

DETECTION OF ALPHA AMYLASE USING CORE SHELL NANOPARTICLE AS SENSOR

Submitted in partial fulfillment of the requirements for the award of Bachelor of
Technology in B. Tech Biomedical Engineering

By

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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)
Accredited with Grade "A" by NAAC**

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the Bonafide work of **Renitta D (39240031)** and **Akshara S (39240006)** who carried out the final year project entitled "**Detection of Alpha Amylase using Core Shell Nanoparticle as Sensor**" under our supervision from December 2022 to May 2023.

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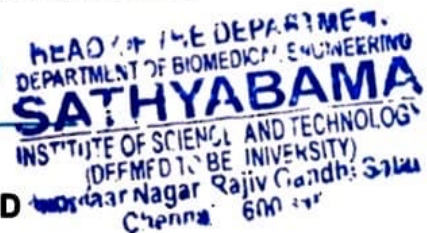
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We, **RENITTA D (39240031)** and **AKSHARA S (39240006)**, hereby declare that the Project Report entitled "**DETECTION OF ALPHA AMYLASE USING CORE SHELL NANOPARTICLE AS SENSOR**" done by us under the guidance of **Dr. ANIMA NANDA** and **Dr. S. KUMARAN** and is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in B. Tech Biomedical Engineering.



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ABSTRACT

The development of low-cost biosensors has a lot of potential for smart biocompatible materials that react to various external stimuli. The invention of intelligent enzyme-responsive core-shell nanoparticle-based sensors as low-cost diagnostics for alpha amylase detection is described in the current work. Ionic gelation will be used to construct a chitosan-tripolyphosphate core, which will then be covered in a shell made of starch and iodine to create biocompatible core-shell nanoparticles. In the presence of specific concentrations of amylase, the starch-iodine shell will rupture, revealing the core. This application explains how to recognize salivary alpha amylase at the point of care by an observable color change from blue to white (sAA). This biomarker can be used to identify autonomic problems like stress. Alpha amylase can also be used in forensic investigations to find latent saliva at crime scenes. There won't be any interference from other compounds in the saliva, and the nanoparticles will be stable and repeatable. The new study offers a proof-of-concept technology that shows how smart nanoparticles can be used in flexible, low-cost biosensing platforms like paper-based and swab-based formats for uses as varied as forensics and stress diagnostics.

Key words – Alpha amylase, chitosan, core-shell nanoparticle, forensic, stress, biomarker, sensitivity, immunosensor.

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LIST OF ABBREVIATIONS

CTS	Chitosan Nanoparticles
DNSA	Dinitro Salicylic Acid
EDX	Energy Dispersive X-Ray Analysis
FEGTEM	Field Emission Transmission Electron Microscope
FESEM	Field Emission Scanning Electron
FTIR	Fourier Transform Infrared Spectroscopy
GOD	Glucose Oxidase Peroxidase
HPA	Hypothalamus Pituitary Adrenal System
LOD	Limit of Detection
LMW	Low Molecular Weight
PDI	Poly Dispersity Index
RGB	Red Green Blue
SAA	Salivary Alpha Amylase
SEM	Scanning Emission Microscope
SNS	Sympathetic Nervous System
STPP	Sodium tri poly phosphate
TEM	Transmission Electron Microscope
TPP	Tri poly phosphate
UV	Ultraviolet
WHO	World Health Organization
XRD	X-Ray Diffraction
XRF	X-Ray Fluorescence

CHAPTER 1

INTRODUCTION

1.1.1 Nanomaterials

Nanomaterials are usually considered to be materials with at least one external dimension that measures 100 nanometers or less or with internal structures measuring 100 nanometers or less. They may be in the form of tubes, rods or fibers. One of the most crucial aspects of the broad field of nanotechnology is the creation, analysis, and use of nanoparticles domains covered by the broad term "nanotechnology." The focus of research in recent years has been on nanoparticles. Researchers in the field noticed significant changes in a material's physical and chemical properties as a result of the shift from microparticles to nanoparticles. (Jeevanandam., et al, 2018)

1.1.2 Core Shell Nanoparticles

Nanoparticles can be divided into simple, core shell and composite types based on a single or combination of materials. In general, the core shell nanoparticles and composite nanoparticles are made up of two or more components, in contrast to simple nanoparticles, which are typically made of a single material. The broad definition of type of core shell nanoparticles is that they comprise of an inner core material and an outer shell material (outer layer material). They can be made up of a variety of distinct mixtures working together, like organic-organic, organic-inorganic, inorganic-organic, inorganic-inorganic components. (Baig., et al, 2021).

The choice of the shell material is greatly influenced by the final use and application of the nanoparticle. Since core shell nanoparticles have evolved at the interface between chemistry and number of other fields including biomedicine, electronics, pharmaceuticals and so on, they have been steadily gaining more attention. Core/shell nanoparticles have altered characteristics and are extremely useful materials. Occasionally the characteristics resulting from the core or shell materials can be very dissimilar. The core to shell ratio or the constituent materials can be changed to alter the characteristics. (Baig., et al, 2021).

1.1.3. Biosensors

The biosensor can be defined as an analytical device which is used for the detection of a chemical substance which combines a biological component with a physicochemical detector. Utilizing Core Shell Nanoparticles has accelerated recently in the development of biosensor related research, but it has been discovered that the CSNs with a particular shape and size depends on physical and chemical properties and have been receiving significant attention for the use in the fabrication of chemo sensors and biosensors. (Sadhucharan., et al, 2022).

An integral component of modern living is the quantitative measurement of biomarkers to track health issues. (Chen et al., 2015). To meet some of the needs, glucometers, blood pressure monitors, and pregnancy kits are already on the market. Some significant biomarkers, on the other hand, may not have a quantitative estimation technique or may have one but be more expensive and time-consuming than other methods. Moreover, the facilities necessary for the testing are only accessible through centralized pathology organizations, which fall short of meeting the immediate needs of a sizable portion of the world's population (Pullano et al., 2022).

The development of affordable point of care diagnostic tools supported by the advantages of micro or nanotechnology may represent the next “tipping point” in the current situation. Healthcare devices should be ASSURED – affordable, sensitive, user friendly, quick, sturdy and deliverable according to the World Health Organization (WHO). (Kumar., et al,2022).

1.1.4. Chitosan

Chitosan nanoparticles are cationic in nature, biocompatible, largely non-toxic, and degradable. They are therefore widely used in biomedical applications like drug delivery. The most used technique for creating chitosan nanoparticles is ionic gelation. In this procedure, sodium tripolyphosphate is used to cross-link chitosan precursors (TPP). The technique typically produces large-sized (100–300 nm) polydisperse particles. Even though ionic gelation is a widely used technique and that variables influencing the size and dispersity of chitosan nanoparticles (such as reactant concentration, temperature, pH, and deacetylation level) are well known,

our fundamental understanding of the process at a mechanistic level is lacking. Chitosan molecules are crosslinked by TPP during the ionic gelation process, which also connects them to other cross-linked moieties. This kind of unregulated intra and intermolecular cross-linking results in polydispersity in the synthesized product. (Vivek., et al., 2016).

Chitosan [poly-1,4)-amino-2-deoxy-D glucan] is a natural, non-toxic, microbially resistant and biodegradable polymer that is a deacetylated derivative form of chitin. It has been thoroughly researched and stated in the literature that chitosan exhibits antibacterial properties. To increase its antibacterial action, a number of chitosan derivatives with various modifications have been created. Since nanoscience and technology have advanced, a variety of studies are being conducted by R&D, academics and business in the field of preparing and utilizing chitosan nanoparticles as a carrier for medicine, proteins and peptides. Recently it is discovered that chitosan nanoparticles might have a unique property that could have antibacterial activity by Wen Li., et al. and Lifeng Qi., et al.

The study focused on examining the antibacterial activity of chitosan nanoparticles even though chitosan in nano form is extremely active due to the high surface area to volume ratio. Chitosan is said to have favorable bioactivity even at low concentrations. (Subbiyan., et al., 2017)

1.1.5 Starch

Starch is a white, odorless, tasteless, granular or powdery complex carbohydrate ($C_6H_{10}O_5$)_x that is the primary storage form of carbohydrate in plants, which is an essential food. Starch is also used in pharmacy, laundry, adhesives and sizes and in medicine. It is a natural polymer or polysaccharide which means that it is a long chain comprising one type of molecule throughout the chain. Starch consists of glucose molecules that can occur in two forms: amylose and amylopectin. The composition of starch is of long strands of sugar molecules linked together.

Amylose, a linear polymer, is the most fundamental form of starch, whereas amylopectin is the branched polymer. Amylase, a starch breaking enzyme found in the saliva and pancreas, is used by mammals to break down starch. It is made up of *n* glucose monomers linked together by (1,4) links in chains and (1,6) linkages at

branching points. For the creation of a starch polymer, two glucose molecules combine to lose one molecule of water (H₂O).

Our diet contains a lot of starch, which the body uses to produce energy. Alpha amylases from the pancreas and saliva as well as alpha glucosidases from the small intestine's brush border are the principal enzymes involved in the digestion of starch. (De Sales et al., 2019).

1.1.6 Saliva

Saliva is a multipurpose biofluid that has qualities that are antibacterial, lubricative, digesting, buffering, and tissue coating. Amylase, lipase, and lysozyme are three important proteins that make up saliva and are involved in digestion. The enzymes amylase and lipase have been identified to be potential molecular marker of pancreatic cancer when detected in blood. Because pancreatic and salivary α -amylase are 97% homologous, which is proven approach for α -amylase biosensing would work for screening without significant adjustment. Pancreatic cancer, Ovarian cancer and liver cancer are referred to as "silent killers" due to the extreme delayed development of symptoms. Less than 5% of people with pancreatic cancer who are diagnosed go into remission and survive for more than five years. Hence, early illness detection using chemical markers is critical. (Hassan., et al, 2019).

The salivary glands generate salivary α -amylase, which has a physiological function in the digestion of starch starting in the mouth. Salivary and pancreatic α -amylase are the two primary isozymes of alpha amylase found in humans. Several bodily fluids have been shown to contain both alpha amylases. This has historically made it challenging to document the presence of salivary alpha amylase in forensic case work. Specificity and sensitivity are the most important shortcomings of current technologies for saliva detection, and it can be challenging to incorporate them into profiling approaches. (Wang., et al, 2014).

1.1.7. Alpha Amylase

Amylase is a forensic tool that can be used to presumptively screen stains for relevant genetic material during crime scene investigations. Among other crucial indicators, alpha amylase activity (endo-1,4-D-glucan glucanohydrolase) in various

bodily fluids needs to be regularly monitored since abnormal levels of the alpha amylase enzyme in saliva, urine, sebum or blood serum signifies the pathological health state of the person. Human salivary glands and pancreas produce amylase, which hydrolyzes starch into simple sugars like glucose, maltose and dextrin. (Costa et al., 2014).

Amylase levels can also be used to diagnose pancreatic dysfunction or kidney disease. There are a variety of techniques available to determine the amount of amylase in body fluids, including spectrophotometry, colorimetry, fluorometry, isoelectric focusing, electrokinetic processes, chromatography, weight-based detections, electrochemical methods, both immunological techniques. (Fana et al., 2021).

1,4- alpha- D- glucan glucanohydrolase or the salivary alpha amylase, the vital enzyme, helps in the conversion of starch into simple sugars in the oral activity. In addition to its function in carbohydrate digestion, sAA is regarded as a biomarker to identify changes in stress-related sympathetic nervous system (SNS) activity. When a stressor is present, the SNS reacts almost immediately, but the hypothalamus pituitary adrenal system (HPA) axis doesn't respond for a long time. (Muritala 2018).

1.1.8. Intelligent Materials

Smart materials or intelligent materials are those which reacts and responds to a stimulus given that were created in the 20th century and has the capability of changing from one or more physical properties such as color, form, stiffness, opacity or porosity as a response to minute changes in the environment. (Nasseri, et al., 2020). In order to take note of these changes in features caused by the change in stimulus for use as actuators and sensors or for the strategies for self-assembly, smart materials are being thoroughly researched in the field of biosensors and diagnostics. (Verma, et al., 2016).

Recently, a range of stimulus responsive materials that respond to pH, temperature, electric fields or chemical changes have been used in the development of sensor applications. Smart nanoparticles for diagnostics can especially make use of the materials' intelligence, which results from conformational changes caused by the presence or absence of specific compounds. For instance, fluorescence based

magnetic (Fe/Fe₃O₄) core shell nanoparticles have been used in cancer diagnostics to identify the activity of proteases in biospecimens. (Guo et al., 2020).

CHAPTER 2

LITERATURE SURVEY

Rishi and Rinti (2021) says that the development of biosensors for affordable diagnostics holds enormous potential to smart biocompatible materials that are responsive to numerous external stimuli. The invention of intelligent enzyme responsive core shell nanoparticle-based sensors as low-cost diagnostics for alpha amylase detection. The chitosan tripolyphosphate core of the biocompatible core shell nanoparticles had a size range of 200-250 nm. The core was covered in a shell made of starch and iodine. The shell of the starch-iodine was broken when certain amylase concentrations were present, exposing the core. To identify salivary alpha amylase at the point of care, this application describes a noticeable color change from blue to red (sAA). By evaluating this biomarker, stress and other autonomic abnormalities can be identified. Alpha amylase can also be employed at crime scenes to find latent saliva for forensic investigations. The current platform technology was used to develop a paper-based diagnostic for salivary alpha amylase detection showing a limit of detection (LoD) of 140 units/ml (70 mg/ml) at 5 minutes, whereas a coated swab made from 12 nanoparticles for crime scene investigations could achieve a LoD of 2.5 units/ml (1.25 mg/ml) over 30 mins. The nanoparticles showed stability and repeatability, while other compounds in saliva did not interfere with them. The proof-of-concept technology presented in this study highlights the potential of smart nanoparticles in platforms for biosensing that are affordable and versatile, such as paper based and swab-based formats, for a wide range of applications.

Divya and Jisha (2018) discussed how the preparation of shellfish is a highly prevalent activity in marshy areas. Degradation is a gradual process due to the sheer amount of waste, which leads to accumulation of waste over time. Recycling shell trash to create useful materials like chitin is a relatively straightforward and efficient way to address this environmental threat. Chitosan is the N-acetyl derivative of chitin produced via N-deacetylation. In the food and bioengineering sectors, chitosan is frequently used to encapsulate active food ingredients, immobilize enzymes, deliver drugs under regulated conditions, and boost plant

development. Chitosan is an antibacterial agent as well as a defense elicitor. Interesting characteristics of chitosan include its polycationic nature, biodegradability, biocompatibility, bioactivity, and nontoxicity. Chitosan's structural traits and physicochemical makeup are discussed in this review. Chitosan nanoparticle preparation techniques are described in depth. Chitosan nanoparticle applications were described. Some examples of applications are drug delivery, encapsulation, antibacterial agent, plant growth promoting agent and plant protection.

Satarupa Dutta, et al., (2016) describes the development of a paper-based sensor for measuring the activity of alpha amylase in human blood serum. The coating of the filter papers was achieved by coating with starch iodine solution, which caused the surface to be colored in deep blue. The application of starch iodine in the paper lead to starch hydrolysis caused by the amylase resulted in the decrease in the intensity of the color. Three steps were taken to estimate the relationship between the variation in color's intensity and the concentration of amylase. When a particular bio-reaction was used to identify amylase activity, the sensor was discovered to be equally effective at identifying unidentified concentrations of amylase in human blood serum. The proposed sensor can develop into a point-of-care tool for α -amylase detection.

Zhang, et al., (2015) proposes a smartphone-based potentiometric biosensor for salivary α -amylase (sAA) point-of-care testing. Saliva is one of the most sensitive indicators of autonomic nervous system function, making it a promising noninvasive biomarker for mental health. A potentiometric reader, with sensing chips with preloaded reagents and a smartphone with a SAA detection app make up the biosensing system. When the saliva sample gets into the reaction zone on the detecting chip and the SAA combines with the preloaded agents, an electron mediator, $\text{Fe}(\text{CN})_6^{3-}$ is transformed into $\text{Fe}(\text{CN})_6^{4-}$. The reaction is subsequently mixed into the detection zone for the potentiometric measurement by pressing the sensor chip with fingers. A calibration curve is used to translate the potential recorded by the potentiometric reader powered by a smartphone into SAA concentration, which is then delivered to the smartphone App via the USB port. Within 5 minutes, our technique allows for the quantitative analysis of SAA in a real

human sample. The results are associated to the respondents' psychological states and are in good accord with those obtained using a reference approach.

Rizliya, et al., (2016) using the glucose test kit, a reliable, easy-to-use, quick, and high-performance analytical approach for the identification and measurement of alpha amylase inhibitory activity was developed. The novel technique makes it possible to quickly screen a large number of samples, cuts down labor, time and chemicals. This process is based on the formation of a red quinone from the interaction of maltose with glucose oxidase (GOD). The assay takes 40 minutes not complete, including the pre incubation stages, and is carried out in microtiter plates with a total capacity of 260L. Dimensionality, sensitivity, precision, reproducibility, and application of the novel method are examined. The most popular 3,5 Dinitro salicylic acid (DNSA) approach and the new technique are constructed in order to assess the activity of alpha amylase.

David Cassey and Judy Price (2010) shows how samples commonly used in forensic casework can be used to test for human salivary α - amylase using the RSIDTM- saliva test. They demonstrated the Phadebas 1 Quantitative test intensifies salivary α - amylase at higher concentrations than the RSIDTM saliva test, which can be successfully integrated into entire swab semen extraction technique.

Pietro, et al., (2013) indicates that the purpose of the study was to measure and contrast the discomfort experienced by epileptic children during an invasive procedure to collect venous blood. This was done by measuring salivary alpha-amylase (sAA) activity. Twelve children with epilepsy and eleven healthy controls, totaling 23, were included in the study. One saliva sample was taken from each child in each group using a non-invasive device 15 minutes before (t0), during (t1), and 15 minutes after (t2) blood withdrawal. The sAA activity was then assessed using a kinetic-colorimetric assay. According to the study, epileptic children were more sensitive to pain. The results obtained suggest that SAA activity might be a brand new noninvasive and objective biomarker for the evaluation of pain perception in epileptic children.

Kalpana, et al., (2010) explains how the conventional medication delivery system has been transformed by nanoparticles' ability to modify molecules and their

architectures. Chitosan nanoparticles provide a useful tool to innovative drug delivery systems in the current environment due to their biodegradability, biocompatibility, improved stability, low toxicity, simple, and mild manufacturing procedures. In addition to the ionotropic gelation technique, other techniques are also in use. Additionally, it has been noted that the chitosan nanoparticles have important uses in the controlled delivery of drugs through mucosa, electrodeposition, parenteral drug administration, oral drug administration, non viral gene delivery, vaccine distribution, ophthalmic drug delivery, brain targeted drug delivery, stability enhancement, tissue engineering and the effective administration of insulin. The current review discusses the history, characteristics, and applications of chitosan and its nanoparticles in new drug administration, as well as the numerous ways it is prepared.

Wen Fan, et al., (2012) claims chitosan nanoparticles have been extensively explored for medication and delivery of genes. This study presented a novel technique for producing monodispersed, low molecular weight (LMW) chitosan nanoparticles employing sodium tripolyphosphate (TPP) as a cross linking agent, this study's goals were to find a solution to the issue of creating chitosan with TPP nanoparticles with high degree of mono dispersity and stability and to investigate the impact of various parameters on the development of LMW chitosan and TPP nanoparticles. It was also discovered that by lowering the ambient temperature and acetic acid concentration during cross linking process, the nanoparticle's distribution of particle size could be dramatically constricted. The nanoparticles demonstrated excellent room temperature storage stability for at least 20 days.

Andreia, et al., (2014) indicates that it is crucial to research the ideal circumstances to produce nanoparticles with the required size and superficial charge because there are various types of chitosan that are derived from various sources of chitin and methods. The scientific community of how to make chitosan nanoparticles (nano CTS) with the characteristics are shown in this study. Tripolyphosphate (TPP) and chitosan were linked to create nanoparticles using ionic gelation process. The effects of the pH, CTS: TPP ratio, acetic acid concentration and particle size were examined using 23 factorial designs. Analysis using FTIR, TGA, EDX and TEM

revealed the characteristics of the optimal formulation. The ratio of CTS:TPP was the primary factor in the nanoCTS production.

Nida Ali and Nater (2020) describes how behavioral medicine research has shifted its attention in recent years to better understanding the effects of chronic and acute stress on health outcomes. The physiological stress systems of the body are triggered by stress. These systems have a strong relationship with the immune system and play a significant role in the development and continuation of disease conditions. While the hypothalamic-pituitary-adrenal axis and its downstream biomarker, cortisol, have received considerable attention in behavioral medicine research that has examined the effects of stress on disease, the autonomic nervous system also plays an important role. The biological process of stress, as well as the emergence and maintenance of stress-related symptoms, are all significantly influenced by (ANS).

Salivary alpha-amylase (sAA) is an important biomarker to take into account in behavioral medicine because it has recently emerged in stress research as a relevant and reliable marker of ANS activation. In this editorial, we will highlight studies pertinent to behavioral medicine that have examined ANS function in clinical populations using sAA measures, both at rest and in response to stress. Also, we will review the results of studies that looked at how various targeted therapies affected changes in sAA levels. With this, we hope to show that sAA can serve as a reliable biomarker of ANS (dys)function in both health and disease. We will also highlight key methodological considerations that readers should be aware of when adopting sAA assessments into their own study in order to achieve this. The major goal of this brief article is to illustrate how a multimodal approach to evaluating physiological stress may contribute to researchers' understanding of physical health and disease states.

CHAPTER 3

AIM AND SCOPE

3.1 AIM

To develop a salivary amylase detecting kit for crime scene trace swabs, utilizing amylase sensitive paper. It can be used as a presumptive test for crime scenes to locate the presence of saliva and stains on the surface.

3.2 SCOPE

- Amylase testing can be used as a presumptive test for crime scene saliva for over three decades, mainly to locate saliva stains on surfaces.
- The Salivary Alpha-Amylase Assay Kit is specifically designed to standardize the detection of alpha-amylase in saliva samples for research and biomedical laboratories.
- A presumptive kit is a one that establish the possibility that a specific bodily fluid is present.

CHAPTER 4

MATERIALS AND METHODS USED

4.1 MATERIALS

Low Molecular- weight chitosan with a molecular weight of 7000 Da and 85% degree of acetylation, Soluble Starch, Potassium Iodide, Iodine, Glacial acetic acid, Sodium hydroxide, Sodium tripolyphosphate (TPP), Agarose and N-acetyl-D-glucosamine and Whatman qualitative filter paper grade 1.

4.1.1 Chitosan

Chitosan is a linear polysaccharide made up of randomly dispersed -(1,4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine. (Acetylated unit). It is produced by treating prawns and other crustaceans' chitin shells with an alkaline substance like sodium hydroxide. Chitosan has a number of commercial and possible biomedical uses. Chitosan is produced from chitin, a fibrous substance found primarily on crustaceans' hard outer skeletons and in the cell walls of some fungi. Chitosan is very difficult to dissolve in water, but it can be dissolved in a corrosive solution. Its insolubility in water is a significant barrier to its use in medical uses. Chemical methods such as acylation, alkylation, sulfation, hydroxylation, quaternization, esterification, graft copolymerization, and etherification can be used to produce chitosan derivatives. Chemical properties of modified chitosan are better than those of unmodified chitosan.

4.1.2 Starch

A whitish, odorless, tasteless, granular or powdery complex carbohydrate ($C_6H_{10}O_5$)_x that is the primary storage form of carbohydrate in plants, is an essential food, and also used in adhesives and sizes, laundry, pharmacy, and medicine. Starch is a natural polymer, or polysaccharide, meaning that it is a long chain comprising one type of molecule. Starch consists of glucose molecules. It can occur in two forms: amylose and amylopectin. Starch is composed of long strands of sugar molecules linked together. The most fundamental form of starch is amylose, a linear polymer, while amylopectin is a branched polymer. Amylase, a starch-breaking enzyme found in saliva and the pancreas, is used by mammals to break down starch. It is made up of n glucose monomers linked together by (1,4)

links in chains and (1,6) linkages at branching points. For the creation of a starch polymer, two glucose molecules combine to lose one molecule of water (H_2O).

4.1.3 Iodine

Iodine is a chemical element with the symbol I and the atomic number 53. At standard conditions, it exists as a semi-lustrous, non-metallic solid. Iodine exists in a variety of oxidation states, including iodide (I⁻), iodate (IO₃⁻), and the different periodate anions. The iodine test is a molecular test that is used to differentiate mono or disaccharides from polysaccharides such as amylose, dextrin, and glycogen. This test has a variant known as the starch-iodine test, which is used to detect the presence of glucose produced by plants in their foliage. The presence of polysaccharides, mainly starch, must be detected. The Iodine Test Procedure, 1 mL of a specified sample should be placed in a clean, dry test tube. In another container, control 1 mL of distilled water. Add about 2-3 droplets of Lugol's solution to each tube and vortex them. Examine how the color appears in the test containers. In the water bath, heat the test containers until the color fades.

4.1.4 TPP

Sodium tripolyphosphate (STP), also sodium triphosphate (STPP), or tripolyphosphate (TPP), is an inorganic compound with formula $Na_5P_3O_{10}$. It is the sodium salt of the polyphosphate penta-anion, which is the conjugate base of triphosphoric acid. It is the sodium salt of the polyphosphate penta-anion, which is the conjugate base of triphosphoric acid. Under carefully controlled conditions, a stoichiometric mixture of disodium phosphate, Na_2HPO_4 , and monosodium phosphate, NaH_2PO_4 , is heated to create sodium tripolyphosphate. Tripolyphosphate (TPP) was used as the physical cross-linking agent in the production of chitosan-based microparticles and nanoparticles. It is discovered that raising the weight ratio of chitosan to TPP increases the chitosan concentration in the solution and the viscosity of the solution, thereby increasing the liquid phase resistance to particle dispersion and the size of the produced nanoparticles.

4.2 METHODS

4.2.1 Preparation of Solutions:

The solutions used in the experiments are newly prepared. The studies were conducted using starch and iodine solutions at their optimal concentrations of 2% (w/v) and 0.2% (w/v), respectively. The 2% (w/v) starch solution was made by mixing 0.2 g of starch with 10 ml of distilled water, then carefully boiling the mixture to make it translucent. The 10% KI and 5% iodine were dissolved in water (w/v) to create 5% iodine stock solution. Before further usage, the stock solution was diluted to 0.2% (w/v) with distilled water.

4.2.2 Preparation and Characterization of Core Shell Nanoparticle:

In 1% acetic acid, 100 ml of chitosan solution (1 mg/ml) was made. Separately, as indicated in Fig. 4.2, a solution of sodium tripolyphosphate (TPP) (1 mg/ml) was prepared in deionized water. When 30 ml of TPP solution was pumped at 5000 rpm for 20 minutes at room temperature in a dropwise manner, ionic gelation resulted in particle formation spontaneously. Centrifugation was used to separate the chitosan TPP nanoparticle pellet for 15 minutes at 4000 rpm. The collected pellet is as shown in Fig 4.3. In a volume ratio of 5:1, Lugol's iodine and soluble starch solution (5 mg/ml in 1 N NaOH) were combined to resuspension the pellet. The resulting nanoparticle was vortexed and then subjected to a probe sonicator for two minutes of sonication. The coating of the core was anticipated to be achieved by electrostatic binding of the negatively charged starch-iodine complex and the positively charged chitosan nanoparticles (white suspension) and (blue suspension) respectively. The resulting core-shell nanoparticles were then centrifuged once more for 20 minutes at 4000 rpm. The pellet was then dissolved in 2 ml of deionized water, sonicated for 2 minutes, and then refrigerated at 4 °C.

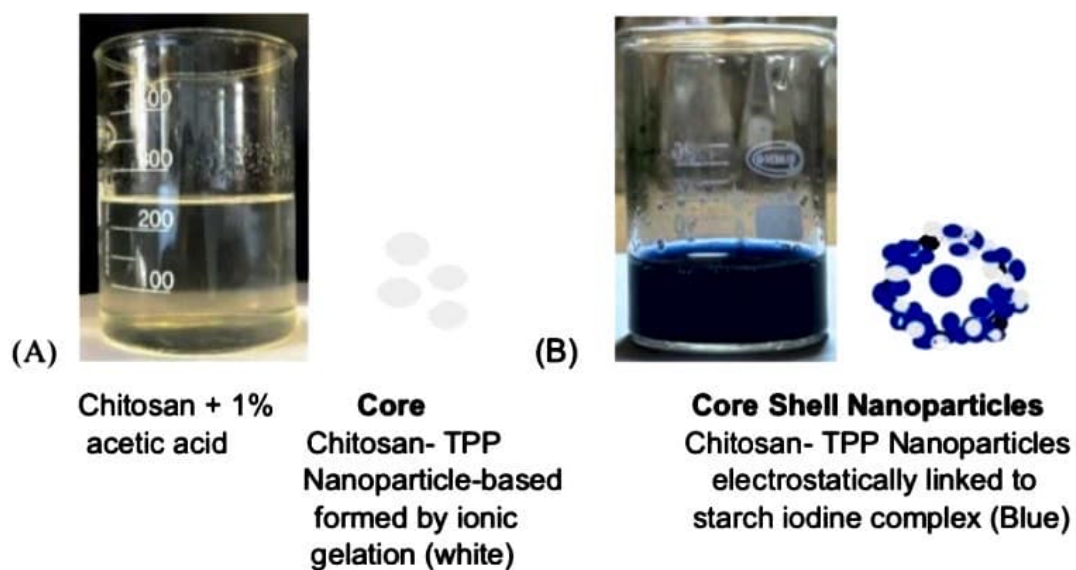
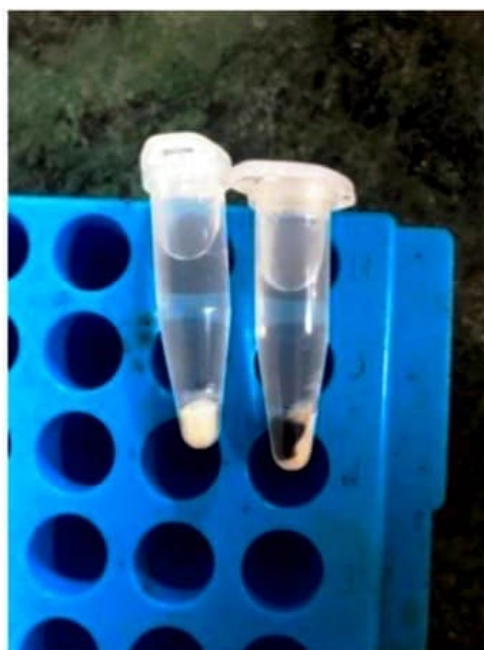


Fig. 4.1 Schematic diagram representing the process of preparation of the core and core-shell nanoparticles



Fig 4.2 Synthesis of Chitosan Nanoparticle



**Fig 4.3 Core Shell Nanoparticles Pellet
(Chitosan + TPP)**

The field emission gun-transmission electron microscope JEM 2100 and the FEI, Tecnai G2, F30, 300 kV, along with the JSM-7600 F Field Emission Gun (FEG) Scanning Electron Microscope (SEM), were used to characterize the core-shell nanoparticles.

4.2.3 Spectroscopic Analysis of Nanoparticles in tube-based format:

UV-Vis spectroscopy was used to measure the absorbance of core-shell nanoparticles that were in suspension. Alpha amylase was diluted in simulated saliva at various concentrations. Lyophilized samples were subjected to Fourier-transform infrared (FTIR) spectroscopy to ascertain the chemistry involved in the starch-iodine reaction in the shell as well as the whole nanoparticles as well as the impact of amylase on these samples.

4.2.4 Development of paper-based kit:

The procedures for creating the paper based α -amylase sensor are shown in the schematic depiction Fig 5.4. The filter papers were initially cut into pieces that were 1 cm by 5 cm in size. After that, to create the dark blue SI solution, starch and Lugol's iodine were combined in equal parts (v/v). Firstly, the core shell

nanoparticles are coated in paper using spray method and let to dry completely at room temperature. Following this, 60 ml of the SI solution was dispensed over the paper, which caused a dark blue coating to appear on its surface, as seen in image of Fig. 5.3. The positive and negative results are shown in the image of Fig 5.4 which depicts the presence of alpha amylase in the paper. The vacuum desiccator was then used to dry and preserve the dark blue colored paper kit that was filled with SI.

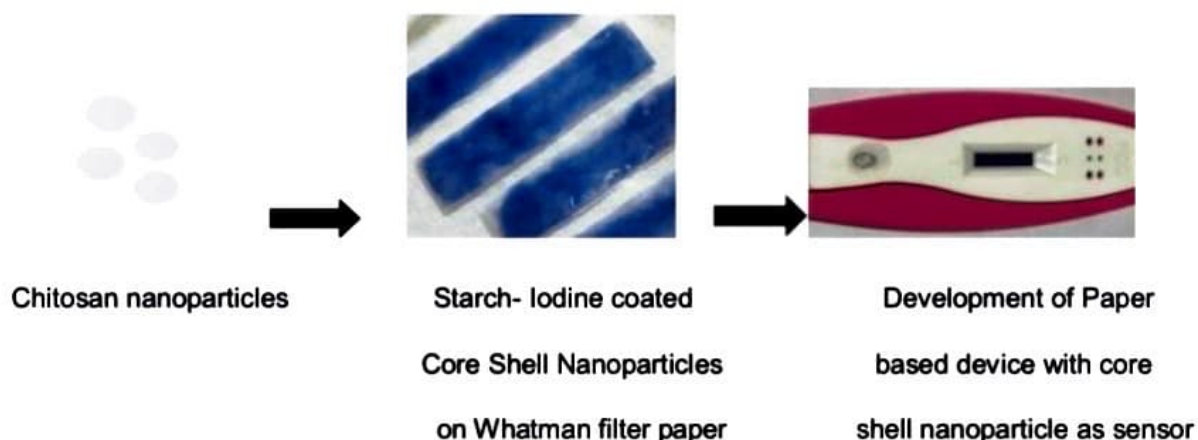


Fig 4.4 Process of development of paper based device

4.2.5 Application of the Nanoparticles in a paper-based format:

Nanoparticles were put to widely used Whatman Qualitative Grade 1 filter paper in order to create an economical point-of-care device, and the same was evaluated using different amylase concentrations on paper. In brief, 5 ml of the nanoparticle suspension was applied to the filter paper and left to completely dry. After that, 5 ml of the sample was added. The color changes were noticed 5 minutes to an hour later. It was discovered that 10 minutes was the ideal amount of time for results to be observed, and this time frame was also employed for subsequent investigations on 12 sAA concentrations ($n = 5$ each concentration; total $n = 60$). The transition of the color from blue to white was regarded as a positive reaction. The paper remains blue in color in the absence of alpha amylase.

4.3 CHARACTERIZATION METHODS:

4.3.1 UV Spectroscopy:

UV spectroscopy, also known as ultraviolet-visible spectroscopy, is a technique used to analyze the interaction of a sample with ultraviolet or visible light. The resulting UV spectroscopy graph shows the absorbance or transmittance of the sample at different wavelengths of light. The shape and characteristics of the UV spectroscopy graph provides valuable information about the sample analyzed. Specifically, the graph shows one or more peaks or valleys, which correspond to the absorption or transmission of light by the sample. The position of the peaks on the UV spectroscopy graph indicates the presence of certain functional groups or chromophores in the sample. For example, a peak near 280 nm indicates the presence of aromatic amino acids like tryptophan or tyrosine, while the peak near 200 nm indicates the presence of peptide bonds in the sample. The intensity of the peaks on the UV spectroscopy graph can be used to determine the concentration of the sample. The intensity of the peaks is proportional to the amount of sample present in the solution, making UV spectroscopy a valuable tool for quantitative analysis. Overall, the results of a UV spectroscopy graph provide information about the composition, concentration, and structural properties of a sample.

4.3.2 Fourier Transform Infrared Spectroscopy:

FTIR (Fourier transform infrared spectroscopy) (Fig 4.5) is an instrumental technique used to identify the functional groups present in organic and inorganic compounds by measuring their absorption of infrared radiation over a range of wavelengths. The FTIR method first collects an interferogram of a sample signal using an interferometer, and then it performs a Fourier transform (a mathematical algorithm) on the interferogram to obtain the infrared spectrum. Thus, FTIR identifies compounds and confirms them using functional groups. An FTIR graph usually shows the intensity of infrared light absorbed by a sample over a range of wavelengths, typically from 4000 to 400 cm^{-1} . The x-axis represents the wavenumber (the inverse of the wavelength), and the y-axis represents the intensity of the absorbance.

4.3.3 Field Emission Scanning Electron Microscopy

The topography of objects is studied using field emission scanning electron microscopy (FESEM), which uses electrons rather than light sources. The electrical field emission source produces electrons that are propelled in the direction of a strong electrical field gradient. The primary electrons are directed towards a high vacuum column, where an electronic lens deflects the electrons that are bombarded towards each object. Every object emits secondary electrons as a result. Every object's surface characteristic strongly influences the secondary electrons' angle and speed. An electronic signal is produced once a detector captures these electrons. A video-scan image of this signal is created and preserved for later processing, allowing it to be viewed on a monitor. Thus, FESEM confirms that the given core shell nanoparticle sample contains nanoparticles.

4.3.4 X Ray Diffraction:

In XRD, the generated X-rays are collimated and directed to a nanomaterial sample, where the interaction of the incident rays with the sample produces a diffracted ray, which is then detected, processed, and counted. A method for examining the atomic or molecular structure of materials is called X-ray diffraction, or XRD. It is non-destructive and functions best with fully or partially crystalline materials. X-ray powder diffraction is a common name for the method because the material being studied is frequently uniformly crushed down to a fine powder. When light strikes an obstruction, an aperture, or the edge of an object, it gently bends, which is known as diffraction. The extent to which it happens is determined by how big a wavelength is in relation to how big an obstacle or aperture is.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 RESULTS:

5.1.1 Preparation and Characterization of core shell Nanoparticle:

After collecting the chitosan TPP pellet, in a volume ratio of 5:1, Lugol's iodine and soluble starch solution (5 mg/ml in 1 N NaOH) were combined to resuspension the pellet. Fig 5.1 shows the result of adding starch iodine solution to the prepared chitosan pellet. The pellet had changed its color to dark blue color as a reaction of SI in chitosan. The coating of the core was anticipated to be achieved by electrostatic binding of the negatively charged starch-iodine complex and the positively charged chitosan nanoparticles (white suspension) and (blue suspension) respectively.



Fig 5.1 Starch Iodine added in Core Shell Nanoparticle at different proportions

5.1.2 Spectroscopic Analysis of Nanoparticles:

The resulting UV spectroscopy graph shows the absorbance or transmittance of the sample at different wavelengths of light. Specifically, the graph (fig 5.2) shows one or more peaks or valleys, which correspond to the absorption or transmission of light by the sample. The position of the peaks on the UV spectroscopy graph

indicates the presence of certain functional groups or chromophores in the sample. Here, a peak near 280 nm indicates the presence of aromatic amino acids like tryptophan or tyrosine, while the peak near 200 nm indicates the presence of peptide bonds in the sample. The intensity of the peaks on the UV spectroscopy graph can be used to determine the concentration of the sample.

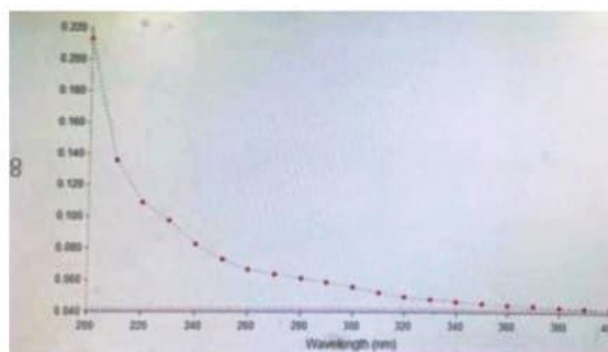


Fig 5.2 Spectrum of UV-visible absorption of the core and core-shell nanoparticles, as well as other core-shell nanoparticle components

5.1.3 Application of the Nanoparticles in a paper-based format:

While the goal of the study was to create a sensor with equivalent sensitivity to human bodily fluids including saliva, urine, and blood serum, we used a unique bio-reaction to detect the activity of α -amylase. The colorimetric effects resulting from two distinct reactions were used to develop the sensors. The interaction between starch and Lugol's iodine resulted in the creation of the dark blue SI complex. The top view of the sensors when SI solutions were coated on the paper is depicted in the first image in Fig. 5.8(A). The α -amylase analyte was then used to measure the enzyme applied to these paper sensors with SI coating, and it broke down simple starch molecules via their -1,4 glycosidic linkages forming sugars. The blue color's intensity is in addition to the SI's shattering, the colored complex was diminished. Iodine's evaporation is a result of both the basic and complex processes. The reaction's sugar products did not interact with it in any way. The fading of the blue color with an increase in α -amylase concentration in the analyte is shown in the images in Fig. 5.8(B). To investigate the responses on the paper surface, a thorough FTIR investigation was conducted. For this, two SI films were deposited on glass substrates, one on top of the other, and then α -amylase was added. By removing an equal mass from each of these two films, samples for the FTIR studies were

created, which later allowed for comparison of the peak intensities during the study which is shown in Fig 5.3.

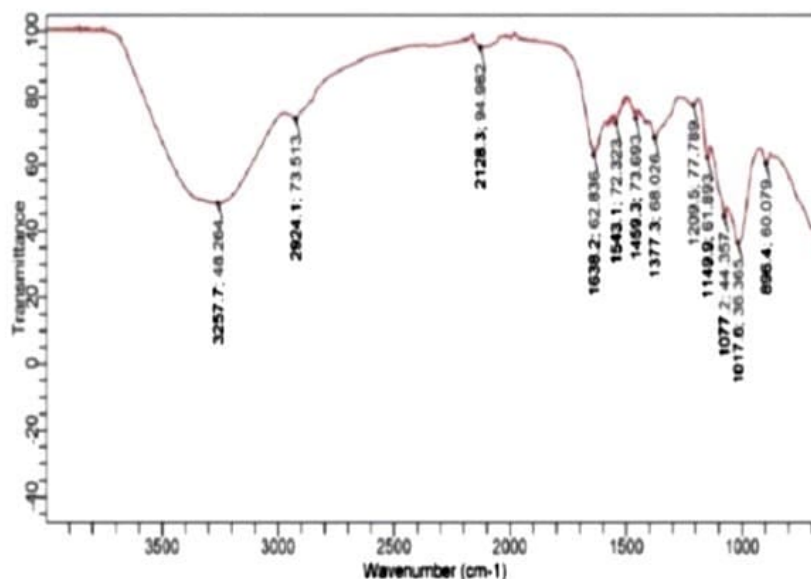


Fig 5.3. FTIR Graph indicating the peaks corresponding to different functional groups

In the above FTIR graph (Fig 5.3), we observe peaks or bands corresponding to the different functional groups of the sample. For instance, a peak near 3257 cm⁻¹ indicates the presence of C-H bonds in the sample, while a peak near 1638 cm⁻¹ indicates the presence of a carbonyl group (C=O) in the sample. The shape and position of the peaks provides information about the molecular structure and chemical composition of the sample. A sharp peak indicates a strong absorption, while a broad peak indicates a weaker absorption due to multiple overlapping peaks.

5.1.4. Physicochemical characterization of core-shell nanoparticles:

The obtained nanoparticles, which had a center and a shell, were between 20 and 100 μm in size, as determined by an electron microscope. (Fig. 5.4). Chitosan-tripolyphosphate nanoparticles were used to make the core. These were observed as uniform particles by ionic gelation (Fig. 5.4 A, and C), whereas the core-shell nanoparticles gave the surface an uneven texture. Simultaneously, XRD results and

graph are shown in Fig 5.4(E) and Fig 5.5 respectively. The diameter of the core was decreased by combining high shear homogenization at high rotational speed with controlled, slower addition of TPP using a peristaltic pump. Other elements that were altered were the proportion of chitosan to TPP, the temperature and pH of the chitosan solution, the concentration of the acetic acid solution, and other elements that have been demonstrated to be essential for the creation of smart nanoparticles, even in the context of drug delivery. The starch-iodine compounds were added to create core-shell nanoparticles, which were measured to be 830 nm (8×10^{-9} m) in diameter. The change in diameter of the core-shell nanoparticles further proved that the chitosan core was encased in a shell. Using specifically created core-shell nanoparticles that exhibit a change in the core's charge with the addition of the starch shell, our lab has published on the application of starch-iodine chemistry to detect enzymes like beta-lactamases.

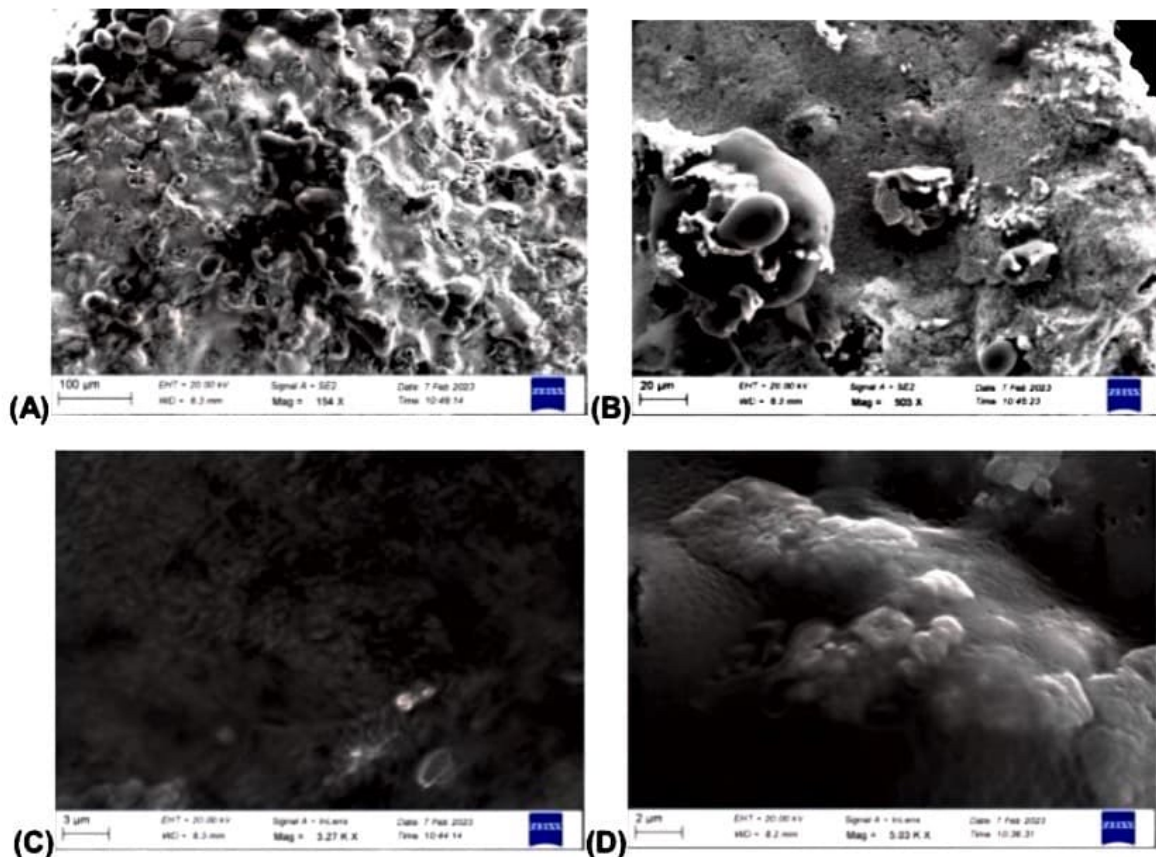




Fig.5.4 Electron microscopic image of FESEM with EDAX (FESEM: A-D and XRD: E) showing the chitosan core and the core-shell nanoparticles

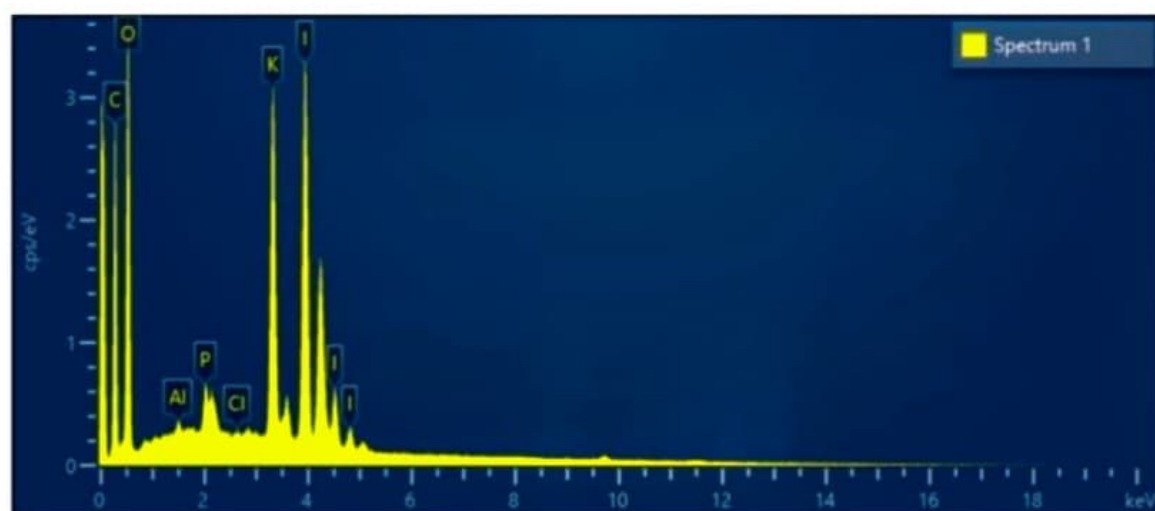


Fig 5.5. The graph shows the XRD pattern of the sample with O as its highest peak and I as its lowest peak

5.1.5 Results of X Ray Diffraction:

Table 5.1. The tabular column represents the elemental composition of a sample as determined by X-ray diffraction (XRD).

Spectrum 1				
Element	Line Type	Weight %	Weight % Sigma	Atomic %
C	K series	32.48	0.44	55.01
O	K series	27.73	0.28	35.27

Al	K series	0.23	0.04	0.18
P	K series	0.54	0.04	0.35
Cl	K series	0.12	0.03	0.07
K	K series	8.02	0.09	4.17
I	L series	30.88	0.26	4.95
Total		100.00		100.00

The sample was analyzed using X-ray fluorescence (XRF) and the resulting spectrum is labeled "Spectrum 1". Table 5.1 includes six columns: Spectrum 1, Element, Line Type, Weight %, Weight % Sigma, and Atomic %. The first column simply identifies the spectrum being analyzed. The second column lists the elements detected in the sample. The third column identifies the X-ray emission line used to detect each element. The fourth and fifth columns show the weight percent and weight percent sigma, respectively, for each detected element. The weight percent is the percentage of each element in the sample by weight, while the weight percent sigma represents the uncertainty in the weight percent measurement.

The last column, Atomic %, gives the atomic percentage of each element in the sample. The atomic percentage is the percentage of each element in the sample by number of atoms. The sum of the atomic percentages should add up to 100%. The table shows that the sample contains primarily carbon (55.01%) and oxygen (35.27%), with smaller amounts of potassium (4.17%), iodine (4.95%), phosphorus (0.35%), aluminum (0.18%), and chlorine (0.07%).

5.1.6 Screening of Alpha Amylase:

The screening of alpha amylase was done in the following method, 0.5g of Agarose was added in 50 ml of distilled water. The solution was mixed well and kept in hot water bath for 30 mins at 100-degree C. Then, 0.5 g of starch and 0.5ml of Iodine solution was added to the solution and mixed well in the magnetic stirrer. The agarose SI solution was then transferred to a petri plate and allowed to cool. Solidification occurs due to the addition of agarose. Two wells are cut open in the solidified gel where one acts as the control and the other as the positive to which the saliva is added. In the first well, 0.2 ml of water was added where no color change occurs and acts as the control. When 0.2 ml of saliva was added in the second well the color change was observed as the disappearance of blue color.

This color changes indicates the presence of alpha amylase and acts as the screening test for detection of alpha amylase. (Fig 5.6).

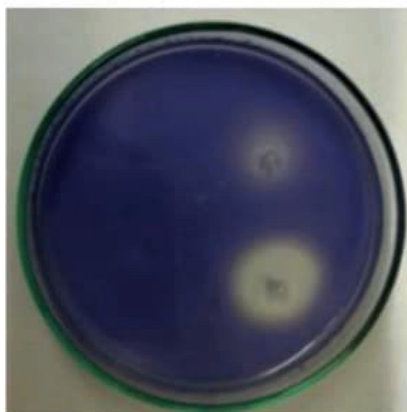


Fig 5.6. Screening of alpha amylase

5.1.7 Development of paper of paper-based kit:

The procedures for creating the paper based α - amylase sensor is shown in 4.2.4. Fig 5.7 shows the result of starch iodine coated core shell nanoparticles on Whatman filter paper. The starch reacts with iodine and leads to the formation of blue color. Firstly, the nanoparticles were coated on the Whatman filter paper and dried. Followed by this, the paper was dipped in the starch iodine solution and kept for 10 mins. Later, the paper is taken and kept in the hot air oven for 10 mins at 50-degree C. After complete drying of the paper, the paper is taken for the experiment.

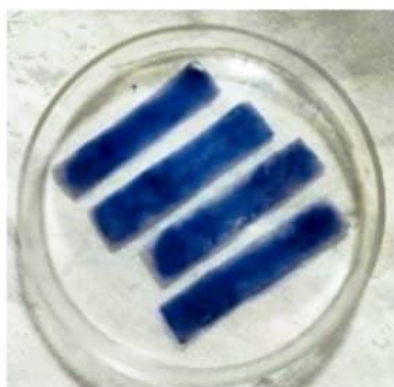


Fig 5.7 Core shell nanoparticles with SI coating on Whatman filter paper

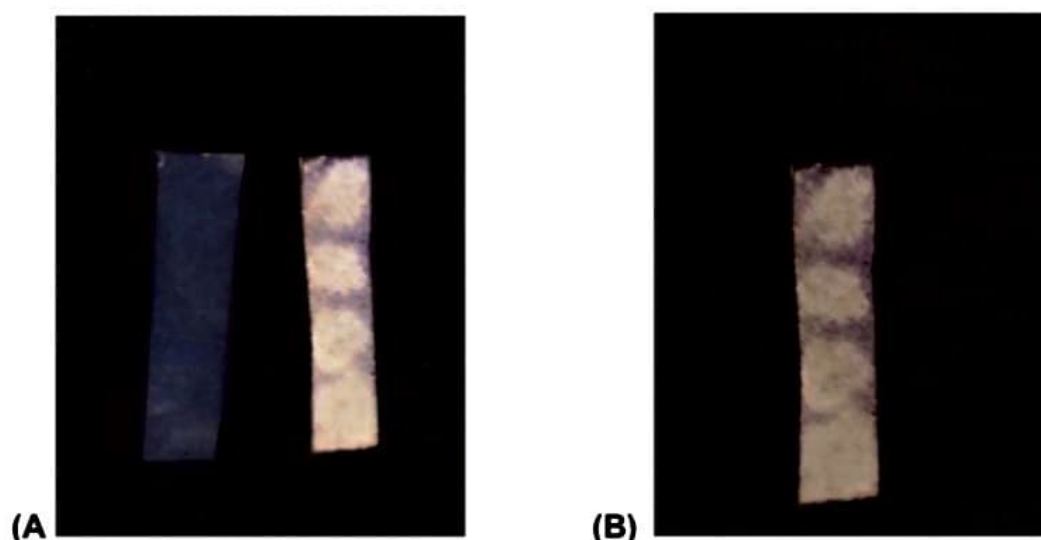


Fig 5.8(A) Negative and positive results respectively shown in the nanoparticle coated SI papers in addition of saliva and (B) shows a closer look of the positive paper in which the color faded from blue to white color in the presence of alpha amylase.

In the above figure 5.8, we observe 2 images: (A) 2 nanoparticle coated SI papers were placed, in which, the first paper shows the negative result where we do not observe any color change. In the second image of fig.5.8 (A) we observe color change of white color from blue color. In brief, 0.5 ml of saliva was added to the paper. The starch iodine complex broke and lead to the white color. The shell ruptures revealing the core, indicating the color from blue to white. A closer look can be seen in fig.5.8(B). This confirms the presence of alpha amylase in the paper.

5.2 DISCUSSION

From the results of Adhikary, et al., 2021, we find that the resulting nanoparticles, were made up of a core and a shell, were 200–250 nm in size, according to an electron microscope. The surface of the core-shell nanoparticles was given an irregular texture, as opposed to the core, which was formed as chitosan-tripolyphosphate nanoparticles by ionic gelation. the size range of 200–250 nm nanoparticles as observed under an electron microscope. The concentration of the acetic acid solution, the temperature and pH of the chitosan solution, and the relative ratio of chitosan to TPP were also altered. All of these variables had been demonstrated to be significant in the creation of smart nanoparticles, although in the context of drug delivery. The produced core-shell nanoparticles with the addition

of starch-iodine complexes were measured to have a diameter of 384.4 45.3 nm. The core-shell nanoparticles' shift in diameter further demonstrated that the chitosan core was covered by a shell.

From the results of Satarupa Dutta, et al., the graphs depict the FTIR Spectra of the Starch iodine complex before and after the addition of alpha amylase have peaks at 1153 and 905 cm⁻¹ for the C-O and C-C stretching modes, respectively. (Satarupa Dutta, et al., 2015).

5.2.2 Spectroscopic analysis of nanoparticles in tube-based format:

UV-visible spectroscopy was used to examine the nanoparticles in solution. The substance used to create these core-shell nanoparticles was also investigated. The core-shell nanoparticles had clear peaks at 375 nm and 549 nm. When amylase was added at the specific concentration, the nanoparticles produced after the addition of amylase had displayed recognizable absorbance peaks at 375 nm and 507 nm. (Adhikary, et al., 2021)

5.2.3 Development of the amylase test for forensics:

This was subsequently done; however, three parameters were not included in the subsequent analysis since they varied so much between their positive and negative samples. Also, it enabled the use of RGB data for cellphone-based condition detection, which is very important in today's life. In order to help with the qualitative identification of amylase and aid in the detection of saliva for forensic purposes, the platform was developed as an improvement to the available technology. Much less starch was supplied to the shell as an iodine-starch combination. The core and core-shell nanoparticles had much thinner shells, according to FEGTEM imaging of the particles. It was anticipated that it would be simpler to extract DNA from the saliva stains if necessary for a crime scene investigation because all the chemicals utilized were biocompatible. Even after 30 minutes, the tip's color changed noticeably. Moreover, a suspension that was applied to fabric and produced a positive reaction, such as a change in color, was devised. Their result was a decreased limit of detection (LoD) at a salivary dilution to the ratio of 1:40 and an amylase concentration of 2.5 units/ml due to the shift in the diameter of the core-shell

nanoparticle. Also, even two hours after saliva was deposited on the skin surface, alpha amylase was able to be detected, indicating that the test may be able to identify latent salivary stains. (Adhikary, et al., 2021)

CHAPTER 6

SUMMARY

Summarizing all the methods and results, it can be said that the use of alpha amylase is an interesting approach in forensic science and biomedical laboratories. The evaluation of synthesis of core shell nano particles, coating of nanoparticles in paper and development of kit provided with satisfactory results. The characterization methods used to study the nanoparticles also demonstrated with good results. When UV Spectroscopy was performed, we obtained results with 200 nm indicating the presence of peptide bonds in the sample. FTIR Spectra was performed with results of 3257 cm^{-1} indicating the presence of C-H bonds. Then SEM Analysis with EDX was performed. It showed surface morphology with 20 – 100 micrometers. Additionally, XRD analysis was performed with results Carbon at its peak and Chlorine with least. Lastly, a kit was developed with the use of HCG kit. Overall, these findings suggest that this research provides significant contributions in the field of Forensic science and future has great scope in kit development for alpha amylase detection.

CONCLUSION

Based on the findings presented, it can be concluded that the use of alpha amylase in forensic investigation is a promising approach. The in vitro studies evaluating the synthesis of core shell nanoparticle, coating of paper, kit development has demonstrated satisfactory results. Additionally, the XRD analysis revealed a satisfactory level of crystallinity, while the SEM analysis provided insight into the surface morphology. The FTIR Spectra revealed the composition of solids, liquids and gases whereas the UV Spectroscopy measured how much the chemical substance absorbs light. Overall, these findings suggest that this research provides important contributions to the field of forensic science and biomedical engineering and serves as a foundation for future studies in this area.

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