

Development of smart core-shell nanoparticles-based sensor for diagnostics and forensics

Submitted in partial fulfillment of the requirements for the award of Bachelor of Technology Degree in Biotechnology

by

Kajal Purohit (38230019)



**DEPARTMENT OF BIOTECHNOLOGY SCHOOL OF BIO AND
CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A" by NAAC

JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600 119.

MAY 2022



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY (DEEMED TO BE UNIVERSITY)

Accredited with Grade “A” by NAAC JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI- 600 119

www.sathyabama.ac.in

DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Kajal Purohit** (Reg. No. **38230019**), who carried out the project entitled “**Development of smart core-shell nanoparticles-based sensor for diagnostics and forensics**” under our supervision from **December 2021 to March 2022**.

Internal Guide

Dr. Kiruthiga. B

Department of Biotechnology Sathyabama Institute of Science and Technology

External Guide

Dr. S. Kumaran

Department of Biotechnology Sathyabama Institute of Science and Technology

Head of the Department Dr. V. Ramesh Kumar

Department of Biotechnology

Sathyabama Institute of Science and Technology

Submitted for Viva voce Examination held on _____

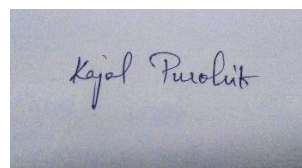
Internal Examiner

External Examiner

DECLARATION

I, **Kajal Purohit (38230019)**, hereby declare that the Project Report entitled “**Development of smart core-shell nanoparticles-based sensor for diagnostics and forensics**” done by me under the guidance of **Dr. Kiruthiga. B, Department of Biotechnology, Sathyabama Institute of Science and Technology** and **Dr. S. Kumaran Centre for Drug discovery and development, Sathyabama Institute of Science and Technology**, is submitted in partial fulfilment of the requirements for the award of Bachelor of Technology Degree in Biotechnology.

DATE: 21-04-2022

A rectangular box containing a handwritten signature in blue ink that reads "Kajal Purohit".

PLACE: Chennai, Tamil Nadu, India

SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

We are pleased to acknowledge our sincere thanks to **Board of Management of SATHYABAMA** for their kind encouragement in doing this project and for completing it successfully. We are grateful to them.

We convey our thanks to the **Head of the Department Dr. V RAMESH KUMAR, Department of Biotechnology** for providing us necessary support and details at the right time during the progressive reviews.

We would like to express our sincere and deep sense of gratitude to our Project Guide **Dr. Kiruthiga. B, Department of Biotechnology, Sathyabama Institute of Science and Technology and Dr. S. Kumaran Centre for Drug discovery and development, Sathyabama Institute of Science and Technology**, for valuable guidance, suggestions and constant encouragement paved way for the successful completion of our project work.

We wish to express our sincere thanks to all **Teaching and Non-Teaching** staff members of the **Department of Biotechnology** who were helpful in many ways for the completion of the project.

Finally, we express our sincere thanks to our parents, friends and others who helped us to complete this work.

ABSTRACT

Smart biocompatible materials that respond to a variety of external stimuli have a lot of potential in the creation of low-cost diagnostic biosensors. The present work describes the creation of smart enzymeresponsive core-shell nanoparticle-based sensors as low-cost diagnostics for alpha amylase detection. A chitosan-tripolyphosphate core was generated via ionic gelation and was coated with a starch-iodine shell to create biocompatible core-shell nanoparticles. The starch-iodine shell was ruptured in the presence of certain amounts of amylase, exposing the core. This application explains a noticeable colour change from blue to red that can be used to identify salivary alpha amylase at the point of care (sAA). This biomarker can be used to diagnose stress and other autonomic disorders. In forensic investigations, alpha amylase can also be employed to detect latent saliva at crime scenes. A paper-based diagnostic for salivary alpha amylase detection was developed using the current platform technology, with a limit of detection (LoD) of 140 units/ml (70 mg/ml) at 5 minutes, and a coated swab developed from nanoparticles for crime scene investigations with a LoD of 2.5 units/ml (1.25 mg/ml) over 30 minutes. The nanoparticles were stable and reproducible, with no influence from other substances in the saliva. The current research presents a proof-of-concept technology demonstrating the applicability of smart nanoparticles in low-cost, adaptable biosensing platforms such as paper-based and swab-based formats for applications as diverse as stress diagnostics and forensics.

Keywords: Amylase, Chitosan, Core-shell nanoparticle, Forensic, Stress.

CONTENTS

S.No	Content	Page. No
1. Chapter 1 – Introduction		10-14
1.1 Biosensors		12
1.2 Nano biosensors		13
1.2.1 Alpha amylase and its detection		14
2. Chapter 2 - Literature survey		17
3. Chapter 3 - Aim and Scope		22
3.1 Aim		22
3.2 Scope		22
3.3 Plan of work		22
4. Chapter 4 - Materials and methods		23-24
4.1 Materials required		23
4.2 Preparation and characterization of core shell NPs		23
4.3 Characterization of core shell nanoparticles		24
4.4 Spectroscopic analysis of tube based format		25
4.5 Application of NPs in paper-based format		25
4.6 Development of the paper-based device		25
4.7 Application of a-amylase for the detection of saliva		25

Chapter 5 - Results & discussions	26-32
5.1 Physiochemical characterization of core-shell NPs	26
5.2 Spectroscopic analysis of NPs in tube-based format	28
5.3 Analysis of the α -amylase on paper-based device	29
5.4 Development of paper-based device	30
5.5 Development of amylase test for forensics	31
5.6 Advantages and limitations	32
 6. Chapter 6 - Summary & conclusion	 33-34
6.1 Summary	33
6.2 Conclusion	34
 7. References	 35-40

LIST OF ABBREVIATIONS

Abbreviation	Expansion
μ	Micro
Ab value	Absorbance value
FTIR	Fourier-transform infrared spectroscopy
g	gram
Hcl	Hydrochloric acid
hr	hours
ml	milliliter
NPs	Nanoparticles
UV-vis	Ultraviolet visible spectroscopy
TEM	Transmission electron microscopy
SEM	Scanning electron microscope

LIST OF FIGURES

S.No	Figure.no	Content	Page. No
1.	1	Preparation of core-shell nanoparticles	26
2.	2	SEM analysis	27
3.	3	FTIR analysis	27
4.	4	UV-vis analysis	28
5.	5	Spectroscopic analysis	29
6.	6	Paper based device	31
7.	7	Swab based test	31-32

CHAPTER 1

INTRODUCTION

The historical context of criminological research remains elusive, as scientific technology as a discipline has received little attention until recently. As one has a better understanding of forensic science's historical significance, one can't help but feel more awe for this branch of science. The term "forensic" comes from the Latin word "forensis," which means "forum" (Della et al., 2017). In general, forensic science is employed in conjunction with any discipline that has ties to the legal system. In a nutshell, forensic science is the application of scientific concepts and methods to legal issues. To put it another way, forensic science is a branch of research that the forensic investigators use to solve crimes (Shi et al., 2018, Elman et al., 1929).

Forensic science is still in its infancy as a component of today's criminal justice system. The importance of forensic science may be traced back to ancient civilizations. The use of forensic science can be traced back to ancient Greece and Rome. Those civilizations contributed much to medicine, especially in the field of pharmacology. Their studies of toxin manufacture, use, and symptoms allowed them to look into their use in earlier killings. A forensic scientist's primary responsibility is to help determine whether a crime has been committed and, if so, to assist in the identification of the perpetrator (Bernfeld, 1951). Any procedure's analytical component, as well as the formation of hypotheses and the interpretation of results in the context of the case, is critical. The majority of forensic studies and comparisons are fundamentally a reduction process in which the scientist attempts to differentiate between multiple sources of the same sample or substance. In this context, it is widely acknowledged that certain types of forensic evidence are more important than others, with fingerprints and DNA at the top of the scale (Somgyi, 1938). Analytical sciences, on the other hand, have advanced dramatically in recent years. Trace evidence has long been at the heart of forensic science, having played a pivotal part in the development of the field in the twentieth century, spurred on by pioneers such as Gross, Reiss, and Locard's important work. In the last 20 years, however, the face of trace evidence has evolved considerably (Merritt et al., 1977).

Analytical sciences, on the other hand, have advanced dramatically in recent years. Trace evidence has long been at the heart of forensic science, having played a pivotal part in the development of the field in the twentieth century, spurred on by pioneers such as Gross, Reiss, and Locard's important work. In the last 20 years, however, the face of trace evidence has evolved considerably. The results obtained in this manner are absolutely admissible in an intelligence or tactical environment, but they still need to be confirmed in a laboratory before they can be used in court (McGeachin and Lewis, 1959). Current and future technical advancements are expected to close

the gap, and a growing number of tests with a high degree of dependability will be used on the scene in the near future. Smart materials are stimulus-responsive or trigger-responsive materials created towards the end of the twentieth century that can change one or more physical or chemical properties (e.g. colour, form, stiffness, opacity, porosity) in response to tiny changes in the surrounding environment (Zhou et al., 2013). Smart materials are being extensively investigated in the field of biosensors and diagnostics to leverage these changes in characteristics caused by changes in stimulus, for use as actuators and sensors, or for self-assembly methods (Verma et al., 2016).

Sensor applications have recently been developed using a variety of stimulus-responsive smart materials that respond to temperature, pH, electrical field, or chemical changes such as enzymes (Murphy, 2010). Because of changes in conformation in the presence or absence of particular chemicals, the smartness of the materials involved can be employed in smart nanoparticles for diagnostics (Fleige et al., 2012; Verma et al., 2016). Fluorescence-based magnetic (Fe/Fe₃O₄) core-shell nanoparticles, for example, have been shown to work as nanobiosensors in cancer diagnostics to detect protease activity in biospecimens (Wang et al., 2014). In other experiments, biocompatible nanoparticles such as liposomes were mixed with standard labels such as gold nanoparticles. The use of liposome-gold nanodot hybrids for phospholipase C biosensing was described, which led liposomes to hydrolyze membrane phospholipids, generating byproducts that boosted the stability of gold nanodots and reduced fluorescence quenching in an oxygen-rich environment (Chen et al., 2013). In addition, a recent example described the use of biopolymers alone in core-shell nanoparticles to construct a device for detecting antimicrobial resistance by observing changes in starch-iodine chemistry in the presence of beta-lactamases (Punjabi et al., 2020). The goal of this research is to create enzyme-responsive core-shell biocompatible nanoparticles that can be used as point-of-care biosensors for low-cost diagnostics.

Salivary alpha amylase (sAA) (EC 3.2.1.1), also known as 1,4-alphaD-glucan glucanohydrolase, is an enzyme in the oral cavity that aids in the breakdown of starch into simple sugars (Nater and Rohleder, 2009). Apart from its involvement in carbohydrate digestion, sAA is also used as a biomarker to identify changes in sympathetic nervous system (SNS) activity caused by stress (Nater and Rohleder, 2009). The SNS responds very instantly in the presence of a stressor, while the hypothalamus pituitary adrenal system (HPA) axis is activated after a time lag (Ali and Pruessner, 2012; Chrousos 2009, Dreiling et al. 1963, Wilding, 1965, Searcy et al., 1964, Searcy et al, 1965). Catecholamines (epinephrine and norepinephrine) regulated by the systemic and adrenomedullary SNS, as well as glucocorticoids largely regulated by the HPA axis, are the main peripheral effectors of these two systems. (According to Chrousos, 2009). Autonomic dysfunction markers that alter the stress response may have multiple uses in the diagnosis and prognosis of stress and autonomic

dysfunction in neurological disorders. Salivary alpha amylase has been used as a marker for SNS and stress in a variety of settings, including nursing care (Tokunaga and Fukai, 2016), burn injuries (Brown et al., 2014), surgery-related stress (Robles et al., 2012), and pain perception in children (Tokunaga and Fukai, 2016). (Ferrara et al., 2013). In addition to forensic biology, point-of-care amylase detection is being used in foods and drinks (honey, malt, eggs), as well as detergents and textiles (Ludwig et al., 2018). Amylase can be employed in forensics to examine suspicious stains for relevant genetic material (deoxyribonucleic acid, DNA) during crime scene investigations. This is especially important because saliva contains 263–376 IU/ml of the enzyme, which is hundreds of times more than other stains formed by urine, sperm, perspiration, or nasal secretions (Wittstein et al., 2005). The current study describes the creation of smart core-shell nanoparticles that can detect the presence of amylase in saliva for stress diagnosis and forensic applications. The use of smart materials on paper-based point-of-care devices and on smart swabs to detect amylase for the aforementioned applications is discussed in this study.

1.1 BIOSENSORS

A biosensor is a sensing device or measurement system that is designed to estimate the properties of a material using biological interactions. A biosensor's principal function or goal is to detect a biologically unique substance. Antibodies, proteins, enzymes, immunological compounds, and other similar materials are frequently used. Since a long time, biosensing has been one of the trendiest topics drawing scientific minds. It's because biological entities are extremely complex and are intimately linked to the existence of a healthy environment. Biosensor design has also undergone considerable advancements in recent years. Biosensors are now being used for a wide range of applications, including food quality estimate, environmental monitoring, and the identification of clinical and metabolic issues. Nanotechnology has provided several extremely fascinating components for improving sensory phenomena. The usage of various nanomaterials such as nanoparticles, nanotubes, nanorods, and nanowires has greatly improved detection speed and reproducibility. Nanomaterials' distinctive properties, such as strong electrical conductivity, greater shock resistance, and sensitive responses, such as piezoelectric and diverse color-based detecting methods, are the outcome of a confluence of nanomaterial qualities (Florkin, 1972).

In the current setting of ever-changing environmental developments and corresponding changed homeostatic events occurring at both in vivo and ex vivo levels, sensing biological reactions has taken enormous importance. The study of the behaviour of constantly changing materials has become more important in areas such as pharmacological diagnosis, food quality testing, and environmental applications. The development of effective biosensors that can evaluate the tiniest details of biological interactions even at a very small scale with exceptional precision and maximal

ever achievable sensitivities merits immediate attention in this reference. The transduction mechanisms are an important part of biosensing since they are responsible for turning the responses of bioanalyte interactions into an identifiable and repeatable manner by converting specific biochemical reaction energy into an electrical form. Nanomaterials can be fantastic occupants in this dimension because they have high surface area to volume ratios, allowing the surface to be exploited in significantly more diversified ways. Furthermore, their electromechanical features are fantastic advantages for biosensor technology. The nanostructural marvels given by nanotechnology have transformed molecular biology by allowing for the manipulation of atoms and molecules as well as the monitoring of biological phenomena at the physiological level with far more precision. In the sense that it includes the word nano appended to it, the name nanobiosensors is a misnomer (Skude,1977). To get to the real technology, it's important to first understand what a biosensor is. Because nanoscience is interdisciplinary in nature, using the prefix nano to denote use or manipulation at a size of one billionth of a metre is common.

1.2 NANO BIOSENSORS: THE MERGING OF NANOTECHNOLOGY WITH BIOSENSORS

Understanding the notion of biosensing lays the groundwork for investigating and developing nanobiosensors. Nanobiosensors are nanomaterial-based sensors that do not detect nanoscale events. The foregoing description raises the question of why nanoparticles are intended to be utilised in the manufacture of biosensors and whether they will make a major change in overall technology. Nanomaterials are a one-of-a-kind gift from nanotechnology to humanity; they are materials with one of their dimensions ranging from 1 to 100 nanometers (Wohlgemuth, 1908). These materials are unique because to their size restrictions, as the majority of their constituent atoms are located at or near their surface, and all important physicochemical properties are vastly different from those of the identical materials at the bulk scale (Henri, 1902). They have the potential to play critical roles in biosensor sensing mechanisms. Nanoelectromechanical systems (NEMS) are electrical transduction mechanisms that combine nanomaterials and electrical systems. Several nanomaterials have been investigated for use in better biological signalling and transduction mechanisms based on their electrical and mechanical characteristics. Nanotubes, nanowires, nanorods, nanoparticles, and thin films comprised of nanocrystalline matter are examples of commonly used materials. Among these, the usage of nanoparticles has received the most attention and research to date. Nanobiosensors have proven to be incredibly effective developing inroads in biosensor technology, made possible solely by the miracles of nanotechnological implications. Several studies have looked into a wide range of biosensing devices that use nanoparticles or nanostructures all around the world. These can range from amperometric devices for enzymatic glucose detection to quantum dots as fluorescent agents for binding detection and even bioconjugated nanomaterials for particular biomolecular detection. Colloidal nanoparticles, for

example, can be conjugated with antibodies for immunosensing and immunolabelling purposes. These materials can also be utilised to improve the detection of electron microscopy. Furthermore, metal-based nanoparticles are good materials for electronic and optical applications, and their optoelectronic capabilities may be effectively employed for nucleic acid sequence identification. There are numerous criteria that govern or influence the utilisation of specific nanomaterials for biosensing applications. Their physical and chemical properties, as well as their energy sensitive and selective responses, are all based on these parameters. Nanobiosensor categorization is a complicated topic. This is because of the nature of the nanoparticles used in the biosensing procedure. Furthermore, unlike biosensors, the classification is not as straightforward (Michaelis and Pechstein,1914).

1.3 ALPHA AMYLASE AND ITS DETECTION

For more than 150 years, researchers have been fascinated by the study of amylase. When Kirchhoff discovered the digestion of starch by wheat extracts in 1811, and Dubrunfaut discovered a similar activity in barley malt extract in 1830, it was the first enzymic action ever recorded. In Payen and Persoz's subsequent experiments in 1833, this extract was dubbed "diastase," a name that stuck until it was displaced by "amylase" in the early twentieth century. Leuchs initially reported the enzyme from saliva in 1831, and it was later discovered in serum by Magendie in 1846 and urine by Cohnheim in 1863. Kjeldahl described a saccharogenic assay in 1880, but Wohlgemuth's amyloclastic assay for diastase determination in urine, was the first to use amylase measurement as a clinical tool. Amylase was used in the early studies of classical enzyme kinetics by Henri in '1902 and Michaelis and Pechstein in 1914 due to its relative simplicity of isolation and testing.' Elman's report of the link between raised amylase levels and severe pancreatitis⁸ in 1929 sparked increased interest in amylase testing and clinical utility. Somogyi's 1938 description of two assays, one saccharogenic and the other amyloclastic, had a long-term impact on clinical biochemistry. His amyloclastic method, as well as subsequent variations, became the most often used amylase assay in clinical chemistry. Despite the availability of newer and better assays and unit definitions, several studies still report amylase activity in Somogyi units. Despite the fact that amylase has a lengthy history of scientific study, evidence of the presence of isoenzymes was not discovered until the late 1950s. Ironically, these early paper electrophoresis methods were really separating and detecting amylase and albumin, both of which produced a visible reaction in the commonly used starch-iodine assay at the time. It wasn't until 1964 or 1965 that it was discovered that these methods were not capable of detecting actual amylase isoenzymes. Norby electrophoretically revealed the first real separation of pancreatic and salivary amylase isoenzymes" in 1964, and Muus and Vnenchak demonstrated isoenzymic forms of salivary amylase in 1965. ¹⁶ This was quickly followed by the application of these procedures to urine samples within a few years. Today, less than 20 years later,

the study of amylase isoenzymes and their clinical value in a number of settings has grown into a burgeoning field of research in which staying current and educated can be difficult. We hope that this review will assist the reader in gaining a better grasp of progress and results in what may be a complex subject, at least for the time being.

The random hydrolysis of internal α 1,4 glycosidic links in starch, glycogen, and other glucose polymers is catalysed by α -amylase. It's an endoglycosidase that produces reaction products with the α conformation intact. It should not be confused with exoglycosidases like α -glucoamylase (or α -glucosidase), which cleaves glucose residues progressively from the nonreducing end, or with β -amylase, which produces reaction products in the β configuration. Except as otherwise specified, when we talk about amylase, we're talking about α -amylase, and specifically human α -amylases. Except for the terminal bond on the reducing end and the penultimate bond on the nonreducing end, Amylase can cleave practically any internal α -1,4 glycosidic bond.' 3 Maltose, maltotriose, and other dextrans are the results of amylase digestion of amylose, a linear glucose polymer of starch with solely α -1,4 links, however some glucose can also be generated under specific α -1,6 conditions. Amylase does not hydrolyze links, hence products containing unhydrolyzed α -1,6 linkages, known as limit dextrin, result from the digestion of amylopectin, the branched-chain component of starch. Variable sources and compositions of starches have different susceptibilities to amylase digestion. Amylopectin is a better substrate for total amylase assay than glycogen, which is a better substrate than amylose. "Activity levels of starch substrates from various sources vary as well. 'y~3' The starches, on the other hand, all have higher activity than the oligosaccharides with lesser molecular weights." On the other hand, the pancreatic and salivary amylase isoenzymes have differing substrate specificities for starch and oligosaccharide substrates. Potato starch is digested faster by the salivary isoenzyme than corn starch, but corn starch is digested faster by the pancreatic isoenzyme. Even with the uncommon cyclic substrate cyclooctaamylose (γ -cyclodextrin), the K_m and V of the pancreatic and salivary isoenzymes differ, possibly indicating separate modes of action. 2h The salivary isoenzyme has a higher specific activity than the pancreatic isoenzyme³.^{7.2H}, therefore substrate comparisons without this factor can be deceptive. When comparing amylase activity with various substrates on a per unit weight of enzyme basis, the salivary isoenzyme is usually more active than the pancreatic isoenzyme with any particular substrate. The pancreatic and salivary isoenzymes, on the other hand, have identical relative substrate specificities when compared to some arbitrary substrate. Amylases have been isolated from numerous species' pancreas and salivary glands. The various amylase purification strategies will not be discussed in length here; instead, the reader is directed to Merritt and Kam's review for more information. Salt fractionation, starch or glycogen precipitation, gel permeation and ion-exchange chromatography, and crystallisation have all been used in amylase purification processes.

The invention of a portable diagnostic equipment for detecting Human Salivary-Amylase (HSA) is extremely opportune, as this enzyme is an important biomarker for diagnosing and monitoring human stress. The proposed immunosensors' reliability has been demonstrated by the validation of the one-step protocol with a standard laboratory method such as the Phadebas test, which can be used to measure amylase concentration in body fluids such as blood serum and urine where the physiological level is greater than 20 U/L. (Della et al., 2017).

α -Amylase plays a crucial role in the human body's physiological cycle; its function is continually being researched and employed as a major indicator of disorders such as acute pancreatitis, acute organophosphorus pesticide poisoning, and anxiety or depression. Existing approaches, including the assay kit, have limited sensitivity and time consumption, or are indirect assays that require the use of a tool enzyme or a competitive substrate inhibitor, making them unsuitable for low activity and nondestructive detection of α -amylase in human fluids. It is currently difficult to perform a quick, highly sensitive, and easy direct α -amylase determination in human bodily fluids. An AIEgen-based small molecule α -amylase sensing system was initially constructed in this study. Because of its high solubility, the probe emits little light in aqueous environments, but the insoluble AIE residues can be liberated following hydrolysis by α -amylase, dramatically increasing fluorescence. The detection limit in this unique sensing system is 0.14 U L⁻¹ in MES buffer with a linear range of 0–45.5 U L⁻¹, with a test time of only 3 minutes and excellent selectivity to α -amylase compared to other proteins (Shi et al, 2018).

CHAPTER 2

LITERATURE SURVEY

Amylase was first explained by Kircchoff (1811). For more than 150 years, researchers have been fascinated by the study of amylase. When Kirchhoff discovered the digestion of starch by wheat extracts in 1811, and Dubrunfaut discovered a similar activity in barley malt extract in 1830, it was the first enzymic action ever recorded. In Payen and Persoz's subsequent experiments in 1833, this extract was dubbed "diastase," a name that stuck until it was displaced by "amylase" in the early twentieth century. Leuchs described the saliva enzyme in 1831, then Magendie discovered it in serum in 1846, and Cohnheim discovered it in urine in 1863. Wohlgemuth's amyloclastic assay for determining diastase in urine led to the introduction of amylase measurement as a clinical tool. Kjeldahl described a saccharogenic assay in 1880, but it was Wohlgemuth's amyloclastic assay for determining diastase in urine that led to the introduction of amylase measurement as a clinical tool. Amylase was used in the early studies of classical enzyme kinetics by Henri in 1902 and Michaelis and Pechstein in 1914 due to its relative simplicity of purification and testing. Elman's report of the link between raised amylase levels and severe pancreatitis⁸ in 1929 sparked increased interest in amylase testing and clinical utility. Somogyi's 1938 description⁹ of two assays, one saccharogenic and the other amyloclastic, had a long-term impact on clinical biochemistry. His amyloclastic method, as well as subsequent modifications, became the most widely used amylase assay in clinical chemistry. Despite the availability of newer and better assays and unit definitions, several studies still report amylase activity in Somogyi units (Jack and David, 2009).

In a recent study done by Rishi RajatAdhikary and RintiBanerjee, 2021, the stimulus-responsive core-shell nanoparticles were developed that can be used as affordable and accessible biosensing elements for the point-of-care testing for diagnostic and forensic purposes in paper-based and swab-based formats for detection of salivary alpha amylase (sAA). Further investigations would be needed to validate the use of these technologies in human samples. It can be concluded from the present study that such novel nanoparticles can be developed towards biosensing analytes like enzymes through color-switching behavior to enable simple, instrument-free visual readout in detecting stress, autonomic dysfunction and latent saliva for multiple applications.

Another study highlighted the sensitivity of reactive AUC sAA over reactive AUC cortisol as an effective marker of the two stress systems, in an at risk sample. Moreover, this research suggests that inclusion of information about the coordination between HPA and SNS activity may be particularly important for biosocial models of risk and resilience in developmental psychology. The

results provide encouragement to continue exploring stress as a multivariate construct and allow to continue testing the complementary relationship between the two stress systems. Anand et al., 2018 showed that the findings indicated the extraction of chitosan from shrimp shells and CS NPs synthesized with an aid of TPP as a reducing agent. The percentage of mortality was significantly correlated with the CS NPs exposed to *A. aegypti* ($R^2 = 0.939$). These results may be extrapolated to comment that CS NPs had greater larvicidal effect than raw chitosan samples and could serve selectively as a potential larvicide. As the exoskeleton of shrimp shells waste dumped along the sea shore gets decomposed and poses a great environmental threat, it is established from this study that these shell waste materials could be potentially employed for biomedical applications.

Alpha-amylases are digestive enzymes that break down starch into glucose, maltose, maltotriose, and dextrin by hydrolyzing glycosidic linkages. They are used in a wide range of industries, including food, textiles, paper, and detergents, and account for roughly 30% of global enzyme output. The range of amylase applications has broadened as new horizons in biotechnology have opened up, including clinical, pharmaceutical, and analytical chemistry. To increase the production of amylases, traditional strain enhancement methods such as mutation and selection, as well as recombination, can be used. Microorganisms manufacture amylases. The study examines the microbiological origins of this enzyme as well as its use in industry and biotechnology. Alpha-amylase has been used in starch-based businesses for decades, and while there are several microbiological sources for effective production of this enzyme, only a few strains of fungi and bacteria match the standards for commercial production. The search for new microbes that can produce amylase is a never-ending activity. Many authors have lately shown promising results in developing Alpha-amylase purification procedures, paving the way for applications in the pharmaceutical and healthcare industries that demand high purity amylases. Amylases are among the most significant enzymes utilised in industry, and they are now being considered interesting for biopharmaceutical applications because to advances in biotechnology. In medicinal and therapeutic chemistry, they are useful instruments. The use of low-value agro-industrial residues as enzyme substrates should be prioritised, since this will lower the cost of manufacturing while also addressing disposal and pollution issues. Because thermostability is a desirable property of amylases for industrial usage, efforts should be undertaken to create enzymes from thermophilic and extremely thermophilic bacteria. Amylases are expected to continue to give new prospects in biotechnology as biocatalysts, as well as new uses in the biopharmaceutical industry (Mobini et al., 2012).

Amylases are one of the most commonly employed enzymes in business. Enzymes like these hydrolyze starch molecules into polymers made up of glucose units. Amylases have the potential to be used in a variety of commercial activities, including food, fermentation, and pharmaceuticals. Plants, animals, and microbes all produce alpha-amylases. In the industrial sector, however, enzymes derived from fungi and bacteria have dominated. Alpha-amylase production is required for

the conversion of starches to oligosaccharides. Starch is a key component of the human diet and a major storage product in a variety of commercially important crops, including wheat, rice, maize, tapioca, and potato. Maltodextrin, modified starches, and glucose and fructose syrups are all made with starch converting enzymes. A significant range of microbial alpha-amylases are used in a variety of industries, including food, textiles, paper, and detergents. Submerged fermentation has traditionally been used to produce alpha-amylases, although solid state fermentation systems look to be a promising approach. The thermostability, pH profile, pH stability, and Ca-independency of each alpha-amylase are critical in the development of the fermentation process. The use of alpha-amylase in starch-based businesses has been widespread for decades, and while there are a variety of microbiological sources for effective synthesis of this enzyme, only a few selected fungi and bacteria strains match the standards for commercial production. The search for new microorganisms that can produce amylase is a never-ending process. Many authors have lately shown promising results in developing alpha-amylase purification procedures, which can be used in pharmaceutical and clinical applications that demand high purity amylases (Souza et al., 2010).

Alpha-amylases are most commonly found in the starch industry, where they are employed for starch hydrolysis in the starch liquefaction process, which turns starch into fructose and glucose syrups. Gelatinization, which involves the disintegration of starch granules and the formation of a viscous suspension; liquefaction, which involves partial hydrolysis and the loss of viscosity; and saccharification, which involves the synthesis of glucose and maltose by additional hydrolysis. Initially, *Bacillus amyloliquefaciens* amylase was utilised, but it has now been replaced with *Bacillus stearothermophilus* or *Bacillus licheniformis* -amylase. Because of their outstanding thermostability and the availability of effective expression techniques for these enzymes, *Bacillus* species enzymes are of particular relevance for large-scale biotechnological activities (Gupta et al., 2003, Prakash and Jaiswal, 2009).

Enzymes are mostly consumed by the detergent industry, both in terms of volume and value. Enzymes are used in detergent formulations to improve the detergent's ability to remove tough stains while also making it environmentally friendly. Amylases are the second type of enzyme employed in enzymatic detergent formulation, and they are found in 90% of all liquid detergents. These enzymes are used in laundry detergents and automatic dishwashing machines to break down starchy food residues such as potatoes, gravies, custard, chocolate, and other oligosaccharides into dextrans and other smaller oligosaccharides. Amylases have activity at lower temperatures and alkaline pH, allowing them to maintain the requisite stability in detergents. Amylases' oxidative stability is one of the most critical criteria for their usage in detergents with a highly oxidising washing environment. Because starch is an attractant for many forms of particle soils, removing starch from surfaces is also significant for delivering a whitening benefit. *Bacillus* or *Aspergillus* amylases are examples of amylases utilised in the detergent business (Mitidieri et al., 2006, Mukerjee et al.,

2009).

The most widely used liquid biofuel is ethanol. Starch is the most commonly utilised substrate for ethanol synthesis due to its inexpensive cost and availability in most parts of the world. In order to obtain fermentable sugars, starch must first be solubilized and then subjected to two enzymatic processes. Liquefaction and saccharification, in which starch is converted to sugar using an amylolytic microorganism or enzymes such as alpha-amylase, are followed by fermentation, in which sugar is converted to ethanol using an ethanol fermenting microorganism such as yeast *Saccharomyces cerevisiae* and fermentation, in which sugar is converted to ethanol using an ethanol fermenting microorganism such as yeast *Saccharomyces cerevisiae* (Sanchez and Cardona, 2008). Brazil's economy relies heavily on the manufacturing of ethanol through yeast fermentation. Protoplast fusion between the amylolytic yeast *Saccharomyces fibuligera* and *S. cerevisiae* was used to create a new yeast strain that can directly make ethanol from starch without the requirement for a second saccharifying step. During the first step of starch hydrolysis, bacteria employ alpha-amylase derived from thermoresistant bacteria like *Bacillus licheniformis* or modified strains of *Escherichia coli* or *Bacillus subtilis* (Öner, 2006).

Amylases are widely used in the processed food business, including baking, brewing, making digestive aids, making cakes, fruit juices, and starch syrups. In the baking sector, Alpha-amylases are commonly used (Conto et al., 2006). These enzymes can be used into bread dough to break down the starch in the flour into smaller dextrins, which the yeast then ferments. The addition of alpha-amylase to the dough improves the volume and texture of the finished product by increasing the rate of fermentation and lowering the viscosity of the dough (Gupta et al., 2003).

Furthermore, it produces additional sugar in the dough, which improves the bread's flavour, crust colour, and toasting properties. In addition to creating fermentable chemicals, alpha-amylases have an anti-staling impact in bread baking and increase the softness retention of baked goods, extending their shelf life. In the baking business, a thermostable maltogenic amylase from *Bacillus stearothermophilus* is currently employed commercially (Ahlawat et al., 2009). Amylases are also used to clarify beer and fruit juices, as well as to improve the digestibility of fibre in animal feed by pretreatment (Fitkenhauer, 2003)

Amylases are employed in the desizing process in the textile industry. To ensure a rapid and secure weaving process, sizing agents such as starch are applied to yarn prior to fabric manufacture. Starch is an appealing size since it is inexpensive, widely available in most parts of the world, and readily removed (Bruinenberg et al., 1996). In the textile finishing industry, starch is later removed from the woven cloth using a wet-process. Desizing is the process of removing starch from a fabric, which acts as a strengthening agent to prevent the warp thread from breaking during the weaving process (Saxena et al., 2004). The alpha-amylases remove the size in a selective manner and do not harm the fibres. Amylase derived from *Bacillus stain* has long been used in the textile industry (Van der

Maarel et al., 2002).

In the pulp and paper business, Alpha-amylases are used to modify the starch of coated paper, resulting in low-viscosity, high molecular weight starch. The coating procedure improves the writing quality of the paper by making the surface sufficiently smooth and robust. The native starch's viscosity is too high for paper sizing in this application, but this can be changed by partially decomposing the polymer with alpha-amylases in batch or continuous operations. In addition to being an excellent coating for the paper, starch is a good sizing agent for the finishing of paper, boosting the quality and erasability. Paper stiffness and strength are improved as a result of the size. includes Amizyme® (PMP Fermentation Products, Peoria, USA), Termamyl®, Fungamyl, BAN® (Novozymes, Denmark) and -amylase G9995® (Enzyme Biosystems, USA).

CHAPTER 3

AIM AND SCOPE

3.1. AIM

To develop a smart core-shell nanoparticle based sensor for diagnostics and forensics

3.2. SCOPE

- The main scope of the study is to presents a proof-of-concept technology that demonstrates the applicability of smart nanoparticles in inexpensive, adaptable biosensing platforms such as paper-based and swab-based formats for applications as diverse as stress diagnostics and forensics.

3.3 PLAN OF WORK

- Collection of materials
- Preparation and Characterization of core-shell nanoparticles
- Spectroscopic analysis of nanoparticles in a tube-based format
- Application of the nanoparticles on a paper-based format
- Development of the paper-based device
- Application of α -amylase detection for the detection of saliva

CHAPTER 4

MATERIALS AND METHODS

4.1. MATERIALS REQUIRED

Low-molecular-weight chitosan with a molecular weight of 7000 Da was used. Soluble starch, potassium iodide, iodine, glacial acetic acid, sodium hydroxide, sodium tripolyphosphate (TPP) were used from Center for drug development and discovery, Sathyabama Institute of Science and Technology. Food grade dye was obtained from essence shop (Chennai). Whatman qualitative chromatography paper grade 1 was purchased from Whatman, India. High purity distilled water, was used in all experiments. All the tissue culture plates and flasks were used from Center for drug development and discovery, Sathyabama Institute of Science and Technology.

4.2. PREPARATION AND CHARACTERIZATION OF CORE-SHELL NANOPARTICLES

Chitosan solution (1 mg/ml) was prepared in 1% acetic acid and a hydrophilic red colored food-grade dye was added to the chitosan solution. Sodium tripolyphosphate (TPP) solution (1 mg/ml) was prepared separately in deionized water. Ionic gelation caused particle formation spontaneously when 10 ml of TPP solution was added dropwise into 30 ml chitosan solution under high shear homogenization at room temperature for 15 minutes at 910 rpm. The pellet of chitosan-TPP nanoparticles were separated by centrifugation at 5000 rpm for 10 minutes. Soluble starch solution (5 mg/ml in 1 N NaOH) was mixed with Lugol's Iodine in a volume ratio of 5:1 and this mixture was used to resuspend the pellet. The nanoparticle thus formed was vortexed and then sonicated for 2 minutes using a probe sonicator. The coating of the core was expected to be achieved by electrostatic binding of the positively charged chitosan nanoparticles (red suspension) with the negatively charged starch-iodine complex (blue suspension). The core-shell nanoparticles so formed were again centrifuged at 6000 rpm for 10 minutes, and then the pellet was dispersed in deionized water (2 ml), sonicated for 2 minutes and then stored at 4°C under refrigeration.

4.3 Characterization of core shell nanoparticles

4.3.1 Characterization using Scanning Electron Microscope

The core shell nano particles were characterize using scanning electron microscope. A scanning electron microscope (SEM) is a type of electron microscope that produces images of a sample by scanning the surface with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that contain information about the surface topography and composition of the sample. The electron beam is scanned in a raster scan pattern, and the position

of the beam is combined with the intensity of the detected signal to produce an image. In the most common SEM mode, secondary electrons emitted by atoms excited by the electron beam are detected using a secondary electron detector. The number of secondary electrons that can be detected, and thus the signal intensity, depends, among other things, on specimen topography. Some SEMs can achieve resolutions better than 1 nanometer.

4.3.2 Characterization using FTIR

The core shell nano particles were subjected to Fourier transform infrared. FTIR stands for Fourier transform infrared, the preferred method of infrared spectroscopy. When IR radiation is passed through a sample, some radiation is absorbed by the sample and some passes through (is transmitted). The resulting signal at the detector is a spectrum representing a molecular 'fingerprint' of the sample. The usefulness of infrared spectroscopy arises because different chemical structures (molecules) produce different spectral fingerprints.

4.3.3. Characterization using Zeta potential

Zeta Potential was performed to find out the particle size of the nano particles. Zeta potential is a physical property which is exhibited by any particle in suspension, macromolecule or material surface. It can be used to optimize the formulations of suspensions, emulsions and protein solutions, predict interactions with surfaces, and optimise the formation of films and coatings.

4.3.4. Characterization using UV-vis spectroscopy

The core shell nano particles were analyzed using UV-vis spectroscopy. UV spectroscopy or UV-visible spectrophotometry (UV-Vis or UV/Vis) refers to absorption spectroscopy or reflectance spectroscopy in part of the ultraviolet and the full, adjacent visible regions of the electromagnetic spectrum. This means it uses light in the visible and adjacent ranges. The absorption or reflectance in the visible range directly affects the perceived color of the chemicals involved. In this region of the spectrum, atoms and molecules undergo electronic transitions. Absorption spectroscopy is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions of electrons from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state.

4.3. SPECTROSCOPIC ANALYSIS OF NANOPARTICLES IN A TUBE BASED FORMAT

Core-shell nanoparticles present in suspension were analyzed for the absorbance by UV-Vis spectroscopy (Center for drug and discovery, Sathyabama Institute of Science and Technology) between 350 and 600 nm. Different dilutions of alpha amylase were prepared in simulated saliva. Freshly prepared alpha-amylase was used as the simulated saliva (Viennot et al., 2006). Besides the red food color present in the core of the core-shell nanoparticles as well as the core-shell

nanoparticles were also analyzed.

4.4. APPLICATION OF THE NANOPARTICLES ON A PAPER-BASED FORMAT

In order to develop an affordable point-of-care device, the nanoparticles were applied on the commonly used Whatman Qualitative Grade 1 filter paper and the same was assessed using various concentrations of amylase on paper (Yetisen et al., 2013). In brief, 5 μ l of the nanoparticle suspension was placed on the filter paper and allowed to dry at room temperature. This was followed by addition on 5 μ l of the sample. The color changes were observed after 15-20 minutes. The optimized time for the observation of results was found to be 5 minutes.

4.5. DEVELOPMENT OF THE PAPER BASED DEVICE

A paper-based device was developed to allow inexpensive and easy detection of salivary alpha amylase. The core-shell nanoparticles were placed on a Whatman Qualitative Grade 1 filter paper and was suitably sealed for the prevention of loss of iodine and subsequent loss of color. In both these cases, the samples were not in the area of interest as obtained from the sample set.

4.6 APPLICATION OF α -AMYLASE FOR THE DETECTION OF SALIVA

In order to modify the application, the core-shell nanoparticles were developed with 1 mg/ml soluble starch for the shell. 1 g of nanoparticles was suspended in a dilutions of 0.5, 1, 1.5 and 2 g of amylase in 10 ml of deionized water. The suspension therein was in used for the testing of saliva on skin surface and cloth. For the purpose of detection of saliva skin surface.

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1 Making of nanoparticles, tube based format and Physicochemical characterization of core-shell nanoparticles

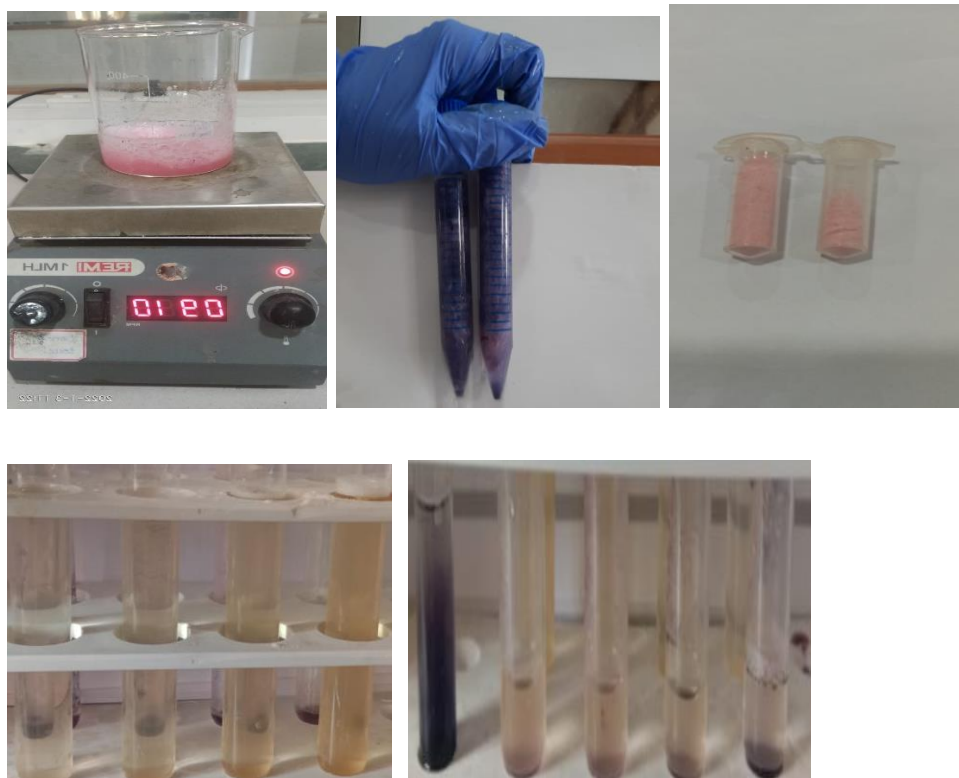


Fig.1. The preparation of core shell nano particles and tube-based adsorption

The nanoparticles were obtained and lyophilized. The alpha amylase was varied in different concentrations from 0.5, 1, 1.5, 2 mg in 10ml of water. The core shell nanoparticles were added in concentration of 0.1 mg. Starch-iodine was added in concentration of 0.1 mg in 10 ml of water. The tubes were let undisturbed for 1 hour. Colour change is observed and the adsorption increases with increase in concentration of alpha amylase. The dilutions are taken for spectroscopic analysis.

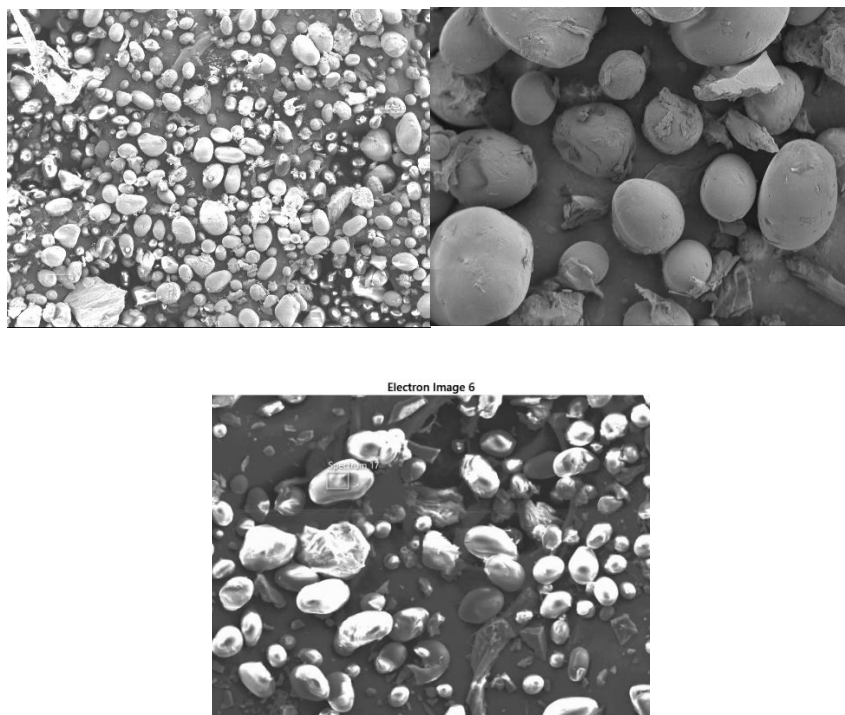


Fig.2. SEM and Zeta potential showing core shell nano particles

The nanoparticles obtained, consisting of a core shell, were in the spherical shape and size range of 90 μm as seen through electron microscope. Core-shell nanoparticles were imparted unevenness to the surface to the surface. This could be due to addition of alpha-amylase. In a similar study the size range of nanoparticles were found to be of 200-250 nm. The core shell prepared as chitosan-tripolyphosphate nanoparticles by ionic gelation were seen as unevenness to the surface (Rishi and Rinti, 2021). By scanning electron microscopy, olfactory cells and sustentacular cells can be seen lining the luminal surface of the nasal cavity. About 10 to 20 $\times 10^6$ olfactory cells comprise the olfactory epithelium; they are interspersed with more numerous sustentacular cells. A lipid-rich layer of mucus, about 60 μm thick, which is produced by *glands of Bowman*, normally bathes the epithelial surface. Besides protecting the epithelium from desiccation and turbulent airflow, the mucus also aids in transporting lipid-soluble odorant molecules to the olfactory receptors *viachaperone (odorant-binding proteins)* (William, 2021).

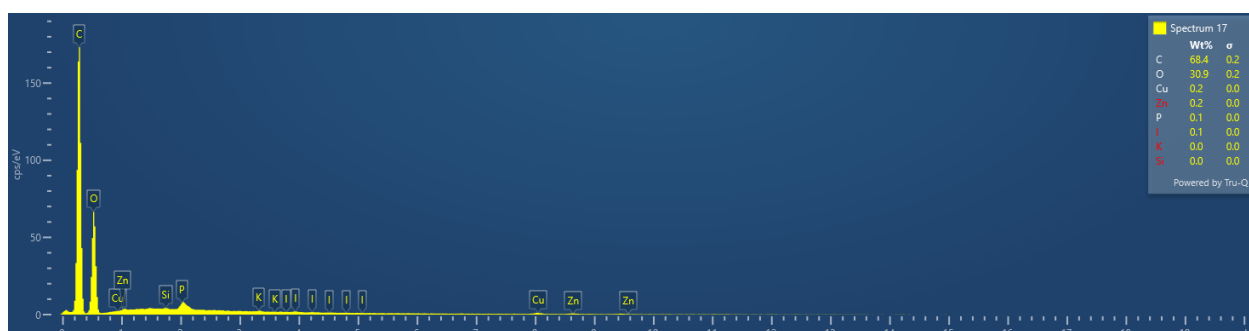


Fig.3. FTIR analysis of the core shell nanoparticles

FTIR was performed on the lyophilized sample. The characteristic peak of C stretch was seen at 170 cm^{-1} . The Raman out-of-plane bending modes are found at 194 and 170 cm^{-1} . Out of plane bending moments are those which are caused by out of plane forces (Frost et al., 2002). The forces might be caused because of the carrying out of the lyophilization process. Fourier transform ir (FTIR) spectra of 21 globular proteins was obtained at 2 cm^{-1} resolution from 1600 to 1700 cm^{-1} in deuterium oxide solution. Fourier self-deconvolution was applied to all spectra, revealing that the amide I band of each protein except casein consists of six to nine components. The components were observed at 11 well-defined frequencies, although all proteins do not exhibit components at every characteristic frequency. The root mean square (RMS) deviation of 124 individual values from the 11 average characteristic frequencies is 1.9 cm^{-1} (Byler, 1986).

5.2 Spectroscopic analysis of nanoparticles in tube-based format

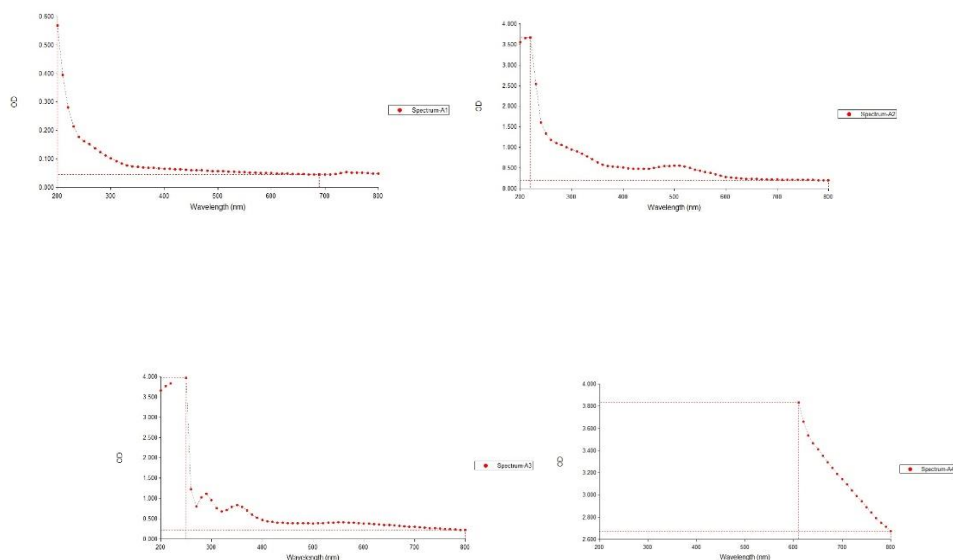


Fig.4 Uv-vis analysis of Blank solution, Chitosan-TPP nanoparticles, Starch Iodine complex and core-shell nanoparticles

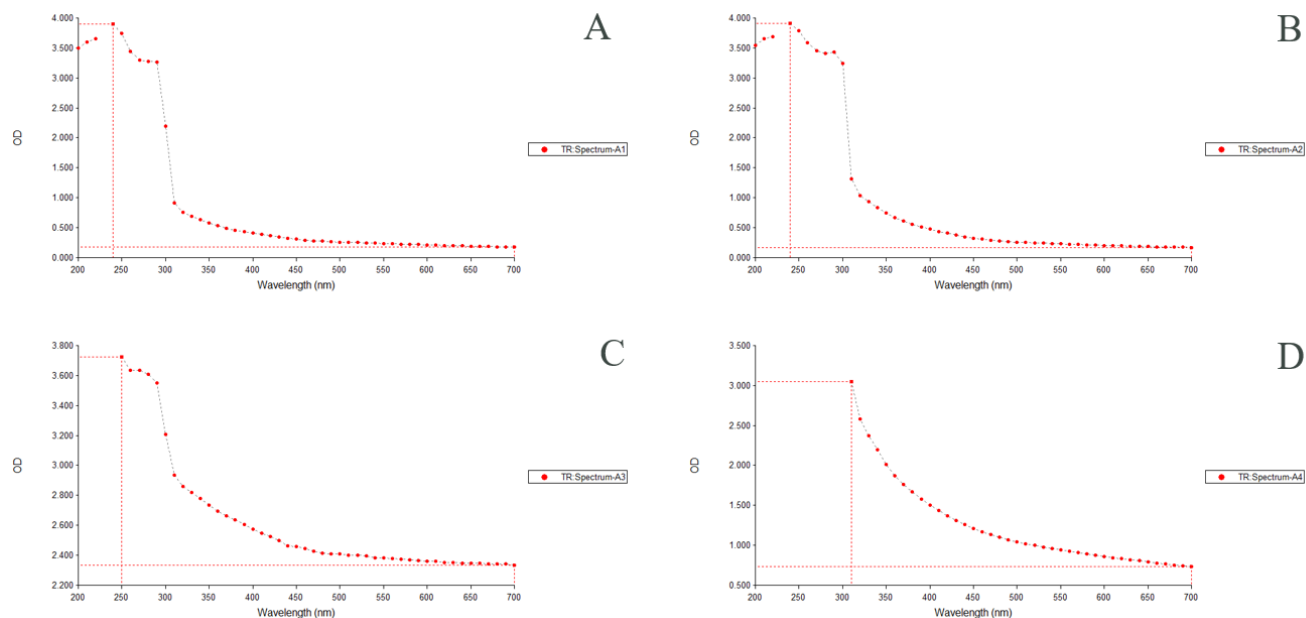


Fig.5 Spectroscopic analysis of tube based format

The UV-vis analysis were taken from 200-700 nm. The above UV-vis analysis shows results similar to the results (Rishi and Rinti, 2021), where the distinct peaks present at 375 nm and 549 nm for the core-shell nanoparticles while the red food dye displayed a peak at 507 nm. The 375 nm peak was due to the shell of the nanoparticles containing Lugol's iodine which shows an absorption peak at 355 nm due to triiodide ions. The interaction with other components of the nanoparticles led to a red shift of the peak to 375 nm as has been described for other polymers as well. The addition of amylase led to an increase in the amount of free triiodide resulting in an increase in absorbance peak at 375 nm. The corresponding release of red dye from the core showed an increase in absorbance at 507 nm. Thus, on addition of amylase, the nanoparticles obtained had characteristic absorbance peaks at 375 nm and 507 nm. The transition of blue to red on exposure to salivary alpha amylase was thereby confirmed through spectroscopic analysis. As explained previously, the iodine bound shell of starch in the core-shell nanoparticle leads to the formation of triiodide anion complexes at the helices of starch producing an intense blue/purple color. This color disappears with the degradation of starch by sAA. This change in color can be further observed by the naked eye or through image analysis. Thus, the addition of amylase led to a gradual reduction in the blue color and increase in the red color.

The above UV-vis analysis shows clearly that the ab value increases with increase in the amylase concentration.

5.3 Analysis of the alpha amylase on paper-based format

In order to develop an affordable point-of-care device from the tube-based assays, Whatman filter

paper qualitative Grade 1 was used as has been used by major research organizations across the world. It is affordable, accessible, easy to wick and has the property of biocompatibility with most biological samples (Yetisen et al., 2013). The use of paper-based diagnostics is gaining much impetus and is being used for a number of healthcare applications in point-of-care testing (Yetisen et al., 2013). Filter papers have also been used for the development of photoresistor-based amylase sensors that show changes in electrical resistance in response to enzymes (Dutta et al., 2016). Besides, the use of starch-iodide papers, starch-iodate papers and potassium iodate-starch papers prepared on filter papers have a long history as indicator papers (Troy et al., 2006). It has been previously shown that sAA present in concentrations above 150 units/ml as diagnostic of stress (Nater et al., 2005). It was observed that the test was able to provide a diagnostic cut-off of 140 units/ml for sAA where the blue to red transition was evident to the naked eye. While the details of sample collection are beyond the scope of the present study.

5.4 Development of the paper-based device

The device was suitably sealed with coating and blocking membranes to prevent the loss of iodine over time. The coating was achieved with a scotch tape while the backing membrane had a 200 GSM (grams per square meter) glossy paper. 1 cm × 6.5 cm Whatman filter paper 1 was used with 5 µl of nanoparticle suspension air-dried at the center and the sticker paper was attached to the same. The sticker paper with the filter paper were placed on a blotting paper for the absorption of extra saliva. The arrangement was then placed within the seal of the scotch tape and the backing membrane to reduce the loss of iodine as mentioned previously.

Paper-based devices have been developed since the turn of the 21st century and are becoming increasingly relevant, affordable, accessible diagnostic tools, especially in resource-limited settings. Recent examples of the use of starch-iodine chemistry towards the detection of alpha amylase in the serum have been developed but require additional equipment towards the quantification of the enzyme (Dutta et al., 2016; Mandal et al., 2019). Development of a mobile application serves the purpose of an objective method of quantification without using such expensive, complex technologies or desktop scanners or cameras (Shen et al., 2012). Hence a mobile application was developed for the quantification of the results displayed by the device. Further advantages of using the mobile application is the increased use of smart phones even in developing countries and the use of such a platform technology for use in other healthcare applications relying on the change in color (Shen et al., 2012). Besides, loading the nanoparticles on paper and suitable sealing of these nanoparticles prior to use can further reduce the leaching and explain the stability of the device. Also, the device was not prone to contamination due to the presence of Lugol's iodine in the shell. Thus, the use of an inexpensive paper-based device has been made possible using a smart

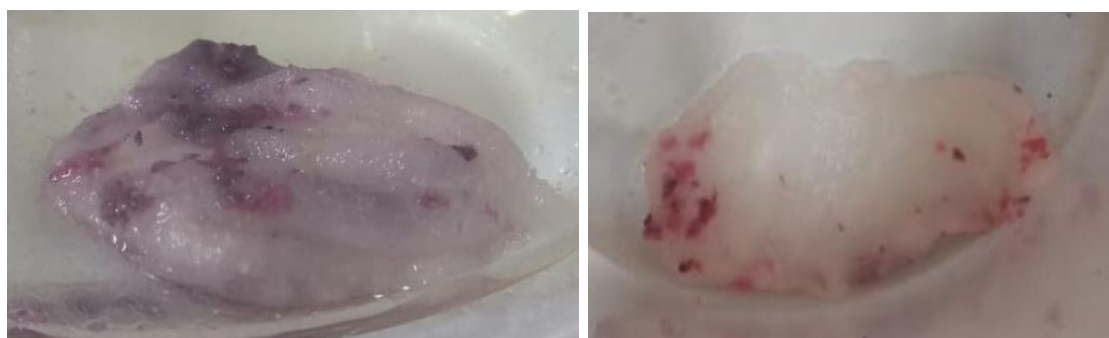
nanoparticle-based biosensor system allowing for affordable, instrument-free detection of salivary alpha amylase.



Fig.6. Paper based device for detection of saliva samples

5.5 Development of the amylase test for forensic purposes

As a modification of the present technology, the platform was developed in the qualitative detection of amylase to aid the detection of saliva for forensic purposes. Since all the compounds used were biocompatible, it was supposed to be easier to obtain DNA from the saliva stains if needed in crime scene investigations. For this, there were two tests developed. The swab-based test for the identification of presence of saliva on skin or a surface. The change in color of the tip was noticeable even after 30 minutes. Also, a suspension was developed that could be deposited on fabric and a positive reaction could be observed with a change in color.



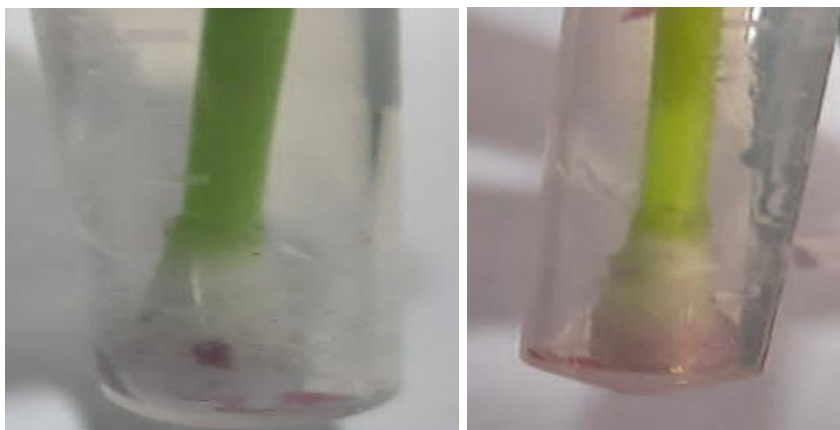


Fig.7 Swab based test

5.6 Advantages and limitations of the smart core-shell nanoparticle sensors

The core-shell nanoparticle-based sensors can reduce the cost of antibodies or labels (e.g. gold, silver, fluorophore, enzymes) traditionally used in point-of-care devices. Besides, the facile method of preparation using homogenization and mixing, allows the process to be scaled up easily. These nanoparticles involve the use of biocompatible compounds like chitosan, TPP and starch that can be used with various body fluids like saliva which can facilitate sample preservation to obtain DNA for critical forensic applications. Also, the nanoparticles are robust, stable and can be modulated with different concentrations of starch in the shell to tune the limit of detection in various substrate materials like paper or swabs. These nanoparticles are shown to be sensitive with a limit of detection (LoD) of 140 units/ml (70 mg/ml) in the paper-based format and 2.5 units/ml (1.25 mg/ml) in the coated swab format as well as specific showing reproducible results in the presence of various interfering substances that may be present in the saliva. This also allows for the development of sAA as a biomarker through non-invasive methods of collection using protocols like the passive drool technique or using swabs followed by suitable pretreatment for the detection of amylase in various samples. The devices developed herein allowed quick reading of the results in the first 5–30 minutes through either a visual readout by a distinct change in color from blue to red in the presence of amylase or through objective methods by a mobile application allowing analysis of various color spaces which precludes the need for expensive instruments like desktop scanners or cameras in diagnostics. The present study also has certain limitations and scope for further investigations. While this technology is tested for interference and has been found to be specific for amylase in the saliva, further sample preparation and testing may be needed for samples like serum. In case of salivary detection, specific instructions of rinsing the mouth and taking the test at least 10 minutes after a meal, will prevent interference due to residual starch in the oral cavity. This is a proof-of-concept qualitative test that can help identify the presence of certain conditions based on a tunable cut-off dependent on the amount of substrate in the shell of the nanoparticles.

CHAPTER 6

SUMMARY AND CONCLUSION

6.1 SUMMARY

In this study, stimulus-responsive core-shell nanoparticles for salivary alpha amylase detection were designed as economical and accessible biosensing elements for point-of-care testing for diagnostic and forensic reasons in paper-based and swab-based formats (sAA). Validation of the usage of these technologies in human samples would require more research. The current study concludes that unique nanoparticles with color-switching behaviour can be produced for biosensing analytes such enzymes, allowing for a simple, instrument-free visual readout in detecting stress, autonomic dysfunction, and latent saliva for a variety of applications. The nanoparticles were prepared using chitosan and TPP. They were homogenized and the core shell nano particle was formed. It was achieved by electrostatic binding. The starch iodine complex was added and the mixture was sonicated. The core-shell nanoparticles were analyzed using UV-vis for absorbance, FTIR, SEM and Zeta potential. The results of the same were interpreted. The nanoparticles were applied to a paper-based format. The paper-based device was developed. The absorbance of starch iodine complex was studied using the tube based format. The absorbance value was interpreted using UV-vis spectroscopy. The alpha amylase was applied for the detection. The results were interpreted.

6.2 CONCLUSION

The present study, stimulus-responsive core-shell nanoparticles were developed that can be used as affordable and accessible biosensing elements for the point-of-care testing for diagnostic and forensic purposes in paper-based and swab-based formats for detection of salivary alpha amylase (sAA). Further investigations would be needed to validate the use of these technologies in human samples. It can be concluded from the present study that such novel nanoparticles can be developed towards biosensing analytes like enzymes through color-switching behavior to enable simple, instrument-free visual readout in detecting stress, autonomic dysfunction and latent saliva for multiple applications.

7. REFERENCES

1. Della Ventura, Bartolomeo, Nikola Sakač, Riccardo Funari, and Raffaele Velotta. "Flexible immunosensor for the detection of salivary α -amylase in body fluids." *Talanta* 174 (2017): 52-58.
2. Shi, Jie, Qianchun Deng, Ya Li, Mingming Zheng, Zhaofei Chai, Chuyun Wan, Zhe Zheng, Lu Li, Fenghong Huang, and Bo Tang. "A rapid and ultrasensitive tetraphenylethylene-based probe with aggregation-induced emission for direct detection of α -amylase in human body fluids." *Analytical chemistry* 90, no. 22 (2018): 13775-13782.
3. Bernfeld, P., Enzymes of starch degradation and synthesis. in Adirrrices iri €/r:wro/oKy. Vol. 12. Nord. F. F., Ed.. Interscience. New York. 1951. 379.
4. Merritt, A. D. and Karn, R. C., The human α -amylases. in Ac/rctric.e.s iri Hirrncirr Gerrcricx. Vol. 8. Harris. H. and Hirshhom. K.. Eds.. Plenum Press. New York. 1977. 135.
5. Florkin, M., A history of biochemistry. in Coirpreherisiw Biodtemisrn. Vol. 30. Florkin. M. and Stotr. E. H., Eds.. Elsevier North Holland. Amsterdam. 1972. 265.
6. Skude, G., On human amylase iscxnzymes. Sctrrrtl. J. Gtrsroc~rirc~rc~1.. 1344). I. 1977.
7. Wohlgemuth, J., Uber eine neue Methode zur quantitaven Bestimmung des diastatischen Ferments. Bioclicwi. 2.. 9. I. 1908.
8. Henri, V., Theorie generale de l'action de quelques diastases. C. R. Actrccl. 5ci. Puris. 135. 916. 1902.
9. Michaelis, L. and Pechstein, H.. Die Wirkungsbedingungen der Speicheldiastase. Birdicwr. Z.. 59. 77. 1914.
10. Elman, R., Arneson, N., and Graham, E. A., Value of blood amylase estimations in the diagnosis of . pancreatic disease: a clinical study. Arch. SirrK., 19. 943. 1929.
11. Somogyi, M., Micromethods for the estimation of diastase. J. Bid. Clicw~.. 125. 399. 1938.
12. McGeachin, R. L. and Lewis, J. P., Electrophoretic behavior of serum amylase. J. Biol. C/ierrr.: 234. 795. 1959.
13. Dreiling, D. A., Janowitz, H. D., and Josephberg, L. J., Serum iso-amylases: an electrophoretic study of the blood amylase and the patterns observed in pancreatic disease. Am. Irirerti. Mecl.. 58. 235. 1963.
14. Wilding, P., The electrophoretic nature of human amylase and the effect of protein on the starch-iodine reaction. Clin. Chiin. Acr cr, 12. 97. 1965.
15. Searcy, R. L., Ujihira, I., Hayashi, S., and Berk, J. E., An intrinsic disparity between amyloclastic and saccharogenic estimations of human serum isoamylase activities. Cliri. Chirn. Acw. 9. 505.

1964.

16. Searcy, R. L., Hayashi, S., Hardy, E. M., and Berk, J. E., The interaction of human serum proteins with the starch-iodine complex. *Clin. Chim. Acta*. 12. 631. 1965.
17. Norby, S., Electrophoretic non-identity of human salivary and pancreatic amylases. *Exp. Cell Res.*, 36. 663. 1964.
18. Muus, J. and Vnenchak, J. M., Isotypes of salivary amylase. *Nature (London)*, 204. 283. 1964.
19. Berk, J. E., Hayashi, S., Searcy, R. L., and Hightower, N. C., Differentiation of parotid and pancreatic amylase in human serum. *Am. J. Dig. Dis.* 11. 695. 1966.
20. Kamaryt, J. and Laxova, R., Amylase heterogeneity: some genetic and clinical aspects, *HitrtrunKeic,rik*. 1. 579. 1965.
21. Aw, S. E., Separation of urinary isoamylases on cellulose acetate, *Ncrritre (Londoi)*. 209. 298. 1966.
22. Bernfeld, P., Amylases. a and p. in *Methods in Enzymology*. Vol. 1. Colowick, S. P. and Kaplan, N. O., Eds., Academic Press, New York. 1955. 149.
23. Fischer, E. H. and Stein, E. A., α -Amylases, in *The Enzymes*. Vol. 4. 2nd ed., Boyer, P. D., Ed., Academic Press, New York. 1960. 313.
24. Thoma, J. A., Spradlin, J. E., and Dygert, S., Plant and animal amylase, in *The Enzymes*, Vol. 5. 3rd ed., Boyer, P. D., Ed., Academic Press, New York, 1970. 115.
25. Bird, R. and Hopkins, R. H., The action of some α -amylases on amylose. *Biochem. J.* 56. 86, 1954.
26. Lalegerie, P., Pourci, M. L., and Bailly, M., Hydrolysis of maltotetraose by human pancreatic α -amylase. with liquid-chromatographic determination of the products. *Clin. Chim. Acta*. 28. 1791. 1982.
27. Kaczmarek, M. J. and Rosenmund, H., The action of human pancreatic and salivary isoamylases on starch and glycogen, *Clin. Chim. Acta*. 79. 69, 1977.
28. Walker, G. J. and Whelan, W. J., The mechanism of carbohydrase action. VI. Stages in the salivary α -amylolysis of amylose, amylopectin. and glycogen, *Biochem. J.*, 76. 257. 1960.
29. Nordin, P. and French, D., I-Phenyl-flavonol derivatives of starch dextrans. *J. Am. Chem. Soc.* 80, 1445. 1958.
30. Roberts, P. J. P. and Whelan, W. J., The mechanism of carbohydrase action. V. Action of human salivary α -amylase on amylopectin and glycogen. *Biochem. J.* 76. 246. 1960.
31. Henry, R. J. and Chiamori, N., Study of the saccharogenic method for the determination of serum and urine amylase, *Clin. Chem.* 6. 434. 1960.
32. Meites, S. and Rogols, S., Serum amylases. isoenzymes. and pancreatitis. 1. Effect of substrate variation, *Clin. Chem.* 14, 1176. 1968.
33. Zakowski, J. J., Gregory, M. R., and Bruns, D. E., Amylase isoenzyme from human serum

- ovarian tumors: purification and characterization, *Clin. Chem.* 30. 62. 1984.
34. Adhikary, R.R., Koppaka, O., Banerjee, R., 2020. *Nanoscale* 12 (16), 8898–8908. Ali, N., Pruessner, J.C., 2012. *Physiol. Behav.* 106 (1), 65–72.
 35. Anand, M., Sathyapriya, P., Maruthupandy, M., Hameedha Beevi, A., 2018. *Frontiers in Laboratory Medicine* 2 (2), 72–78.
 36. Armbruster, D.A., Pry, T., 2008. *Clin. Biochem. Rev.* 29 (Suppl. 1), S49–S52. Suppl 1.
 37. Baldevbhai, P.J., Anand, R.S., 2012.
 38. Brown, N.J., Kimble, R.M., Rodger, S., Ware, R.S., McWhinney, B.C., Ungerer, J.P., Cuttle, L., 2014. *Burns* 40 (5), 887–895.
 39. Chen, S.L., Kapoor, N., 2014. *Method and Apparatus for Performing Color-Based Reaction Testing of Biological Materials*. US.
 40. Chen, W.-Y., Chen, L.-Y., Ou, C.-M., Huang, C.-C., Wei, S.-C., Chang, H.-T., 2013. *Anal. Chem.* 85 (18), 8834–8840.
 41. Chrousos, G.P., 2009. *Nat. Rev. Endocrinol.* 5 (7), 374–381.
 42. Costa, E.M., Silva, S., Veiga, M., Baptista, P., Tavaría, F.K., Pintado, M.E., 2021. *Carbohydr. Polym.* 251, 117120.
 43. Dutta, S., Mandal, N., Bandyopadhyay, D., 2016. *Biosens. Bioelectron.* 78, 447–453.
 44. Fan, W., Yan, W., Xu, Z., Ni, H., 2012. *Colloids Surf. B Biointerfaces* 90, 21–27.
 45. Ferrara, P., Bottaro, G., Angeletti, S., Gatto, A., Vitelli, O., Battaglia, D., Del Re, M., Ruggiero, A., Dicuonzo, G., 2013. *Acta Neurol. Belg.* 113 (3), 279–283.
 46. Fleige, E., Quadir, M.A., Haag, R., 2012. *Adv. Drug Deliv. Rev.* 64 (9), 866–884.
 47. Kalita, D., Kaushik, N., Mahanta, C.L., 2014. *J. Food Sci. Technol.* 51 (10), 2790–2796.
 48. Krieg, P., Lendl, B., Vonach, R., Kellner, R., 1996. *Anal. Bioanal. Chem.* 356 (8), 504–507.
 49. Lathwal, S., Sikes, H.D., 2016. *Lab Chip* 16 (8), 1374–1382.
 50. Lindbloom, B., 2019. *Color Difference Calculator*.
 51. Ludwig, L., Pasma, J.A., Nicholson, T., Aybek, S., David, A.S., Tuck, S., Kanaan, R.A., Roelofs, K., Carson, A., Stone, J., 2018. *The Lancet Psychiatry* 5 (4), 307–320.
 52. Mandal, N., Bhattacharjee, M., Chattopadhyay, A., Bandyopadhyay, D., 2019. *Biosens. Bioelectron.* 124, 75–81.
 53. Momenzadeh, H., Tehrani-Bagha, A.R., Khosravi, A., Gharanjig, K., Holmberg, K., 2011. *Desalination* 271 (1), 225–230.
 54. Moulay, S., 2013. *J. Polym. Eng.* 33 (5), 389–443.
 55. Murphy, E.B., Wudl, F., 2010. *Prog. Polym. Sci.* 35 (1–2), 223–251.
 56. Naorem, H., Devi, S.D., 2013. *Spectrochim. Acta Mol. Biomol. Spectrosc.* 101, 67–73.
 57. Nater, U.M., Rohleder, N., 2009. *Psychoneuroendocrinology* 34 (4), 486–496.
 58. Nater, U.M., Rohleder, N., Gaab, J., Berger, S., Jud, A., Kirschbaum, C., Ehlert, U., 2005. *Int. J.*

- Psychophysiol. 55 (3), 333–342.
59. Pavia, D.L., 2005. Introduction to Organic Laboratory Techniques: A Small Scale Approach. Thomson Brooks/Cole.
 60. Punjabi, K., Adhikary, R.R., Patnaik, A., Bendale, P., Singh, S., Saxena, S., Banerjee, R., 2020. J. Mater. Chem. B 8 (29), 6296–6306.
 61. Rauf, S., Ali, Y., Hussain, S., Ullah, F., Hayat, A., 2018. PloS One 13 (7), e0199978.
 62. Robles, T.F., Sharma, R., Park, K.S., Harrell, L., Yamaguchi, M., Shetty, V., 2012. J. Oral Maxillofac. Surg. 70 (10), 2256–2263.
 63. Sharma, G., Bala, R., 2002. Digital Color Imaging Handbook. CRC press.
 64. Shen, L., Hagen, J.A., Papautsky, I., 2012. Lab Chip 12 (21), 4240–4243.
 65. Tokunaga, N., Fukai, K., 2016. Jpn. J. Nurs. Sci.
 66. Troy, D.B., Remington, J.P., Beringer, P., 2006. Remington: the Science and Practice of Pharmacy. Lippincott Williams & Wilkins.
 67. Verma, R., Adhikary, R.R., Banerjee, R., 2016. Lab Chip 16 (11), 1978–1992.
 68. Viennot, S., Lissac, M., Malquarti, G., Dalard, F., Grosgeat, B., 2006. Acta Biomater. 2 (3), 321–330.
 69. Wang, H., Udukala, D.N., Samarakoon, T.N., Basel, M.T., Kalita, M., Abayaweera, G., Manawadu, H., Malalasekera, A., Robinson, C., Villanueva, D., Maynez, P., Bossmann, L., Riedy, E., Barriga, J., Wang, N., Li, P., Higgins, D.A., Zhu, G., Troyer, D.L., Bossmann, S.H., 2014. Photochem. Photobiol. Sci. 13 (2), 231–240.
 70. Wittstein, I.S., Thiemann, D.R., Lima, J.A.C., Baughman, K.L., Schulman, S.P., Gerstenblith, G., Wu, K.C., Rade, J.J., Bivalacqua, T.J., Champion, H.C., 2005. N. Engl. J. Med. 352 (6), 539–548.
 71. Wyatt, J.C., 2018. BMC Med. 16 (1), 225.
 72. Yang, R., Cheng, W., Chen, X., Qian, Q., Zhang, Q., Pan, Y., Duan, P., Miao, P., 2018. ACS Omega 3 (9), 12141–12146.
 73. Yetisen, A.K., Akram, M.S., Lowe, C.R., 2013. Lab Chip 13 (12), 2210–2251.
 74. Zhou, Y., Yang, L., Huang, Y., 2013. Micro- and Macromechanical Properties of Materials. CRC Press.
 75. Zakowski, Jack J., and David E. Bruns. "Biochemistry of human alpha amylase isoenzymes." *CRC Critical reviews in clinical laboratory sciences* 21, no. 4 (1985): 283-322.
 76. Mobini-Dehkordi, Mohsen, and Fahime Afzal Javan. "Application of alpha-amylase in biotechnology." *J. Biol. Today World* 1, no. 1 (2012): 39-50.
 77. Souza, Paula Monteiro de. "Application of microbial α -amylase in industry-A review." *Brazilian journal of microbiology* 41, no. 4 (2010): 850-861.
 78. Gupta, R.; Gigras, P.; Mohapatra, H.; Goswami, V.K.; Chauhan, B. (2003). Microbial -amylases:

- a biotechnological perspective. *Process Biochem* 38, 1599 – 1616.
79. Prakash, O.; Jaiswal, N. (2009). α -Amylase: An Ideal Representative of Thermostable Enzymes. *Appl Biochem Biotechnol*.
 80. Mitidieri, S.; Souza Martinelli, A.H.; Schrank, A.; Vainstein, M.H. (2006). Enzymatic detergent formulation containing amylase from *Aspergillus niger*: a comparative study with commercial detergent formulations. *Bioresour Technol* 97, 1217-1224.
 81. Mukherjee, A.K.; Borah, M.; Raí, S.K. (2009). To study the influence of different components of fermentable substrates on induction of extracellular α -amylase synthesis by *Bacillus subtilis* DM-03 in solidstate fermentation and exploration of feasibility for inclusion of α -amylase in laundry detergent formulations. *Biochem. Eng. J.* 43, 149– 156. 55.
 82. Öner, E.T. (2006). Optimization of ethanol production from starch by an amylolytic nuclear petite *Saccharomyces cerevisiae* strain. *Yeast* 23, 849–856.
 83. Sanchez, O.J.; Cardona, C.A. (2008). Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresour Technol* 99, 5270-5295.
 84. Gupta, R.; Gigras, P.; Mohapatra, H.; Goswami, V.K.; Chauhan, B. (2003). Microbial α -amylases: a biotechnological perspective. *Process Biochem* 38, 1599 – 1616.
 85. Couto, S.R.; Sanromán, M.A. (2006). Application of solid-state fermentation to food industry- A review. *Journal of Food Engineering* 76, 291-302.
 86. Ahlawat, S.; Dhiman, S.S.; Battan, B.; Mandhan, R.P.; Sharma, J. (2009). Pectinase production by *Bacillus subtilis* and its potential application in biopreparation of cotton and micropoly fabric. *Process Biochemistry* 44, 521–526.
 87. Feitkenhauer, H. (2003). Anaerobic digestion of desizing wastewater: influence of pretreatment and anionic surfactant on degradation and intermediate accumulation. *Enzyme Microb. Technol.* 33, 250–258.
 88. Bruinenberg, P.M.; Hulst, A.C.; Faber, A.; Voogd, R.H. (1996) A process for surface sizing or coating of paper. In: European Patent Application.
 89. Saxena, R.K.; Malhotra, B.; Batra, A. (2004). Commercial Importance of Some Fungal Enzymes. In: Arora, D.K. (ed) *Handbook of fungal biotechnology*. Marcel Dekker, New York, USA, p 287-298.
 90. van der Maarel, M.J.; van der Veen, B.; Uitdehaag, J.C.; Leemhuis, H.; Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the α -amylase family. *J Biotechnol* 94, 137-155.
 91. Frost, Ray L., Wayde N. Martens, Llewellyn Rintoul, Emir Mahmutagic, and J. Theo Kloprogge. "Raman spectroscopic study of azurite and malachite at 298 and 77 K." *Journal of Raman Spectroscopy* 33, no. 4 (2002): 252-259.
 92. Ovalle, William K., and Patrick C. Nahirney. *Netter's Essential Histology E-Book: With Correlated*

Histopathology. Elsevier Health Sciences, 2020.

93. Byler, D. Michael, and Heino Susi. "Examination of the secondary structure of proteins by deconvolved FTIR spectra." *Biopolymers: Original Research on Biomolecules* 25, no. 3 (1986): 469-487.