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ABSTRACT

Trichlorfon (TF) is considered the most frequently used organophosphorus compound among all compounds used for agricultural purposes. As such, TF can be quite effective in controlling insects that damage crops in several different ways. However, TF is poisonous. When individuals consume food items contaminated with TF, it blocks the function of acetylcholinesterase, resulting in a massive buildup of neurotransmitter at the synapses of nerves, ultimately disrupting normal nerve signal transmissions and possibly producing severe neurological disorders. To address this problem, we have created a rapid, low-cost electrochemical biosensor using acetylcholinesterase (AChE) for detecting Organophosphate pesticide (TF) through employment of Graphite Carbon Nitride (g-C_3N_4)/CuS-based nanocomposite. Electrostatic interaction between the g-C_3N_4/CuS nanocomposite enhances the transfer of electrons to the electrode surface, this supports the covalent immobilization of the AChE enzyme onto the electrode surface by the use of glutaraldehyde as a cross-linker to prevent leaching of the enzyme from the electrode. Biosensor (AChE/g-C_3N_4/CuS/ITO) demonstrated an ultrahigh sensitivity toward trichlorfon over the linear range 0.1 pM - 1 μM. The developed biosensor showed stability, interference capability, good reproducibility and improved sensitivity. These characteristics enabled us to successfully detect trichlorfon in two real samples: apple and cucumber.

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ABBREVIATIONS

OP	Organophosphorus Pesticide
TF	Trichlorfon
g-C_3N_4	Graphitic Carbon Nitride
CuS	Copper Sulphide
ATCl	Acetyl thiocholine chloride
PBS	Phosphate Buffer Saline
ITO	Indium tin oxide
XRD	X-Ray Diffraction
FT-IR	Fourier Transform Infrared
CV	Cyclic Voltammetry
DPV	Differential Pulse Voltammetry
EPD	Electrophoretic Deposition
RSD	Relative Standard Deviation
EDAX	Energy Dispersive X-Ray Spectroscopy
FESEM	Field Emission Scanning Electron Microscope
TEM	Transmission electron Microscope
AChE	Acetylcholinesterase

CHAPTER 1

Introduction

1.1 Introduction to pesticides

Agriculture is an essential part of humanity and will continue to be so for years to come. In order to ensure the world's increasing number of people can eat, farmers are using a lot of chemicals to keep their crops from being destroyed by insects, fungus, weeds and other pests. Pesticides originated from the Latin words pestis (plague) and caedere (to kill). A pesticide is generally defined as any substance which may be applied either directly to plants or sprayed into the environment around them for preventing, destroying, repelling, or controlling any pest including insects, mites, nematodes, slugs, snails, rodents, birds, bacteria, viruses, weed seedlings etc., but excluding organisms present in or on commodity crops at the time of harvest [1].

Synthetic pesticides have been used in modern agriculture since World War II (1939–1945). During this time scientists identified the insecticidal properties of DDT (dichlorobiphenyl-trichloroethane). Following World War II many new synthetic pesticides were developed and introduced commercially. As stated earlier by the FAO (Food and Agriculture Organization), the amount of pesticides used globally increased over 100 percent since 1990. The total amount of active pesticide ingredient was estimated at approximately 3.73 million tons in 2023[2].

Although pesticides greatly increased production of food in many areas of the world, overuse and lack of regulation of pesticides has produced a wide variety of very serious problems for the environment and public health. Pesticides will not remain in the location where they were applied, they move through the soil, into our rivers, lakes, groundwater and into the foods that we eat. Many non-target animals are harmed by pesticides, including fish, birds, pollinating insects (bees), and the microorganisms which live in soils. Human exposure to pesticides has also been linked to several serious diseases, including cancer, Parkinson's disease, endocrine disruption and to nerve disorders. The people who are at highest risk from pesticide exposure include farmers, young children and rural residents [3].

1.2 Classification of pesticides

The main classifications of pesticides based upon the organism being targeted as a pest are Insecticides, Herbicides, Fungicides, Bactericides, Ovicides, Rodenticides, Acaricides, and Nematicides. Within each category, there are many different chemical subcategories of pesticides which vary greatly from one another in terms of how long they last in the environment, how toxic they are and how they act on living organisms [1].

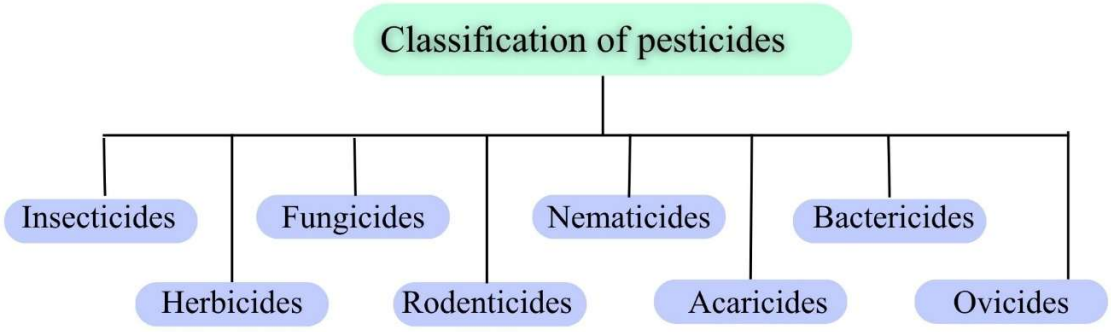

Fig. 1.1 Classification of pesticides

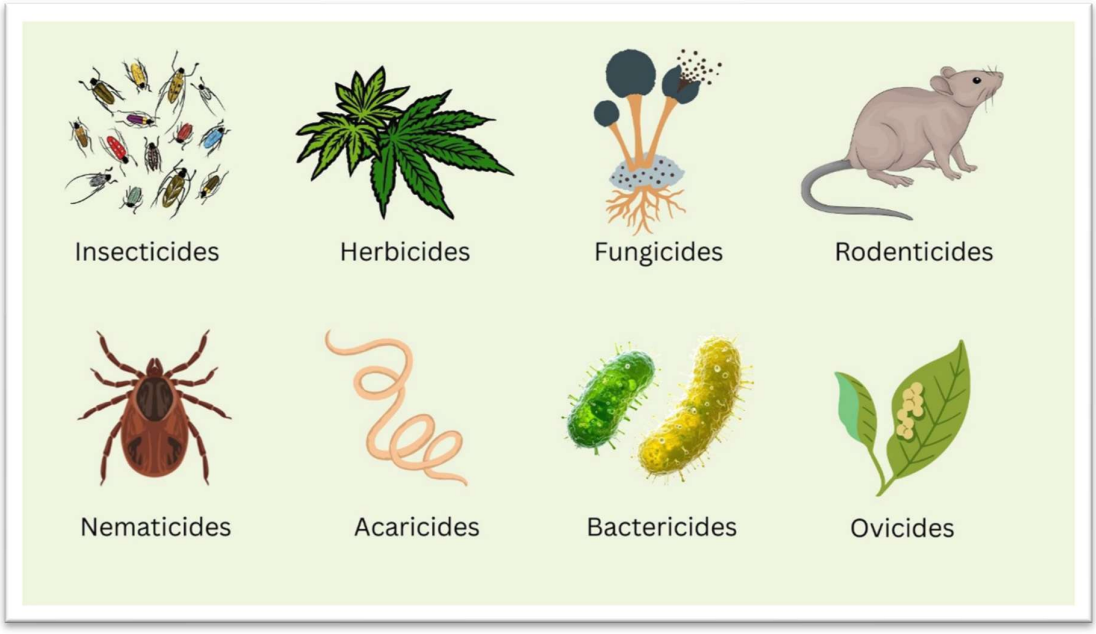

Fig. 1.2 Types of pesticides and their target pests

a) Insecticides: Pesticides that have been designed to kill or repel insects are called insecticides. Their action can be based on the disruption of a pest's metabolism or its developmental cycle. The major chemical classes among these insecticides include organochlorine (e.g., DDT, aldrin), organophosphate (e.g., trichlorfon, chlorpyrifos, malathion) and carbamate (e.g., carbofuran) compounds as well as pyrethroid (e.g., permethrin) and neonicotinoid (e.g., imidacloprid) compounds [4].

b) Herbicides: Herbicides kill undesired plants like weeds by interrupting a necessary function of all plants like growth through photosynthesis or amino acid production, or hormone activity. Some of the most common types of herbicide chemicals include Chlorophenyl compounds (such as 2,4-D), Triazines (like Atrazine), Glycine Derivatives (Glyphosate) and Urea Derivatives (Diuron) [4].

c) Fungicides: Fungicides are chemicals that have the ability to either destroy fungal spores or stop them from growing. They are also important in preventing disease in plants, including rust, blight and mildew. There are several types of fungicides, which fall into the categories of dithio-carbamates, phthalimides, benzimidazoles and triazoles [5].

d) Rodenticides: Rodenticides are a class of pesticide products developed to manage and regulate rodent populations such as rat and mouse populations. Rodenticides can be very toxic in nature and can be utilized for agricultural or urban purposes. Rodenticides fall into three main categories: First Generation Anticoagulants (e.g. Warfarin), Second Generation Anticoagulants (e.g. Brodifacoum) and Non-anticoagulant Rodenticides (e.g. Zinc Phosphide) [6].

e) Acaricides: Acaricides are specifically designed to target and kill mites and ticks (Acari subclass) that exist as pests of both agricultural crops and livestock. They also have a role in controlling ectoparasites in humans. Most acaricides fit into one or another of the many common families of insecticides such as the organophosphate family (trichlorfon), the pyrethroid family (fenpropidin), and the carbamate family [6].

f) Nematicides: Nematocides are chemical compounds intended for use as pesticides, which target and destroy nematode organisms (which are also known as roundworms), which prey on the roots of plants. Most nematicide products are formulated into either a solid granule product

or a gas. The most commonly used nematicides are carbamate pesticides (such as carbofuran and aldicarb) and organophosphate pesticides. Due to their high toxicity many nematicides have a moderate to long term persistence in the soil [6].

g) Bactericides: Bactericides can be defined as a group of pesticides that are produced for killing or limiting the reproduction of bacteria. The use of these is most common within agricultural applications to manage bacterial infections on crops like fire blight in apple trees and bacterial leaf spot in tomato and pepper plants and as disinfectants and antiseptic agents in public health and industrial environments. There are several main categories of chemically classified bactericides including, but limited to copper-based formulations (copper hydroxide, copper oxychloride), antibiotics (streptomycin, oxytetracycline), phenols (triclosan), and sulpha-based formulations [6].

h) Ovicides: Ovicides are a specific type of pesticide that will destroy egg stages of all insect pests, as well as mite and arachnid eggs. Most pesticides kill at the adult stage while ovicides kill the early embryo before it ever has an opportunity to hatch out. Therefore, ovicides break the reproductive cycle of the pest. Some common types of ovicides include benzoyl urea derivatives (diflubenzuron) and these chemicals stop chitin production in developing embryos [6].

1.3 Classification by Chemical Structure

There are many different classifications for pesticides based upon their chemical composition and groupings. These include how a pesticide's structure influences its environmental fate as well as its biological activity [7]. Each of the three major classes listed below:

a) Organochlorine: Organochlorines represent a class of man-made pesticides which contain one or more carbon-chlorine (C-Cl) linkages bonded to either an aliphatic or aromatic backbone. Due to the high electronegative nature of chlorine atoms and the relatively strong covalent bonding energies present in C-Cl linkages, organochlorines exhibit great resistance towards hydrolysis, photodegradation, and microbial biodegradation. Organochlorines also possess very low water solubility levels as indicated by high octanol-water partition coefficients (log K_{ow}), generally greater than four, resulting in significant accumulation in lipid rich tissue [7].

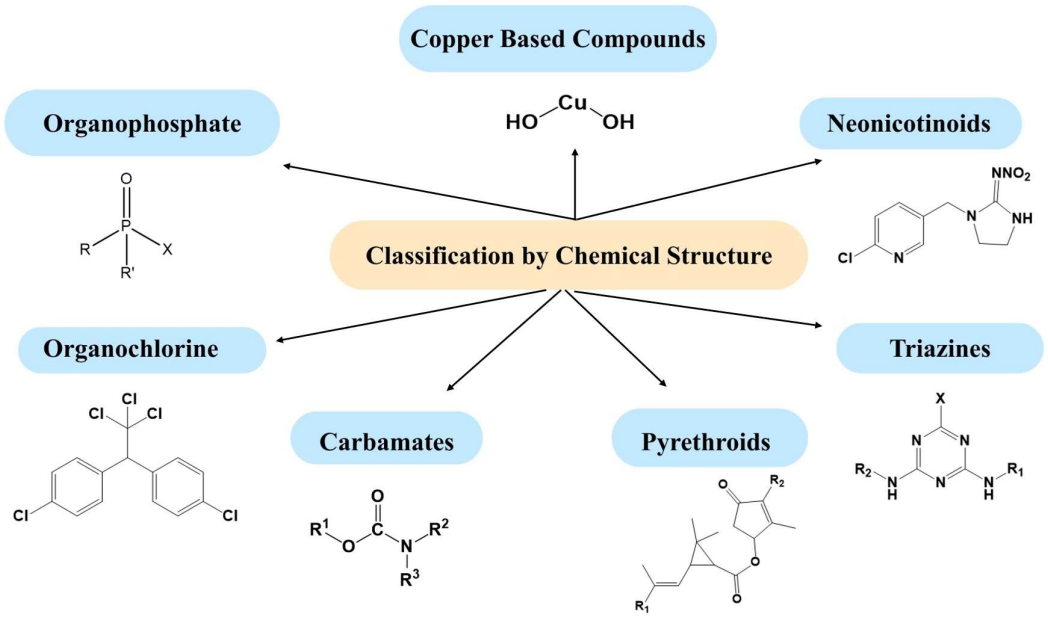

Fig. 1.3 Classification by Chemical Structure

b) Organophosphate: Organophosphate (OP) compounds are typically derived from either phosphorus (III) containing acids such as phosphoric acid. These have side chains that can provide a variety of functional groups such as alkyl, aryl, alkoxy, aryloxy, etc. However, regardless of the composition of these side groups, all OPs have one thing in common, they are all esterified forms of phosphoric acid and therefore contain a phosphoryl (P=O) or a thio-phosphoryl (P=S) linkage [7]. OPs act by causing irreversible inhibition of acetylcholinesterase (AChE), the enzyme responsible for the breakdown of the neurotransmitter acetylcholine. AChE uses the amino acid serine to catalyse this reaction.

c) Carbamates: Carbamate compounds are esters of carbohydric acid (NH_2COOH). The carbamate functional group has been attached to an aromatic ring (naphthyl or phenyl) via a labile.

d) Ester linkage: As with the organophosphates, carbamates inactivate AChE by forming a covalent link to the serine hydroxyl, however, this inactivation can be reversed. The carbamoyl group will bind to the serine hydroxyl and then be removed rapidly (minutes to hours) via decarboxylation. Although reversible inhibition may make carbamates less acutely toxic to mammals than organophosphates based on a mole-for-mole comparison, they still represent a significant hazard.

e) Pyrethroids: Pyrethroids are artificial versions of the natural compounds pyrethrins, those being naturally occurring insecticides produced by the Chrysanthemum flower (Chrysanthemum cineraria folium). The fundamental structure is that of a cyclopropane carboxylic acid linked as an ester to an alcohol with a phenoxy benzyl or similar grouping. Pyrethroids cause neuronal action potential to repeatedly fire because they keep the sodium channel open longer than normal. Eventually this will lead to the death of the neuron, or at least it will render the neuron unable to function [8].

f) Neonicotinoids: Neonicotinoids represent a recent structural class of compounds, with their chemical backbone based on the nitroguanidine or nitro methylene groups attached to an aromatic or heteroaromatic cycle (i.e., thiazole, tetrahydrofuran, etc.) or pyridine. A common structural representation of neonicotinoids describes them as "pharmacophores" that interact with high affinity with the nicotinic acetylcholine receptors (nAChRs) found in insects. Neonicotinoids cause excessive activation of the nAChR due to agonism and this results in continuous stimulation of neurons leading to paralysis and eventual death [8].

g) Triazines: Triazines are heterocyclic compounds that consist of a six-membered ring system including three nitrogen atoms (a 1,3,5-triazine). Some chloro-substituted triazines contain one or more chlorine atoms on their rings (i.e. atrazine, simazine), while others can be substituted at their ring positions by either a methyl group or an oxygen atom. The triazines interfere with photosynthesis in plants, specifically the PSII apparatus of photosynthesis. They bind directly to the D1 protein which is a critical part of this mechanism and block electron transfer so as to cause oxidative damage and kill the plant [8].

h) Copper-Based Compounds: Copper is an inorganic pesticide because it releases a chemical called Cu^{2+} which is the active compound. The most common forms of copper pesticides include Copper Hydroxide. When proteins are denatured, copper will interfere with the enzyme site, especially iron-Sulphur cluster enzymes. Also, when bacteria and fungi undergo oxidative stress from ROS (reactive oxygen species) and they die due to non-specific cell damage [8].

Table 1.1 Major classes of pesticides by target organism, with target pests and chemical subclasses.

S.No.	Target Class	Target Pests	Chemical Subclass	Example
1.	**Insecticides**	Insects like beetles, moths, and mosquitoes	Organochlorines	DDT, Aldrin
			Organophosphates	Chlorpyrifos
			Carbamates	Carbofuran
			Pyrethroids	Permethrin
			Neonicotinoids	Imidacloprid
2.	**Herbicides**	Weeds	Triazines	Atrazine
			Glyphosate-based	Glyphosate
			Urea derivatives	Diuron
3.	**Fungicides**	Fungi like rusts and blights	Dithiocarbamates	Mancozeb
			Phthalimides	Captan
			Benzimidazoles	Carbendazim
			Triazoles	Tebuconazole
4.	**Rodenticides**	Rodents like rats, mice, etc	Anticoagulants	Warfarin
			Non-anticoagulant	Zinc phosphide
5.	**Acaricides**	Mites and ticks	Organophosphates	Trichlorfon
			Pyrethroids	Fenpropathrin
6.	**Nematicides**	Nematodes like roundworms	Carbamates	Carbofuran
7.	**Bactericides**	Bacteria	Copper-based compounds	Copper hydroxide
			Antibiotic bactericides	Streptomycin
			Sulfone bactericides	Fubianezuofeng
8.	**Ovicides**	Eggs of insects	Benzoylureas	Diflubenzuron
			Natural products like neem	Azadirachtin

1.4 Useful impacts of Pesticides

Increasing crop yields and protecting global food security: Crops are protected from being destroyed by various types of pests including insects, weeds, fungi, etc., through the use of pesticides [9]. These same pesticides provide the farmer with the ability to produce significantly higher yields on their existing land. If we were to eliminate pesticide use it is estimated that there could be an additional 50-80% loss in total crop yield and create another serious problem with providing enough food globally [10].

a) Protecting human health: There are several types of pesticides (especially insecticides) that will reduce populations of vectors that carry diseases such as malaria (mosquitoes), Lyme disease (ticks), typhoid fever (flies) etc. Historically programs utilizing DDT and many other insecticides have been able to save millions of people in less developed areas of the world from dying due to vector borne diseases [10].

b) Reducing populations of non-native or aggressive weed species and other plant species: Herbicides allow farmers to control non-native or aggressive plant species that can outcompete crops for resources such as sunlight, water and nutrients. If herbicide use was eliminated then crop production rates would dramatically decrease and native ecosystems could become overrun by non-native or aggressive plant species.

c) Controlling household pests (both domestic and urban): Pesticides are commonly used within residential settings, commercial/office settings, and public spaces to control household pests such as cockroaches, termites, bed bugs, rats, etc. The use of pesticides helps prevent destruction of property and also reduces the potential for transmitting disease within urban communities [9].

d) Providing veterinary or livestock related Benefits: The agricultural industry utilizes pesticides (specifically acaricides such as trichlorfon) to control tick or mite, fly and parasitic infestation problems in animals. These pest control measures improve the health status and productivity of animals resulting in improved quality meat and milk products [10].

1.5 Harmful impacts of pesticides

Pesticides are an environmentally threatening hazard of the modern age. As effective as they may be in increasing crop yields and managing plant diseases, pesticides also carry many environmental and health risks. Pesticides can enter the earth's air for years to come and contaminate our water, food, and soil and this has created numerous health problems, both short- and long-term toxicities. There are three major areas of concern that are all connected: the impact on human health (acute poisonings to long-term health issues or cancer), contamination of the environment (long-lasting presence in soils and waters) and the destruction of beneficial organisms and the harm caused to ecosystem services [11].

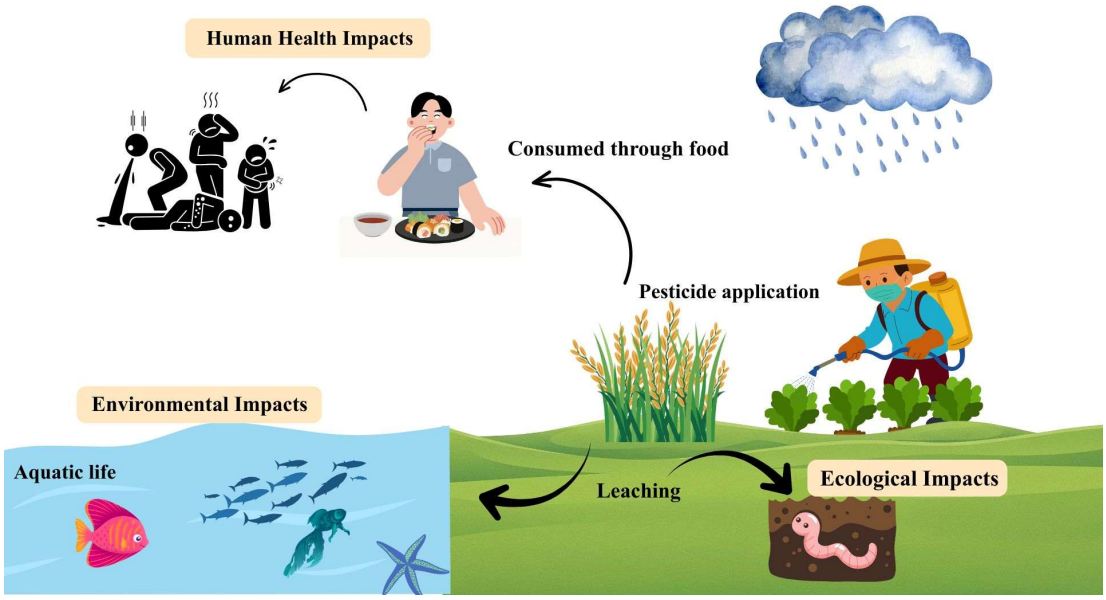

Fig. 1.4 Harmful impacts of pesticides

a) Human Health Impacts: The toxicology of pesticides has created a multitude of harmful health impacts in people, both acute and chronic. Acute poisoning occurs when a person ingests, inhales or comes into contact with pesticides at high doses, which can lead to symptoms such as dizziness, tremors, convulsion, and respiratory arrest. As well, some chemicals, specifically the organophosphate and carbamate families, work to block an enzyme called acetylcholinesterase, which is used by nerve cells to send signals. Chronic toxicity occurs over time due to lower dose exposure to pesticides. The chronic toxicities include neurotoxicity, immunotoxicity, hormonal imbalance (endocrine disruption), developmental disorders (e.g., reproductive issues), and several forms of cancer [12]. Many pesticides have demonstrated

carcinogenic properties: DDT and other related products are listed as "probable human carcinogens" (cancer-causing agents) and are associated with liver cancer. Atrazine is likely to be carcinogenic to humans according to the World Health Organization (WHO) and may be associated with developing prostate and lung cancer earlier in life than normal. Hormone disruption is one of the largest concerns regarding pesticide usage, DDT, atrazine, and neonicotinoids all disrupt hormone homeostasis, which contributes to birth defects, infertility, thyroid disease, and increases the risk of developing type II diabetes, obesity, and breast cancer. Chemical categories of pesticides demonstrate unique patterns of toxicity. For example, organochlorine pesticides are persistent, bioaccumulate and affect neural development, neonicotinoids affect multiple body systems and form covalent bonds with DNA, thereby creating genetic mutations, and atrazine contaminates drinking water supplies of millions of people, contributing to the occurrence of reproductive and metabolic diseases [10].

b) Environmental Impacts: Pesticide contamination affects virtually every part of our planet's environment and can last anywhere from a few days to hundreds of years. Pesticides that persist longer than 10 years (i.e., organochlorines) continue to build up in the soil each year they are applied, resulting in decreasing soil biota populations and decreased fertility. Contamination of surface waters occurs primarily through direct runoff from treated fields, through seepage of contaminants from treated fields into groundwater aquifers (leachate), and indirectly through aerial drift. While most pesticides do not adhere to sediment particles, organochlorines are known to strongly bind to sediment particles [13]. Neonicotinoids are very soluble in water and have been found in extremely small amounts (parts per trillion to parts per billion) in surface waters throughout much of North America. Surface water contamination will occur if the agricultural practice involves spraying pesticides near bodies of water. Persistence and bioaccumulation are key characteristics of POPs.

c) Ecological Impacts: Agricultural practices involving pesticides result in significant detrimental effects to non-target species as well as disruptions in fundamental ecosystem processes. One of the most notable examples of the negative effects of pesticides on non-target species is the impact on pollinator populations particularly honeybee (Apis mellifera) colonies and wild bee populations. Neonicotinoids and fipronil inhibit reproductive behaviour (female honeybees stop laying eggs), navigation behaviour (orientation within the hive), memory formation (learning of floral scents), and immune response (ability to fight off infection) resulting in colony collapse disorder (CCD). Field-realistic concentrations of neonicotinoid

pesticides have been shown to suppress the immune system of adult honeybees making them more susceptible to parasitic infections and infestations by varroa mite. Aquatic organisms are also significantly impacted by pesticide applications [14]. Runoff containing pesticides entering freshwater stream channels can kill fish and other aquatic animals. Organochlorines produce reproductive failure in fish whereas neonicotinoids and pyrethroids are lethal to aquatic insects and thus disrupt entire aquatic food webs. The persistence of pesticide residues in soils can negatively affect soil organisms like earthworms, microorganisms, arthropods which reduce both biological diversity and functional diversity. The death of earthworms reduces the mixing and movement of nutrients in soils and impairs soil structure. Reduced microbial populations diminish decomposition rates and nutrient availability. Losses in biodiversity are occurring rapidly and largely driven by increased agricultural intensity combined with widespread use of pesticides. Non-target plant contamination, surface water contamination, and contamination of food chains create continuous exposure pathways that compromise ecological resilience [13].

1.6 Techniques for Detection of Pesticides

Over the years, there has been an extensive development of numerous analytical techniques to detect and quantify residues of pesticides in food, water, soil and biological samples for public health safety and environmental protection. In general terms, these many different types of analytical techniques can be categorized as either: traditional or conventional, and advanced or modern [15].

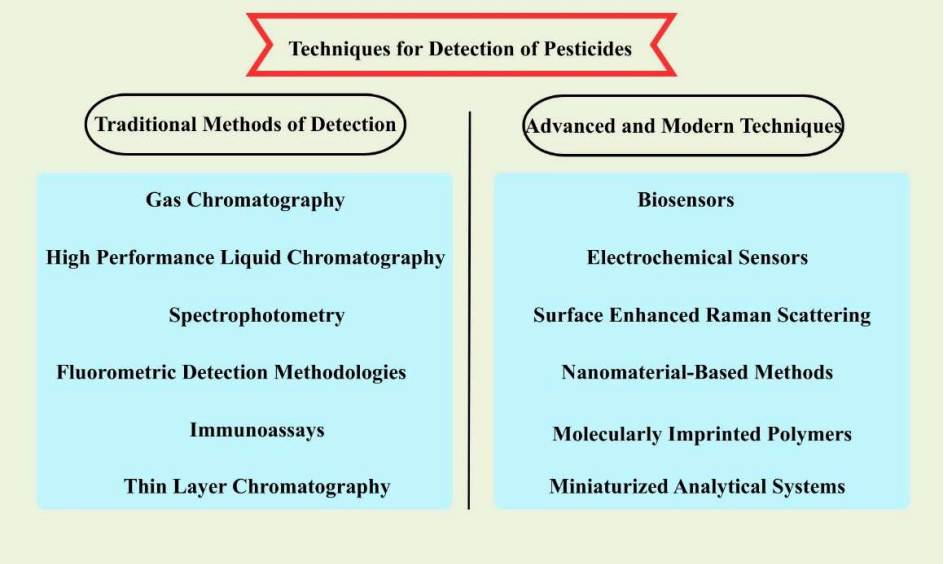

Fig. 1.5 Different Techniques for Detection of Pesticides

1.6.1 Traditional Methods of Detection

a) Gas Chromatography: Gas chromatography is a well-established, conventional laboratory technique that can be utilized to analysis many types of pesticide residues. The basic principle behind this technique is that the sample must first be vaporized, then the vapor will move through a column of stationary phase material by an inert carrier gas. Pesticide compounds will separate from each other along the length of the column based upon their volatility and ability to interact with the stationary phase. GC is typically coupled with various types of detectors including: Flame Photometric Detectors (FPDs), Electron Capture Detectors (ECDs), and Mass Spectrometers. FPDs are particularly sensitive for organophosphorus and organosulfur-containing pesticides. ECDs are especially sensitive toward chlorinated pesticides. GC-MS offers both accurate quantitative information and unequivocal identification of pesticide residues. As such, GC-MS is the "gold standard" for pesticide residue analysis. However, this technology is quite expensive and requires a significant amount of technical expertise to operate. Sample preparation prior to analysis also tends to be rather labour-intensive [16].

b) High Performance Liquid Chromatography: HPLC is utilized when the pesticide compound being analysed is either too thermally unstable or does not volatilize sufficiently under normal conditions. This limits the use of GC. When separated based upon their retention times, these compounds interact with a stationary phase located within the HPLC column while simultaneously moving through a liquid mobile phase. HPLC is frequently combined with UV-VIS spectrophotometers, fluorescence detectors, or mass spectrometers (LC-MS/MS). HPLC has become a common tool in food safety laboratories for detecting pesticide residues in fruit, vegetable, and water samples [17].

c) Spectrophotometry: Spectrophotometric detection involves measuring the absorption of light at specific wavelengths by pesticide residues or their chemical transformation products. These methodologies are generally less expensive than chromatographic detection methods, however, they tend to lack the higher sensitivity and selectivity found in some chromatographic methods [18].

d) Fluorometric Detection Methodologies: Some pesticide residues or their metabolic byproducts naturally fluoresce or can be chemically modified into fluorescent substances. By measuring the fluorescent radiation produced during excitation, fluorometric analyses can

provide very low limits of detection. Matrix effects can still negatively affect the precision of this methodology [18].

e) Immunoassays (ELISA): Immunoassays involve using monoclonal antibodies designed to selectively bind to target pesticide residues. Due to their simplicity and speed, immunoassays are generally employed for screening large quantities of samples. They are widely applied in food safety monitoring programs. Since ELISA can produce positive results that do not reflect actual levels of pesticide residues present in samples, the reliability of ELISA can vary depending upon the application. Furthermore, ELISA's sensitivity to a single pesticide residue makes it impractical for simultaneous multiresidue determination [19].

f) Thin Layer Chromatography: TLC is a relatively easy-to-use, low-cost technique used primarily for qualitative screening of pesticide residues. TLC employs a solid-coated plate where separations occur via capillary action using a solvent-based eluent. Although TLC is much less sensitive than many chromatographic techniques, it is capable of providing quick turnaround data for initial field screenings.

1.6.2 Advanced and Modern Techniques

a) Biosensors: Biosensors represent a new generation of detection tools for pesticides. Biosensors combine a biological sensor element (enzymes, antibodies, aptamers, DNA) with a transducer which will convert the biologic reaction into a quantifiable signal. There are several reasons why biosensors are superior to older technologies for pesticide detection: Rapid response time, High specificity and sensitivity, Portability and application in the field, Cost-effective and Minimal need for sample preparation. Among all the types of biosensors that exist, electrochemical biosensors are by far the most common for pesticide detection [20]. The type of biosensor works by measuring changes in current (amperometric), voltage (potentiometric) or impedance (impedimetric) when the pesticide interacts with the biosensor. Enzyme-based biosensors using Acetylcholinesterase (AChE) are especially good for detecting organophosphate and carbamate pesticides because both classes of compound inhibit enzyme activity.

b) Electrochemical Sensors: Electrochemical sensors work by detecting the presence of pesticides based on an oxidation or reduction reaction occurring at an electrode. In addition to

being very sensitive they can also be made very small and built using nanotechnology materials like carbon nanotubes, graphene, MXene (Ti_3C_2Tx), metal particles (such as Au NPs) and metal oxides (like CeO_2). Some examples of how data from electrochemical sensors are generated includes: Cyclic Voltammography (CV), Differential Pulse Voltammography (DPV), Square Wave Voltammography (SWV) and Electrochemical Impedance Spectrometry (EIS), All of the methods offer very low detection limits ranging from picomoles (pM) to nanomoles (nM) [21].

c) Surface Enhanced Raman Scattering: SERS is an optical method that uses nanostructured metallic surfaces (most often gold or silver nanoparticles) to increase the intensity of the Raman scattering signal produced when molecules adsorb onto the surface. SERS is able to achieve extremely high sensitivities allowing for the detection of pesticides at very low levels of contamination without needing to prepare the samples extensively. As a result, SERS is now being examined for its ability to rapidly screen for pesticides in remote locations [21].

d) Nanomaterial-Based Methods: The incorporation of nanomaterials such as gold nanoparticles (AuNPs), quantum dots, carbon nanotubes, graphene oxide and MXenes has greatly improved pesticide detection. These materials offer a large surface area, enhanced electrical conductivity and increased molecule adsorption, resulting in higher sensitivity, selectivity and lower detection limits than traditional sensor methods.

e) Molecularly Imprinted Polymers: MIPs are synthetic polymer receptor molecules engineered to specifically bind to particular pesticide molecules with greater affinity than natural biological receptors. MIPs are used as recognition elements in sensors and have been shown to have long-term stability, can be reused many times and are inexpensive compared to their biological counterparts [22].

f) Miniaturized Analytical Systems: They consist of microfluidic devices or lab-on-chip devices. These miniaturized devices contain all the necessary components needed for performing chemical analyses including sampling preparation, separation and detection. They require minimal amounts of sample volume and yield fast results making them well-suited for applications.

1.7 Biosensors

1.7.1 Definition and History of Biosensing

Biosensors are devices used for measuring the presence of biochemicals (analytes), through combining a biological component and an electronic or physical component. They produce a measurable signal related to the amount of biochemical present. The term biosensor was created by Prof. Leland C. Clark Jr., and is often referred to as the "father of biosensors". In 1962, he invented the first enzyme electrode (glucose biosensor), and this technology was later commercially available in 1975, and it was used in the first commercially available product designed to measure blood glucose levels [20]. Over the last few decades, there have been tremendous advancements in many fields, including biotechnology, nanotechnology, materials science and microelectronics, resulting in exponential growth of biosensor development.

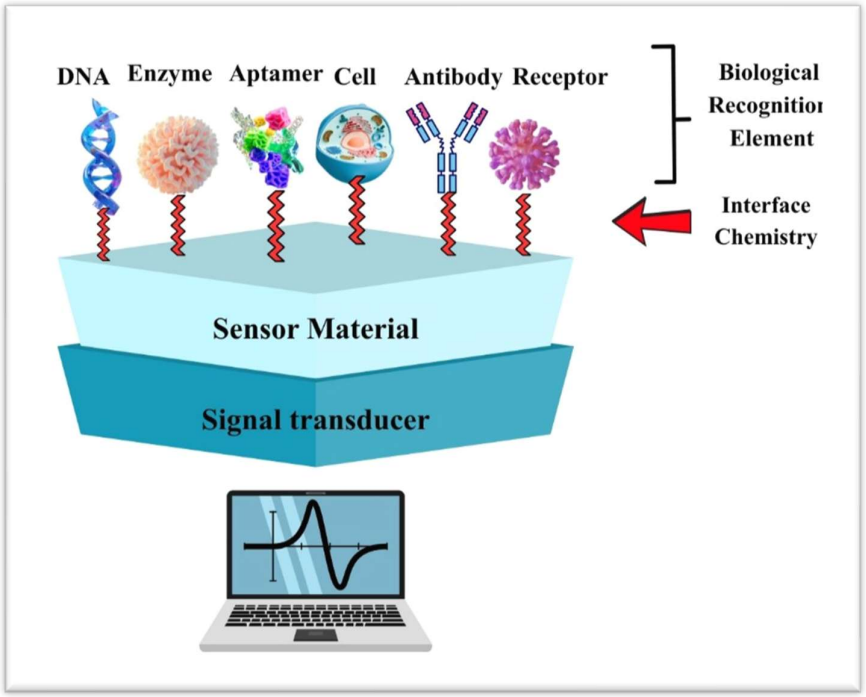

Fig. 1.6 An overview of Biosensor

1.7.2 Principles of Operation for a Biosensor

The basic premise for a biosensor is the high degree of specificity associated with the interaction between a biochemical receptor and its corresponding analyte. Upon interaction between the

receptor and analyte, a biological event occur i.e. a chemical reaction takes place, the receptor undergoes a shape change or other type of interaction occurs, which is then transformed into an electrical, optical, thermal or mass-related signal by the transducer [23].

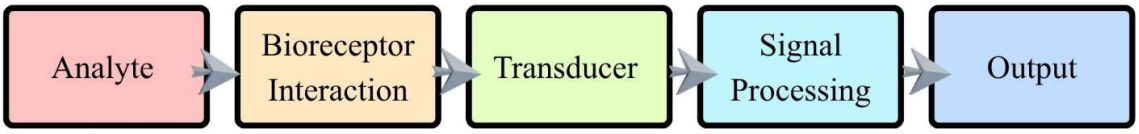

Fig. 1.7 The basic working principle of biosensors

1.7.3 Components of a Biosensor

The basic components in making up a biosensor are comprised of five main components:

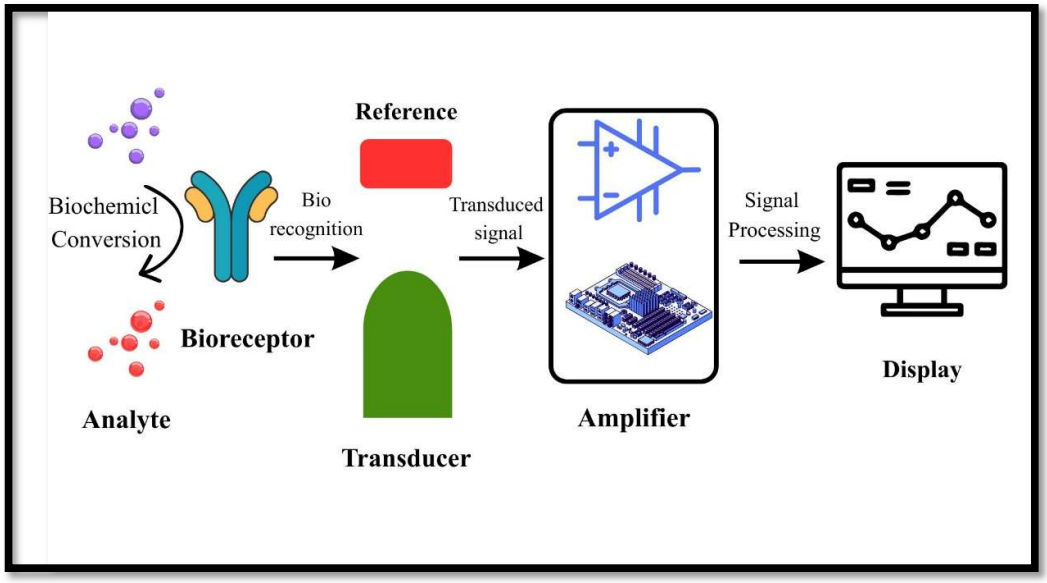

Fig. 1.8 The components of biosensors

a) Analyte: The analyte is the target substance being identified and quantified by the biosensor. This could be any type of molecule which has biological, chemical, or environmental importance. For instance, this could be a glucose molecule (to monitor for Diabetes), pesticide molecules (for use in Food Safety), heavy metal ions (for use in Environmental Monitoring), pathogenic organisms (for use in Clinical Diagnostics), or tumour biomarkers (for use in Oncology) [24].

b) Biorecognition Unit or Bioreceptor: The biorecognition unit/bioreceptor is the biological element that recognizes and bonds to the analyte [25]. The biorecognition unit/bioreceptor provides specificity and sensitivity to the overall performance of the biosensor. Examples of biorecognition units/bioreceptors include:

- Enzymes: They have catalytic action on their substrate,
- Antibodies or Antigen: Highly specific immunological reactions occur when antibodies bind to antigens.
- Nucleic Acids (DNA/RNA/Aptamer): Hybridization based binding occurs between nucleic acid strands.
- Cells/Microorganisms: Whole cells are used as biorecognition elements in some types of biosensors.
- Molecularly Imprinted Polymers (MIPs): Synthetic polymers that mimic the binding properties of biological systems.
- Lectins: Carbohydrate binding proteins.

c) Transducer: The transducer transforms the biochemical interaction between the analyte and the biorecognition unit/bioreceptor into a detectable physical or chemical signal. The transducer acts as an intermediary between the biochemical part of the biosensor and its electronic part. Depending on what type of transducer was chosen will determine how you measure and classify your biosensor.

d) Signal Processor or Amplifier: Once the transducer generates a raw signal, it is sent to a signal amplifier that will amplify and clean the signal from background noise. The amplified and cleaned signal is then converted from an analogue to a digital signal ready for final processing and analysis [26].

e) Display or Output Device: Once the data has been processed it is shown through one or multiple display/output devices such as computers, smart phones, or even a stand-alone read-out device.

1.7.4 Classification Based on Bioreceptor

There are seven classifications of biosensors based on bioreceptors:

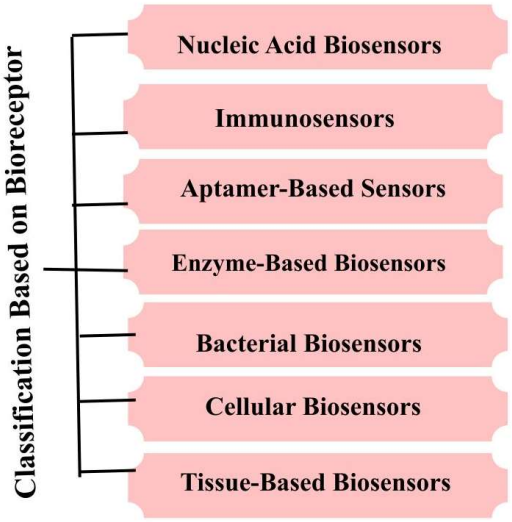

Fig. 1.9 The Classification Based on bioreceptor

a) Enzyme-Based Biosensors: Although enzyme-based biosensors contain an enzymatic reaction that leads to the formation of electrochemically active substances like O_2, H_2O_2, or NADH, the transducer then measures those substances, this is the largest category of biosensors in terms of commercial success. Although there are many different enzymes that have been utilized for various types of biosensors including glucose oxidase (for glucose determination), acetylcholinesterase (to determine organophosphate pesticides), horseradish peroxidase (for H_2O_2 quantitation), urease (urea determination), and lactate oxidase (lactate determination), the advantages of using enzyme-based biosensors include a large number of possible targets, very rapid response time, and a high degree of sensitivity. Disadvantages of enzyme-based biosensors include unstable enzymes, potential interference by other molecules that can inhibit the enzyme's activity, and high cost due to the requirements for producing and purifying the enzymes [26].

b) Immunosensors: These are based on the highly specific interaction between antigens and antibodies. The immobilized antibody on the surface of the transducer binds to its corresponding antigen, resulting in a detectable signal. As such, immunosensors are commonly

used for the detection of pathogens, hormones, drugs, toxins, and cancer biomarkers. Immunosensors are generally classified into two subcategories: labelled (sandwich ELISA format) and unlabelled (direct binding detection). Although one of the major advantages of immunosensor is their exceptional specificity that enables them to function reliably in samples containing multiple components and they are often expensive because antibodies can be costly to produce [27]. Additionally, the shelf-life of antibodies is typically short-lived and it is difficult to regenerate the sensing surface after each use.

c) Nucleic Acid Biosensors: Genosensors have ss-DNA or ss-RNA probes attached directly on the surface of a transducer. Hybridization occurs when a complementary nucleic acid sequence is present in the sample and generates a detectable signal. Genosensors can be utilized for pathogen detection, genetic disease diagnosis and food safety analysis. There are several types of genosensors including DNA biosensors, RNA biosensors and aptamer-based sensors (aptasensors). One advantage of genosensors is they are highly specific and stable and can perform multiple assays at once. However, one disadvantage is some formats will need amplification of the target sequence prior to performing the assay [28].

d) Aptamer-Based Sensors: An aptamer is a small (usually less than 30 bases), single-stranded DNA or RNA molecule that has a distinct 3-dimensional structure allowing it to selectively bind to a specific molecular target much like an antibody does. Aptamers were developed using an in vitro method called SELEX (Systematic Evolution of Ligands by Exponential Enrichment). In comparison to immunosensors, aptasensors have higher stability, increased reproducibility and decreased production costs.

e) Bacterial Biosensors: They contain whole living organisms (bacteria, yeast, etc.) as the biorecognition elements. When the biorecognition element detects a target analyte, it modifies its metabolic response which results in a measurable signal such as altered oxygen utilization rates, bioluminescence or changes in electrical properties. Bacterial biosensors can be applied to monitor environmental factors (e.g. biochemical oxygen demand measurements and toxicities) and also to determine food safety.

f) Cellular Biosensors: In cellular biosensors the biorecognition elements are entire living cells or subcellular structures. These biosensors are particularly useful for identifying chemical substances that affect cellular processes, i.e. toxins, drugs, pollutants and other hazardous

chemicals [29]. These biosensors measure cellular responses to identify how the target analyte affects cell function.

g) Tissue-Based Biosensors: They consist of thin slices or extracts of various types of biological material (animal or plant) containing the necessary enzymes. Because tissue-based biosensors utilize whole enzyme systems within their normal cellular matrix and protective co-factors, they tend to be more stable than purified enzyme systems and generally cheaper.

1.7.5 Classification Based on Transducer Type

a) Electrochemical Biosensors: The best-researched and commercialized type of biosensor is the electrochemical biosensor. These sensors work by measuring the effect on the electrical properties of a system (like current, potential, impedance, etc.) which occurs during biological recognition events at the surface of the electrode. There are several types of electrochemical biosensors:

- Amperometric biosensors measure the amount of electrically active species being oxidized/reduced at a constant voltage (potential). This type of sensor is extremely popular and has been used in glucose meters.
- Potentiometric biosensors measure the difference in voltage between a reference and working electrode. Potentiometric sensors follow the Nernst Equation and are also known as ion-selective electrodes.
- Voltametric biosensors are able to measure how much electrically active species is being oxidized/reduced as a function of changing voltage.
- Voltametric sensors have three main configurations: cyclic voltammetry (CV), differential pulse voltammetry (DPV) and square wave voltammetry (SWV).
- Impedimetric biosensors measure how much the resistance and/or capacitance at the electrode-electrolyte interface changes due to the presence of an analyte. Impedimetric sensors offer very high sensitivity and allow for label-free detection.
- Conductometric biosensors simply measure how much the conductivity of a solution has changed due to binding events.

The biggest advantage of electrochemical biosensor includes high sensitivity, low detection limits, miniaturization, low cost and the ability to provide data in real time. Electrochemical biosensors have many uses including glucose monitoring, pesticide detection, heavy metal analysis, and pathogen identification [30].

b) Optical Biosensors: Optical biosensors detect changes in the physical characteristics of light - absorbance, fluorescence, luminescence, refractive index or surface plasmon resonance - caused by interactions between an analyte and biorecognition molecules. Of all the types of optical biosensors,

- Surface plasmon resonance biosensors are among the most interesting, these measure changes in refractive index when an analyte binds to a biorecognition molecule attached to a thin layer of metal (usually gold) at its surface, allowing them to detect analytes in real-time and without using labels.
- Fluorescence-based biosensors measure either fluorescent labelling or intrinsic fluorescence changes.
- Chemiluminescent biosensors measure light produced through a chemical reaction without requiring an outside energy source.
- Colorimetric biosensors create visible colour changes that may be seen with the unaided eye or measured with a spectrophotometer.
- Fiber-optic biosensors use optical fibres to transmit light signals from the sensing element to a remote detector.

The primary advantages of optical biosensors include their high sensitivity, lack of electrical noise and suitability for remote sensing [31]. Optical biosensors have numerous applications in environmental monitoring, food safety, medical diagnostics and drug discovery.

c) Piezoelectric Biosensors: Piezoelectric biosensors measure the change in resonant frequency of a piezoelectric crystal, most often a Quartz Crystal Microbalance (QCM) as a result of small changes in the mass on its surface due to analytes' attachment. These are among the most sensitive devices available with the ability to detect changes in mass as small as nanograms. Due to the fact that they do not need labels and respond in "real-time," piezoelectric biosensors are widely utilized for detecting proteins, DNA/RNA, viruses, and bacteria. They are primarily used for identifying pathogens, studying protein-protein interactions, and screening drugs [32].

d) Thermal (Calorimetric) Biosensors: Thermal biosensors (also referred to as enzyme thermistors or calorimetric biosensors) measure the amount of heat produced or consumed through biological reactions, including enzyme-substrate conversions or antibody-antigen binding. Nearly all biological reactions generate a change in enthalpy. thus, these sensors will

work with nearly all biological assays. The limitation of this technology is relatively poor sensitivity and susceptibility to interference by varying temperatures. As a consequence of both limitations, thermal biosensors are far less common than electrochemical or optical technologies. Nevertheless, they continue to be useful in a variety of research-based environments [32].

e) Magnetic Biosensors: Magnetic biosensors represent an evolving category that utilize magnetic nanoparticles or detect variations in magnetic field strength to quantify analyte. Through the use of bioreceptor-functionalized magnetic labels, target molecules may be captured and the resulting magnetic signal detected using magnetoresistance sensors, SQUIDS (Superconducting Quantum Interference Devices), or MRWS (Magnetic Relaxation Switches). Biological samples rarely contain inherent magnetization. Thus, background noise is virtually non-existent providing high levels of sensitivity. The increasing interest in magnetic biosensors for clinical diagnostic applications and point-of-care testing is expected to increase significantly over time, as well as potentially extend into areas related to environmental monitoring and food safety [33].

1.7.6 Characteristics of an Ideal Biosensor

The table given below shows the characteristic of an ideal biosensor,

Table 1.2 The characteristics of an ideal biosensor

S.No.	Characteristic	Description
1	**Sensitivity**	The ability of an assay to measure extremely low levels of an analyte in a sample (ng/mL to fg/mL LOD).
2	**Selectivity**	The ability of an assay to specifically measure the presence or quantity of one analyte while excluding all others.
3	**Reproducibility**	The results obtained through an assay are consistent when it is performed on different days, at different times, using different personnel, etc.
4	**Stability**	The resistance of the measurement system to changes in temperature, pH, humidity, etc., over time.

5	**Linearity**	A linear response exists over a broad concentration range between the signal generated by the measurement technique and the amount of analyte present.
6	**Limit of Detection (LOD)**	The lowest concentration of an analyte that can be accurately measured.
7	**Biocompatibility**	The material properties of a device or instrument that are non-toxic to living organisms and compatible with biological materials.
8	**Cost-effectiveness**	An affordable cost per unit for devices such that they can be deployed in sufficient quantities to provide adequate coverage.
9	**Reusability**	An assay's ability to make repeated measurements without loss of analytical performance.
10	**Response Time**	The speed at which a measurable signal is produced by the measurement system upon introduction of the analyte into the system.

1.7.7 Immobilization Techniques for Biosensors

Fabrication of the biosensor relies heavily on the immobilization of the bioreceptor on the surface of the transducer. To ensure that the bioreceptor maintains its biological activity throughout the immobilization process and provides stable contact with the sensor, it is essential to utilize an immobilization technique which fulfils these two requirements. Some of the key techniques utilized in the immobilization of bioreceptors include:

a) Adsorption (Physical): Adsorption is a physical binding method which utilizes weak van der Waals, hydrophobic, and electrostatic forces to bind the bioreceptor to the surface of the electrode. This approach is simple, does not disrupt the bioreceptor's enzymatic activity however, since this method is based upon weak bonds, the bioreceptor can be removed from the surface by elution or degradation over time [34].

b) Chemical Coupling (Covalent Attachment): Chemical coupling involves covalently attaching the bioreceptor to the electrode surface through chemical linkages formed between the bioreceptor and functional groups present on the electrode. Since this type of linkage is much stronger than those involved in adsorption-based immobilizations, chemical coupling provides strong and relatively permanent attachment of the bioreceptor to the electrode. This is the preferred immobilization method in commercial biosensors. The use of glutaraldehyde as a crosslinking agent is particularly prevalent in biosensor construction [34].

c) Encapsulation: Entanglement occurs when the bioreceptor is physically entrapped within a polymeric matrix (such as acrylamide gel, sol-gel derived matrices, etc.) at the electrode surface. Biocatalysts such as enzymes and even intact cells have been effectively immobilized using entrapment techniques. However, because diffusion pathways are limited within an entrapment matrix, enzymatic activity may also be affected.

d) Cross Linking: Cross linking refers to the formation of links between bioreceptor molecules themselves and/or between bioreceptor molecules and a solid support matrix using a bifunctional cross linker (for example, glutaraldehyde). While cross linking provides a highly effective means of immobilizing biocatalysts, it often alters the structure of the immobilized enzyme and thus affects its catalytic activity [35].

e) Affinity Based Immobilization: Affinity based immobilization uses specific biological interactions (streptavidin-biotin, nickel-histidine tags, protein A/G-antibody) for oriented immobilization of biocatalysts. As this method is extremely selectivity-based immobilization minimizes disruption to the bioreactor's natural biological functions.

1.7.8 Nanostructures in Biosensors

The application of nanostructured materials in biosensing platforms significantly enhances the analytical capabilities of biosensors. In particular, several types of nanostructured materials are being investigated including:

a) Carbon Nano Structures:

- Carbon nanotubes (CNTS): They exhibit high electrical conductance and very high surface areas.
- Graphene and reduced graphene oxide (rGO): These structures display high electron mobility and high surface areas.
- Carbon quantum dots: They possess fluorescence characteristics and are bio-compatible.

b) Metallic Nano Particles:

- Gold nano particles (AuNP): They exhibit good biocompatibility along with surface plasmon resonance characteristics.
- Silver nano particles (AgNP): They show anti-microbial effects along with optical characteristics.
- Platinum nano particles: They display good electro-catalytic behaviour.

c) Metal Oxide Nano Particles:

- Zinc oxide (ZnO): It displays semiconductor characteristics and piezoelectricity.
- Titanium dioxide (TiO_2): It shows photo catalysis and good biocompatibility.
- Cerium oxide (CeO_2): It exhibits redox cycle properties (Ce^{3+}/Ce^{4+}), it has good electro-catalytic properties and has been found useful for constructing non-enzymatic sensors.

d) Two-Dimensional Materials:

- $Ti_3C_2T_x$ MXene: Excellent electrical conductivity (6000-8000 S/cm) and very large surface area. Its surface chemistry is tuneable and it is excellent for supporting growth of biological components. Currently, $Ti_3C_2T_X$ is emerging as one of the most promising platforms for developing electrochemical biosensors [36].

e) Quantum Dots:

- Quantum dots represent a class of semiconductor nanocrystal whose size dependent fluorescence can be engineered to allow multiple analytes to be detected simultaneously.

1.7.9 Applications of Biosensors

Applications of biosensors are used in many areas of medicine, environmental monitoring, agriculture and pharma.

a) Medical Diagnostics

- Blood glucose monitoring: The use of glucose sensors has become widespread as they monitor diabetes patients by continuously measuring their blood glucose levels.
- Cardiac biomarkers: They detect cardiac biomarkers which allow clinicians to rapidly identify acute myocardial infarction.
- Cancer biomarkers: Specific proteins (PSA, CEA, AFP) are detected in order to screen for cancer.
- Infectious diseases: Biosensors are used to rapidly diagnose infections (HIV, COVID-19, Hepatitis, Malaria), and also to detect blood borne pathogens.
- Therapeutic drug monitoring: Concentration of therapeutic drugs is measured in patients' bloodstreams to determine if dosage is correct and if there are side effects.
- Hormone & vitamin levels: Biosensors can measure the exact number of important vitamins and hormones in a person's blood [37].

b) Environmental Monitoring

- Pesticide residues: Biosensors can find residues of pesticides from contaminated soil, groundwater, or food.
- Heavy metal testing: Water samples can be tested using biosensors for toxic heavy metals (Lead, Mercury, Cadmium, Arsenic).
- Pollutant detection: Industrial pollutants and endocrine disruptors in an ecosystem can be found using biosensors.
- Wastewater management: BOD can be monitored at the wastewater treatment facility using biosensors.

- Toxic algae: Biosensors can detect the presence of toxic algae in waterways [37].

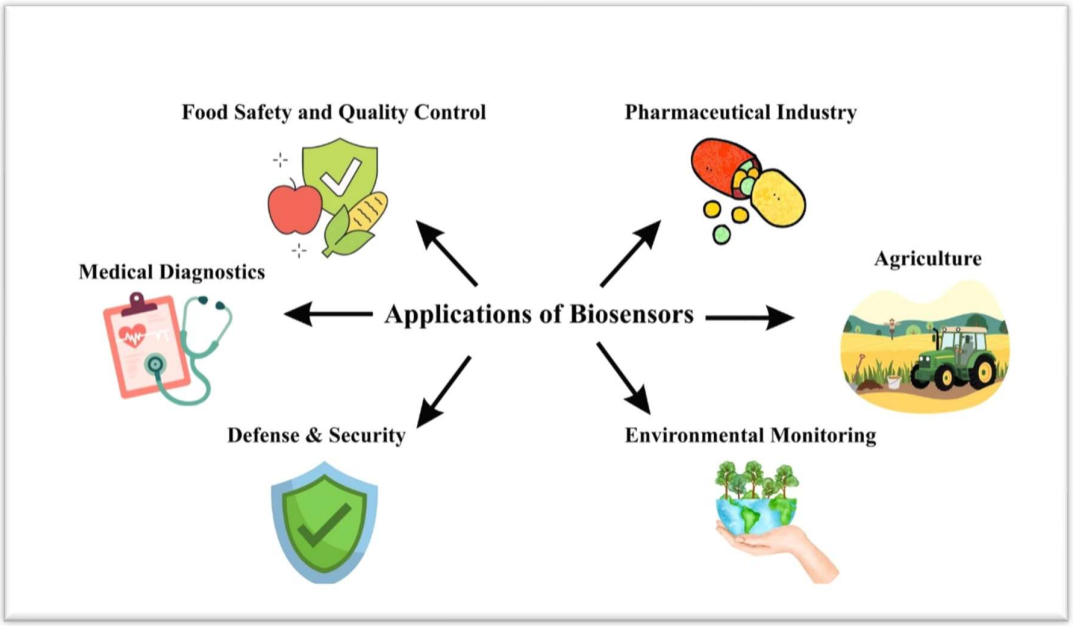

Fig. 1.10 The application of biosensors

c) Food Safety and Quality Control

- Pesticide detection: Fresh produce can have residues of pesticides from farming practices, while other types of produce may contain residues from processing. These residues can be detected using biosensors.
- Pathogen screening: Foodborne pathogens (Salmonella, E. coli, Listeria) can be identified in food using biosensors.
- Freshness indicators: Chemical signals that occur when food spoils or rots can be detected by biosensors.
- Contaminant identification: Unwanted contaminants in commercial food products can be identified by biosensors.
- Nutritional analysis: Amounts of nutrients in certain food items (glucose, lactate, cholesterol) can be determined through biosensor technology [38].

d) Defence & Security

- Chemical warfare agents: Trace amounts of chemicals such as nerve agents or mustard gas can be identified rapidly using biosensors.
- Biological warfare agents: Anthrax and Botulinum Toxin can be screened for as biothreats using biosensors.
- Explosive detection: Small quantities of explosives can be found using biosensors in security applications.
- Radiation measurement: Biosensors can be used to track high levels of radiation in an area.

e) Pharmaceutical Industry

- Drug discovery: In addition to finding new medicines, biosensors assist researchers in identifying potential compounds to test further.
- Quality control: Many manufacturers utilize biosensors to check the quality and purity of manufactured pharmaceuticals.
- Fermentation monitoring: Biosensors help companies to track and optimize the results of large-scale fermentation.
- Drug interactions: Researchers study how medications react with biological targets by using biosensors.

f) Agriculture

- Soil health: Conditions of the soil (nutrient content etc.), can be monitored by tracking pH level and nutrient availability.
- Plant pathogen detection: Early detection of diseases/plant pathogen allows farmers to treat problems early to avoid losses.
- Plant hormone levels: By tracking hormone levels farmers can determine if plants are stressed or growing properly.
- Crop monitoring: Some biosensors can detect pesticide residue on farm grown crops before they are harvested [39].

1.7.10 Future Scope and Emerging Trends in Biosensors

The future of biosensors is incredibly bright due to the integration of many emerging technology trends:

a) Wearable Biosensors: Future biosensors are anticipated to be wearable biosensors that include flexible skin worn devices such as biosensor patches, tattoo biosensors, or smart watch devices that provide continuous and real time health parameter tracking (i.e., glucose, lactate, cortisol, etc.) without drawing a drop of blood.

b) Implantable Biosensors: In addition to wearable biosensors there will also be miniaturized implantable biosensors used to continuously monitor glucose levels in diabetic patients, drug levels, and physiological parameters in severely ill patients.

c) Internet of Things (IoT) Connected Biosensors: As part of the Internet of Things (IoT), biosensors will have the capability of transmitting real time data to healthcare providers using a variety of methods, i.e., via smartphones, cloud computing platforms, and/or artificial intelligence systems. This will enable remote monitoring, data analysis, and telemedicine services.

d) Artificial Intelligence & Machine Learning: Additionally, artificial intelligence and machine learning systems are becoming more prevalent in analysing biosensor-generated data. The ability of these advanced analytical systems to identify patterns in data sets generated by biosensors will enable early disease diagnosis, prediction of future diseases based on trends in data collected over time, and the elimination of false positive readings which may occur when analysing large amounts of complex multi-analyte data [40].

e) CRISPR Based Biosensors: Another area where significant advancements are being made involves integrating CRISPR-Cas systems into biosensors. These advanced systems use RNA guides to target very specific sequences of nucleic acids within samples. Two examples of these types of systems are SHERLOCK and DETECTR. Both systems exhibit an unprecedented level of sensitivity and specificity in terms of detecting target nucleic acids [40].

f) Point-of-Care (POC) and Lab-On-A-Chip Devices: Point-of-care and lab-on-a-chip microfluidic biosensors are another example of how technology continues to advance in this field. These tiny chips contain all of the necessary components required to analyse samples and produce results in just minutes. As a result, they can be used at the patient's bedside or even in remote locations. They eliminate the need for traditional laboratory equipment.

g) Nanomaterial Enhanced Next Generation Biosensors: Researchers are currently exploring various forms of nanomaterials as potential materials for next generation biosensors. Some examples of materials being studied include MXene, metal-organic frameworks (MOFs), covalent organic frameworks (COFs), black phosphorous, and boron nitride. Each type of material has the potential to improve the performance characteristics of biosensors and allow them to detect smaller quantities of analytes than current biosensors [41].

h) Multiplexed Biosensors: An additional emerging trend is multiplexed biosensing. Multiplexed biosensing refers to the use of arrays of biosensors that can measure multiple analytes simultaneously. This allows researchers and clinicians to obtain detailed information about diseases and assess environmental conditions more quickly and efficiently than would otherwise be possible.

i) Self-Powered Biosensors: Lastly, self-powered biosensors represent yet another exciting area of development. Biofuel cells are one type of self-powering device. Unlike most other biosensors, biofuel cells do not require an external power source to operate. Instead, they generate their own electrical energy through biochemical reactions occurring inside the device. This makes them well-suited for long-term deployment at remote sites or for use in implanted medical devices [42].

CHAPTER 2

Literature Review

2.1 Introduction to Organophosphate

Organophosphate (OP) pesticides are a wide-ranging and structurally diverse group of man-made compounds that were developed from phosphoric acid and related phosphorous acids. They represent the most used class of insecticides globally with an estimated 40-50 % of total global use, and 30 % of global pesticide sales. The majority of OP compounds began to be developed in the early 1930's and mid-1940's. These developments occurred in part through advances made in organophosphorus nerve agents as part of war-time efforts in WWII. OP compounds have since become essential tools in agricultural applications, public health vector control, veterinary medicine and aquaculture [43]. The large-scale use of OP compounds was largely due to their relatively low environmental persistence compared to those of the organochlorine pesticides which they displaced. The half-lives of OP compounds generally range from days to weeks when subjected to hydrolysis in soils and aquatic environments. Although OP compounds may degrade rapidly in the environment, the potential for acute toxicity remains high, and some of the more common OP compounds remain highly toxic relative to other man-made substances. As such, the misuse or overuse of OP compounds has raised increasing concerns for scientists, regulators and public health officials around the world [44].

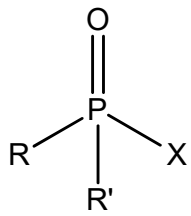

Fig. 2.1 Structure of OP

In terms of chemical structure, most OP compounds contain a central phosphorus atom with bonds to four substituent groups, often two alkoxy groups (e.g., methoxy or ethoxy), one double-bonded oxygen or sulphur atom, and one leaving group that will be displaced once the

compound is exposed to water via hydrolysis or enzymes is shown in **Fig. 2.1**. Due to the various possible combinations of these four functional groups, OP compounds can be grouped into several different chemical classes including phosphate, phosphorothioates, phosphonodithioates, phosphonates and phosphonamidites. The variety of structures exhibited by members of the OP family explains the differences in physical characteristics, environmental behaviour and biological effects observed between individual compounds within this family [43].

2.2 Health Consequences of Organophosphate Pesticide Exposure

a) Acute Toxicity: The vast majority of all acute poisonings from pesticides throughout the world result directly from the use of OP pesticides. According to WHO, it is estimated that each year there are approximately three million people who suffer from acute pesticide poisoning. This number represents the largest portion of such poisonings involving OP pesticides. Intentional self-poisoning using very high toxicity levels of OP pesticides has become a major public health issue within many developing countries especially within South and Southeast Asia resulting in an average annual death toll of hundreds of thousands of people [45].

b) Chronic Exposure: There is substantial evidence that exposure to low-dose levels of OP pesticides over long periods of time via occupational routes (such as farm workers, or pesticide applicators) and diet from consuming food products contaminated with these pesticides may lead to a wide range of negative health consequences among individuals participating in various epidemiologic studies:

- Neurotoxicity: Various epidemiologic studies have indicated that there exists a positive relationship between chronic exposure to low-dose levels of OP pesticides and the risk of suffering from Parkinson's disease, Alzheimer's disease, and additional neurodegenerative conditions. The California-based CHAMACOS study and the FARM study provide considerable evidence showing a positive association between exposure to organophosphates as measured by biomarkers and the risk of Parkinson's disease [45].
- Developmental Neurotoxicity: Prenatal and early postnatal exposure to low-dose amounts of OP pesticides have been positively correlated with neurodevelopmentally-related developmental problems including decreased IQ, diminished ability to perform working memory tasks, increased occurrence of ADHD, and increased occurrences of autism-

spectrum disorders. The CCCEH birth cohort study showed significant negative relationships between prenatal chlorpyrifos exposure (as measured by cord blood biomarkers) and cognitive performance measures at age 7.

- Carcinogenicity: The IARC has identified several OP compounds as being either "probable" (Group 2A) or "possible" (Group 2B) human carcinogens. Studies conducted to assess the relationship between exposure to OP pesticides and human cancer have shown statistically-significant associations between the two variables. These associations include non-Hodgkin lymphoma, brain cancer, and breast cancer [46].
- Endocrine Disruption: Many different biochemical processes are capable of being negatively impacted by OP compounds, including interference with endocrine function through direct actions on the nucleus which are involved in the breakdown of both OP compounds and oxidized lipids interaction with nuclear hormone receptors and disruption of thyroid hormone biosynthesis. Adverse effects on reproductive function have also been observed in individuals who work in environments where they are exposed to OP compounds, including reductions in male fertility (reduced sperm counts, reduced sperm motility) alterations in female menstrual cycle, etc.
- Immunotoxicity: Suppression of cell-mediated immunity and humoral immunity, increased susceptibility to infection, and increased incidence rates for autoimmune disorders have been documented as potential immunologically-related adverse health effects associated with prolonged exposures to OP pesticides [46].

c) Vulnerable Populations: Children are much more susceptible to the adverse neurological effects associated with exposures to OP pesticides due to several factors related to their physiology: a) their blood-brain barriers are less well-developed, b) their brains are experiencing rapid growth and synaptogenesis, c) their metabolic capabilities to detoxify OP compounds are lower than those in adult humans, d) their food intake relative to body mass is greater than that experienced by adults [47]. Urinary OP metabolites were significantly higher in children residing in agricultural areas compared to urban children as determined by a landmark study published by Eskenazi et al. (2007). Therefore, the pathways used by children to be exposed to OP pesticides include dietary and environmental residential pathways.

2.3 Characteristics of the material

Electrode materials chosen appropriately are among the most important elements of the sensitivity, selectivity, and overall electro-analytical performance of a biosensor based on electrochemistry. Over the past decade or so, due to superior physicochemical properties such as high surface area, modifiable electronic structure, numerous catalytically-active sites, and better than typical bulk materials, highly-efficient electrocatalysis, considerable interest has been directed toward use of 2D and nano-structured electrode modifiers. This research employs a composite made from nano-scaled particles of graphitic carbon-nitride (g-C_3N_4) and copper sulphide (CuS) for construction of an enzymatic electrochemical sensor [48]. The detailed descriptions provided in the sections below describe the structural, physical, chemical, optical and electrochemical properties of each individual material and also those of their composite.

2.3.1 Graphitic carbon nitride (g-C3N4)

Structure and Physical Properties:

The allotropes of Carbon Nitride include g-C_3N_4, the most stable allotrope of carbon nitride under normal temperature and pressure.

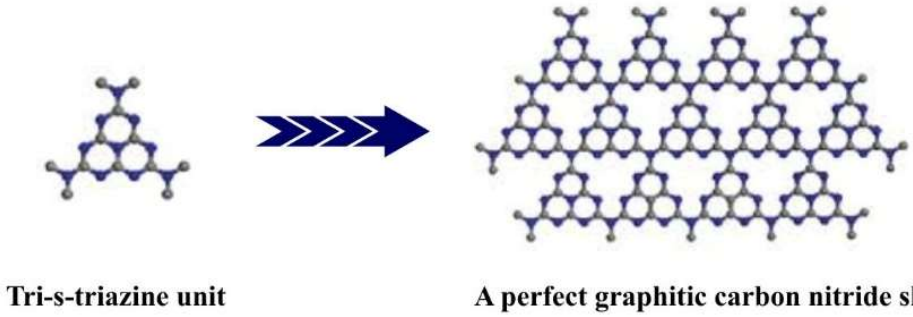

Fig.2.2 Structure of g-C_3N_4

The carbon and nitrogen atoms are arranged in a 3:4 molar ratio and include residual hydrogen as terminal amino (-NH_2) and imino (-NH-) groups along the edges of their polymeric layers. Each layer consists of heptazine (tri-s-triazine) units bonded together via planar tertiary nitrogen bridges as shown in **Fig. 2.2**. These layers are then stacked together through Van Der Waal's

forces and pi-pi interactions similar to those found in graphite with an interlayer distance of about 0.326nm [49].

Optical Properties:

The g-C_3N_4 is a visible light absorbing semiconductor with a very narrow bandgap of approximately 2.7eV and therefore absorbs light at wavelengths less than 460nm. Therefore g-C_3N_4 can absorb visible light and utilize energy from the sun, unlike titanium dioxide (TiO_2) that only utilizes UV light due to its larger bandgap size of 3.2eV. The absorption of UV-VIS radiation demonstrates significant absorption bands at approximately 320nm due to π-π^* electronic transition in the aromatic heptazine ring structures. There is also a secondary absorption band centred near 460nm originating from n-π^* transitions due to the lone pair electrons of the bridged nitrogen atoms. In addition, g-C_3N_4 emits strong photoluminescence in the blue-green region from approximately 430 to 530nm when excited at wavelengths ranging from 350-380nmv [50]. Photoluminescence arises from the recombination of photo-generated electrons and holes produced by the absorbed photons in the heptazine framework, a feature commonly used in fluorescent based optical sensors.

Electrochemical Properties:

Due to its numerous surface nitrogen rich functional groups such as terminal amino (-NH_2) and bridged imino (-NH-) groups that act as electro-catalytically active sites facilitating analyte adsorption on the working electrode surface and promoting electron transfer between the analyte and the electrode, g-C_3N_4 makes an ideal additive material for developing electrochemical sensor electrodes. Additionally, g-C_3N_4 has a wide electrochemical operating window thus significantly reducing background currents, and greatly improving signal-to-noise ratios, ultimately decreasing detection limits [51]. Although pure bulk g-C_3N_4 has inherently low electrical conductivity limiting its use as a sole electrode material, it is typically paired with electrically conductive nano-materials such as copper sulphide, graphene, single-walled carbon nanotubes, or metallic nanoparticles to create high-performing nanocomposites that possess enhanced electron transfer rates and improved electrochemical capabilities while maintaining the surface-functionalized nitrogen rich characteristics of g-C_3N_4 [52].

2.3.2 Applications of g-C_3N_4

a) Electrochemical Sensors and Biosensors: g-C_3N_4 has become a very popular material used to modify electrodes in order to improve the sensitivity of detecting various analytes including pesticides, heavy metals, pharmaceuticals, dopamine, uric acid, glucose and H_2O_2. The unique properties of g-C_3N_4 such as high surface area, numerous nitrogen active sites on the surface and ability to adsorb analytes provide an ideal combination to increase electrode sensitivity and decrease the detection limits which are why g-C_3N_4 has emerged as one of the most commonly studied non-enzymatic electrode materials for electrochemical sensing applications [51].

b) Photocatalysis: g-C_3N_4 has shown great potential as a metal free, visible light activated photocatalyst for the production of hydrogen gas from the decomposition of water via solar energy, conversion of CO_2 to solar fuels and photocatalytic degradation of organic pollutants, pharmaceutical residues, dye contaminants and heavy metals present in waste water. As of today, g-C_3N_4 is still the most heavily researched metal-free photocatalyst globally with many thousands of research studies published that demonstrate its photocatalytic activity under visible light irradiation.

c) Energy Storage: g-C_3N_4 has been incorporated into lithium-ion batteries as anode materials, super-capacitors as electrode materials and in fuel cells as catalyst materials. In each case, the layered structure of g-C_3N_4 facilitates the insertion or de-insertion of ions, the large surface area of g-C_3N_4 provides ample site for charge storage, and the functional groups containing nitrogen within the molecular structure of g-C_3N_4 enhances both wetting characteristics and electron transfer across the electrolyte/electrode interface. Furthermore, g-C_3N_4-based electro-catalysts have also exhibited good ORR activity (oxygen reduction reaction) in alkaline fuel cells [53].

d) Fluorescence or Optical Detection: Utilizing the inherent blue-green fluorescence emission at 430-530 nm of g-C_3N_4 nano-sheets, researchers have successfully developed fluorescent sensors and assays based upon g-C_3N_4 nano-sheets to detect a variety of analytes including metal ions (Hg^{2+}, Pb^{2+}, Cu^{2+}), pesticides, antibiotics and nucleic acids using fluorescence quenching or turn-on mechanisms. Additionally, due to their narrow band gap and small particle sizes (typically <10nm), g-C_3N_4 QDs display extremely stable and tuneable fluorescence that can be utilized for biological imaging applications.

e) Biomedical Applications: The biocompatibility, intrinsic photoluminescence, large surface area and adjustable surface chemistry properties of g-C_3N_4 enable the use of g-C_3N_4 in biomedical applications including drug delivery systems, PDT treatment of cancer cells, antimicrobial coating materials, and biosensors designed to detect disease biomarkers. Drug delivery systems incorporating g-C_3N_4 can encapsulate drugs that can then be released from the system in response to external stimuli (e.g., pH or light) [53].

f) Environmental Cleanup: Recent studies have demonstrated that g-C_3N_4-based composite nanomaterials can effectively degrade a wide range of environmental pollutants, textile dye, endocrine disruptors and heavy metals from polluted waters using visible light for activation. The photo-stability of g-C_3N_4-based composites also enables these materials to remain effective throughout repeated uses.

2.3.3 Copper Sulphide (CuS)

a) Structure and Physical Properties

A naturally occurring mineral called covellite has been found to have properties similar to those of a p-type inorganic semiconductor. Covellite has a hexagonal crystal structure. The complex structure of covellite is composed of alternating layers of trigonal-planar CuS and tetrahedral CuS units. These two types of units are connected by S-S bonds. Therefore, the same lattice contains Cu^{+} and Cu^{2+} oxidation states, leading to a mixed valency. This mixed state leads to many unusual electronic and electrochemical properties in CuS [54].

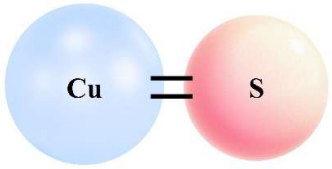

Fig. 2.3 Structure of CuS

As compared to other semiconductor materials, the CuS semiconductor has a relatively small direct band gap of about 1.2–2.2 electron-volts. Depending upon how the material was synthesized, what shape and size particles were produced, and how well the crystals formed, the band gap will vary. However, this range includes much of the visible and near infrared (NIR) part of the electromagnetic radiation spectrum, which means that CuS is a good

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broadband absorber of light. This property makes it very interesting for use in many different forms of photocatalytic, photothermal, and photoelectrochemical applications.

b) Optical Properties

There are two factors that determine the optical properties of CuS, its small bandgap, and its ability to produce localized surface-plasmon-resonance (LSPR). Unlike LSPR in gold and silver nanoparticles where the resonance occurs primarily in the visible part of the electromagnetic radiation spectrum, LSPR in CuS nanoparticles produces absorption peaks in the near-infrared (NIR) region of the spectrum between approximately 900–1200 nm. The reason for this behaviour is due to the collective oscillations of positive charge carriers (holes) located at the surface of the CuS nanoparticles. These oscillations are similar to those seen in gold nanorods, however, they occur at much lower costs, making them potentially useful for various applications including photothermal therapy, photo-acoustic imaging, and NIR-driven photocatalysis [55].

Although there are no sharp peaks present in either the UV-visible absorption spectrum or the IR transmission spectrum of CuS, it does show broad absorption extending across the entire UV-visible-NIR spectral range. The broad absorption spectrum is responsible for its typical dark blue/black color. In addition to being used for identification purposes, the FTIR spectrum of CuS provides information regarding the presence of specific chemical bonds in the material. Bands associated with stretching vibrations involving the Cu-S bond appear at approximate wavenumber values of 620 cm^{-1} [56].

c) Electrochemical Properties

The potential uses of CuS in electrochemical sensing and energy storage applications depend largely on its electrochemical properties. Due to its ability to undergo rapid reversible oxidation-reduction processes, CuS exhibits favourable redox characteristics. The redox cycles result from the simple conversion between Cu^{+} and Cu^{2+} oxidation states within the CuS lattice. By allowing CuS to take an active role in catalysing electrochemical reactions (e.g., oxidizing organic analytes, reducing dissolved O_2 and reducing pesticide compounds), it generates measurable currents during operation. The resulting electrochemical responses include both amperometric and voltametric signals [55]. The high electrical conductivity exhibited by CuS results from hole-carrier mediated p-type conduction through the Cu-S bonding network. The

high electrical conductivity allows CuS to serve as an effective conductive scaffold when combined with electrically insulating materials such as g-C_3N_4. The low charge-transfer resistance, fast electron-transfer rates, and high electroactive surface areas observed in CuS-modified electrodes enhance their electrochemical sensitivities in sensing applications.

2.3.4 Applications of CuS

a) Electrochemical Sensing: CuS has been heavily utilized as a sensor electrode modifier for the ultra-sensitive electrochemical detection of pesticides, heavy metals (Hg^{2+}, Pb^{2+}, Cd^{2+}, As^{3+}), drugs (antibiotics etc.), dopamine, H_2O_2, and glucose. The very good electrical conductivity of CuS along with the excellent redox behaviour, the high electroactive surface area and the strong interaction between the analytes and the CuS surface allow CuS to be among the best transition metal chalcogenide electrode materials for electrochemical sensing. This is demonstrated by the extremely low detection limit that can be achieved in the nanomolar to picomolar range.

b) Photocatalysis: The photocatalytic activity under visible light and near-infrared light of CuS has been studied for many years in relation to the degradation of chemical contaminants in wastewater including antibiotics and textile dyes. The narrow band gap of CuS allows for the optimal use of the sun's radiation, additionally, the p-type character of CuS enables the oxidation reaction by holes generated during illumination [57].

c) Photothermal Therapy (PTT): The superior absorption capability of CuS nanoparticles in the near-infrared region converts this wavelength efficiently into localized heat. As a result, CuS nanoparticles are being used increasingly as photothermal agents in the treatment of cancer via near-infrared laser-induced thermal destruction. The selective heating of cancerous tissues occurs upon exposure to NIR laser light by CuS nanoparticles accumulated in these regions thereby protecting nearby normal cells [57].

d) Energy Storage: Supercapacitor and lithium or sodium ion battery applications of CuS have received considerable interest lately. When functioning as pseudocapacitive electrode material, CuS stores electric charge through rapid, reversible faradaic redox reactions of the Cu^{2+}/Cu^{+} redox couple.

e) Solar Cells and Photo-electrochemistry: Counter electrode in dye-sensitized solar cells (DSSCs), Hole transport layer in perovskite solar cells, Photoelectrodes in

photoelectrochemical water splitting devices. High electrical conductivity, catalytic activity towards I^-/I_3^- redox pair, and cost-effective production process render it a substitute for expensive Pt-counter electrodes in DSSC applications [[58].

f) Biomedical and Antibacterial Applications: Due to their inherent ability to exhibit antibacterial and antifungal activities resulting from the controlled release of toxic amounts of Cu^{2+} ions capable of disrupting cellular membranes of bacteria and inhibiting essential biological processes, CuS nanoparticles have been integrated into various types of antimicrobial coatings, wound dressings, and biomedical implant materials. Additionally, they possess favourable optical properties suitable for application in photoacoustic imaging (PAI) and NIR fluorescence bioimaging (NIRFBI) techniques [59].

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CHAPTER 3

Material and Methods

3.1 Introduction

This chapter is a general overview of the materials used to create Graphitic Carbon Nitride (g-C_3N_4), Copper Sulphide (CuS) and their respective Nanocomposite material as it relates to the detection of Organophosphates (OP). The Chapter will cover all Morphological, Structural and Electrochemical Characterization Techniques for g-C_3N_4, CuS and the respective Nanocomposite Materials created from these two individual materials [60]. In addition, this Chapter will discuss various methods used to Immobilize Acetylcholinesterase (AChE) onto the Surface of the Electrode.

3.2 Materials

The details of the materials used in the experiment are as follows:

3.2.1 Chemicals

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Acetylthiocholine Chloride (ATCl), Trichlorfon (TF), Glutaraldehyde Solution, Glucose (Glu ≥ 99.5 %), Ascorbic Acid (AA 99 %) and Uric Acid (UA ≥ 99 %) were obtained from Sigma Aldrich Pvt Ltd., India. Chemicals such as Acetone, Ethanol, Sodium Chloride (NaCl), Melamine and Copper (II) Sulphate Pentahydrate ($CuSO_4.5H_20$), Sodium Thiosulphate Pentahydrate ($Na_2S_2O_3.5H_2O$) were purchased from Thermo-Fisher Scientific Pvt Ltd., India. The purity of all analytical grade chemicals is at least 98 %. The chemicals have been used in their pure form [61].

3.2.2 Solutions and Buffers

- 0.1 M Phosphate buffer saline (PBS), pH 7
- 5mM $[Fe(CN)_6]^{3-/4-}$ used in PBS solution as redox initiator.

3.3 Characterization Methods

Several methods are available to determine the structural properties of a material which include the use of Fourier Transform Infrared Spectroscopy (FTIR) and X-ray Diffraction (XRD). Therefore, the morphology and elemental composition of all developed electrodes and synthesized materials will be characterized by various analytical techniques. Those analytical techniques used to characterize the physical and elemental characteristics of the materials studied included Transmission Electron Microscopy (TEM). Field Emission Scanning Electron Microscopy (FESEM) and Energy-Dispersive X-ray Spectroscopy (EDX) for the morphological and elemental characterization. Electrochemical analytical characterization techniques including Cyclic Voltammetry (CV) and Differential Pulse Voltammetry (DPV) were also used to perform electrochemical characterization [62].

3.3.1 Fourier transform infrared spectroscopy (FTIR) spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) is a powerful non-destructive technique that is capable of identifying the presence of different types of functional groups and molecular environments within a solid material. It works according to the principles of molecular vibrations where molecules vibrate when exposed to infrared radiation at energies equivalent to those of the vibrational modes of their chemical bonds. This vibrational energy may be absorbed by the molecule if the infrared radiation has an appropriate frequency, which reduces the amount of radiation transmitted through the sample. Each type of bond like stretching, bending, twisting or rocking, will have its own characteristic absorption frequency resulting in a unique 'fingerprint' spectrum for each compound [63].

The use of FTIR involves irradiating the sample with infrared light having a wide range of wavelengths from a broadband infrared source. A Michelson interferometer modifies the wavelength of this light into an interference pattern referred to as an interferogram. An interferogram is then mathematically transformed (Fourier transformed) to obtain the absorption spectrum of the sample as a function of wavenumber. The mid-infrared spectral region (wavelengths: 2.5-100μm or 400-4000 cm^{-1}) is preferred because most common organic and inorganic compounds exhibit their fundamental vibrational modes here. As such, FTIR spectra provide information about the presence of different types of chemical bonds and

functional groups in materials. For example, g-C_3N_4 displays two distinct absorption regions: one related to C-N stretching and C=N stretching modes in the range 1200-1650 cm^{-1}, and another due to a very broad N-H stretch absorption at higher wave numbers (>3000 cm^{-1}). A Diagrammatic representation of FTIR is shown in **Fig. 3.1.**

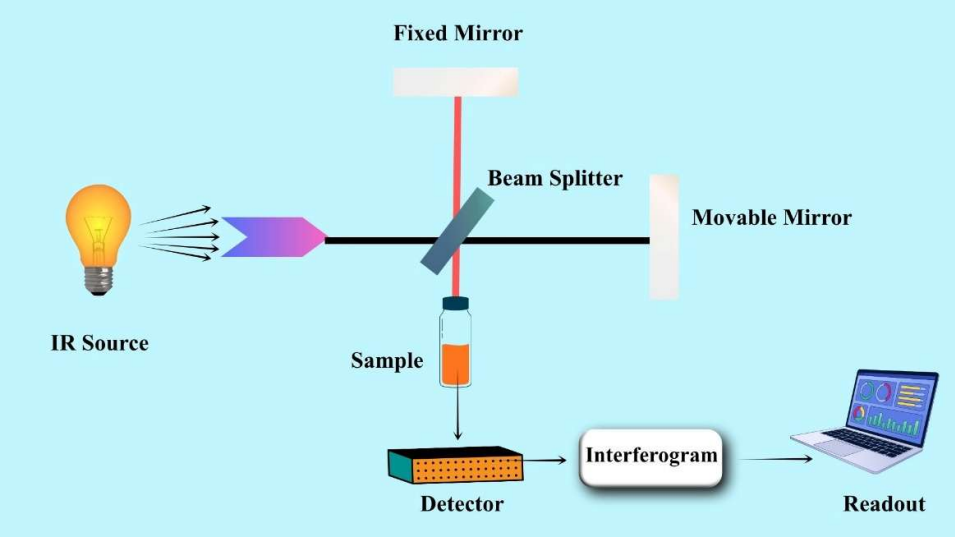

Fig. 3.1 Diagrammatic representation of FTIR

Lattice vibrations are expected to dominate the FTIR spectra of CuS samples. Therefore, FTIR studies of these nanomaterials are essential for confirming the formation of the composite, assessing the nature of bonding between components and determining whether any significant changes occur to functional groups during processing. This research uses a FTIR instrument to collect IR data for g-C_3N_4, CuS and g-C_3N_4/CuS composites in the range 400-4000 cm^{-1} at a resolution of 4 cm^{-1} [63].

3.3.2 X-ray diffraction (XRD)

The main method used for identifying the crystallographic structures, phases, and particle sizes of solid products through X-Ray Diffraction (XRD) uses Bragg's Law of diffraction. According to Bragg's Law, constructive interference of the -rays that have been diffracted off a crystal lattice will occur whenever the wavelength of the x-ray diffraction will produce a path length difference equal to an integer number of times the x-ray wavelength. This can be represented mathematically as follows:

$$n\lambda = 2d\sin(\theta)$$

where:

- n is an integer representing the order of diffraction
- λ represents the x-ray wavelength
- d represents the distance between adjacent layers of atoms in the crystal lattice
- θ represents the angle at which the diffracted x-rays emerge from the sample

A plot of x-ray intensity versus 2θ produces a diffractogram pattern, which contains sharp peaks. The position of these peaks is indicative of both the arrangement of atoms within a unit cell and the distances between layers of atoms in the crystal lattice. Identification of the phases present in a sample is determined by comparing experimental diffractograms to known reference patterns available in databases such as those developed by the International Centre for Diffraction Data [64]. A Diagrammatic representation of XRD is shown in **Fig. 3.2**.

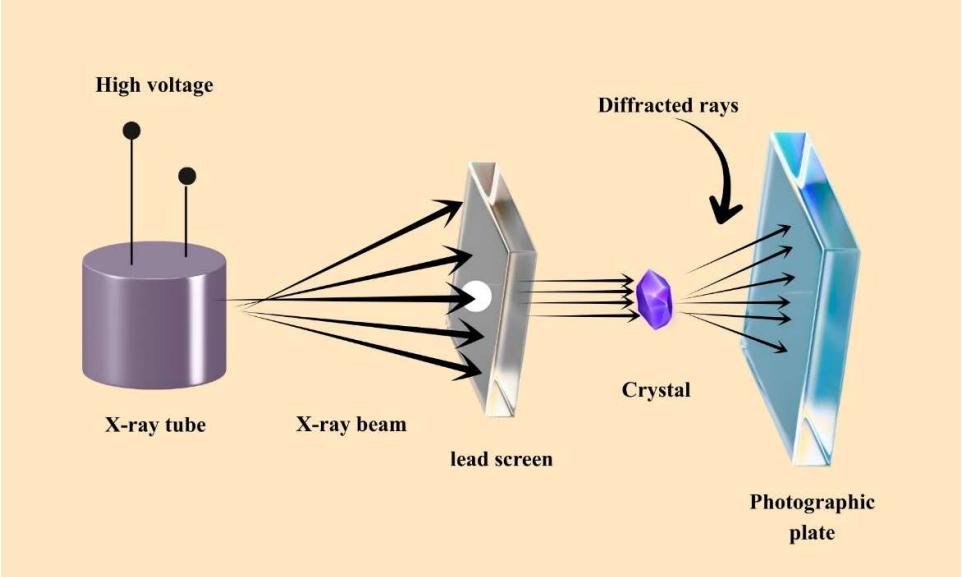

Fig. 3.2 Diagrammatic representation of XRD.

3.3.3 Field Emission Scanning Electron Microscopy (FESEM)

Field Emission Scanning Electron Microscopy (FESEM) is a highly accurate method for obtaining surface image data based on both the size, shape, and the textured nature of nanomaterials. While FESEM provides similar information to Conventional Scanning Electron Microscopy (CSEM) regarding the surface characteristics of materials, CSEM utilizes a thermionic tungsten filament as its electron source. The FESEM system uses a field emission

gun instead [65]. The metal tip used in the field emission gun has a sharp end. By placing an extremely high electric potential difference across this metallic tip, it causes quantum mechanical tunnelling of electrons through the metallic barrier. As a result, the FESEM produces a very coherent, low energy electron beam with minimal chromatic aberrations. This results in high spatial resolution capabilities for FESEM when compared to CSEM systems. Spatial resolutions can be achieved with FESEM ranging from 1-5 nm. A Diagrammatic representation of FESEM is shown in **Fig. 3.3**.

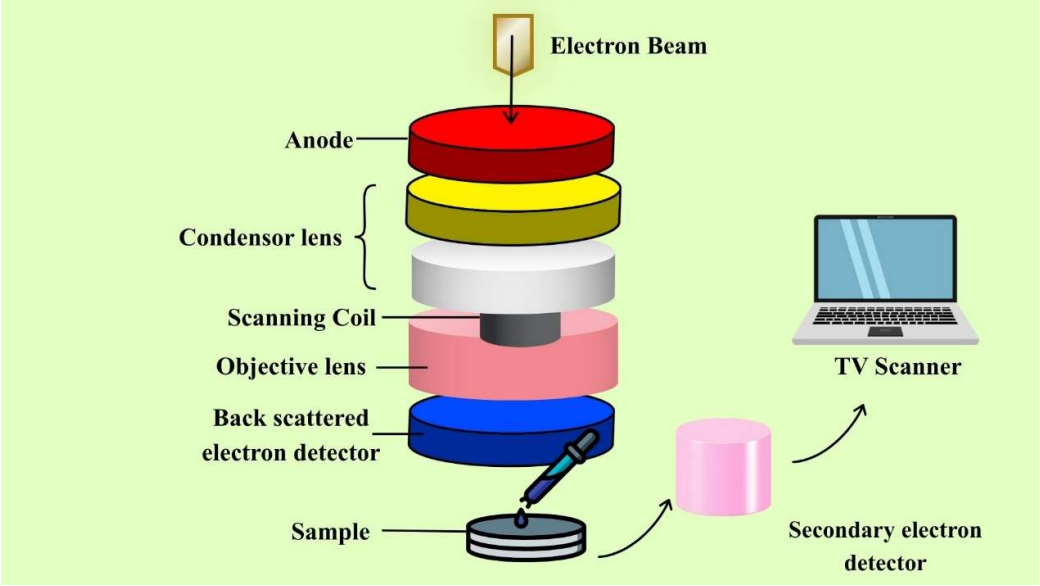

Fig. 3.3 Diagrammatic representation of FESEM machine

In FESEM, the primary electron beam produced from the field emission gun is directed onto the sample surface so that it raster scans the entire surface. When the primary electron beam interacts with near-surface atoms on the sample, secondary electrons (SE) are produced. These SE are relatively low energy electrons produced from the first few nanometres of the surface. An Everhart-Thornley detector collects these SE producing high contrast topographic images. From these topographic images, researchers can see the surface morphology of their samples, how particles have aggregated together and what types of nano or micro structural features exist. When studying electrochemistry and electrochemical applications, the morphology of your samples also determines the amount of electroactive surface area present as well as how easily analyte molecules will be able to access the surface of the working electrode. Therefore, in order to determine the electrochemical behaviour of our synthesized materials, we chose to

use FESEM to study the surface morphology of our g-C_3N_4, CuS and g-C_3N_4/CuS nanocomposite samples [65].

3.3.4 Energy Dispersive X-ray Spectroscopy (EDX)

EDX, also called EDS or EDXA, uses a primary electron beam that strikes the sample to eject inner shell electrons from its constituent atoms, this leaves the atom with characteristic electron vacancies. The outer shell electrons drop down to replace the missing electrons creating a difference in energy levels and when they do so, they emit characteristic x-ray photons. These x-rays have energies that correspond specifically to the atomic numbers of their respective emitting elements. EDX spectra represent energy dispersive detectors sorting x-rays by their energy, each spectral peak represents a specific element contained in the sample. EDX has significant value in characterizing nanomaterials due to the ability to obtain both morphological information and elemental composition simultaneously on the same sample using one instrument [66]. This is a particular advantage compared to traditional methods where samples would require additional processing steps to prepare the samples for each analytical technique. While EDX provides point analysis information, elemental mapping can provide visual representation of how individual elements distribute throughout the field of view demonstrating whether there is compositional homogeneity or heterogeneity in nanocomposites. Therefore, we anticipate EDX will demonstrate the presence of C, N, Cu and S as the major elements for the g-C_3N_4/CuS nanocomposite, and elemental mapping will show the close proximity and uniform mixing of the g-C_3N_4 and CuS phases throughout the nanocomposite structure. The EDX analysis utilized a detector connected to our FESEM instrument. The elemental mapping was done in order to determine if the various elements were spatially distributed in relation to each other [64].

3.3.5 Transmission Electron Microscopy (TEM)

The atomic scale structural and morphological information of Transmission Electron Microscopy (TEM) is provided by the high energy electron beam (usually 80–300 kV) that passes through the thin sectioned specimen and then collects all of the electrons that pass through and are deflected from the thin sectioned specimen to produce a magnified image. In comparison to Field Emission Scanning Electron Microscope (FESEM) which examines the surface morphology of the specimen, TEM is capable of examining the internal nanostructure, lattice fringes, particle size distribution and crystallographic relationships at sub-nanometre

resolutions. The contrast in TEM images comes from variations in electron scattering due to variations in sample thickness, density and atomic number. Heavy elements scatter stronger and therefore appear darker in bright field images [67].

High Resolution TEM (HRTEM) extends the capability of conventional TEM imaging capabilities so as to provide atomic plane level resolution. Therefore, it is possible to directly observe the crystal lattice fringes and measure the d-spacings and compare these measurements to X-ray Diffraction (XRD) results. A Diagrammatic representation of TEM is shown in **Fig. 3.4**.

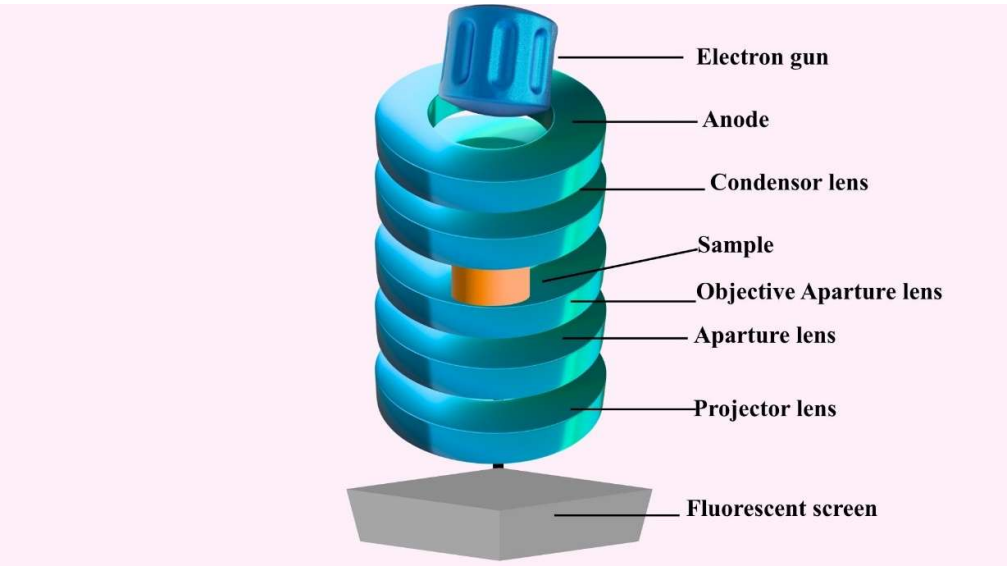

Fig. 3.4 Diagrammatic representation of TEM machine

For nanocomposites, TEM is required to examine the interfacial relationship between phases in the composite material, determine if particles have been anchored to the other phases in the composite material and evaluate if quantum size effects occur when dimensions approach nano-meter scale. In this research study, TEM was used to analyse the dispersion and anchorage of CuS nanoparticles on g-C_3N_4 nanosheets, verify lattice parameters using High Resolution TEM (HRTEM) [68].

Sample preparation involved the use of ultrasonic agitation to disperse a small amount of powdered material into ethanol followed by the deposition of a few microliters of the resulting solution onto carbon coated copper TEM grid support films. Following drying of the deposited liquid, samples were analysed.

3.4 Electrochemical Characterization Techniques

Electrochemical analysis is a branch of analytical chemistry where electrochemical concepts are applied for assessing the chemical reaction, identifying some of the properties of substances, and determining their concentration. The advantages of electrochemical analysis over other surface modification technologies include its ability to preserve the surface features during electrochemical dissolution. The crystal structure and orientation remain intact. Cyclic voltammetry and differential pulse voltammetry are among the main types of electrochemical analysis. The basic unit of an electrochemical system includes at least one working electrode, one reference electrode, and one counter electrode. These electrodes are connected with a potentiostat which measures the resulting current. When we run this type of electrochemical test, the working electrode is subjected to a certain potential and plots the corresponding current against time. In this work, each electrode was analysed with respect to its electrochemical responses utilizing an Autolab, Pt or Ag/AgCl as counter-electrodes [69].

3.4.1 Cyclic Voltammetry (CV)

A variety of different voltammetric methods can be used to measure changes in charge and mass transport across an electrode interface. One of the most common and versatile methods is cyclic voltammetry (CV). CV is often utilized to analyse the thermodynamic and kinetic properties of electrode reactions, to identify electrochemically active species within a solution environment, or to evaluate how modifying the electrode surface affects its electrochemical characteristics.

When performing a CV experiment, the working electrode will have a triangle-shaped voltage signal applied to it. The initial potential (E_i) that the electrode starts at is first scanned linearly toward the maximum potential (E_λ) of the triangular shape at a specific scan rate (v) [70]. After reaching E_λ, the direction of the applied potential is reversed, returning the working electrode to either the starting point. As the potential is changed during this scanning procedure, a current is produced due to the reaction occurring at the working electrode. This current is measured and plotted as a function of the applied potential. The resulting plot is called a cyclic voltammogram (CV) [71].

The CV obtained for a reversible redox couple contains two distinct features: an anodic peak (I_{pa}) when the working electrode is being moved in the positive direction that represents

oxidation and a cathodic peak (I_{pc}) when the working electrode is moving in the negative direction that represents reduction. If all of the electrochemistry occurring at the working electrode is controlled by diffusion (all of the reactants are diffusing through the solution to reach the working electrode), then both the anodic and cathodic peak currents should depend upon the square root of the scan rate. If there are no diffusion limitations involved in the electrochemistry occurring at the working electrode, then both of these peak currents should depend directly on v [70].

In this research project, we also made use of CV. We used CV to investigate several aspects of our system to obtain information about the chemical steps involved in each of our three sequential modifications to create the composite material that makes up our working electrodes to obtain values for the effective area of bare glassy carbon electrodes (GCE) and those which had been subjected to our modifications and to understand how increasing the scan rate would affect the ability to oxidize trichlorfon and finally to establish whether diffusion control or adsorption control mechanisms were controlling electron transfer at the surface of our modified working electrodes. All of our CV experiments were done under potentiostatic conditions over a range of scan rates [71].

3.4.2 Differential Pulse Voltammetry (DPV)

Differential Pulse Voltammetry (DPV) is an extremely sensitive electroanalytic method that can be used to analyse very low concentrations of electrochemically active species. DPV was created due to limitations found using Linear Sweep Voltammetry (LSV) where the significant non-faradaic (charge transfer) current associated with double layer capacitance obscures the much smaller faradaic (reduction or oxidation) currents associated with the analytes. Thus, reducing the overall sensitivity and increasing the noise or signal ratio [72].

A series of small-potential pulses (typically 10-100 mV) are placed on top of a staircase shaped potential ramp with increasing voltage each pulse. Two current values are taken during each pulse cycle: one immediately prior to applying the pulse (t1) when primarily non-faradaic current is observed and one closer to the end of the pulse (t2) when a pseudo-steady-state condition exists. The two current values are then subtracted from one another to produce a difference current value (ΔI) that is proportional to the concentration of the analyte. This ΔI value is then measured versus the applied potential. Since the charging current is eliminated through this process, it results in a symmetrical, bell-shaped curve with its peak being

52

proportional to the concentration of the analyte [73]. Additionally, the peak potential of the DPV curve closely approximates the formal reduction potential (E°') of the redox couple allowing for information regarding the mechanism of electron transfer.

Limits of Detection (LOD) and Limits of Quantitation (LOQ) obtained via DPV are generally 1-3 orders of magnitude lower than those achieved via Cyclic Voltammetry (CV) or Linear Sweep Voltammetry (LSV) for the same analyte and electrode system. Due to these characteristics, DPV has become the preferred method for determining traces of pesticides in samples [72].

CHAPTER 4

Graphitic carbon nitride decorated copper sulphide-based Electrochemical biosensor for Trichlorfon Detection

4.1 Introduction

This Chapter covers an innovative and highly effective AChE based electrochemical biosensors by utilizing g-C_3N_4 decorated CuS for detection of Trichlorfon. Due to synergy effects on the synthesized nanohybrid (g-C_3N_4/CuS), there is substantial increase in electrochemically active surface areas, improved catalytic activity, fast charge transfer, and significant bio-compatibility. A Diagrammatic representation of XRD is shown in **Fig. 4.1**.

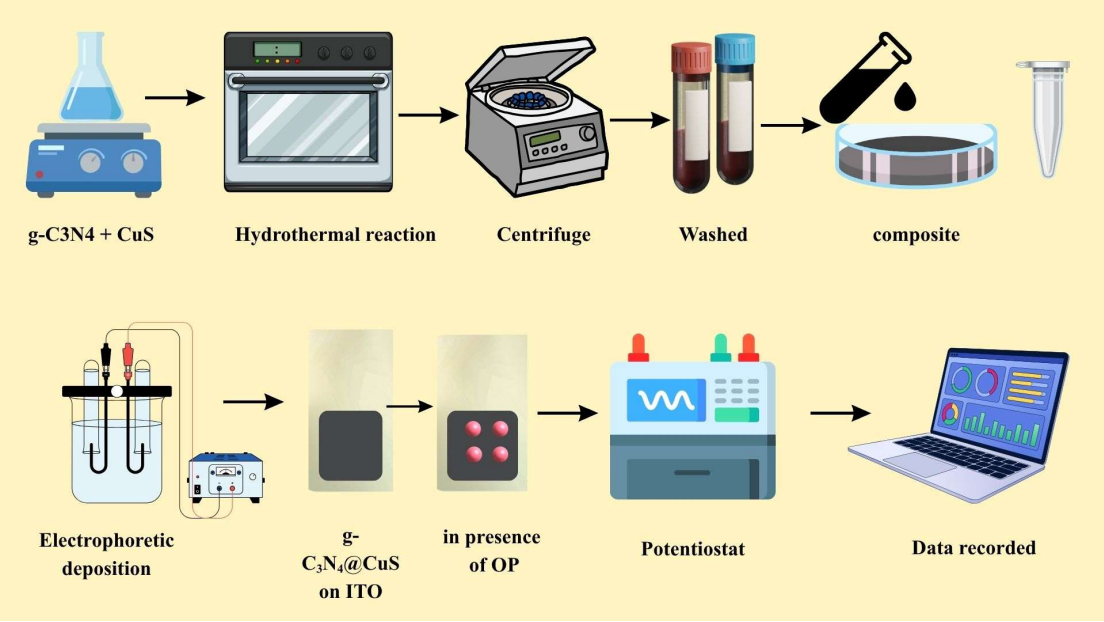

Fig. 4.1 Schematic representation of TF detection using fabricated g-C_3N_4/CuS/ITO bioelectrode.

The fabricated biosensor showed good specificity, very high sensitivity, low detection limit, good reproducibility as well as long-term stability [74]. Moreover, the biosensor can be used for detecting Trichlorfon with acceptable results in two different real samples.

4.2 Experimental section

4.2.1 Synthesis of g-C_3N_4 nanosheets

To produce g-C_3N_4 powder, a typical high-thermal-condensation process is performed under ambient conditions of pressure. Generally, 10g of Melamine is placed in a Muffle Furnace at 550°C for 4 hours. After heating, the pale-yellow product will be first treated by washing it with Distilled Water and Ethanol in equal portions. Finally, it can be dried using a Vacuum Oven [75].

4.2.2 Synthesis of CuS

In beaker, 1 mmol of $CuCl_2.2H_2O$ were dissolved into 40 mL of DI H_2O, resulting in a light-green solution. Then, an additional 3 mmol of thiourea which acts as the redox agent were added to the solution and it was then mixed at 450 r.p.m for 30 min to form a clear colourless homogeneous solution [76].

4.2.3 Synthesis of g-C_3N_4/CuS nanocomposite

To make the nanocomposite, 2.5g of Sodium Thiosulfate ($Na_2S_2O_3.5H_2O$), 24.8g of Copper (II) sulphate ($CuSO_4.5H_2O$) and 50 mL of deionized water were combined in a beaker and vigorously shaken until a homogeneous solution formed. After this point, 1.9g of g-C_3N_4 was added to the solution. This solution was then continuously agitated and ultrasonic treated for 1/2 hours. Then the solution was transferred into a 100mL Teflon lined hydrothermal autoclave and heated at 180 °C for 20 h. A brown solid was obtained after heating. The solid was centrifugated at 4000 r.p.m. and washed several times using an equal volume ratio of distilled water to ethanol, and finally dried under a vacuum at 80 ° C [77].

4.2.4 Electrophoretic Deposition (EPD) of g-C_3N_4/CuS on ITO

The g-C_3N_4/CuS hybrid created using synthesis is electrochemically deposited on to the previously hydrolysed ITO (glass substrate). A Two-electrode setup is utilized where platinum wire is employed as the counter electrode and placed 1cm away from the working electrode which is the ITO-coated glass substrate. Prior to deposition, 5 mg of g-C_3N_4/CuS is sonicated in de-ionised water (15 mL) in order to create a colloidal solution [76]. Subsequently,

electrochemical deposition of the colloidal solution is performed by applying an optimum direct current voltage of 10 V for 8 s.

4.2.5 Fabrication of g-C_3N_4/CuS Nanohybrid-Based Biosensor

The fabrication of the biosensor began with incubation of the g-C_3N_4/CuS/ITO electrode in a solution made from 10µL glutaraldehyde at room temperature for 60 minutes. The next step was to add 20µL of AChE solutions at different concentrations (0.06, 0.04, 0.08, and 0.10 mg/mL^{-1}, prepared in PBS at pH 7.5) to the surface of the previously modified electrode and incubate it for another 12 hours at 4°C. Finally, the biosensor was washed with 50µL of PBS before performing electrochemical measurements in order to remove the free enzyme [77].

4.3 Results and Discussion

4.3.1 Structural Characterisation

The two methods were made through which structural characterisation are analysed are XRD and FTIR.

4.3.1.1 XRD Analysis

The phase composition and crystallographic structures of the synthesized materials were investigated through an examination of their X-ray powder patterns. The X-ray diffractograms for g-C_3N_4, CuS and the g-C_3N_4/CuS nanocomposite are given as **Fig. 4.2 (A)**. The X-ray powder diffraction pattern of pure CuS contained several sharp diffraction maxima that could be indexed based on the hexagonal covellite phase of CuS. The positions of these diffraction maxima indicating that phase-pure CuS had been formed without any observable impurity phases. In addition to the characteristic diffraction maxima of both phases, the X-ray powder diffraction pattern of the g-C_3N_4/CuS nanocomposite showed evidence of both phases being present. Therefore, it can be concluded that this material has successfully been prepared as a binary nanocomposite [78]. As a result of confinement effects exerted by the g-C_3N_4 matrix during the in-situ co-precipitation preparation process, there appeared to be some slight broadening of the CuS diffraction maxima relative to those produced from CuS alone. This observation may indicate that smaller sizes of CuS nanoparticles have resulted. Using the Scherrer equation to determine the average crystallite size of CuS in the nanocomposite by

application to its most intense diffraction maximum yielded an estimate of approximately [X] nm. This calculated size is similar to those observed in the electron microscope images. Therefore, together, the X-ray powder diffraction data provide evidence that a crystalline, phase-pure g-C_3N_4/CuS nanocomposite has been generated that should function effectively in electrochemical sensing applications.

4.3.1.2 FTIR Analysis

Fourier Transform Infrared Spectroscopy (FTIR) was used to examine the structural and functional group properties of the synthetic materials. The infrared spectra for g-C_3N_4, CuS and g-C_3N_4/CuS nanocomposites are depicted in **Fig. 4.2 (B)**. The infrared spectrum of pure g-C_3N_4 showed an absorption band in the range of 3000-3300 cm^{-1} due to N-H and O-H stretching resulting from the incomplete condensation of the melamine precursor during heat treatment. The most diagnostic peak observed in the IR spectrum at about 808 cm^{-1} corresponds to the triazine ring breathing mode through the out-of-plane bend vibration and thus confirmed the existence of the heptazine-based conjugated framework.

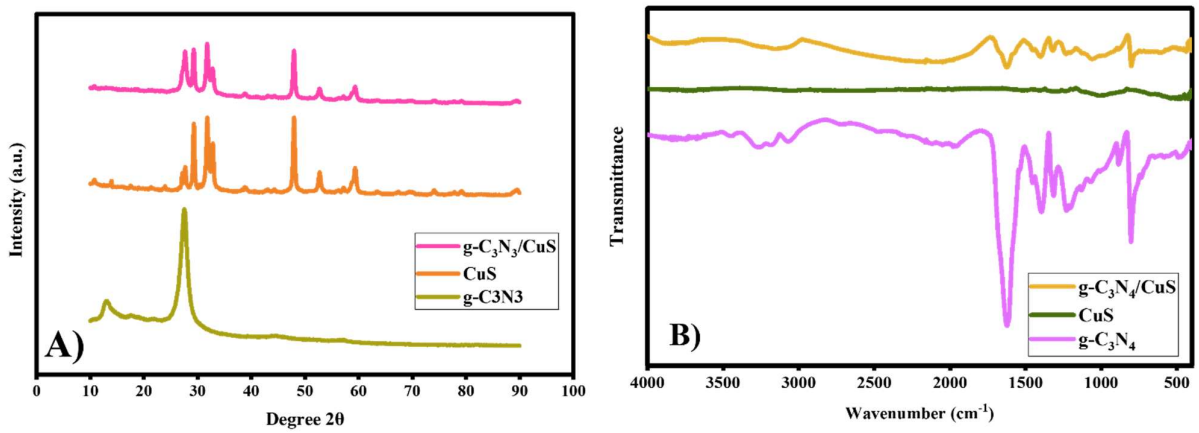

Fig. 4.2 (A) XRD and (B) FTIR of (a) g-C_3N_4/CuS (b) CuS and (c) g-C_3N_4

The several bands occurring between 1200 and 1640 cm^{-1} are ascribed to the C-N and C=N stretches, respectively these are common in the aromatic heterocyclic ring structure of g-C_3N_4 and arise from sp^2-hybridized C-N networks. The low-wavenumber finger print region between 400 and 700 cm^{-1} shows distinct absorption bands for pure CuS due to Cu-S stretching and bending vibrations of the metal-sulphide lattice and this verifies the formation of the covellite CuS phase [79]. Furthermore, there was a slight shift toward lower wavenumbers of some of

the characteristic bands of the g-C_3N_4/CuS composite relative to those for the individual components. This shift suggests a degree of electronic interaction between the g-C_3N_4 nanosheet layers and the CuS nanoparticle surface.

4.3.2 Morphological Studies

4.3.2.1 FESEM

The micro-architecture of the produced nanomaterials has been studied by means of FESEM [89]. The FESEM image of the synthesized g-C_3N_4 represents an example of 2D nanosheets with the appearance of thin, folding, crumpling and stacking on top of each other, typically seen in these kinds of materials for the reduction of the free surface area represented in **Fig 4.3 (A)**. On the contrary, CuS nanostructures have shown a different 3D hierarchical structure consisting of rose-shaped micro-flowers made up of closely-packed nanoflakes. Such kind of interconnected flower-like structure is typical of CuS synthesized through different methods displayed in **Fig 4.3 (B)**.

Finally, the FESEM image of the composite CuS/g-C_3N_4, demonstrates directly that it is possible to obtain a 2D/3D type heterostructure. In fact, from this image we can see how the 3D CuS micro-flowers are well attached to the 2D matrix formed by the g-C_3N_4 nanosheet avoiding thus its aggregation [80]. We can also observe that the intimate contact at the interface between the 3D CuS structures and the 2D g-C_3N_4 substrate creates a “flowers on stalk” structure shown in **Fig 4.3 (C)**.

4.3.2.2 EDX Analysis

EDX tests are conducted on a sample to identify what elements it contains, and how pure it is [81]. The EDX test of g-C_3N_4/CuS a representation of an element peak for carbon, copper, sulphur, and nitrogen is shown in **Fig 4.3(G)**.

4.3.2.3 TEM

The transmission electron microscope (TEM) was used to provide additional information regarding the morphology and how the different components were structurally integrated into the produced samples [82]. As seen in **Fig 4.3 (D)**, the TEM picture of g-C_3N_4 shows the typical characteristics of a 2D nanosheet, an extremely thin, pleated shape for each flake of g-C_3N_4. On

the other hand, The TEM image shown in **Fig 4.3 (E)** shows well-formed spherical morphologies of CuS having a relatively small range of sizes for these spheres. Importantly, the TEM pictures taken of the CuS/g-C_3N_4 composite, illustrated in **Fig 4.3 (F)**, provided unequivocal proof of successful construction of the heterostructures. The pictures indicate that each of the spherical CuS nanoparticles are firmly attached or "anchored" to the surface of the g-C_3N_4 sheets. Thus, they are also uniformly dispersed throughout the composite structure.

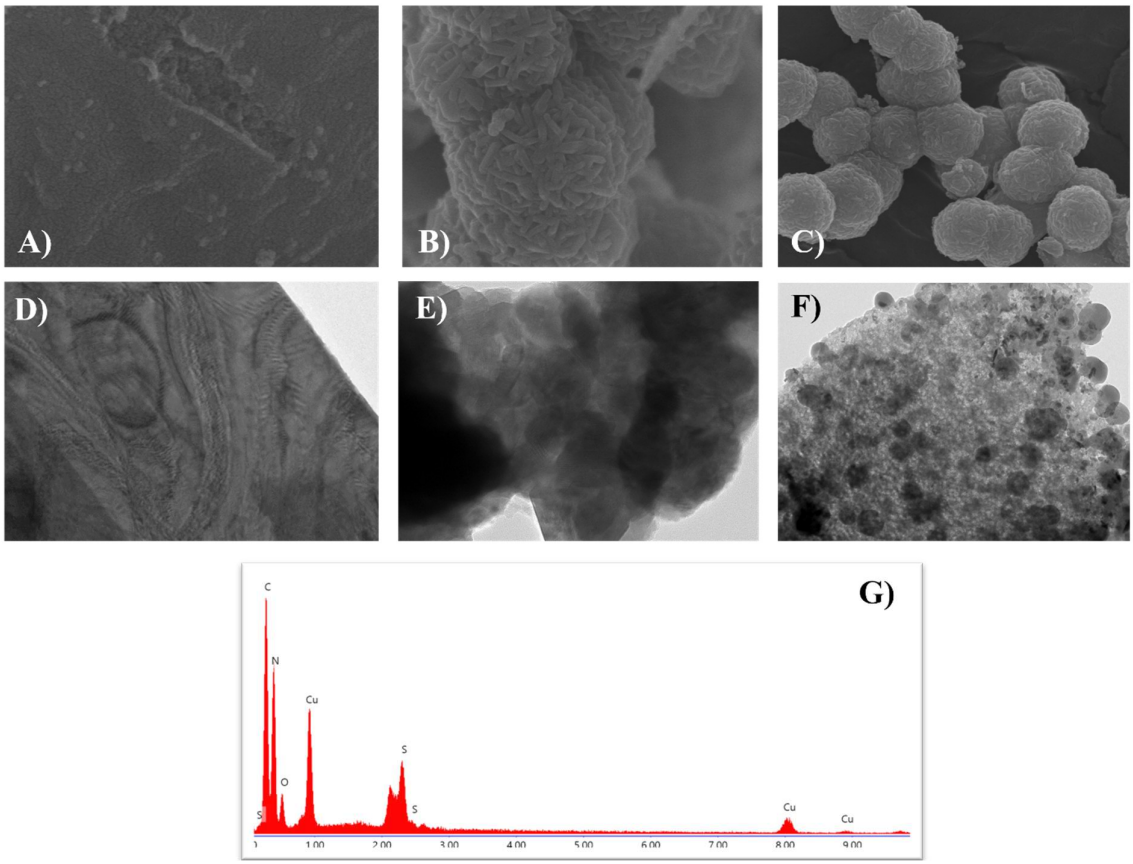

Fig. 4.3 FESEM images of (A) g-C_3N_4, (B) CuS, (C) g-C_3N_4/CuS, TEM images of (D) g-C_3N_4, (E) CuS, (F) g-C_3N_4/CuS and EDAX of (G) g-C_3N_4/CuS

4.3.3 Electrochemical Characterisation

Cyclic Voltammograms (CVs) are used to measure how well an electrode works. The CV plots shown in **Fig. 4.4(G)** demonstrate the cyclic voltammogram studies done for the fabricated (a)g-C_3N_4/CuS /ITO, (b)CuS/ITO, (c)g-C_3N_4/ITO and (d)AChE/g-C_3N_4/CuS/ITO using a scan rate of 50 mV/sec in 0.1M PBS solution that contained 5 mM $[Fe(CN)_6]^{-3/-4}$. It can be seen from **Fig. 4.4(G)** that the largest redox peak current was found for g-C_3N_4/CuS/ITO (curve a, 0.301

mA) compared to CuS/ITO (curve b, 0.233 mA), g-C_3N_4/ITO (curve c, 0.209 mA). The large redox peak current associated with g-C_3N_4/CuS/ITO is thought to arise because of both its high surface area and excellent electronic conductivity provided by the g-C_3N_4/CuS composite [83]. Conversely, it is expected that the lowest currents and least mobilities will be present for AChE/g-C_3N_4/CuS/ITO (curve d, 0.193 mA) due to the poor conductivity exhibited by the enzyme AChE.

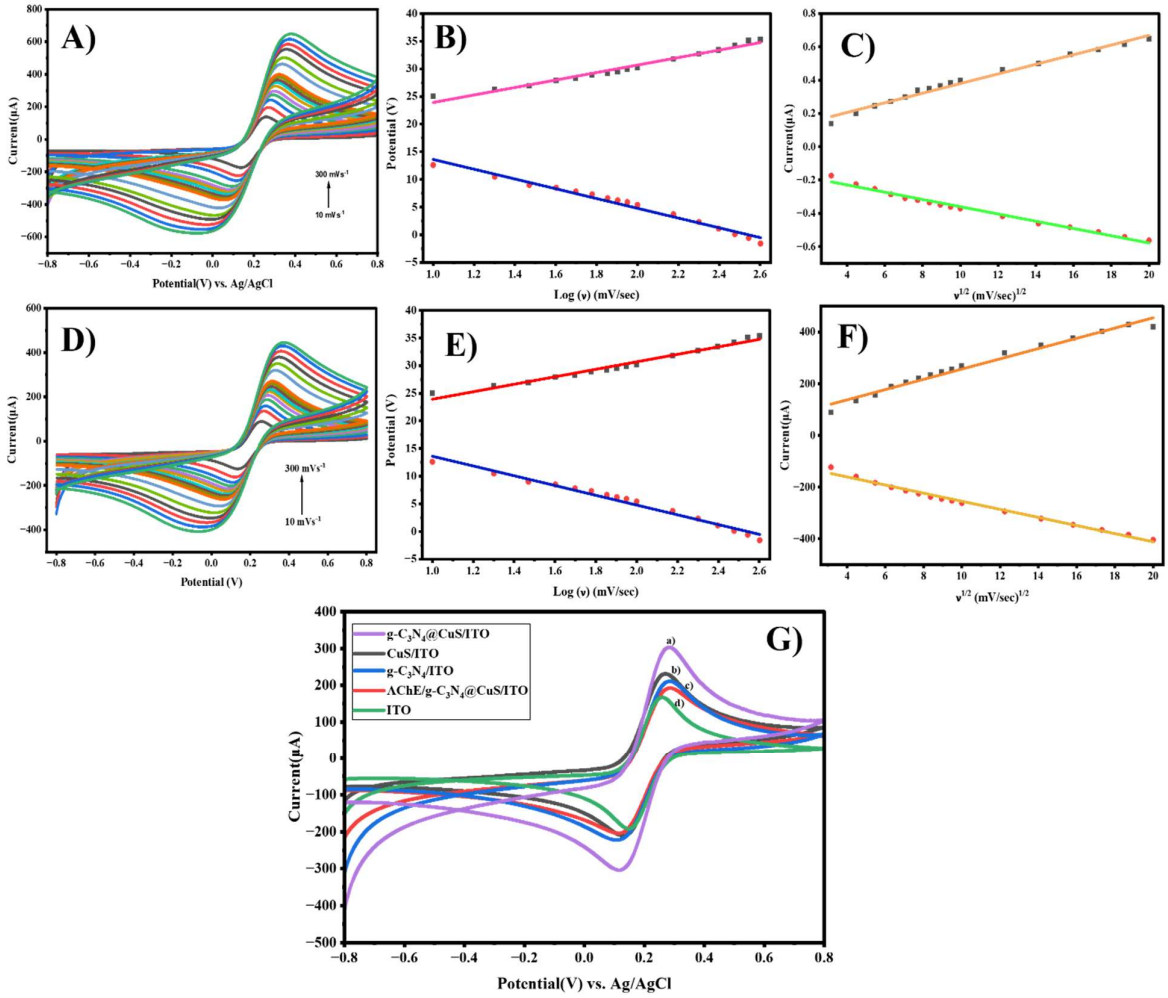

Fig. 4.4 Different scan rate of (A) g-C_3N_4/CuS/ITO and (D) g-C_3N_4 electrode (10-400 mV/sec), (D) Graph of current v/s square root of scan rate of (C) g-C_3N_4/CuS/ITO and (F) g-C_3N_4, (E) Graph for potential v/s log (scan rate) of g-C_3N_4/CuS/ITO, (G) Cyclic voltammogram for different developed electrodes(a) g-C_3N_4/CuS/ITO, (b) CuS/ITO, (c) g-C_3N_4/ITO, (d) AChE/g-C_3N_4/CuS/ITO

The CV has been used in order to investigate the g-C_3N_4/CuS/ITO and g-C_3N_4/ITO electrode material with a varying range of scanning speeds of 10-400 mV/sec. The **Fig. 4.4(C)** and **Fig. 4.4(F)** clearly shows how the anodic peak current (I_{pa}) becomes larger and moves towards a more positive potential, while the cathodic peak current (I_{pc}) becomes larger and moves toward a more negative potential when increasing the scan speed from 10 to 400 mV/sec respectively. Thus, this indicates that both the anodic and cathodic peak currents (E_{pa} and E_{pc}) are linear versus the logarithm value of the scan rate (log v) **Fig 4.4(B) and Fig. 4.4(E)**, which is supported by equations (4.1) and (4.2).

$$E_{pa}\ [g\text{-}C_3N_4/CuS/ITO] = 0.763\ (V)\ log\ (v) + 0.165\ (V),\ R^2 = 0.98323 \quad \textbf{(4.1)}$$

$$E_{pc}\ [g\text{-}C_3N_4/CuS/ITO] = -0.0855\ (V)\ log\ (v) + 0.236\ (V),\ R^2 = 0.98292 \quad \textbf{(4.2)}$$

We also observed that there exists an inverse square root relationship between Ipa and Ipc and $v^{1/2}$[84]. These relationships can be described using the following equations (4.3), (4.4), (4.5) and (4.6).

$$I_{pa}\ [g\text{-}C_3N_4/CuS/ITO] = 28.93\mu A(s/mV)^{1/2}\ v^{1/2} + 90.25\mu A,\ R^2 = 0.98323 \quad \textbf{(4.3)}$$

$$I_{pc}\ [g\text{-}C_3N_4/CuS\ /ITO] = -21.82\mu A(s/mV)^{1/2}\ v^{1/2} - 142.08\mu A,\ R^2 = 0.98292 \quad \textbf{(4.4)}$$

$$I_{pa}\ [g\text{-}C_3N_4/ITO] = 19.86\mu A(s/mV)^{1/2}\ v^{1/2} + 58.4\mu A,\ R^2 = 0.9773 \quad \textbf{(4.5)}$$

$$I_{pc}\ [g\text{-}C_3N_4/ITO] = -15.69\mu A\ (s/mV)^{1/2}\ v^{1/2} - 97.54\mu A,\ R^2 = 0.98822 \quad \textbf{(4.6)}$$

By using Randles - Sevcik equation,

$$(I_p = 2.69\times10^5\ n^{3/2}ACD^{1/2}\ v^{1/2})$$

we found the values of effective surface area to be 0.35 cm^2 and diffusion coefficient to be 7.77 $\times10^{-6}$ $cm^2\ s^{-1}$, where n is number of electrons, C is concentration of $[Fe(CN)_6]^{3-/4-}$ in mole cm^{-3}, v is scan rate in V/s.

4.3.4 Optimization studies

The DPV method determines optimal conditions of electrolyte solution's pH, quantity of AChE, and the length of incubation. In order to find the best enzyme concentration, the DPV response of the AChE/g-C_3N_4/CuS/ITO electrode to the AChE/g-C_3N_4/CuS/ITO electrode in the

presence of 2 mM of ATCl and 1 nM TF were examined [85]. **Fig. 4.5 (A)** illustrates how the DPV response of the AChE/g-C_3N_4/CuS/ITO electrode varies as function of increasing concentrations of AChE from 0.04 mg/mL to 0.1 mg/mL while keeping the amounts of ATCl and TF constant. When the concentration of AChE is increased from 0.04 mg/mL up to 0.06 mg/mL, there is an increase in current signal, beyond this concentration level there will be a decrease in current signal due to excessive mass transfer resistance. For these experiments therefore, an amount of 0.06 mg/mL of AChE are used.

In **Fig. 4.5 (B)**, it demonstrates the influence of pH values of the electrolyte solution ranging from 6 to 8, on the sensor's performance. The largest current signal was observed at a pH of 6 as demonstrated by the data provided below. This is the pH value where all subsequent electrochemical analyses were conducted.

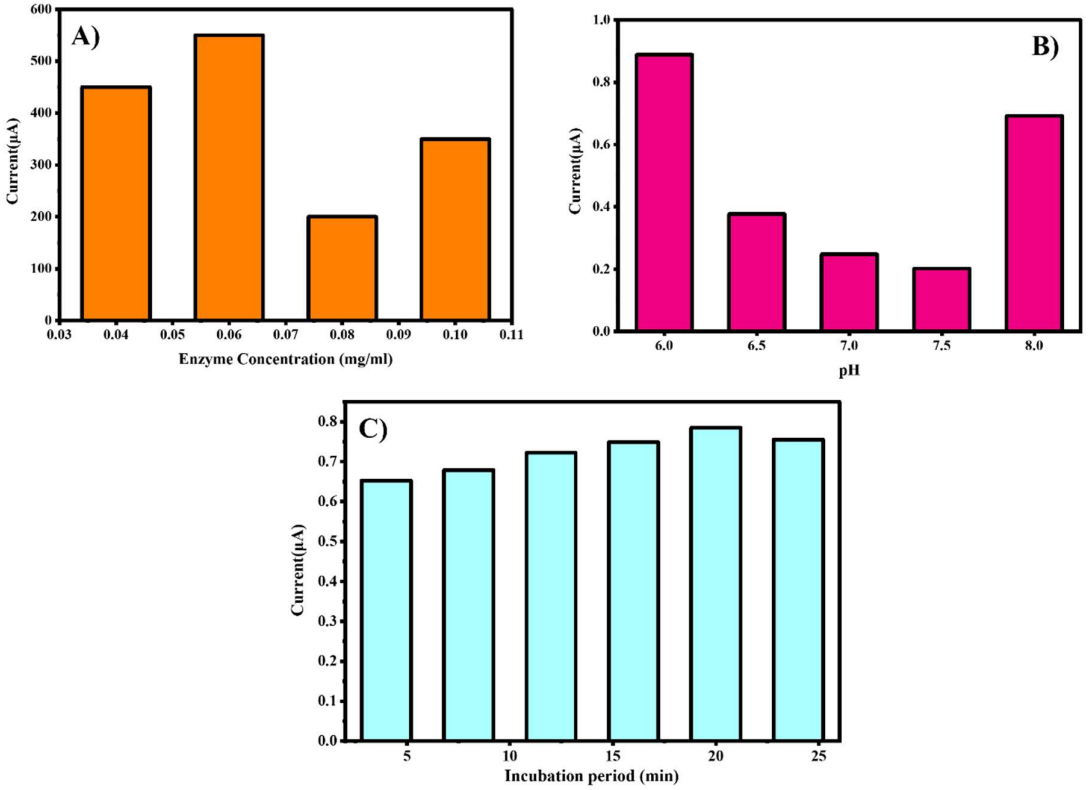

Fig 4.5 Optimization studies of (A) Concentration of an enzyme, (B) Effect of pH, (C) Incubation time

Further studies related to the optimum incubation period have also been conducted. These studies involved measuring the peak current as a function of time for durations of incubation

ranging from 4 min to 24 min as depicted in **Fig. 4.5 (C)**. Results indicate that longer times of incubation lead to decreased peak currents until saturation occurs at 20 min.

4.3.5 Electrochemical Biosensing Study

DPV was used for the electrochemical characterization of the AChE/g-C_3N_4/CuS/ITO electrode to measure the response of the developed sensor toward TF. The peak current of the fabricated bioelectrode at various concentrations of TF from 0.1pM up to 1µm were measured using DPV in 0.1 M PBS with a pH = 6.0 and ATC1(2mm) [86].

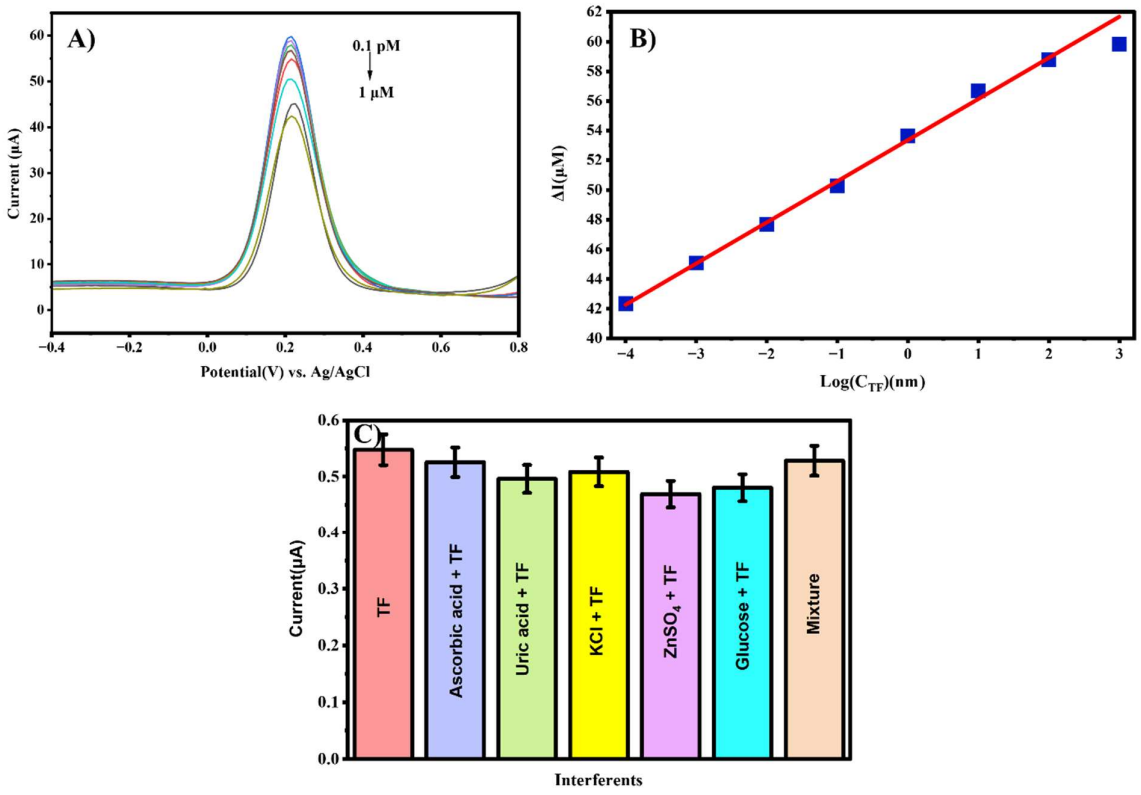

Fig. 4.6 (A) DPV response study of AChE/g-C_3N_4/CuS/ITO developed electrode at different concentrations of TF (0.1 pM - 1 µM), (B) Linearity plot for current v/s TF concentration, (C) Study of selectivity of AChE/ g-C_3N_4CuS/ITO electrode

An increase in TF is associated with an observed decrease in peak current shown in **Fig. 4.6(A)**, showing that TF will inhibit the AChE enzymatic reaction. The calibration plot in **Fig. 4.6(B)** shows the linearity relationship between I peaks and TF concentration, where it can be seen from this plot that the peak current is proportional to the TF concentration.

$I = 2.77375\ \mu A/pM + 53.342\ \mu A \times C_{TF},\ R^2 = 0.93503$

The sensitivity of the biosensor (AChE/g-C_3N_4/CuS/ITO) was 7.925μA(pM)$^{-1}$cm^{-2}, The regression coefficient was 0.9242. The LOD is 0.757 pM, using the equation 3σ / S, in which σ represents the standard deviation of the bioelectrode and S represents its sensitivity [90].

4.3.6 Interference Study and Real Sample Analysis

The selectivity of the fabricated biosensor (AChE/g-C_3N_4/CuS/ITO) is demonstrated through an incubation of the prepared biosensor with 100nM glucose, uric acid, ascorbic acid, $ZnSO_4$ and KCl in addition to 1 nM TF. The data illustrate a lack of a resultant change in current after exposure to interfering analyte concentrations and this illustrates a high degree of selectivity toward TF detection [87].

The utility and practicality of the AChE/g-C_3N_4/CuS/ITO biosensor have been evaluated using spiked samples (apple & cucumber) at established concentrations of 1, 10 and 100nM. The recovery values from the spiked sample experiments ranged from 90% to 103%, providing evidence for the reliability of the AChE/g-C_3N_4/CuS/ITO biosensor. The TF detection data from the spiked samples are found in table 4.1.

Table 4.1 Detection of TF in two real samples using AChE/g-C_3N_4/CuS/ITO electrode.

Samples	Added amount (nM)	Found amount (nM)	Recovery (%)	RSDs (%)
Apple	1	0.987	98.73	0.899
	10	9.347	93.47	4.76
	100	103.18	103.18	2.21
Cucumber	1	0.932	93.26	4.92
	10	9.285	92.85	5.23
	100	90.68	90.68	6.91

4.3.7 Reproducibility and Stability Studies

The reproducibility of the AChE/g-C_3N_4/CuS/ITO electrode was evaluated with the DPV method to measure the response of five similarly made electrodes in the presence of 1 nM TF shown in **Fig. 4.7 (A)**. The results show an acceptable level of variability, as evidenced by the low Relative Standard Deviation (RSD) of 6.29%.

Stability testing of the AChE /g-C_3N_4/CuS/ITO electrode has been conducted by storing the bio-sensing electrode at a temperature of 4°C in a refrigeration unit and measuring its electrochemical activity once per six-day interval shown in **Fig. 4.7 (B)** for a total time period of 30 days. After 30 days, the electrochemical activity measured was 69.96% of the initial electrochemical activity.

CHAPTER 5

CONCLUSION

In conclusion, an Electrochemical Biosensor using a g-C_3N_4/CuS composite was successfully fabricated. This new biosensor has excellent sensitivity toward TF. Factors that affect its performance have been optimized including, the pH of PBS at 6, the amount of AChE, ATCI, and the time of the incubation period at approximately 20 minutes. The use of g-C_3N_4/CuS modified transducers increases the rate of electron transfer between AChE and the electrode surface. These improvements increase the sensitivity of the proposed biosensor. The developed biosensors demonstrate superior performance based on sensitivity (7.925μA $(pM)^{-1}cm^{-2}$), lower limits of detection (LOD) are 0.757 pM and wider dynamic ranges (0.1pM - 1μM) than all but one. Good recoveries (between 90 % to 103%) and small relative standard deviations (R.S.D.) are also demonstrated for two different food matrices, demonstrating the feasibility of utilizing the fabricated biosensor.