

Analytical Methods

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

1
2
3
4 1 **Selective choline biosensors based on choline oxidase co-immobilized with self-**
5
6 2 **assembled monolayers onto micro-chips at low potential**
7
8
9

10 3
11
12 4 **Mohammed M. Rahman*, Abdullah M. Asiri**
13
14 5

15
16
17 6 *Center of Excellence for Advanced Materials Research (CEAMR) & Chemistry Department,*
18
19 7 *Faculty of Science, King Abdulaziz University, P.O. Box 80203, Jeddah 21589, Saudi Arabia*
20
21 8

22 9
23
24 10
25
26 11
27
28 12
29
30
31
32
33 13 ***Corresponding address:**
34

35
36 14 **Dr. Mohammed M. Rahman,**

37
38 15 Center of Excellence for Advanced Materials Research &

39
40 16 Chemistry Department, Faculty of Science,

41
42 17 King Abdulaziz University, Jeddah 21589,

43
44 18 P.O. Box 80203, Saudi Arabia

45
46 19 Email: mmrahman@kau.edu.sa; Phone: +966-59-6421830;
47
48
49
50
51 20

52
53
54 21
55
56
57 22
58
59
60

Abstract:

The fabricated choline-biosensor exhibited excellent specific and selective recognition to selected biological molecule among coexistence with other analytes in the buffer system at low potential. This novel effort is initiated a well-organize way of efficient enzyme immobilized microchip biosensor development for selective choline (Ch) detection using choline oxidase (ChOx) enzymes in large scales. Here, reusable and sensitive Ch biosensor is developed based on mediator-free ChOx by self-assembled monolayer (SAM) onto tiny micro-chip. The simple cyclic voltammetry (CV) technique was employed with the enzyme fabricated chips in phosphate buffer solution (PBS, 0.1M) at room conditions. The analytical parameters of ChOx fabricated electrode displayed a lower detection limit (DL, 0.012 ± 0.005 nM), a wide linear dynamic range (LDR, 0.05 nM to 10.0 uM), good linearity ($R = 0.9938$), and higher sensitivity ($3.5 \mu\text{A}\mu\text{M}^{-1}\text{cm}^{-2}$), where a tiny sample volume (50.0 μL) was analyzed. The micro-chip system exhibits a simple and efficient approach to immobilize the oxidative enzymes onto thioglycolic acid (TGA) SAM modified surfaces, which can improve the biosensor performances for a large group of biomolecules in broad scale of biomedical applications in health-care fields. This integrated microchip provides a promising low-cost platform for the sensitive and rapid detection of biomolecules using miniature samples.

Key words: Choline; Choline oxidase; Self-assembled monolayer; Cyclic voltammetry; Sensitivity; Thioglycolic acid; Selectivity; Micro-chips

1 Introduction:

2 Choline is an essential nutrient that body makes in small amounts, however it must
3 consume it through the diet to get enough. In adults, choline helps keep cell membranes
4 functioning properly, plays a role in nerve communications, prevents the buildup of
5 homocysteine in blood (elevated levels are linked to heart disease) and reduces chronic
6 inflammation. In pregnancy, choline plays an equally helping to prevent certain birth defects,
7 such as spina bifida and brain development. It is a nutritional prerequisite of various animal
8 species (dog, cat, rat, and guinea pig), and only lately [1,2] it was recommended that a nutritional
9 source of choline may be requisite for adult humans. Choline, classified as being “vitamin-like”,
10 acts a rather considerable function for the synthesis of the neurotransmitter acetylcholine
11 precursor which is disseminated in both central and peripheral nervous systems of mammals
12 [3,4]. Ch insufficiency results in various syndromes, such as liver-damage and brain-disorders.
13 Thus, the significance of ch has produced much attention in emerging a Ch-biosensor. Enzyme-
14 based Ch-biosensors have emerged in the past several years as the most promising methods. Ch
15 is converted by ChOx in the presence of oxygen, generating H_2O_2 . The electroactive hydrogen
16 peroxide can be consequently identified with various modified sensors or electrodes. A Ch-
17 biosensor based on a bielelectrocatalytic property of amorphous MnO_2 nanoparticles modified flat-
18 electrodes to hydrogen peroxide was fabricated via a straight and reliable electrochemical
19 deposition of a biocomposites that was prepared of chitosan hydrogel, ChOx, and MnO_2
20 nanoparticles onto a glassy carbon electrode [5]. Ch is a significant component of phospholipids
21 (lecithin and sphingomyelin), it is essential for the preparation of the neurotransmitter
22 acetylcholine, it acts as a source of labile methyl groups, and it is a component of pulmonary
23 surfactant [6]. This stuff is usually generated in human tissues in adequate amounts to meet

1 human requirements, and it is confidential as being “vitamin-like”. The endogenous preparation
2 of Ch, however, needs sufficient amounts of the amino acids serine and methionine, along with
3 adequate amounts of folic acid and vitamins B12 and B6. Ch is extensively disseminated in
4 foods; the requisite is commonly contented by both nutritional and endogenous sources, although
5 Ch deficiency has been published. The most universal signs of ch insufficiency are fatty liver and
6 hemorrhagic kidney necrosis [7]. Confirmation for free radical activity in liver with Ch
7 deficiency is reported, and this may be associated to the carcinogenesis method [8]. Ch is
8 ingested mostly in the form of phosphatidylcholine rather than free base. Ch-chloride and Ch-
9 bitartrate are supplemented to infant formulas and milk products to make sure the presence of Ch
10 at levels found in milk.

11 Rapid in situ determination of Ch is important for the characterization of cholinergic
12 transmission in normal and pathological physiology. Choline metabolite at select nicotinic
13 receptor types [9] are involved in various functions, e.g., learning and memory formation [10],
14 development and maintenance of addiction [11], and degeneration of cholinergic neuronal
15 systems in Alzheimer’ disease [12]. An effective method for Ch measurement should facilitate
16 examination of basic mechanisms of cholinergic transmission and evaluation of pharmaceuticals
17 that affect cholinergic activity [13,14]. An important confront to development of biosensors for
18 in situ measurement is the high level of selectivity required due to the complexity of the
19 physiological environment. Enzyme-based biosensors offer selectivity via indirect recognition of
20 the products of a specific enzymatic reaction. Amperometric enzyme microsensors suitable for in
21 situ measurement of Ch has been developed that utilize choline oxidase [15], and several have
22 been tested in rat brain [16]. Recently, Ch biosensor were made with PD-films to which ChOx
23 was immobilized on Pt with a detection scheme similar to that used for the present sensors.

1
2
3 1 However, the previously reported Ch biosensors were used for detection of organophosphorus
4
5
6 2 pesticides and are rather large for use in vivo [17].
7

8 3 Ch-ion is an significant biological cation that assists control the structure of cell
9
10 4 membranes and has functions in cell signaling and as a neurotransmitter [18,19]. Ionic liquids
11
12 5 based on choline ion guarantee long-term stability of biomolecules like DNA and proteins
13
14 6 [20,21]. Both cations and anions included in ionic liquid may impinge on the chemical stability
15
16 7 of these molecules. Fujita et al. illustrated that the character of anion shows to be more
17
18 8 significant to the stability of proteins than the type of cation [22]. It is likely, however, that
19
20 9 cations are more significant to the stability of DNA, because cationic molecules are needed to
21
22 10 decrease the repulsive forces between the phosphate groups of DNA strands. Recognition of the
23
24 11 interactions that happen between Ch-ion and DNA at the atomic level will offer imminent into
25
26 12 how these ions control the stability of DNA duplexes in living cells where ch-ions play active
27
28 13 functions and will further our ability to control DNA duplex stability in molecular machines.
29
30 14 Enzyme based detection techniques have become a common tool for detecting and quantifying
31
32 15 substances that are difficult or impossible to measure through standard analytical techniques
33
34 16 alone, especially when a short time scale of seconds or milliseconds is required [23]. In this
35
36 17 work, it is developed the choline biosensors using TGA self-assembled monolayer modified tiny
37
38 18 micro-chip. A highly-sensitive and low-detective Ch-biosensor was fabricated with ChOx on
39
40 19 TGA-SAM, which perfectly designed and fabricated onto tiny micro-chips.
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 **1 Experimental section:**

4
5
6 **2 *Materials and Methods***

7
8 Choline, Choline oxidase, monosodium phosphate, disodium phosphate, Thioglycolic
9 acid (TGA), ethanol, and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
10 (EDC) were purchased from Sigma-Aldrich company. All other chemicals were analytical grade
11 and used without further purification. 0.1 M PBS (pH ~7.2) is prepared by mixing the proper
12 ratio of 0.2 M NaH₂PO₄ and 0.2M Na₂HPO₄. Required solutions are prepared with distilled
13 water, which obtained from a water purifying apparatus (12.0 M.Ω.cm) (AQUA MEDIA). Cyclic
14 voltammetry (CV) is a type of potentiodynamic electroanalytical measurement. In CV, the
15 significant working electrode potential is ramped linearly against time, like linear-sweep
16 voltammetry. CV takes the experiment a step further than linear sweep voltammetry, which ends
17 to achieve a set-potential. When CV attains a set-potential, the working electrode's potential
18 ramp is reversed. This inversion can occur several times in a single experiment. The resultant
19 current at the working electrode is plotted against the applied potential to confer the CV trace.
20 CV is usually used to investigate the electro-analytical properties of target analytes in reaction
21 medium in reaction conditions. The utility of CV is directly depended on the analytes
22 concentration. The analyte has to be redox active within the experimental potential window. It is
23 also highly enviable for the analyte to display a reversible wave. A reversible wave appears,
when an analyte is reduced or oxidized on a forward scan and then re-oxidized or re-reduced in a
predictable route. The technique uses a reference electrode (RE), working electrode (WE),
and counter electrode (CE), in which the combination is sometimes referred to as a three-
electrode system. Here, the electrochemical analyses were performed using a votammetric
analyzer (CV-50W, BAS) at room condition. All investigations were carried out on

1
2
3 1 electrochemical micro-chip (5.0 mm × 5.0 mm), which sensing area is close to 0.0805 cm². The
4
5
6 2 total investigations were carried out with ChOx-enzyme modified SAM-chips composed as
7
8 3 working, Pt layer as counter, and Ag/AgCl (sat. KCl) as reference electrodes. CVs were recorded
9
10 4 at Choline Oxidase/SAM of TGA/Gold surface of Chip (i.e., ChOx/TGA-SAM/AuE) electrode
11
12 5 from -0.1 to +0.5 V (versus Ag/AgCl; sat KCl) in 0.1M PBS (pH ~7.2) at 0.1 V/s scan rate.
13
14 6 Fabrication assembly of Ch/ChOx biosensor was prepared onto tiny micro-chip, which already
15
16 7 fabricated by self-assembled monolayer of TGA. Analyte solution was prepared with different
17
18 8 concentration ranges of target Ch from 0.05 nM to 50.0 uM. Calibration experiment is performed
19
20 9 by using the various concentration of target Ch analyte using enzyme immobilized micro-chips.
21
22 10 The calibration curve is plotted using the resultant current (from the applied potential) versus
23
24 11 various Ch concentrations. The ratio of current and concentration (slope of calibration curve)
25
26 12 was used to measure the sensitivity of Ch. Limit of detection was also calculated from the ratio
27
28 13 of noise (3N) versus sensitivity (S) in the linear dynamic range of the calibration plot (shortly
29
30 14 SNR).
31
32
33
34
35
36
37
38

39 ***Construction of micro-chips by photolithography method:***

40
41 17 The tiny microchips are fabricated by conventional photolithographic technique.
42
43 18 Electrodes and passivation layers are developed on silicon wafer followed by dicing and
44
45 19 packaging. N-doped Si wafers are prepared and over-flowed by extra-pure water. In this step, the
46
47 20 contaminants on the surface and native SiO₂ layer are removed. At first, the wet oxidation is
48
49 21 processed, and then dry oxidation is executed. Wafers are annealed in the condition of nitrogen.
50
51 22 Aluminum is sputtered with Al-1% on Si target. Then the photolithograph processes are applied.
52
53 23 Resist coating, baking, exposure, and development are done by Kanto chemicals, and then it is
54
55
56
57
58
59
60

1 rinsed by ionic water. Al is etched by etching solution. Resist is removed by plasma etching
2 instrument. Then wafers are cleaned by acetone, methanol, and finally by plasma simultaneously.
3 SiN layer is deposited by chemical vapor deposition. Surface of pad electrodes are etched by
4 reactive ion etching. Finally residual resist layer is removed by plasma acing. After
5 photolithographic process, Pt is sputtered by SP150-HTS. Then it is patterned by lift-off
6 technique, in which wafers are immersed into the remover, and then washed with IPA.
7 Photolithographic process is again investigated, where Ti is sputtered as a binding layer, and then
8 Au is evaporated by deposition. Finally, Au layer is patterned by lift-off process. Parylene
9 passivation layer is formed for the protection of the chip from water. Photolithographic process
10 is executed again for pad protection. Then parylene-dimer is evaporated by deposition apparatus.
11 Photolithography process is done again for patterning. Parylene layer is patterned by etching.
12 Finally, unnecessary resists are removed by acetone and then wafer is cleaned by IPA. Resist is
13 coated on a whole surface of the wafer for protection during dicing process. Si wafer is diced
14 into pieces by dicing apparatus and stored into the desiccators. Resist on chip surface is removed
15 by acetone and cleaned with IPA. The backside of the chip is roughed by a sheet of sandpaper
16 for better adhesion and electrical stability. The chip is bonded with die and packaged by silver
17 paste. It is dried in a drying oven. Pads on chip are connected to the package through gold wire
18 with bonding machine. Finally, Si-based adhesive is put on the periphery of the chip to protect
19 pads and gold wire from sample solution. Adhesive is dried at room temperature for 24 hours.
20 The composition and thickness of each fabricated layer into micro-devices are mentioned in

21 **Table 1.**

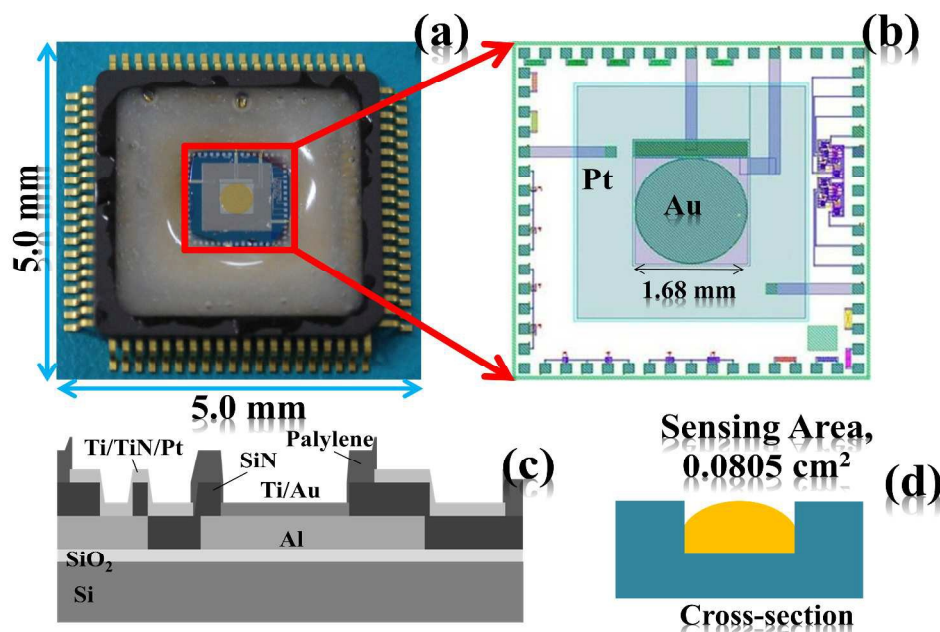
22
23

Table 1. Function and thickness of every layer on the micro-chip

Material	Function	Thickness (μm)
Si	Wafer material	500.00
SiO ₂	Insulation	0.40
Al	Electric wiring	1.00
SiN	Protection/separation	1.00
Ti/TiN	Binding	0.15
Ti	Binding	0.10
Pt	Counter Electrode	0.25
Au	Working Electrode	0.30
Parylene	Passivation/protection	1.00

Then, the semiconductor micro-chips were prepared on silicon wafer. Al was sputtered to fabricate the wiring and bonding pads. Pt/Ti/TiN was sputtered on thermal oxide of silicon and patterned by photolithography to fabricate CE. Ti/TiN layers were used for strong adhesion. Au/Ti was sputtered and lithographed, which made circular WE with a diameter of 1.6 mm in the center of the chip. After electrodes fabrication, parylene layer was fabricated by evaporation method as a passivation layer. The wafer was diced to 5.0 mm square chips. This chip was bonded by silver paste to make the package. Aluminum pads were connected to the package by gold wire. Finally, adhesive (Araldite, Hantsman, Japan) was put on the periphery of chip, which prevented target solution from contacting pads (Figure 1a). The magnified construction view of internal chip-center (sensing area) is presented in the Figure 1b. A cross section of the sensor

1 chip is shown as well in Figure 1c. The cross-section of the center of microchip (sensing area) is
 2 also presented in Figure 1d and calculated the active sensing area (0.0805 cm^2).



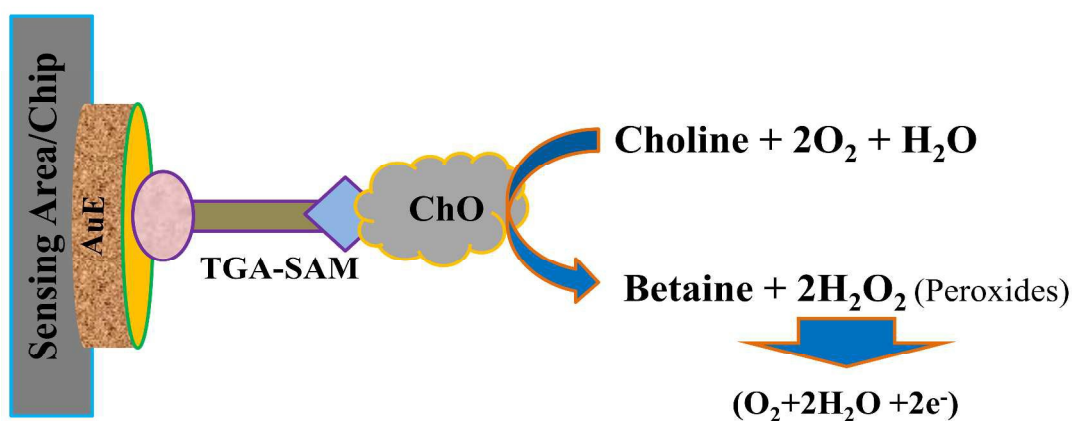
3
 4 **Figure 1.** Schematic diagram of (a) camera-view from top of micro-chip, (b) magnified
 5 schematic view of micro-chip sensing-area, and (c) cross-sectional view of micro-chip, and (d)
 6 cross-section of only sensing area. Calculated active sensing area is 0.0805 cm^2 .

7 **Results and discussions:**

8 **Fabrication of ChOx/TGA-SAM/AuE onto micro-chip**

9
 10 The micro-chip biosensor especially sensing-area is chemically cleaned several times
 11 with cleaning agents and dried with acetone. The gold-sensing area is finally cleaned with
 12 nitrogen (flow of nitrogen gas) and then deposited TGA solution onto the center micro-chip and
 13 kept for 6 hours. The TGA-modified micro-chip is cleaned slightly with ethanol and then dried
 14 and prepared for the enzymatic steps. The ChOx enzyme is dropped onto the TGA-SAM sensing

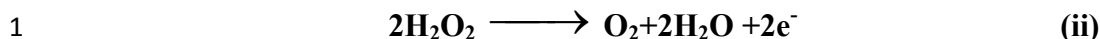
1 area for 12 hours. Figure 2 outlines the sensing protocol using the ChOx/TGA-SAM/AuE-
 2 modified chip. It is prepared for covalent-bond formation to immobilize the ChOx enzyme on the
 3 TGA-SAM via peptide-conjugation in presence of EDC activating agent. First, self-assembled
 4 monolayer of TGA is formed by drop wise adding the TGA solution onto the sensing area of
 5 micro-chips for two hours. Then ChOx enzyme is immobilized on TGA-SAM by amide bond
 6 formation between the terminal-unbound carboxylic acids groups on TGA film and the amine
 7 groups of the ChOx enzyme.



9 **Figure 2.** Fabrication way of Ch/ChOx biosensor is prepared on tiny micro-chip. Step-1: SAM
 10 of TGA on gold-surface of centered chips for 6 hr; Step-2: Activation of TGA-COOH group
 11 using EDC (12 hours, 4.0 °C); Step-3: Immobilization of ChOx for 12 hours at 4.0 °C.

12
 13 For the stable attachment of ChOx onto TGA-SAM, the chip is placed for 12 hours into the
 14 refrigerator at 4.0 °C. The ChOx/Ch enzymatic reactions involved in the bio-sensing system for
 15 the detection of target Ch are given in below (Fig. 2),



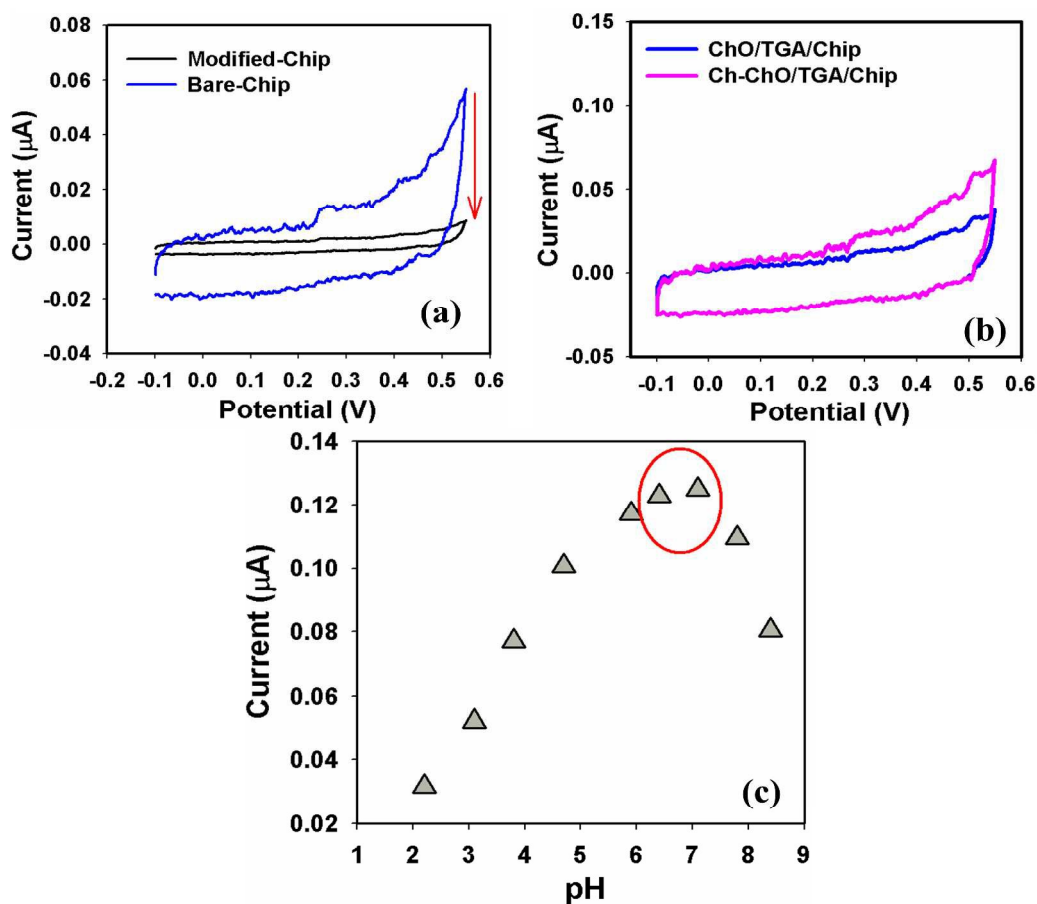


Reaction (i) is directly related on Ch concentration in the reaction medium. On the micro-chip, Ch is oxidized to form betaine and H_2O_2 . Then H_2O_2 is dissociated to produce the current (Reaction ii). This current is directly proportional to the Ch concentration in the solution system. Based on thin-layer of TGA-SAMs, it was successfully fabricated of Ch sensor using ChOx on the sensing area of micro-chip at room conditions. Conventional electrochemical method, CV is the most versatile electro-analytical method for the study of bioactive materials and species, which is widely used in industrial applications, academic or bio-chemical researches or R&D approaches. CV is also an important method to assess the coating/blocking property of the monolayer-coated electrodes using diffusion controlled redox couples. Micro-chip surface is cleaned by Piranha solution [$\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2$ (3:1)] and washed with pure water, then dried sufficiently by nitrogen. TGA is dissolved in ethanol to make 10.0 mM stock solution. The prepared TGA solution was dropped onto sensing area of micro-chip, and then kept for 6 hours at room temperature.

Controlled experiment and Optimization

Figure 3 displays the CVs of un-modified and TGA-SAM modified micro-chip electrodes in 0.1 M PBS as the supporting electrolyte at 0.1 V/s potential scan rates. It can be observed from the Figure 3a, that the bare micro-chip (blue-curve) exhibits a voltammogram for the in 0.1 M PBS electrolytes. This is indicated that the electron-transfer reaction is totally diffusion controlled. In contrast, the absence of any peak formation in the CVs of TGA monolayer-modified micro-chip (black-curve) shows the reaction is slightly inhibited. CVs for TGA are indicated a good blocking behavior for the electron-transfer reaction, which means that a highly

1 ordered, compact self-assembled monolayer formed on the sensing surface of the micro-chips
 2 [24,25].



3
 4 **Figure 3.** (a) CVs of 0.1M PBS for bare (blue-line) and TGA-SAM (black-line) modified micro-
 5 chips; (b) CVs recorded in 0.1M PBS of bare micro-chip (blue-line), ChOx modified micro-chip
 6 in presence of 0.1 μM Ch (pink-line) solution of micro-chip; (c) Effect of pH of
 7 ChOx/TGA/micro-chip in 0.1 μM Ch solution at room conditions. Scan rate: 0.1 V/s, RE:
 8 Ag/AgCl (sat^d. KCl), Supporting electrolytes: 0.1M PBS.

9 ChOx enzyme was immobilized onto the TGA-SAM surface via peptide conjugation.
 10 First 10.0 mM EDC in 0.1 M PBS was put onto the TGA-SAM chip and then kept at 4.0 °C in
 11 the refrigerator to activate carboxylic group of TGA for 12 hours. Then EDC-treated electrode

1 was washed gently with 0.1 M PBS to remove excess EDC on the electrode surfaces. Then ChOx
2 solution was dropped on the sensing area of chip and incubated in the refrigerator at 4.0 °C for
3 12 hours. ChOx was successfully immobilized onto TGA-SAM via covalent bond, which
4 confirmed by the current change in Figure 3b. It showed that the CVs recorded for the bare
5 microchip (blue curve), ChOx/TGA-SAM/AuE with 0.1 mM Ch solution (pink curve) of
6 fabricated chip in 0.1 M PBS (pH 7.2) at 0.1 V/s scan rates. According to the control experiment,
7 no significant change was observed when the CV recorded with the bare-microchip for 0.1 mM
8 Ch in PBS due to the absence of ChOx enzyme. A small current change was observed at
9 approximately +0.55 V versus Ag/AgCl for 0.1 mM Ch solution, due to the enzymatic reaction
10 occurred with Ch in presence of ChOx enzyme on the sensing surface of the micro-chip. The
11 enzymatic current (approximately +0.55 V) was increased due to the increasing of Ch
12 concentration in the PBS solution. The experimental parameters which affecting the
13 performances (detection limit, sensitivity, and response time) of the biosensor were optimized in
14 term of pH and presented in Figure 3c. The pH of the buffer is exhibited a strong effect on the
15 activity of the sensing layer on the microchips. The effect of the pH of the buffer on the current
16 variation is studied over the pH range of 2.2 to 8.5. Figure 3c shows the peak-current is obtained
17 at different pH values for 0.1 μM Ch in 0.1 M PBS system. The peak-height is increased from
18 pH 2.2 to 7.2 and then decreased above pH 7.2. The peak-current is decreased above pH 7.2, due
19 to the poor ChOx enzyme activity at higher basic medium. Therefore, the pH of the PBS system
20 is set at 7.2 throughout the experiments.

1 **Detection of choline by enzyme immobilized micro-chip**

2 Cyclic voltammetric technique using mediator-free PBS system was employed to confirm
3 the detection of Ch with immobilized chip. 50.0 μL of each Ch solution with PBS was dropped
4 on the sensing area of enzyme immobilized micro-chip and investigated the oxidation current.
5 Figure 4a shows a typical CV (current-voltage) plot for the addition of various concentration of
6 Ch solution in 0.1 M PBS (pH 7.2). The current is increased gradually with increasing the
7 concentration of Ch (0.05 nM to 50.0 μM ; by sequential addition) to a stable and saturated value.
8 The ChOx/TGA-SAM/Au modified microchip electrode is achieved 90% of steady state currents
9 with in 10.0 sec. The increase of oxidation current is significantly observed in this investigation.
10 This is because of Ch oxidization in presence of ChOx and the current-changes lead to the
11 positive current increased. In Figure 4b, it was plotted the calibration plots for the Ch-current at
12 +0.5V, which exhibits from the current-voltage responses with fabricated microchips. Under the
13 optimized conditions, the steady-state current is showed a linear relationship with the Ch
14 concentration in the range from 0.05 nM to 10.0 μM (Fig. 4 inset). The linear dependence of Ch
15 concentration is yielded with a correlation coefficient (~ 0.9938). The detection limit for Ch is
16 calculated to be 0.012 ± 0.005 nM, based on signal to noise ratio ($\sim 3S/N$). The Ch sensor is also
17 measured the higher sensitivity, which is close to $\sim 3.5 \mu\text{A} \mu\text{M}^{-1} \text{cm}^{-2}$. The observed sensitivity is
18 much higher than the previously reported Ch sensors [26-28].

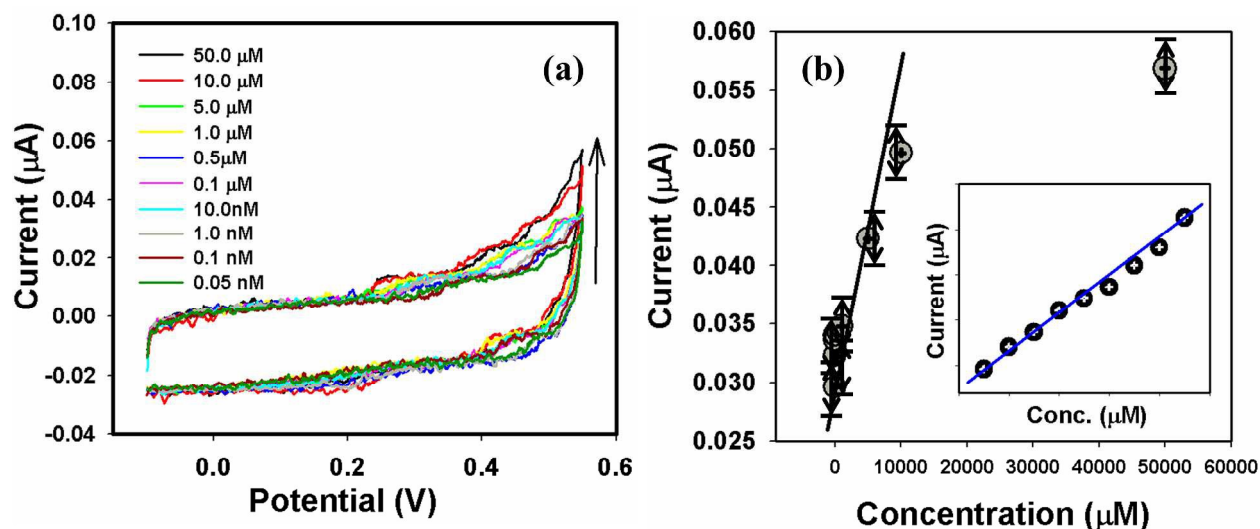
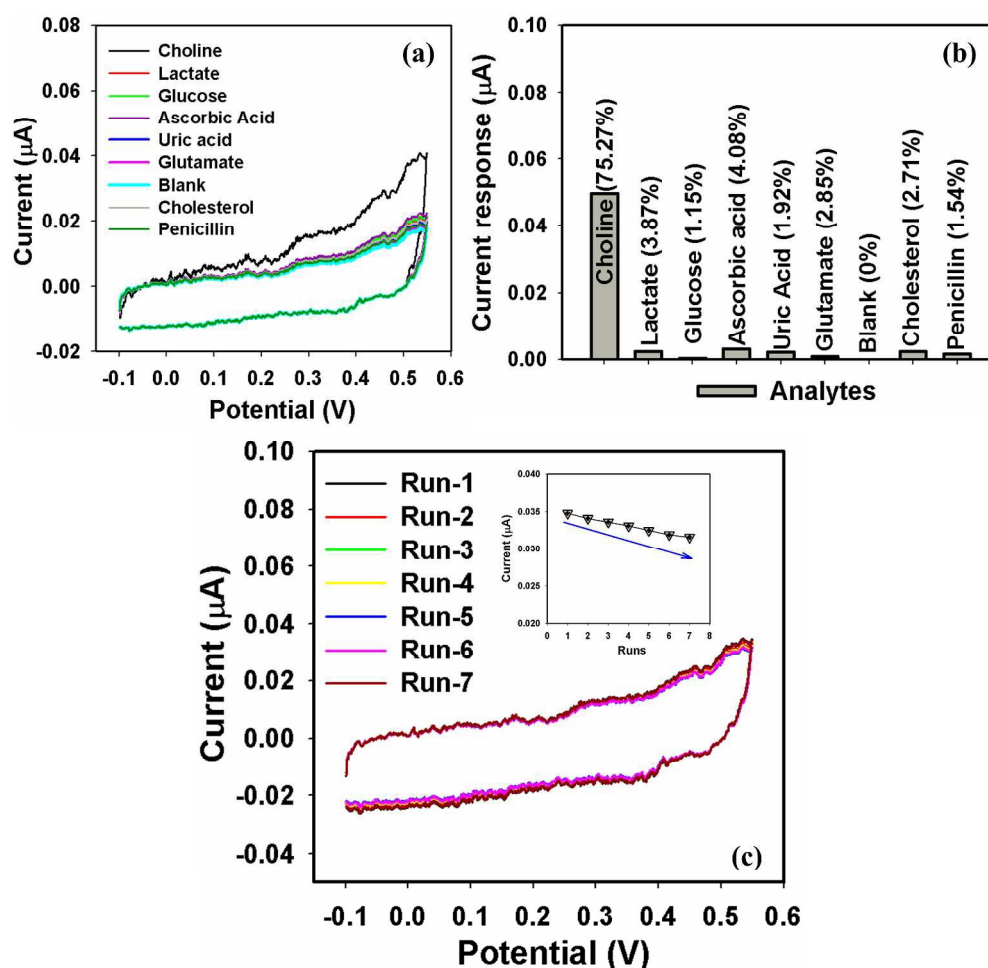


Figure 4. Electrochemical responses of (a) variation of analyte concentration (0.1 M PBS system), (b) calibration plot (at 0.5 V), and (inset) linearity of ChO_x fabricated electrode on micro-chip at room conditions. Analytes: 0.05 nM to 50.0 μM ; Scan rate: 0.1 V/s; Reference electrode: Ag/AgCl (sat. KCl).

The selectivity (interference-effect) of the ChO_x/TGA/Au-chip sensor is evaluated by voltammogram in the presence of other electro-active species for choline. It was also investigated the sensing selectivity performances (interferences) with other biological stuffs like lactate, glucose, ascorbic acid, uric acid, glutamate, cholesterol, and penicillin etc (Figure 5a). No significant current response is found, when 0.1 μM lactate, glucose, ascorbic acid, uric acid, glutamate, cholesterol, and penicillin were added into the 0.1 M PBS buffer. But when 0.1 μM Ch solution injected to the electrolyte solution, a clear current response is executed, indicating the selective detection of Ch with the ChO_x/TGA/Au-chip sensor layer. At this concentration level, lactate, glucose, ascorbic acid, uric acid, glutamate, cholesterol, and penicillin have no significant interference for 0.1 μM Ch detection. Choline exhibited the higher current response by I-V system using ChO_x/TGA fabricated micro-chip electrodes compared in the similar

1 phenomena of reported biosensors [29-38]. By calculating the percentile of current responses
 2 each chemical, Ch exhibited the maximum current response by I-V system using
 3 ChOx/TGA/AuE-chips compared to others biological stuffs, which is presented in Figure 5b. By
 4 deducting the current value of blank solution (at +0.55 V), it is found the current value is less
 5 than 5% for all chemicals (lactate 3.8%, glucose 4.52%, ascorbic acid 4.08%, uric acid 1.92%,
 6 glutamate 2.85%, blank 0%, cholesterol 2.71%, and penicillin 1.54%) compared to target choline
 7 (75.27%). It is specific and selective towards choline compared to all other chemicals with
 8 ChOx/TGA-SAM/AuE-chip sensor in phosphate buffer system.



9
 10 **Figure 5.** I-V responses of ChOx/TGA/ μ -chips are presented for choline biosensors (a)
 11 selectivity study, and (b) percentile of analytes responses by subtracting the blank current

1 responses (at +0.55 V), and (c) reproducibility study. Ch and other chemicals concentration are
2 taken as 0.1 μM for selectivity study. Delay time: 1.0 s. PBP was taken 0.1 M (pH = 7.2);
3 Potential range: -0.1 to +0.55 V.

4 To investigate the reproducibility and storage stabilities, I-V response for ChOx/TGA/ μ -
5 chips sensor was examined (up to 2 weeks). After each experiment, the fabricated ChOx/TGA/ μ -
6 chips substrate was washed gently and observed that the current response was not significantly
7 decreased (Figure 5c). A series of five successive measurements of 0.1 μM Ch in solution
8 yielded a good reproducible signal at ChOx/TGA/ μ -chips sensor in different conditions with a
9 relative standard deviation (RSD) of 2.5%. The sensitivity was retained almost same of initial
10 sensitivity up to week (1st to 2nd week), after that the response of the fabricated ChOx/TGA/ μ -
11 chips gradually decreased. The sensor-to-sensor and run-to-run repeatability for 0.1 μM Ch
12 detection were found to be 1.5% using ChOx/TGA/ μ -chips. Current loss of each run-to-run (1 to
13 6th run) is calculated and presented in the Figure 5c (inset). There is not any significance loss of
14 the sensor signal with the run-to-run variations. To investigate the long-term storage stabilities,
15 the response for the ChOx/TGA/ μ -chips sensor was determined with the respect to the storing
16 time. The long-term storing stability of the ChOx/TGA/ μ -chips sensor was investigated
17 significantly at room conditions. The sensitivity retained 92% of initial sensitivity for several
18 days. The above results clearly suggested that the fabricated sensor can be used for several days
19 without any significant loss in sensitivity. The dynamic response (0.05 nM to 10.0 μM) of the
20 sensor was investigated from the practical concentration variation curve. The sensor response
21 time is mentioned and investigated using this sensor system at room conditions. The
22 performances of ChOx/TGA-SAM/AuE micro-chip sensor are investigated for Ch in presence of
23 common interfering objectives in normal physiological levels. The analytical parameters of the

1 constructed Ch biosensors are relatively better than previously reported biosensors [39-47],
 2 based on various methods and materials modified electrodes presented in **Table 2**.

3
 4 **Table 2:** Comparison of the analytical performances of some Ch biosensors fabricated based on
 5 different materials and modification on electrode surfaces by electrochemical approaches.

Materials/Methods	LDR	LOD	Sensitivity	Response time	Linearity, r^2	Ref.
Multienzymes-ChOx/pmPD/Pt/CV	0~100 μ M	0.33±0.09 μ M	---	<1 s	0.997	[39]
Polymer/Pt/DAB/ChOx Amperometry probe	0.03±0.11 mM	0.3 μ mol/L	---	15s	0.9975	[40]
ChOx/ α -MnO ₂ /Chit/GC/CV	2.0×10^{-6} ~5.8 $\times 10^{-4}$ M	1.0 μ M	---	25 s	---	[41]
ChOx/Polyacrylamide Microgels/Etd	2×10^{-5} M to 2×10^{-4} M	8.0 μ M	1.745 mA M ⁻¹ cm ⁻²	---	0.9944	[42]
Fe ₃ O ₄ magnetic NPs/SWV	10^{-9} to 10^{-2} M	0.1 nM	---	---	0.995	[43]
Os-gel-HRP/CHOx/CV	10^{-7} ~ 10^{-5} mol/L	10^{-8} mol/L	100 μ mol/L/cm ²	---	---	[44]
Ceramic/Microelectrodes	---	0.2 μ M	---	1s	---	[45]
Gold screen-printed/Silica Biocomposites	---	6.0 μ M	6.0 μ AmM ⁻¹	---	---	[46]
Ch-ChOx/Microbiosensors	---	16.0 nM	324±46 nAuM ⁻¹ cm ⁻²	1.4s	---	[47]
Ch-ChOx/TGA-SAM/Chip/CV	0.05nM to 10.0uM	0.012nM	3.5 μAmM⁻¹cm⁻²	~10s	0.9938	Current work

1

2 Conclusions:

3 Successful fabrication of sensitive Ch biosensor based on the immobilization of ChOx on
4 TGA modified micro-chip has been investigated using mediator-free system. Sensor chips are
5 constructed by photolithographic technique, which feasible to detect in micro-level of target Ch
6 analyte. The analytical performances of the fabricated Ch-ChOx/TGA/ μ -chips sensors are
7 excellent in terms of sensitivity, detection limit, linear dynamic ranges, and in short response
8 time. ChOx/TGA/ μ -chip is exhibited higher-sensitivity ($\sim 3.5 \mu\text{A}\mu\text{M}^{-1}\text{cm}^{-2}$) and lower-detection
9 limit ($\sim 0.012 \pm 0.005 \text{ nM}$) with good linearity in short response time, which efficiently utilized
10 as biosensor for Ch-detection onto tiny ChOx/TGA/ μ -chips. The simple fabrication technique of
11 the biosensor has many advantages such as ease of fabrication, enhanced electro-catalysis, and
12 efficiently preserving the activity of Ch biomolecules. It would have potential applications in Ch
13 determination in biomedical and health care fields.

14

15 Acknowledgements:

16 Author is thankful to Center of Excellence for Advanced Materials (CEAMR) and
17 Chemistry department, King Abdulaziz University, Saudi Arabia. Toyohashi University of
18 Technology, Japan is also highly acknowledged for fabrication of devices.

19

20

21

22

23

24

25

1
2
3 **1 References:**
4

- 5
6
7 2 1. G. Panfili, P. Manzi, D. Compagnone, L. Scarciglia, G. Palleschi. Rapid Assay of
8
9 3 Choline in Foods Using Microwave Hydrolysis and a Choline Biosensor. *J. Agric. Food*
10
11 4 *Chem.* 2000, 48, 3403-3407.
12
13 5 2. S.J. Fomon, D.B.B. McCormik, Vitamins and choline. In *Nutrition of Normal Infants*;
14
15 6 Fomon, S. J., Ed.; Mosby: St. Louis, MO, 1993.
16
17 7 3. G. Panfili, P.J. Manzi, *Agric. Food Chem.* 2000, 48, 3403–3404.
18
19 8 4. E.M. Cornford, L.D. Braun, W.H. Oldendorf, *J. Neurochem.* 1978, 30, 299–308.
20
21 9 5. Y.H.; Bai, Y.; Du, J.J.; Xu, H.Y. Chen, *Electrochem. Commun.* 2007, 9, 2611–2616.
22
23 10 6. G.P. Zaloga, M.D. Bortenschlager, *Vitamins*. In *Nutrition in Critical Care*; Mosby-Year
24
25 11 *Book*: St. Louis, MO, 1994.
26
27 12 7. M.M. Chan. Choline. In *Handbook of Vitamins*; Machlin, L. J., Ed.; Dekker: New York,
28
29 13 1984; p 11.
30
31 14 8. E. Farber, A.K. Ghoshal, Lipotropes and chemical carcinogenesis. In *Nutrition and*
32
33 15 *Chemical Toxicity*; Costas, I., Ed. Wiley: Baffins Lane, U.K., 1998.
34
35 16 9. M. Alkondon, E.F. Pereira, W.S. Cortes, A. Maelicke, E.X. Albuquerque, *Eur. J.*
36
37 17 *Neurosci.* 1997, 9, 2734-2742.
38
39 18 10. D. Jerusalinsky, E. Kornisiuk, I. Izquierdo, *Neurochem. Res.* 1997, 22, 507-515.
40
41 19 11. J.A. Dani, D. Ji, F. M. Zhou, *Neuron.* 2001, 31, 349-352.
42
43 20 12. P. Kasa, Z. Rakonczay, K. Gulya, *Prog. Neurobiol.* 1997, 52, 511-535.
44
45 21 13. J. Lemiere, D.V. Gool, R. Dom, *Acta Neurol. Belg.* 1999, 99, 96-106.
46
47 22 14. R. Lenigk, E. Lam, A. Lai, H. Wang, Y. Han, P. Carlier, R. Renneberg, *Biosens.*
48
49 23 *Bioelectron.* 2000, 15, 541-547.
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3
4 1 15. M.G.; Garguilo, N. Huynh, A. Proctor, A.C. Michael, *Anal. Chem.* 1993, 65, 523-528.
- 5
6 2 16. J. Cui, N.V. Kulagina, A.C. Michael, *J. Neurosci. Methods* 2001, 104, 183-189.
- 7
8 3 17. A. Curulli, S. Dragulescu, C. Cremisini, G. Palleschi, *Electroanalysis* 2001, 13, 236-242.
- 9
10 4 18. M. Nakano, H. Tateishi-Karimata, S. Tanaka, N. Sugimoto. Choline Ion Interactions with
11 DNA Atoms Explain Unique Stabilization of A–T Base Pairs in DNA Duplexes: A
12 Microscopic View. *J. Phys. Chem. B* 2014, 118, 379–389.
- 13
14 5
15 6
16
17 7 19. S.H. Zeisel, K.A. da-Costa, Choline: An Essential Nutrient for Public Health. *Nutr. Rev.*
18 2009, 67, 615–623.
- 19
20 8
21
22 9 20. R. Vijayaraghavan, A. Izgorodin, V. Ganesh, M. Surianarayanan, D.R. MacFarlane,
23 Long-Term Structural and Chemical Stability of DNA in Hydrated Ionic Liquids. *Angew.*
24 *Chem., Int. Ed. Engl.* 2010, 49, 1631–1633.
- 25
26 10
27 11 21. A. Chandran, D. Ghoshdastidar, S. Senapati, Groove Binding Mechanism of Ionic
28 Liquids: A Key Factor in Long-Term Stability of DNA in Hydrated Ionic Liquids? *J. Am.*
29 *Chem. Soc.* 2012, 134, 20330–20339.
- 30
31 12
32 13
33 14
34 15 22. K. Fujita, D.R. MacFarlane, M. Forsyth, Protein Solubilising and Stabilising Ionic
35 Liquids. *Chem. Commun.* 2005, 38, 4804–4806.
- 36
37 16
38 17 23. J.D. Keighron, S. Åkesson, A.S. Cans. Coimmobilization of Acetylcholinesterase and
39 Choline Oxidase on Gold Nanoparticles: Stoichiometry, Activity, and Reaction
40 Efficiency. *Langmuir* 2014, 30, 11348–11355.
- 41
42 18
43 19 24. M.M. Rahman, A.M. Asiri. One-step electrochemical detection of cholesterol in presence
44 of suitable $K_3Fe(CN)_6$ /phosphate buffer mediator by an electrochemical approach.
45 *Talanta* 2015, 140, 96-101.
- 46
47 20
48 21
49 22
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3 1 25. M.M. Rahman. Development of mediator-free acetylcholine sensor co-immobilized with
4
5 2 acetylcholine oxidase using micro-chips. *Current Proteomics* 2012, 9, 272-279.
6
7
8 3 26. K.L. Baker, F.B. Bolger, J.P. Lowry. A microelectrochemical biosensor for real-time in
9
10 4 vivo monitoring of brain extracellular choline. *Analyst* 2014, 140, 3738-3745.
11
12 5 27. M.S. Dehlawi, A.T. Eldefrawi, M.E. Eldefuawi, N.A. Anis, J.J. Valdes. Choline
13
14 6 Derivatives and Sodium Fluoride Protect Acetylcholinesterase against Irreversible
15
16 7 Inhibition and Aging by DFP and Paraoxon. *J. Biochem. Toxicology* 1994, 9, 261-268.
17
18 8 28. M.M. Rahman. Reusable and mediator-free cholesterol biosensor based on cholesterol
19
20 9 Oxidase immobilized onto TGA-SAM modified smart bio-chips. *PLOS ONE* 2014, 9,
21
22 10 e100327.
23
24
25 11 29. Z. Zhang, X. Wang, X. Yang. A sensitive choline biosensor
26
27 12 using Fe₃O₄ magnetic nanoparticles as peroxidase mimics. *Analyst*, 2011, 136, 4960-
28
29 13 4965.
30
31
32 14 30. K.L. Baker, F.B. Bolger, J.P. Lowry. A microelectrochemical biosensor for real-time in
33
34 15 vivo monitoring of brain extracellular choline. *Analyst*, 2015, 140, 3738-3745.
35
36
37 16 31. M.M. Rahman, A.M. Asiri. Development of Penicillin G biosensor based on Penicillinase
38
39 17 enzymes immobilized biochips. *Biomed. Microdev.* 2015, 17, 9.
40
41
42 18 32. A. Guerrieri, F. Palmisano. An Acetylcholinesterase/Choline Oxidase-Based
43
44 19 Amperometric Biosensor as a Liquid Chromatography Detector for Acetylcholine
45
46 20 and Choline Determination in Brain Tissue Homogenates. *Anal. Chem.* 2001, 73, 2875–
47
48 21 2882.
49
50
51 22 33. M.M. Rahman. Fabrication of Mediator-free Glutamate Sensors Based on Glutamate
52
53 23 Oxidase using Smart Micro-devices. *J. Biomed. Nanotech.* 2011, 7, 351-357.
54
55
56
57
58
59
60

- 1
2
3
4 1 34. S. Pati, F. Palmisano, M. Quinto, P.G. Zambonin. Quantitation of
5
6 2 Major Choline Fractions in Milk and Dietary Supplements Using a Phospholipase D
7
8 3 Bioreactor Coupled to a Choline Amperometric Biosensor . J. Agric. Food Chem. 2005,
9
10 4 53, 6974–6979.
11
12 5 35. M.M. Rahman. Fabrication of L-lactate biosensor based on redox species mediated
13
14 6 lactate oxidase using micro-device. Inter. J. Biolog. Med. Res. 2010, 1, 9-14.
15
16 7 36. G. Panfili, P. Manzi, D. Compagnone, L. Scarciglia, G. Palleschi. Rapid
17
18 8 Assay of Choline in Foods Using Microwave Hydrolysis and a Choline Biosensor. J.
19
20 9 Agric. Food Chem. 2000, 48, 3403–3407.
21
22 10 37. M.M. Rahman, A. Umar, K. Sawada. Development of Amperometric Glucose Biosensor
23
24 11 Based on Glucose Oxidase Enzyme Immobilized with Multi-Walled Carbon Nanotubes
25
26 12 at Low Potential. Sens. Actuator. B Chem. 2009, 137, 327-333.
27
28 13 38. J. Wang, G. Liu, Y. Lin. Amperometric choline biosensor fabricated through electrostatic
29
30 14 assembly of bienzyme/polyelectrolyte hybrid layers on carbon nanotubes. Analyst, 2006,
31
32 15 131, 477-483.
33
34 16 39. K.M. Mitchell. Acetylcholine and Choline Amperometric Enzyme Sensors Characterized
35
36 17 in Vitro and in Vivo. Anal. Chem. 2004, 76, 1098-110.
37
38 18 40. A. Curulli, S. Dragulescu, C. Cremisini, G. Palleschi. Bienzyme Amperometric Probes
39
40 19 for Choline and Choline Esters Assembled with Nonconducting Electrosynthesized
41
42 20 Polymers. Electroanalysis 2001, 13, 236-242.
43
44 21 41. Y.H. Bai, H. Zhang, J.J. Xu, H.Y. Chen. Relationship between Nanostructure and
45
46 22 Electrochemical/Biosensing Properties of MnO₂ Nanomaterials for H₂O₂/Choline. J.
47
48 23 Phys. Chem. C 2008, 112, 18984–18990.
49
50
51
52
53
54
55
56
57
58
59
60

- 1
2
3
4 1 42. M. S.P. Lopez, J.P.H. Perez, E. Lopez-Cabarcos, B. Lopez-Ruiz. Amperometric
5
6 2 Biosensors Based on Choline Oxidase Entrapped in Polyacrylaide Microgels.
7
8 3 Electroanalysis 19, 2007, No. 2-3, 370 – 378.
9
10 4 43. Z. Zhang, X. Wang, X. Yang. A sensitive choline biosensor using Fe₃O₄ magnetic
11
12 5 nanoparticles as peroxidase mimics. Analyst, 2011, 136, 4960-4965.
13
14 6 44. G. Shi, K. Yamamoto, T. Zhou, F. Xu, T. Kato, J. Ji-ye, L. Jin. On-line biosensors for
15
16 7 simultaneous determination of glucose, choline, and glutamate integrated with a
17
18 8 Microseparate ion system. Electrophoresis 2003, 24, 3266–3272.
19
20 9 45. J.J. Burmeister, F. Pomerleau, P. Huettl, C.R. Gash, C.E. Werner, J.P. Bruno, G.A.
21
22 10 Gerhardt. Ceramic-based multisite microelectrode arrays for simultaneous measures of
23
24 11 choline and acetylcholine in CNS. Biosens. Bioelectron. 2008, 23, 1382-1389.
25
26 12 46. I. Mazurenko, O. Tananaiko, O. Biloivan, M. Zhybak, I. Pelyak, V. Zaitsev, M. Etienne,
27
28 13 A. Walcarius. Amperometric biosensor for choline based on gold screen-printed electrode
29
30 14 modified with electrochemically-deposited silica biocomposite. Electroanalysis. 2015.
31
32 15 27, 1 – 9.
33
34 16 47. R.M. Santos, J. Laranjinha, R.M. Barbosa, A. Sirota, Simultaneous measurement of
35
36 17 cholinergic tone and neuronal network dynamics in vivo in the rat brain using a novel
37
38 18 choline oxidase based electrochemical biosensor. Biosens. Bioelectron. 2015, 69, 83-94.
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Graphical Abstract:1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60