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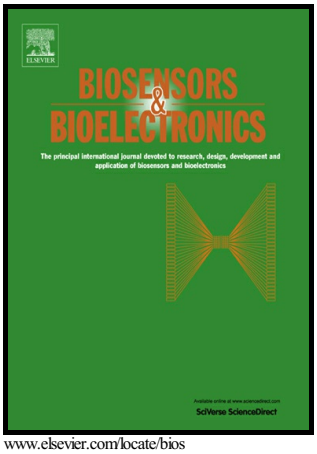


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# Off surface matrix based on-chip electrochemical biosensor platform for protein biomarker detection in undiluted serum

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**Abstract**

The manuscript describes a concept of using off surface matrix modified with capturing biomolecule for on-chip electrochemical biosensing. 3D matrix made by laser engraving of polymethyl methacrylate (PMMA) sheet as off surface matrix was integrated in very close vicinity of the electrode surface. Laser engraving and holes in PMMA along with spacing from surface provide fluidic channel and incubation chamber. Covalent binding of capturing biomolecule (anti-TNF-α antibody) on off-surface matrix was achieved via azide group activity of 4-fluoro-3-nitro-azidobenzene (FNAB), which act as cross-linker and further covalently binds to anti-TNF-α antibody via thermal reaction. Anti-TNF-α/FNAB/PMMA matrix was then integrated over comb structured gold electrode (CSGM) array based sensor chip. Separate surface modification followed by integration of sensor helped to prevent the sensor chip surface from fouling during functionalization. Nonspecific binding was prevented using starting block T20 (PBS). Results for estimating protein biomarker (TNF-α) in undiluted serum using Anti-TNF-α/FNAB/PMMA-CSGM reveal that system can detect TNF-α in 100 pg/ml to 100 ng/ml range with high sensitivity of 119 nA/(ng/ml) , with negligible interference from serum proteins and other cytokines. Thus, use of off surface matrix may provide the opportunity to electrochemically sense biomarkers sensitively to ng/ml range with negligible nonspecific binding and false signal in undiluted serum.

Keywords: electrochemical sensor; off-surface 3D matrix; PMMA, FNAB; TNF-α; serum

## 1. Introduction

Ever increasing requirement for sensitive and specific detection of desired biomolecules has necessitated the need for development of more advanced systems with newer methods for biomolecular binding and detection to achieve faster, real-time and reliable medical diagnosis.(Arya et al. 2013; Arya et al. 2012; Bosnjakovic et al. 2012; Liu et al. 2015; Mazloum-Ardakani and Hosseinzadeh 2015; Sun et al. 2013). Till date for real sample testing optical ELISA has shown great success and is most commonly used method of screening target proteins in real samples (Bettazzi et al. 2012). However, due to its limitation in sensitivity, high cost of instrumentation and non-portability, electrochemical ELISA is desirable. Detection using electrochemical techniques has shown huge promise owing to simpler system and ability of transduce signal directly from biomolecular interaction into electric signal (Fei et al. 2015; Gan et al. 2011b; Li et al. 2015; Mazloum-Ardakani et al. 2015; Pan et al. ; Sadik et al. 2009; Wei et al. 2016). However, in development of such advanced sensors, stability, nonfouling, nonspecific response and false signal in real sample is still posing the major challenge in their wider use. Usually electrochemical ELISA involves the binding of biomolecule and electrochemical signal recognition on same sensor surface (Kokkinos et al. ; Pan et al. ; Zhang et al. 2016), however binding on biomolecule on sensor surface may result in degradation of its electrochemical properties and application of input electrochemical signal also can affect sensing layer.

Binding of recognition molecule on biosensor surface and the biosensor surface activity are well known to play a crucial role in biosensor development (Solanki et al. 2007). However, binding of recognition molecule generally involve the modification of electrochemical sensor surface. Such modification may compromise the electrochemical property of sensor, which in turn cause lower sensitivity, sensor response variation and false signals especially for real sample analysis. In one such

approach, Gan et al, developed DNA derived magnetic nano-chain probes modified chip surface based sandwich ELISA. Study revealed the requirement of 50 to 100 times dilution of serum samples to minimize matrix effect (Gan et al. 2011a). In other study to measure thyroid stimulating hormones using electrochemical ELISA approach, Wang et al, modified surface between sensor electrodes, however process involved in such modification may also result in sensor surface modification, thus causing passivation of active surface and variation in results (Wang et al. 2014). Further, the patents from Andcare, Inc., Durham, N.C (US005391272A) (O'Daly et al. 1995); Abbott Point of Care Inc., (US) (US 2013/0224775 Al) (Davis et al. 2013) and Centre National de la Recherche Scientifique, Paris (US 7045364 B2) (Limoges et al. 2006) for electrochemical biosensor platforms also require electrode surface modification for electrochemical detection. In other approach, Yaku et al, from Osaka: Panasonic Corporation, Osaka (JP) described bio assay in liquid phase at different places on electrode (Yaku and sugihara 2009). As there is no matrix involved for recognition molecule binding, there are higher chances of detecting large background signal, thus making this approach suitable mainly for enzymatic sensors. Furthermore, few magnetic beads and off chip based electrochemical ELISA has been reported (DiagnoSwiss ; Liu et al. 2013), however, miniaturization and automation of such system is not easy and involve manual handling for testing. Thus, to improve the characteristics of electrochemical biosensor there exists a scope to develop a system for efficient, sensitive, cost effective, and specific detection of desired target protein in real samples.

To avoid the mentioned issues and to improve signal sensitivity with low background noise, present work describes the concept of integrated sensor chip, where off chip matrix is employed for on-chip electrochemical biosensing. The new platform fabrication process involves the selection of matrix followed by its off-site modification with sensing molecule and its integration near electrochemical sensor surface. In this work, concept has been established using antibody modified

off-surface 3D matrix of PMMA for on-chip electrochemical biosensing in estimation of protein marker (Tnf-α in present case) in complex undiluted serum sample. Off surface 3D matrix made by engraving polymethyl methacrylate (PMMA) polymer sheet has been modified with capturing biomolecule in separate location and then integrated on top of electrochemical sensor for sensitive and interference free on-chip electrochemical biosensing in undiluted serum samples. Such a biosensor platform has potential to replace most commonly used optical ELISA based detection platforms.

## 2. Materials and Methods

### *2.1 Chemicals*

TNF-α, recombinant peptides, primary antibodies, biotin-conjugated secondary antibodies were procured from Biolegend (USA); streptavidin-conjugated alkaline phosphate (ALP) solution from Mabtech AB; 4-Aminophenyl phosphate monosodium salt (4APP) from Santa Cruz Biotechnology (USA); Starting Block PBST20 and starting block TBST20 were from fisher scientific, FemtoTBS is from gbioscience. FNAB was from Setareh Biotech, LLC. Gold nanoparticles from AC Diagnostics G-010-P-2 10 nm, OD 1 (520 nm abs max); nanocomposix G-50-100, 50 nm 1 OD (535 nm abs max). Other analytical grade chemicals were utilized as received.



### *2.2 Gold sensor chips development*

Gold sensor chips with comb structure were developed via regular micro-fabrication methods on silicon with silicon oxide layer wafers as mentioned in previous report (Pui et al. 2013). For comb design, 5 μm wide and 3200 μm long electrode bands with 25 μm spacing, distributed over 5500 μm

length were fabricated and utilized. The developed comb shaped gold sensor chips were washed with ethyl alcohol, acetone and water, and then cleaned using UV-ozone for 30 min before use.

*2.3 Formation of PMMA chip and its modification*

To make off-surface matrix 11 mm x 12 mm PMMA chip of 3 mm thickness was laser engraved (Universal Laser Systems, Arizona, USA) with Power 100, Speed 6 in area of 6.5 mm x 7.5 mm to cover active area on sensor. Such engraving result in the formation of cuboidal pillars (Fig 1a: black dots on white background), thus making 3D structures. Two holes were also made at diagonal end of engraving to create fluidic channel and incubation chamber. Prior to FNAB modification, the PMMA matrix was washed and sonicated in IPA for 10 min and then washed using water jet before drying using nitrogen blow. For FNAB modification, back end of holes in chip were blocked and 2 mg of FNAB in 50 µl of methanol was dropped on 3D matrix and allowed to dry in dark (Fig 1b). After drying, yellow colored chips were exposed to UV irradiation system from Dyna tech (DT-UIS 1020) (Fig1c: chips after exposure) and washed with methanol to remove extra FNAB (Fig 1d). FNAB during UV exposer produces highly reactive nitrene group which undergoes grafting on hydrocarbon chain of PMMA surface via covalent bond formation (Bora et al. 2006). Change in color of matrix (Fig 1) confirms the PMMA chip modification. Fig 1e shows the schematic for off-surface matrix modification with capturing biomolecule.



**Figure 1**

*2.4 Immobilization of anti-TNF-α antibody on FNAB modified PMMA matrix and chip assembly.*

For Anti-TNF-α antibody binding, 10 µg/ml solution of antibody was prepared in phosphate buffer (1x) as a stock solution. For covalent immobilization, 50 µl of Anti-TNF-α antibody solution in phosphate buffer was poured on each chip and was kept at 37 °C for 2 hr, where covalent binding takes place via replacement of thermally active fluoro group (Arya et al. 2006; Arya et al. 2007; Bora et al. 2006). During incubation, amino group of antibody act as nucleophile and at 37 $^{\circ}$C replace fluoro group of FNAB molecule to get covalently linked on phenyl ring. Afterward antibody binding chips were washed with PBS tween solution and with PBS to remove physically bound antibodies. Anti-TNF-α/FNAB/PMMA chip was then integrated on sensor surface using 140 micron thick double side tape as spacer to keep matrix in very close vicinity of sensor surface for high sensitivity. Starting block PBS T20 blocker via 30 min incubation was utilized for blocking of extra free spaces on matrix. Fig. 2 shows the image of sensor chip and schematic for chip assembly and modified matrix. The Anti-TNF-α/FNAB/PMMA/Au electrodes were thus formed and stored in fridge in humid chamber till use.

**Figure 2**

*2.5 Sandwich electrochemical immunoassay and signal detection*

For antigen (TNF-α) detection, Anti-TNF-α/FNAB/PMMA/Au electrode was incubated with desired concentration of TNF-α in undiluted serum. Incubation timing was optimized using 10, 15, 20 and 30 min incubation and sufficiently high, reproducible and distinctive response was observed around 20 min. Even though the current was found to increase even after 20 min of incubation, 20 min was selected to minimize the assay time. Thus, 20 min incubation was used for each step unless otherwise stated. After antigen incubation electrode was washed using 1x Femto TBST washing

buffer by flowing 1 ml solution through the incubation chamber. The washing buffer was then sucked and the chip was incubated for 20 min with 50 µl of 10 µg/ml biotin tagged secondary antibody solution in starting block TBST20. After incubation chip was again washed with 1x Femto TBST washing buffer followed by incubation for 20 min with 50 µl of 10 µg/ml streptavidin-alkaline phosphatase solution in starting block TBST20. After completion chip was again washed with1x Femto TBST washing buffer. Electrode was then finally incubated with 2 mg/ml of 4APP in deoxygenated tris-HCl buffer (100 mM, pH 9) containing 4 mg/ml $MgCl_2.6H_2O$ for 20 min followed by voltammetric (differential pulse voltammetric; DPV) signal recording using DPV scan from -0.3 to 0.4 V at step potential of 10 mV, amplitude of 25 mV, and interval time of 0.1 s. DPV scans were performed using EcoChemie Autolab Potentiostat/Galvanostat running on General Purpose Electrochemical System (GPES) software v4.9. Undiluted serum without TNF-α was used as control and serum spiked with other cytokines such as IL6, IL8 and IFN-γ were used for interference studies. All studies were repeated at least three times and data's were represented via error bars. Fig. 2f shows the schematic for bioassay at each step

## 3. Results and discussions

### *3.1 Off-surface matrix with on-chip detection platform and its working principle*

Fig. 1 shows the optical images of 3D laser engraved PMMA matrices at various step of FNAB modification along with the general schematic for matrix modification and antibody binding. The change in colors at each stage indicated successful FNAB modification of the PMMA matrix. The FNAB acts as crosslinker that helps bind anti TNF-α antibody on PMMA matrix. When the surface modified PMMA matrix is assembled on sensor electrode using double side tape, it provides off surface matrix for on-chip detection. Anti TNF-α antibody binding on PMMA matrix was

achieved at 37 °C, where amino group of antibody undergoes nucleophilic reaction and replace the fluoro group on FNAB modified PMMA and result in its covalent binding (Arya et al. 2006; Arya et al. 2007; Bora et al. 2006). After antibody binding, the change in visible hydrophobic character to hydrophilic character suggested successful immobilization. Fig. 2 shows the general schematic for modified matrix and sensor chip assembly along with the schematic for immunoassay process as described in section 2.5. As the reaction happen on matrix, which is separate from sensor, it results in higher sensitivity with low background response.

*3.2 Blank Gold electrodes with comb structure chip characterization*

Blank gold electrode with comb structure was characterized using cyclic voltammetry (CV) in 5 mM potassium ferrocynide - 5mM potassium ferricynide ($FeCN_6^{3-/4-}$) in PBS. Fig 3a shows the CV spectra of blank electrode indicating semi reversible behavior with strong oxidation and reduction peaks with almost similar currents. Chip was further tested for different scan rates (10 - 80 mV/s) (Fig 3b). CV scans clearly suggested that peak current and potential varies with scan rate and separation between oxidation and reduction peak increases with increased scan rate. Linear rise in oxidation peak current with $(\text{scan rate})^{1/2}$ revealed the diffusion controlled behavior of electrode.



**Figure 3**

*3.3 TNF-α concentration in serum studies*

Fig. 4 (a) reveals the DPV scans recorded using Anti-TNF-α/FNAB/PMMA/Au electrode for TNF-α concentrations in undiluted serum 100 pg/ml–100 ng/ml. It is clear from the figure that sensor show almost no response to serum, indicating no nonspecific binding or reaction. Further, the sensor

shows almost no response to 100 pg/ml, however peak current increased with increasing concentration starting from 1 ng/ml, revealing successful binding of antigens and secondary tags. With increasing concentration, more and more antigen binds, which on interaction with 2nd antibody and ALP-Streptavidin resulted in higher conversion of 4-APP to 4-amino phenol which on oxidation releases 2 electron to produce quinonimide. Fig. 4 (b) shows the zoom image of lower concentration indicating clear signal upto 1 ng/ml. Fig.4 c reveal linear relationship of $\Delta I\ (\mu A) = -0.19\ \mu A + 0.13\ \mu A/$(ng/ml) $C_{\text{TNF-}\alpha}$ (ng/ml) between the difference in peak current values for increasing TNF-α in 100 pg/ml to 100 ng/ml in serum. Anti-TNF-α/FNAB/PMMA/Au electrode exhibit high sensitivity of 130 nA/(ng/ml), standard deviation (SD) of 4.86 nA and detection limit of 112.1 pg/ml when estimated using 3SD/sensitivity. Error bars representing repeated measurements reveals the consistency within 5% error. Measurements for TNF-α in undiluted serum using Anti-TNF-α/FNAB/PMMA/Au electrode revealed that the off surface matrix with on-chip measurement based system may provide new and better biosensor platform for measuring of protein markers in serum. Further, to enhance sensitivity and ability to detect lower concentration clearly, gold nanoparticles of 10 nm and 50 nm with 2nd antibody and streptavidin-ALP, respectively were utilized and tested.

**Figure 4**

Fig. 5 (a) reveals the DPV scans recorded using Anti-TNF-α/FNAB/PMMA/Au electrode for TNF-α concentrations in undiluted serum (100 pg/ml to 100 ng/ml), when used with 2nd antibody-Au (10 nm), and ALP-Au (50 nm). To tag AuNPs, 100 µl of AuNPs were centrifuged at 14000 RPM for 20 min and extra solution was removed. 20 µl of 2nd antibody (500 µg/ml) or 10 µl of streptavidin-ALP (1 mg/ml) was added to precipitate, mixed and left for 1 hr at 4 °C before diluting in starting

block TBST20 to obtain final concentration of 10 µg/ml. Fig. 4b showing DPV spectra's for lower concentration clearly suggest that use of AuNP result in better and differentiable signal for even 100 pg/ml and 1 ng/ml, which is much better than that without AuNPs, however there was slight increase in background signal for serum alone. Higher and sharper signals suggested successful interaction between various reagents producing final product. Fig. 5c reveal linear relationship of $\Delta I\ (\mu A) = 0.068\ \mu A + 0.119\ \mu A/$(ng/ml) $C_{\text{TNF-}\alpha}$ (ng/ml) between the difference in peak current values for increasing TNF-α in 100 pg/ml to 100 ng/ml in serum. Electrode exhibit sensitivity of 119 nA/(ng/ml), standard deviation (SD) of 2.65 nA and detection limit of 66.8 pg/ml when estimated using 3SD/sensitivity. Error bars representing repeated measurements reveals the consistency within 5% error. Measurement of TNF-α in undiluted serum concentration using Anti-TNF-α/FNAB/PMMA/Au electrode with AuNPs tagged reagents revealed that off surface matrix with on-chip measurement based system may be utilized for highly sensitive biosensor for measuring protein markers in serum with selectivity and sensitivity.

**Figure 5**

*3.4 Interference from other cytokines and shelf life studies*

The interference from other cytokines was tested using IL6 and IFN-γ at concentration of 1µg/ml and compared with TNF-α at concentration of 50 ng/ml. Following the procedure developed and mentioned above. Fig. 6a reveals the DPV scans for interference measurements recorded for undiluted serum (i), 1 µg/ml of IL6 in serum (ii), 1 µg/ml of IFN-γ in serum (iii), and 50 ng/ml of TNF-α in serum (iv). From Fig. 6a, it is clear that electrode does not exhibit substantial change in peak current for 1 µg/ml of IL6 or IFN-γ in serum; however, it shows prominent signal for TNF-α in

serum. Thus, Anti-TNF-α/FNAB/PMMA/Au electrode in off surface matrix with on-chip measurement offers a new method for binding recognition molecule near sensing electrode for and testing of protein biomarkers in undiluted serum without interference. The stability of prepared Anti-TNF-α/FNAB/PMMA/Au electrode was investigated for 2 month via DPV measurements once every week. Fig. 6b shows the stability graph for measurement of 50 ng/ml of TNF-α in serum on electrodes made in same set and kept at 4 °C in humid chamber. Results suggests that electrode retained more than 96% of initial value till 6 weeks, after which the biosensor started to lose its activity and reached 90% by $9^{th}$ week. These results indicated that the sensor can be kept stable when stored at 4 °C. The sensor stability can even be improved with industrial grade packaging and storing, which involve packaging in $O_2$ free environment and in controlled humidity and temperature.

**4. Conclusion**

In summary, off surface matrix with on-chip measurement based system provides new biosensor platform that is able to reduce the effect of background interference and result in selective and enhance detection sensitivity and specificity. Anti-TNF-α/FNAB/PMMA/Au electrode with gold nanoparticle tagged reagents exhibited linearity over 100 pg/ml–100 ng/ml range, with sensitivity of 119 *nA/*(ng/ml) *and* minimum detectable concentration of 66.8 pg/ml when estimated using 3SD/sensitivity. The Anti-TNF-α/FNAB/PMMA/Au electrode showed selectivity over serum proteins, IL2 and IFN-γ. System is found to be stable for 2 months and can also be employed for other biomarkers. Further, the off-surface detection scheme can be employed with various different types of matrices and their compatible chemistries for wider application. Thus, proposed system holds high potential replace presently used optical ELISA for biomarker sensing. Team is in process of making prototype where whole detection process can be automated to generate results for desired

analyte (TNF-α) estimation in real sample. Once developed prototype will be tested and results will be published elsewhere.

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**Figure captions**

Fig. 1. PMMA chip at various stages of FNAB modification (a) blank laser engraved chip, (b) after dropping FNAB and drying in dark, (c) after UV exposure and (d) after washing with methanol, (e) schematic for off-surface matrix modification with capturing biomolecule.

Fig. 2. Schematic for top view of (a) sensor chip, (b) sensor chip with spacer tape, (c) sensor chip with modified PMMA matrix on spacer tape, (e) side view for 3D matrix assembly and (e) zoom view of modified matrix on sensor surface and (f) Schematic of Immunoassay procedure.

Fig. 3. Characterization of blank gold electrodes using CV recording (a) at 50 mV/s scan rate and (b) at different scan rates

Fig. 4 (a) DPV spectra obtained on Anti-TNF-α/FNAB/PMMA/Au electrode for TNF-α concentrations in undiluted serum 100 pg/ml – 100 ng/ml, (b) DPV spectra for different concentration upto 1 ng/ml, (c) TNF-α concentration linearity curve for normalized current obtained by subtracting serum current value.

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Fig. 6 (a) DPV scans for undiluted serum (i), 1 µg/ml of IL6 in serum (ii), 1 µg/ml of IFN-γ in serum (iii), 50 ng/ml of TNF-α in serum (iv). (b) Shelf life studies using TNF-α (50 ng/ml) over two month period.

**Highlights**

A new 3D off surface matrix combined with on-chip detection has been developed for biosensors

4-Fluoro-3-nitro azido benzene as cross linker has been employed for covalent binding of antibodies

Anti-TNF-α/FNAB/PMMA-CSGM was developed by integrating Anti-TNF-α/FNAB/PMMA on to CGGM surface using spacer

TNF-α spiked in undiluted serum was detected selectively with high sensitivity.

Electrode showed linear detection for TNF-α in 100 pg/ml to 100 ng/ml range with high sensitivity of 119 nA/(ng/ml), with negligible interference from serum proteins and other cytokines.

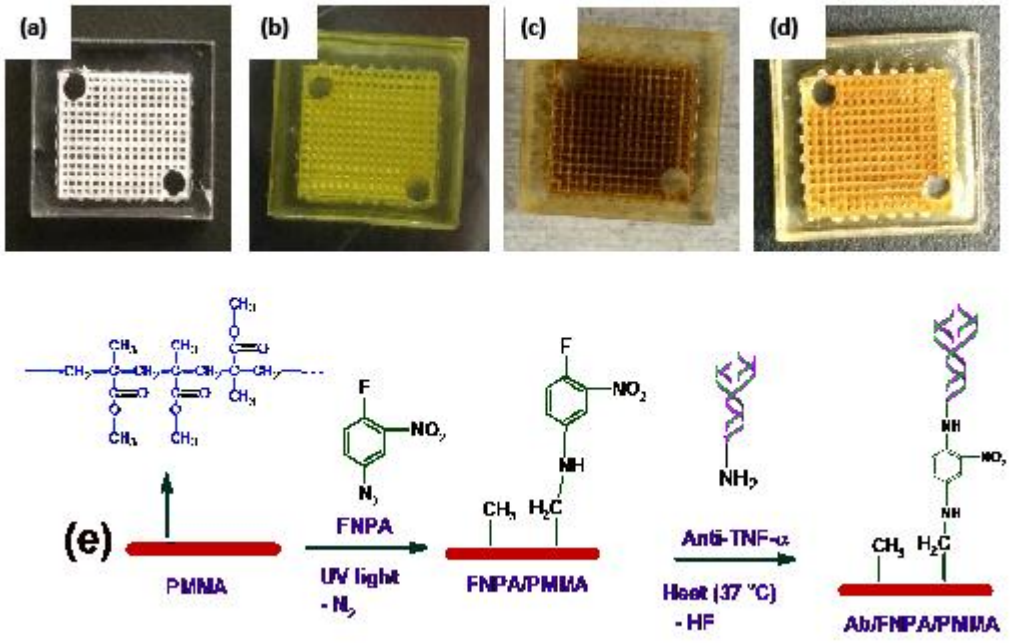
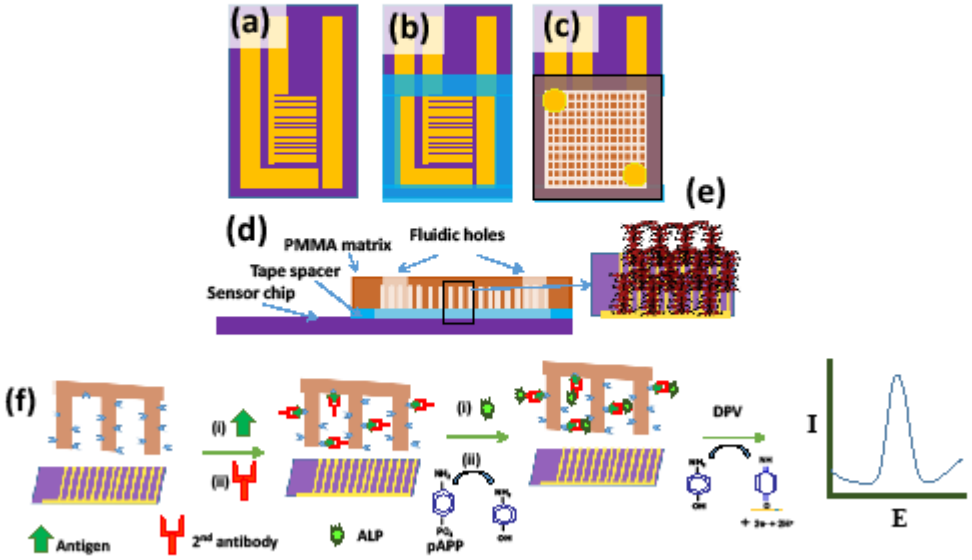



Fig. 1. PMMA chip at various stages of FNPA modification (a) blank laser engraved chip, (b) after dropping FNPA and drying in dark, (c) after UV exposure and (d) after washing with methanol, (e) schematic for off-surface matrix modification with capturing biomolecule.

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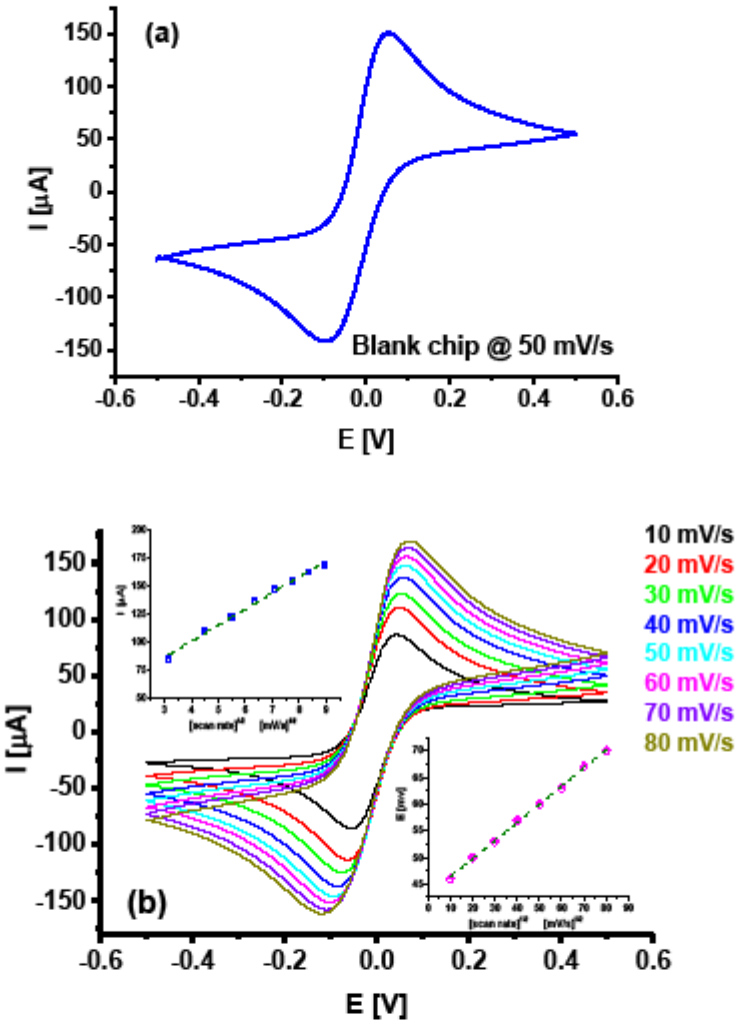


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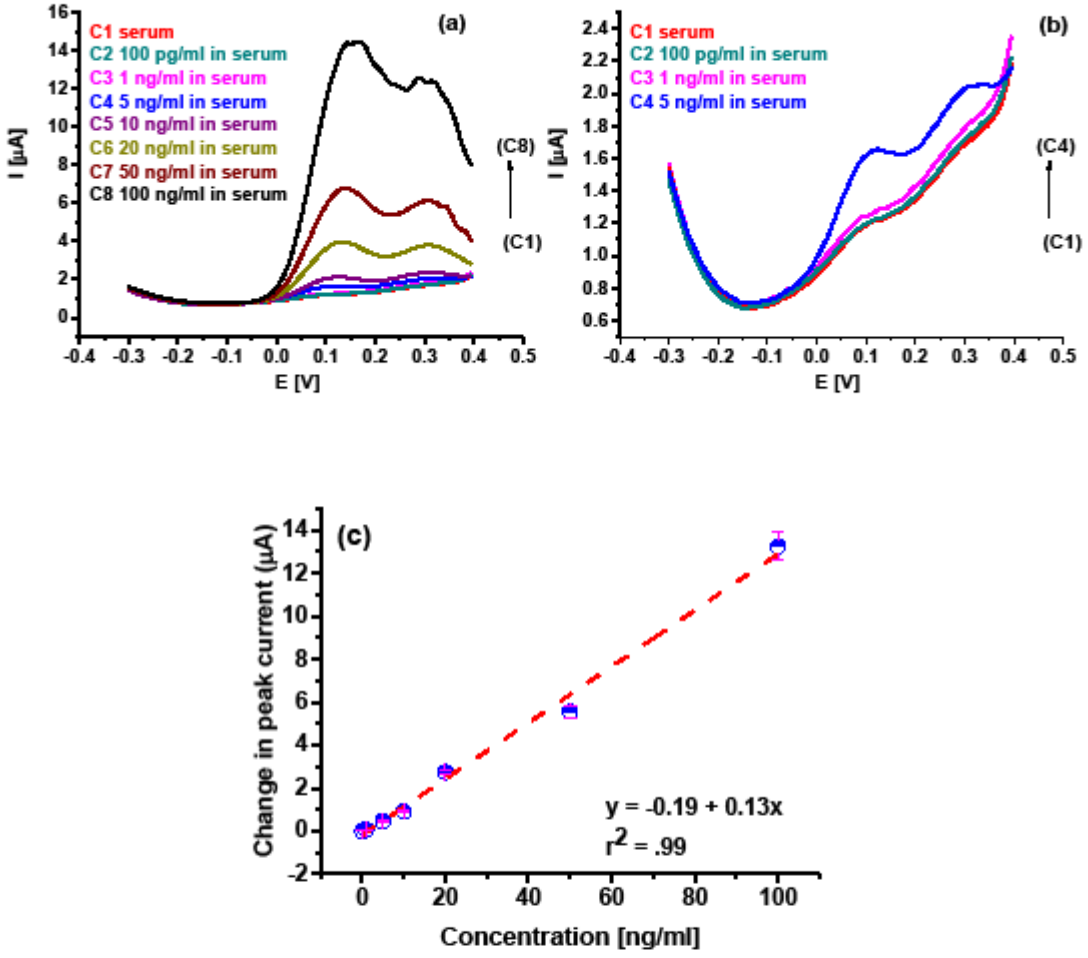


Fig. 4 (a) DPV spectra obtained on Anti-TNF-α/FNAB/PMMA/Au electrode for TNF-α concentrations in undiluted serum 100 pg/ml – 100 ng/ml, (b) DPV spectra for different concentration up to 1 ng/ml, (c) TNF-α concentration linearity curve for normalized current obtained by subtracting serum current value.

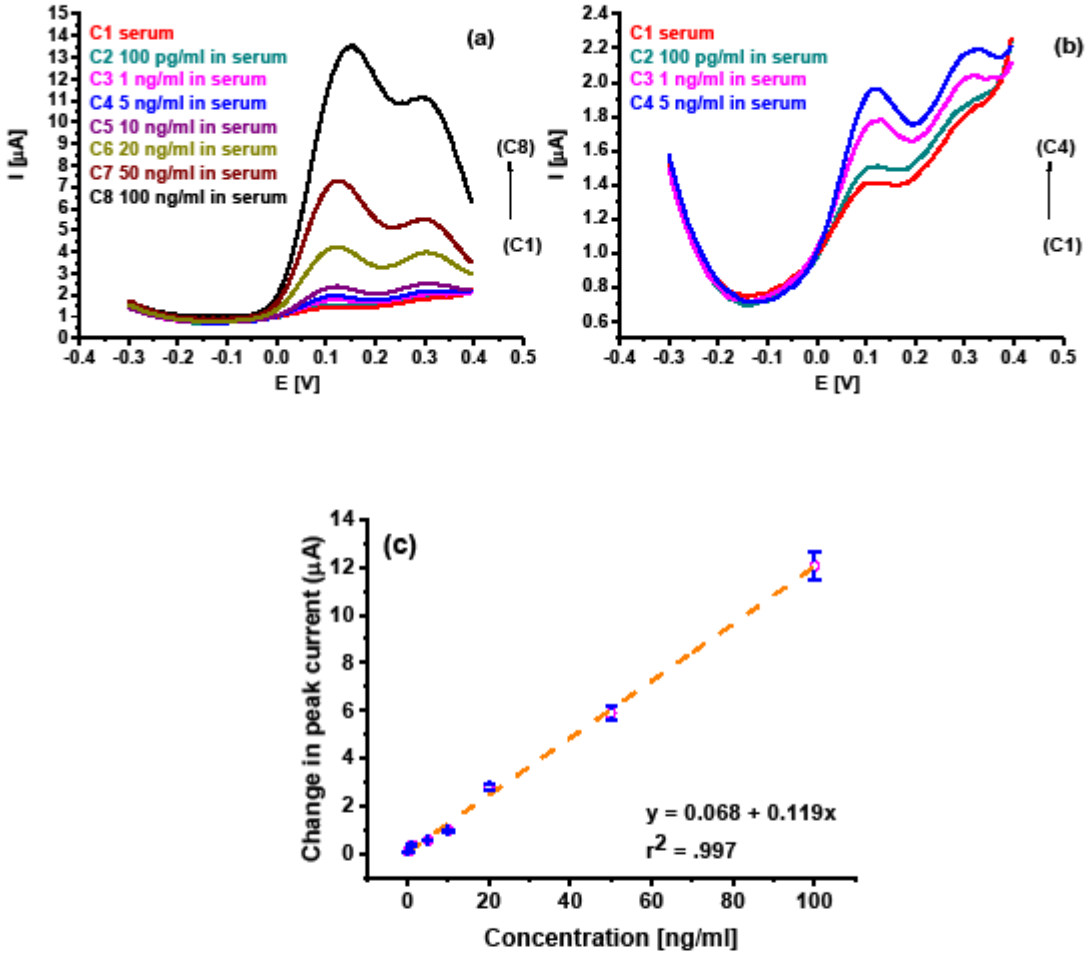


Fig. 5 (a) DPV spectra obtained on Anti-TNF-α/FNAB/PMMA/Au electrode for TNF-α concentrations in undiluted serum (100 pg $ml^{-1}$ – 100 ng $ml^{-1}$), when used with 2nd antibody-Au (10 nm), and ALP-Au (50 nm) (b) DPV spectra's at lower concentration and (c) TNF-α concentration linearity curve for normalized current obtained by subtracting serum current value.

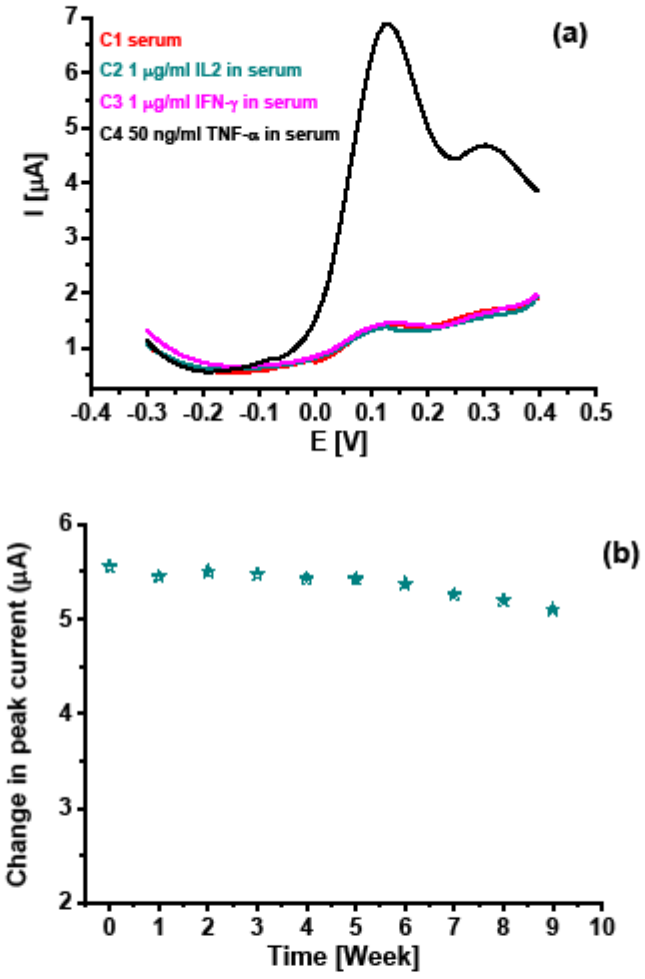


Fig. 6 (a) DPV scans for undiluted serum (i), 1 μg/ml of IL6 in serum (ii), 1 μg/ml of IFN-γ in serum (iii), 50 ng/ml of TNF-α in serum (iv). (b) Shelf life studies using TNF-α (50 ng/ml) over two month period.