Folic-Acid-Modified Conducting Polymer: Electrochemical Detection of the Cell Attachment

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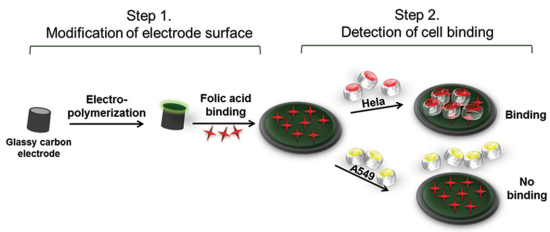
Here, postfunctionalization and bioapplication of a π-conjugated polymer named 4-[4H-dithieno(3,2-b:2′,3′-d)pyrrol-4-yl]aniline (DTP-aryl-NH_2) are reported, which is successfully synthesized via electropolymerization onto the glassy carbon electrode. Folic acid (FA) is used to modify the amino functional polymer via *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride/*N*-hydroxysuccinimide chemistry for the further steps. The selective adhesion of folate receptor positive cells on the surface is followed by the electrochemical methods. Cyclic voltammetry and electrochemical impedance spectroscopy have been used to characterize stepwise modification of the electroactive surface. After optimization studies such as scan rate during the polymer deposition, FA amount for the efficient surface targeting, incubation time with the cells etc., analytical characterization is carried out. The surface morphologies at each step are imaged by using fluorescence microscopy.



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1. Introduction

In the ever expanding field of organic materials consisting of polymers, ceramics, carbonated structures, biologically derived natural polymers, composites, and conducting polymers have gained an increased attraction in the use of biological applications.[1–6] Beside the other functional organic materials, conducting polymers (also known as synthetic metals) have taken a place in several research areas such as sensors, batteries, anticorrosion films actuators, and optical devices.[7,8] Conducting polymers, which can be deposited on electroactive surfaces by electropolymerization, provide suitable environment for immobilization and stabilization of biomolecules and have some advantages such as ease of preparation, good stability as well as conductivity.[9–11] Cultivation, adhesion, and proliferation of mammalian cells on the suitable platforms are the important points for the experimental process of biomedical researches. The surface

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must have high mechanical stability and also compatibility with the biological materials.[12] Various natural and synthetic polymeric structures have been designed and applied as a matrix for cell cultivation to examine in vitro toxicity of newly developed drug candidates, medical devices such as microarrays and scaffolds for tissue regeneration, etc.

Folic acid (FA, folate) also known as vitamin B_9 has gained much attention as a targeting ligand. In the concept of a general FA-targeting strategy, a 38 kDa glycosylphosphatidylinositol-anchored glycoprotein referred as a folate receptor (FR) is considered by overexpressing in the cancer cell lines whereas in normal tissue the expression of FRs is limited. It is worthy of note that folate has a high affinity to FRs as $K_d \approx 10^{-10}$ M.[13,14] On behalf of this, FA-conjugated materials have been used in drug delivery as a "trojan horse," bioimaging, and selective cell adhesion studies. Recently, FA-modified montmorillonite clay was prepared, characterized and tested as a cell culture material for the selective cell adhesion.[15] Furthermore, FA-modified poly(ε-caprolactone)/clay nanocomposite resulting in selective adhesion and proliferation of FA-receptor rich cells, was synthesized and characterized and applied as a cell biosensing platform.[16] In another study, the electropolymerization of 3-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl)aniline monomer on indium tin oxide (ITO) glass and its use as a coating material for the cell culture applications were investigated. In that case, functional amino groups on the conducting polymer provide postmodification of the surface with arginylglycylaspartic acid (RGD) peptide sequence via EDC Chemistry.[17] On the other hand, electroactive polymers such as polypyrrole are highly attractive for various bioinvestigations including their use as coating materials for electroactive surfaces or neural probes and as scaffolds for tissue regeneration. Besides, surface modification of these materials with biological moieties is required to create the interface for the sufficient cell–surface interaction.[18] Here, we describe the synthesis and the bioapplication of 4-(4H-dithieno[3,2-b:2′,3′-d]pyrrol-4-yl)aniline (DTP-aryl-NH_2) as a cell adhesion platform. In other to provide selectivity of modified surfaces against to FA (+) cells, FA conjugation was carried out and electrochemical methods were used for the analysis of cell amount. To achieve expanded application of this conducting polymer, which has both fluorescence and electrochemical characteristics and verify the presence of the selective cell binding, first electrochemical detection of cell attachment was performed and then fluorescence microscopy images were collected. Voltammetry such as differential pulse voltammetry or cyclic voltammetry is a powerful electrochemical technique to study electrochemical behavior analytes and the amount of an unknown analytes in samples is determined by plotting a calibration curve of peak current height versus analyte concentration.[9,12,17,19,20] Among other methods, electrochemical techniques are generally not interfered the colorful nature of the matrix. Also, electrochemical methods are useful to characterize the layer-by-layer-modified surfaces.[9,12,17,19,20] Dithione [3,2-b:2′,3′-d] pyrroles (DTP) as fluorescent thiophene material has been the application of first reported by Zanirato.[21–23] Functionalization of DTP-NH_2 with electron-releasing group also supports the development of high-energy electronic transitions based on materials such as the preparation of OLEDs, OFEDs as well as biosensors. This property helps to get fast direct electron transfer to electrode. Recently, novel π-conjugated monomers containing(4-(4H-dithieno[3,2-b:2,3-d]pyrrol-4-yl) aniline) (DTP-aryl-NH_2) were synthesized and characterized successfully.[24] In this case, after electropolymerization of DTP-aryl-NH_2 on the glassy carbon electrode, amine containing polymer was modified with FA via EDC/NHS chemistry. Then obtained DTP-aryl-NH_2/FA modified surfaces were used for the electrochemical detection of HeLa cells where FRs are overexpressed on the cell surfaces, which is called as FA receptor positive.[15] To evaluate the capturing efficiency on the surface and the selectivity, results were then compared with A549 cells, which is described as FR-negative cell line and used as a control.[20,25] The electrochemical data for the cell binding were also confirmed by using fluorescence microscopy images of the electroactive surface.

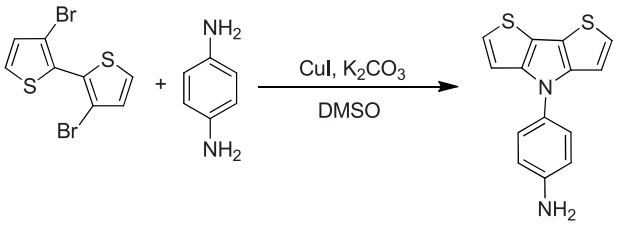
2. Experimental Section

2.1. Reagents

N-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), glutaraldehyde (GA) solution (25%), potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$), 4,6-diamino-2-phenylindol (DAPI), and FA were obtained from Sigma–Aldrich. *N*-hydroxysuccinimide (NHS) was obtained from Fluka. The phosphate buffer saline (PBS) containing 8.0 g L^{-1} NaCI, 0.2 g $LKCI^{-1}$, 1.44 g L^{-1} Na_2HPO_4, and 0.24 g L^{-1} KH_2PO_4 at pH 7.4 (50×10^{-3} M) was used. ITO glass (24×24 mm, sheet resistance of 8–10 ohm sq^{-1} with the thickness of 150–170 μm) was purchased from TEKNOMA, Turkey.

2.1.1. Cell Lines and Cultivation

A549 The human alveolar type-II (ATII)-like cell lines and human cervical cancer cell line (HeLa) were cultured in Dulbecco's modified Eagle medium (DMEM) with L-glutamine (Lonza, Switzerland) supplemented with 10% (v/v) FCS (Lonza, Switzerland), 10 000 U mL^{-1} penicillin and 10 000 U mL^{-1} streptomycin (Lonza, Switzerland). Cells were cultivated in 75 cm^2 tissue culture flasks and maintained under standard cell culture conditions (5.0% CO_2, 95% humidity and 37 °C in incubators). Cells were passaged two times each week. Under the light microscope (Olympus BX51M) cell count was done. After required solutions were prepared, they were stored at +4 °C for a short time.



Scheme 1. Synthesis of 4-[4Hdithieno(3,2-b:20,30-d)pyrrol-4-yl]-aniline; (DTP-aryl-NH_2).

2.2. Construction of Biofunctional Surfaces

The DTP-aryl-NH_2 monomer (4-(4H-dithieno[3,2-b:2′,3′-d]pyrrol-4-yl)aniline) was synthesized as previously described.[24] The monomer was synthesized by using traditional Ullman Reactions method. Using Chem Draw program, the molecular weight of the DTP-aryl-NH_2 monomer was calculated as 270 g mol^{-1}. Synthetic route for the preparation of poly(4-(4H-dithieno[3,2-b:2,3-d]pyrrol-4-yl)aniline) is described in Scheme 1.

The electrodeposition of the monomer solution was performed with a CV technique in a 0.1 M solution of tetrabutylammoniumhexafluorophosphate ($TBAPF_6$)/dichloromethane (DCM)/acetonitrile (ACN) (1:2) supporting electrolyte under a nitrogen atmosphere at a scan rate of 100 mV s^{-1}. Poly (DTP-aryl-NH2) was functionalized by FA to construct electrochemical biodetection system for following mammalian cells. After pretreatment of glassy carbon electrode with alumina slurry and further sonication steps, electropolymerization of monomer was carried out. The polymer film was formed on the working electrode after running of five voltammetric cycles between 0.8 and –0.4 V at a scan rate of 0.1 V s^{-1} in $TBAPF_6$ (0.1 M)/dichloromethane medium. Carboxyl groups of FA were activated using EDC/NHS chemistry and covalent binding of FA to free amine groups on the structure of DTP-aryl-NH_2 were carried out. Poly(DTP-aryl-NH_2)-modified surfaces were immersed into 50 µL of FA solution (50×10^{-3} M in dimethyl sulfoxide, DMSO), 200 µL of EDC solution (0.2 M in pH 6.0, MES buffer) and 200 µL of NHS solution (0.05 M in pH 6.0, MES buffer) for 2 h at ambient conditions. After rinsing thoroughly via distilled water and PBS buffer solution within immersing the electrodes, cells were dispersed on the modified electrode surface in pH 7.4, PBS.

2.3. Measurements

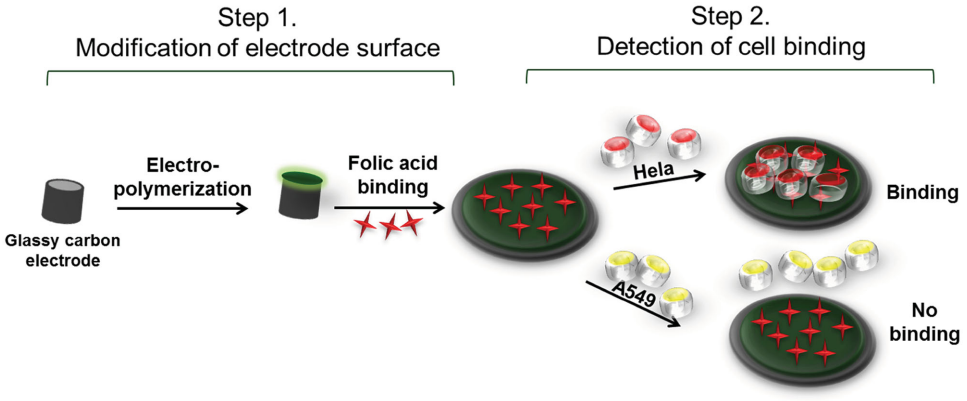
Electrochemical experiments were carried out in a three-electrode cell using a PalmSens electrochemical measurement unit for cyclic voltammetry (CV) and CH Instruments (CH-600 model) for electrochemical impedance spectroscopy (EIS) measurements. CV and EIS were used to characterize the stepwise modification of electrode surface. The three-electrode system consists of the modified glassy carbon electrode, an Ag/AgCl electrode used as a reference electrode, platinum as a counter electrode. CV measurements were performed between –0.4 and 0.8 V potentials. Apart from CV, ITO was used in EIS analysis as a working electrode. In the EIS measurements, an excitation signal of 10 mV (peak amplitude) was applied to the electrodes at 0.18 V potential and the frequency of the signal was varied between 0.031 and 1000 kHz. All measurements were carried out in pH 7.4 PBS containing 5.0×10^{-3} M, $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl. A two-step biosensor cell attachment protocol was constructed as shown in Scheme 2. Initially, the electrode surface were coated with the poly(DTP-aryl-NH_2) and subsequently modified with FA, as mentioned above. Second, cells were introduced onto the surface in which the cells containing FA receptors have the opportunity to bind selectively with the immobilized FA.

Cell binding to the surface resulted in a drop in CV signals, which were correlated with the cell capturing. Differences between the current signals were calculated as follows:

$$\Delta I = I_o - I_c$$

where *Io* is the mean cathodic current at zero cell concentration and *Ic* is the mean cathodic current at any concentration after cell binding onto the FA-modified surfaces.

For the cell imaging, ITO glasses were used as an electroactive surface for the polymer coating and FA binding. Modified ITO glasses are placed in six-well plate. Afterwards, 40.000 HeLa and A549 cells were seeded on them in 3.0 mL media. After 2 d incubations, the cells fixed with 4.0% paraformaldehyde and then, washed three times with PBS. After fixation step, DAPI solution was added for staining cell's nucleus for 15 min. Then cells were washed with PBS again. Images were taken with Fluorescence microscope (Olympus BX53F) equipped with a CCD camera (Olympus DP72) under green and DAPI filter.



Scheme 2. Schematic illustration of the principle of cell attachment by proposed biosensor.

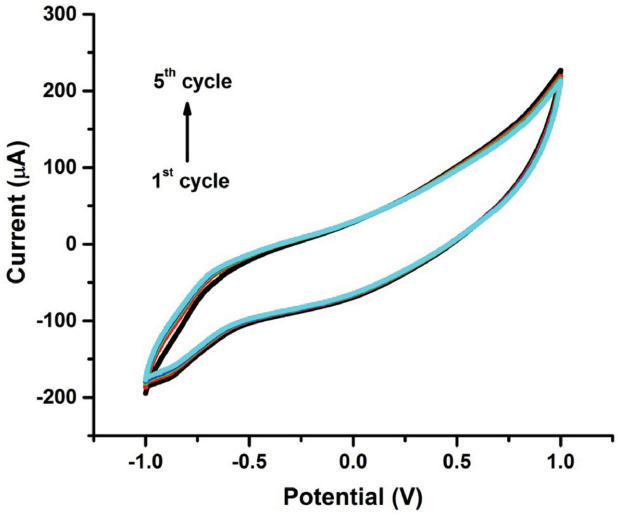


Figure 1. Electropolymerization of poly(DTP-aryl-NH_2) between –1.0 and 1.0 V, at the ambient conditions [at a scan rate of 100 mV s^{-1} in 0.1 M $TBAPF_6$/DCM:ACN (1:2) electrolyte solution].

3. Results and Discussion

Electrodeposition of π-conjugated polymers has been lead to an innovative approach with the convergence of two main areas as electrochemistry of modified electrode and conjugated systems. As a result from this approach, conducting polymer-modified electrodes were enriched in electrochromism, electrocatalysis, energy storage, and electroanalysis studies.[25–29] Moreover, many biosensor studies have been shown up by using electropolymerization of different monomers. Despite the conventional enzyme and microbial-based biosensor systems, such polymers and composite materials have been conducted to cell sensing platforms. Hereby, DTP-aryl-NH_2 film was generated onto GCE by electropolymerization via CV. In the cyclic voltammogram of the electropolymerization steps (Figure 1), monomer oxidation and reduction peak potentials for DTP-aryl-NH_2 was obtained at 0.302 and 0.245 V, respectively. Within the formation of deposition, the increased thickness of the film was noticed the increased peak current after each scan.

Electrochemical characterization of poly(DTP-aryl-NH_2)/FA-modified GCE electrodes were performed at each step of the biosensor construction and cell binding. Figure 2 illustrates the CV (A) and EIS graphs (B) as the evidence of the surface modification with poly(DTP-aryl-NH_2) and FA as well as the cell binding. EIS has been used to analyze charge transfer resistance and hereby demonstrate the surface modification. EIS spectra of the modification steps were accomplished using $[Fe(CN)_6]^{3-/4-}$ as a water-soluble redox mediator. Nyquist plots of EIS spectra that were obtained by step-by-step modification onto the electrode were shown in Figure 2B. According to the

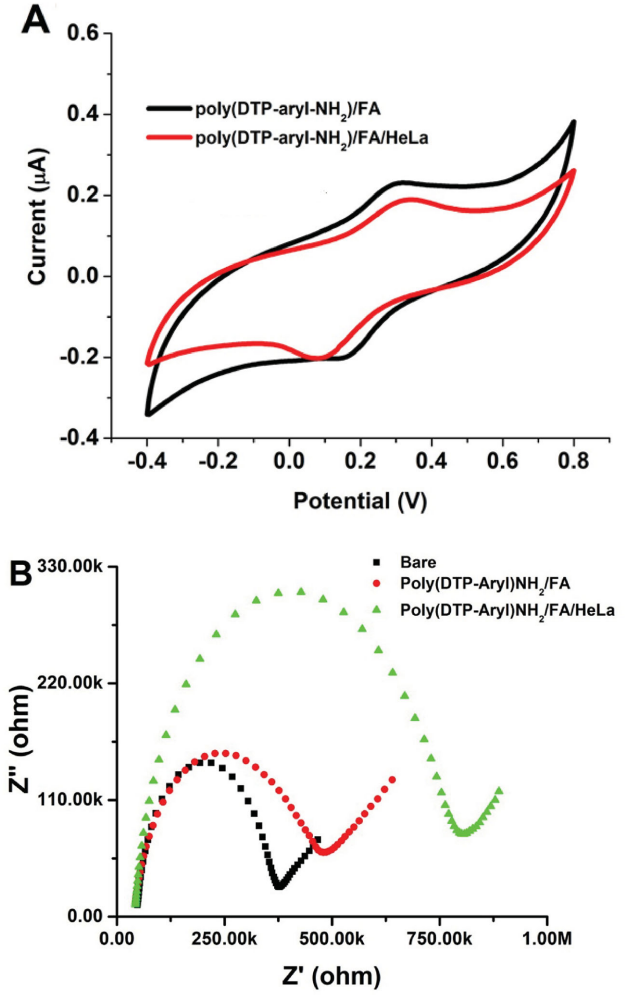


Figure 2. A) CV and B) EIS diagrams of bare GCE, poly(DTP-aryl-NH_2)/FA, poly(DTP-aryl-NH_2)/FA/HeLa (3.00×10^3 cells mL^{-1}), [0.1 M KCl in PBS (pH 7.4). 5.0×10^{-3} M $[Fe(CN)_6]^{3-/4-}$ was used as redox probe].

Nyquist plots, the diameter of the semicircle that was obtained by bare ITO is smaller than poly(DTP-aryl-NH_2)/FA-modified electrodes. Also, addition of the HeLa cells to the modified surface increased the diameter of the semicircle in EIS spectra. The increased diameter of the semicircles proves the blocking behavior of the modified electrode after each modification step.

3.1. Cell Sensing

In various kinds of cancer types such as breast, ovaries, and kidney, FR was overexpressed and used as a tumor marker to detect these cells.[30] To recognize mammalian cells which FR overexpressed, FA has been utilized and can bind to FR with high affinity.[31] After conjugation of material to FA, it can be used as active targeted carrier and label-free detection of cancer cells.[32] Here we report

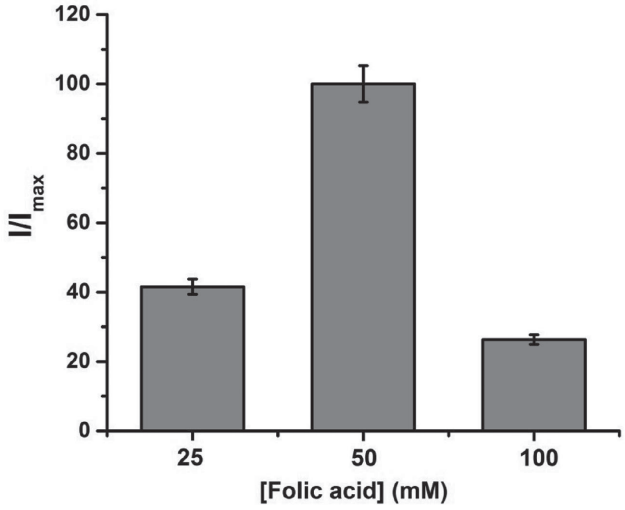


Figure 3. Influence of FA concentration for capturing of HeLa cells on poly(DTP-aryl-NH_2) deposited GCE surfaces. [Similar conditions were applied as given at Figure 2].

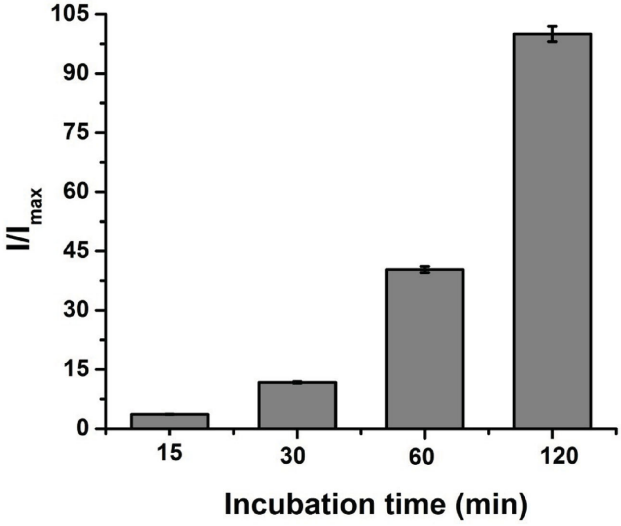


Figure 4. Effect of incubation time with cells (10^3 cells mL^{-1}). [Similar conditions were applied as given at Figure 2].

a newly synthesized conducting polymer and modification with FA. The covered material with FA have two tasks in this assay: one of them was the usage as a biocompatible surface for cancer cells and the second one was support for the electrochemical and fluorescence sensing of cells. To investigate the influence of scan number for the electropolymerization on the signal was evaluated at different scan numbers of electropolymerization (from 5 to 20 scans). The greatest current differences from CV measurements were belong to 5 scans of poly(DTP-aryl-NH_2) in the presence of $[Fe(CN)_6]^{3-/4}$. In the electropolymerization step, it is well known that increase of the cycles leads to thicker polymer film layers. Moreover, this thickness mostly generates building blocks for electron transfer on the interface of bulk solution (electrolyte) and electrode surface. Hence, electropolymerization of DTP-aryl-NH_2 monomer was carried out as five scans for the further optimization and characterization studies (data not shown).

To optimize the amount of FA on the response, 25–100 $\times$ 10^{-3} M of FA was used to modify the surfaces, which was covered with poly(DTP-aryl-NH_2). Among three different concentration of FA (25 $\times$ 10^{-3}; 50 $\times$ 10^{-3}; 100 $\times$ 10^{-3} M), results were recorded as relative current response against maximum current difference between those concentrations (Figure 3). Since the best and reproducible signals were obtained by using 50 $\times$ 10^{-3} M FA, further experiments were conducted with this modifier amount. On the other hand, nonspecific bonds may have been formed during FA modification when 100 $\times$ 10^{-3} M concentration was applied to the poly(DTP-aryl-NH_2) film.

Incubation time of cells with the poly(DTP-aryl-NH_2)/FA-covered surfaces was also optimized. Incubation time of cell adhesion to poly(DTP-aryl-NH_2)/FA covered surfaces was varied from 15 to 120 min (Figure 4). It can be clearly seen that longer incubation time enabled stronger cell adhesion. Since the maximum response was obtained after 120 min incubation, it was selected as optimum for the cell sensing experiments.

After the optimization parameters of FA amount and incubation time of cells, a calibration curve was generated as the analytical characterization of poly(DTP-aryl-NH_2)/FA biorecognition layer. As can be seen from Figure 5, the changes in electrochemical signals were linearly over a wide cell concentration range of 10 to 10^5 cells mL^{-1} with the equation of $y = 0.057x + 0.121$, ($R^2 = 0.996$). The comparison of the some characteristics of the functionalized surfaces for cell detection, which were obtained using various techniques, were summarized in Table 1.

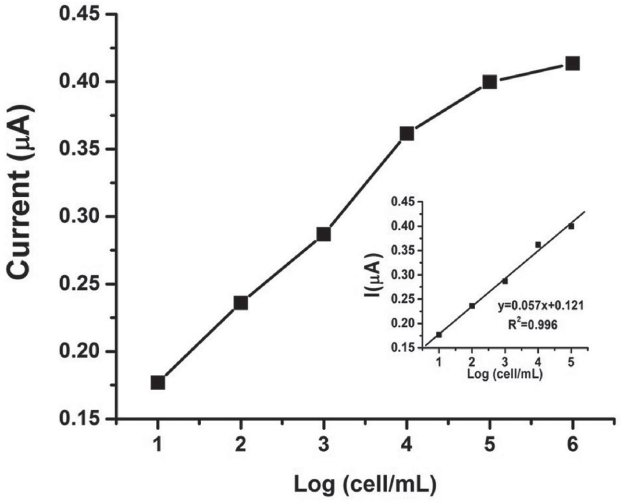


Figure 5. Relationship between electrochemical response and cell concentration. [Similar conditions were applied as given at Figure 2].

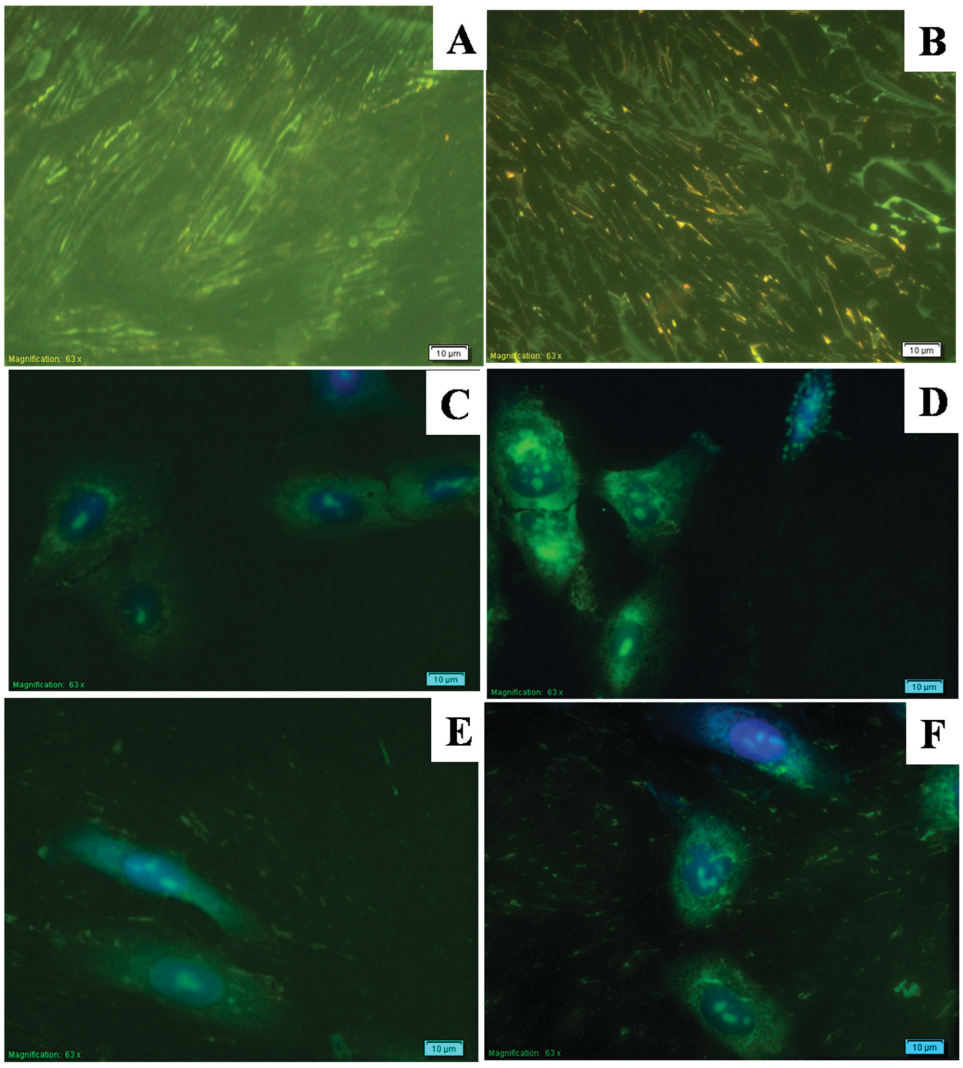


Figure 6. Fluorescence microscope images of A) poly(DTP-aryl-NH_2); B) poly(DTP-aryl-NH_2)/FA-modified ITO electrodes; HeLa cells on C) poly(DTP-aryl-NH_2); D) poly(DTP-aryl-NH_2)/FA and A549 cells on E) poly(DTP-aryl-NH_2), F) poly(DTP-aryl-NH_2)/FA surfaces (Proliferation behaviors of HeLa and A549 cell lines after 72 h incubation on the surfaces were performed via DAPI (blue) staining. Scale bar: 10 µm).

The poly(DTP-aryl-NH_2)/FA-modified surfaces were also tested for different cell line attachments. As shown in Figure S1 (Supporting Information), relative current responses were obtained for A549 cells. Also to test the influence of the surface modifications on proliferation properties of HeLa and A549 cell lines for 72 h, poly(DTP-aryl-NH_2)- and poly(DTP-aryl-NH_2)/FA-modified ITO surfaces were prepared. Because of fluorescence characteristics of polymer, the adhesion of cells on the modified surfaces was monitored via fluorescence microscopy techniques. Figure 6 shows that both of cell lines adhered on the FA-modified surfaces. And also same experiments were repeated after 48 h incubation of cells on poly(DTP-aryl-NH_2)- and poly(DTP-aryl-NH_2)/FA-modified ITO surfaces. After DAPI staining, the obtained images were given in Figure S2 (Supporting Information).

4. Conclusions

π-conjugated polymers have been taken a considerable place in the area of biological processes. Till now, different electroactive monomer with having a functional group (–NH_2, –COOH and/or –SH etc.) are the fundamental of conducting polymer based bioapplications such as biosensing and targeted drug delivery. Thereby, a novel cell sensing platform using poly(DTP-aryl-NH_2) was created by targeting with FA as the biorecognition element of

Table 1. Comparison of poly(DTP-aryl-NH_2)/FA system with some recent studies for the detection of cancer cells.

Detection technology	Support	Ligand	Cell line	Linear range [cells mL^{-1}]	References
Electrochemical	Au/ITO	Arg-Gly-Asp-Sertetrapeptide (RGDS)	HeLa	3.0×10^2 to 10^7	[33]
EIS	Gold disk electrode	Concanavalin A (Con A)	Bel-7404	10^3–10^5	[34]
EIS	Glassy carbon electrode	Aptamer against nucleolin	HeLa MDA-MB-231 K562 NIH3T3	10^3–10^6 cells mL^{-1} (HeLa)	[35]
Chronocoulometric	Disk gold electrode	Small molecule-linked DNA	MCF-7	10^2–10^6	[36]
Electrochemical	Glassy carbon electrode	FA	HeLa	10–10^5 cell	This study

the electroactive biofilm layer. After the optimization of different parameters such as electropolymerization, FA amount and incubation time for cell adhesion, a calibration curve was generated for HeLa cells, which are known as FR-rich cell line. In the characterization of biorecognition layer, both CV and EIS techniques were used and the step-by-step modification was in an accordance with each other. On the other hand, the selectivity of FA-modified biofilm was proved by using A549 (FR poor cell line) cells in both electrochemical and fluorescence microscopy studies. In brief, it can be claimed that poly(DTP-aryl-NH_2)/FA surface could be a good candidate in the electrochemical selective cell sensing applications.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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