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An Improved Amperometric Acetylcholine Biosensor by Integrating Enzyme Nanoparticles with Graphene Oxide Nanoparticles

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ABSTRACT

An improved amperometric acetylcholine (ACh) biosensor was developed by co-immobilizing enzyme nanoparticles (ENPs) i.e. acetylcholine esterase (AChENPs) and choline oxidase (ChONPs) onto graphene oxide nanoparticles (GrONPs) electrodeposited onto pencil graphite electrode (PGE). The GrONPs were prepared from graphite rod and characterized by cyclic voltammetry (CV) transmission electron microscopy (TEM), Fourier-transform infrared spectroscopy (FTIR), UV and visible spectroscopy. ENPs/GrONPs/PGE was studied by scanning electron microscopy (SEM) and FTIR at different stages of its construction. The biosensor showed optimum current response at an applied potential of 0.5V within 2s at pH 7.0 and 30˚C in acetylcholine concentration range, 0.001µM to 1000µM, with a detection limit (LOD) of 0.001 µM. The analytical recovery of added acetylcholine in serum, as measured by this bio sensor was 98%. Within and between batch coefficient of variations were 0.04% and 0.06%, respectively. A good correlation (R2 = 0.989) was found between sera ACh level measured by standard enzymic colorimetric method and the present biosensor. The biosensor measured ACh level in sera of apparently healthy persons and patients suffering from Alzheimer's. The working electrode lost 25% of its initial activity, over 240 days, when stored dry at 4˚C.

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Introduction

Acetylcholine (ACh) is an important neurotransmitter, which is present at neuromuscular junction (synapse) in human body and required to send signals to other cell types in both central and peripheral nervous system [1]. The biosynthesis of acetylcholine occurs at nerve ending, from acetyl coenzyme A and choline. Various disorders like cognition, memory, sleep, focus, and learning capacity are affected by ACh level [2]. Evidences showed that ACh has a role in diseases like Alzheimer's, Parkinson's disease, as well as paranoid schizophrenia [3]. As compared to various conventional methods available for detection of ACh, biosensing methods are comparatively more simple, sensitive, specific and rapid for detection of Ach in neurological disorders [4, 5]. In enzyme-based biosensors, the direct immobilization of native enzymes on electrodes and nanomaterials may cause their denaturation and thus affect activity. This problem was solved by aggregating enzyme molecules into nanoparticles. The use of enzyme nanoparticles (ENPs) is more effective than native enzyme in enzyme biosensors, due to their large surface area, unique catalytic and thermal properties [6-11].

Graphene nanoparticles (GrONPs) due to their exceptional mechanical, electrical, optical, and thermal characteristics are perfect for a range of biological sensing applications [12]. Few reports are available on the use of GrONPs in the improvement in of analytic performance of glycerol, lactate and L- lysine biosensors [12-14] respectively, due to their large surface area, thermal stability, mechanical properties, conducting properties, electrochemical behaviour [15].

We have reported recently an amperometric acetylcholine (Ach) biosensor based on co-immobilization of nanoparticles of acetylcholine esterase (AChENPs) and choline oxidase (ChONPs) onto nanocomposite of platinum nanoparticles (PtNPs) and grapheme oxide nanosheets (GrONS) electrodeposited onto pencil graphite electrode (PGE) [16]. However the synthesis of PtNPs produce toxic chemicals, which are hazardous to human health and environment. Further, it requires chloroplatinic acid hexahydrate, which has a high cost [16]. In the present work, we have replaced PtNPs-GrONS nanocomposite by GrONPs to get the improvement in analytical properties of Ach biosensor.

Materials and Methods

Reagents and Materials

Purified acetylcholine esterase (EC 3.1.1.7) from electric eel and choline oxidase (EC 1.1.3.17) from Alcaligenes sp.,

glutaraldehyde, horseradish peroxidase (HRP), 4-aminoantipyrine, and were purchased from Sigma Aldrich (St. Louis, MO, USA). Graphite powder, acetylcholine chloride, sodium nitrate ($NaNO_3$), sulphuric acid 98% (H_2SO_4), potassium permanganate ($KMnO_4$), hydrogen peroxide (H_2O_2), hydrochloric acid 5% (HCl) were from SRL, Mumbai (India). A 6B pencil with a graphite lead of 2mm diameter was from local market. The left over/unused sera samples of apparently healthy persons and Alzheimer's patients were collected from the hospital of Pt. Bhagwat Dayal Sharma Post Graduate Institute of Medical Sciences (PGIMS), Rohtak, which had its ethical clearance for collecting human sera and used for ACh determination. The double distilled water (DW) and analytical reagent (AR) grade chemicals were used throughout the study.

Techniques

Fourier transform infra-red (FTIR) spectrophotometer by Bruker was used to study the chemical bonds and functional groups present in the synthesized nanoparticles. UV-vis spectrophotometer (Lab India Analytical, UV 3092) was used for recording UV-Visible spectra. Transmission electron microscope (TEM) manufactured by JEOL 2100 F was used to scan the morphology and size of the nanoparticles. Scanning electron microscope (SEM) by Zeiss EV040, USA was used to observe the surface structure of the nanoparticles. A potentiostat instrument by Autolab, Model: AUT83785 and contrived by Ecochemie, The Netherlands was used for electrochemical analysis. The instrument consisted of three electrodes, including a platinum wire as an auxiliary electrode, an Ag/AgCl electrode as a reference electrode, and AChENPs/ChONPs/GrONPs/PGE as a working electrode.

Preparation of Graphene Oxide Nanoparticles (GrONPs)

GrONPs were synthesized by Hummer's method with modification. A 6B pencil graphite powder was used as the starting material. The synthesis process involved the mixing of 0.5g of graphite powder, 0.5g of sodium nitrate ($NaNO_3$) and 25 ml of concentrated sulphuric acid (H_2SO_4) in a 500 ml reaction flask under continuous stirring for 15 minutes in an ice water bath. Then, 4.0g of potassium permanganate ($KMnO_4$) was added slowly to the reaction mixture within 15 minutes at room temperature (20°C). The mixture was then stirred for 90 minutes at 40°C in a water bath, while 20 ml of distilled water (DW) was added slowly. After 90 minutes, a brown-coloured suspension was formed, which was treated with 6 ml of 30% hydrogen peroxide (H_2O_2) solution. The resulting mixture was washed with 5% hydrochloric acid (HCl) and DW to remove excess manganese (Mn) until the suspension reaches neutrality. The purified GO suspension was then dried at 60°C for 24 hours in an oven. Thus, graphite was oxidized to GrONPs using a strong oxidizing agent and acid mixture.

Preparation of AChENPs/ChONPs

AChENPs and ChONPs were prepared by desolvation method and characterized as described Ref. [16].

Characterization of AChENPs/ChONPs and GrONPs

The characterization of AChENPs/ChONPs and GrONPs was carried out using TEM, UV-vis and FTIR spectroscopy.

Electrodeposition of GrONPs onto PG Electrode

The PG electrode (2cm x 5mm) was polished manually with alumina slurry using a polishing cloth. The polished electrode was washed systematically with DW to remove any residual particles. The cleaned electrode was then immersed into 4 ml of piranha solution (3 parts concentrated sulphuric acid (H_2SO_4) and 1-part hydrogen peroxide (H_2O_2)) for 10 minutes. After the piranha treatment, the electrode was washed with DW to remove any remaining piranha solution. GrONPs (200 µl) were added to 25 ml of 5 mM $K_3Fe(CN)_6$/$K_4Fe(CN)6$ (1:1) solution. GrONPs were electrodeposited onto the PG electrode by operating 5 polymerization cycles at a potential range of -1.0V to +1.0V as shown in Figure 1. This process involved the use of an electrochemical cell system i.e. the Autolab instrument.

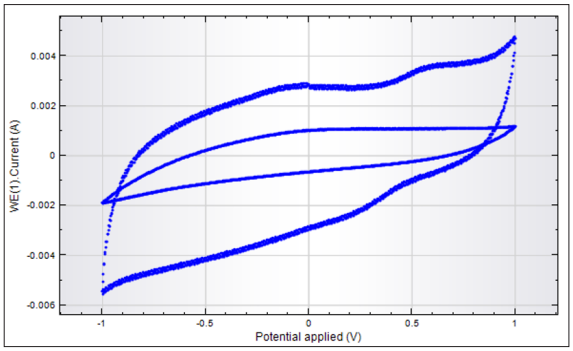


Figure 1: Cyclic Voltamogram (CVs) of bare PGE and Electro Deposition of GrONPs onto PG Electrode. Supporting Electrolyte: 25 ml of 5 mM K3Fe (CN)6/K4Fe (CN)6 (1:1) Solution; Scan Rate: 25 mV/s. (PG= Pencil Graphite), GONPs= Graphene Oxide Nanoparticles)

Co-Immobilization of AChENPs and ChONPs onto GrONPs/PG Electrode:

The resulting GrONPs/PG electrode was rinsed with DW and excess of unbound GrONPs was removed using a DW sponge. The GrONPs/PG electrode was immersed in 4 ml of AChENPs and ChONPs suspension for 24 hours at 4°C. After immobilization, the AChENPs/ChONPs/GrONPs/PG electrode was washed 3-4 times with DW to remove any unbound AChENPs and ChONPs (Figure 2).

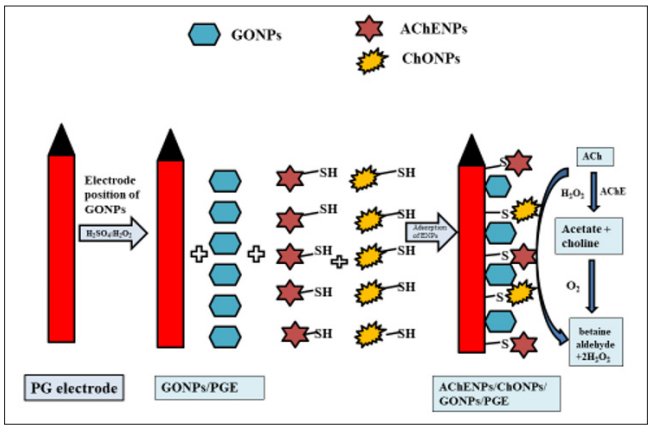


Figure 2: Diagrammatic Representation of AChENPs/ChONPs/GrONPs/PG Electrode based Acetylcoline Biosensor. (AChENPs= Acetylcholine Esterase Nanoparticles; ChONPs= Choline Oxidase Nanoparticles; GrONPs= Graphene Oxide Nanoparticles; PG= Pencil Graphite)

Characterization of Working Electrode

The AChENPs/ChONPs/GrONPs/PG electrode was used as the working electrode for further experiments. The electrode was characterized by SEM and FTIR spectroscopy before and after the immobilization of AChENPs and ChONPs. The resulting modified electrode (AChENPs/ChONPs/GrONPs/PGE) provided a platform for electrochemical analysis and bio sensing applications.

Construction of Acetylcholine Biosensor and its Response Measurements

Three electrode cell systems consisting AChENPs/ChONPs/GrONPs/PG as working electrode, silver/silver chloride (Ag/AgCl) as reference electrode and Pt wire as auxiliary electrode was used as an amperometric acetylcholine biosensor set up. All three electrodes were attached by potentiostat/galvanostat.

Optimization of Acetylcholine Biosensor

The kinetic properties like pH, optimum temperature, and acetylcholine concentration of the AChENPs/ChONPs/GrONPs/PG electrode were studied to optimize the optimum working conditions of the acetylcholine biosensor. To determine the optimum pH, the pH range of 6.0 to 9.0 was investigated using suitable buffers with a strength of 0.1 M. The biosensor response was measured at different pH intervals of 0.5. Similarly, to determine the optimum temperature and incubation time, which includes the buffers and substrate, was incubated at different temperatures ranging from 20°C to 50°C. The incubation time was varied from 1 to 10 seconds. The effect of acetylcholine concentration on the biosensor response was determined by varying the concentration of acetylcholine in the range of 0.001µM to 1000 µM.

Results and Discussion

Characterisation of GrONPs by TEM and FTIR

The size of GrONPs was in the range of 1-100 nm as measured by TEM (Figure 3). UV and visible spectra of GONPs showed absorbance peak at 230 nm (Figure 4). The FTIR analysis of GrONPs showed distinctive peaks at 3317.91 and 3355.97 which corresponds to N-H, functional group, 2334.19, 2367.56, and 2398.69 cm^{-1} corresponds to C≡C bonds, 10 and 1635.55 cm^{-1} corresponding to different functional groups C=C bonds as shown in figure (Figure 5).

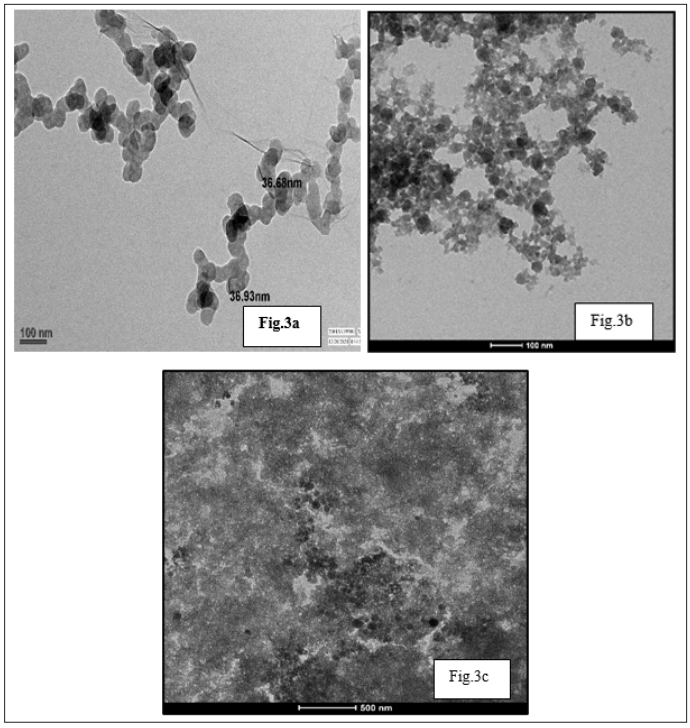


Figure 3: Transmission Electron Microscope (TEM) Images, of (a) GONPs, (b) AChENPs and (c) ChONPs. (AChENPs= Acetylcholine Esterase Nanoparticles; ChONPs= Choline Oxidase Nanoparticles; GONPs= Graphene Oxide Nanoparticles)

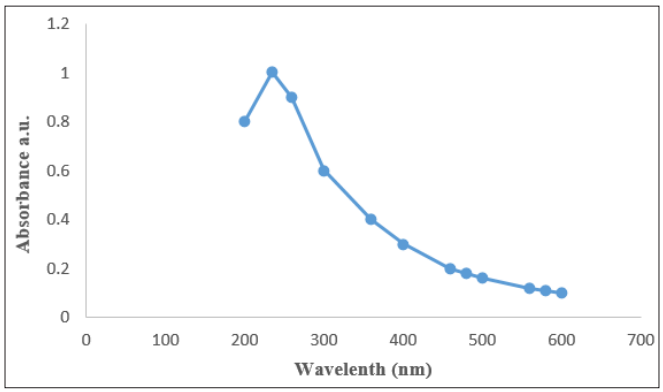


Figure 4: UV-Visible Spectra of GrONPs Showing Absorbance Peak at 235nm Wavelength. (GrONPs= Graphene Oxide Nanoparticles)

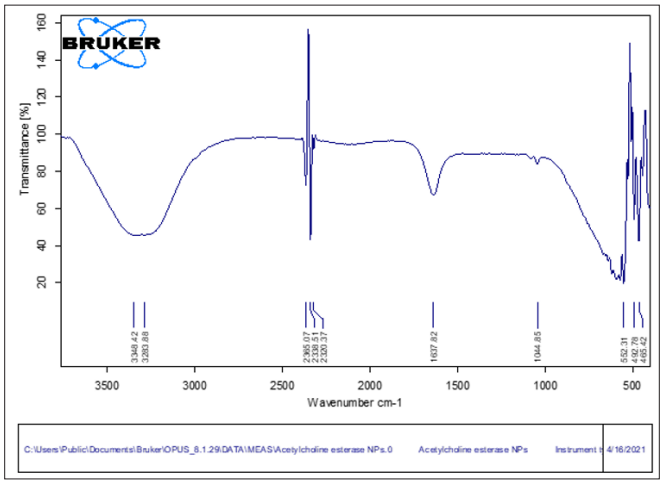


Figure 5a: Fourier Transforms Infra-Red Spectroscopy (FTIR) of (a) GrONPs and ChONPs. (AChENPs= Acetylcholine Esterase Nanoparticles; ChONPs= Choline Oxidase Nanoparticles; GONPs= Graphene Oxide Nanoparticles)

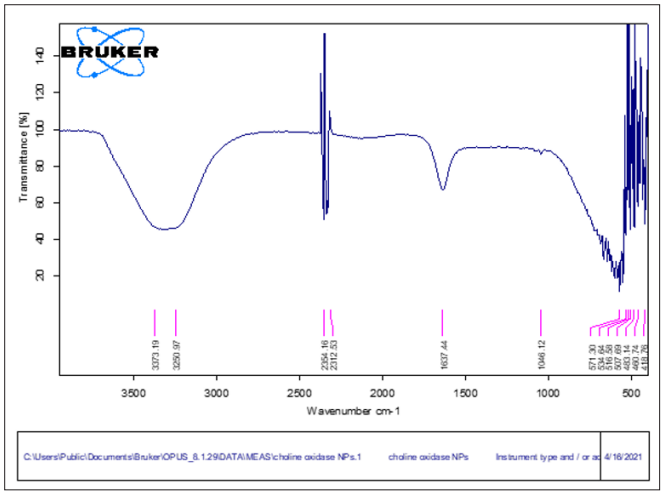


Figure 5b: Fourier Transforms Infra-Red Spectroscopy (FTIR) of (b) AChENPs and ChONPs. (AChENPs= Acetylcholine Esterase Nanoparticles; ChONPs= Choline Oxidase Nanoparticles; GONPs= Graphene Oxide Nanoparticles)

Characterization of AChENPs/ChONPs/GrONPs/PGE by SEM and FTIR Spectroscopy

The SEM image of the bare PG electrode showed a smooth and flat morphology, indicating the surface of the electrode without

any modifications or adsorbed materials (Figure 6a).In contrast, the SEM image of the AChENPs/ChONPs/GrONPs/PGE revealed a globular structural morphology due to the presence of enzyme nanoparticles (AChENPs and ChONPs) that were clearly adsorbed onto the surface of the transparent PGE as showed in Figure 6b. Earlier immobilisation of ENPs on PGE has been reported [Malik et al.,]. Due to electrodeposition of GrONPs on PGE, the surface area was increased followed by increased entrapment of ENPs onto modified PGE, which improved the electrochemical performance and sensitivity of the biosensor. The bare PGE showed peaks at 1025.43cm^{-1} and 1363.49 (C = C bonding), 2158.17 cm^{-1} and 2633.96 cm^{-1} (C≡C extending), and 3224.39cm^{-1} (C-H bonding), as shown in Figure 7a. The GONPs/PG electrode showed bands at 997.29cm^{-1}and 1473.60 (C = C stretching) cm^{-1}, 2135.35 cm^{-1}(C≡C stretching) and 2909.08 cm^{-1} (C-H bonding) 3229.98 cm^{-1}, and 3519 cm^{-1} (O-H bonding) (Figure 7b). The AChENPs/ChONPs/GrONPs/PG electrode showed various bonds at different peaks of 1236–1955 cm^{-1}, 2127–2871cm^{-1} and 1520-1894 cm^{-1} and 965-1454 cm^{-1} which confirms the presence of enzyme nanoparticles (Figure 7c).

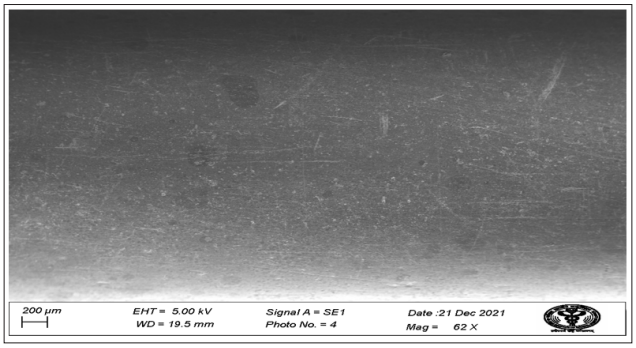


Figure 6a: SEM Images of (a) Bare PG Electrode. (AChENPs= Acetylcholine Esterase Nanoparticles; ChONPs= Choline Oxidase Nanoparticles; GONPs= Graphene Oxide Nanoparticles; PG= Pencil Graphite)

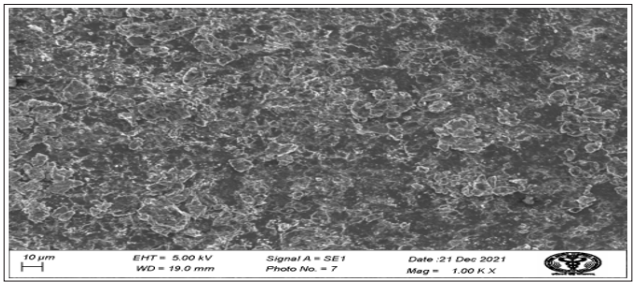


Figure 6b: SEM Images of (b) Electrode. (AChENPs= Acetylcholine Esterase Nanoparticles; ChONPs= Choline Oxidase Nanoparticles; GONPs= Graphene Oxide Nanoparticles; PG= Pencil Graphite)

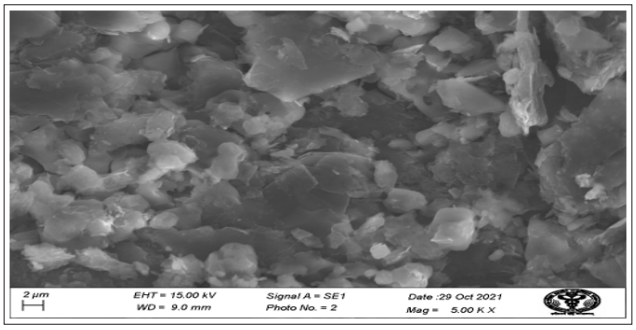


Figure 6c: SEM Images of (c) AChENPs/ChONPs/GrONPs/PG Electrode. (AChENPs= Acetylcholine Esterase Nanoparticles; ChONPs= Choline Oxidase Nanoparticles; GONPs= Graphene Oxide Nanoparticles; PG= Pencil Graphite)

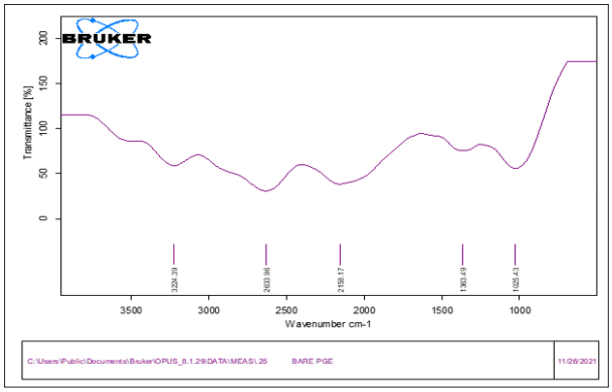


Figure 7a: Fourier Transforms Infra-Red Spectroscopy (FTIR) of (a) Bare PG Electrode and (AChENPs= Acetylcholine Esterase Nanoparticles; ChONPs= Choline Oxidase Nanoparticles; GONPs= Graphene Oxide Nanoparticles; PG= Pencil Graphite)

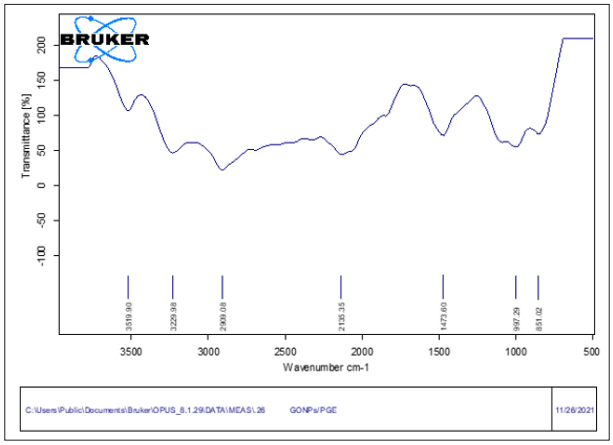


Figure 7b: Fourier Transforms Infra-Red Spectroscopy (FTIR) of (b) Electrode and AChENPs= Acetylcholine Esterase Nanoparticles; ChONPs= Choline Oxidase Nanoparticles; GONPs= Graphene Oxide Nanoparticles; PG= Pencil Graphite)

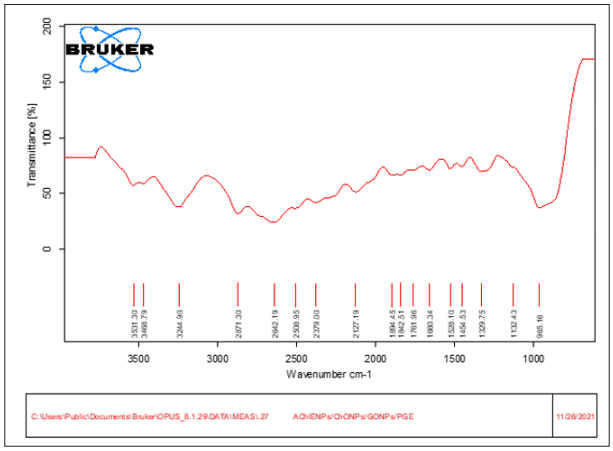
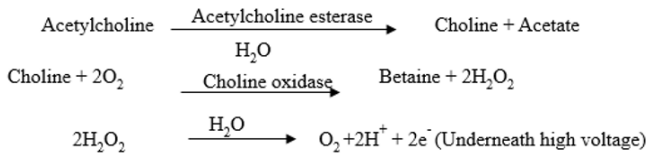


Figure 7c: Fourier Transforms Infra-Red Spectroscopy (FTIR) of (c) AChENPs/ChONPs/GrONPs/PG Electrode. (AChENPs= Acetylcholine Esterase Nanoparticles; ChONPs= Choline Oxidase Nanoparticles; GONPs= Graphene Oxide Nanoparticles; PG= Pencil Graphite)

Current Response Measurement of AChENPs/ChONPs/GrONPs/PG Electrode

The working of the amperometric Ach bio-sensor is based on

the electrochemical oxidation of H_2O_2, which is generated by oxidation of choline by ChONPs, which in turn, is produced from ACh (as AChCl) by AChENPs. The said electrochemical oxidation of H_2O_2 at working electrode generates current, which is measured by Auto lab PGSTAT(Potentiostat). The biosensor utilizes AChENPs ChONPs immobilized on a GrONPs decorated PGE (as working electrode), along with a Pt wire (as counter electrode) and an Ag/AgCl electrode (reference electrode) in a saturated 3.5 M KCl electrolyte solution. Cyclic voltammetry experiments were conducted in a solution containing 25 ml of 5 mM K3Fe (CN)6/K4Fe (CN)6 (1:1) as the redox probe, along with 200 µL of acetylcholine chloride (AChCl) at a concentration of 0.05 M. The applied voltage ranges from -0.1 to +0.1 V, and a scan rate of 20 mV/s is used.The electrochemical oxidation of H_2O_2 occurs at the working electrode generates in this high potential range. Thus, the generated/measured current is directly proportional to the acetylcholine (Ach) concentration in the sample., allowing for the quantitative detection of acetylcholine. By monitoring the generated current, the biosensor provides the ACh concentration in a sample.



Optimization of Acetylcholine Biosensor

The amperometric acetylcholine biosensor response was affected by change in incubation temperature, pH and substrate (Ach) concentration. The optimum current was obtained at pH 7.0 (Figure 8a), temperature 30°C (Figure 8b). The response time was 2s, which is lower than earlier Ach biosensor. The linearity for substrate concentration was 0.001–1000 µM with a limit of detection (LOD) of 0.001 µM, which is better than earlier ACh biosensor, indicating the improvement in the analytic performance of present ACh biosensor (Figure 8c) [16].

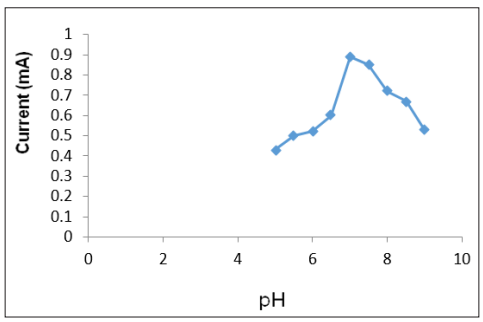


Figure 8a: Effect of pH on AChENPs/ChONPs/GrONPs/PG electrode

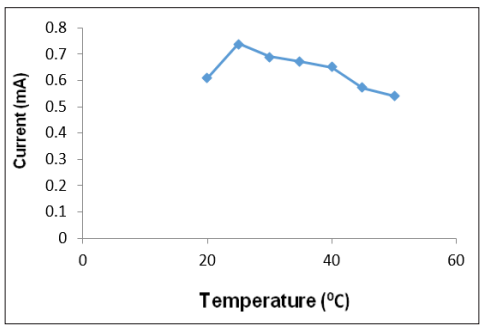


Figure 8b: Effect of Incubation Temperature on AChENPs/ChONPs/GrONPs/PG Electrode

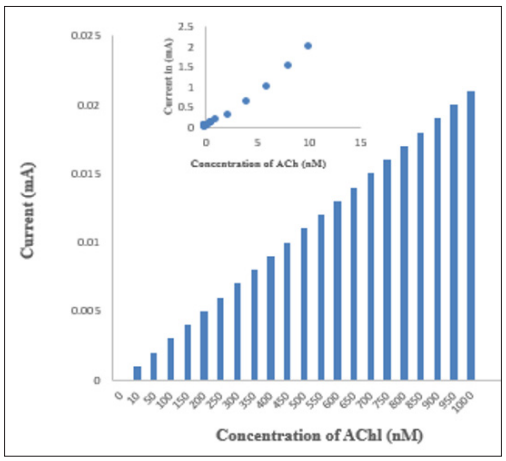


Figure 8c: Standard Curve Ofacetylcholine Concentration by Acetylcholine Biosensor based on AChENPs/ChONPs/GrONPs/PG Electrode

Evaluation of Acetylcholine Biosensor

The performance of this biosensor was evaluated by following parameters.

Reproducibility

Th e analytical recoveries of added (5 nM and 10 nM) acetylcholine concentration were found as 98.39% and 98.23%, respectively (Table 1). The within and between-batch coefficients of variation for the determination of acetylcholine in sera on the same day and after one week of storage were 0.04% and 0.06%, reported. These high precisions highlighted the good reproducibility and consistency of the present biosensor (Table 2). This can be attributed to the electrochemical attraction of ENPs onto GONPs, electrodeposited onto PG electrode.

Table 1: Analytical Recovery of ENG/GrONP/PGE

Acetylcholine added (nM)	Acetylcholine found (nM)	% Recovery
–	6.2	–
5	11.9	98.39 ± 0.3
10	12.4	98.23 ± 0.5

Table 2: Precision Study of ENG/GrONP/PGE

Acetylcholine (nM)	Mean	CV (%)
Within assay		
9.7		
9.8		
9.9	10.06	0.04
10.3		
10.6		
Between assay		
5.1		
5.3		
5.4	5.46	0.06
5.7		
5.8		

Repeatability

The procedure of washing the working electrode with 10 mM, pH 7.0 potassium phosphate buffer (PB) solution is an effective method to remove any residual substances or contaminants from the surface of the electrode, allowing for its reuse in subsequent assays. This step helps to maintain the integrity and functionality of the immobilized enzymes. The loss of 25% of the initial activity of enzyme electrode after its use for more than 6 months on a weekly basis suggests that the GrONPs composite film used in the biosensor has good long-term stability and biocompatibility. This means that the film retains its properties and supports the activity of the immobilized enzyme over an extended period of time, demonstrating its durability and suitability for long-term use. Additionally, storing the enzyme electrode at 4 °C in a dried condition when not in use helps to preserve its biological activity.

Correlation Study

The value of sera Ach levels by present biosensor were correlated with those by standard colorimetric assay and the coefficient of correlation was found R2 = 0.989, which is similar to our earlier study using AChENPs/ChONPs/GONS/PtNPs/PGE [16]. This confirms the reliability of the present Ach biosensor.

Application of Acetylcholine Biosensor in Sera

The ACh content was determined in serum samples (1ml each) obtained from both healthy individuals (n=30) in the age group 25-68 yrs, ranged from 8.1- 12.9 nM and Alzheimer's individuals (n=30) in the age group 25-85yrs, ranged from 1.0-6.2 nM, which is significantly lower ($p<0.01$) in Alzheimers patients compared to apparently healthy individuals (Table 3). Previous study also shows the decreased level of serum ACh in Alzheimer's patients similar to our earlier report [16].

Table 3: Blood sera acetylcholine determination of apparently healthy and Alzheimer's patients by ENG/GrONP/PGE

S.no.	Sex	Age (year)	Apparently healthy persons (nM)	Sex	Age (year)	Alzheimer's patients (nM)
1	M	49	10.3±0.7	M	62	4.0±0.8
2	M	55	10.7±0.3	M	65	4.4±0.5
3	M	47	10.8±0.2	M	81	3.0±0.4
4	M	52	10.6±0.4	F	68	3.5±0.7
5	F	61	9.2±0.5	M	60	4.8±0.6
6	M	40	11.8±0.2	M	46	5.4±0.8
7	F	65	9.0±0.1	M	39	5.7±0.9
8	M	45	9.8±0.3	M	76	1.6±0.5
9	M	50	9.9±0.5	M	83	3.4±0.2
10	F	67	9.9±0.9	M	85	3.1±0.3
11	M	39	12.1±0.4	F	61	3.7±0.5
12	M	34	12.7±0.6	M	78	3.2±0.3
13	M	25	12.9±0.4	F	72	1.0±0.5
14	M	44	9.7±0.5	M	76	3.6±0.4
15	M	39	10.3±0.7	M	66	4.8±0.7
16	M	27	12.7±0.5	M	37	6.2±0.9
17	M	30	12.7±0.3	M	80	3.2±0.5
18	M	37	11.5±0.5	F	62	2.9±0.8
19	M	29	12.7±0.9	M	61	4.2±0.7
20	F	55	10.3±0.8	M	54	6.5±0.6
21	F	24	12.1±0.9	F	25	5.1±0.2
22	F	30	12.5±0.3	F	30	5.7±0.3
23	F	35	11.4±0.6	F	35	5.8±0.6
24	F	40	11.3±0.7	F	40	5.8±0.5
25	F	45	10.8±0.3	F	44	5.3±0.2
26	F	50	10.7±0.5	F	51	4.2±0.2
27	F	60	9.2±0.2	F	56	3.7±0.3
28	M	60	8.8±0.3	F	65	1.6±0.4
29	M	65	8.2±0.6	F	70	1.4±0.6
30	M	68	8.1±0.2	F	78	1.1±0.9

Interference Study of Acetylcholine Biosensor

The interference study conducted by the acetylcholine biosensor demonstrated that interfering components commonly found at their physiological concentrations, such as glucose, glutamic acid, uric acid, ascorbic acid, urea, sodium chloride, potassium chloride had minimal effect on the biosensor response. The interference obtained was 5-10% (Figure 9), which is insignificant, similar to the earlier study based onAChENPs/ChONPs/GrONS/PtNPs/PGE [16].

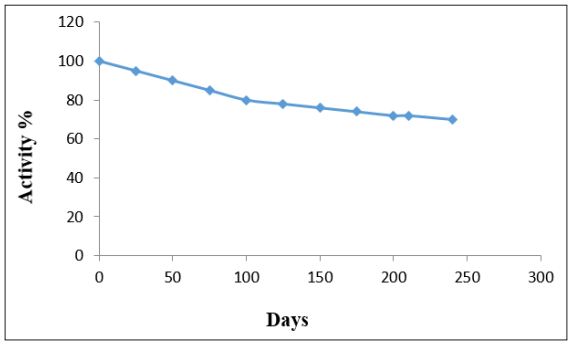


Figure 9: Storage Stability of AChENPs/ChONPs/GrONPs/PG Electrode at 4◦C in Dry Condition

Storage Stability

The present biosensor investigated the response of the acetylcholine concentration in 0.1 M PBS for a time period of 240 days (8months). During this time, the initial activity of the biosensor was decreased by 25% upon continuous use for 250 times. Therefore, the storage stability of the present biosensor was higher than earlier biosensors based on AChE/ChO/MWCNT-MnO2/PtNPs/Au (90 days), AChE-ChO/GrNP/PtNPs/ITO (120 days), AChE-ChO/ePAMAM-Sal/CPE (30days) and AChENPs/ChONPs/GrONS/PtNPs/PGE (180days) [16-19].

Conclusion

The co-immobilization of AchENPs and ChONPs onto GrONPs modified PGE has resulted into improved analytic performance of Ach biosensor in terms of detection limit (0.001 µM), broader linear range (0.001-1000 µM), lower response time (2s) and increased storage stability (240 days). The laboratory model of present biosensor could be miniaturized using MEMS (micro-electromechanical system) for the construction of portable biosensor for point of care (POC) diagnosis.

Competing Interest: Authors declare no competing interest

Ethical Approval: In the present work, we have collected left over human serum samples from PtBDS PGIMS Rohtak hospital, affiliated to Pt. BDS University of Health Sciences, Rohtak under

MoU between MDU Rohtak and Pt. BDS University of Health Sciences, Rohtak for research purpose. PtBDS PGIMS Rohtak hospital has its own ethical clearance from competent ethical committee for collecting biological samples for diagnosis of various diseases and their treatment.

Consent for Publication: All authors have given their consent for publication if accepted.

Consent to Participate: All authors have given their consent if required.

Availability of Data and Materials: All relevant data and materials, if required, are available.

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Authors Contributions: Prof. CS Pundir and Prof. M Sharma planned the research work and supervised and guided the whole work. Practical work and initial writing of research was done by Dr. J Ahlawat, Dr. Shikha Pundir checked and prepared the final draft.

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