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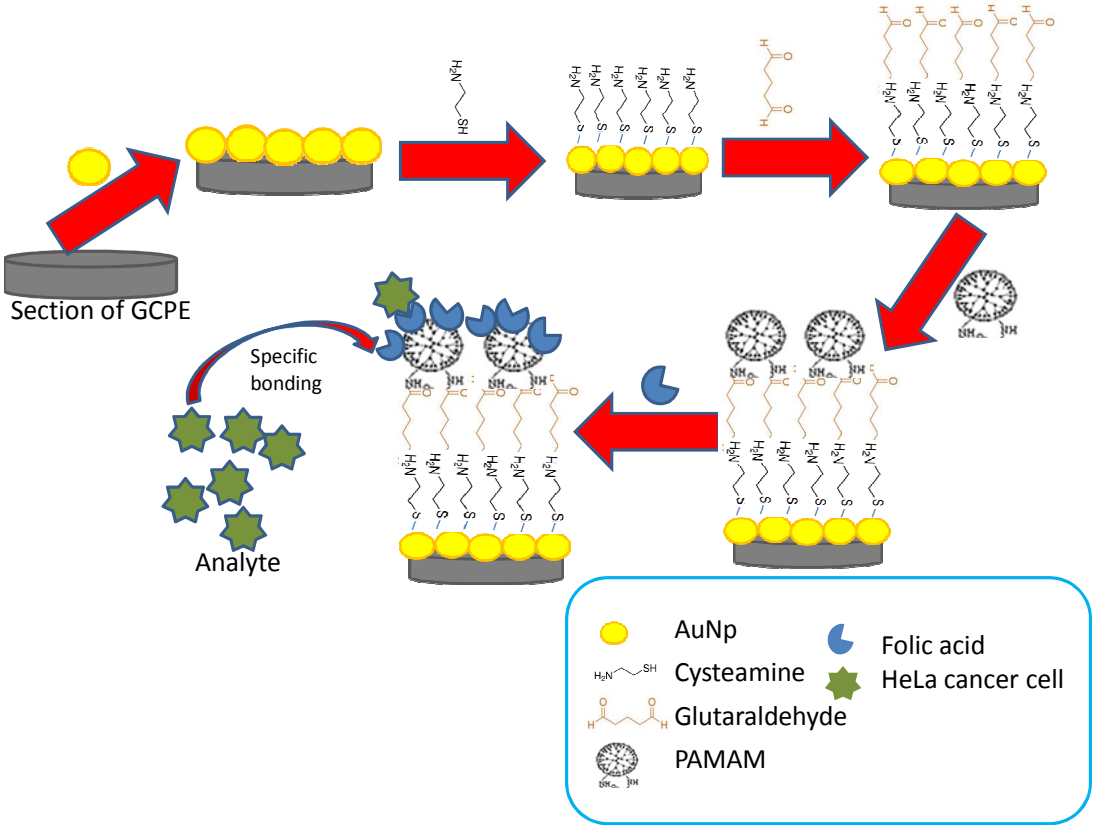

Electrochemical detection of HeLa cancer cells with GCPE/AuNp/Cys/Glu/PAMAM/FA cytosensor.

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ARTICLE

An Electrochemical Cytosensor based on PAMAM Modified Glassy Carbon Paste Electrode

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A novel electrochemical cytosensor was developed based on PAMAM and folic acid (FA) modified glassy carbon paste electrode (GCPE) where HeLa cells were utilized as model cancer cells. For this purpose, gold nanoparticles (AuNp), cysteamin (Cys), glutaraldehyde (Glu), PAMAM and FA was immobilized onto GCPE respectively. After the characterization of GCPE/AuNp/Cys/Glu/PAMAM/FA cytosensor and optimization of experimental parameters, analytical characteristics were examined. Linear range was found between 10^2 cells/mL and 10^6 cells/mL. LOD value was calculated as 100 cells/mL with RSD value of 1.55 % (for $5.0x10^4$ HeLa cells/mL (n=3)). The selectivity of GCPE/AuNp/Cys/Glu/PAMAM/FA cytosensor was tested by using folate negative cell line A549. Also cytosensor's performance was compared with similar previous studies. As a result, a selective, sensitive and practical system was developed.

Introduction

Increasing in life expectancy, urbanization and changes in environmental conditions make cancer a growing health problem around the world [1,2.] The early detection and proper treatment of metastases are very important in terms of saving cancer patients life [2,3]. Conventional detection methods, like cytologic testing, fluorescent imaging, magnetic resonance imaging, computerized tomography, X-ray radiography and ultrasound are complex techniques [4]. They are time consuming and often require special technical skills and instruments that mean high diagnostic costs. As a result, it is obligatory to develop a practical and convenient method for cancer cell detection. Electrochemical biosensors are sensitive, efficient and simple systems [5-7]. An electrochemical cytosensor is a kind of biosensor which can detect the cells by using electrochemical technics. Cytosensors were especially used for selective detection of cancer cells. Depending on the type of cancer cell, appropriate cytosensors have easily been prepared by

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modifiying biosensor's selective surface as being specific to particular cancer cell [4]. Various electrochemical approaches have been developed for monitoring cancer cell. For example, Wang et. al developed a simple electrochemical method which involved indium tin oxide (ITO) as the support electrode. They modified ITO electrodes with nanoparticles and tetrapeptides by using thioctic acid (TA), N'-ethylcarbodiimide hydro-chloride (EDC) and N-Hydroxysuccinimide (NHS). Differential puls voltammetry (DPV) was applied for the detection of HeLa cells. Developed cytosensor showed limit of detection value (LOD) of 300 cells/mL [8]. Additionally, Castillo et al. fabricated a new cytosensor for the selective detection of human cervical cancer cells. The group modified graphene electrode with a new conjugate of peptide, nanotubes and folic acid (FA). They obtained LOD of 250 cells/mL [9]. Yang et. al developed an electrochemical cytosensor based on carboxymethyl chitosan-functionalized graphene (CMC-G) modified glassy carbon electrode (GCE) for specific determination of the human leukemic cells (HL-60) and obtained LOD of 500 cells/mL [10]. Recently, a novel electrochemical lab-on-paper-cyto-device was fabricated for determination of specific K562 cancer cells by Su et. al. For this purpose, they used gold-paper working electrode and DPV. Developed cyto-device showed LOD value of 400 cells/mL [11]. Lastly, Coa et. al. developed an electrochemical cytosensor based on protein-inorganic hybrid nanoflowers modified GCE for sensitive determination of human colon cancer cells (CLC-1). They used electrochemical impedance spectroscopy (EIS) for detection of CLC-1 cells and obtained LOD of 40 cells/mL[12].

The alpha isoform folate receptor which is a kind of cancer associated antigen has been commonly over-expressed on the cancer cells while it has been highly limited on normal cells. For this reason, it can be used as a marker for cancer cells and can tightly bind with folate or folate-conjugated compounds like FA [13].

In this study, a new assay was developed and applied for detection of HeLa cells which were chosen as model cells. The assay was based on sequential immobilization of gold nanoparticle (AuNp), Cysteamine (Cys), Glutaraldehyde (Glu), PAMAM and FA onto glassy carbon paste electrode (GCPE). As far as we know GCPE was used for the first time as support electrode for fabrication of electrochemical cytosensor. Since its composite nature, GCPE provides practicality in preparation, modification and renewal of the electrode's surface. For this reason, this electrode was utilized in many applications by our group and also by other groups [14-19].

On the other hand, although there is one work that contains PAMAM dendrimer in the construction of breast cancer cytosensor[20], this material was used for the first time for determining HeLa cells.

Experimental

Reagents

Glassy carbon micron powder (GC, %99.95, 2-12 micron), mineral oil, chloroauric acid ($HAuCl_4$, 99%), (25%), PAMAM (25%) C_{12} dendrimer Generation 4 solution, MES monohydrate, sodium sulphate (Na_2SO_4, 99%), sodium nitrate ($NaNO_3$), potassium ferricyanide (III) ($K_3Fe(CN)_6$, 99.9%), potassium chloride (KCl), sodium chloride (NaCl), FA (97%), EDC, NHS, Glu, 2-(4-Amidinophenyl)-1*H*-indole-6-carboxamidine (DAPI) were obtained from Sigma-Aldrich. Hydrochloric acid (HCl, 37%), sodium hydroxide (NaOH), potassium dihydrogen phosphate (KH_2PO_4), sulphuric acid (H_2SO_4, 95-97%) were obtained from Merck and Cys was purchased from Fluka. Cell culture materials including Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum (FCS Gold) and penicillin/streptomycin (P/S, 100x) were purchased from Lonza (Basel, Switzerland). All the solutions were prepared in deionized water. All chemicals were analytical grade and were used without further purification.

Apparatus

Voltammetric measurements were carried out with a µ-AUTOLAB Type III electrochemical measurement system from Metrohm B.V. controlled by NOVA 1.10 software. The experiments were conducted in a 1.0 mL voltammetric cell, at room temperature (25 °C), using a three-electrode cell configuration. GCPE was used as the working electrode, an Ag/AgCl electrode (in saturated KCl) served as the reference electrode and a Pt wire served as the counter electrode. Finally, ITO electrodes that were used for fluorescence images were provided from Sigma-Aldrich.

Fabrication of the electrochemical cytosensor

GCPE was prepared with 80/20 (w/w)% glassy carbon microparticles/ mineral oil ratio by hand-mixing. Then a portion of the resulting paste was packed firmly into the Teflon body of electrode cavity (2.0 mm radius and 5 mm depth). The paste surface was smoothed on a weighing paper and rinsed carefully with double distilled water.

Firstly, AuNps were deposited onto GCPE by applying cyclic voltammetry (CV) with scanning potentials of 1.2 V to 0.3 V via solution containing 0.01 M Na_2SO_4, 0.01 M H_2SO_4, and 1.0 mM $HAu(III)Cl_4.3H_2O$. The CV procedure was repeated for five times at a scan rate of 60 mV/s. 0.1 M $NaNO_3$ solution was employed as a supporting electrolyte [21]. In this method, GCPE/AuNps electrode was obtained and characterized by using SEM. AuNps sizes were obtained as average 45 nm (Figure 1.). After the deposition of AuNp, electrode was washed with distilled water in order to remove impurities.

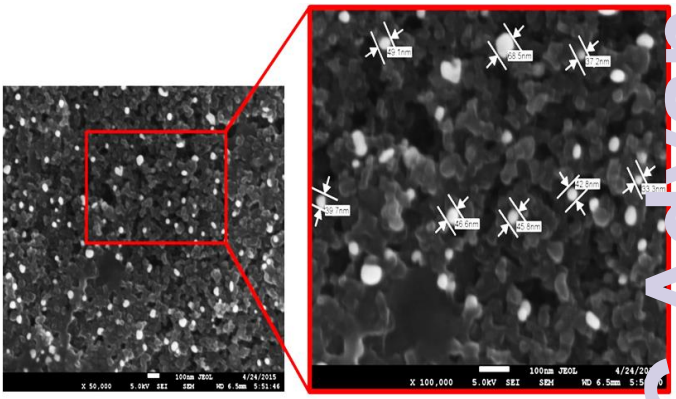
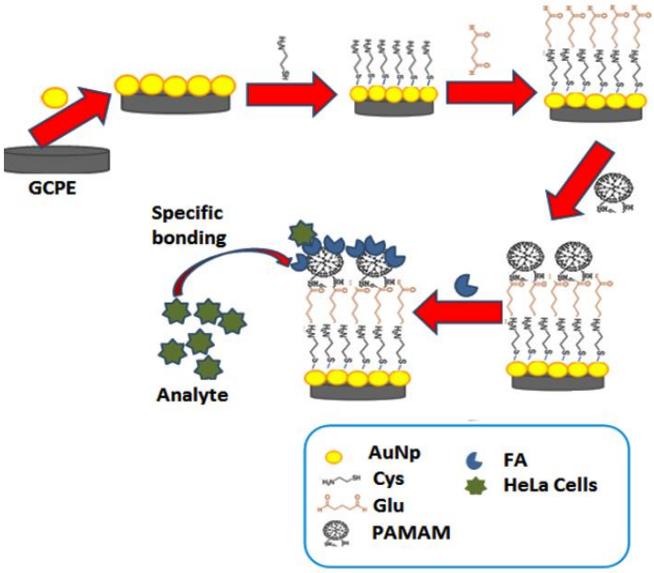


Figure 1. SEM İmages of the surface AuNps/GCPE.

Then, AuNp/GCPE was immersed into 100 mM Cys solution for 1 h. Electrode surface was cleaned with double distilled water. Resulting electrode was immersed into Glu solution (%5.0; in pH 7.0, 50 mM phosphate buffer system (PBS)) for croslinking for 30 min. Then the electrode surface was cleaned again with PBS. After the electrode was dried at room temperature, 35 µL PAMAM solution (%1.0; in pH 7.0, 50 mM PBS) was dropped onto the electrode surface. Then electrode was left to dry for 1 h. After that, modified electrode was cleaned with PBS again. At the last step of the modification procedure, the electrode surface was modified to be selective to folate receptor positive HeLa cells. For this purpose, 25 mM FA solution containing EDC (25 mM), NHS (25 mM), (in 25 mM MES solution) was prepared and then 35 µL of FA solution was dropped onto the electrode surface and left to dry for 30 min. After that, the electrode surface was washed with double distilled water and PBS and then HeLa specific cytosensor was obtained (Scheme 1.).



Scheme 1. Schematic illustration of step by step preparation of the GCPE/AuNp/Cys/Glu/Pamam/FA cytosensor.

Preparation of cell culture and cell capture

In order to achieve a selective biofilm layer, A549 (lung cancer cell) which is termed as folate receptor negative cell line and HeLa (ovarian cancer cell) cell which is termed as folate receptor positive cell line were used all through the experiments. Both cell types were grown in the DMEM containing 10% FCS and 1.0% P/S medium at 37°C in a humidified environment with 5.0% CO_2. After the enough confluence (about 80%) of cells, trypsinization was carried out with 1.0% trypsin to dissociate the adhered cells on the flask surface and they were counted and adjusted to known number of cells.

For immobilization procedure, the cells were separated from the medium by centrifugation at 3000 rpm for 5 min and then washed twice with PBS which contains 100 mM NaCl and 50 mM KH_2PO_4. Then the GCPE/AuNp/Cys/Glu/PAMAM/FA electrode was immersed into the cell suspensions for 60 min.

Fluorescence imaging

Fluorescence microscope (Olympus BX53F) equipped with a CCD camera (Olympus DP72) were used for the fluorescence images. Also, ITO electrodes were used instead of GCPE where the same preparation steps were applied onto them. For the control experiments, A549 cells as folate receptor negative cell line were used instead of HeLa cells. After incubation step with the cancer cells, the electrodes were washed gently for removal of excess reagents with PBS. Then the surface of the electrodes was treated with DAPI, for making cells nuclei visible under the fluorescence microscope. The cell number was quantified for each surface by using Image J (NIH) software. Student's T test using GraphPad Prism version 5.03 software (GraphPad Software, San Diego, CA) was utilized to compare relative cell numbers per surface area among different surfaces.

Results and discussion

Characterization of prepared cytosensor

GCPE/AuNp/Cys/Glu/PAMAM/FA cytosensor was characterized by using both fluorescence microscope and electrochemical techniques. As can be seen from the following sections, both methods demonstrate that, developed cytosensor was prepared as it is thought.

Fluorescence images: For obtaining fluorescence images, ITO electrodes were prepared as mentioned in the experimental part. Then necessary images were provided as shown in Figure 2. As can be seen from Figure 2.A, when there is no modification onto the electrode surface, no possible fluorescence image was obtained. At Figure 2.B, the surface of ITO was modified with AuNp/Cys/Glu/PAMAM and FA. Because of strong fluorescence properties of AuNps, a fluorescence signal was obtained despite of multi layers found on the electrode surface. Meanwhile, A549's (folate receptor negative cell line) DAPI stained nuclei provide fluorescence signal showing small numbers of these cells onto the electrode's surface (Figure 2.C). Figure 2.D, demonstrates complete coverage of developed electrode surface with HeLa cells. This fluorescence image was obtained when HeLa cells nuclei were treated with DAPI. From these results, it can be concluded that developed cytosensor is very selective to HeLa cells. Furthermore, cell density analyses were accomplished between FA receptor negative and positive cell lines to obtain more informative data from the cell images by using Image J Software. According to the data, the density for HeLa cells due to the selective capture on the ITO/AuNp/Cys/Glu/PAMAM/FA electrode was about 3886 cells/mm^2. On the other hand, this value was calculated as 400 cells/mm^2 for A549 cells. Therefore, It can be claimed that the selectivity of FA modified surface is significantly higher for HeLa cells compared to A549 cell lines, ($p<0.05$).

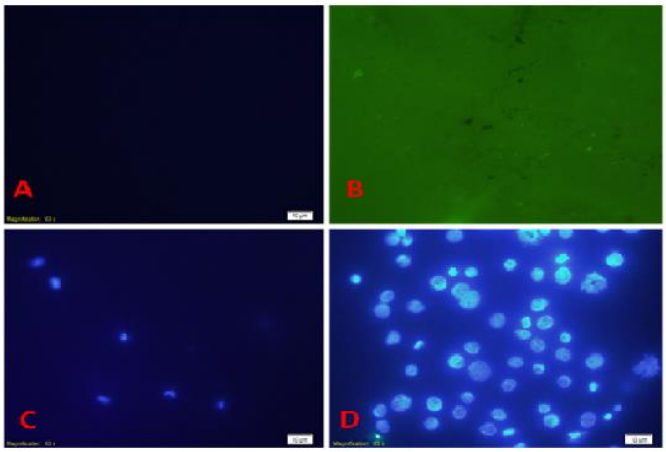


Figure 2. Fluorescence microscopy imagine of **A)** bare ITO electrode, **B)**FA/PAMAM/Glu/Cys/AuNp/ITO modified electrode, **C)** A549/FA/PAMAM/Glu/Cys/AuNp/ITO electrode and **D)** HeLa/FA/PAMAM/Glu/Cys/AuNp/ITO electrode. [Image B was obtained under the green filter for monitoring the fluorescence of AuNps and Image C and D were obtained under the blue filter for monitoring the DAPI treated samples (Scale of the each image was 10 µm).]

Electrochemical Characterization: The characterization of electrochemical cytosensor was monitored step by step using CV and EIS techniques. These electrochemical methods were used for layer by layer characterization of cytosensor (Figure 3.A,B). A bare GCPE showed normal redox waves when CV was applied to this electrode (Figure 3.A curve a). When the electrode was covered with AuNps, which can greatly enhance the charge transfer, the pair of redox peaks becomes more visible (Figure 3.A curve b), hence the diameter that represents the resistance on the electrode surface decreases (Figure 3.B, b). After cysteamine was bounded to AuNp layer from its –SH end, the current increases and resistance decreases (Figure 3.A,B curve c). This can be attributed to the electrostatic attraction between positively charged cysteamin and negatively charged $[Fe(CN)_6]^{3/4}$ [22-23]. For cross-linking procedure to obtain better chemical bonding, glutaraldehyde was used. Since it covers the active surface, the charge transfer became more difficult and the current decreases while surface resistance increases (Figure 3.A, B curve d). Then PAMAM was attached onto the surface. In this step the current increases, because of conductive structure of PAMAM dendrimers (Figure 3.A, B curve e) [20]. Lastly, when FA immobilized onto upper layer of modified electrode's surface, the charge transfer ability of the electrode was gradually reduced, accordingly exhibiting a decrease in the current response and increase in surface resistance (Figure 3.A, B curve f). This might be due to the inhibition of interfacial electron transfer when FA is attached onto the concuctive PAMAM dendrimer.

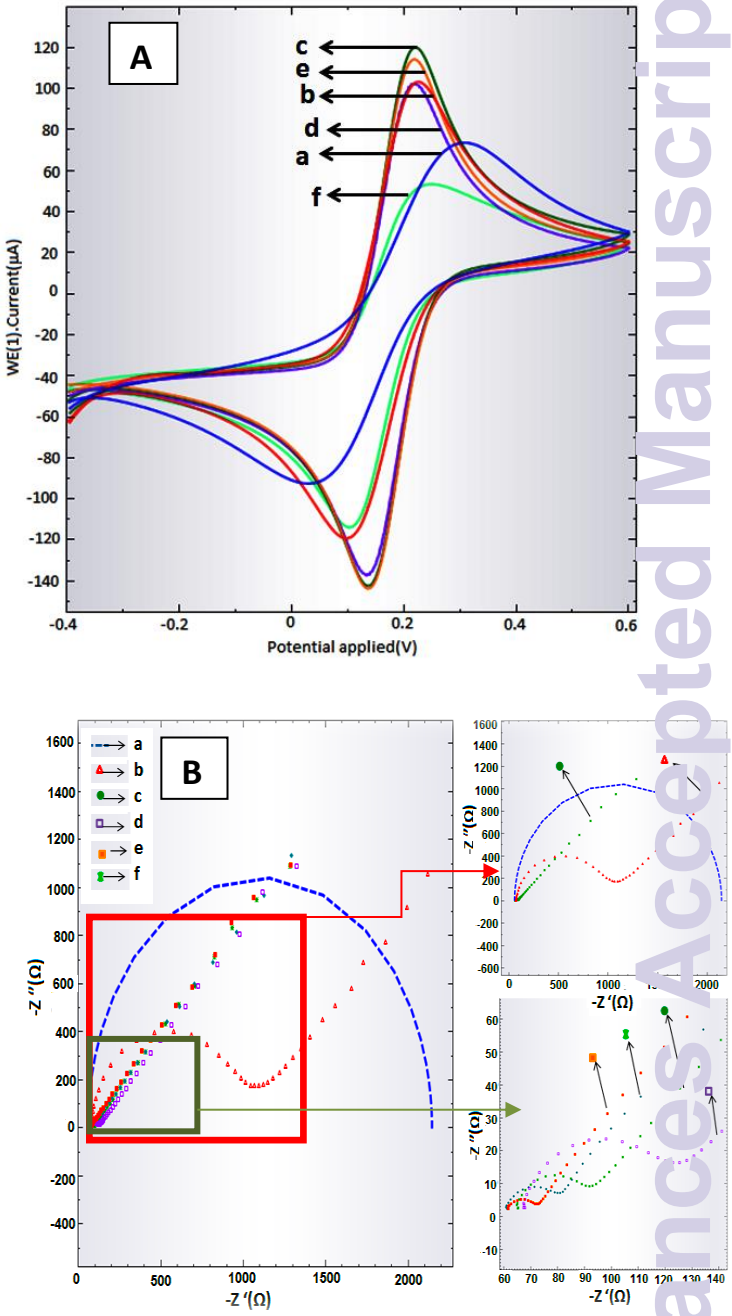


Figure 3. Characterization of GCPE/AuNp/Cys/Glu/Pamam/FA cytosensor, **A)** by using CV technique at 0.4 V to -0 .1 V working potential and scan rate 0.05 v/s **B)** by using EIS at 0.179 V, 10^{-2} Ω to 10^{5} Ω and the 0.005 amplitude step. a) plain GCPE; b) AuNp/GCPE; c) Cys//AuNp/GCPE; d) Glu/Cys//AuNp/GCPE; e) PAMAM/Glu/Cys/AuNp/GCPE; f) FA/PAMAM/Glu/Cys/AuNp/GCPE. For the characterization all electrochemical measurements were performed in 50 mM PBS pH 7.0 including 50 mM $[Fe(CN)_6]^{3/4}$.

Optimization of experimental parameters

Optimizations of experimental parameters are important in order to achieve high sensitivity and better reproducibility. The incubation time and FA amount were important parameters for capturing cells onto cytosensor surface. For this reason, optimizations of these two parameters were done.

Optimization of incubation time: 30, 60, 90 and 120 min were used as incubation times for the optimization of incubation time and obtained results are presented in Figure 4.A. From the Figure 4.A, it can be seen that the current value increases up to 60 min of incubation time and then a sharp decrease was obtained. From this result, it can be concluded that 30 min was not enough for immobilization of all the cells onto electrode surface while after 60 min, the accumulation of the cells onto each other rather than onto electrode surface might occur which results with current decrease. As a result, 60 min was used as optimum incubation time for further studies.

Optimization of FA amount: For the optimization of FA amount on the surface, the response signals of the cytosensor that were prepared by using 1.0 mM, 3.0 mM, 5.0 mM, 15 mM, 25 mM and 50 mM FA concentrations were investigated (Fig. 4.B). The current value increases up to 25 mM and then a decrease was obtained. This might be attributed to the coverage of the electrode surface up to 25 mM. After that concentration, because of the occurence of non-specific bonding of FA, a decrease was observed. Following these findings, 25 mM FA was selected as the optimum FA amount and used for further studies.

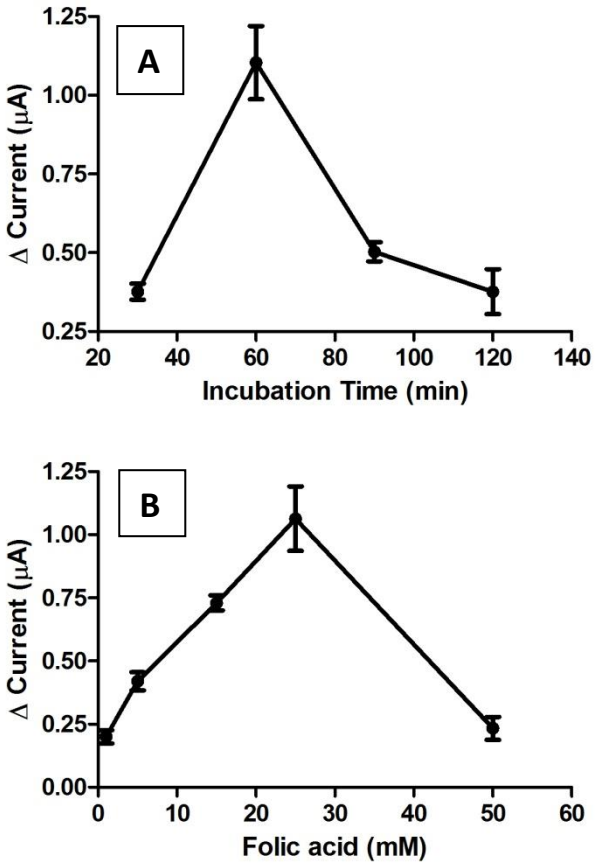
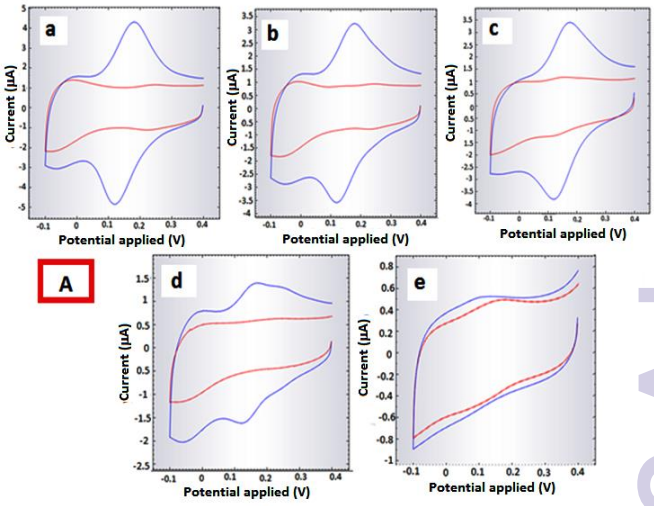


Figure 4. The effect of **A)** Different incubation times (30 min, 60 min, 90 min and 120 min); **B)** different amounts of FA (1.0 mM, 3.0 mM, 5.0 mM, 15 mM, 25 mM and 50 mM) on the current values for $5x10^4$ cells/mL at GCPE/AuNp/Cys/Glu/Pamam/FA cytosensor in 5 mM PBS pH 7.0, temperature of 25°C, working potential 0.4 V to -0.1 V and scan rate 0.05 v/s.



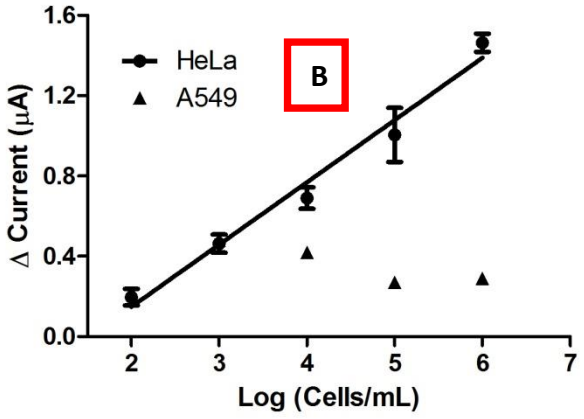


Figure 5. A) CV voltammograms for the linear range. (a) 10^6 , (b) 10^5, (c) 10^4, (d) 10^3 and (e) 10^2 cells/mL. **B)** Calibration graph was obtained for HeLa and A549 cells with GCPE/AuNp/Cys/Glu/Pamam/FA cytosensor under optimized parameters (25 mM FA, 60 min). These measurements were performed in 50 mM PBS pH 7.0, temperature of 25°C, working potential 0.4 V to -0 .1 V and scan rate 0.05 v/s.

Analytical characteristics

After the optimization of experimental parameters, analytical characteristics were examined. When concentration of HeLa cells for immobilization process increases, obtained CV shows a decrease in peak current indicating higher amounts of HeLa cells onto the cytosensor. The linear range for detection of HeLa cells was obtained between 10^2 - 10^6 cells/mL with the equation of y = 0.206x - 1.028 and correlation coefficient of R^2 = 0.9616 respectively. Voltammograms were presented at Fig 5.A. R.S.D value was calculated for $5.0x10^4$ HeLa cells/mL (n=3) and found as 1.55 %. LOD value was also obtained as 100 HeLa cells/mL by taking the smallest value in the calibration graph in terms of these cells

To evaluate the selectivity of the prepared cytosensor, CV measurements were performed to monitor current signal changes after incubating the cytosensors in A549 cells suspensions which are folate negative cells. As a result, a decreasing straight line with poor R^2 as 0.8388 was obtained. This result is in accordance with fluorescence image that was obtained with A549-cells (Figure 2.C). Once again it has been proved that developed approach is selective for HeLa cell detection.

GCPE/AuNp/Cys/Glu/Pamam/FA was compared with similar electrochemical HeLa cytosensors as presented in Table 1. As can be seen from the Table, presented work's analytical characteristics are in accessible limits and have better sensitivity than most of other works. Also the incubation time provides practicality to the developed system compared to some other works that were listed in Table 1. On the other hand usage of a composite electrode GCPE, also makes the developed protocol practical and economic.

Table 1. Comparison of performance of GCPE/AuNp/Cys/Glu/Pamam/FA cytosensor with previous HeLa cytosensors.

Electrode	LOD	Linear Range	Incubation time	References
GC/AuNpChit/AuNp/ integrinβ1/BSA/ integrin β1	$3.5x10^3$ cells/ mL	($1.0x10^4$ - $2.0x10^6$) cells/mL	24 h	24
GCE/AuNps/TH^+/ $PDCN_X$	500 cells/ mL	(8.0×10^2 - 2.0×10^7) cells/mL	120 min	25
ITO/Au/TA/RGDS	300 cells/ mL	($3x10^2$ - $1x10^7$) cells/mL	90 min	8
GE/MPA/(FcPEI/ $SWNT)_5$/FA	10 cells/ mL	(10 - 10^6) cells/mL	20 min	4
GE/Au/MUA/FA	6 cells/ mL	($6x10^0$ - $1x10^3$) cells/mL and ($1x10^3$ - $1x10^5$) cells/mL	Not presented	13
GCPE/AuNp/Cys/Glu/Pamam/FA	100 cells/ mL	(10^2 - 10^6) cells/mL	60 min	this work

GC; glassy carbon, GE; gold electrode , SWNT; single-walled carbon nanotube, Chit; chitosan, integrin β1; antibody, BSA; bovine serum albumin, TH^+; thionine, CN_x;carbon nanotube, $PDCN_x$; poly(diallydimethylammonium chloride-functionalized carbon nanotube, RGDS; Arg-Gly-Asp-Ser tetrapeptide, MPA; 3-mercaptopropionic acid, Fc; ferrocene, PEi; poly(ethylene imine), MUA; 11- mercaptoundecanoic acid.

Conclusions

A new electrochemical cytosensor has been developed where HeLa cells were utilized as model cancer cells. As far as we know this is the first study where GCPE and PAMAM were used together for HeLa cell detection. Obtained LOD value and linear range were compared with similar electrochemical cytosensors. As a result, developed system's values were found in acceptable limits proving that practical and sensitive electrochemical cytosensor was

developed for HeLa cell detection. Also the selectivity of developed approach was tested by using A549 cells instead of HeLa cells. As can clearly be seen from characterization studies and also from calibration graphs, GCPE/AuNp/Cys/Glu/Pamam/FA is very selective for HeLa cell detection. We believe that usage of composite natured electrode, GCPE, brings practicality to this area. Combination of GCPE together with PAMAM results with selective and sensitive cytosensor which has potential for effective cancer cell detection in the future.

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