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A Novel Capacitance Electrochemical Method for Determination of Trace amounts of t-NMP22 by Adsorptive FFT Continuous Fast Cyclic Voltammetry at Au-Pt Ultramicroelectrode in a Flowing Solution

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A novel technique has been developed for the analysis of trace amount of Nuclear Matrix Protein 22 (t-NMP22) using adsorptive fast Fourier transform fast cyclic voltammetry (AFFTFCV) in a flow system. The measurements involved continuously applying a cleaning, stripping potential steps together with a potential ramp onto a gold platinum (Au/Pt) ultramicro disk electrode in a flow system, and measuring a signal based on changes in the electrode capacitance as a result of the adsorption and/or desorption of t-NMP22. The effects of different factors including eluent pH, potential scan rate, and accumulation potential and time were also evaluated. The response was determined to be linear from 5 to 100 pg mL^{-1} (r^2 = 0.996) and the detection limit was determined to be 1.1 pg mL^{-1}. The accuracy, precision and sensitivity of the technique make it a promising candidate for the measurement of t-NMP22.

Keywords: t-NMP22, Fast Fourier transformation, Cyclic voltammetry, Capacitance sensor, Flow injection analysis, Ultramicroelectrode

1. INTRODUCTION

After prostate cancer, bladder cancer (BC) is classified as one of the most frequent genitourinary malignancy [1]. For its medication, the main challenge in such diseases is difficulty in diagnostic of the disease at early stages [2]. On this purpose, cystoscopy method is considered as a gold standard, while it suffers from draw backs such as being invasive, expensive, and inapplicable for several specific patients [3, 4]. Therefore, in today's developed world having for a novel diagnostic method for such highly spreading diseases has triggered intense attention.

High concentration of Nuclear Matrix Protein 22 (NMP22) are considered as a sign of tumor growth in cancer patients [4, 5]. Thus, its recognition can provide a simple sensitive direct way to identify the BC patients [6]. Recent publications report that that electrochemical immunoassays such as enzyme-linked immunosorbents [1] and urine cytology can be used in the analysis of biomolecules. Yet the complexity of these methods, their low sensitivity and high times required are among the disadvantages of the techniques [7]. Thus, highly sensitive and precise detection of NMP22 is significant obsession and the area has the value to be explored. It was reported that part of NMP22 (truncated t-NMP22) can be used as a marker for BC [8].

As a possible solution, electrochemical biosensors could be applied for quantitative and qualitative detection of bio-analytes as they have advantages including cheaper and easier analysis [9]. At this stage, electroanalytical techniques including DPV (differential pulse voltammetry), CV (cyclic voltammetry), and chronoamperometry could be qualified choices [10-16]. Determinative parameters here could be the target nature, the working electrode type and its probable modifiers [17].

Considering the high importance of solid working electrode in the electroanalytical measurements, Ultramicroelectrodes (UMEs) have a unique position, due to their considerable surface area, catalytic effects and biocompatibility [18-20]. Thus, biomolecules as analytes are possible to be immobilized on them using electrostatic force. Among various types of UMEs, Au and Pt electrodes have displayed considerable properties such as nontoxicity, adequate biocompatibility and high chemical stability [21-23].

This work describes a novel detection method for the trace analysis of t-NMP22 through the application of an AFFTCFCV approach [24-33]. The analytical method can provide sensitive measurements in combination of advantages of using UME. Hence, the electrochemical signal would not be affected by the random noises. Yet AFFTCFCV can distinguish and filter the voltammetric signal from the background noise in the frequency domain based on a fast Fourier Transformation (DFFT) approach, which makes it suitable for analyses of compounds like t-NMP22.

2. EXPERIMENTAL SECTION

2.1. Chemicals

The first 300 amino acids of NMP22 is named truncated (t-NMP22), which was selected based on "In silico" studies and reported data [8], as following sequence;

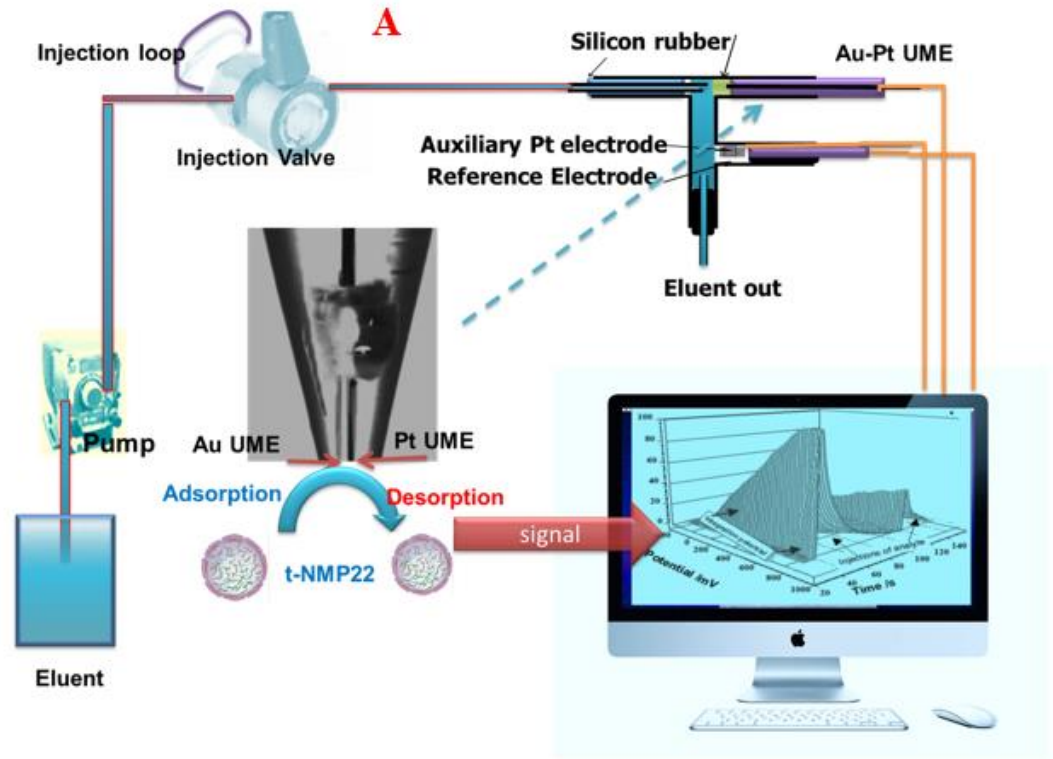
MTLHATRGAALLSWVNSLHVADPVEAVLQLQDCSIFIKIIDRIHGTEEGQQILKQPVSE
RLDFVCSFLQKNRKHPSSPECLVSAQKVLEGSELELAKMTMLLLYHSTMSSKSPRDWEQFEY
KIQAELAVILKFVLDHEDGLNLNEDLENFLQKAPVPSTCSSTFPEELSPPSHQAKREIRFLELQK
VASSSSGNNFLSGSPASPMGDILQTPQFQMRRLKKQLADERSNRDELELELAENRKLLTEKDA
QIAMMQQRIDRLALLNEKQAASPLEPKELEELRDKNESLTMRLHETLKQCQ

All other employed chemical materials were at analytical grade and purchased from Merck.

2.2. Instrumental setup

Flow injection analysis (FIAs) were performed using a Supelco Rheodyne 5020 four–way injection valve with a 80 µL injection loop, a peristaltic pump (8 rollers) and a four–way electrochemical cell (Figure 1A). The electrochemical cell has three-electrodes; a Pt-Au UMEs working electrode, a Pt wire as the counter electrode (1 cm length and 1 mm in diameter); and an Ag/AgCl reference electrode. The dead volume of the cell was 200 µL). The eluent was injected into the loop using a plastic syringe.

The 50 µm in radius Pt-Au UMEs were prepared by sealing Pt and Au micro-wires (Good fellow Metals Ltd.) into a soft glass capillary by heating and then cutting the assembly to obtain disks UMEs. Next silver epoxy (Johnson Matthey Ltd., UK) was used to connect the micro wires to a copper wire to establish electrical contact (Fig. 1A). The surface of the electrode was polished using extra-fine carbonado-paper for 3 minutes and then with 0.3 µm alumina for 10 minutes.



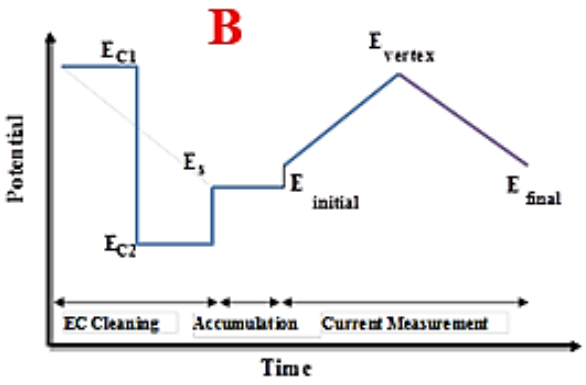


Figure 1. A) The plot of the setup for FFTFCV and FIAs, **B)** The potential waveform applied in the AFFTCFCV technique.

FFTCFCV runs were performed by a homemade potentiostat and an electrochemical software, which controlled a 12-bit PCL-818H analog to digital board ((Advantech Co.) [28, 29, 34-37]. By means of this, the FFTCFCV waveform was applied and acquiring the data current. The data acquisition was performed using a home-made software developed in Delphi 6.0 environment. In a flow analysis, the potential waveform for a limited time window was repeatedly applied to the electrochemical cell. The diagram of the potential waveform is shown in Figure 1B. As shown, the waveform consists of potential pulses, E_{c1} and E_{c2} (used for electrochemical cleaning of the electrode surface, and E_s, used for adsorption of the protein (which follows with a potential scan).

3. RESULTS AND DISCUSSION

Figure 2 demonstrates three dimensional graph of AFFTCFCV plot progression measurements for 100 s (the time axis a number subtracted cyclic voltammograms were recorded during the FIA runs. The applied potentials ranged from -200 to 800 mV and the scan rate was 40000 mV/s.

In the measurement, the injected volume was 200 µL of 0.2 and 0.1 µg mL^{-1} of t-NMP22 solutions in PBS (0.1 M, pH=7.4) into an eluent solution (0.1 M PBS pH=7.4). The voltammograms were subtracted to eliminate the background current, and minimizing the noises according to fowling equation:

$$\Delta i_n(E) = i_n(E) - i_{rf}(E) \qquad (1)$$

where $\Delta i_n(E)$ represents the subtracted current, $i_n(E)$ current sampled at potential E during the n^{th} potential scan , $i_{rf}(E)$ is the reference current (the first recorded voltammogram of experiment.).

Even though this current calculation seems to be simple, but it can increase the sensitivity of the detection.

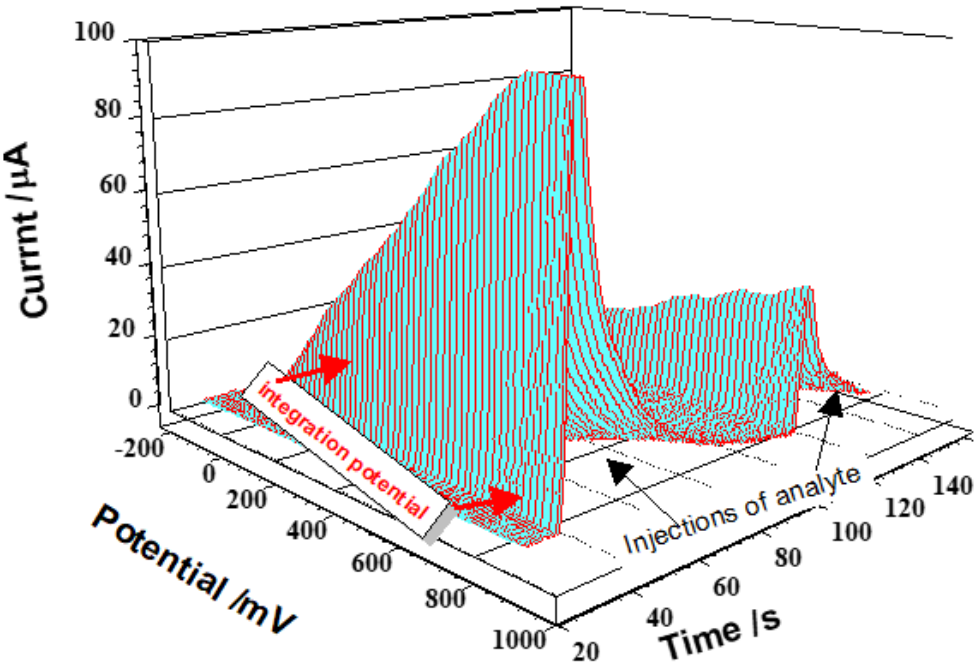


Figure 2. Subtracted AFFTCCV voltammograms of the Au-Pt UME in the potential range of -200 to 800 mV at 40000 mV s^{-1} in absent and presence of 200 µL of 0.2 and 0.1 µg mL^{-1} t-NMP 22 in 0.1 M PBS pH=7.4

Here, it is shown that with blank samples not containing t-NMP22, the voltammograms do not contain there is considerable peak currents, yet the curves show current changes all over the potential. In fact, when t-NMP22 exposes to the Au-Pt-solution interface, it adsorbs on the electrode surface, and changes the double layer structure, as well as the value of its capacitance. Moreover, the value of the current changes increase with potential during potential scan, and hence an intense changes in the shape of voltammograms take place. The sensitivity of such measurement in this mode is very advantageous for microfluidic analysis.

In AFFTCFCV experiments, the current was only recorded while the potential was scanned. Then, the data processing operation was carried out simultaneously with data acquisition during the measurement.

The response calculation is based on the obtaining the capacitance change over selected potential of the cyclic voltammogram. For this calculation at first, the net current was integrated digitally of over the scanned potential range by calculation to obtain total charge:

$$Q_t = \frac{1}{\nu}\int_{E_1}^{E_2} i_{(E)} dE \qquad (2)$$

In fact, by sampling current in equal time intervals voltammogram were numerically recorded. So the value of Q is

$$Q(n\Delta t) = \frac{\Delta E}{\nu}\left(\sum_{e1}^{e2} i(n, j)\right) \qquad (3)$$

In order to remove the background current $Q(n\Delta t) = Q(kt) - Q(n_{rf})$ where n is the scan number, v is the scan rate. Δt expresses the time between subsequent scans, e_1 and e_2 is the potential range for integration, $i(n,j)$ represents the recorded current during the k^{th} scan, ΔE is the potential difference of two successive points at the voltammogram. In the computer program, the algorithm was used for the current integration was,

$$\Delta Q(n\Delta t) = \frac{\Delta E}{\nu}\left(\sum_{e1}^{e2} i(n, j) - \sum_{e1}^{e2} i(n_{rf}, j)\right) \qquad (4)$$

Where $i(n_{rf},j)$ is the reference current, which recorded at voltammogram (i.e. before injecting the analyte).

The capacitance change ΔCp (the analyte response) is,

$$\Delta Cp = \Delta Q / E \qquad (5)$$

The calculated electrode response is shown in Figure 3. To evaluate the maximum effect of the adsorbed target species on ΔCp, during the measurements, the rate of potential scan should be very high r(e.g. >20000 mV s^{-1}). Also, another of the important aspects of the measurements, which could improve the sensitivity, is using of a DFFT digital filtration, in which he existing possible high frequency noises is removed.

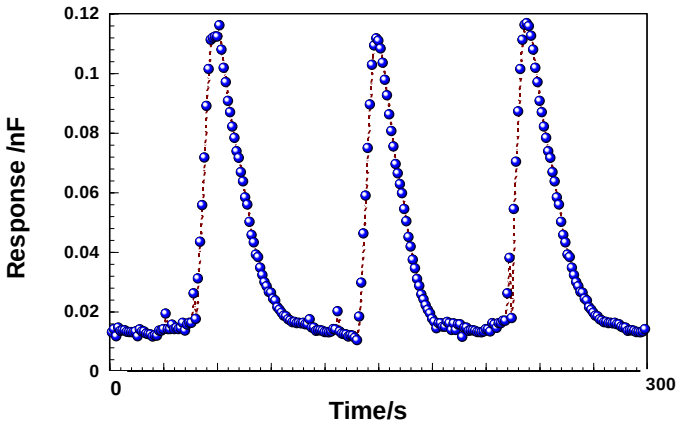


Figure 3. Response ΔCp of Au-Pt UME to the consecutive injection of 200 µL of t-NMP22 standard solution. The integration rang was from 100 to 800 mV. The experimental condition was as shown in Fig. 2.

In these determinations, it is well known that, the nature of the adsorption bond at the protein/ electrode interface is related to the surface coverage. Hence, the electrode response the AFFTCFCV method seems to correlate with the surface coverage and the analyte the sensitivity of the signal. Adsorption of most protein and organic compounds at Pt or Au electrodes can be compared with the complex formation reactions. For visualization of the adsorption process of the protein, the π orbitals of the adsorbed specie may undergo a type of bound platinum d-orbitals.

Due to this fact that adsorption of the protein is the key mater in the measurement, , the response is expected to be influenced by kinetic factors like adsorption, mass transport rate and the electrochemical properties of the adsorbed protein on Au-Pt surface. However, to a degree, the selection of the concentration and type of the eluent can changes the rate of analyte adsorption. Therefore, for a measurement condition, in order to achieve maximum sensitivity of the detector, the influence of operational factors like flow rate, potential scan rate, accumulation potential and time o should be optimized.

3.1. Optimizing the experimental parameters

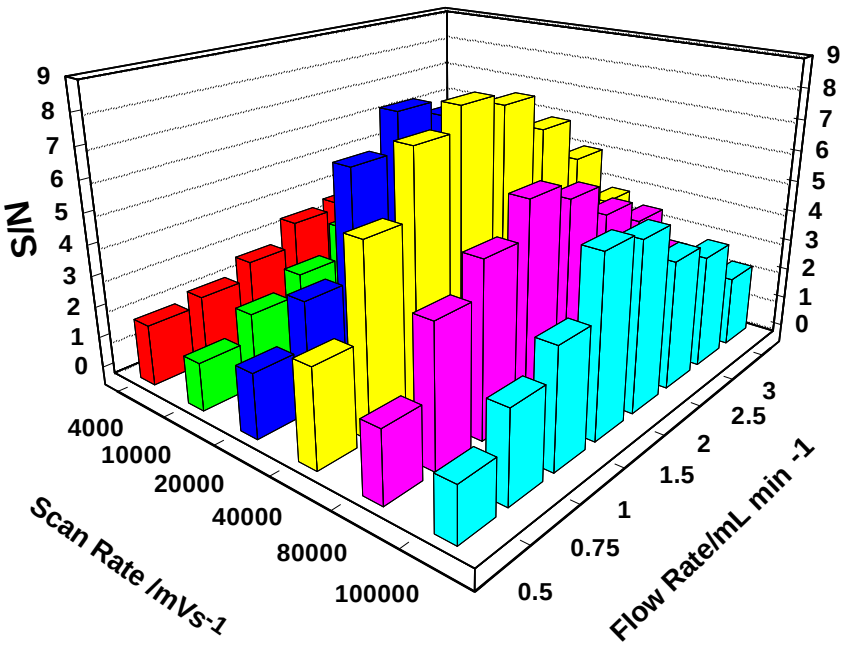


Figure 4. The influence of the scan rate and eluent flow rate on the signal to noise ratio of Au-Pt UME electrode to injection of 200 µL of 0.2 µg ml^{-1} NMP22 in 0.1 M PBS at pH=7.4

In any voltammetric measurement, the sensitivity is mainly a function of the rate of the potential excitation. The highest possible response in such cases t could be due to the limitation of the rate of the electrochemical processes by electrode-solution interface for analyte (e.g. kinetic factors of the adsorption process of the protein), and instrumental conditions. Likewise, in a flow analysis, the effects

of other factors must be taken into consideration such as; speed of the speed of data acquisition (the A/D board), parameters of adsorption kinetics of the protein, and the eluent flow rate, which control the retention time window of the sample zone in the solution should be considered.

The results obtained for injecting 0.2 µg ml^{-1} solutions of t-NMP22 are presented in Figure 4. Where, the effects of the potential scan rate, from 4000 to 100000 mV/s, and the eluent flow rate (from 0.5 to 3.5 mL min^{-1}) on the sensitivity of the t-NMP22 response was tested. Clearly the maximum sensitivity (S/N) could be obtained at a scan rate of 40000 mV/s and a 2 mL min^{-1} eluent flow rate. At scan rates over 40000 mV/s, the sensitivity diminished due to the restriction of the adsorption rate of the protein by electrode surface. Likewise, when the flow rates are higher over 2 mL/min, the retention time of the analyte is not enough for the adsorption to take place on the electrode surface. Hence due to the lower data point, S/N decreases. Hence the activity of the protein considerably depends on pH, therefore the change of S/N of Au-Pt UME injection of 200 µL of 0.2µg mL^{-1} t-NMP22 in 0.1 M PBS at was considered in several pH values. The charge response was evaluated in the pH window of 5.0–9.0. The results indicate that the best S/N is at pH of 7.4.

Figure 5 shows, the influences of accumulation time and potential on value of S/N. In AFFTCFCV method, similar to stripping voltammetric methods, the sensitivity of detection is changes by analyte pre-concentration condition. It is well known that, organic compounds or protein adsorb on electrodes at suitable potentials (here is E_s, see Figure 1B). Consequently, the extent of pre-concentration time can be affected by the accumulation potential.

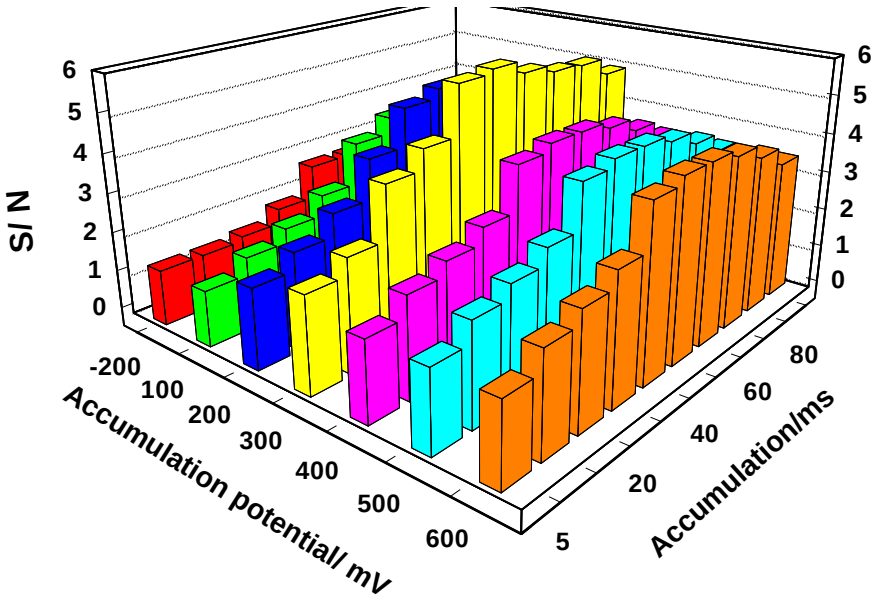


Figure 5. The influences of accumulation potential and time on the S/N of the Au-Pt UME to injecting 200 µL of 0.2µg mL^{-1} t-NMP22 in PBS (0.1 M, pH7.4) in an integration rang extending from 0 to 800 mV and accumulation time, 5-100ms.

To optimize the performance of AFFTCFCV measurement in the flow injection analysis, the influences of accumulation time and potential on the response of the electrode to injecting 200 µL of 0.2

µg mL^{-1} t-NMP 22 solutions in 0.1 M PBS (pH=7.4) were investigated (The ranges of accumulation time and potential were 5-100 ms and 0 to 800 mV) . Naturally the higher accumulation times should be preferred, yet longer accumulation times can also decrease the number of data points for plotting the voltammograms (A minimum of 10 data points are required for plotting the analyte peak)., Consequently, the best accumulation time and potential were determined to be 200 ms and 300 mV respectively.

3.2. Calibration curve and characterization of the UMEs

To find the sensitive and the quantitative range of the t-NMP22 immunoassay, asset of different concentrations of the analyte from 0.1 to 500 pg mL^{-1} in 0.1 M PBS solution (pH 7.4) was prepared. Under optimal conditions. The results obtained for different of t-NMP22 standard solutions, are shown in Figure 6. Where, the potential scan rate 4 V/s and the eluent flow rate was 2 mL/min, the results shown in this figure represent the integrated signal (from 0 to 800 mV), ΔCp, for 3 to 5 consecutive injection of the standard solution of t-NMP22. It shows typical calibration curve (response versa concentration) [38,39]. Moreover , the response of the detector, in one injection, about 10s, reaches 90% of the steady state, which indicates a fast response [40]. The linearity was evaluated by linear regression analysis, which calculated by the least square regression method. The regression equation is $\Delta Cp(\mu F)=0.0033C(pg/mL)+0.002$ with $R^2= 0.996$. The detection limit, estimated based on signal to noise ratio (S/N=3), was found to be 1.1 pg mL^{-1}.

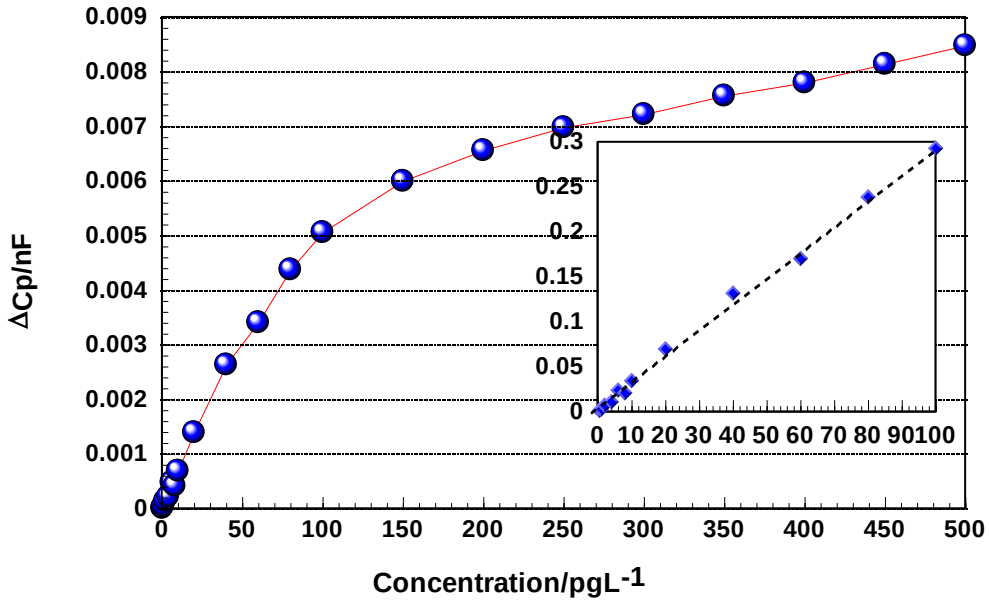


Figure 6. Response of the electrode to the t-NMP22 concentrations: 0.2 to 500 pg mL^{-1} in buffer solution at pH 7.4, in the potential range of 0 to 800 mV; the inset graph of this figure shows linear range. Integration potential range for the admittance is 0 to 800 mV

In evaluation, the performances of the sensor is compared with some of the best previously reported cholesterol biosensors based on the utilization of different materials as the working electrode and different detection techniques (Table 1) and it was confirmed that the presented Au-Pt UME with ACFFTCFCV exhibited an excellent and reproducible sensitivity.

Table 1. A comparison on the detection limits of formerly reported biosensor with the developed UME

Method	**Limit of detection**	**Ref.**
Au@Pd/Agyolk-bimetallic shell nanoparticles and amination graphene	3.3 pg/mL	[1]
Gold nanoparticles enhanced electrochemiluminescence of graphite	10.0 pg/mL	[3]
Co(III) Phthlocyanine/Fe_3O_4/Au Collide Coimmobilized Electrode	0.5 ng/mL	[41]
Au-Pt UME	1.1 pg/mL	This work

4. CONCLUSION

A very sensitive t-NMP22 detection method was developed based on the application of an Au-Pt UME electrode. In assessment, it was confirmed that the presented Au-Pt UME combined with AFFTCFCV method demonstrates excellent reproducible responses for the analysis of t-NMP22 . The response of the electrode was the capacitance change during adsorption of the analyte, which was calculated by numerical method and integration of the current in a selected potential range. The results showed sensitivity of the detector retained 94.8% of initial sensitivity up to 120 days, and then gradually decreases afterwards due to changes in electrode surface. This selective method opens a new area for development of portable microfluidic detector for t-NMP22.

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