. coli* by this bacteriophage biosensor is based on capacitance changes. The layout of this biosensor is patterned as two Au electrodes in a 2 mm diameter, configuration as shown in Fig.1. Two Au electrodes were connected to the LCR meter by Ag wire. The performance of the electrodes was investigated by measuring the capacitance change of *E. coli* bacteria which is prepared with a concentration range between $0\text{-}160\times10^5$ N/mL by serial dilution. Each solution was stirred and the capacitance was recorded, as the solution became stable, and then plotted as a function of *E. coli* concentration.

Equivalent circuit fit for biosensor

Figure 1 illustrates the correlation between each immobilized layer on the Au electrode with the capacitance intensity. The insulating self-assembled monolayer pattern on the Au electrode caused a constant capacitance and prevents any faradic processes and the capacitance got the highest level (Figure 2). According to the image illustrated in Scheme 2, two planar bacteriophage-modified Au electrodes stand as two capacitor plates, and the buffer between them is an insulating layer. By developing each layer on the bacteriophage-modified Au electrodes, different dielectric coefficients, thicknesses, and surface area reach a new capacitor value. Therefore, by attachment of *E. coli* bacteria on the left and right bacteriophage-modified Au electrodes (C1 and C2), the total capacitance was changed [10]. As presented in Figure 1, the total capacitance (Ct) at this level can be calculated through Eq.1:

$1/Ct=1/C1+1/C_2$ (1)

***E. coli* bacteria and bacteriophage culture**

***E. coli* bacteria culture**

Briefly, in 1000 mL deionized water, 200 mg LB broth was added. Then it was boiled and sterilized in the autoclave with 1.5 kg/cm^2 pressure on the liquid cycle for 20 min at 121 ^{0}C. In the next step, 100 µL fresh *E. coli* were added to the mix under sterile conditions. So, it was shaken at 37 ^{0}C at 150 rpm and allowed bacteria to grow until reached an optical density (OD) equal to 0.5 in 600 nm wavelength ($\lambda_{max}=600_{nm}$) [11].

Bacteriophage culture

200 µL of λ-bacteriophage was added to *E. coli* cultured in LB Broth (OD=0.5, λ=600 nm), and shaken at 37 ^{0}C at 150 rpm overnight. Supernatants containing bacteriophage were isolated from carcasses of bacteria using 10 min at 5000 rpm & 4 ^{0}C. Then, the concentration of bacteriophage was boosted by adding PEG to the supernatant and stored at 4 ^{0}C after dewatering with PBS buffer.

Biotinylation of bacteriophage

Biotinylation of bacteriophage is the first step to immobilization. In this experiment, NHS-Biotin solution (22 mg/mL) was added to the total volume of the bacteriophage 10% and stirred gently, and incubated at room temperature for 4 hours. NHS-Biotin reacts with the phage surface protein through primary amines in this step.

Evaluation of *E. coli* bacteria and bacteriophage binding by FESEM

Fig. 2, 3, and 4 show FESEM images of streptavidin/Au electrode, streptavidin/ bacteriophage Au electrode, and *E. coli* bacteria trapped by bacteriophage, respectively. In the streptavidin/Au electrode, very small dots are clearly seen, which indicates the modification of the gold electrode surface with biotin. In Fig. 3, the Biotinylated bacteriophage was attached to the Au electrode by streptavidin, and the bacteriophage image is visible on the Au electrode surface. Also, Fig. 4 shows that *E. coli* bacteria are trapped by bacteriophage.

Characterizations of biotin/phage by DLS

The size of biotin and biotin/bacteriophage was investigated by dynamic light scattering (DLS) technique. As shown in Fig. 5, the biotin/bacteriophage spectrum is flattened. So, it is confirming biotin attachment to bacteriophage.

Biotin/phage study by UV-Vis spectroscopy

The UV-Vis spectroscopy of biotin (before and after binding to bacteriophage) was studied for the evaluation of biotin binding (Fig. 6).

***E. coli* bacteriophage assay:**

In order to bacteriophage assay of *E. coli* the double agar overlay assay (DLA) was performed on disposable plastic petri dishes (in sterile condition). The DLA is a technique that allows localized phage-bacteria contact in a Petri dish containing two layers of agar (fig 7). The bottom layer was prepared with Luria–Bertani (LB) medium containing 1.5% agar and the top layer contains the LB medium with a lower concentration of agar (0.5%) containing 10 mM $MgCl_2$ [12], and it is mixed with kanamycin (50 mg/L) and five microliters of optimal phage dilutions were added to 1ml of E. coli bacteria (an equivalent of 1× 109 CFU) and immediate pouring onto the bottom agar layer. In the top-agar, diffusion allows the bacteria to occupy the lawn completely and bacteriophage to bind to the bacteria. This is followed by incubation at 37°C for 24 h (plates are incubated overnight). [13]. The titer of infective phages in the sample was established by spotting serial dilutions of the sample as a plaque-forming unit (PFU). Each one of the ploughs is riffed to one bacteriophage that infected an *E. coli* bacterium (Fig. 7). So, the phage was able to lyse the *E. coli* bacteria, and plaque is formed.

Optimization of biotin concentration

As mentioned, the surface of the gold electrode was modified by streptavidin, and biotin was used for phage binding to immobilize it on the gold electrode through streptavidin. Ideally, it is necessary that the head protein of the bacteriophage be biotinylated and the phage tail remains free to the attachment of host bacterial cells. So, the biotin concentration is important to optimize phage binding on the electrode surface and retain maximum phage activity. Then, the optimum biotin concentration attachment on phage for maximum activity was studied by plaque-forming units, which was done according to the section "*E. coli* bacteriophage assay". We used different concentrations of biotin (0, 5, 10, 20, 50, 70, 100 & 200 mg/mL) for biotin binding on bacteriophage and then performed a phage assay. It seems that the concentration of 5 mg/mL biotin is an optimal concentration (Fig. 8).

The investigation of conductivity by Cyclic Voltammetry

Cyclic voltammograms (CVs) were used to follow the electron conductivity of the bare Au, streptavidin/Au, bacteriophage/streptavidin/Au, and *E. coli*/bacteriophage/streptavidin/Au electrodes (Figure 9). In this experiment, $K_3[Fe(CN)_6]$ solution in PBS (0.1 M) is applied as a redox probe. As shown in Fig. 8, the CVs of bare and modified electrodes were seen in the potential from -0.2 to 0.5 V. When the streptavidin and oriented biotinylated bacteriophage were immobilized on the Au electrode, they act as insulators, and the current intensity was decreased. And of course, the current decrease by *E. coli*/bacteriophage/streptavidin/Au electrode is even more.

Optimization of capacitance response toward *E coli*/bacteriophage/streptavidin/Au electrode by pH changes

To investigate the pH dependency of capacitance coefficient response toward the *E. coli*/bacteriophage/streptavidin/Au electrode, the electrode was exposed to PBS (0.1 M) at various pHs ranging from 5.4 to 8.0. for this, different volumes of *E. coli* bacteria (1µL=2×10^5 N/mL), were added to the solution, and capacitance signals were recorded. As shown in Fig. 10, the biosensor can be active in a wide range, but it seems to represent a maximum current response at pH 7. Thus, it was selected as the optimal pH.

Calibration curve for *E coli* biosensor

For measuring *E. coli* the capacitance method was used. Figure 11 demonstrates the association of the change of capacitance besides *E. coli* number by bacteriophage-modified Au electrodes. It is obvious that the bare Au (without bacteriophage) fixed on electrodes does not show any response to *E. coli* concentration change. Also, oriented bacteriophage-modified Au electrodes, has high sensitivity and a wide concentration range, because modified electrode with bacteriophage immobilization creates a high sensitivity-to-area ratio for capacitance storage. In bacteriophage-modified Au electrodes, *E. coli* was detected in a linear range from 4×10^2 to 1.4×10^4 N/mL by the correlation coefficient of 0.997. The detection limit (DL) of this biosensor was calculated at a signal-to-noise ratio of 3 (S/N=3) according to Eq.2:

DL: 3.3 σ/S

In Eq. 2, σ stands for the standard deviation of the response and S presents the slope of calibration curve [14]. According to Eq. 2, the detection limit was calculated equals to 12 N/mL. Finally, the analytical parameters of this biosensor were compared with other similar biosensors reported for the detection of *E. coli* bacteria (Table 1).

Selectivity of the *E. coli* bacteria biosensor

The selectivity in medical diagnosis is so vital in analyzing biological samples which are within a complex matrix. In the present report, the selectivity of the designed biosensor was considered in the existence of five several gram-negative and gram-positive bacteria such as *Klebsiella pneumoniae*, *Shigella dysenteriae*, *Vibrio cholera* and *Staphylo coccusaureus* and *Salmonella typhi*. It does not significant response to oriented biotinylated bacteriophage biosensors, as shown by the selectivity of the system to *E. coli* bacteria detection (table. 2)

CONCLUSION

Detection and determination of analytes such as biomaterial and microorganisms are very important in biomedicine and the medical field. The traditional methods are complicated and time-consuming, so biosensors entered extensive and power volume to the diagnostics field. So far, many rapid and expensive methods including electric DNA chips, amperometric biosensors, quartz crystal microbalance, optofluidic ring resonator sensors, imaging ellipsometry, and surface plasmon resonance (SPR) were developed. In fact, the design of miniature, portable, simple user and cheap biosensors will be helped to promote public health. Therefore, we tried to design of simple and cheap biosensor based on bacteriophage for *E. coli* bacteria detection. In this work, we designed the gold template biosensor. It can be connected to an LCR meter for capacitance change monitoring. The biotinylated bacteriophage was immobilized in a chemically

oriented method on a biosensor by Au/streptavidin electrode. The *E. coli* bacteria trapped by bacteriophage and the *E. coli* bacteria concentration determined to record of by capacitance coefficient changes.

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Figure legend:
Figure 1. Capacitance measurement illustration. The interaction between bacteriophage and *E. coli* followed on the electrodes modified through streptavidin on the Au electrode.
Figure 2. FESEM image of Au electrode modified by streptavidin.
Figure 3. FESEM image of Au electrode modified by streptavidin/ bacteriophage.
Figure 4. FESEM image of *E. coli* bacteria trapped by bacteriophage.
Figure 5. DLS spectra of bacteriophage (a) and biotin/bacteriophage (b).
Figure 6. The UV-Vis spectroscopy of biotin (…), biotin/ bacteriophage (---) and bacteriophage (—).
Fig 7. the left sample is LB medium cultured by *E. coli* alone as control and the right one is LB medium cultured by *E. coli* containing bacteriophages, each one of the plows is riffed to one phage that infected a bacterium.
Figure 8. Optimization of biotin concentration for bacteriophage binding. (0, 5, 10, 20, 50, 70, 100, 200 µL) from (200 mg/mL) was added to 1 mL phage solution.
Figure 9. Comparison between CVs. Au electrode (—), streptavidin /Au electrode ‹(. -. -.)bacteriophage/streptavidin/Au electrode (---), and *E. coli*/bacteriophage/streptavidin/Au electrode (…). CVs are recorded in PBS solution (0.1 M, pH =7) with a scan rate of 100 mV/s at air-saturated conditions.
Figure 10. pH dependency of capacitance coefficient changes in bacteriophage/streptavidin/Au electrode, (1µL=2×10^5 N/mL).
Figure 11. Comparison of the calibration curves for *E. coli* determination. Bare Au electrodes (Δ) and bacteriophage/streptavidin/Au electrode (♦) in PBS solution (0.1 M, pH=7) by capacitance method, (1µL=2×10^5 N/mL).

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Table 1. Comparison of the parameters obtained in this work with others reported in the literature

Biosensors type based	**Material function based**	**Detection of bacteria type**	**Further details**	**LOD**	Ref
microfluidic	smartphone imaging for monitoring the color change of the AuNPs	*Ecoli*O157:H7	Research is done in piked chicken samples	50 CFU/mL	[15]
Conformable surface acoustic wave (SAW)	aluminum nitride (AlN)-based conformable SAW immunosensor	*E-coli*	SAW devices for bacteria contamination control in the food chain, water, and smart packaging.	$1.04*10^6$ CFU/ml	[16]
impedance-based biosensor	Interdigitated Electrode (IDE) arrays	*E-coli*	The molecular composition of the self-assembled monolayer (SAM) formed on the top of the IDEs	9 cfu mL^{-1}.	[17]
Surface-enhanced Raman spectroscopy (SERS) analysis	aptameric DNA sequences as bio-capture molecules	*E. coli* O157:H7	non-modified citrate reduced GNPs are promising for potential low-cost, high-throughput applications	$\sim10^1$ CFU mL^{-1}) and ground beef samples ($\sim10^2$ CFU mL^{-1}).	[18]
Impedimetric biosensor	Self-assembled gold nanoparticles and protein G	*-E.coli*	the Au NP-modified biosensor is ascribed to the synergistic effect between the Au NPs and the PrG-thiol scaffold	140 cfu mL^{-1}	[19]
Fluorescent magnetic biosensor b ased on DNAzyme	-Fluorescent sensor based on triple signal amplification of magnetic beads, DNAzyme and photoluminescence	*E-coli*	the *E. coli*-specific RNA-cleaving DNAzyme can specifically identify the target protein in CIM,	1.57 CFU mL^{-1}	[20]
bacteriophage capacitive free label biosensor	*E. coli*/bacteriophage/strept avidin/Au electrode	*E-coli*	streptavidin immobilized on 11-MUA modified Au electrode and biotinylated bacteriophage was oriented fixed on electrode	12 N/mL	This work

Table 2. Comparison of several bacteria capacitance response to *E. coli*/bacteriophage/streptavidin/Au electrode

Bacteria	ΔC/nF for 7000 N/mL bacteria
E. coli	65
Salmonella	0-
Klebsiellapneumoniae	0-
Shigelladysenteriae	0-
Vibrio cholerae	0-

Figures:

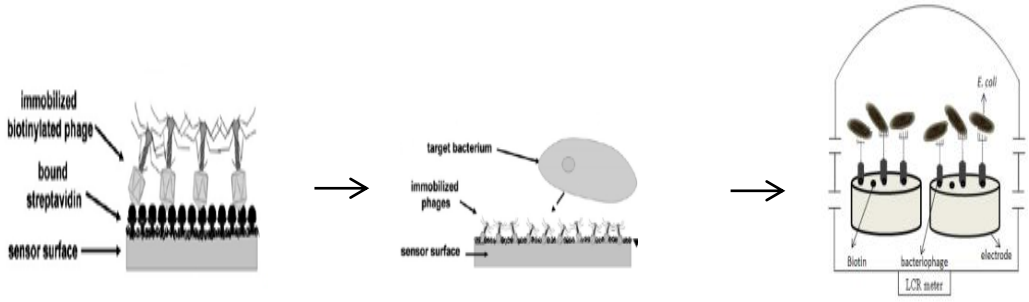


Fig. 1

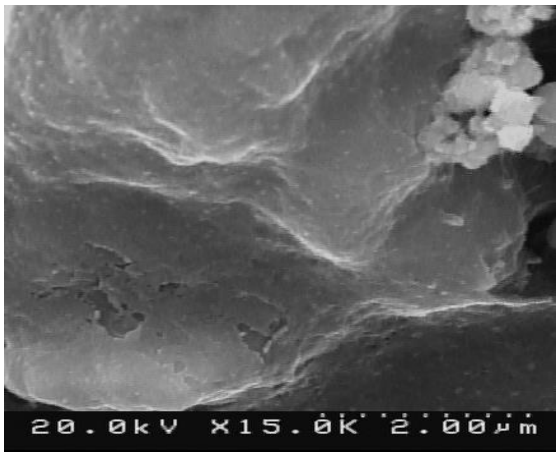


Fig. 2

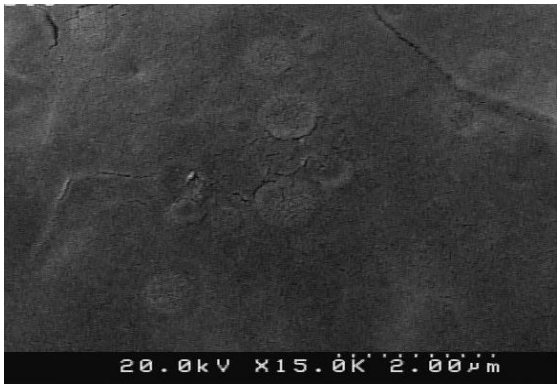


Fig. 3

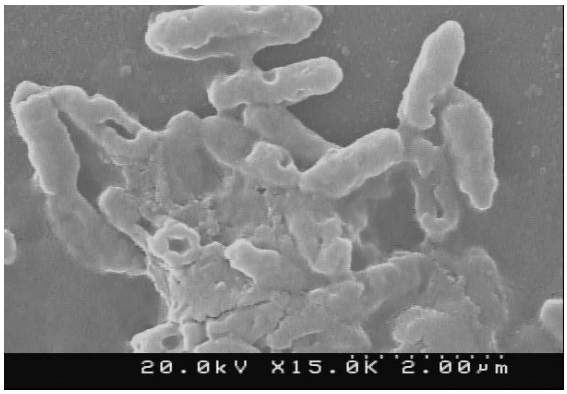
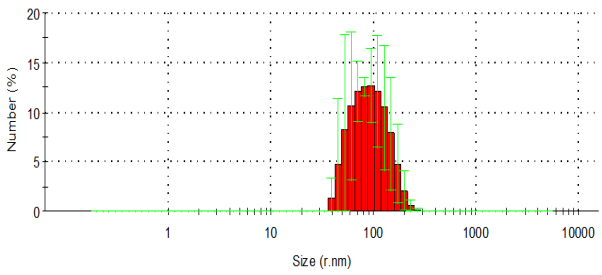
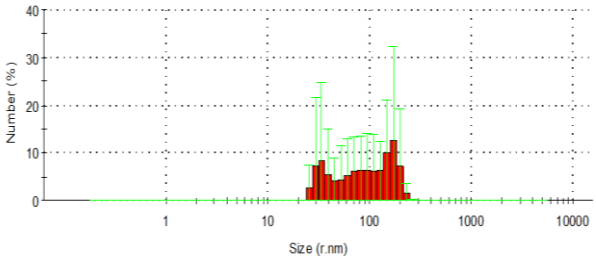


Fig. 4



a



b

Fig. 5

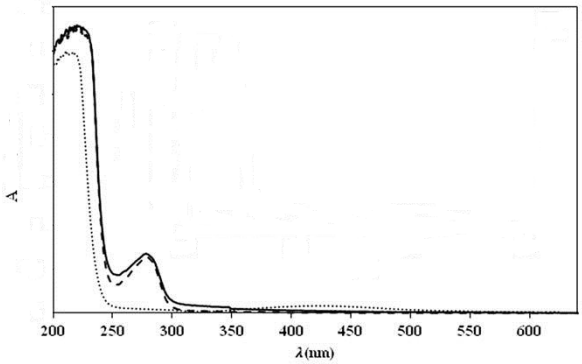


Fig. 6

Fig. 7

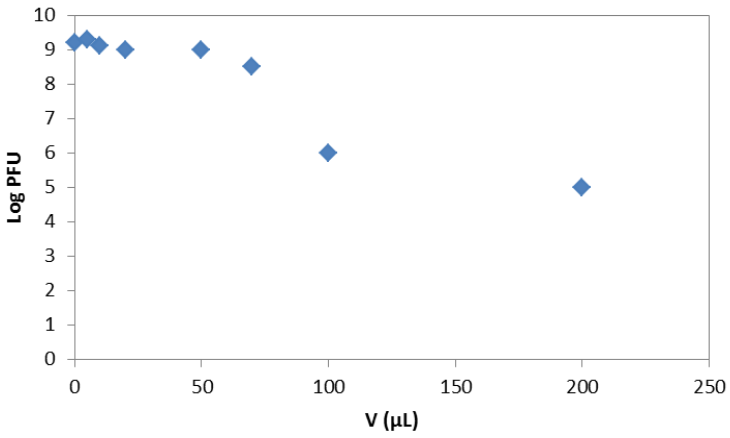


Fig. 8

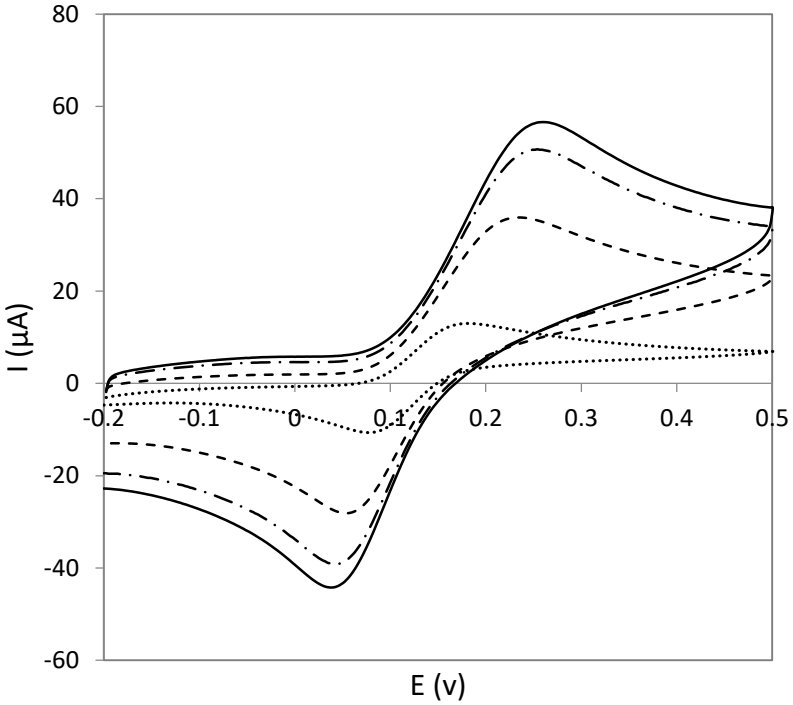

80
60
40
20
0
-20
-40
-60
I (µA)
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
E (v)

Fig .9

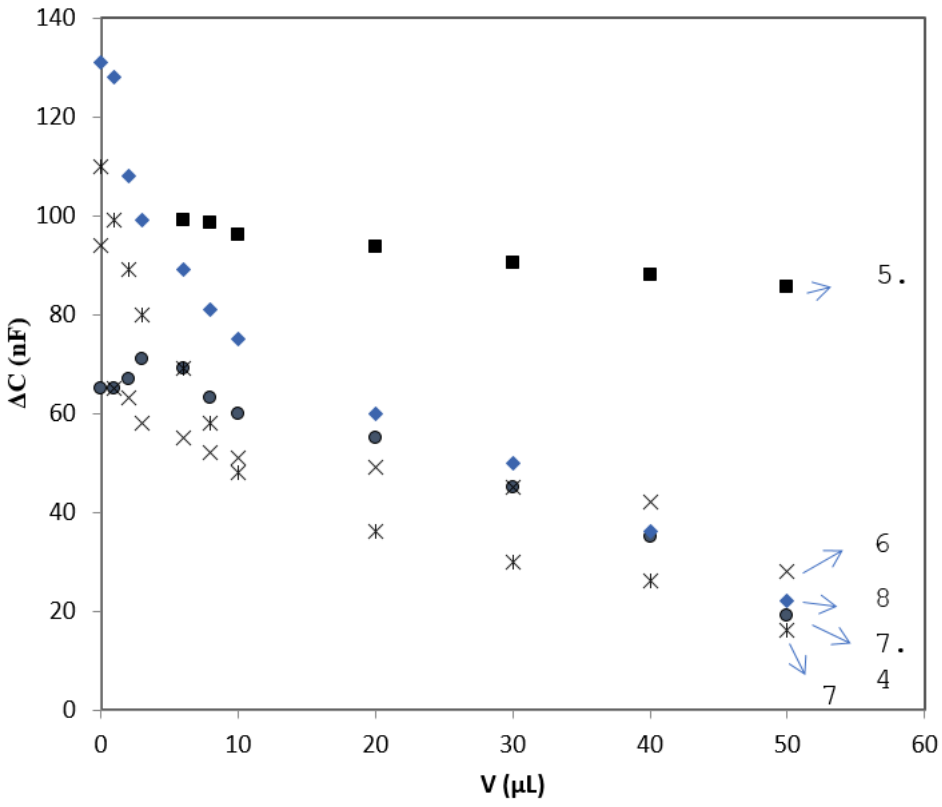

140
120
100
80
60
40
20
0
ΔC (nF)
0
10
20
30
40
50
60
V (µL)
5.
6
8
7.
4
7

Fig .10

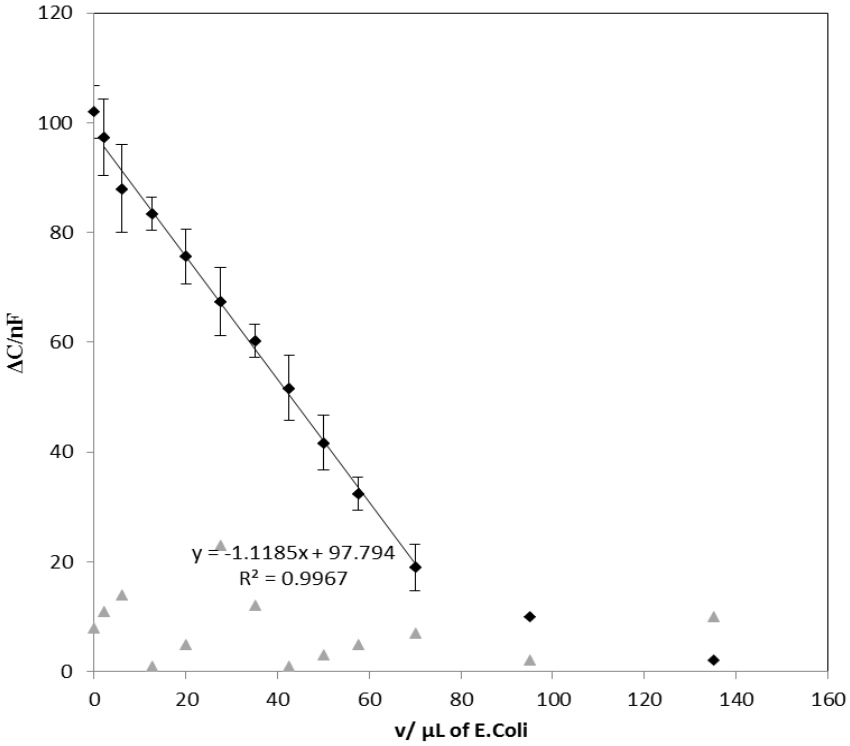

120
100
80
60
40
20
0
ΔC/nF
y = -1.1185x + 97.794
R² = 0.9967
0
20
40
60
80
100
120
140
160
v/ µL of E.Coli

Fig. 11