Microencapsulation techniques towards building a sensor: A study of factors affecting enzyme encapsulation and stability

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MICROENCAPSULATION TECHNIQUES TOWARDS BUILDING A SENSOR: A STUDY OF FACTORS AFFECTING ENZYME ENCAPSULATION AND STABILITY

Suman R. Nayak, B.E.

A Dissertation Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy

COLLEGE OF ENGINEERING AND SCIENCE LOUISIANA TECH UNIVERSITY

May 2005
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We hereby recommend that the dissertation prepared under our supervision
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ABSTRACT

Minimally and non-invasive glucose sensing techniques are instrumental in improving the quality of life of diabetics, point-of-care testing, critical care monitoring, development of new anti-diabetic drugs or therapeutic compounds, and the study of basic physiology. The overall goal of this project was to build a fluorescent glucose sensor by incorporating relevant sensing chemistry within microcapsules. The specific aims for this research were to study various processes used for the fabrication of micro-containers, the use of these for encapsulation purposes, and the application of the encapsulated system towards glucose sensing. The following is a brief description highlighting the key areas of the research.

Different materials such as polystyrene sulfonate (PSS), polyallylamine hydrochloride (PAH), polydiallyldimethyl ammonium chloride (PDDA), chitosan (CHT), and polyvinyl sulfate (PVS) were used to construct the capsule walls. Fluorescence recovery after photobleaching (FRAP) was used to study the effect of capsule wall architecture on the permeability of capsules to FITC labeled GOx (FITC-GOx). Long-term stability and activity studies proved that \( (PVS/CHT)_2 - (PSS/PAH)_2 \) capsules were the most stable and showed minimal leaching (79% of enzyme retained) and the activity of the encapsulated enzyme did not change significantly over four weeks. Hence, they were selected for demonstration of glucose sensing. Fluorescence sensors for glucose using pH-sensitive Pyrene 8-hydroxy-1,4,6-trisulfanylchloride (HPTS), which facilitates
ratiometric monitoring of pH changes, was used to label GOx (HPTS-GOx), and encapsulated in these capsules. Changes in glucose concentration were monitored as a function of the pH changes during glucose consumption by GOx, and the sensors showed a linear response to sequential additions of glucose in the physiological region of interest (0mM-30mM).

In summary, the ideas, techniques and discussion of the results presented in this dissertation are a significant contribution to the science of enzyme encapsulation. The preliminary data demonstrating glucose sensing using a labeled pH-sensitive dye is an important contribution to this application. Some of the novel techniques presented here serve as a foundation for future application of these to other areas. The experimental techniques and methods of characterization described in this dissertation can serve as a comprehensive guide to researchers involved in similar study.
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Author

Date MAY 12, 2005
DEDICATION

I dedicate this work to my parents, Shiela and Ramesh Nayak, my brother, Atul, my cousin and her husband, Vishula and Venugopal Shenoy, and my close friend Sireesha Krishna.
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What was only a figment of the imagination of American author and biochemist Issac Asimov has become today’s quest for understanding our macroscale world with microscale innovations. Though living beings have yet not been shrunk and injected into the body as was envisaged by Asimov, there have been Promethean strides made in the field of micro- and nano-technology. Scientists and researchers are developing artificial cells that can replace damaged cells in the body, and miniaturized sensors have been injected into cells to monitor cellular events. Nanorobots—which were earlier dismissed
as mythical monsters are now being considered for diverse applications from mouthwashes to skin disease cures to medical nanodevices that can augment the human immune system and nibble away atherosclerotic plaques. The concept of tattooing, viewed as a form of body art, fashion, and rebellion is now also being used by researchers for embedding smart sensors for monitoring vital analytes in the body.

Dr. Gregory Fahy of the American Red Cross aptly said in the paper “Nanotechnology in Medicine” presented at the U.S. Pharmacopeial Convention in fall 1992. “Living organisms are naturally-existing, fabulously complex systems of molecular nanotechnology.” With that in mind, it seems that the development of micro- and nanoscale sensors and actuators might prove to be instrumental in curing the human body of its various ills. Among the various devices that are in great demand both in terms of improvement of the quality of life of patients, as well as in terms of scientific challenge, is an ongoing quest for the “ideal” glucose sensor. Sensing glucose is imperative in a wide spectrum of applications ranging from food industry to personal management of diabetes. Many systems have been developed for ex vivo analysis of glucose; however, there is still a need for development of a reliable, implantable or minimally implantable glucose sensor for in vivo monitoring of physiological levels of glucose. Such a sensor needs to satisfy a number of stringent criteria, such as selectivity, linear range, and response time, biocompatibility, storage and operational stability at body temperature, reversibility of signal, and reproducibility of sensor fabrication, which current endeavors have not been able to completely satisfy.

Advances in the micro- and nano-fabrication industries together with rapid progress in the photonics industry have created a tremendous potential for development
of portable, minimally invasive sensors for monitoring a wide spectrum of micronutrients and analytes in the body\textsuperscript{2}, especially the market driven demand in the glucose sensing industry for in vivo glucose monitoring of diabetic patients. Self-monitoring of blood glucose (SMBG) is the bedrock of successful diabetes management. It has the potential to improve the quality of life of millions of diabetics all over the world essentially by ameliorating the pain associated with the traditional finger prick method, and by developing a continuous and reliable in-vivo glucose monitoring system that would help the patient to optimize insulin therapy and metabolic control\textsuperscript{3}. This method would also prevent the dangers associated with acute metabolic deterioration, such as hypo and hyperglycemia.

There have been several minimally invasive and non-invasive techniques developed for continuous monitoring of blood glucose. Implantable subcutaneous glucose sensors and subcutaneous interstitial fluid sampling by microdialysis are examples of techniques based on analysis of interstitial fluid. Electrodes developed for this purpose and which are inserted into the subcutaneous tissue cannot be used for long periods of time in humans due to fouling of the electrode, which results in drift of signal, and hence a loss of glucose sensitivity. The microdialysis methods have a significant time lag associated with the process of measurement, which can vary between 5 and 45 minutes\textsuperscript{4}. Transdermal glucose monitoring systems and optical glucose sensors have also been developed. Most non-invasive approaches use optical techniques the basic principle being measurement of the properties of light reflected from the skin, either as a result of direct interaction with glucose using spectroscopic techniques, or due to indirect effects by changes in the physical properties of the skin (scattering). The non-invasive
techniques suffer from insufficient precision\textsuperscript{4}. Implantable glucose sensors have the obvious major applications in diabetic self-monitoring, but other applications include critical care monitoring and the study of basic physiology. As an example of the latter, real-time monitoring of neurochemical dynamics, including glucose and lactate, is important to understanding neural processing, cell biology, and drug activity. To date, some minimally invasive methods have been developed for sensing blood glucose concentrations, but all of the accurate devices on the market still require extraction of blood samples. Fully-implanatable systems with wireless communication are an ideal solution; however, while many such devices are under development, the stability of enzyme-electrochemical systems remains a difficult obstacle to overcome. Sensors based on optical measurements are an attractive alternative, as they may be analyzed spectroscopically.

Among the various non-invasive glucose biosensors reported in literature, several optical techniques have been cited, such as, near-infrared spectroscopy\textsuperscript{5}, polarimetry\textsuperscript{6}, Raman spectroscopy\textsuperscript{5}, and fluorescence spectroscopy\textsuperscript{7}. Optical sensors are generally advantageous in terms of minimal analyte consumption and can potentially be used to monitor real-time events. Fluorescence sensors provide a non-invasive method of detection of analytes, which is fast, reagentless, and versatile\textsuperscript{1}. However, if the fluorescent dyes are directly introduced into the body, their location cannot be controlled. Moreover, there can be problems of cytotoxicity and compartmentalization of the indicators in organelles. Hence, encapsulation of the sensor chemistry is vital in order to overcome all these drawbacks. A number of fiber-optic sensors for sensing various
analytes (oxygen, carbon dioxide, glucose and NADH) have been developed; however, the measurement technique is invasive.

The study of enzyme encapsulation has been of significant interest both from the standpoint of academic research as well as in several industrial applications. The primary objective behind encapsulation of enzymes is to use a semi-permeable support matrix to entrap the enzyme, prevent its leaching out of the matrix, while allowing exchange of analytes, products, and co-factors between the encapsulated enzyme and the surroundings. Various methods of encapsulation in micro and nanoparticles have been developed for applications in pharmaceutics, cosmetics, agriculture and biosensors, among others. Liposomes, microspheres prepared by microemulsion techniques, and silica matrices are among the various carrier systems used for various chemicals, drugs and biomacromolecules. Medical applications of such micro- and nanocontainers include using them as protective shells for cell and tissue encapsulation or as carriers for drug-delivery. Some of these techniques suffer from the lack of homogeneity of particles, and lack of repeatability in terms of the quantity encapsulated within and between batches. In the field of fluorescence biosensing, analyte specificity is achieved by co-encapsulation of indicator and reference dyes and various receptor systems, such as synthetic receptors (i.e. boronic acid derivatives), naturally occurring (i.e. enzyme glucose oxidase (GOx), lectin, Concanavalin A, sugar binding proteins) and genetically engineered binding proteins (i.e. glucose binding protein, GBP).

Recently, encapsulating polymeric microcapsules with specialized multilayer compositions and controllable wall properties has been proposed. Layer-by-Layer
(LbL) self assembly has proved to be a versatile method by which both planar thin film architectures and hollow core shells can be produced\textsuperscript{35,36,37}. This method is advantageous due to the ease and repeatability of the fabrication process, and because it does not require sophisticated and expensive equipment. Various templates have been used to fabricate microcapsules with different sizes and wall architectures. Among the various templates that have been used for fabrication of microcontainers are silica, polymeric particles like polystyrene and melamine formaldehyde (MF)\textsuperscript{38,39,40}, metal particles\textsuperscript{41}, biological cells\textsuperscript{42,43}, and inorganic crystals\textsuperscript{44}. Using micro/nanocapsules made by LbL methods, several methods of encapsulation have been developed. These methods include encapsulation of both biomolecular and non-biomolecular materials.

To date, there have been no reports of the use of pH sensitive ratiometric dyes encapsulated using micro/nanoscale carriers, for the measurement of glucose. Pyrene 8-hydroxy-1,4,6-trisulfonylchloride (HPTS), is a pH indicator dye that has been used by several groups to demonstrate pH sensitivity in thin films\textsuperscript{45,46,47}. It has numerous advantages such as large Stokes shifts, high quantum yield, high absorbance, excellent photostability, and lack of toxicity\textsuperscript{45}.

The fundamental aim of this dissertation was to develop a glucose sensor based on sensing pH changes associated with oxidation of glucose by glucose oxidase to indicate changes in glucose concentration. With this goal aim in mind, two specific aims were identified. Aim1, various enzyme encapsulation techniques were studied and compared with the purpose of identifying the best method (percentage encapsulation efficiency, activity, and stability) for the encapsulation of GOx. In the process of developing methods of encapsulation for GOx a novel method of encapsulation of HRP
was discovered. This method involved fabrication of a charged matrix of acrylic acid inside the hollow capsule volume. The method is rather simple and can be extended to encapsulation of macromolecules with slightly basic isoelectric points (pI). A short study of the effect of encapsulation parameters on the encapsulation efficiency and stability of the encapsulated enzyme was performed. Aim 2, based on comparison of the relative merits of the various methods of encapsulation in specific aim 1, a method was selected and used to encapsulate HPTS-GOx. The effect of labeling on the pH sensitivity of the dye was studied and compared with the response of the free dye. The glucose sensitivity of the dye was studied in solution and after encapsulation, both in DI water and in PBS buffer.

A novel method of sensing glucose has been demonstrated. The marriage of fluorescence spectroscopy and nanotechnology has produced glucose sensors which can potentially be used to monitor glucose changes in the body as a function of changes in pH. To date, there has been no reported research on glucose sensors based on translation of pH changes into changes in glucose concentration. Sensors were fabricated by encapsulating glucose oxidase (GOx), labeled with a pH sensitive fluorophore pyrene 8-hydroxy-1,4,6-trisulfonylchloride (HPTS) in microcapsules. The microcapsules were fabricated using LbL assembly of generic polyelectrolytes and chitosan on an MF template. The glucose sensitivity of the encapsulated chemistry was demonstrated. The conceptual schema of sensor fabrication and desired optical response is shown in Figure 2. From the figure it can be seen that increasing glucose concentrations causes an increase in excitation ratio of HPTS, which is labeled to glucose oxidase. The principal advantage of this method has been the simplicity of the encapsulation procedure, as well
as the lack of a need for an additional reference indicator due to the inherent ratiometric nature of HPTS.

![Graph](image)

**Figure 2. Schema of sensor fabrication and optical response**

The following chapters of this dissertation describe the background, experimental theory and methods, materials and techniques, results and conclusions of the work done to fulfill the aforementioned specific aims. Chapter II is an overview of the literature and background pertaining to glucose regulation in the body, previous and current research in non-invasive and minimally invasive, transdermal, fluorescence glucose sensing techniques; skin optics and the concept of smart tattoos have also been reviewed. Chapter III gives details of the chemical techniques (cross-linking, labeling reactions),
the optical imaging and spectroscopic techniques used for measurements and the surface characterization techniques used to achieve the aims of the project. The materials and methods used to perform the experiments have been described in Chapter IV. Chapter V describes the experimental results with a detailed discussion of the outcomes and the rationale behind them. Finally, the conclusions derived from the work have been described in Chapter VI.
CHAPTER II

LITERATURE REVIEW

This chapter describes a wide range of topics related to glucose regulation in the body, diabetes mellitus and its treatment options and the need for continuous glucose monitoring. Current research in implantable, non-invasive and minimally invasive, transdermal, fluorescence glucose sensing techniques, skin optics and the concept of smart tattoos have been reviewed. Further, it deals with layer-by-layer (LbL) assembly and various encapsulation techniques using this technique.

2.1 Physiology of Glucose Metabolism

2.1.1 Anatomy of the Pancreas

The pancreas (Figure 3), a large compound gland with an internal structure similar to that of the salivary glands, lies parallel to and beneath the stomach. It is mainly composed of two main types of tissues: the acini, which secrete digestive juices into the duodenum; and the islets of Langerhans, which secrete insulin and glucagon directly into the blood. The human pancreas has one to two million islets of Langerhans organized around small capillaries into which its cells secrete their hormones. The islets contain three major types of cells: alpha cells, which secrete glucagons; the beta cells, which secrete insulin; and the gamma cells, which secrete somatostatin. The close interrelations
among these cells types in the islets of Langerhans allow direct control secretion of some of the hormones by the other hormones; i.e., insulin inhibits glucagon secretion, and somatostatin inhibits the secretion of both insulin and glucagon\textsuperscript{49}.

Figure 3. The pancreas-- an important organ for insulin regulation of the body. 
2.1.2 Effect of Various Enzymes on Glucose, Fat and Protein Metabolism

Though insulin secretion has been associated with energy abundance, i.e. with consumption of foods rich in carbohydrates and proteins, it has also been found to affect fat and protein metabolism almost as much as carbohydrate metabolism. The secretion of insulin in great quantities is especially true for excess carbohydrate consumption and is less for proteins and only slightly for fats.

Excess carbohydrates are stored as glycogen, mainly in the liver and muscles. Fat storage takes place in the adipose tissue; insulin causes all excess carbohydrates that cannot be stored as glycogen in the liver and muscles to be converted into fat. In the case of proteins, insulin promotes amino acid uptake by cells and their conversion into protein. Insulin also inhibits the breakdown of the proteins that are already in the cells.

Mainly, four hormones play an important role in switching between carbohydrate and lipid metabolism. The growth hormone from the anterior pituitary gland, cortisol from the adrenal cortex, epinephrine from the adrenal medulla, and glucagon from the alpha cells of the islets of Langerhans. Growth hormone and cortisol are secreted in response to hypoglycemia and inhibit cellular utilization of glucose while promoting fat utilization. Epinephrine has both lipolytic effects increasing the fatty acids in the blood and glucogenolytic effects increasing glucose concentration of blood, but mostly the former. The effect of epinephrine is particularly important during periods of stress, such as exercise, anxiety, and circulatory shock.

Glucagon encourages gluconeogenesis during periods of hypoglycemia by activating several enzymes that promote amino acid uptake by the liver and their
conversion into glucose. It also promotes glycogenolysis, which entails the breakdown of glycogen in the liver into glucose and its release as glucose into the blood.

2.2 Diabetes Mellitus

Type I and Type II diabetes account for 99.9% of all prevalence of diabetes and affect about 135 million people worldwide. Type I diabetes results from a chronic autoimmune destruction of the pancreatic \( \beta \)-cells and usually starts from childhood or early adulthood (<35 yrs). Type II diabetes results when organs become insensitive to the action of insulin and/or the production of insulin by the beta cells is insufficient (dysfunction of \( \beta \) cells). Heredity plays a vital role in diabetes, both Type I and Type II\(^49\).

2.2.1 Pathophysiology of Diabetes Mellitus

Diabetes Mellitus is characterized by the following consequences of lack of insulin: increase in blood glucose concentration up to 300-1200mg/dl (16mM-66mM) and increased fat immobilization from fat deposit areas, causing abnormal fat metabolism and deposition of cholesterol in arterial walls leading to atherosclerosis and protein depletion in the tissues of the body\(^49\).

When blood glucose concentration rises above 180mg/ml (blood threshold), a significant portion of this glucose entering the kidney tubules in the glomerular filtrate cannot be reabsorbed and hence spills into the urine. This leads to osmotic diuresis, or the increased loss of water in urine due to osmotic effect of glucose decreasing tubular reabsorption of fluid. It also leads dehydration of cells. Extracellular and intracellular dehydration can lead to circulatory shock. Increased fat metabolism can also lead to acidosis and hence coma and eventually death. Polyuria (excessive elimination of urine),
polydipsia (excessive drinking of water), polyphagia (lack of energy), weight loss and asthenia (lack of energy) are the earliest signs of diabetes.

At elevated levels of glucose (>5mM), glycosylation of proteins (hemoglobin, collagen etc.) takes place due to reaction of glucose (without enzyme catalysis) with proteins. This can result in tissue damage and failure of vital organs such as the kidneys, which is a long-term complication of diabetes.

2.2.2 Treatment of Diabetes Mellitus

For type II diabetes, a diet, oral hypoglycemic agents and/or the administration of insulin is required and sufficient to regulate the blood glucose concentration. In addition to increasing glucose levels in the blood, there are also other concerns in type II diabetes. The emphasis in the treatment of type II diabetes is on the reduction of chronic hyperglycemia and the long-term degenerative conditions that are associated with it.

Type I diabetes is associated with absolute insulin deficiency. Hence, treatment specifically involves insulin injections in the subcutaneous tissues of arms, legs or abdomen (IDDM). An intensive regimen of short-acting insulin before meals, followed by an additional injection of intermediate-acting insulin before bedtime are required to mimic the normal insulin profile in blood and assists in improvement of the metabolic profile of the patient. The patient needs to check his/her blood glucose regularly, currently by using a finger-prick method, and adjust the insulin dosage accordingly. Continuous monitoring can help achieve even better glucose regulation. Insulin delivery using implantable pumps to the peritoneal cavity can further improve metabolic control for type I patients who are difficult to regulate.
A combination of insulin injections and frequent self-monitoring of blood glucose (SMBG) have improved metabolic control in type I patients; normoglycemia is difficult to achieve because subcutaneous insulin injections cannot mimic non-diabetic insulin secretion patterns. For example, high concentrations of peripheral insulin are needed to achieve sufficient insulin concentrations in the portal vein to slow down the production of glucose by the liver. Also, short-acting insulin is resorbed much more slowly than insulin secreted by the beta cells, and there is no feedback control for adjusting the insulin delivery rates according to the prevailing glucose level.

Yet another approach to improve metabolic control of type I diabetic patients is transplantation of pancreas or isolated islets of Langerhans. The first transplantation of pancreas was done in 1967. By the 1970s, due to significant improvement of the surgical technique and better control of immunosuppression and infection post transplantation, the success rate of transplantations greatly improved. Today, transplantations of the pancreas are carried out in combination with kidney transplantation; this procedure has a success rate of 75%. However, it still remains to be seen if majority of the type I diabetic patients will benefit from this procedure; patients have to be on immunosuppression for the rest of their lives. There is also a paucity of suitable donors. Hence, the American Diabetes Association (ADA) has proposed that transplantation of pancreas be considered the appropriate therapy only for type I diabetic patients with end-stage renal disease who have to plan a kidney transplant or as an alternative therapy for patients who have a history of frequent acute and severe metabolic complications.
The success rate of islet-transplantation is only 10% as opposed to 75% for the combined pancreas-kidney transplantation. This rate results from the rejection of the transplanted islets due to the immuno response of the acceptor site. Also, non-specific inflammatory responses occurring at the time of implantation may alter islet function. The normal procedure for islet transplantation involves isolation of islet tissue by collagenase digestion of the pancreatic donor tissue, then purification of the tissue followed by injection of a sufficient mass of islet tissue into the hepatic parenchyma via injection through the portal vein. This procedure requires the use of a large quantity of immunosuppressant; an alternative is to use islets encapsulated in a porous material that is permeable for insulin and glucose but impermeable to leukocytes. Also, encapsulated animal islets can be considered as a solution to shortage of donor islets from humans. However, though encapsulation makes the cells less vulnerable to immune response, it does alter the reaction dynamics of the islets resulting in delayed insulin secretion. This alteration results from the elongation of the diffusion pathway to the encapsulated cells since they are not directly provided by blood capillaries. A potential solution to this problem is the use of a solid support for the encapsulated cells that can be placed in the peritoneal cavity. This placement will hasten the accommodation of the implant with blood capillaries and thus reduce the diffusion pathway.

2.2.3 Need for Continuous Glucose-Monitoring Systems (Glucose Sensors) and Glucose Measurements in the Interstitial Fluid (ISF)

The ultimate goal of glucose regulation regimens should be preferably to achieve the same degree of regulation of blood glucose concentrations as non-diabetics. A new
milestone in insulin therapy has been achieved by the development of self-monitoring
devices. A combination of self-monitoring of glucose and an intensified insulin regimen
has proved effective in managing the occurrence of hyperglycemic events. However, the
same cannot be said about hypoglycemia. It has been found that the incidence of
occurrence of hypoglycemia increases with tightly metabolic controlled patients.
Hypoglycemic attacks are unpleasant and can lead to loss of consciousness. Though
hypoglycemia is not restricted to nocturnal attacks, they are especially dangerous because
the patient is asleep and hence unaware of the low blood glucose levels. A major focus
of interest for diabetologists is the “hypoglycemia unawareness syndrome” which is
characterized by the occurrence of sudden and unpredictable hypoglycemia without clear
warning symptoms.

A major drawback of self-monitoring is the simple fact that current methods are
discontinuous. Most patients are deterred from making multiple measurements because of
the pain and boredom associated with the procedure and simple dislike because the
patient is forced to confront the disease everytime he/she makes a measurement. Only
highly motivated patients measure their blood glucose more than six times a day, and this
is a miniscule fraction of the total number of diabetics. Hence, a continuous glucose
monitoring system would prove to be a boon for diabetics in order to better control
hypoglycemic events. A major factor in the regulation of blood glucose is the delay time
between the actual change in blood glucose concentration and the glucose measurement;
this delay should be minimized. In a healthy person, a complex series of events involving
gut hormones and neural factors lead to anticipatory insulin secretion, prior to insulin
secretion from a rising blood glucose level. Hence, a more realistic design for continuous
monitoring would be a closed-loop glucose sensor for operation in the "non-meal" or basal periods, with additional insulin delivered at or just prior to the start of a meal.

Though glucose monitoring had been confined mainly to measurement of blood glucose for historical reasons, there are obvious risks involved in inserting a glucose sensor in the intravascular compartment for prolonged time periods such as embolism, thrombosis, and septicemia. Hence, currently several methods involve placing the sensor in the extravascular spaces or the interstitial fluid (ISF). Minimally invasive sensors measure glucose levels in the ISF (Figure 4), and non-invasive ones measure a composite of glucose levels in the intracellular, interstitial, and intravascular compartments.

![Figure 4. A glucose electrode inserted in the subcutaneous tissue to measure glucose in the interstitial fluid.](image)

Microcirculation, which is the body's capillary system performs one of the most important functions of circulation—transport of nutrients to the tissues and removal of cellular excreta. The diameters of the arterioles are controlled by the local conditions in the tissues themselves. Capillaries are extremely thin structures with single layered walls of highly permeable endothelial cells to permit free interchange of nutrients and cellular excreta between the tissues and circulating blood.
About one sixth of the body consists of spaces between cells containing fluid called the interstitial fluid. It has solid structures composed of collagen fibers and proteoglycan filaments that give it its gel-like constitution. Figure 5(a) shows the lymphatic system, which represents an accessory route for fluid flow from the interstitial spaces into the blood, especially for large proteins and particulate matter from the tissue spaces that cannot be removed by absorption directly into the blood capillary. Though most of the fluid filtering from the arterial capillaries flows among the cells and is finally reabsorbed back into the venous ends of the blood capillaries, one tenth of it enters the lymphatic capillaries instead and returns to the blood through the lymphatic system rather than the venous capillaries. Though this amount is a minute quantity, it is extremely important because this route is the only one allowing high molecular weight substances such as proteins to be reabsorbed into the blood. As shown in Figure 5(b), the walls of the lymphatic capillaries have a special structure. The endothelial cells are attached by anchoring filaments, and the edge of one endothelial cell overlaps the edge of the adjacent cell in such a way that the overlapping end is free to flap inward, thus forming a valve. These valves can be pushed open by the interstitial fluid, and backflow is prevented because of the closing of the valve.
A prominent volume fraction of the human skin is interstitial fluid (45%), and blood vessels represent only 5%. Hence, glucose changes in skin tissue for the most part represent changes in the extravascular spaces rather than blood glucose. However, in the adipose tissue, the proportion of ISF is lower than in the skin and varies according to the size of adipocytes. There is constant free and rapid exchange of glucose molecules between blood plasma and interstitial fluid. Though there is much debate about the amenability of measuring ISF glucose as a measure of blood glucose, it has been reported that changes in blood glucose and interstitial glucose are strongly correlated. However, there is a delay between changes in the ISF and the blood vessels, which can vary between few seconds to 15 minutes. This delay reduces reliability of ISF glucose measurements, and can lead to dangerous situations if blood glucose levels decline to hypoglycemic values more rapidly than ISF. Also, diabetic patients have altered capillary walls due to glycosylation of wall proteins by excess amounts of glucose, which may
increase the diffusion barrier and hence the time lag. It also remains to be established as to how much the intra- and inter-individual variability of the delay between glucose changes in the intra- and extravasal compartments typically is. The agreement of absolute glucose values between the two compartments is also debatable. It has been reported that there is 50-100% variation between glucose values in the two compartments\textsuperscript{55}. Hence, even the percentage of variation between glucose values in the ISF and blood shows a wide variability from one region of the body to another. This variability is mainly due to local factors determining ISF clearance in different body regions. Finally, the relationship between the two during non-steady-state conditions, i.e. exercise or postprandially, still need to be determined.

2.3 Smart Tattoos and the Use of Human Skin for Implantation of Minimally Invasive Sensors

2.3.1 The Human Skin

The human skin (Figure 6) is divided into two main regions: the epidermis, and the dermis (which is attached to the underlying hypodermis)\textsuperscript{57}. The epidermis is the most superficial layer of the skin, principally composed of cells called keratinocytes. It is composed of five layers: the stratum corneum, the stratum lucidum (present in areas of very thick skin like the palms of the hands and the soles of the feet), the stratum granulosum, the stratum spinosum, and the stratum germinatum. The epidermis is constantly regenerated. Newly formed cells from the stratum germinatum undergo a progressive maturing process called keratinization and migrate to the outer layer, the stratum corneum.
The dermis is the innermost layer of the skin. It supports the vascular network that supplies the avascular epidermis with nutrients. The dermis contains mostly fibroblasts which secrete collagen, elastin and ground substance that give the support and elasticity of the skin. It is made out of two layers: the outer papillary layer, which contains the vascular network, and the inner reticular layer, which houses hair follicles and glands among other things. The hypodermis is a subcutaneous layer of connective tissue which connects the skin to deeper structures.

![Cross-section of the skin](http://www.agen.ufl.edu/~chyn/age2062/lect/lect_19/lect_19.htm)

Figure 6. Cross-section of the skin.
(Source: http://www.agen.ufl.edu/~chyn/age2062/lect/lect_19/lect_19.htm)
2.3.2 Optics of the Skin

The structure and chemical composition of the skin is reflected by the optical properties of the skin. As shown in Figure 7, when a beam of light reaches the skin surface, a part of it is specularly reflected (4%) by the surface; the rest of it will be reflected and transmitted into the skin (96%). The transmitted light will be scattered and absorbed by the skin tissue. After multiple scattering, some of the transmitted light will re-emerge through the air-stratum interface into the air, which is called diffuse reflectance. This scattering contributes to a diffuse distribution of light in the tissue that extends beyond the boundaries of the collimated incident beam.

Figure 7. Tissue Optics- What happens to light in the skin
The amount of diffuse reflection is determined by both scattering and absorption properties of the skin. A back scattered photon from the diffuse part of the light distribution that reaches the tissue-air boundary at an angle with the inward normal larger than the so called critical angle for total reflection, is reflected back into the tissue. A much smaller diffuse back-reflectance coefficient may be more appropriate for laser irradiated skin because light close to boundaries may not be perfectly diffuse but more forwardly directed. After a photon is absorbed by the skin, an electrically excited absorbing molecule may rapidly return to a more stable energy state by re-emission of a photon with lower energy, i.e. fluorescence emission. Autofluorescence is caused by native fluorophores inside the skin.

2.3.2.1 Tissue Absorption and Scattering. An endogeneous or exogeneous fluorescentophore is required for interrogation of tissue biochemistry and to obtain useful information from a reemitted optical signal. Endogeneous chromophores are oxy- and deoxyhemoglobin, melanin, myoglobin, and water. These chromophores are primarily responsible for absorption of light in the wavelength region of 600-1000nm, or the "therapeutic window," where tissue scattering predominates over absorption. Because of the large absorbance of hemoglobin and myoglobin at wavelengths <600nm and the predominant absorbance of water in the IR range (>1000), optical characterization beyond a millimeter of tissue is impractical when outside the therapeutic window of 600-1000nm. Hence, back-scattering measurements at these wavelengths characterize surface and subsurface tissues. Typically, endogeneous tissue absorption coefficients range from 0.1 to 10,000 cm\(^{-1}\) from NIR to UV range, and correspond to an optical penetration depth of 10cm to 0.1mm before absorption. NIR light can propagate several centimeters, which
enables one to extract information non-invasively and deeply. The elastic tissue scattering arises from the microscopic inhomogeneities of refractive indices between extracellular, cellular, and subcellular components.

Water is the main tissue constituent of the skin. Along with many other components of the skin, it absorbs MIR radiation very effectively. Hence, in-vivo penetration of MIR light is low. However, light in NIR and VIS regions penetrates deeper into the blood perfused skin layers allowing for glucose monitoring. If tissue thickness is low, transmission spectra can be recorded, otherwise, only diffusely reflected light intensity can be used. For the most part, light scattering in tissue is caused by Mie scattering. Mie scattering happens when the size of the scattering particles and the wavelength of the light are comparable or of the same order of magnitude. This idea has been illustrated in Figure 8. Mie scattering mainly happens due to the passage of light through the boundary between media with different refractive indices. The scattering properties strongly depend on the ratio of the refractive indices of the scattering and the solute in a suspension. A decrease in the refractive indices of the scattering particles causes a decrease in the scattering and vice versa. The scattering suspension becomes optically transparent when there is a perfect match of both the refractive indices.
2.3.3 Tattoos and Smart Tattoos

As discussed in the previous section, the skin is divided into three basic areas, epidermis, dermis, and the hypodermis. The epidermis constantly renews itself from the bottom up; new skin cells are created by stem cells at the bottom of the epidermis, pushing the older cells up towards the surface. The cells at the surface are the oldest, and they die and flake off. Hence, for a tattoo to be permanent, ink must be poked deeper into the skin, past the basement membrane and into the dermis. Ink molecules are too big and so unlike other foreign objects that are hauled away by leukocytes, the ink molecules can remain in the dermis for decades. The process has been illustrated in Figure 9. Tattoos are usually inserted in the skin’s dermal layer with needles.
The concept of “smart tattoos” to monitor blood glucose was proposed as a minimally-invasive method for improved metabolic control in diabetics. Polyethylene glycol (PEG) beads coated with fluorescent molecules are injected beneath the surface of the skin, and the glucose in the ISF interacts with the fluorescent molecules of the sensors. When the glucose concentration is low, the tattoo is highly fluorescent; when the concentration increases, the glucose binding causes displacement of fluorescent molecules, and the overall fluorescence of the tattoo decreases.

The excitation and detection system consists of a fiber optic probe containing fibers for delivery of excitation light and collection of emission light or some other appropriate optical system that can be held to the skin at the implantation site.
2.4 In-vivo Glucose Sensing: Totally Implanted, Minimally Invasive, Non-invasive Monitoring and Fluorescent Sensing Techniques

2.4.1 Totally Implanted Sensors

Needle-type sensors implanted transcutaneously for a short term have been found to be unstable because acute inflammation resulting in cellular accumulation, changing protein and fluid composition at the site of implantation results in variable glucose and oxygen concentrations. Long term implantation, on the other hand, was found to be a more stable sensing environment after an encapsulating foreign body response. Glucose sensors implanted in the subcutaneous tissues of dogs were inactive for the first few days, unstable for the next 7-14 days after which they became relatively stable for the next several weeks; the stability was insufficient for satisfactory clinical use.59,60

2.4.2 Minimally Invasive Techniques

2.4.2.1 Transdermal Methods. Transdermal methods are based on removal of interstitial intradermal fluid (ISF) across the skin, thus avoiding penetration of the skin61,64. Reverse iontophoresis is a popular method that is being used in a commercially available glucose sensor, GlucoWatch, (Cygnus, Redwood City, CA, USA). In this device, a low current is applied to the skin to drag ions through the skin, thereby extracting ISF across the skin. Small amounts of glucose (1/1000 of blood levels) are also transferred with the fluid. Feasibility studies have shown that acceptable results for accuracy of measured glucose levels can be obtained from 40 to 400 mg/dl. However, the glucose oxidase reaction used to measure glucose must be pushed to its limits for accurate measurements due to the small quantities of glucose available. Also, the system needs a three hour warm-up period and can provide up to three glucose readings per hour
for 12 hour after a single-point calibration with a conventional self-blood glucose measurement\textsuperscript{54}. The current applied to the skin can cause a mild to moderate skin irritation that clears within 3-27 days, and the reliability of the device is questionable during sweating and varying degrees of skin hydration.

Other methods involve using transdermal patches (TCPI, Fort Lauderdale, FL, USA) with permeation enhancers. The patch is placed on the skin for five minutes, after which time a meter is placed in the tabbed area of the patch to take a spot glucose reading. Yet another method involves application of ultrasound to increase skin permeability resulting in an increase in transdermal glucose flux\textsuperscript{56}.

2.4.2.2 Electrochemical Methods and the Glucose Electrode. In the 1980s, biosensors were among the most promoted technologies both by researchers and industry, and were touted to have significant clinical applications\textsuperscript{62}. Reagentless probes or "biosensors" for measuring glucose in vitro were first mentioned in the 1960s\textsuperscript{63}, and it was in the early 1970s\textsuperscript{64} that glucose sensors were first tested in vivo in animals.

Figure 10 shows one of the most studied type of glucose sensors called "amperometric enzyme electrodes". Glucose oxidase is immobilized by chemical cross-linking at a charged electrode, and glucose changes are monitored by the change in current flow caused by the enzyme catalyzed production of hydrogen peroxide\textsuperscript{65,66,67} or by the consumption of oxygen\textsuperscript{68}. The electrode itself is constructed of two different metals with an insulating material between them. A modified enzyme electrode is now being used in most commercial devices for self monitoring of finger prick samples for blood glucose concentration (MediSense)\textsuperscript{69}. Sensor configuration usually comprises a fine needle or flexible wire, the active sensing element being at the tip; it is implanted in
the subcutaneous tissue. Such an implantation is regarded “minimally invasive,” and it avoids septicaemia, fouling and blood clot, and embolism associated with intravascular placement\textsuperscript{62}.

![Diagram of an electrochemical/amperometric electrode](image)

Figure 10. Typical construction of an electrochemical/amperometric electrode\textsuperscript{62}.

Although there have been encouraging test results with needle type sensors in animals and humans\textsuperscript{65,70} clinical development has been slow. The sensor output in vivo is suppressed by a variable amount compared with the in vitro signal at the same glucose concentration, requiring careful calibration procedures\textsuperscript{71}. Also, there is an unpredictable drift in output over time\textsuperscript{72}. Since the exact cause of these erratic responses is unclear, a rational modification of the design has been precluded. Presently, evidence points towards a reversible coating of the implanted sensor or a diffusion of low molecular weight inhibitors into the sensor; the chemical nature of the interference is still unclear\textsuperscript{62}.

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2.4.2.3 Microdialysis. Microdialysis has been the most popular among the tissue sampling methods, which has undergone considerable testing in humans\textsuperscript{60,73}. It presents a possible alternative to the implantable and transcutaneous measurement methods, which have biocompatibility problems. Microdialysis imitates capillary function\textsuperscript{4}. The procedure involves insertion of a semi-permeable dialysis fiber into the subcutaneous tissue and perfusion of the fiber with isotonic glucose-free fluid. Due the concentration gradient, glucose diffuses from the interstitial fluid through the dialysis membrane into the perfusate. By including a glucose sensing mechanism outside the body in the path of the perfusate, the glucose concentration can be measured continuously.

This method is advantageous in that a foreign body reaction is avoided and hence continuous monitoring without a pronounced signal drift is possible. A significant disadvantage, especially because applications such as continuously monitoring of brain analytes mandates the use of a method that can rapidly detect transient changes in concentrations, is that there is an inherent time lag of 5-45 minutes associated with this technique\textsuperscript{74}. This delay depends on the length, the inner diameter of the tubing, and the perfusion flow rate selected (between 0.1 and 10\textmu{l/min}). This method also requires recalibration at least once daily. Also, the procedure may buffer transient changes\textsuperscript{75}, is imprecise because the dialysate concentration with respect to those in the body concentration never reaches 100\% (the ratio depends on instrumental variables such as perfusion rate, surface area, and molecular weight cutoff of the membrane, and physiological factors such as temperature, analyte species, analyte metabolism etc.), also the sampling procedure may cause a depletion of the analytes and hence is an underestimation of the concentrations. Although incorporation of capillary
electrophoresis (CE) and laser-induced fluorescence (LIF) detection\textsuperscript{76}, and microfabricated microdialysis\textsuperscript{77} have been suggested to improve temporal resolution, none are commercially available to date for \textit{in vivo} applications.

2.4.2.4 Fiber and Submicron Fiber Probes. Fiber optic probes for glucose sensing based on both glucose oxidase and competitive binding assays have been developed by various groups of researchers\textsuperscript{78-83}. The sizes of these fiber probes range from millimeter to submicron diameter. The sensing chemistry is immobilized at one end of the fiber, and a light source and detector are connected to the opposite end. The larger probes suffered from disadvantages such as limited spatial resolution and sensing applications due to size. Also, the response time and sensitivity are reduced because methods of immobilization of sensing chemistry increase diffusion lengths. These problems were alleviated by the development of submicron fiber-optic probes. However, these probes still had a limited lifetime (4-6 days) because of the loss of sensing chemistry from the immobilized polymer matrix.

2.4.3 Non-Invasive Sensing Techniques

The human skin allows penetration of light up to some centimeters into the skin only in the near infrared region (NIR, 600-1300nm) - the so called “optical window.” Above or below this optical window, light is absorbed by water, skin pigments, or blood within the outer layers of the skin\textsuperscript{84}; primarily, light in the NIR region can penetrate to the deeper blood-perfused skin layers, potentially allowing glucose monitoring.

Some of the other technologies which are at the preclinical stage and have not yet been established as precise methods are polarimetry, far infrared radiation spectroscopy, radio wave impedance, and pulsed photoacoustic spectroscopy\textsuperscript{85,86,87}. 

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2.4.3.1 Spectroscopic Techniques. Glucose shows a characteristic absorption spectrum called “fingerprint” when a light beam is directed through a cuvette with a clear solution (otherwise, light scattering causes an additional attenuation of light) of glucose in water. Spectrophotometry, which is a well established method for quantification of solutes in liquids, is based on solute-specific absorption bands in the visible (VIS), near infrared (NIR) or mid infrared (MIR) spectral range. Using this method, solutes are quantified by determination of light attenuation caused by absorption at a single wavelength (taking light path length into account). Hence, using high performance spectroscopic equipment in combination with sophisticated mathematical calibration procedures, ex vivo quantification of glucose in complex matrices such as plasma, serum, or whole blood is possible using this approach. MIR radiation is particularly appropriate for this purpose because glucose exhibits specific absorption bands in this region. In vivo measurements in the skin, however, are difficult to make using MIR because of absorption by water. Hence, the in vivo penetration depth of MIR light in the skin is low. Light in the NIR and VIS regions penetrates into deeper blood perfused skin layers and provides an optical window for glucose monitoring. However, glucose does not exhibit specific absorption properties in this region.

Despite more than 25 years of research, patents and publications, the major challenge with this approach has been to achieve sufficient precision within the clinically relevant blood glucose range over prolonged periods of time. The various problems associated with optical spectroscopy have been 1.) glucose has no specific absorption pattern in the near infrared region; 2.) low glucose concentration occurs in the tissue; 3.) scattering greatly reduces the absorption signal; 4.) high concentrations of other light
absorbers such as water interfering with the signal; 5.) light absorption depends on temperature; 6.) heterogeneous distribution of glucose occurs in different tissue compartments, 7.) light absorbing structures are heterogeneously distributed in the skin. Due to these reasons very small and unspecific changes in the absorption spectra can be seen by changes in blood glucose.

2.4.3.2 Scattering. The major optical interaction mechanism in tissue is light scattering, caused by different structures in skin tissue such as cell membranes, cell organelles, collagen fibers. A photon in the NIR range is more likely to be scattered than absorbed in tissue because in this range, the wavelength of light is comparable to the size of the scattering structures. “Mie scattering” is the principal scattering mechanism that takes place in tissue when the size of the scattering particles (cell membranes, collagen, organelles, etc.) is comparable with the wavelength of light. The human skin comprises a turbid system, and the scattering of light depends on the ratio of the refractive indices of the particles to the solution. Each change in the ratio of the refractive indices of the scattering particles and solute leads to a variation in the “transparency” of the tissue.

When blood glucose increases, there is a subsequent rise in the refractive index of blood and ISF (solution), whereas the refractive index of scattering particles in the skin is not changed. Thus, the ratio is reduced and so is scattering. Thus, since the proportion of light scattered in the skin is slightly lower, the intensity of light reflected back into the skin is also lower.

The sensitivity of this method of glucose monitoring (based on light scattering properties of the human skin) is small in absolute terms but was found to be much higher than those due to glucose-specific light absorption in the NIR region. This method does
not measure glucose concentration per se, but derives glucose concentration indirectly from glucose-induced changes in the refractive indices of components of the human skin. Hence, other blood analytes and physiological factors, such as shifts in the water/plasma distribution between the intra- and extravasal compartments, may influence the scattering coefficient. Due to a significant variability of the baseline scattering coefficient, in vivo calibration of the scattering signal with respect to the prevailing blood glucose is required at regular intervals.

2.4.3.3 Fluorescence Techniques. Numerous glucose-sensing mechanisms based on fluorescence spectroscopy have been proposed and are advantageous because of their inherent characteristics of specificity and sensitivity. Numerous fluorescence-based optical sensors have been developed94-96, most use GOx for oxidation of glucose, and the fluorescence intensity is measured as a function of dissolved oxygen concentration. For oxygen sensitive dyes that exhibit quenching of fluorescence in the presence of oxygen, the relationship between the fluorescence intensity and oxygen concentration is given by the Stern-Volmer equation:

\[ I/I_c = 1 + K[O_2] \]

where \( I_0 \) is the fluorescence intensity of the sensor in absence of oxygen,

\( I_c \) is fluorescence in a given dissolved oxygen concentration \([O_2]\),

\( K \) is the Stern-Volmer quenching constant.

Some of the advantages of optical sensing schemes over electrochemical schemes are absence of electrical connection to the subject and potential for simultaneous multi-analyte sensing using spectroscopy. The rapid development of optical communication systems has produced cheap and reliable sources and detectors. Among the
disadvantages are the limited penetration depth of the optical signal due to scattering and absorption by the human skin tissue, and difficulty in calibrating changes in optical signals due to changes in analyte of interest.

Fluorescence measurements are particularly useful for totally implanted sensors, particularly by using NIR dyes (dyes that are excited and emit in the NIR region). Light in the NIR region can penetrate through several centimeters of tissue and hence; NIR probes implanted in the subcutaneous tissue can be excited and interrogated from outside the body more efficiently, as they are less affected by interference from scattering or absorption by water or other components in the skin (collagen, organelles, membranes etc.). Also, lifetime measurements are advantageous over measurements of fluorescent intensity because lifetime measurements are less affected by changing concentrations of the fluorescent label, photobleaching or light scattering in the tissues.

The following is an overview of the types of fluorescent sensing mechanisms that can be used when a certain combination of glucose receptor/enzyme and indicator can be co-immobilized. The items in the overview have been segregated on the basis of receptor/enzyme type and further subdivided into various transduction mechanisms. Finally, there is a section on some of the immobilized fluorescent sensing techniques currently being researched.

2.4.3.3.1 Fluorescence Resonance Energy Transfer (FRET) and Competitive Binding Sensors. Fluorescence resonance energy transfer (FRET) is based on the transfer of excitation energy from one fluorescent molecule (donor) to a nearby molecule (acceptor) that has overlapping spectral properties. Changes in fluorescence intensity or lifetime give an estimate of the changing distance between the donor and acceptor. Shultz\textsuperscript{97} demonstrated the basic principle of this system as illustrated in Figure 11.

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Concanavalin A (Con A) was used as a receptor for competing species of FITC labeled dextran and glucose. TRITC-Con A binds with the FITC-dextran in the absence of glucose, excitation of FITC results in TRIC emission due to FRET. With the increase in glucose concentration, FITC-dextran is displaced by glucose causing increased FITC emission. FRET systems for glucose sensing in vitro have also been developed using NIR fluorescent molecules coupled with Con A\textsuperscript{29}.

Unlike the sensing scheme using glucose oxidase, there is no consumption of substrates or production of byproducts in the competitive-binding scheme. However, it is debatable whether the sensing scheme involving the Con A-dextran system is reversible\textsuperscript{98}.

2.4.3.3.2 Boronic Acid Receptor Based Fluorescence Sensing. Synthetic boronic acid derivatives are able to complex glucose rapidly and reversibly by means of covalent interactions\textsuperscript{99}. Synthetic receptor 1,2- anthrylboronic acid was used, with the boronic acid
moiety being responsible for the binding of glucose, and the anthracene moiety for the transduction of the fluorescent signal. The major disadvantages of this system are small analytical signal, poor selectivity in the order- D-fructose>D-allose>D-galactose>D-glucose, and the ability to work only in basic media (pKa of 2-anthrylboronic acid =8.8).

The pKa (4.8) of the boronic acid receptor was improved by introducing a tertiary amine in the proximity of the boronic acid moiety\textsuperscript{100}. This improvement lead to the use of another phenomena, photoinduced electron transfer (PET) for transduction of the fluorescent signal. The fluorescence signal is quenched by the nitrogen atom via PET in the absence of glucose. Upon binding of glucose, PET from the nitrogen to the fluorophore is suppressed, which "switches on" the fluorescence signal of anthracene. The selectivity of the synthetic receptors towards glucose was also improved by a designed alignment of the two boronic acid moieties. The following selectivity was presented by this arrangement with the binding constants presented in paranthesis: D-glucose (3981 M\(^{-1}\))> D-allose (630 M\(^{-1}\))> D-fructose (316 M\(^{-1}\))> D-galactose (158 M\(^{-1}\)).

However, all these improvements do not really favor the use of this form of the acid for physiological applications due to the limited solubility of the receptors. This situation, too, was improved upon by Norrild et al by developing the ionic receptor\textsuperscript{101}. This modification however, reduced the sensitivity of the system. Another aqueous soluble system was introduced by another group\textsuperscript{102}. It comprised of two distinct parts, a pyranine playing the role of the fluorescent transducer and a discrete diboronic acid receptor. A dual-fluorescence system was designed by Heagy et al which displayed two emission bands (430nm and 550nm) after excitation at 370nm\textsuperscript{103}. It, however, had limited sensitivity at physiological glucose levels.
Lifetime based sensing was introduced by Lackowicz's group using phase-modulation methods. Upon interaction of the boronic acid receptor with glucose, there is a shift toward low-modulation frequencies indicating longer mean lifetimes\textsuperscript{104}. The same group is working on a glucose sensing contact lens for ex vivo glucose monitoring\textsuperscript{105}. They designed boronic acid based receptor which was found to respond to physiological tear glucose within disposable contact lens. Even though it showed comparable selectivity for both glucose and fructose, the authors state that the interference for such an application was minimized due the fact that concentration of fructose is tenfold lower than glucose.

2.4.3.3.3 Lectin-Based Fluorescence Sensing. Concanavalin A (Con A) is a plant sugar-binding protein which is mannose and glucose specific. It has been used in numerous fluorescence resonance energy transfer (FRET) glucose sensing mechanisms\textsuperscript{106}. In this work, two sets of dextran polymers, one labeled with rhodamine and the other with fluorescein, were used. In the absence of glucose, a multivalent lectin interacted with the dextrans of both types of polymer beads bringing them close together. FRET occurs due to the close proximity of the two fluorescent labels. These affinity sensors are based on fluorescense quenching of the donor emission by FRET. When a high concentration of glucose is added, the lectin binds to glucose instead and as a result the FRET decreases. Other work involves using TRITC-apo-GOx instead of Con A with FITC-dextran\textsuperscript{107}.

The attachment of ruthenium to Con A to form a metal-ligand complex (RuCon A) permits the selective detection of glucose. This method of detection involves using a FRET based system with RuCon A as the donor and maltose-insulin-malachite green
(MIMG) complex as the acceptor. The maltose offers the affinity of an acceptor for ConA. The method utilizes the long lifetime of ruthenium, and changes in the decay time of the complex due to the presence of glucose can be measured\textsuperscript{29}. Frequency domain intensity decays showed a decrease in the average lifetime of the RuCon A emission, which was attributed to energy transfer. Steady-state fluorescence measured for 2\textmu M RuCon A and 4 \textmu M MIMG demonstrated a consistent increase in the RuCon A emission signal as the glucose increased up to 150mM.

Another sensing mechanism used has addressed donor/acceptor distribution in FRET assays. The donor-acceptor distribution function was assumed to remain unchanged throughout a monitored FRET process (glucose sensing). Structural information can be obtained by measuring the fluorescent decay with nanosecond resolution, called distribution sensing. The donor/acceptor distribution changes were calculated at a single Forster radius for glucose concentrations ranging from 0-30mM\textsuperscript{29}.

2.4.3.3.4 Glucose Sensing Based on GBP (Glucose Binding Protein) Fluorescence Sensing. Proteins can be used as biorecognition elements because protein-based biomolecular recognition events are normally accompanied by conformational changes of the protein due to binding with the ligand. Reporter groups can be introduced into the protein structure, which respond to ligand binding. Also, a pair of fluorophores can be arranged on selected sites of the protein to allow detection of ligand binding by energy transfer.

GBP is a glucose/galactose binding protein that has binding affinity for glucose in the micromolar range. The protein has two conformations, an “open” conformation where the domains are far apart and a “closed” conformation where they come together. Thus,
biosensing involves monitoring conformational changes that occur when proteins and ligands bind to each other. Incorporation of a fluorescent reporter near the ligand-binding site allowed sensing glucose due to conformational changes when the GBP binds glucose. The labeled GBP mutant was found to be stable at 37°C for over three months, and the secondary structure of the protein is stable up to 64°C. Hence, this system was claimed to be as an ideal system for in vivo monitoring of glucose\textsuperscript{108,109}.

2.4.3.3.5 Other Protein Based Fluorescence Sensing. Thermostable proteins that are isolated from bacteria are active at room temperature and do not lose activity at shelf temperature; e.g., BSGK is a thermostable glucokinase isolated by Lakowicz's group and can be used to as a binding protein to develop a sensor for the substrate. This development is done in the absence of ATP whereby it does not convert hexose to hexose-6-phosphate (utilizing ATP).

Among the different sensing mechanisms that have been cited so far, FRET-based sensing mechanisms and those involving monitoring GBP conformational changes seem most promising. FRET-based sensing mechanisms do not require any referencing system for calibration, they have been shown to be linear in the physiological range with good response times, and analyte consumption can be avoided by using FAD deactivated enzymes. A variety of donor-acceptor pairs with long Stokes shifts are available. By using NIR fluorophores, the sensor can be made amenable to skin tissue measurements.

GBP can be used by incorporating a wide variety of reporter groups at appropriate sites on the protein. Again, either intensity changes or FRET changes can be monitored with changes in conformation of the protein as it binds the substrate. Genetic engineering of GBP has made it possible to produce more robust forms of the protein. Also, with
genetically engineered GBPs, detecting perturbations in glucose homeostasis by monitoring glucose measurement in living cells with temporal and cellular resolution is possible. Mutants can be engineered with varying binding constants to sense different concentrations of glucose at different locations in the body. For example, Formmer et al\textsuperscript{110} have developed GBP labeled with a FRET pair with a binding affinity of 0.17\textmu M for glucose to monitor cellular glucose. Yet another mutant has a glucose binding constant of 0.59\textmu M and can monitor physiological glucose (0.07-5mM)\textsuperscript{109}.

2.4.3.3.6 Enzyme Based—Glucose Oxidase, Hexo- and Glucokinases. Apoenzymes Fluorescence Sensing. Glucose oxidase (GOx) catalyzes the conversion of D-glucose and oxygen to D-glucono lactone and hydrogen peroxide. The detection of oxygen consumption, hydrogen peroxide production, or local pH change has been widely used in the development of GOx-based glucose sensors. GOx has been used in numerous biosensors due to its high selectivity over other sugars to glucose, ex vivo enhanced enzyme stability, and low cost.

This inner filter effect between FAD and GOx-FC (GOx is labeled with fluorescein) can be used as a transduction mechanism\textsuperscript{111}. The molecular adsorption of FAD overlapped with the molecular adsorption of GOx-FC (creating the inner filter effect) but not with that of FADH\textsubscript{2}. This inner filter effect is canceled in the presence of glucose due to conversion of FAD to FADH\textsubscript{2}, increasing the fluorescence of GOx-FC. However, this method is found to be insufficient in terms of sensing the physiological range of glucose values.

Among the various transduction mechanisms of glucose oxidase-based optical sensors, the sensing of oxygen consumption by using an oxygen-sensitive dye, or use of
pH sensitive polyacrylamide polymer gels which were functionalized with fluorescent dyes have been reported\textsuperscript{112}. Also a number of fiber-optic probes for pH sensing applications have been developed\textsuperscript{113,114}. However, to date, there have been few reports of the use of pH sensitive ratiometric dyes for measurement of glucose. Pyrene 8-hydroxy-1,4,6-trisulfonylchloride (HPTS), is a pH indicator dye that has been used by several groups to demonstrate pH sensitivity in thin films\textsuperscript{46,47}. It has numerous advantages such as large Stokes shifts, high quantum yield, high absorbance, excellent photostability and lack of toxicity\textsuperscript{45}.

Some of the other pH sensitive dyes that have been used to demonstrate pH sensitivity in thin films are pyranine\textsuperscript{115}, which is based on the analyte causing a wavelength shift in the excitation and emission spectrum; the amount of analyte can thus be correlated with the ratio of fluorescence intensities at the two wavelengths. Other pH sensitive probes include Benzo[c]xanthenes such as carboxy seminaphthorhodarfluors (SNARFs) and carboxy seminaphthofluoresceins (SNAFLs), which are long wavelength, dual emission dyes and can be excited in the green region\textsuperscript{116}. All of these have been used in fiber-optic configurations for pH sensing and not for glucose sensing. But with the incorporation of GOx, they can potentially be used for glucose sensing. The main disadvantage in using pH sensing mechanism is that it is subject to interference from global pH changes and local pH changes due to factors other than the glucose-sensing reaction. In order to overcome this problem, in addition to sensing pH changes due to oxidation of glucose, pH changes due to other reasons need to be monitored simultaneously. This can be achieved by using sensors incorporating pH-sensitive dyes with spectral properties different from those used in the glucose sensor without co-
immobilizing GOx or other enzymes which catalyze the oxidation of glucose; a combination of these, together with the glucose sensors will help differentiate between pH changes due to glucose oxidation and otherwise.

To monitor oxygen consumption, an oxygen sensitive fluorophore (ruthenium (II),4,7-diphenyl-1,10-phenanthroline)-dodecylsulfate- Ru(dpp) or Ru[dpp(SO$_3$Na)$_2$]$_3$Cl$_2$) has been used by some groups$^{25,26}$. The basic principle is the monitoring of quantity of oxygen consumed by the enzymatic reaction. The excitation and emission wavelengths of the dye are 465 and 610nm, respectively. The drawback is that they should be accompanied by an oxygen sensor to compensate the partial oxygen pressure variations from sample to sample and during the continuous monitoring of glucose.

In the case of hydrogen peroxide based sensors, not much work has been reported in optical sensing of hydrogen peroxide produced during glucose consumption by GOx. Most references to H$_2$O$_2$ sensing have been with respect to amperometric sensors. Some work has been done regarding fiber-optic biosensors for H$_2$O$_2$ and glucose$^{117}$. Arnold et al have developed two different configurations. In the first biosensor used homovanillic acid as the substrate; the H$_2$O$_2$ diffused across a microporous PTFE (Teflon) membrane and entered an internal solution of the substrate and horseradish peroxidase (HRP) and the fluorescence of a dimeric product formed was monitored as a function of time$^{118}$. The rate of the fluorescence intensity was related to the H$_2$O$_2$ concentration in the sample. In the second configuration, the substrate was luminol and the HRP-catalyzed chemiluminescent reaction between hydrogen peroxide and luminol produced light, which was measured and related to the HPR concentration. The second configuration produced faster response times and lower limits of detection. The resulting H$_2$O$_2$
biosensor was used as an internal sensing element of a glucose biosensor by immobilizing GOx on the outer surface of the gas-permeable membrane. Sukhorukov et al. reported the use of an Amplex red reagent to visualize the localization of the active enzyme into microcapsules produced by LbL assembly of biodegradable polyions, dextran sulfate, and protamine on MF cores, followed by dissolution of the core. In the presence of peroxidase (HRP), Amplex Red reacts with H₂O₂ in a 1:1 stoichiometry to produce red-fluorescent resorufin (ex 563nm and em 587nm). Though this scheme was not used to sense glucose, it could be applied in the following way. By co-encapsulation of GOx, HRP and Amplex Red, the changes in fluorescent emission of the dye can be monitored with changes in glucose consumption by GOx resulting in production of H₂O₂.

Researchers from the University of Illinois at Urbana-Champaign have created a near-infrared nanoscale glucose sensor by using carbon nanotubes as the fluorescent indicators. This sensor was fabricated by assembling a monolayer of GOx on the surface of nanotubes and then functionalizing the surface with ferricyanide (an ion sensitive to hydrogen peroxide). When glucose encounters GOx, hydrogen peroxide is produced, which reacts with ferricyanide to modulate the electronic structure and optical characteristics of the nanotube and cause it to fluoresce in the NIR region, which is very advantageous for measurements from deeper tissues or blood. The more glucose that is present, the brighter it will fluoresce. However, carbon nanotubes have been proven to be toxic.

Glucokinases in the liver and Hexokinases in most other cells are responsible for phosphorylation of glucose by utilization of ATP. Hexokinases, unlike glucokinases,
have a broad specificity and are sensitive to D-mannose, D-fructose, sorbitol, and D-glucosamine in addition to glucose\textsuperscript{123,124}. Glucokinases, unlike hexokinases, are insensitive to inhibition by glucose-6-phosphate and can respond to changes in glucose concentration in the physiological range\textsuperscript{125}. Hexokinases, on the other hand have a Km of 0.1mM and get 95% saturated at 5mM glucose; a further increase in glucose does not significantly affect glucose binding. Hence, hexokinase is ruled out as a possible glucose sensor. Only a few species, especially bacteria, are known to contain true glucokinases, i.e., enzymes specific for glucose\textsuperscript{126}. However, glucokinases with true glucose specificity are not easily available and are, therefore expensive.

An insignificant amount of work has been done in the use of hexokinases and glucokinases for glucose biosensing applications\textsuperscript{127,128,129} perhaps due to high cost and lack of availability. However, if recombinant forms of these enzymes could be produced, it may aid in mass production and hence decrease in cost. One group has reported (though the work has not been published yet) the use of a conformational change in heterokinase on glucose binding, and the optical monitoring (intrinsic fluorescence or FRET) of this change, both in solution and after entrapment, in a sol-gel matrix. The intrinsic fluorescence has been attributed to tryptophan (ex 290nm, em 330-340nm); it decreased with increasing glucose concentration. Also, extrinsic covalent labeling of the enzyme with RB1TC (donor) and Cy5.5 (acceptor) led to changes in FRET with changes in glucose concentration\textsuperscript{130}.

D'Auria et al presented an approach for reversible GOx fluorescent sensors by using apo-GOx (obtained by removal of FAD, which still binds glucose with comparable affinity to that of the holoenzyme, but does not consume glucose)\textsuperscript{24}. Apo-GOx interacts
non-covalently with the fluorophore ANS (8-anilino-1-napthalene sulfonic acid) with excitation wavelength at 325nm and emission at 480nm. The fluorescence intensity and mean lifetime of ANS decrease with an increase in glucose concentration. These give reversible sensors; however, the dynamic range of the sensors was not found to be satisfactory.

The same group demonstrated a similar mechanism with glucose dehydrogenase (GD)\textsuperscript{131}. The main advantage of the apoenzyme sensors is that there is no consumption of the analyte and no formation of byproducts such as gluconic acid and hydrogen peroxide, which may cause inflammatory responses in the body.

Hence, an ideal system for glucose sensing must not consume analytes, is non-toxic, is unperturbed by changes in local oxygen or pH, and yet has the range and sensitivity to measure physiological glucose changes. If glucokinases can be mass produced by DNA recombinant technology, which will also reduce the cost, then it may be ideal to label it with a FRET donor-acceptor pair of NIR dyes (cy5 and cy5.5). In the absence of ATP, glucose is not consumed (phosphorylated), and there are no reaction products. Since the transduction mechanism has nothing to do with sensing changes in oxygen or pH, it is not prone to interference due to random changes in oxygen partial pressure or pH. It has also been reported that glucokinases can respond to changes in glucose in the physiological range.
2.5 Immobilization Methods for Dyes and Enzymes Used in Optical Sensors

2.5.1 Fiber-Optic and Non-Fiber Optic Immobilization Methods

The early use of fluorescent probes involved injection of liquid fluorescent indicator dyes into the sample and analysis using a spectrometer or microscope. This procedure has numerous disadvantages including toxicity to the biological sample, compartmentalization of the dye and leakage over time, and hence requires reintroduction of the dye. For these reasons, sensor systems involving immobilization of the dye using microelectrodes and special sub-micron fiber optic probe tips have been developed\textsuperscript{132,133,134}. Encapsulation of enzymes is primarily aimed at using a semi-permeable support matrix to entrap the enzyme, prevent its leaching out of the matrix, while allowing exchange of analytes, products and co-factors between the encapsulated enzyme and the surroundings\textsuperscript{135}. Liposomes\textsuperscript{136}, microspheres prepared by microemulsion techniques\textsuperscript{137,139}, and silica matrices\textsuperscript{20} are among the various carrier systems used for various chemicals, drugs and biomacromolecules. Medical applications of such micro- and nanocontainers include using them as protective shells for cell and tissue encapsulation or as carriers for drug-delivery.

Among the non-fiber optic micro and nanosensors are the probes encapsulated by biologically localized embedding (PEBBLEs), ranging in sizes from 20 nm to 200 nm in diameter that have been developed by the Kopelman group at the University of Michigan. Microemulsion techniques were used to fabricate these probes, which consist of a polymer matrix containing dyes and/or enzymes. The main advantages of these sensors...
are protection of the dye from the environment and vice versa, ability to immobilize multiple dyes for multianalyte monitoring and ratiometric monitoring, a good correlation between in vitro and in vivo calibrations, and a biocompatible surface provided by the polymer matrix. Though PEBBLEs have been used for intracellular monitoring (pH\textsuperscript{138}, glucose\textsuperscript{138}, sodium\textsuperscript{139}, calcium\textsuperscript{142}, zinc\textsuperscript{140}, oxygen\textsuperscript{141}, potassium\textsuperscript{142}), they have a few significant disadvantages. There is no guarantee of uniformity from sensor to sensor within the same batch and from batch to batch fabricated using the same process, due to the nature of the emulsion process. Another problem is leaching of the encapsulated chemistry over time\textsuperscript{143}. Since, PEBBLEs are typically less than 200nm in diameter, they are useful for intracellular monitoring but unsuitable for use as “smart tattoos” because the size of implants plays an important role in phagocytosis\textsuperscript{144}. Implants or substances in particulate form are of a size that can easily be phagocytosed by macrophages.

### 2.5.2 Layer-by-Layer Assembly (LbL)

Electrostatic layer-by-layer self-assembly (LbL) has been employed for highly controlled assembly of charged polyions (polymers), nanoparticles, proteins, dyes and other supramolecular species onto arbitrary surfaces of any shape and size. It is possible to design ultrathin ordered films in the range of 5 to 1000 nm, with precision better than 1nm, and a definite knowledge of their molecular compositions. The method consists of alternate deposition of oppositely charged polyions after re-saturation of the terminal charge after each layer is deposited. The forces governing spontaneous assembly of layers are primarily ionic and covalent in nature; however, they can also include hydrogen bonding, hydrophobic, and other types of interactions. This idea also implies that there are no major restrictions on the choice of polyelectrolytes.
There have been numerous reports demonstrating assembly of polyions and dyes, viruses and charged nanoparticles, and organized protein multilayers on charged substrates. Proteins have been assembled by alternate electrostatic adsorption with positively charged polyions such as polyethyleneimine (PEI), poly(allylaminehydrochloride) (PAH), poly(dimethyldiallyl ammoniumchloride) (PDDA), and chitosan or with negatively charged PSS, DNA, and heparin. Proteins are maintained sufficiently charged under experimental conditions by setting the protein solution pH apart from the isoelectric point. There are numerous references to assembly of water soluble proteins in alternation with oppositely charged polyions, including cytochrome c and P450, lysozyme, histone type YIII-S, myoglobin (Mb), pepsin, horseradish peroxidase (POD), hemoglobin, glucoamylase (GAM), concanavalin A (Con A), albumin, glucose oxidase (GOx), catalase, invertase, diaphorase, acteriorhodopsin, and immunoglobulin IgG.

The pioneering work of Iler first demonstrated the principle of film formation by alternate adsorption of charged colloids and proteins; a related method was later introduced for film assembly by alternate adsorption of linear polycations and polyanions. This method has been illustrated in Figure 12. Polyions are used at pHs that ensure a high degree of ionization.
A negatively charged solid planar substrate is immersed in a solution of cationic polyelectrolyte, thus adsorbing a layer of polycation. Since a relatively high concentration of polyelectrolytes is used, a number of ionic groups remain exposed, thus reversing the surface charge and preventing further polycation adsorption. The substrate is then rinsed and immersed in a solution of anionic polyelectrolyte. The adsorption of the anionic polyelectrolyte layer reverses the original surface charge.

Various templates have been used for the fabrication of microcontainers such as polystyrene, melamine formaldehyde (MF)\textsuperscript{156,157,158}, metal particles\textsuperscript{159}, inorganic crystals\textsuperscript{44} and biological cells\textsuperscript{42,160}. Figure 13 illustrates how the assembly of polyions on
flat supports can be extended to charged solid spherical supports (polystyrene, MF and silica particles 0.1-5μm).

![Diagram of layer-by-layer film assembly on spherical solid supports](image)

Figure 13. Schema of layer-by-layer film assembly on spherical solid supports (Source: Dissertation, Mengyan Li, 2003)

This method can be used to create hollow microcapsules by removal of the solid template post polyion multilayer assembly for encapsulation of dyes$^{161,162}$, enzymes$^{163}$ and other macromolecules$^{62}$, or the template can be retained for creation of complex catalytic particles. The process involves addition of polyelectrolyte solution to a suspension of particles. After adsorption saturation is reached, the particles are separated from the polyelectrolyte solution by centrifugation or filtration and then rinsed and exposed to the oppositely charged polyelectrolyte solution.

2.5.2.1 Encapsulation Techniques for Macro/Biomacromolecules. Over the last ten years, the use of electrostatic layer-by-layer assembly to assemble particles and molecules in a highly controlled manner has advanced rapidly$^{164,165,166}$. Multilayer coatings of thickness in the range of 5 to 1000nm, with precision better than 1nm and a definite knowledge of their molecular compositions, have been fabricated on both solid supports as well as charged micro- and nano-sized particles to produce hollow core
shells. This method is also advantageous in that the fabrication process is easy and repeatable and does not require complicated and expensive equipment. Various templates have been used for the fabrication of microcontainers such as polystyrene, melamine formaldehyde (MF)\textsuperscript{167,168,169}, metal particles\textsuperscript{170}, inorganic crystals\textsuperscript{44} and biological cells\textsuperscript{42,43}. Also, several groups have demonstrated encapsulation of biomolecular and non-biomolecular materials by different methods.

The following is an elaboration of the use of LbL in encapsulation of macromolecules. Although some of these techniques have been used to encapsulate non-enzymatic macromolecules, the techniques have been described as potential methods for the purpose.

Encapsulation of urease\textsuperscript{163} and FITC-dextran\textsuperscript{171} into prefabricated capsules by solvent and pH induced variations in arrangement of polyelectrolytes in the capsule wall has been reported. The capsule architecture used was \{PSS/PAH\}_4 and the MF template was used. It was shown that the capsule walls are in the open state at low pH (pH 3) and closed state at high pH (pH 10). Hence, the opening of pores allows the FITC-dextran (MW 45000) to penetrate the capsule interior. The closing of pores at pH 10 ensured that the capsules remained loaded with FITC-dextran. The open state for FITC-dextran was observed for pH values up to 6. Most of the capsules were closed from pH 8 and above. The capsules were initially exposed to a FITC-dextran solution at pH 3. Then the pH was shifted to 10, and the rest of FITC-dextran was removed from the bulk by centrifugation. Similar results were obtained for MW 2000000 FITC-dextran.

It was observed that the transition between the open and closed state of the polyelectrolyte multilayer capsules introduced by adding ethanol in aqueous solution is
The permeability of capsules \( \{\text{PSS/PAH}\}_4 \) (MF template) for high molecular weight compounds changes from closed to open by adding water and ethanol respectively to a capsule suspension. For encapsulation of urease, the enzyme was first exposed to polyion capsules in a water/ethanol mixture, then ethanol was removed after centrifugation, and the capsules were resuspended in water. At this point, their walls were closed and the urease was captured inside. This process has been illustrated in Figure 14.

It has been speculated that there is a segregation of the polyion network in water/ethanol media. This segregation leads to defects in the shell, and the pores might be open big enough to pass 5-nm diameter urease globules through the wall. Returning the capsules into pure water relaxes the polyion walls to a closed state again. The encapsulated urease was found to retain 13% of its activity compared with the free enzyme.

Figure 14. Illustration of encapsulation of materials by changing solvent\(^{161}\)

The **advantages** of the above methods include the ease with which enzymes and drugs can be encapsulated for sustained release and other drug-delivery applications after treatment with pH or salt treatment. Selective wall permeability allows for substrates and
reaction products to diffuse rather easily through the walls while the encapsulated enzymes are kept in the capsules. The disadvantages of the methods include the inherent reversibility of the mechanism and dependence of the processes on external environment. Hence, they are not suitable for all applications, especially in the case of biosensors where an irreversible change in the physico-chemical properties of the wall architecture is warranted in order for the encapsulated material to be retained.

Another method of encapsulation involves the use of microcapsules as reaction cages for formation of a charged internal matrix by polymerization of monomeric units by enzyme-catalyzed or free-radical reactions¹⁷² and the use of the matrix to attract and encapsulate oppositely charged macromolecules. This technique is based on the principle that microcapsule walls are impermeable for polymers whereas monomers penetrate the wall easily. This procedure is called “ship in bottle” synthesis. Polymers and functionalized copolymers have been synthesized inside the capsules (\{PSS/PAH\}_9, MF template) from water-soluble anionic, cationic, and neutral monomers. Polymerization was carried out at various sodium styrene sulfonate (SS) concentrations in the presence of capsules for 4 h at 80°C under nitrogen atmosphere. 1% potassium peroxodisulfate was added as initiator. There was increased polymerization in the walls.

This approach was based on the selective permeability of capsule walls for monomer molecules, while the biocatalyst and the polymeric chains formed in the capsule interior were trapped inside because of their higher molecular weight. In another similar approach, horseradish peroxidase (HRP) was encapsulated into \{PSS/PAH\}_4 capsules (MF template) using pH driven pore regulation. Tyramine was used as a
monomer\textsuperscript{173}. It started fluorescing after polymerization upon addition of hydrogen peroxide to the system to catalyze the reaction.

The advantages of these techniques include use of these capsules in micromechanics by inducing a reversible swelling and collapse of the capsules by application of a high concentration of polyelectrolytes in the bulk solution and the subsequent application of a counter pressure. Due to synthesis of polymers in and on the capsule walls, it can be strongly charged and hence used as an ion exchange membrane. Moreover, they can be used to attract and entrap enzymes and proteins.

The technique is limited by the availability of suitable monomeric units which will form polymers that are oppositely charged to the enzyme of interest. Moreover, the polymers should not hamper the conformation and hence the activity of the encapsulated enzymes.

Dyes have been precipitated into hollow polyelectrolyte capsules in a controlled manner by changing the ionic or solvent composition\textsuperscript{174,175}. Also, DNA has been precipitated on manganese carbonate templates after which polyelectrolytes were adsorbed to form a protective biocompatible shell, followed by dissolution of the core material to entrap the enzyme in the hollow interior\textsuperscript{176} as shown in Figure 15.
Capsules with architecture \( \{ \text{PSS/PAH} \}_4 \) were used to demonstrate the precipitation of water soluble dyes, acid 6-carboxyfluorescein (6-CF), and base 6G rhodamine (R-6G). 6-CF was precipitated from basic solution in the presence of empty capsules by dropwise addition of \( 10^{-3} \) M HCl until supersaturation took place at pH 6. R-6G was precipitated by increasing the value of pH \( (2 \times 10^{-2} \) M NaOH). Fast and random precipitation of the dye occurs at pH 12. The cationic cyanine dye 1,1'-diethylcarbocanin (pseudoisocyanine, PIC) was precipitated by the addition of \( 10^{-3} \) M solution of ammonium hexafluorophosphate to a solution of PIC bromide \( (10^{-3} \text{M}) \) and capsules. The crystals showed a narrow fluorescence band after aggregation.

Yet another method of encapsulation involved formation of protein (chymotrypsin) aggregates followed by LbL adsorption of polyelectrolytes\(^{177-179} \). This encapsulation was performed by salting out the proteins by mixing with appropriate volume of saturated solution of NaCl \( (0.01 \text{N HCl and NaCl 1:1 by volume}) \) yielding

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particles between 0.1\(\mu m\) to 0.4\(\mu m\) in dimension. The advantages of these methods include the fact that they are simple, and they obtain high encapsulation efficiency.

These methods, however, may cause denaturation of enzyme and other biomolecules due to the use of extreme pHs and solvents. Also, crystallization may lead to an unfavorable conformation of the enzymes and hence render them inactive (though the protein crystals were found to retain 73% of their activity as compared with free enzymes, which lost 56% of activity after six days. This result may not hold true for all proteins, enzymes and biomolecules).

Low molecular weight dyes have been encapsulated by first coating them as weak inner sacrificial layers capped by layers of strong polyelectrolytes (the weak layers dissociate when the template is dissolved, whereupon they get encapsulated in the capsule interior)\(^{180}\). Multilayer films composed of Tb3+/polystyrene sulfonate (PSS) and 4-pyrene sulfate/polyallylamine (PAH) were successfully assembled on polystyrene sulfonate (PS) and MF latex particles.

Dissolution of the MF particles leads to formation of hollow capsules with the release of multivalent ions and the entrapment of the inner polyelectrolyte layers, due to selective permeability of the walls. The simplicity of the method is its main advantage. If this method were used to encapsulate biomolecules, they would be subjected to very low pHs. Hence, they may be selectively appropriate for encapsulation of biomolecules.

Some groups have reported the encapsulation of macromolecules into capsules fabricated using MF template due to the formation of a gel-like matrix comprising of the inner polyelectrolyte layers and MF. A case in example is the loading of horseradish peroxidase (HRP) into capsules “spontaneously” due to electrostatic attraction between
the positively charged enzyme and the negatively charged complex of the innermost anionic layer, namely, poly (styrene sulfonate, sodium salt) (PSS) and MF oligomers formed during the dissolution of the core\textsuperscript{181}.

However, it has also been pointed out that this matrix formation does not occur in all cases, and that to a large extent it is dependant on the nature of polyelectrolytes used to coat the MF\textsuperscript{16} and in some cases the age of the capsules\textsuperscript{182}. Hence, depending on the nature of interaction between the template and the multilayers, microcapsules that are completely hollow or those with internal charged matrices are produced. Other examples include encapsulation of $\alpha$-chymotrypsin in capsules formed by assembling 8 bilayers of negatively charged alginate/protamine followed by formation of an alginate matrix in the interior by dissolution of the core\textsuperscript{183}.

The above method has high encapsulation efficiencies since the loading is spontaneous and driven by electrostatic attraction of the dense charged inner matrix, rather than just by diffusion. A possible disadvantage is that there is some MF in the capsule interior and may be toxic. However, long-term studies have not been done with in-vitro experiments to prove it.

Diazoresin has been used in the past to demonstrate cross-linking with polyelectrolytes having sulfonate or carboxyl groups by photocrosslinking reaction as shown in Figure 16. Recently, hollow capsules were fabricated with 5$\mu$m manganese carbonate (MC) templates, by depositing \{PSS/PAH\}\textsubscript{1}+\{PSS/PAH\}\textsubscript{5}+\{PSS/PAH\}\textsubscript{1} and dissolving the core with 0.1M HCl. Loading of RITC-GOx, RITC-HRP, and FITC dextran was demonstrated followed by irradiation with UV lamp for five minutes to crosslink the multilayer wall (refer Figure 17)\textsuperscript{184}. This method is useful for biosensors,
where a permanent alteration of wall properties is desired for stable encapsulation of the molecules. However, diazoresin is a known carcinogen.

![Diagram of crosslinking of diazoresin]

Figure 16. Crosslinking of diazoresin

![Diagram of fabrication, loading, and crosslinking of capsules with DAR in their walls]

Figure 17. Fabrication, loading and crosslinking of capsules with DAR in their walls.

Though alginate-based hydrogels have been used for encapsulation of materials before, the high porosity of the matrix has made it difficult to retain macromolecules
inside the alginate matrix, leading to leaching out of the encapsulated material over time. However, recently, it has been shown that by coating alginate microspheres with different combinations of polyelectrolyte bilayers, it is possible to reduce leaching and maintain activity of encapsulated glucose oxidase\textsuperscript{185}. Alginate microspheres (<5μ) were fabricated using an emulsification technique and were ionically crosslinked with calcium ions; glucose oxidase was encapsulated inside. It was found that with a single bilayer of {PSS/PAH}, the total loss of enzyme was reduced to 20%. Alginate is biocompatible, and alginate microspheres have high encapsulation efficiency.

Almost all methods described above have certain advantages and disadvantages. The parameters on the basis of which they can be compared are the application/purpose, cost, repeatability, ease of fabrication, mono/polydispersity, biocompatibility, stability and activity of the encapsulated enzyme, ease and feasibility of commercialization, and effect of the location of entrapped enzyme on desired activity. Some of the methods involve use of harsh conditions such as low or high pHs, high salt concentrations, and/or organic solvents when the biomolecule is present in the capsule, or encapsulation is carried out using harsh conditions. The capsules fabricated after coating the MF and removing the core are rinsed after acid treatment and hence are under milder conditions before encapsulation of biomolecules. Some of these methods have higher encapsulation efficiency than others. However, this difference does not directly translate to higher activity of the enzyme. A tight packing of the enzyme can actually hamper enzyme activity due to diffusion constraints and the unfavorable conformations of enzymes. Also, a stable entrapment of enzymes in the walls may actually lead to higher activity due to easy availability of the enzyme to the substrate and assay chemistry. On the issue of
biocompatibility, none of these methods have been tested in-vivo to confirm biocompatibility or toxicity. The use of DAR in capsules for medical applications is questionable because of its known carcinogenicity. As far as cost is concerned, MF is expensive compared with the inorganic crystal templates. However, it does offer other advantages such as formation of charged internal matrices when certain materials are coated on it, whereas with inorganic crystals, other techniques have to be used for the same purpose.

In summary, this chapter has described the pathophysiology of diabetes, which is not the only though the single most important reason for the development of inexpensive and reliable minimally invasive glucose sensors. The exciting prospect of using “smart tattoos” for glucose sensing has also been discussed. Various totally implanted, minimally invasive, non-invasive and fluorescent sensing techniques have also been elaborated. The need for reliable encapsulation techniques for immobilization of the sensing dye and the enzyme required for sensor fabrication has been underlined; some methods for encapsulation have been discussed and application of the versatile LbL self-assembly technique to encapsulation of molecules has been elaborated. The next chapter describes the techniques used to characterize the fabrication of microcapsules and the encapsulation, activity and stability of enzyme. Also discussed are the techniques for characterization and testing of glucose sensors fabricated by encapsulation of GOx labeled with a pH sensitive fluorophore HPTS.
CHAPTER III

EXPERIMENTAL DESIGN AND THEORIES

The ultimate goals of this project were to fabricate and test glucose sensors using GOx for glucose specificity, and to delineate a method to optically sense the reactants consumed or the products of the reaction involving the enzymatic consumption of glucose. Another purpose of this research was to demonstrate the use of nanotechnology, particularly the versatile LbL technique to construct hollow microcapsules and use them as microcontainers for the encapsulation of the sensing chemistry.

With the above aims in mind, numerous experiments were performed; capsules with different wall architectures were compared for loading, stability, and activity of encapsulated GOx. The wall permeabilites of capsules with these architectures were also compared using fluorescent recovery after photobleaching. In the course of this study, it was discovered that the presence of a charged internal matrix in some capsules aided the attraction and encapsulation of oppositely charged GOx. These matrices were produced during the capsule fabrication process (during LbL assembly of polyions) due to the interaction of the polyions with the template material.

For capsules that did not have internal matrices, a novel method of polymerization of internal matrices using free-radical polymerization of acrylic acid monomers was
found to be suitable for encapsulation of HRP but not for GOx. Another method involved
the use of amino acid arginine and its polymerization using a heterobifunctional cross-
linker EDC. Finally, HPTS (a pH sensitive excitation ratiometric dye) was used to label
GOx, and the glucose sensitivity of the system was studied both in solution and after
encapsulation.

This chapter gives a detailed description of chemical techniques and
instrumentation used to perform the above experimental procedures. The first section
describes the chemical cross-linking techniques used in the fabrication of charged capsule
internal matrices. The second section describes the optical techniques used for imaging
the capsules and sensors, obtaining fluorescence spectra for glucose sensitivity tests, and
for obtaining fluorescence recovery profiles for photobleaching studies used to compare
permeabilities of capsules with different wall architectures. The third section describes
the various surface characterization techniques for studying surface topographies of
capsules, and the instrumentation and methodology for studying the coating of charged
materials on surfaces (thin films and on particles). The last section is an account on the
working of enzymes, the pH sensitive dye HPTS used in the sensor and theory behind
absorbance measurements used to assess efficiency of encapsulation, stability, and
activity of the encapsulated enzyme.
3.1 Chemical Techniques for Fabrication of Internal Matrices of Capsules

3.1.1 Cross-linking Techniques

Cross-linking is the process of using a covalent bond to chemically join two or more molecules; they contain reactive ends to specific functional groups (primary amines, sulfhydryls, etc.) on proteins and other molecules. Due to the availability of reactive chemical groups in proteins and peptides, they can be easily conjugated and studied using cross-linking methods. Among the various applications of cross-linking techniques are determination of three-dimensional structures of proteins; near-neighbor relationships; hapten-carrier protein conjugation; molecular associations in cell membranes; and preparation of enzyme-antibody conjugates, immunotoxins, and other labeled protein reagents. Cross-linking techniques have also been used to modify nucleic acids, drugs, and solid surfaces.

By performing cross-linking studies before and after the interaction occurs, conformational changes of a protein with a particular reaction can be studied. Distances between molecules can be determined by comparing cross-linkers with different arm lengths. Information can be obtained about the exposure of specific protein domains and amino acids in the tertiary and quaternary structure and may be interpreted as conformational changes in proteins by examining which cross-linkers effectively conjugate to specific domains of the proteins. Cross-linking techniques also made the study of receptors easier. Isolation of the receptor-ligand complex was possible by derivatizing a receptor with a cross-linker before and after contact with a ligand.²⁸⁶
3.1.2 Homo- and Heterobifunctional Cross-linkers

Homo-bifunctional cross-linkers have two identical reactive groups. They are used in one-step reactions to crosslink proteins, to each other, or to stabilize quaternary structures, in solution. This procedure is unsuitable when the goal is to cross-link two different proteins, as it often results in self-conjugation, intramolecular cross-linking and/or polymerization. Such procedures can be performed effectively using heterobifunctional cross-linkers, which have two different reactive groups that allow two-stage sequential conjugations. This method helps minimize undesirable polymerization or self-conjugation. Hetero-bifunctional cross-linkers are especially useful when modification of amines is problematic due to the locations of these reactive groups at the active sites. In such cases, other moieties such as sulfhydryls, carboxyls, phenols and carbohydrates are more appropriate targets. For example, cross-linkers that are amine-reactive at one end and sulfhydryl-reactive at the other end are particularly useful in such cases.

In sequential reactions involving heterobifunctional reactions, the cross-linker is reacted with one protein using the most labile group of the cross-linker first, followed by removal of excess non-reacted cross-linker and addition of the modified first protein to a solution containing the second protein where reaction through the second reactive group of the cross-linker occurs. The most widely-used heterobifunctional cross-linkers have an amine-reactive succinimidyl ester (NHS ester) at one end and a sulfhydryl-reactive group on the other end. The sulfhydryl-reactive groups are usually maleimides, pyridyl disulfides, and a-haloacetyls. Since NHS-ester stability is less in aqueous solution and is usually reacted first in sequential procedures. NHS-esters react with amines to form amide bonds. Carbodiimides are zero-length cross-linkers (EDC) and cause direct

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coupling between carboxylates (-COOH) and primary amines (-NH₂) and have been used in peptide synthesis, hapten-carrier protein conjugation, subunit studies and protein-protein conjugation.

Some heterobifunctional reagents have one reactive group that is photoreactive instead of thermoreactive. These groups are useful in case of protein-protein interaction studies where the availability of thermoreactive target groups is a factor. This option allows for specific attachment of the labile thermoreactive group first, followed by conjugation to any adjacent N-H or C-H sites, which may be initiated through the photoreactive group by UV light activation. The reactivity of the photochemical reagent allows for formation of a conjugate that may not be possible with a group-specific reagent. Photoreactive cross-linkers have low efficiency; yields of 10% are considered acceptable.

N-Hydroxysuccinimide-Esters (NHS-Esters) react with primary amines with relatively efficient coupling at physiological pH and yield stable products (a covalent amide bond is formed when the NHS-ester cross-linker reacts with a primary amine, releasing N-hydroxysuccinimide) as shown in Figure 18.

Carbodiimides couple carboxyls to primary amines or hydrazides resulting in the formation of amide or hydrazone bonds (Figure 19). No spacer exists between the molecules being coupled. Both carboxyl as well as glutamic and aspartic acid residues can be targeted, and in the presence of excess cross-linkers, polymerization occurs because all proteins contain carboxyls and amines. EDC reacts with carboxylic acid group and activates the carboxyl group to form an active O-acylisourea intermediate, allowing it to be covalently attached to the amino group in the reaction mixture. The O-
acylisourea intermediate is unstable in aqueous solutions. This instability makes it ineffective in two-step conjugation procedures without increasing the stability of the intermediate using hydroxysuccinimide. Failure to react with an amine results in hydrolysis of the intermediate and regeneration of the carboxyls. The reaction is performed between pH 4-5, though the yield of the reaction is similar between pH 4.5-7.5.

![Figure 18. Reaction of NHS cross-linker](Source: www.piercenet.com/Proteomics/)

![Figure 19. Reaction of EDC cross-linker](Source: www.piercenet.com/Proteomics/)

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3.1.3 Free Radical Polymerization

Free-radical initiators are chemical substances that, under certain conditions, initiate chemical reactions by producing free radicals. These free radicals are the starting point of the free radical polymerization process which can be broken down into three main steps: *Initiation, Propagation and Termination*.

Prior to the aforementioned steps, the initiator is first decomposed into free radicals. This decomposition is followed by initiation, which involves reaction of the free radical with double-bonded monomers. This reaction leads to generation of free radical monomeric units, which further propagate the reaction by attacking other double bonded monomers. The termination of the reaction happens by a combination reaction (two free radicals react and combine to a single molecule) or a disproportionation reaction (transfer of a proton from one monomer to another leads to formation of a double bond in one).

These steps have been illustrated in the following reactions:

**Radical Generation**

\[
R-R \rightarrow 2R^* \\
\text{Initiator} \quad \text{Radicals}
\]

**Initiation**

\[
R^* + C=C \rightarrow R-C-C^* \\
\text{Monomers}
\]

**Propagation**

\[
R-C-C^* + C=C \rightarrow R-C-C-C^* \\
\]

**Termination**

\[
R-C-C^* + \overset{\text{C}}{C-C-C-R} \rightarrow R-C-C-C-C-C-R \\
\text{Polymer}
\]
In summary, this section described the chemical cross-linking techniques (homo- and heterobifunctional crosslinking agents and free-radical polymerization technique) used in the fabrication of charged capsule internal matrices.

3.2 Optical Imaging Techniques

3.2.1 Fluorescence

The past 15 years have seen a sensational growth in the use of fluorescence techniques in biological sciences. What was confined to the fields of biochemistry and biophysics until a few years ago is now being extended to applications in environmental monitoring, clinical chemistry, flow cytometry, genetic analysis by fluorescence in situ hybridization (FISH), DNA sequencing, and the study of intracellular substances by fluorescence microscopy to name a few. The high costs and handling difficulties associated with radioactive substances, combined with the sensitivity of fluorescence detection, have led to continuous development of medical tests based on fluorescence principles. Enzyme linked immunoassays (ELISA) and fluorescence polarization immunoassays are examples of this development.

Fluorescence is the result of a three-stage process that occurs in certain molecules (generally polyaromatic hydrocarbons or heterocycles) called fluorophores or fluorescent dyes. Fluorescent probes are fluorophores designed to localize within a specific region of a biological specimen or to respond to a specific stimulus. The Jablonski diagram (Figure 20) demonstrates the process responsible for the fluorescence of fluorescent probes and other fluorophores. It is a simple electronic-state diagram illustrating the processes involved in the creation of an excited electronic singlet state by optical absorption and subsequent emission of fluorescence. For polyatomic molecules in solution, the discrete electronic
transitions represented by $S_0$, $S_1$, $S_2$, etc., are replaced by rather broad energy spectra called the fluorescence excitation spectrum and fluorescence emission spectrum, respectively. The bandwidths of these spectra are parameters of particular importance for applications in which two or more different fluorophores are simultaneously detected.

![Jablonski diagram](http://elchem.kaist.ac.kr/vt/chem-ed/quantum/jablonsk.htm)

**Figure 20. The Jablonski diagram**
(Source: http://elchem.kaist.ac.kr/vt/chem-ed/quantum/jablonsk.htm)

The entire fluorescence process is cyclical; the same fluorophore can be repeatedly excited and detected unless the fluorophore is irreversibly destroyed in the excited state (an important phenomenon known as photobleaching). The fact that a single fluorophore can generate many thousands of detectable photons is fundamental to the high sensitivity of fluorescence detection techniques. With few exceptions, the fluorescence excitation spectrum of a single fluorophore species in dilute solution is identical to its absorption spectrum. Under the same conditions, the fluorescence emission spectrum is independent of the excitation wavelength, due to the partial dissipation of excitation energy during the excited-state.
lifetime. The emission intensity is proportional to the amplitude of the fluorescence excitation spectrum at the excitation wavelength as shown in Figure 21. Currently, fluorophores used as fluorescent probes offer ample permutations of wavelength range, Stokes shift, and spectral bandwidth to satisfy demands imposed by instrumentation, while allowing flexibility in the design of multicolor labeling experiments. The fluorescence output of a given dye depends on the efficiency with which it absorbs and emits photons and its ability to undergo repeated excitation/emission cycles.

![Excitation and fluorescence emission spectra](http://www.zeiss.com/C12567BE0045ACF1/allBySubject/A0ACC4A12EA4EF86C1256AEA0050794E)

Figure 21. Excitation and fluorescence emission spectra

Important parameters for quantification of absorption and emission efficiencies are the molar extinction coefficient ($\varepsilon$) for absorption and the quantum yield (QY) for fluorescence, both of which are constants under specific environmental conditions. The value
of $\varepsilon$ is specified at a single wavelength (usually the absorption maximum), whereas QY is a measure of the efficiency of conversion from excitation to emission.

With the availability of a wide spectrum of reagents, fluorophores, and derivatization methods for the development of fluorophores with improved sensitivity, selectivity and applications, the use of dye indicators for ions ($\text{Na}^+$, $\text{K}^+$, $\text{Cl}^+$, $\text{Ca}^{2+}$, $\text{Mg}^{2+}$, $\text{Zn}^{2+}$), pH etc. to study intracellular events and related extracellular dynamics has grown tremendously. However, there are limitations to the current technology, in that liquid-based indicators after introduction into cells leak over time, get compartmentalized within organelles, are toxic and cannot be used for long-term monitoring without being reintroduced. Hence, encapsulated-dye-based monitoring systems offer several advantages over liquid indicators and, for this reason, great potential. In combination with analyte specific receptors or enzymes, they can respond to specific reactions in the body.

3.2.2 Confocal Microscopy

New and improved synthetic fluorescent probes and genetically engineered fluorescent proteins, a wide spectrum of laser light sources coupled to very accurate acousto-optic tunable filters, and more advanced software packages combined with high performance computers have led to one of the most significant advances in optical microscopy, namely, the refinement of laser scanning confocal microscopy (LSCM). Confocal microscopy has several advantages over conventional wide-field optical microscopy, namely, ability to control the depth of field, ability to take serial optical sections from thick specimens, and reduction of background information from planes away from the focal plane.
The basic principle of the confocal approach involves the use of spatial filtering techniques to eliminate out-of-focus light or glare in thick specimens from planes other than the plane of focus. The tremendous popularity of confocal microscopy in recent years has been due to the ease with which high quality images can be obtained, and the growing number of applications in cell biology for imaging both fixed and living cells and tissues.

Figure 22 shows a schematic of the optical train in a typical confocal microscope, indicating all the vital optical components. Point A in the specimen is in the focal plane. The laser beams focus at the observation point; fluorescent light generated by the specimen (red line) enters the overhead detector.189

![Figure 22. Schematic of a Confocal Microscope](http://www.nikon.co.jp/main/eng/society/tec-rep/confocal_02_e.htm)
Light from the specimen’s surface and the underside (green and blue lines) also heads towards the detector. The Z axis indicates the depth of the specimen, which is crucial mapping information. The image blurs if light from anywhere other than the focal plane enters the detector. The pin-hole, which allows light only from the observation point (point A) to enter the detector, prevents such blurring.

3.2.2.1 Optical slicing of 3-D specimens. As shown in Figure 23, numerous points along the Z axis of the specimen can be imaged or examined in order, just as if the specimen has been sliced into segments. This information can be fed into a computer, and 3-D reconstruction software can be used to obtain a 3-D image of the specimen by combining all the sections.

![Figure 23. Concept of optical slicing of specimen using confocal microscope (Source: http://www.nikon.co.jp/main/eng/society/tec-rep/confocal_02_e.htm)](image)

The confocal system’s ability to discriminate between slices of the specimen made it invaluable in imaging capsules and identifying the area of localization of the
enzyme. Imaging with a fluorescent microscope made it impossible to distinguish between entrapment of the enzyme in the walls and encapsulation in the interior because of the out-of-focus blur.

3.3 FRAP – Application to Studying Capsule Wall Permeabilities

3.3.1 FRAP History and Applications

Fluorescent recovery after photobleaching experiments were started over 30 years ago as a non-invasive technique to visualize the lateral diffusion and dynamics of fluorescent proteins in living cells, especially over the last 10 years after non-invasive fluorescent tagging became popular using the green fluorescent protein (GFP)\textsuperscript{190,191}. This research facilitated the performance of FRAP on living cells without disruption by microinjection. FRAP has been used extensively to study the lateral diffusion of membrane-associated proteins has been used to study protein localization, dynamics, and interactions with other components in living cells. Also, membrane continuity and mobility of molecules within specific cell compartments has been visualized.

FRAP can also be used to study events during cell division and signaling as well as to measure protein interactions, conformational changes, and protein dynamics. FRAP has also been used to study diffusion of macromolecules in cross-linked polymer solutions and gels\textsuperscript{192}. An important example is the study of effect of enzyme treatment on diffusion of macromolecules in guar galactomannan and titanium guar hydrogels. These hydrogels are extensively used in petroleum, textile, and food industries because of advantages such as, natural abundance, biodegradability, low cost, and unique ability to alter rheological properties.
In most of the studies involving the study of lateral diffusion of adsorbed proteins, data acquisition was performed by using a photomultiplier tube (PMT), which measures the total intensity in a single spot\textsuperscript{192}. The diffusion coefficient was obtained by fitting the plot of the total fluorescence intensity versus time after bleaching to a diffusion model. This method did not provide spatially resolved fluorescence intensities, and hence was an exchange of protein between the surface and the bulk. Thus, changes in total intensity contributed from factors other than lateral diffusion would also be attributed to lateral diffusion. Using a charge-coupled device (CCD) camera helps in direct visualization of the bleached and unbleached areas and the measurement of intensity profiles showing surface mass transfer and fluorescence recovery. These intensity profiles can be normalized relative to those prior to bleaching. Hence, by changing the measurement method, the reliability of can be improved and non-standard transport mechanisms can be detected by making use of a more complete data set. However, the potential to obtain and analyze the intensity profiles within the microscope filed of view has not been fully utilized to date. Another method of improving the reliability of FRAP is by obtaining spatially resolved fluorescence data by scanning confocal fluorescence microscopy, by which the collected intensity can be limited to the focal plane. It thus enables the performance of 2D diffusion measurements in bulk samples.

Yet another application that merits the use of using CLSM is the study of diffusion of macromolecules through cartilage\textsuperscript{193}. This study is important because the transport of solutes is critical to the normal function of articular cartilage and is affected by the local structure and composition of the cartilage, which is dependent on depth from the tissue surface. Magnetic resonance imaging (MRI) and nuclear magnetic resonance
(NMR) have been used to track the movement of only very small molecules and ions. Tracking of radiolabeled solutes and fluorescence desorption allow for tracking of a variety of molecules. They measure spatially averaged diffusion coefficients over large volumes and for this reason are not reliable.

3.3.2 What is FRAP?

FRAP (also microphotolysis) is the process by which irreversible photobleaching of fluorescent molecules (permanent destruction of the fluorescence of molecules) is carried out in a small area of the region of interest by a high-powered focused laser beam. The geometry of the bleached spot can be either a spot or parallel stripes (P, or pattern photobleaching)\textsuperscript{194}. Subsequent diffusion of non-bleached fluorescent molecules from the surrounding area into the bleached area leads to fluorescence recovery in the previously photobleached area, which is recorded at low laser power, and the diffusion coefficient (D) of the fluorescent molecules can be derived. The rate of recovery of the fluorescent signal is a measure of the mobility of the species in the system. The amount of recovery is a measure of the fraction of the species that is mobile in the observed region\textsuperscript{195}. The lateral diffusion of fluorescent molecules into the bleached spot can be modeled on the basis of two assumptions: all fluorescence recovery is due to two-dimensional diffusion in an infinite plane, and that there is no diffusion in or out of the spot during bleaching\textsuperscript{194}. Bleaching during measurements is assumed to be also ignored.

3.3.3 Principle of FRAP

Two parameters regarding mobility of a fluorescent molecule in a defined compartment can be obtained from, namely, the mobile fraction of fluorescent molecules
and the rate of mobility, which is related to the characteristic diffusion time $\tau_D$. The mobile fraction can be determined by comparing the fluorescence in the bleached region after full recovery ($F_\infty$) with the fluorescence before bleaching ($F_i$) and just after bleaching ($F_0$), as shown in the typical fluorescence recovery curve in Figure 24.

![Fluorescence recovery after photobleaching (FRAP).](image)

The mobile fraction is given by $R$ defined by:

$$R = \frac{(F_\infty - F_0) / (F_i - F_0)}$$

The mobile fraction is affected by the interaction between fluorescent molecules with other molecules and by presence of membrane barriers and microdomains in membranes, which are discontinuities that can prevent or temporarily restrict the free diffusion of membrane molecules. In the context of study of cellular proteins in living cells, when motion due to active transport or unidirectional flow can be ruled out, protein mobility is due to Brownian motion. The mobility is expressed in terms of diffusion
coefficient, $D$, which is related to the characteristic diffusion time $\tau_D$, which is given by the two-dimensional diffusion equation described by Axelrod et al.

$$\tau_D = \frac{\omega^2 \gamma}{4D}$$

$\omega$----radius of focused circular laser beam at the $e^{-2}$ intensity

$\gamma$----correction factor for the amount of bleaching

The equation holds good for unrestricted two-dimensional diffusion in a circular bleached area and assumes that there is no recovery from above and below the focal plane. The Stokes-Einstein formula describes unrestricted diffusion of a particle in a free-volume model:

$$D = \frac{kT}{6\pi\eta R_h}$$

$T$------absolute temperature

$\eta$------viscosity of solution

$k$------Boltzmann constant

$R_h$------hydrodynamic radius

The diffusion coefficient $D$ is determined mainly by $R_h$ because the temperature and viscosity remain constant during bleaching. The diffusion coefficient is directly proportional to the inverse of the cube root of the molecular mass ($D \sim M^{-1/3}$), when the diffusing molecule is assumed to be a sphere with a volume proportional to its molecular mass.

New FRAP recovery equations for three-dimensional apertured scanning using a Gaussian approximation for the axial beam profile have been successfully developed and used to extract lateral diffusion coefficients from CLSM experimental data. Two-
dimensional diffusion coefficients of fluorescent species can be successfully measured by FRAP using CLSM; a big advantage of using this technique is that bleaching can be targeted at a well-defined volume element in bulk samples. Diffusion coefficients as high as $2 \times 10^{-6}$ cm$^2$/s can be measured. FRAP can be performed within a conventional microscope. A laser beam is focused into a specimen placed on a stage of a light microscope. The fluorochrome is excited as well as bleached by the laser; an acousto-optic device switches between low laser intensity for measuring and high laser intensity for bleaching. A photomultiplier is used for measuring the intensity of the fluorescent signal. This system suffers from the dearth of good imaging and probing techniques for the bleach experiment; the laser beam cannot be positioned for exact local bleaching. On the other hand, using a CLSM, images can be obtained from a two-dimensional area located at a precise depth inside a specimen. The CLSM also has the advantage of three-dimensional laser beam probing and three-dimensional imaging. Thus FRAP can be performed on well-defined volume elements in a sample.

Axelrod et al (1976) developed the FRAP theory, and this theory is valid for the idealized case of pure two-dimensional diffusion monitored by a laser beam with a Gaussian intensity profile$^{195}$. The normalized recovery is given by:

$$f(t) = \sum_{n=0}^{\infty} \frac{\lambda^n}{n!} \frac{1}{1 + \left(\frac{t}{\tau_D}\right)^2}$$

(5)
k is the bleach constant given by

\[
\frac{F_0}{F_i} = e^{-k}
\]  

(6)

τ₀ is the 2-D characteristic diffusion time, which is related to the 2-D diffusion coefficient, D by

\[
τ₀ = \frac{ω^2}{4D}
\]  

(7)

ω → half the width of the Gaussian profile of the focused laser spot determined at \(e^{-2}\) times the height of the profile. D(cm²/s) is defined by

\[
D = kT/6\pi\eta R_h
\]  

(8)

k → Bolzmann’s constant

T → temperature (K)

\(\eta\) → viscosity (N.s/m²) of the medium

\(R_h\) → effective radius (m) of the diffusing particles

The fluorescent recovery when an immobile fraction is present, e.g. due to irreversible adsorption, is given by

\[
F(t) = F(i)[1 - R[1 - f(i)]]
\]  

(10)

Where F(i) is the intensity of the bleach spot before bleaching, and R is the mobile fraction defined by equation x above.

By using FRAP and the inherent advantages of a CLSM system, permeabilities of various hollow capsule architectures to labeled GOx were compared. This comparison is essential for understanding how the composition of the wall affects the loading of enzyme and the leaching after loading.
3.4 Surface Characterization Techniques

3.4.1 Atomic Force Microscopy (AFM)

There are about two dozen types of scanned proximity probe microscopes that work by measuring a local property (height, magnetism, optical absorption, etc) with a probe or "tip" placed close to the sample. The ability of these devices to image at atomic resolution together with the ability of most of these devices to image a wide variety of samples under a wide variety of conditions has facilitated its usage in the study of biological structures.\footnote{196} Typical applications include materials evaluation (surface roughness of silicon wafers, surface profiles, and magnetic field mapping of recording media or reading heads), failure analysis (defect analysis of compact disk stampers), and quality control (surface finish of substrates for thin film deposition).\footnote{197} The AFM works by measuring attractive or repulsive forces between a tip and the sample. Unlike its predecessor the scanning tunneling microscope (STM), which maps the surface topography by measuring the tunnel current between an atomically sharp tip and a metallic sample, the AFM can be used to image insulated samples. Measurements can be taken over a small area because of the small probe-sample separation (on the order of the instrument's resolution, which is typically of nanometer range\footnote{198} and can even go as low as 10pm\footnote{199}). Image acquisition is done by a raster scanning the probe over the sample while measuring the local property in question.

The concept of the AFM and the optical lever is illustrated in Figure 25. In its repulsive "contact" mode, the instrument lightly touches a tip at the end of a leaf spring or "cantilever" to the sample. A detection apparatus measures the vertical deflection of the cantilever as a raster-scan drags the tip over the sample; this scan indicates the local sample height. Hence, hard-sphere repulsion forces between the tip and sample are measured in the contact mode.
Though in principle, AFM resembles the record player as well as the stylus profilometer, it incorporates a number of refinements that enable it to achieve atomic scale resolution. Also, because AFM is based on interaction between the tip and sample, as well as surface topography, local properties, such as stiffness and friction can be determined.
Figure 25. Working of the Atomic Force Microscope. Inset (Feedback control of the AFM). (Source: http://www-ermm.cbcu.cam.ac.uk/00001587h.htm)

The high resolutions attainable using the AFM make it ideal for the study of surface topography of capsules. It was used to assess the change in surface structure and the overall structure of capsules before and after loading, as well as to study the effect of formation of internal matrices inside a capsule on its overall structure. While
transmission microscopy can give this information by indicating changes in optical properties (such as opaqueness of capsule interior when it is not hollow), it cannot give fine details of the surface and the overall topographical (or physical) information.

3.4.2 Zeta-potential Analysis

A charged particle suspended in an electrolytic solution attracts ions of opposite charge to those at its surface, where they form the Stern layer (Figure 26). To maintain the electrical balance of the suspending fluid, ions of opposite charge are attracted to the Stern layer. The potential at the surface of that part of this diffuse double-layer of ions that can move with the particle when subjected to a voltage gradient is the zeta potential. This potential measured is very much dependent upon the ionic concentration, pH, viscosity, and dielectric constant of the solution being analyzed.

Figure 26. The electrical double layer—Stern layer
(Source: http://www.desicca.de/index.html?/international/engl and/zeta-potential/hauptteil_zeta-potential.html)
Thus, the zeta potential is based on the measurement of migration rate of dispersed particles under the influence of an electric field.

\[ \frac{v}{E} = \mu_E \]  \hspace{1cm} (11)

\( v \) = observed velocity

\( E \) = strength of the applied electric field

\( \mu_E \) = electrophoretic mobility

The zeta potential can be calculated from mobility using the Henry equation:

\[ \mu_E = \varepsilon \zeta f(k_a) / 6\pi \eta \]  \hspace{1cm} (12)

\( \varepsilon \) = dielectric constant of the medium

\( \eta \) = viscosity of the medium

\( f(k_a) \) = correction factor which takes into account the thickness of the double layer and particle diameter.

The unit \( k \) is a reciprocal length. 1/k is frequently described as the thickness of the double layer. In practice an approximation can be made for \( f(k_a) \), this is \( f(k_a) = 1.0 \) for non-polar media,

\( f(k_a) = 1.5 \) for particle dispersions in polar media. This number is a good approximation for particles >100nm in aqueous solutions with an ionic strength >10^{-3} \text{ M}.

This formula is known as the Smoluchowski approximation.

\[ \mu_E = \varepsilon \zeta / 4\pi \eta \]  \hspace{1cm} (13)

The second limiting case \( f(k_a) = 1.0 \) applies for very small particles in media with a low permittivity. This equation is known as the Hückel approximation.
Charged particles in a liquid suspension can be made to move by applying an electric field to the liquid through two electrodes as shown in Figure 27. By alternating the charge between the electrodes, the particles move back and forth between the electrodes at a velocity relative to their surface charge and the electrode potential. This velocity can be determined by measuring the Doppler shift of laser light scattered off the moving particles.

![Figure 27. The sensing portion of the zeta potential instrument (Source: http://www.rpi.edu/dept/chem-eng/Biotech-Environ/COAG/zeta.htm)](http://www.rpi.edu/dept/chem-eng/Biotech-Environ/COAG/zeta.htm)

This instrument is particularly useful in determining the feasibility of using charged polymers for coating charged templates and was used to confirm the assembly of polyions on charged templates.
3.5 Absorbance Measurements for Characterization of Efficiency, Stability, and Activity of Encapsulated Enzyme

3.5.1 Beer’s Law

The absorbance, $A(\lambda)$, of a species at a particular wavelength of electromagnetic radiation, $\lambda$, is proportional to the concentration, $c$, of the absorbing species and to the length of the path, $L$, of the electromagnetic radiation through the sample containing the absorbing species.

$$A(\lambda) = e(\lambda)Lc$$

The proportionality constant $e(\lambda)$ is called the absorptivity of the species at the wavelength, $\lambda$. The way in which $e(\lambda)$ depends on wavelength defines the spectrum of the substance in question. The wavelength at the maximum value is called the analytical wavelength of the substance. Figure 28 shows the absorbance spectrum of DNA with the wavelength at the absorbance maximum indicated as the analytical wavelength. Normally, the Beer’s law applies at the analytical wavelength. Hence, the sensitivity to concentration changes is the highest at that wavelength.
To experimentally obtain the Beer's Plot, the following steps can be followed:

- Prepare a series of samples of known concentration of the substance, the analytical wavelength of which is known ($\lambda_{\text{anal}}$).

- Measure the absorbance of each of the solutions of known concentration at the analytical wavelength and plot the values of the absorbance as a function of the concentration (Figure 29).

- Verify that, within experimental error, the absorbance is a linear function of the concentration. If linear, the slope of the best straight line through the experimental points in the absorbance vs. concentration plot gives the Beer's Law slope. The Beer's law slope has the value $e(\lambda_{\text{anal}}) l$, where $l$ is the path length through the sample.
The slope can be used to determine the concentration corresponding to an absorbance for samples of unknown concentration if the same experimental arrangement (same spectrometer, same cell) are used. If a different experimental arrangement will be used (e.g., a different cell), the Beer's Law slope will need to be adjusted by the path length through the cell used in the Beer's Law determination. This slope will provide the value of the absorptivity, $e(\lambda_{\text{max}})$, which should be characteristic only of the substance and the wavelength, and independent of the experimental arrangement used to determine it.

### 3.6 Glucose Sensor Components

An ideal glucose sensor needs to satisfy a number of criteria, such as selectivity, linear range and response time, biocompatibility, storage and operational stability at body temperature, reversibility of signal, reproducibility of sensor fabrication, which current endeavors have not been able to completely satisfy. Moreover, the sensor components must be inexpensive and fabrication method must be simple.
This section describes the components of the glucose sensor designed in this research. The sensor is selective to glucose because of the use of flavoenzyme GOx. The numerous advantages of this enzyme have been enumerated in the following sections. The incorporation of HPTS in the sensor makes it a ratiometric sensor of glucose based on changes in pH. Being ratiometric, it is less affected by fluctuations in instrumental parameters. The sensor fabrication procedure, which will be described in the Chapter IV is simple and repeatable.

The following sections expound on the sensor components and their characteristics, which made them amenable to the construction of a glucose sensor.

3.6.1 Enzymes and Enzymatic Activity

Enzymes bind to one or more ligands (substrates) and convert them into one or more chemically modified products. This binding happens over and over with phenomenal speed. They speed up reactions by a factor of a million or more, without themselves changing. They act as catalysts that permit cells to make or break covalent bonds in a controlled way. Catalysis is a vital function that creates and maintains the cell by aiding organizing sets of chemical reactions, making life possible. Enzymes work in teams, with the product of one enzyme becoming the substrate for the next. Thus an elaborate network of metabolic pathways provides the cell with energy and produce molecules, both large and small that the cells need.

The basic reaction path of an enzyme is as follows:

$$E + S \rightarrow EP \rightarrow E + P.$$ 

There is a limit to the amount of substrate that a single enzyme molecule can process in a given time. Figure 30 shows the relationship between substrate concentration
and enzyme activity. Enzyme activity (reaction rate) is defined as the number of substrate molecules processed per second. With the increase in the concentration of substrate, the rate at which product is formed also increases, up to a maximum value (the enzyme is now saturated with substrate). The rate of reaction $V_{\text{max}}$ at this point depends on how rapidly the enzyme can process the substrate molecule. The turnover number is defined as the enzyme rate divided by the enzyme concentration. It is typically 1000 substrate molecules processed per second per enzyme molecule, though, numbers between 1-1000 are also known. Another important parameter in enzyme kinetics is $K_m$, defined as the concentration of the substrate that allows the reaction to proceed at one-half its maximum rate ($0.5V_{\text{max}}$). If the $K_m$ is low, the enzyme generally binds tightly to the substrate and hence reaches its maximum catalytic rate at a low substrate concentration.

![Diagram of enzyme kinetics](image)

Figure 30. An illustration of enzyme kinetics
The enzyme increases the local concentration of substrate molecules at the catalytic site, holds all the appropriate atoms in the correct orientation for the reaction that is to follow, and most importantly, the binding energy contributes directly to catalysis. Substrate molecules have to pass through a series of intermediate states (altered geometry and electron distribution) before they form the final products of the reaction. Activation energy is the free energy required to attain the most unstable transition state (Figure 31); it is the major determinant of the reaction rate. Enzymes have a much higher affinity for the transition state of the substrate than they have for the stable form. The tight binding lowers the transition state energies tremendously, and hence accelerates a particular reaction by lowering the activation energy that is required.

By experimentally determining the rates of enzyme reactions using standard assays, the effect of various parameters (concentrations of substrates, products, inhibitors, regulatory ligands, effect of binding and entrapment of enzymes in matrices) can be studied. This determination is particularly important in enzyme encapsulation studies because the effect of encapsulation on enzyme conformation and hence activity, and the availability of substrates to the enzyme after encapsulation, can be studied by measuring enzyme activity. By comparing activities of the enzyme before and after encapsulation, the effect of encapsulation on the enzyme can be interpreted both for short and long periods of time.
3.6.2 Theory of Glucose Measurement Using GOx

Glucose oxidase is a slightly elongated globular protein with an axial ratio of 2.5:1, an average diameter of 8nm. The absorbance spectrum has maxima at 278, 382 and 452nm. The enzyme is a dimer composed of two identical subunits. The holoenzyme is made of two identical subunits, each with a MW of 80000. There is a long but narrow contact area connecting the monomers non-covalently with either salt linkages or hydrogen bonds. Each monomeric unit is a compact spheroid with dimensions 60Å x 52 Å x 37 Å. It folds into two structural domains, one binding the FAD and the other involved with substrate binding. The carbohydrate content of the enzyme ranges from 8 to 12, which accounts for variations in the isoelectric point between 3.9 and 4.3. It is anionic at physiological pH.
Figure 32 illustrates the glucose oxidase reaction. Similar to other flavoenzymes, the enzymatic reaction can be divided in two steps\textsuperscript{203}. The reductive half consists of transfer of two protons and electrons from glucose to the enzyme yielding d-gluconolactone. The oxidative half reaction consists of the oxidation of the enzyme by molecular oxygen yielding hydrogen peroxide. Finally, d-gluconolactone can be hydrolyzed non-enzymatically to gluconic acid.

![Chemical Structures](http://www.imb-jena.de/www_bioc/post/GOX/)

**Figure 32.** The glucose oxidase reaction
(Source: http://www.imb-jena.de/www.bioc/post/GOX/)
GOx is easy and cheap to obtain; it is one of the most robust enzymes, which can withstand greater extremes of pH, ionic strength, and temperatures (these properties allow less stringent conditions during manufacturing and relatively carefree storage and use by home-users of the biosensor incorporating it); the concentration range of glucose with which GOx reacts optimally coincidentally agrees with the range of concentrations encountered in human blood.

3.6.3 HPTS as a pH Sensitive Dye

HPTS (Figure 33(a) and (b)) has many attractive features that make it an ideal fluorescent indicator, namely, large Stokes shifts, high quantum yield, high absorbance, excellent photostability, and non-toxicity\textsuperscript{204}. It has a pKa of 7.3, which is in the middle of the physiological pH range. At near neutral pH, HPTS fluoresces green (conjugate base form exclusively) and independent of whether it was excited in its conjugate base or acidic form, or both\textsuperscript{205}. The ratio of fluorescence intensities resulting from excitation (Figure 33(b)) at 470nm and 405nm can be used to quantify pH values between 6 and 9. This ratio is not influenced by temperature and ionic strength\textsuperscript{206}. 

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In summary, this chapter described the techniques used to study the encapsulation efficiency, stability, and activity of encapsulated enzymes, comparison of wall permeability of capsules of different architectures, and the study of response of glucose sensors. The next chapter describes the chemicals, materials and methods used to implement the goals of this project using the techniques described in this chapter.
CHAPTER IV

MATERIALS AND METHODS

This section describes the chemicals, instruments and methods used in experiments. Detailed information about the inventory of these materials and instruments, can be found in Appendices A and B, respectively. Appendix A includes product name, number and vendor information for chemicals and other materials used. Appendix B contains vendor information about the instrumentation used.

The methods described here pertain to capsule preparation, visualization of capsules using CLSM, and characterization of capsule topography before and after loading of enzyme with the AFM. Procedures for testing glucose sensitivity of HPTS-GOx sensors have also been described. Use of specific equipment such as the UV-Vis spectrophotometer in studying the stability and activity of encapsulated enzyme has also been described. Chemical procedures involving labeling of enzymes and polyions, as well as free radical polymerization of acrylic acid monomers have been mentioned.
4.1 Chemicals and Materials

4.1.1 Polvions

Poly (sodium 4-styrenesulfonate) (PSS, MW 70000), Poly (allylamine hydrochloride) (PAH, MW 15kDa), Poly (diallyldimethylammoniumchloride) (PDDA, MW 100-200kDa) and Poly (vinyl sulfate, potassium salt) (PVS, MW 170,000) were purchased from Sigma. Chitosan (CHT, low molecular wt, 50,000 Da) was obtained from Aldrich.

4.1.2 Enzymes and Enzymatic Activity Assays

Glucose oxidase (GOx, 160K and 200000 units/g) was obtained from Sigma. Horseradish Peroxidase (HRP, 40K and 181 purpurogallin units/mg) was obtained from Sigma. Potassium Phosphate Buffer (100mM, pH 6.0 at 20°C), 0.5% (w/w) Hydrogen Peroxide, and 5% (w/v) Pyrogallol solution were used to prepare the enzymatic assay for HRP. 3,3'-Dimethoxybenzidine (O-dianisidine, FW 317.2, Sigma), β-D (+) glucose (Sigma) and HRP were used to prepare the enzymatic assay for GOx. Sodium bicarbonate buffer was used for the labeling reaction and was obtained in powder form from Sigma.

4.1.3 Colloids (template)

Monodisperse melamine formaldehyde (MF) particles (5.25μm, 10% solids weight) with positive surface charges were purchased from Microparticles GMBH, Germany. Monodisperse manganese carbonate (MnCO₃) microparticles (5.8μm) with positive surface charges were synthesized according to a previously established protocol²⁰⁷. Polymethacrylate (PMA) particles (6 μm, 10% solid content) were purchased from Fluka chemicals.
4.1.4 Labeling Reactions

Tetramethylrhodamine-5- (and-6)-isothiocyanate (TRITC) was obtained from Molecular Probes and was used to label GOx to visualize loading of the enzyme. Pyrene 8-hydroxy-1,4,6-trisulfonylchloride (HPTS, MW 513.78) was purchased from Marker Gene Technologies, Inc., and GOx was labeled with HPTS for sensor experiments. PD-10 desalting columns were obtained from Amersham Biosciences and were used for separation of the labeled enzyme from the free dye. Tetramethylrhodamine-5- (and-6)-isothiocyanate (TRITC) was obtained from Molecular Probes and was used to label HRP. All labeling reactions were performed using a standard amine labeling protocol\(^{208}\), which has been described in the methods section. Fluorescein isothiocyanate isomer I was obtained from Sigma and was used to label PAH.

4.1.5 Formation of Poly(Acrylic Acid)

Acrylic acid monomer (99%) and ammonium persulfate ((NH\(_4\))\(_2\)S\(_2\)O\(_8\)) were purchased from Aldrich and Sigma, respectively.

4.1.6 Other Chemicals and Materials

Glacial acetic acid (99.7%) was purchased from J.T. Baker. L-Arginine was obtained from NOW Foods, Bloomingale, IL; N-(3-Dimethyaminopropyl)-N’-ethylcarboxiimide hydrochloride (EDC, MW 191.7) and N-Hydroxysulfosuccinimide Sodium salt (NHS, MW 217.13) were purchased from Fluka. Phosphate buffered saline (PBS) buffer was obtained from Fluka BioChemika. Coverslips and microscope slides were obtained from VWR Scientific and Cole Parmer Instruments respectively. Centrifuge tubes and cuvettes were purchased from Fisherbrand.
All chemicals were reagent grade and used as received. Deionized water (18.75MΩ) was used for all solutions.

4.2 Methods

This section describes the procedure for preparation of capsules for all experiments described in this dissertation. The first section describes the labeling procedures for labeling GOx, HRP and PAH with dyes (FITC, TRITC and HPTS) and the procedure for imaging of capsules using the atomic force microscope. The second section describes the fabrication procedure for capsules with wall architectures \{PVS-PAH\}_4(MF), \{PSS-PAH\}_4(MF), \{PSS-PDDA\}_4(MF), \{PVS-PAH\}_2 + \{PSS-PAH\}_2(MF), \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF) using MF template and \{PSS-PAH\}_4(MC- internal arginine matrix) using the MC template. It also describes the procedure for loading GOx and evaluation of encapsulation efficiency, long-term stability, and activity of encapsulated GOx. The third section describes the FRAP procedure for evaluation of permeabilities of various capsule architectures to FITC-GOx.

The fourth section describes the procedure for fabrication of capsules and formation of internal poly(acrylic acid) (PAA) matrix for encapsulation of HRP, and evaluation of encapsulation efficiency, long-term stability, and activity of the encapsulated HRP. It also explains the experimental procedure for studying the effect of (acrylic acid:(NH₄)₂S₂O₈) concentration on efficiency of enzyme encapsulation and enzyme activity.

The fifth section explains the procedure for using PMMA as the template for fabrication of microcapsules. Finally, the sixth section describes the experimental
protocols for testing the glucose sensitivity of HPTS-GOx both in solution and after encapsulation.

4.2.1 Labeling Reactions

The procedure was used for making FITC-PAH: 0.1 M sodium bicarbonate (NaHCO₃) buffer was prepared and adjusted to pH 9 by HCl and KOH. Next, 60 mg PAH was dissolved in 1 mL 0.1M NaHCO₃ buffer and 0.25mg FITC was dissolved in 100 µL N,N-Dimethylformamide (DMF). FITC solution was added into PAH solution and stirred at room temperature for one hour. Then, the FITC-PAH conjugate was precipitated in 30 mL acetone and centrifuged at 3000 rpm for five minutes. After removing the supernatant, the remaining FITC-PAH conjugate solid was resuspended and dissolved in 30 mL pH 7 tris buffer. FITC-PLL was made in a similar way, but 5mg PLL was dissolved in 1 mL 0.1M NaHCO₃ buffer in place of the PAH.

A protein-labeling procedure for tagging GOx and HRP with FITC was developed from the guidelines provided by Molecular Probes and Sigma-Aldrich. For labeling GOx with FITC and TRITC, 0.1 M sodium bicarbonate buffer was prepared and adjusted to pH 9. Next, 20 mg of the enzyme was dissolved in 1 mL of 0.1 M NaHCO₃ buffer. Then, 2.5 mg FITC powder was dissolved in 5mL DMF. While stirring the enzyme solution slowly, the FITC solution was added. The reaction was incubated at room temperature with continuous stirring for one hour. Finally, the conjugate was separated from unreacted labeling reagent with an Amersham Pharmacia Biotech PD-10 desalting column. For labeling GOx with TRITC, 10mg/ml GOx and 0.5mg/ml TRITC were used. For labeling GOx with HPTS (10mg/ml), 6mg/ml of GOx and 70µL of HPTS were used in the labeling reaction.
4.2.2 Characterization of Capsule Surface Topology Using Atomic Force Microscopy (AFM)

A volume of 10μl of the suspension to be imaged was dispensed onto a mica substrate and incubated at 60°C for five minutes, to remove water from the interior of the capsules. Scanning probe microscopy (AFM Quesant, Model 250) was used to obtain morphological information of the capsules before and after polymerization of acrylic acid, and after encapsulation of enzyme. The intermittent contact mode (Wave Mode) was used to scan the sample with a 40μm scanning head. The NSC16 cantilever used had the following tip specifications: -30° full tip cone angle, 15-20μm tip height, tip curvature radius <10nm.

4.2.3 Capsule Fabrication

Among the architectures studied, \{PSS-PAH\}_4(MF), \{PSS-PDDA\}_4(MF), \{PVS-PAH\}_2 \pm \{PSS-PAH\}_2(MF) were hollow capsules and \{PVS-CHT\}_2 \pm \{PSS-PAH\}_2(MF), \{PSS-PAH\}_4(MC- internal arginine matrix) were believed to have internal matrices due to the capsule preparation procedures described in the next section. PAH, PDDA, PSS and PVS solutions were prepared using DI water, and 0.2M NaCl was added to each of them. For the preparation of CHT solution (1% wt), 0.5g of chitosan was added to 49.5g water, 3ml of 3% (3ml acetic acid and 97ml water) acetic acid was added to CHT solution, and this solution was sonicated at level 2-3 for 15-20 minutes.

The general procedure for coating particles is as follows: Depending on the type of template used, the polyanion was added to 100μL (10% solids) of MF colloid suspension or 30mg of MC particles, and 15 minutes were allowed for complete adsorption saturation of the polyanion. After rinsing with DI water, the sample was
centrifuged at 5000rpm for 3 minutes. Three such rinse cycles followed every polyelectrolyte adsorption step. The particles were then immersed in polycation for 15 minutes. This process of adsorbing alternate layers of polycations and polyanions was continued until the desired numbers of bilayers were deposited on the template. Except for \( \{\text{PVS-CHT}\}_2 + \{\text{PSS-PAH}\}_2 \) architecture with MF templates, the core material (MF or MC) was then dissolved by suspending the coated particles in 0.1M HCl for five minutes and rinsed four to five times with DI water.

Capsules with \( \{\text{PVS-CHT}\}_2 + \{\text{PSS-PAH}\}_2 \) architecture with MF templates were believed to have internal matrix formed during the process of polyelectrolyte coating. MF begins to dissolve partially due to low pH (2-3) of the CHT solution and hence a matrix was formed due to the interaction of the template with the initial bilayer materials, consisting of MF oligomers, CHT and PVS. No additional dissolution steps were required for these particles.

Capsules with \( \{\text{PSS-PAH}\}_4 \) architecture with MC templates were used to test a novel method of formation of internal charged matrices using amino acid arginine using zero-length cross-linking reagent EDC with NHS. Briefly, 300\( \mu \)l of 80mg/ml of arginine monomer solution was added to 250\( \mu \)l of freshly prepared capsules, followed by addition of 300\( \mu \)l each (1:1 ratio) of 15mg/ml EDC and NHS. All solutions were prepared with sodium bicarbonate buffer (pH 8.5). Though the crosslinking action of EDC/NHS is most effective between pH 4-5, the reaction was carried out at an alkaline pH because arginine is mildly protonated and more reactive. The mixture was incubated overnight to allow sufficient reaction time for the slower action of EDC/NHS.
4.2.4 Loading of Capsules

After fabrication of capsules, each capsule suspension was incubated with unlabeled GOx (2mg/ml, pH=5 in DI water) for six hours for long-term stability, and activity studies. At this pH, GOx (pI=4.2) is negatively charged. In the case of {PVS-CHT}$_2$-PSS-PAH$_2$(MF) capsules, the internal matrix inside the capsule was positively charged (likely due to chitosan and MF oligomers) at this pH, and GOx was electrostatically attracted into the capsule and entrapped in the matrix.

TRITC-GOx was used for visualization of {PSS-PAH}$_4$(MF), {PSS-PDDA}$_4$(MF), {PVS-PAH}$_2$-PSS-PAH$_2$(MF), and {PVS-CHT}$_2$+PSS-PAH$_2$(MF) capsules. PAH$_4$(MC) capsules were visualized using FITC-GOx. A Leica TCS SP2 system, Germany (HCXPL APO CS 63x1.4 oil immersion objective) was used to perform confocal imaging. After adding 3 μl of capsule suspension to a coverslip, 3 μl of labeled GOx (1mg/ml, pH=5) was added to the drop of capsules. Imaging was performed with the 488nm line of the Ar : Kr laser set to 50% and the PMT voltage at 500V. The Leica Lite software was used to analyze data. For FRAP studies, 3 μl of capsules in loading solution was pipetted on a coverslip precoated with {PEI-PSS}$_4$ layers (to stabilize capsules on the surface of the coverslip). The edges of a coverslip were smeared with paraffin wax and was then placed on the sample and sealed by gently pressing against the surface of the slide to prevent evaporation of the sample. Sealing was necessary to prevent evaporation during the experiment.
4.2.5 UV-Vis Spectrophotometry for Stability and Activity Studies of Encapsulated GOx

A Perkin Elmer Lambda 45 UV/Vis spectrometer was used for estimation of the quantity of GOx encapsulated, stability of the encapsulated GOx, and its activity. The percentage of GOx encapsulated was estimated by measuring the absorbance at 280nm of the enzyme in the loading solution and the supernate after loading; the difference in the two absorbance values followed by conversion to mass using the Beer's law (in mg) gives an estimate of the mass of enzyme encapsulated. For the activity study, the standard assay protocol from Sigma was used. Upon addition of 60µl of capsules with encapsulated GOx, O-dianisidine, which is one of the constituents of the assay, is oxidized by H$_2$O$_2$ (this reaction is catalyzed by peroxidase), which is a byproduct of the reaction between glucose and GOx to a highly colored substance. The formation of this product can be measured at 500nm. The continuous spectrophotometric rate determination was used to record changes in absorbance at 500nm, at 30-second intervals, for a total of 15 minutes. The initial linear increase in absorbance with time was taken as a quantitative measure of the catalytic activity of glucose oxidase. The temperature was maintained at 25°C for all activity tests using a Quantum Northwest temperature controller.

4.2.6 Long-term Stability and Activity Studies

Two samples per architecture were prepared for long-term stability and activity studies; one sample was used for activity study and the other for stability study. For long-term leaching studies, GOx loaded capsules were stored in deionized water for a period
of four weeks. Both stability and activity measurements were first made on the first week, on the day of loading. Subsequently, measurements were made one and three weeks after loading. For the stability measurements, each of the samples was centrifuged to separate the loaded capsules from the supernate. The supernate was drawn off, and absorbance measurements were made to calculate the amount of enzyme leached into the supernate during the storage period.

4.2.7 Protocol for FRAP Studies

A slide was coated with \( \{\text{PEI-PSS}\}_4 \) layers (to stabilize capsules on the slide surface) and three \( \mu l \) of capsules in loading solution was pipetted on the slide. The edges of a coverslip were smeared with paraffin wax, and it was placed on the sample. The edges of the coverslip were sealed by gently pressing them against the surface of the slide to prevent evaporation of the sample during the experiment.

The Marquardt procedure was used to fit the intensity of the FRAP signal with the theoretical curve given by equation (5). From the MATLAB code, it can be seen that three different models were used to perform curve fitting. Two parameters, \( k \) – bleaching constant and \( \tau_D \) – characteristic diffusion time characterize the theoretical curve. Ideal values of \( k \) and \( \tau_D \) (\( k_{\text{guess}} \) and \( \tau_D \) respectively) were first obtained by random substitution of values for these parameters in the theoretical curve to obtain an ideal representation for the diffusion recovery. To obtain the Least Squares Fit (LSQ) using model0, the value of \( k \) evaluated from equation x1 (\( k_{\text{calc}} \)) was used in the theoretical curve equation and fitted for \( \tau_D \). In model1, \( \tau_D \) was fitted first, followed by \( k \). In model2, both \( \tau_D \) and \( k \) were fitted simultaneously. For all architectures, model1 and model2 provided better fits for the data.
4.2.8 Multilayer and Capsule Preparation

And Polymerization of Acrylic Acid
Matrix for Peroxidase
Encapsulation

Manganese carbonate (MnCO₃) particles were coated with four bilayers of PSS/PAH using the LbL nanoassembly approach. Briefly, 0.5mL of PSS solution (2mg/ml, 0.2M NaCl) was added to 50μL of MnCO₃ particle suspension, and allowed to react for 15 minutes to achieve complete adsorption saturation. The sample was then rinsed with DI water and centrifuged at 5000rpm for 3 minutes. Three such rinse cycles followed every polyelectrolyte adsorption step. The particles were then dispersed in 0.5 ml PAH (2mg/ml, 0.2M NaCl) for 15 minutes and then rinsed thrice with DI water. The consecutive adsorption of alternating polyelectrolytes was continued to achieve deposition of four bilayers of PSS/PAH. After coating, the MnCO₃ core was dissolved by immersion in HCl (0.1M) for 30 minutes, followed by rinsing and immersion in EDTA for 15 minutes. The resulting suspension of microcapsules was then rinsed four times with DI water. These capsules were used in the following process for free radical polymerization of acrylic acid to form a poly (acrylic acid) matrix in the interior, followed by loading of HRP.

Figure 34 contains a schematic illustrating the process of fabrication and spontaneous loading of microcapsules with HRP. An internal anionic poly (acrylic acid) matrix was formed by incubating capsules in a highly concentrated solution (99%) of acrylic acid and 2mg/ml of initiator in a microcentrifuge tube at 60°C.
Hollow microcapsules Loading with AA monomers

Polymerization with initiator, (NH₄)₂S₂O₈

Capsules with PAA matrix and loaded HRP

Loading and encapsulation of positively charged enzyme

Figure 34. Schematic of acrylic acid matrix formation and loading and entrapment of peroxidase inside the capsule

In a pilot experiment, it was discovered that using 100μl each of the monomer (99%) and snainitiator (2mg/ml) produced a highly viscous gel of polyacrylic acid and the capsules indicating a high degree of polymerization; progressively reducing the volumes of the reactants produced less viscous gels, indicating lower degrees of polymerization. Hence, acrylic acid and (NH₄)₂S₂O₈ were added in 1:1 ratio by volume to 100μl of freshly prepared microcapsules (three different volumes of reactants, 10μl, 50μl and 100μl each of monomer and initiator; the corresponding concentrations of acrlylic
were 0.23M, 1.18M and 2.36M, and of the initiator were 0.13mM, 0.65mM, and 1.31mM). The mixture was diluted with DI water to make up a total volume of 600μl. This mixture was then incubated in an oven maintained at 60-70°C for 30-120 minutes. The sample with the highest volumes of the reactants (monomer and initiator) was incubated only for 30 minutes, that with intermediate volumes for 45 minutes and the sample with the lowest volume of reactants was incubated for 120 minutes. The change in viscosity of the mixture, as assessed by visual observation was used as an indicator for polymerization of acrylic acid monomers. After polymerization the capsules were rinsed with DI water and centrifuged (5000rpm for 10 minutes) twice, then dialyzed against DI water to remove unreacted monomers and the initiator from the capsule suspension. These capsules were then loaded with TRITC-HRP as described in the next section.

4.2.9 Spontaneous Loading of Capsules with TRITC-HRP/HRP After Polymerization of Internal PAA Matrix

After polymerization and rinsing, the capsule suspension was incubated with 1mg/ml TRITC-HRP in DI water (pH 5) for 6 hours. At this pH, HRP is positively charged (pI=8.8); since the acrylic acid matrix inside the capsule is negatively charged at this pH, TRITC-HRP was electrostatically attracted and spontaneously loaded into the capsules and entrapped in the matrix. The loaded capsules were then imaged to visualize loading and localization of TRITC-HRP using a confocal microscope as described in the next section. For activity studies and estimation of loading efficiency, unlabeled HRP (1mg/ml) was used instead of TRITC-HRP, and the same loading procedure described above was used.
4.2.10 Assessment of Time-Dependent Loading Behavior of Capsules Using Confocal Laser Scanning Microscopy (CLSM)

A Leica TCS SP2 system, Germany (63X, 1.4 oil immersion objective) was used to perform confocal imaging. After placing three microliters of capsule suspension on a microcover glass (24x60mm, 0.16-0.19mm thickness), three microliters of TRITC-HRP (1mg/ml, pH=5) was added to the drop of capsules. Imaging was performed with the intensity of with the 543nm line of the Ar : Kr laser set to 50% and the PMT voltage at 500V. A single capsule was located and multiple images of the same x-y section of the capsule were collected over time (30second intervals) to record the spontaneous loading of TRITC-HRP. The Leica Lite software was then used to analyze data; data analysis involved representation of loading of the capsule by considering a region of interest (ROI) in the capsule interior and plotting the average intensity in the ROI against time.

4.2.11 Assessment of Encapsulation Efficiency and Activity Using UV-Vis Spectrophotometry

The procedure described previously in this chapter for estimation of percentage encapsulation of GOx in the capsules after loading was also used for HRP. The assessment of activity of encapsulated HRP was done using the following procedure. 60µl of capsules with encapsulated peroxidase is added to the assay; pyrogallol, the dye constituent of the assay, is oxidized by peroxidase to purpurogallin, which is a highly colored substance. The formation of his product can be measured at 420nm. The continuous spectrophotometric rate determination was used to record changes in absorbance at 420nm, at 30-second intervals, for a total of 60 minutes. The initial linear
increase in absorbance with time was taken as a quantitative measure of the catalytic activity of peroxidase.

4.2.12 Effect of Acrylic Acid:(NH₄)₂S₂O₈ Concentration on Efficiency of Enzyme Encapsulation and Enzyme Activity

Six batches of \{PSS/PAH\}_₄ capsules (three pairs, each with a different volume of reactants, acrylic acid and (NH₄)₂S₂O₈ in 1:1 ratio) were prepared and the poly (acrylic acid) matrix was formed as described previously. The capsules were split into three pairs, each pair consisting of one sample for stability tests and the other for activity tests. After dialysis, loading, and rinsing, the capsules were used for activity studies and were assessed for efficiency of encapsulation as described in the previous section. The encapsulation efficiencies and activities of capsules were compared as a function of the volume of reactants used in the preparation of the internal poly (acrylic acid) matrix.

4.2.13 Capsule Preparation Using PMMA Template

The assembly of oppositely charged polyelectrolytes was carried out on negatively charged PMMA. Three methods were attempted to improve the yield and quality of microcapsules produced. In the first attempt, 50μl of 6μm PMMA template was coated with 6 bilayers of PAH-FITC and PSS. The coated PMMA particles were then suspended in acetone overnight. This coating was followed by two more acetone rinses and finally with four rinses with deionized water. Since the first attempt produced a large number of broken capsules, the number of bilayers in the subsequent attempts was increased. Also, since labeling of PAH with FITC reduces the charge density of PAH and hence weakens its electrostatic interaction with PSS, PAH-FITC was used in the
fabrication of only the three outermost bilayers, the other bilayers used unlabeled PAH. In the second attempt 50μl of 6μm PMMA template particles were coated with \{PAH/PSS\}_3 + \{PAH-FITC/PSS\}_3. This coating was followed by immersion in acetone for five hours. A small portion of the sample was then observed with the confocal microscope, while the rest was left immersed in acetone. Another aliquot of the sample was observed after 17 hours, and yet another after 24 hours. In the third attempt, 6μm PMMA template particles were coated with eight bilayers of PAH and PSS followed by three more bilayers of FITC labeled PAH and PSS to help in visualization. They were immersed in acetone for 1.5 hours, followed by gradual titration of DI water into the sample. The volume of supernatant drawn off from the sample was gradually increased and replaced with an equal volume of DI water. The capsules were visualized using confocal microscopy and AFM.

4.2.14 HPTS-GOx for Glucose Sensing

For studying glucose sensitivity of encapsulated GOx, capsules with architecture \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF) were used. The procedure for fabrication of these capsules has been described previously in this chapter. The loading of the capsules has also been described previously. For visualization of loading with confocal microscopy, TRITC-GOx was used to load capsules.

4.2.15 Study of Effect of Titration of 0.1M HCl on the Change in pH of PBS (pH=7.4) Buffered HPTS-GOx, PBS Buffer and HPTS

After mixing 40μl of HPTS-GOx with 1.2ml of PBS 7.4 buffer, the initial pH was recorded with a Thermo Orion pH meter. Aliquots of 0.1M HCl were added successively
to the solution, and the pH was recorded after each increment. The solution was continuously stirred using a magnetic stir bar throughout the titration experiment. The variation of pH of HPTS in PBS was recorded similarly.

4.2.16 Fluorescence Spectroscopy to Record Change in Excitation Peak Ratio of HPTS

A scanning fluorescence spectrometer (QM1, Photon Technology International) was used to record the change in peak ratio of HPTS with the changes in pH. Fluorescence excitation scans were obtained by using the “Excitation scan” mode of the PTI. An emission wavelength of 520nm and an excitation range of 350nm-500nm were used. All solutions and dispersions were continuously mixed (using magnetic stirring) during the titration experiments. Measurements were made in triplicate.

4.2.17 Response of PBS Buffer (pH=7.4), PBS Buffered HPTS and HPTS-GOx to Titration of 0.1M HCl

Initial excitation spectra of HPTS-GOx in PBS buffer before addition of acid were obtained. Measurements were made after each successive aliquot of HCl was added. The acid was added until changes were no longer observed in the acid to base peak ratio (405/454nm) after addition of HCl. The experiment was carried out in a similar way for PBS buffered HPTS.

4.2.18 Glucose Sensitivity Study of HPTS-GOx Both in Solution and After Encapsulation in Chitosan Capsules

Glucose sensitivity experiments were carried out using four different conditions, namely, (1) HPTS-GOx in DI water, (2) HPTS-GOx in PBS buffer, (3) HPTS-GOx
encapsulated in chitosan capsules and suspended in DI water, and (4) HPTS-GOx encapsulated in chitosan capsules and suspended in PBS buffer.

In each case, initial excitation spectra of the solution or dispersion were obtained before addition of glucose. After each addition of glucose measurements were made to observe the change in acid to base peak ratio. All spectra were normalized to 412nm, which is the isobestic point of the two emission peaks.

In summary, this chapter described the methods used to fabricate and visualize capsules and characterize capsule topography before and after loading of enzyme, and procedures for testing glucose sensitivity of HPTS-GOx sensors. Chemical procedures involving labeling of enzymes and polyions, as well as free radical polymerization of acrylic acid monomers have also been described. The next chapter elucidates the results obtained using the aforementioned techniques.
CHAPTER V

RESULTS AND DISCUSSION

Results of the various stages of the project are explained in detail in this chapter. A comparison of microcapsules with different wall architectures with respect to encapsulation, stability, and activity of GOx is presented in the first section. As will become obvious, the type of template material used, together with the wall architecture, determines the internal composition of capsules (presence or absence of an internal matrix). Melamine formaldehyde (MF) and manganese carbonate (MC) were used as templates for the fabrication of microcapsules. Capsules with wall architectures \( \{ \text{PSS/PAH} \}_4 \), \( \{ \text{PSS/PDDA} \}_4 \), \( \{ \text{PVS/PAH} \}_4 + \{ \text{PSS/PAH} \}_4 \), and \( \{ \text{PVS/PAH} \}_4 + \{ \text{PSS/CHT} \}_4 \) were fabricated using MF as template material. Internal matrices were formed in the case of \( \{ \text{PVS/PAH} \}_4 + \{ \text{PSS/CHT} \}_4 \) capsules during the capsule fabrication process, due to the interaction of CHT and PVS with the MF. In the case of \( \{ \text{PSS/PAH} \}_4 \) capsules fabricated using MC templates, internal matrices were fabricated by polymerizing amino acids using crosslinking agents EDC and NHS. The second section deals with the use of fluorescence recovery after photobleaching (FRAP) to compare the effect of capsule wall architecture on mobility of labeled GOx. Finally, the third section describes the results for experiments demonstrating glucose sensitivity of HPTS-GOx both in solution and after encapsulation in \( \{ \text{PVS/PAH} \}_4 + \{ \text{PSS/CHT} \}_4 \) capsules. Also,
while exploring various possibilities for encapsulation of GOx using capsules with charged internal matrices, a novel method of encapsulation of HRP using the "ship-in-a-bottle" concept was developed. The results of this study have been described in APPENDIX A. Also, in APPENDIX B a short study to determine the possibility of using PMMA as an alternative template has been described.

5.1 Effect of Polyelectrolyte Architectures on Microencapsulation - Encapsulation Efficiency, Long-Term Stability and Activity of Glucose Oxidase

5.1.1 Evaluation of \{(PVS/PAH)\}_4-(MF) Microcapsules to Encapsulate GOx

It was previously reported that the formation of a charged matrix inside capsules fabricated by LbL assembly depends on the choice of materials used to coat \(\text{MF}^{210-213}\). Different materials interact with the dissociating template in different ways; the innermost polyelectrolytes that are in contact with the template can detach from the wall structure and participate in matrix formation by interacting with the MF oligomers. However, if the innermost layers are strongly attached to the next layer, the likelihood of dissociation during core dissolution is small and they will remain firmly in the wall structure. Initially, the effect of capsule wall architecture on matrix formation was studied using PVS (Figure 35 [a]) instead of PSS (Figure 35 [b]) as an alternative polyanion in multilayer assembly.
The procedures for fabrication of capsules, loading, and imaging were described in Chapter IV. Figure 36 contains microscope images showing the spontaneous loading of the microcapsules with FITC-GOx. The concentration of FITC-GOx used for the loading experiment was 2mg/ml. At t=0 seconds, prior to addition of FITC-GOx, the fluorescence intensity of the capsule interior was zero. The rapid increase in fluorescence intensity in the capsule interior for the first 20 seconds after addition of the FITC-labeled enzyme can be characterized by the polynomial expression $y = 2.6x^2 - 53x + 270 \ (R^2=1)$, where $y$ is the intensity in the capsule interior which changes polynomially with time ($x$). The intensity then plateaued due to saturation of the PMT.
Figure 36. Loading of \( \{\text{PVS/PAH}\}_4 - (\text{MF}) \) microcapsules with FITC-GOx. Images: Left to right: region of interest (ROI) inside capsule (ROI Area=1.2\( \mu \)m\(^2\)) microcapsule imaged at 0s, 5s, 10s, 15s, 20s and so on after addition of loading solution.

A typical linescan of \( \{\text{PVS/PAH}\}_4 - (\text{MF}) \) capsule in a loading solution of FITC-GOx is shown in Figure 37. It can be seen that the fluorescence intensity of capsule interior is high enough to cause saturation of the PMT of the confocal instrument, while the intensity of the surrounding solution is zero. Though a quantitative estimate of the enzyme encapsulated was not made, the linescan data indicate that a large quantity of enzyme in the loading solution is drawn into the capsule interior and densely packed inside, indicating the presence of an internal matrix.
Figure 37. Linescan of \{PVS/PAH\}_4 \text{-}(MF) microcapsule in a loading solution of FITC-GOx.

The encapsulated enzyme was found to be active. The absolute activity of the encapsulated enzyme was calculated as $2.0 \times 10^{-5}$ absorbance units/sec (not scaled to mass of enzyme). Though the activity was not compared with that of free GOx, it is important to state that the procedure for encapsulation did not inactivate the entire quantity of immobilized enzyme.

An examination of the capsule using the transmission mode of the confocal microscope showed that the capsule interior was not hollow after core dissolution. Transmission images of \{PSS/PAH\}_4 capsules (Figure 38[a]) show that hollow capsules after dissolution of the core have the same contrast both inside and outside the capsule. The \{PVS/PAH\}_4-(MF) capsules (Figures 38[b] and 38[c]), however, have a highly refractive interior indicating that the MF template did not dissolve completely. This observation is an example of how the unpredictable quality of MF can lead to erratic results. The solubility of MF varies inversely with its age. As the MF ages, it develops more stable crosslinks and does not dissolve completely into lower molecular weight
Another influencing factor is the concentration of the acid used. The pH of the acid used determines the rates of two competing reactions—decomposition of MF to form soluble oligomeric products and further polymerization which prevents degradation by acid. A pH lower than 1.1, decomposition surpasses polymerization reaction, and at higher pH values, polymerization surpasses decomposition leading to partial dissolution of MF. Since the acid used had an appropriate pH (1.1), this factor was not responsible for the findings of this experiment. Any of the other aforementioned reasons may have contributed to the behavior of MF. Since a new batch of MF was used for the experiments, age of the capsule was not a factor contributing to the results. It is therefore presumed that the MF was defective, and that either manufacturing or handling conditions were responsible for its poor solubility.

Figure 38. Transmission mode images of (a) & (b) \(\text{PVS/PAH}_4\)–(MF) capsules, (c) \(\text{PSS/PAH}_4\) capsules

An attempt to repeat the fabrication of capsules of the same architecture failed. The capsules collapsed and disintegrated after dissolution of the core. The process of dissolution of these particles after LbL assembly of \(\text{PVS/PAH}_4\) was captured in the form of a time-lapse series of confocal images (Figure 39). This experiment proved that it was difficult to fabricate capsules using only PVS as the polyanion. The reasoning behind this
conclusion is that the bilayers are unstable because of interaction of hydrophobic (PVS) and hydrophilic (PAH) polyions. As a result, the wall is not strong enough to withstand the osmotic pressures experienced during core dissolution. Since the fabrication of stable microcapsules using \{PSS/PAH\} bilayers has been well documented in literature, hybrid capsules were prepared for further studies using two bilayers of \{PVS/PAH\} followed by two bilayers of \{PSS/PAH\}. These capsules did not disintegrate and collapse after core dissolution. With this hybrid architecture, stable capsules were fabricated, and the effect of using PVS instead of PSS on the formation of internal MF matrices during the core dissolution process was studied.

![Figure 39. Time-lapse images of dissolution of MF templates coated with \( (PVS/PAH)_4 \). Time based dissolution shown in succession in images(a)-(c), closeup of clumps of polyelectrolytes after dissolution of core shown in (d) ](image)

5.1.2 Long-term Studies Using Capsules with Different Wall Architectures; Comparison and Determination of Best Capsule Configuration for GOx Encapsulation

5.1.2.1 Zeta-Potential Measurements to Confirm Adsorption of Polyion. Capsules with architectures \( \{PVS-PAH\}_2 + \{PSS-PAH\}_2(MF) \) and \( \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF) \) were fabricated as described in Chapter IV. The alternate assembly of
oppositely charged polyions \{PVS-PAH\}_2 + \{PSS-PAH\}_2(MF) and \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF) was confirmed by zeta potential measurements, as shown in Figures 40 and 41. The MF microparticles possessed strong positive surface charge (+69mV). The surface potential of the coated microparticles changed regularly between positive for PAH and CHT to negative for PSS and PVS. Though surface potential values were not constant for each subsequent positive or negative layer deposited, they indicate that the polyelectrolytes were assembling.

![Graph showing zeta potential measurements](image)

Figure 40. Zeta potential measurement of \{PVS-PAH\}_2 + \{PSS-PAH\}_2(MF)

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5.1.2.2 Estimation of Percentage Encapsulation. The encapsulation or entrapment of GOx was different in terms of both distribution and quantity for each architecture employed, depending upon the wall composition and the presence or absence of an internal matrix. Figure 42 contains confocal images, corresponding line scans, and phase images of each of the samples while still in a loading solution of TRITC-GOx except for \{PSS-PAH\}_4(MC-internal arginine matrix) capsules, which are in a loading solution of FITC-GOx. It can be seen from Figures 42 (a), (b), (d), (e), (g) and (h) that in the case of \{PSS-PAH\}_4(MF), \{PSS-PDDA\}_4(MF), \{PVS-PAH\}_2 + \{PSS-PAH\}_2(MF) capsules, GOx was entrapped mostly in the walls. The line scans show that the concentration of the enzyme was the same inside the capsule as the loading solution, which suggests that loading in these three cases occurred by a process of simple diffusion.

In contrast, for \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF) capsules, it can be seen from Figure 42 (j), (k) and (l) that the loading solution intensity was effectively zero while the

Figure 41. Zeta potential measurement of \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF)
intensity from the capsule interior shows that the concentration of GOx was nearly uniform throughout the interior of the capsule. Also, it can be observed from the phase image of the capsule that, unlike the previous three architectures discussed, there is a refractive character to the interior, which is indicative of the presence of a matrix. For \{PSS-PAH\}_4 capsules with an internal matrix of arginine (Figure 42 [m], [n], [o]), the intensity of the capsule interior was approximately 12 times that in the loading solution, determined by taking the ratio of the average intensities of the linescan of the interior to that of the loading solution. Thus, for capsules without internal matrices studied here, the enzyme was mainly entrapped in the walls due to electrostatic interaction of the charged enzyme with the charges in the capsule walls. On the other hand, capsules with charged internal matrices attracted and encapsulated large quantities of oppositely charged GOx in the capsule interior.
Figure 42. Confocal images of {PSS-PAH}_4(MF), {PSS-PDDA}_4(MF), {PVS-PAH}_2 + {PSS-PAH}_2(MF), {PVS-CHT}_2 + {PSS-PAH}_2(MF) and {PSS-PAH}_4(MC- internal arginine matrix) capsules in loading solution. Figures 1.(a) through (l), in TRITC-GOx loading solution, Figure (m) through (o) in FITC-GOx loading solution.
Capsules of different architectures were evaluated for the quantity of enzyme encapsulated after loading with GOx and separation of capsules from the remainder of the enzyme by rinsing with DI water using the equation:

$$ Enzyme_{per\ capsule} = \frac{Enzyme_{in} - Enzyme_0}{Number\ of\ capsules} $$

$Enzyme_{in}\ is\ the\ initial\ encapsulated\ enzyme\ mass\ and\ Enzyme_0\ is\ the\ enzyme\ mass$ leached out at week0 immediately after loading and rinsing. Figure 43 contains a graph showing the comparison of uptake efficiency of the capsules. It can be seen that \{PVS-CHT\}$_2$ + \{PSS-PAH\}$_2$(MF) capsules, believed to have an internal matrix (comprising of MF oligomers, CHT and PVS), had the maximum encapsulated GOx (37pg/capsule). Among the capsule architectures that did not have an internal matrix, \{PSS-PDDA\}$_4$(MF) had a relatively high mass of encapsulated GOx (31pg/capsule) entrapped, though this was primarily in the walls, as is evident from the confocal images of Figure 42(a).

![Figure 43. Mass of GOx encapsulated per capsule](image-url)
In the case of \( \{\text{PSS-PAH}\}_4(\text{MC}) \) capsules it can be observed from the confocal line scans in Figure 42 (m) and (n) that the maximum entrapment of enzyme occurs to a greater extent around the walls and progressively decreased towards the center of the capsule. This indicates that the polymerization of arginine is higher in the periphery of the walls than the capsule interior. Hence, the mass of GOx encapsulated enzyme is less compared with the \( \{\text{PVS-CHT}\}_2 + \{\text{PSS-PAH}\}_2(\text{MF}) \) capsules, which also have an internal matrix.

5.1.2.3 Leaching of GOx from Microcapsules. Leaching of encapsulated GOx was studied over a four-week period to evaluate the percentage of the enzyme retained in the capsule for each of the different wall architectures. The percentage of enzyme remaining in the capsules at the end of the four-week study is graphically shown in Figure 44. These values were calculated by using the equation:

\[
\text{Enzyme}_{\text{rem}} = \frac{\text{Enzyme}_{\text{in}} - (\text{Enzyme}_0 + \text{Enzyme}_1 + \text{Enzyme}_3)}{\text{Enzyme}_{\text{in}}}
\]

\( \text{Enzyme}_{\text{in}} \) is the initial encapsulated enzyme mass. \( \text{Enzyme}_0 \) is the enzyme mass leached out at week0 immediately after loading and rinsing. \( \text{Enzyme}_1 \) and \( \text{Enzyme}_3 \) are the enzyme masses leached out at week1 and week3 respectively.
Figure 44. Percentage of GOx remaining in capsules with architectures \{(PSS-PAH)\_4(MF), (PSS-PDDA)\_4(MF), (PVS-PAH)\_2 + (PSS-PAH)\_2(MF), (PVS-CHT)\_2 + (PSS-PAH)\_2(MF)\} and \{(PSS-PAH)\_4(MC-internal arginine matrix)\} at the end of the 4-week leaching study.

It can be seen that \{(PSS-PAH)\_4(MC)\} capsules with internal arginine matrices had the least leaching of GOx, with 97% of the enzyme remaining in the capsules. \{(PVS-CHT)\_2 + (PSS-PAH)\_2(MF)\} capsules also retained a large quantity of enzyme (79%). In contrast, \{(PSS-PAH)\_4(MF)\} capsules showed complete leaching of GOx by the second week of study. From these results, it is apparent that capsules with internal matrices retain more encapsulated enzyme over a period of time. Among the hollow capsules, \{(PSS-PDDA)\_4(MF)\} capsules retained the largest percentage of encapsulated GOx (59%); \{(PSS-PAH)\_4(MF)\} capsules showed 100% leaching of the enzyme and \{(PVS-PAH)\_2 + (PSS-PAH)\_2(MF)\} could retain only 14% of the initial encapsulated GOx. Different materials interact with GOx in different ways; some can entrap enzymes more efficiently.
than others. These interactions may be electrostatic, hydrophobic and hydrophilic interactions, hydrogen bonding and coordination bonding or a combination of many or all of these interactions. Also, depending on the elasticity of the materials used, the walls react differently to the osmotic pressures generated during core dissolution, as a result of which wall structures are also different; some capsules may have larger pores than others. The wall structure also determines the quantity of enzyme entrapped.

5.1.2.4 Estimation of Number of Capsules in Each of the Samples. The Coulter counter was used to obtain sizes and concentrations of the capsule samples. The results obtained are shown in Figure 45. Though a distribution of values for size and concentration are indicated in the figures, the mean values of size and concentration have been considered for calculations.
5.1.2.5 Activity of Entrapped/Encapsulated GOx Over a Four-Week Period. The activity/unit mass is a measure of the percentage of active entrapped enzyme; i.e., the fraction of encapsulated enzyme that is active. Higher encapsulation efficiency does not necessarily mean that the entire percentage of encapsulated enzyme is efficiently available for reaction. There are a number of reasons for this. Tight packing of enzymes in the capsule and interaction of enzymes with the matrix in the capsule interior may cause the enzyme to conform unfavorably, which may lead to concealment of its active site. Also, constraints placed on diffusion of the analyte and components of the activity
assay may lead to difficulty in accessibility of the enzyme. Hence, the net activity obtained is either that of a fraction of the active enzyme that is accessible to the analyte or assay, or the activity of the fraction of the encapsulated enzyme that has been able to retain its most stable conformation, or a combination of both factors. A possible reason for variability in results is that the concentration of capsules/active enzyme is not sufficient because of which the signal to noise (SNR) ratio is small. Among the significant contributors to noise are the continuous stirring of the assay using a magnetic stir bar and scattering of light by the capsules themselves. In order to compare activities of each of the capsule architectures over the four-week period (Figure 46), student t-tests were used to determine if the activity/unit mass values differed significantly over the weeks (α value of 0.05).

Figure 46. Activity of effective active encapsulated GOx in capsules over a four-week period. Calculated activity is per unit mass. Symbols indicate significant difference in activity over time, determined using student t-tests using α = 0.5.
According to the tests, capsules with architectures \{PSS-PDDA\}_4(MF) and \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF) did not show a significant change in activity over the four-week period. Except for \{PSS-PAH\}_4(MF) capsules, which completely lose activity by the second week, a distinct trend in change in activity was not identifiable in the case of the other architectures. Since the activities were normalized to enzyme mass the activity/unit mass is indicative of the effects of materials comprising the capsule wall and capsule interior on the encapsulated enzyme. From Figure 46, it is can be concluded that since there were no significant differences in activity/unit mass over the four-week study in the case of both \{PSS-PDDA\}_4(MF) and \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF) capsules, the capsule materials did not have an adverse effect on enzyme conformation.

Summarizing the above results, \{PSS-PDDA\}_4(MF) and \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF) capsules retained a significant portion of encapsulated enzyme at the end of the four-week study (59% and 79%, respectively). However, it has to be underscored that the enzyme was entrapped in the walls and there was no internal matrix in the case of \{PSS-PDDA\}_4(MF) capsules, whereas in the case of \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF) capsules it was encapsulated in the internal charged matrix. This may be the reason why a higher percentage of GOx leached out in the former case.
5.2 Comparison of Diffusion Properties of Different Wall Architectures by Fluorescent Recovery After Photobleaching (FRAP)

5.2.1 FRAP Experiments to Study the Effect of Capsule Wall Architecture on Diffusion of FITC-GOx.

Diffusion coefficients for GOx were obtained in the case of each of the four architectures by performing FRAP experiments and using mathematical models for calculation of diffusion coefficients of GOx as described in Chapter IV. Figure 47 contains a graph showing the comparison of diffusion coefficients for various wall architectures.
Figure 47. Comparison of average GOx diffusion coefficients of the 4 capsule types. Models, M1 and M2 were used to estimate diffusion coefficients from raw data of fluorescence recovery obtained after photobleaching of capsules. \( n=8 \) for \( \{\text{PSS-PAH}\}_4(\text{MF}) \) and \( \{\text{PSS-PDDA}\}_4(\text{MF}) \), \( n=6 \) for \( \{\text{PVS-PAH}\}_2 + \{\text{PSS-PAH}\}_2(\text{MF}) \) and \( \{\text{PVS-CHT}\}_2 + \{\text{PSS-PAH}\}_2(\text{MF}) \).

Student t-tests were performed to statistically verify if the diffusion coefficients were significantly different from each other. Average diffusion coefficients for GOx were three times higher for \( \{\text{PSS-PDDA}\}_4(\text{MF}) \) capsules than for \( \{\text{PSS-PAH}\}_4(\text{MF}) \) capsules indicating that \( \{\text{PSS-PDDA}\}_4(\text{MF}) \) capsule walls are more permeable than \( \{\text{PSS-PAH}\}_4(\text{MF}) \) capsule walls. However, referring back to the leaching results, larger quantities of GOx leached out \( \{\text{PSS-PAH}\}_4(\text{MF}) \) capsules than \( \{\text{PSS-PDDA}\}_4(\text{MF}) \) capsules. The two results cannot be corroborated because the entrapment of GOx in the...
two cases occurs in the capsule walls; though the diffusion coefficients are indicative of the permeability of the walls, they cannot be used to describe the capacity of capsules to retain the enzyme entrapped in the walls. Hence, though \( \{\text{PSS-PDDA}\}_4(\text{MF}) \) are highly permeable to GOx by simple diffusion, they entrap and retain GOx more efficiently in the walls than \( \{\text{PSS-PAH}\}_4(\text{MF}) \) capsules.

Diffusion coefficients of GOx for \( \{\text{PSS-PDDA}\}_4(\text{MF}) \) and \( \{\text{PVS-PAH}\}_2 + \{\text{PSS-PAH}\}_2(\text{MF}) \) capsules were comparable. This result was expected, because \( \{\text{PVS-PAH}\}_2 + \{\text{PSS-PAH}\}_2(\text{MF}) \) capsules had only two bilayers of PSS-PAH, and it has already been mentioned that the inner PVS-PAH layers are weak. Hence, the inner PVS-PAH bilayers may have partially disintegrated during the core dissolution process or may be discontinuous and for this reason, more permeable than \( \{\text{PSS-PAH}\}_4(\text{MF}) \) capsules.

GOx had higher diffusion coefficients in the case of \( \{\text{PVS-CHT}\}_2 + \{\text{PSS-PAH}\}_2(\text{MF}) \) capsules compared with the other three types of capsules. A possible reason for this result is that the internal charged matrix of the capsules played an important role in attracting and hence rapidly drawing the enzyme into the capsule interior, whereas in the other three cases, the enzyme entered the capsules by simple diffusion.

The mobile fraction \( (R) \) was calculated in each of the four cases (Figure 48). The mobile fraction can change due to different circumstances: interaction of the fluorescent labeled molecules with other molecules or materials (capsule wall, internal matrix etc), which could prevent or restrict the free diffusion of molecules. Capsules with architectures \( \{\text{PSS-PDDA}\}_4(\text{MF}) \) and \( \{\text{PVS-PAH}\}_2 + \{\text{PSS-PAH}\}_2(\text{MF}) \) had comparable mobile fractions that were approximately twice that of \( \{\text{PVS-CHT}\}_2 + \{\text{PSS-PAH}\}_2(\text{MF}) \) and \( \{\text{PSS-PAH}\}_4(\text{MF}) \) capsules. For capsules with higher mobile
fractions, diffusion coefficients for GOx were higher, except for \( \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF) \) capsules. A possible reason is that while the presence of a matrix reduces the mobile fraction, the inward movement of the labeled enzyme is faster because of electrochemical attraction by the charged matrix.

![Figure 48](image)

Figure 48. Comparison of average mobile fractions of the 4 capsule types. \( n=8 \) for \( \{PSS-PAH\}_4(MF) \) and \( \{PSS-PDDA\}_4(MF) \), \( n=6 \) for \( \{PVS-PAH\}_2 + \{PSS-PAH\}_2(MF) \) and \( \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF) \)

Recently, a novel LbL assembly of photosensitive diazoresin was demonstrated\(^{215}\). This technique is advantageous in that it can be used to improve stability of multilayer films by converting weak interactions between adjacent layers into strong covalent bonds, and can be applied to a variety of materials containing sulfonate groups, carboxylic acid groups, or phenol groups\(^{216}\). Further, the procedure is uncomplicated and comprises of two steps: first, self-assembly involving ionic (for sulfonate groups) or hydrogen-bonding (for carboxylic acid or phenol groups) forms the initial multilayer;
second, the weak interaction between the neighboring layers is converted to a covalent one by UV irradiation. A previous report described how the stability of hollow capsules based on DAR was improved by this procedure. A useful conclusion drawn from this work was that DAR-based systems can be prospective candidates for permanent encapsulation of materials. Another recent publication reported the stable encapsulation of GOx using DAR-based microcapsules. Confocal microscopy and atomic force microscopy confirmed stable encapsulation of enzyme after UV irradiation, and activity assays revealed that encapsulated enzyme possessed 52.8% of the catalytic activity exhibited by the same amount of free enzyme. The following section describes the application of the FRAP procedure to study the effect of number of DAR bilayers on diffusion of labeled GOx.

5.2.2 FRAP Experiments for Study of Effect of Number of DAR Bilayers in Capsule Wall on Diffusion of FITC-GOx.

Figure 49 shows that alternation of zeta-potential with coating of each charged layer confirms the successful deposition of five bilayers of DAR/PSS.
Figure 49. Zeta-potential measurements of {DAR/PSS}$_5$

FRAP experiments performed to obtain diffusion coefficients for MC-{DAR/PSS}$_5$ and MC-{DAR/PSS}$_8$ showed no significant differences between the values of diffusion coefficients obtained for capsules with five bilayers and eight bilayers (Figure 50).
Figure 50. Diffusion coefficients of MC-{DAR/PSS}$_5$ and MC-{DAR/PSS}$_8$

Figure 51 contains a graph comparing the mobile fractions for MC-{DAR/PSS}$_5$ and MC-{DAR/PSS}$_8$ capsules. Though the diffusion coefficients of the labeled enzyme are comparable in both cases, the mobile fraction reduces by a factor of 5 after addition of three bilayers of DAR/PSS. The reduction of the mobile fraction indicates that the addition of layers does obstruct the diffusion of GOx, which reduces enzyme recovery in the capsule interior.
Figure 51. Comparison of average mobile fractions for MC-{DAR/PSS}_5 and MC-{DAR/PSS}_8. n=6 for MC-{DAR/PSS}_5 and MC-{DAR/PSS}_8.

From the results of the procedures described in this section to compare microcapsules with different architectures, it was concluded that {PVS/CHT}_2-{PSS/PAH}_2 capsules were the most stable and showed minimal leaching (79% of enzyme retained) and the activity of the encapsulated enzyme did not change significantly over four weeks.

Capsules with wall architecture {PVS/CHT}_2-{PSS/PAH}_2 were selected for demonstration of glucose sensing. The experiments performed to characterize these sensors and the corresponding results and discussion are described in the next section.
5.3 Fluorescence Sensors for Glucose Using pH-Sensitive Pyrene 8-Hydroxy-1,4,6-Trisulfonylchloride (HPTS)

Capsules with an internal charged matrix of chitosan, PVS, and melamine formaldehyde oligomers were fabricated by LbL assembly of \( \{\text{PVS-CHT}\}_2 + \{\text{PSS-PAH}\}_2 \) on MF templates. They were loaded with TRITC-GOx for visualization with confocal microscopy. Morphology of unloaded capsules was studied using AFM. The capsules were loaded with unlabeled GOx to study the stability of enzyme-loaded capsules and activity of the encapsulated enzyme over a period of four weeks, using techniques described in Chapter IV. The results of stability and activity studies were described in section 5.1. A pH-sensitive dye (HPTS), was used to label GOx, and HPTS-GOx was loaded into the capsules. Since HPTS has dual excitation peaks and shows ratiometric variation in response to changes in pH, no reference dye was required to account for variation of sensor response from fluctuations in instrument parameters or environmental changes. In order to study the effect of labeling on the pH response of HPTS, pH sensitivities of both conjugated and free HPTS were studied. The sensors were evaluated for glucose sensitivity and response.

5.3.1 Visualization of Loaded Capsules Using CLSM and AFM

Confocal fluorescence and transmission images of capsules in loading solution of TRITC-GOx and a typical line scan of loaded capsules are shown in Figures 52(a) through (d). The transmission image of a capsule shows that it has an opaque interior, which indicates that it is not hollow and empty. The linescan (Figure 52 [d]) proves that the intensity in the interior of the capsules and the walls is 100 times the intensity of the
loading solution, indicating that the quantity of GOx inside the capsules is far more than that in the loading solution. From AFM images of “empty” capsules, (Figure 52 [e] through [g]) it is clear that the capsule is not flat. An estimate of the average height of capsules was calculated by averaging the heights at 40 random points on the imaged capsule. The average height was calculated to be 1.3μm. Assuming the average thickness of the wall to be (4x4nm=16nm), the height should not exceed (2x16nm=32nm) when the capsules collapse after drying if the capsules did not have a matrix. Thus, the height of the capsule with an internal matrix is 41 times the height of a collapsed capsule without an internal matrix, clearly indicating the presence of a matrix.

Figure 52. (a),(b) Confocal fluorescence and (c) transmission mode images, and (d) line scan of chitosan capsule loaded with TRITC-GOx, (e)AFM 3-D and (f),(g) 2-D images of unloaded Chitosan capsule

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5.3.2 Working of HPTS Dye

The pH-sensitive dye HPTS consists of a conjugate acid and conjugate base pair. The excitation spectra of HPTS (Figure x) indicate the presence of two excitation maxima, namely, 405nm (excites the conjugate acid part) and 454nm (excites the conjugate base part). The pH of the solution decides the relative concentrations of the acid and base components, and consequently also decides the intensities of the two peaks. Hence, the peak ratio (405/454nm) varies with the pH.

5.3.3 Buffer Solution Equilibrium: Effect of Addition of Acid on pH of Buffer, HPTS and HPTS-GOx (Both in Buffer) Solutions

The standard equilibrium equation for a phosphate buffer system is

\[ \text{H}_2\text{PO}_4^- + \text{H}_2\text{O} \leftrightarrow \text{H}_3\text{O}^+ + \text{HPO}_4^{2-} \]  \hspace{1cm} (14)
The equilibrium constant $K_a = 6.3 \times 10^{-8}$ is calculated using:

$$K_a = \frac{[\text{H}_3\text{O}^+][\text{HPO}_4^{2-}]}{[\text{H}_2\text{PO}_4^-]} \quad (15)$$

Addition of 0.1M HCl causes the equilibrium to shift to lower pH values due to the reaction of HCl with the conjugate base. This corresponding reaction is:

$$\text{HCl} + \text{HPO}_4^{2-} \rightarrow \text{H}_2\text{PO}_4^- + \text{Cl}^- \quad (16)$$

After estimation of the change in number of moles of the conjugate acid and base from equation (16), the final value of the number of moles of $\text{H}_3\text{O}^+$ is calculated using equations (14) and (15). The pH of the buffer after addition of the acid is given by:

$$\text{pH} = -\log[\text{H}_3\text{O}^+] \quad (17)$$

In this way, values for pH of the buffer following each successive addition of HCl were calculated. These values were also experimentally determined by measuring the pH of solutions of PBS 7.4 buffer, HPTS in PBS 7.4 buffer and HPTS-GOx in PBS 7.4 buffer while titrating 0.1M HCl. Figure 54 contains a comparison of the experimental and predicted values of changes in pH of PBS buffer, and the experimental values of HPTS and HPTS-GOx in PBS buffer during acid titration. The experimental values in all three cases, PBS, HPTS in PBS and HPTS-GOx in PBS indicate that pH changes follow the same trend, irrespective of the presence or absence of the dye or the enzyme. The predicted values agreed with the experimental values with a maximum variation of 18% for the last two datapoints. It was experimentally determined that the pH electrode absorbed 12-15$\mu$l of buffer solution after every measurement. However, the variation
between the predicted and experimental values for the last two datapoints was observed even after accounting for the volume changes of the buffer associated with the measurement process.

![Graph showing pH change with titration of 0.1M HCl solution: comparison of calculated results with experimental result in the case of PBS 7.4 buffer, HPTS in PBS buffer and HPTS-GOx in PBS buffer.]

**Figure 54.** Change in pH with titration of 0.1M HCl solution: comparison of calculated results with experimental result in the case of PBS 7.4 buffer, HPTS in PBS buffer and HPTS-GOx in PBS buffer.

### 5.3.4 Comparison of Response of HPTS Solution and HPTS-GOx in PBS Buffer to Titration of 0.1M HCl

The response of the conjugated dye in solution to changes in pH was studied by recording the fluorescence spectra of HPTS-GOx after subsequent additions of 0.1M HCl. As mentioned in the previous section, the pH of the solution changed significantly with the addition of aliquots of acid. It is also apparent that in both cases pH changes
approximately matched up with each other at every point of titration. The decrease in pH caused a corresponding increase in the excitation peak ratio (405/454nm), as shown in Figures 55(b) and 55(d). The pH changed from 7.4 to 4.4 at the end of the titration experiment. The peak intensity ratio also changes comparably in both cases. The shapes and consequently the ratios obtained in the two cases are different because the number of HPTS molecules in a solution of HPTS is far more than those in the same volume of HPTS-GOx due to which a stronger fluorescence signal is obtained from the former. Also, the process of conjugation of HPTS with GOx can alter the spectra of HPTS because of the change in the local environment of the ligand and its structure after conjugation\textsuperscript{218}.  

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Figure 55 Changes in fluorescence intensities of the acid (405nm) and base (454nm) peaks of (a) HPTS and (c) HPTS-GOx with addition of aliquots of 0.1M HCl. Peak intensity ratios of (b) HPTS and (d) HPTS-GOx vs. addition of 0.1M HCl.

The experiments performed in 5.5.3 and 5.5.4 verified that the pH of the buffer changed in a predictable manner with the addition of HCl, both in the presence and the absence of the labeled enzyme. Also, though the shape of the curve and the peak ratios were altered due to labeling, there was an increase in peak ratio with the addition of acid in both cases. According to the glucose oxidation reaction, for every molecule of glucose oxidized, a corresponding hydrogen ion is generated, and so the pH changes due to the...
addition of glucose can be correlated with those due to addition of HCl. Hence, the equilibrium equations for the PBS buffer system can be used to estimate pH changes due to addition of glucose.

5.3.5 Glucose Titration Test for HPTS-GOx in DI Water

Initial spectra of HPTS-GOx in DI water (measured pH of DI water = 5) showed a prominent acid peak (405nm), and the absence of a basic peak (454nm). Addition of 2µl of 0.2M KOH to the solution sufficiently raised the pH (7.8-8) to produce a strong basic peak. Subsequent addition of small volumes of glucose (100mg/ml) rapidly reduced the pH of the solution. It can be seen from Figure 56 that addition of only 9µmoles of glucose reduced the pH from 8 to 5 and completely suppressed the basic peak.
The final pH of the solution was found to be approximately 5. Hence, HPTS-GOx solution in DI water is not a good medium for testing the glucose sensitivity of the protein-dye conjugate because the protons generated by the catalytic action of GOx on glucose rapidly lower the pH of the entire volume of the solution. Moreover, since the primary application of the sensor is intended to be in vivo, and most regions of the body are buffered to a pH of 7.4, a physiological buffer PBS 7.4 was used to study the sensitivity of HPTS-GOx to glucose.
5.3.6 Glucose Titration Tests for HPTS-GOx in PBS 7.4 Buffer

At the pH of the PBS buffer used (7.4), both the conjugate acid and base forms of HPTS exist. Therefore, both acid and base peaks at 405nm and 454nm, respectively, are prominent in the initial spectra before addition of glucose. Figure 57 shows the effect of serial addition of known aliquots of glucose (100mg/ml), which increased the glucose concentration of the solution from 0mM to 266mM at the end of the titration experiment.

![Normalized intensity vs Wavelength graph](image)

Figure 57. Response of HPTS-GOx in PBS 7.4 buffer to glucose titration.

Since three consecutive measurements were made after addition of glucose, and the error in measurements was within 2% of the average value, it can be assumed that the added glucose reacted completely before each measurement was made. Each addition of glucose reduces the pH of the solution in a controlled manner due to the buffering action.
of PBS, resulting in a finite reduction of the acid-to-base peak ratio. The buffer neutralizes a portion of the protons generated by the enzymatic reaction between the conjugated GOx and glucose. However, addition of large quantities of glucose surpasses the buffering action of PBS, and the overall pH of the solution at the end of the glucose titration experiment was found to be between three and four.

Figure 58(a) shows the sensitivity curve, i.e. the curve showing the change in peak ratio (405/454nm) of HPTS-GOx with titration of known aliquots of glucose. With each addition of glucose to the solution, the cumulative concentration of acid generated due to oxidation of glucose increased. The peak ratio increased approximately linearly between 0mM to 112mM, due to a steady increase in acidity of the solution with each successive aliquot of glucose, caused by the enzymatic catalysis of glucose by GOx with acidic byproducts (gluconic acid). The slope of the sensitivity curve in the linear region was calculated as 3.3x10⁻² ratio/mM. The pH of the solution changes significantly (pH 3-4) at the end of the titration experiment due to addition of a large quantity of glucose and generation of acidic products, which cannot be compensated by the buffering action of PBS. The measured value of the final pH agrees with the predicted value (3.6), which was calculated using the Le Chatelier's principle and the equilibrium equations for PBS buffer. Above the linear range, the signal appears to plateau, since HPTS becomes insensitive to pH changes at very low pH values (pH values around three). The linear region of the sensitivity curve, which was obtained by titrating known aliquots of glucose, can be used as a calibration curve to measure unknown glucose concentrations. To measure unknown glucose concentrations, the measured intensity ratio after adding an
aliquot of glucose is corroborated with the x-axis values (intensity ratios) of the sensitivity curve, and the corresponding y-axis value indicates the glucose concentration.

The estimated error in predicted glucose concentration was calculated as follows for all glucose sensitivity experiments:

The measured glucose (G) is given by

\[
G = A + \frac{\sigma_{\text{ratio}}}{S}
\]  

(18)

A is the actual glucose concentration and \( \sigma_{\text{ratio}} \) is the standard deviation in peak intensity ratio. \( S \) is the sensitivity of measurement given by \( \frac{\Delta \text{ratio}}{\Delta mM} \). The value of \( \frac{\sigma_{\text{ratio}}}{S} \) is a measure of the error in glucose concentration. This value indicates the level of noise in the system and hence the error in the values of predicted glucose concentrations.

Figure 58(b) shows the inverse relation between glucose added and the measured intensity ratio, with glucose values on plotted on the ordinate and the corresponding intensity ratios on the abscissa. This curve indicates the contribution of instrumental noise to glucose measurement. It is apparent from Figure 58(b), that the noise level (standard deviation in the concentration of glucose sensed) is not consistent across all data points. When smaller quantities of glucose are added, the acid generated is correspondingly low. Hence, the change in intensity ratio is small and the measurements are easily affected by noise. The sources of noise can be due to continuous stirring of the sample, the inherent noise contributed by the electronics (PMT), and the measurement process- the two excitation peaks (400nm and 454nm) are not measured at the same time and hence the contribution of instrumental noise at each peak may vary. The normal physiological glucose range is 3.8-6.6mM (70-120mg/dl). It can be observed from

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Figure 58 (b), that there is significant contribution from instrumental noise in this range. Hence, the predicted value of glucose will not be accurate because a single standard deviation in the sensitivity curve can indicate a false-positive for hypo- or hyperglycemic condition. This system is inappropriate for glucose sensing in the physiological range of interest.

Figure 58. (a) Change in peak ratio (405/454nm) of HPTS-GOx with titration of known aliquots of glucose (b) Inverse graph of (a) indicating standard error of calibration due to instrumental noise

5.3.7 Glucose Titration Tests for Encapsulated HPTS-GOx in DI Water

Initial spectra of HPTS-GOx encapsulated in \( \{ \text{PVS-CHT} \}_2 + \{ \text{PSS-PAH} \}_2 \) capsules in DI water (pH=5) showed a prominent acid peak (405nm) and the absence of a basic peak (454nm). Approximately 2μl of KOH was added to raise the pH of the solution (pH=7.8-8) to produce the basic peak. Subsequently, small aliquots of glucose (100mg/ml) were titrated to the suspension. From Figure 59 it can be seen that there is a progressive decrease in 454nm peak with the increase in the 402nm peak with titration of glucose.
Just like the glucose titration test with HPTS-GOx solution in DI water, local changes in peak intensities also lead to global changes in the overall pH of the dispersion. This was verified by measuring the pH of the dispersion after the titration experiment was conducted. The final pH was between 2 and 3.

The variation in peak intensity with consecutive addition of glucose aliquots (the sensitivity curve) is shown in Figure 60(a). The peak ratio increased approximately linearly ($R^2=96\%$) between 0mM and 35mM of glucose addition. The slope of the sensitivity curve in the linear region was $21\times10^{-3}$ ratio/mM. The excitation ratio remains approximately steady beyond the linear region, since HPTS is insensitive to pH changes below pH 3. Figure 60(b) shows the inverse relation between glucose added and the measured intensity ratio, indicating the contribution of instrumental noise to glucose.
measurement, with glucose values plotted on the ordinate and the corresponding intensity ratios on the abscissa. The contribution of instrumental noise to glucose measurement is low in the physiological range of interest (3.8-6.6mM). Unknown glucose concentrations in the physiological range can be determined by using the sensitivity curve as a calibration curve and corroborating the changes in intensity ratio with the x-values and determining the corresponding y-values.

According to confocal images (also indicated at the respective time of acquisition in Figure 60[a]) of the capsules before and after the glucose sensitivity test, the fluorescence intensity from the capsule interior indicate that HPTS-GOx did not leach out of the capsules after the glucose test and that the capsules were intact after the test. Hence, the response to glucose additions was from the capsule interior.

**Figure 60.(a)** Change in peak ratio (405/454nm) of HPTS-GOx encapsulated in \{PVS-CHT\}_2 + \{PSS-PAH\}_2 capsules in DI water with titration of known aliquots of glucose (b) Inverse graph of (a) indicating standard error of calibration due to instrumental noise

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Figure 61 is an illustration of the reaction involving oxidation of glucose by HPTS labeled GOx encapsulated in a microcapsule and suspended in DI water. Changes in local pH (inside the capsule) were responsible for a corresponding change in peak intensity ratio of HPTS. In spite of the possible buffering action of the indicator (HPTS) in the capsule interior during the sensing process, some protons diffuse to the solution surrounding the capsule, and the absence of any kind of buffering action by the DI water raised the pH globally as well as local to the sensor.
H⁺ - protons generated inside the sensor due to oxidation of glucose

Figure 61. Illustration of the working of HPTS-GOx sensors during glucose titration test in DI water.

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5.3.8 Glucose Titration Test for HPTS-GOx Encapsulated in Chitosan Capsules (Capsules Suspended in PBS 7.4 Buffer)

Both acid and base peaks at 405nm, and 454nm, respectively, were prominent in the initial spectra before addition of glucose. Figure 62 contains spectra showing the effect of serial addition of aliquots of glucose.

![Figure 62. Response of HPTS-GOx encapsulated in {PVS-CHT}$_2$ + {PSS-PAH}$_2$ capsules suspended in PBS 7.4 buffer to glucose titration. The peak ratio increases steadily with each successive addition of glucose.](image)

The variation in peak intensity with consecutive addition of glucose aliquots (the sensitivity curve) is shown in Figure 63(a) with the inverse graph in Figure 63(b). There is a linear increase in peak ratio between 0mM and 32mM ($R^2=93\%$) of glucose, which covers the physiological range of interest. The slope of the sensitivity curve in the linear
region was calculated to be $3.7 \times 10^{-3}$ ratio/mM. The contribution from instrumental noise to glucose measurement as indicated in Figure 63(b); given a real value of glucose=5.5mM, the indicated value due to instrumental noise can be anywhere between 4.6 and 6.4mM. Similarly, at the lower end of the physiological range of interest, for actual glucose value=3.3mM, the indicated value can vary anywhere between 2.5 and 4.1mM. For smaller quantities of glucose added, the acid generated is correspondingly low; this factor combined with the low sensitivity of the sensors leads to smaller changes in intensity ratio, and the measurements are more easily affected by noise.

![Confocal images](image.png)

Figure 63. (a) Change in peak ratio (405/454nm) of HPTS-GOx encapsulated in \( \{\text{PVS-CHT}\}_2 + \{\text{PSS-PAH}\}_2 \) capsules in PBS 7.4 with titration of known aliquots of glucose (b) Inverse graph of (a) indicating standard error of calibration due to instrumental noise

Confocal images also indicated at the respective time of acquisition in Figure 63(a) of the capsules before and after the glucose sensitivity test prove that just like the previous case, the capsules were intact and HPTS-GOx did not leach out of the capsules after the test. The pH of the entire suspension was measured with a pH strip both before and after glucose titration test and was found to remain approximately constant. Hence, it is apparent that local pH changes (inside the capsule) were responsible for change in peak concentration.
intensity ratio of HPTS-GOx. Protons that diffuse rapidly into the solution around the sensors are compensated by the buffering action of PBS. Each addition of glucose causes the pH of the encapsulated HPTS-GOx to reduce in a controlled manner due to the buffering action of PBS, resulting in a steady reduction of the acid-to-base peak ratio. In this case, global changes in the overall pH of the suspension were not observed; the pH did not vary significantly when measured before and after the glucose test.

Figure 64 illustrates a possible mechanism by which there are pH induced changes in the response of HPTS inside the capsule, while the pH of the suspension remains approximately constant during the glucose titration experiment. HPTS consists of a conjugate acid- conjugate base pair, and essentially acts as a buffer for protons. The conjugate base reacts with protons thus altering the fluorescent properties of the dye depending on the equilibrium between the conjugate acid-conjugate base pair. Protons generated inside the capsule interact with HPTS before they interact with phosphate buffer due to proximity of the pH-sensitive dye to the reaction products of HPTS-GOx catalyzed oxidation of glucose. Since these protons are bound to the dye producing a change in its response, they can no longer change the pH of the suspension. In this way, HPTS buffers the internal environment of the capsule, and predominates over the effect of PBS buffer. The protons that do not interact with HPTS are buffered by PBS. There are references describing this effect when analyte specific dyes are introduced in cells\textsuperscript{219,220}. Dyes that are pH-sensitive have been shown to buffer the internal cell environment\textsuperscript{221}.  

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The reactions representing a system involving glucose oxidase are:

\[
A + E \xrightleftharpoons[k_1, k_{-1}]{k_2} X_1 \rightarrow F + \text{gluconate}
\]

\[
F + C \xrightleftharpoons[k_3, k_{-3}]{k_4} X_2 \rightarrow F + H_2O_2
\]

where \( k_1, k_2, k_3, \) and \( k_{-1}, k_{-3} \) are the forward and reverse reaction rate constants, respectively, \( A \) and \( C \) are the primary and co-substrates (glucose and oxygen), \( F \) and \( E \) are the reduced and oxidized form of the enzyme. \( X_1 \) and \( X_2 \) are the complexes formed due to binding of enzyme with glucose and oxygen, respectively. Since the total enzyme concentration in the system is constant, and according to the pseudo-steady-state assumption the formation and dissociation of intermediate complexes (\( X_1 \) and \( X_2 \)) occur very rapidly, and the two coupled differential equations representing the system are:

\[
\frac{\partial A}{\partial t} = D_g \frac{\partial^2 A}{\partial x^2} - k_1 AE + k_{-1} X_1 \quad (19)
\]

\[
\frac{\partial C}{\partial t} = D_o \frac{\partial^2 C}{\partial x^2} - k_3 FC + k_{-3} X_2 \quad (20)
\]

where \( D_g \) and \( D_o \) are the diffusion coefficients for glucose and oxygen respectively. Furthermore, (19) can be related to proton (\( H^+ \)) production and diffusion by

\[
\frac{\partial H^+}{\partial t} = k_2 X_1 + D_H \frac{\partial^2 H^+}{\partial x^2} \quad (21)
\]
where $D_H$ is the proton diffusivity. The protons produced during the oxidation of glucose are available for reaction with HPTS. The base form of HPTS reacts with $H^+$ producing the acid form of HPTS.

$$H^+ + \text{HPTS} \leftrightarrow \text{HPTS (H$^+$)}$$

Since the acid and base forms of HPTS are excited at different peaks (405nm and 454nm respectively), the peak ratio changes with the change in pH.
Figure 64. Illustration of the working of HPTS-GOx sensors during glucose titration test in PBS buffer.

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Le Chatelier's principle and using equilibrium equations for PBS described in section 5.5.3 were applied for prediction of change in pH of PBS buffer with addition of aliquots of 0.1M HCl to determine the final internal pH of the sensors suspended in PBS after performing the glucose titration experiment (Figure 65). For every mole of glucose added, correspondingly a mole of H⁺ is generated, with the assumption that all the glucose has reacted. The assumption is valid because after addition of an aliquot of glucose, three consecutive measurements were made and the maximum error in measurements was within 2% of the measured ratio, which indicates that for every measurement all of the added glucose was oxidized. Another assumption was that the concentration of PBS molecules is the same inside the capsule as the surrounding solution. The reason for this assumption is that the sensitivities of free and encapsulated HPTS-GOx in PBS solution are comparable (3.3x10⁻³ and 3.7x10⁻³ respectively) indicating that the availability of PBS to the labeled enzyme is the same in capsules as in solution. The value of pH was calculated to be 3.2 at the end of the glucose titration experiment. Since HPTS is insensitive to pH changes below pH 3, the peak ratio remains steady with the decrease in pH.

A comparison of the properties of the HPTS-GOx sensing system both in solution and after encapsulation is presented in Table 1. It can be seen that encapsulated HPTS-GOx tested in DI water has higher sensitivity than that tested in PBS because in the latter case, protons generated during oxidation of glucose are compensated by PBS. From the experiments performed above, it is apparent that the response of sensors fabricated by encapsulating HPTS-GOx in \{PVS-CHT\}_2 + \{PSS-PAH\}_2 was linear in the physiological range of interest, both when the sensitivity experiments were

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performed in DI water and in buffer. Since most regions of the human body are buffered at approximately pH 7.34, the results of the glucose sensitivity experiment in PBS buffer are more appropriate for \textit{in vivo} applications.

In order to alleviate the effects of instrumental noise in order to improve the response of the sensors in the physiological region of interest, especially for experiments performed in PBS, a number of steps can be taken: optimization of stirring speed of sample during measurement, using higher concentrations of sample to minimize the effects of the inherent noise contributed by the electronics (PMT), and using a dual emission channel fluorometer (the two excitation peaks are not measured at the same time and hence the contribution of instrumental noise at each peak may vary). The sensitivity of HPTS-GOx is 10 times less in PBS than in DI water because of which the effects of noise are more pronounced when experiments are performed in buffer. In order to improve the sensitivity of HPTS-GOx in buffer, the labeling ratio and the strength of the buffer can be optimized. Also, by controlling the surface charge of the outermost layer of the wall, diffusion of PBS buffer molecules into the capsule interior can be prevented by Donnan exclusion$^{22,23}$. 

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Table 1. Range and sensitivity of (a) HPTS-GOx solution in PBS buffer, (b) Encapsulated HPTS-GOx in DI water, and (c) Encapsulated HPTS-GOx in PBS buffer

<table>
<thead>
<tr>
<th></th>
<th>HPTS-GOx solution in PBS buffer</th>
<th>HPTS-GOx encapsulated in (PVS-CHT)$_2$ + (PSS-PAH)$_2$ tested in DI water</th>
<th>HPTS-GOx encapsulated in (PVS-CHT)$_2$ + (PSS-PAH)$_2$ tested in PBS buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear Range (mM)</td>
<td>0-112</td>
<td>0-35</td>
<td>0-32</td>
</tr>
<tr>
<td>Sensitivity (ratio/mM)</td>
<td>3.3x10$^{-3}$</td>
<td>21x10$^{-3}$</td>
<td>3.7x10$^{-3}$</td>
</tr>
</tbody>
</table>

A study of microcapsules with different wall architectures and the effect of capsule wall components on the encapsulation of GOx has been described. Though the HPTS-GOx based sensors described in this dissertation have not actually been used to measure glucose, a novel method of measuring glucose using pH-sensitive dyes immobilized in microcapsules has been demonstrated. The sensors exhibit a linear response in the physiological range of interest. Future work involves conducting experiments to demonstrate measurement of unknown glucose values by using the sensitivity curves obtained in the experiments as calibration curves. Further, the aforementioned measures need to be implemented in order to decrease the effects of instrumental noise on measurements, especially in PBS buffer.

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CHAPTER VI

CONCLUSIONS

This dissertation is a useful contribution to the science of enzyme encapsulation and glucose sensing. The methods described, particularly the application of LbL assembly to develop microdevices for biomedical applications, and experimental techniques to study, compare, and characterize micro-containers serve as a useful guide to students involved in similar research. Capsules with architectures \{PSS/PAH\}_4, \{PSS/PDDA\}_4, \{PVS/PAH\}_4 + \{PSS/PAH\}_4, \text{ and } \{PVS/PAH\}_4 + \{PSS/CHT\}_4 \text{ were fabricated using MF template. Among these, internal matrices were formed in the case of } \{PVS/PAH\}_4 + \{PSS/CHT\}_4 \text{ capsules during the capsule fabrication process, due to the interaction of CHT and PVS with the MF. In the case of } \{PSS/PAH\}_4 \text{ capsules fabricated using MC templates, internal matrices were fabricated by polymerizing amino acids using zero-length crosslinkers EDC and NHS. } \{PSS-PDDA\}_4(MF) \text{ and } \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF) \text{ capsules retained a significant portion of encapsulated enzyme at the end of the four-week study (59% and 79%, respectively). While the enzyme was entrapped in the walls and there was no internal matrix in the case of } \{PSS-PDDA\}_4(MF) \text{ capsules, in the case of } \{PVS-CHT\}_2 + \{PSS-PAH\}_2(MF) \text{ capsules it was encapsulated in the internal charged matrix. This may be the reason why a higher percentage of GOx leached out in the former case.}
FRAP was used to compare the effect of capsule wall architecture on mobility of labeled GOx. Average diffusion coefficients for GOx were three times higher for \( \{\text{PSS-PDAA}\}^4(\text{MF}) \) capsules than for \( \{\text{PSS-PAH}\}^4(\text{MF}) \) capsules indicating that \( \{\text{PSS-PDAA}\}^4(\text{MF}) \) capsule walls are more permeable than \( \{\text{PSS-PAH}\}^4(\text{MF}) \) capsule walls. Though \( \{\text{PSS-PDAA}\}^4(\text{MF}) \) are highly permeable to GOx by simple diffusion, they entrap and retain GOx more efficiently in the walls than \( \{\text{PSS-PAH}\}^4(\text{MF}) \) capsules. Diffusion coefficients of GOx for \( \{\text{PSS-PDAA}\}^4(\text{MF}) \) and \( \{\text{PVS-PAH}\}^2 + \{\text{PSS-PAH}\}^2(\text{MF}) \) capsules were comparable. Diffusion coefficients of GOx were higher in the case of \( \{\text{PVS-CHT}\}^2 + \{\text{PSS-PAH}\}^2(\text{MF}) \) capsules compared with the other three types of capsules. A possible reason for this result is that the internal charged matrix of the capsules played an important role in attracting and hence rapidly drawing the enzyme into the capsule interior, whereas in the other three cases, the enzyme entered the capsules by simple diffusion. For capsules with higher mobile fractions, diffusion coefficients for GOx were higher, except for \( \{\text{PVS-CHT}\}^2 + \{\text{PSS-PAH}\}^2(\text{MF}) \) capsules. A possible reason is that while the presence of a matrix reduces the mobile fraction, the inward movement of the labeled enzyme is faster because of electrochemical attraction by the charged matrix.

Finally, experiments were performed to demonstrate glucose sensitivity of HPTS-GOx both in solution and after encapsulation. Capsules with an internal charged matrix of chitosan, PVS, and melamine formaldehyde oligomers were fabricated by LbL assembly of \( \{\text{PVS-CHT}\}^2 + \{\text{PSS-PAH}\}^2 \) on MF templates. They were loaded with TRITC-GOx for visualization with confocal microscopy. Morphology of unloaded capsules was studied using AFM. A pH-sensitive dye (HPTS), was used to label GOx,
and HPTS-GOx was loaded into the capsules. Since HPTS has dual excitation peaks and shows ratiometric variation in response to changes in pH, no reference dye was required to account for variation of sensor response from fluctuations in instrument parameters or environmental changes. In order to study the effect of labeling on the pH response of HPTS, pH sensitivities of both conjugated and free HPTS were studied. The sensors were evaluated for glucose sensitivity. The response of sensors was linear in the physiological range of interest, both when the sensitivity experiments were performed in DI water and in buffer. Since most regions of the human body are buffered at approximately pH 7.34, the results of the glucose sensitivity experiment in PBS buffer are more appropriate for in vivo applications.

For future work, a number of steps need to be taken in order to alleviate the effects of instrumental noise in order to improve the response of the sensors in the physiological region of interest, especially for experiments performed in PBS. These include optimization of stirring speed of sample during measurement, using higher concentrations of sample to minimize the effects of the inherent noise contributed by the electronics (PMT), and using a dual emission channel fluorometer (the two excitation peaks (400nm and 454nm) are not measured at the same time and hence the contribution of instrumental noise at each peak may vary). The sensitivity of HPTS-GOx is 10 times less in PBS than in DI water because of which the effects of noise are more pronounced when experiments are performed in buffer. In order to improve the sensitivity of HPTS-GOx in buffer, the labeling ratio can be optimized. Also, experiments need to be performed to demonstrate measurement of unknown glucose values by using the sensitivity curves obtained in the experiments as calibration curves.
APPENDIX A

ATTRACTION AND ENCAPSULATION OF HRP BY POLYMERIZING ACRYLIC ACID MONOMERS BY POLYELECTROLYTE MICROCAPSULES

In the process of exploring various possibilities for encapsulation of GOx, a novel method of encapsulation of HRP was developed. This method is unsuitable for encapsulation of GOx for reasons explained later in the chapter. The procedure was developed and studied to demonstrate new and uncomplicated methods to encapsulate macromolecules. The following section describes the results of this short study.

Polyelectrolyte microcapsules with shell architecture \{PAH/PSS\}₄ were fabricated by LbL assembly of polyelectrolytes on MnCO₃, followed by dissolution of the core. After free-radical polymerization of acrylic acid inside the capsules using initiator \((NH₄)₂S₂O₈\), which created a negatively charged matrix inside the capsule, the spontaneous loading of cationic HRP into the capsules was visualized with CLSM, and the effect of using increasing quantities of monomer and initiator on percentage encapsulation of HRP and activity was evaluated.
A1.1 Visualization of Spontaneous Loading of HRP Inside Capsules Using CLSM

The spontaneous loading of TRITC-HRP (1mg/ml) was followed by CLSM as shown in Figure 2. The term "spontaneous" has been used to emphasize the fact that the internal charge of the capsule matrix is responsible for loading of enzyme; the properties of the wall are not altered in any way (by changing pH, salt composition of loading solution etc) to facilitate loading. It can be observed from the first confocal image at t=0 seconds that the capsule interior had zero intensity prior to addition of cationic TRITC-HRP (pI=8). After addition of the labeled enzyme, the intensity in the capsule interior increased rapidly and reached a high intensity within 90 seconds as the TRITC-HRP diffused inside the capsule. Thus, the process of loading was simple and the capsules were loaded quickly, and with high efficiency. It can be observed from Figure 65 that the fluorescence intensity inside the capsule reached steady state within 120 seconds. The charge of the capsule matrix is responsible for loading of enzyme; the properties of the wall are not altered in any way (by changing pH, salt composition of loading solution etc) to facilitate loading.
Figure 65. Loading of microcapsules with TRITC-POx. Images: Left to right: microcapsule imaged at 0s, 30s, 60s, 90s, 120s and 150s after addition of loading solution. Data points on the graph represent change in the averaged intensity in a fixed region of interest (ROI, Area=1.2μm²) inside the imaged capsule.

A1.2 Microcapsules at Various Stages Studied Using Confocal Imaging and AFM

The process of polymerization was not precisely controlled and limited to the capsule interior only. Hence, the monomers polymerized even on the surface of the capsules. However, the fact that there is sufficient polymerization occurring in the capsule interior can be indirectly verified by confocal microscopy imaging of the capsules. Figure 66 contains a capsule with an internal PAA matrix, which was loaded with HRP and rinsed with DI water after loading.
From the image, it is apparent that the capsule interior is filled with the labeled enzyme. The acrylic acid polymerized in the surrounding solution was previously removed by rinsing and dialyzing against DI water. For comparison, capsule without the internal matrix is shown in Figure 68. A linescan of the capsule in a loading solution of TRITC-GOx indicates equilibrium loading, with the intensity being approximately the same inside the capsule as in the loading solution. Further, when these capsules were rinsed, only TRITC-GOx entrapped in the walls was retained and the enzyme in the capsule interior was removed during the rinsing process, indicating that the presence of a charged internal matrix helps encapsulate and retain a larger quantity of enzyme.
AFM imaging was used in order to compare the topographic features of capsules before and after formation of the acrylic acid matrix, and after encapsulation of HRP. Figure 69(a) contains an image of a dried capsule prior to polymerization of PAA internal matrix.

The capsule is flat with folds and invaginations. After polymerization of acrylic acid and loading with enzyme, it can be seen from Figures 69(b) and 69(c) that the
capsule is no longer flat and has a "bean bag" structure. An estimate of the average height of capsules in each of the three cases was calculated by averaging the heights at 20 random points on the imaged capsules. The average heights of the capsules (N=3) before and after formation of the PAA matrix and after loading were calculated to be 163±2.4, 292±0.98 and 757±4.34nm, respectively. Thus, polymerization of acrylic acid inside the capsule interior caused an increase in the average height to approximately twice that of an empty dried capsule. Loading the capsules with HRP increased the average height to five times its original height. The additional increase in the average height after incubation in loading solution and rinsing, as compared to capsules after polymerization but before loading of HRP can thus be attributed to loading of HRP inside the capsules.

A.1.3 Effect of (Acrylic Acid : (NH₄)₂S₂O₈) Volumes on Efficiency of Enzyme Encapsulation and Enzyme Activity

Several groups have reported detrimental effects of free radicals (peroxides, reactive oxides, free radical sulfates, etc.) on enzyme activity²²⁴. Most free radicals are known to inactivate enzymes by oxidizing the enzymes, whereas some act as both oxidizing and reducing substrates, e.g. H₂O₂.²²⁵ Based on this consideration, it was hypothesized that use of higher quantities of initiator may be detrimental to enzyme activity due to higher quantities of residual initiator, post termination of the polymerization reaction, despite careful rinsing of the capsules and dialysis. It has also been shown that larger quantities of monomers produce higher quantities of the polymerized end product and thus, higher internal ionic strength of the capsules²²⁶. Therefore, it was also hypothesized that a stronger internal charge will attract and retain
more enzyme into the capsule matrix. Hence, though higher monomer volumes are necessary to encapsulate more enzyme, if the initiator is not efficiently removed from the capsule suspension after the reaction, it is detrimental to enzyme activity. To test these hypotheses, the effect of using three different volumes of monomers and initiators (1:1 by volume) on the enzyme encapsulation efficiency and activity was studied.

Table 2 contains a comparison of mass encapsulated per capsule and the activity/unit mass in the three cases. From Table 2, it can be seen that there was a correlation between the quantities of the monomer and initiator used and the percentage of HRP in the loading solution encapsulated in the polymerized capsules.

Table 2. Enzyme encapsulation efficiency and activity for loaded capsules with three different volumes (10μL, 50μL and 100μL each) of initiator: monomer (1:1 ratio by volume) used in the polymerization of acrylyc acid to form the internal matrix. Number of capsules=2050000, estimated by Coulter counter

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>10μL(1:1)</th>
<th>50μL(1:1)</th>
<th>100μL(1:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Encapsulated</td>
<td>45%</td>
<td>49%</td>
<td>59%</td>
</tr>
<tr>
<td>Mass/capsule (ng)</td>
<td>0.18</td>
<td>0.2</td>
<td>0.24</td>
</tr>
<tr>
<td>Activity/unit mass</td>
<td>0.007</td>
<td>0.025</td>
<td>0.51</td>
</tr>
</tbody>
</table>

The capsules with 100μL of monomer and initiator encapsulated 59% of the total mass of enzyme in the loading solution, whereas the others encapsulated 45% and 49% of HRP. The estimated mass of HRP encapsulated per capsule was 0.18, 0.2, and 0.24ng, respectively, for capsules with increasing reactant volumes. These data support the hypothesis that higher volumes of reactants attract and entrap higher quantities of oppositely-charged enzyme. There is an approximately linear relationship ($R^2=97\%$)
between the increase in the volumes of the reactants and the mass of enzyme encapsulated.

The activity of the encapsulated enzyme increased exponentially with the increase in volumes of reactants. The sample having capsules with the highest volumes of monomers and initiator and correspondingly the highest percentage of encapsulated enzyme was nearly 70 times as active as one with the lowest volumes of the reactants and a lower percentage of encapsulation of enzyme. Since the activity is normalized to mass, it is independent of the mass of encapsulated enzyme and indicates the effect of other factors, such as effect of material used to create the internal matrix as well as favorability of the internal matrix structure to enzyme conformation, availability of enzyme to substrate and assay. Since the materials used for fabrication of the matrix are the same in all the three cases, the effect of this factor on activity can be ruled out. According to the kinetics of free-radical polymerization, increasing the monomer or initiator concentration increases the rate of polymerization; higher initiator concentrations however produce numerous lower molecular weight polymeric units. Hence, it is possible that some of these may diffuse out over time because of which the internal matrix is not very stable causing the encapsulated enzyme to also leach out. Hence, the effective activity is the activity of two populations of enzymes- free enzyme in the solution and encapsulated enzyme.
Figure 69. Activity/unit mass/unit capsule of encapsulated POx for capsules with three different volumes (10 μl, 50 μl and 100 μl each) of initiator and monomer.

Presence of residual initiator due to inefficient separation of capsules from the initiator after the polymerization reaction can also inactivate the enzyme. However, in this case, apparently, the quantity of the free radical initiator used for the polymerization reaction did not affect the activity, as is evident from the high activity of the sample using the highest volume of initiator. This is because all samples were dialyzed overnight, which removed the unreacted initiator from the capsule solution. Hence, an important consideration for the maintenance of activity of the encapsulated enzyme is an efficient separation of the initiator from the capsules prior to loading of the enzyme.

In this study, a monomer: initiator volume ratio (and hence concentration ratio) has been maintained constant, though the volumes have been changed to study the effect of increasing quantities of the reactants on the encapsulated enzyme. Hence, future work...
will involve studying the effect of factors such as initiator to monomer ratio, and the reaction incubation time on the formation of PAA and activity of the encapsulated enzyme. Also, long-term effects of encapsulation on the enzyme stability and activity (activity/unit mass) will have to be studied. Such a study is important to understand if the entire encapsulated enzyme is equally active or if there are different populations of enzymes with different conformations depending on their position in the matrix. If the enzyme is bound too tightly in the matrix, it may not be able to move or change its conformation during catalysis, and is hence inefficient.

**A1.4 Attempt to Load GOx Into Capsules With Polyacryllic Acid Matrix in the Capsule Interior**

With the successful encapsulation of HRP, the method was tested for the encapsulation of GOx. Various methods, such as varying the pH of the loading solution of FITC-GOx (pH 5 and pH 9), were attempted in order to study the effect of varying the charge of FITC-GOx and the PAA matrix on loading. Figure 71 shows the typical results in each case. The attempts to load FITC-GOx using this method were unsuccessful. The successful loading of HRP and the failure to load GOx can be explained by the fact that GOx(pI=4.2) has an acidic isoelectric point whereas that of HRP is basic (pI=8.1). Also, the isoelectric point of PAA is at a low acidic pH.
So, at basic pHs of loading solution, TRITC-GOx is negative and PAA matrix is also strongly negative. Therefore, the internal matrix electrostatically repels the like-charged enzyme molecules. At acidic pHs, TRITC-GOx is positively charged, and the PAA matrix is close to its pI and hence weakly negatively charged. The results concluded that in both cases GOx is repelled by the capsule interior and cannot be encapsulated by this method.
APPENDIX B

STUDY OF PMMA AS AN ALTERNATIVE TEMPLATE TO M.F

This work was aimed at developing a novel and repeatable method for fabrication of microcapsules using LbL assembly of polyions on PMMA templates, followed by dissolution of the PMMA core using acetone. The main challenge was to have the appropriate number and types of polyions (wall composition and thickness) and most appropriate procedure for titrating water into the suspension of capsules (after dissolution with acetone) in order to counter the tremendous osmotic pressure that resulted during dissolution of the core. As described in the chapter IV, three different methods were used to fabricate the capsules, which were then qualitatively assessed using the confocal microscope to assess the quality of the yield.

The zeta-potential measurements of the assembly of PAH and PSS are shown in Figure 71. The assembly of polyions was confirmed by the alternation of zeta-potential values with the assembly of each consecutive layer.
Figure 71. Zeta-potential measurement of coating of \( \{ \text{PAH/PSS}_5 \} + \{ \text{PAH-FITC/PSS}_3 \} \)

It was observed from confocal images, that approximately 40-50% of capsules produced by the first method (involving overnight immersion in acetone followed by rinses with acetone and DI water) described in chapter IV were ruptured. It was speculated that the overnight immersion in acetone might have damaged the capsules because the acetone may have a detrimental effect on capsule wall materials. Figure 72 contains images of various stages of this method.
Figure 72. Confocal images of capsules with architecture \( \{ \text{PAH} / \text{PSS} \}_3 + \{ \text{PAH-FITC} / \text{PSS} \}_3 \), (a) acetone overnight, (b) acetone rinse1, (c) acetone rinse2, (d) DI water rinse1, (e) DI water rinse2, (f) DI water rinse3, (g) DI water rinse4

Since the first method involving immersion of coated particles in acetone for a prolonged period of time, in second method the capsules were imaged after varying the acetone immersion time. This method did not improve the yield of capsules for all times of immersion (Figure 73).
Figure 73. Confocal images of capsules with architecture \( \{\text{PAH/PSS}\}_3 + \{\text{PAH-FITC/PSS}\}_3 \), (a) undissolved coated PMMA particles, (b) after five-hour immersion in acetone, (c) after 17-hour immersion in acetone, (d) after 24-hour immersion in acetone.

Increasing the wall thickness and titrating DI water into the sample after acetone immersion for an hour resulted in damage to approximately 30-40% of the capsules (Figure 74). Hence, the yields were comparable in all three cases.
Figure 74. Confocal images of capsules with architecture \( \{\text{PAH/PSS}\}_8 + \{\text{PAH-FITC/PSS}\}_3 \)

In the case of MF template, it was previously cited that during the core dissolution process, there is a possibility of the rupture of the walls of capsules, if the MF oligomers do not diffuse out of the interior sufficiently fast\(^{227}\). From Figure 75, it can be seen from the indicated areas in the capsule walls that PMMA dissociated into chunks that were too big to diffuse out of the capsule wall. This effect has two main consequences: first, it increases the osmotic pressure so much so that it causes the capsules to burst; second, large pieces of PMMA are always present in the capsules or capsule walls.
Figure 75. Confocal and transmission images of capsules with architecture \{PAH/PSS\}_8 + \{PAH-FITC/PSS\}_3 in loading solution of GOx-TRITC. The dark regions in (a) and the indicated regions in (b) are undissolved pieces of PMMA.

The integrity of the capsules with architecture \{PAH/PSS\}_8 + \{PAH-FITC/PSS\}_3 was evaluated by a “salt test.” Three μL of a capsule sample was applied to the surface of a bare slide after which five μL of 1M NaCl salt solution were added to the capsule sample. Figure 76(a) contains the initial images of the capsules before the addition of salt. It can be seen that the capsules are spherical and show no signs of collapsing. Figures 76(b) and 76(c) contain images of capsules after addition of salt solution. It can clearly be seen that the capsules collapsed after salt addition. This collapse confirms the integrity of capsules; that is, if the capsules were ruptured, they would be unaffected by the large osmotic forces exerted by the salt solution. Approximately 70% of the capsules collapsed, while the rest remained intact, suggesting that the latter were ruptured.
Figure 76. "Salt test" for confirmation of integrity of $\{\text{PAH/PSS}\}_8 + \{\text{PAH-FITC/PSS}\}_3$ capsules

This study demonstrated that though PMMA proves to be an alternative to MF, much work still needs to be done in order to improve the overall yield of capsules. Other organic solvents such as toluene can also dissolve PMMA, however, they are difficult to handle when compared with acetone. For future work, choices of alternative solvents for PMMA may be considered. Recently, the use of alternative templates that can be simply and inexpensively mass-produced in the lab, and which can be dissolved using acid or non-acid solvents producing a high yield of microcapsules, has been demonstrated\textsuperscript{14,176}. 

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APPENDIX C

CHEMICALS

Polyelectrolytes, buffers and microspheres

- PDDA (Cat number: 40,903-0, Sigma)
- PSS (Cat number: 43,457-4, Sigma)
- PVS (Cat number: 27,196-9, Sigma)
- PAH (Cat number: 283215, Sigma)
- Chitosan (448869, Sigma)
- PEI (P-3143, Sigma)

Enzymes / amino acids

- Glucose Oxidase (G-2133, Sigma)
- Peroxidase (P-8250, Sigma)

Dyes

- FITC (F7250, Sigma)
- TRITC (T-490, Molecular Probes)
- HPTS (M0535, Marker Gene Technologies)

Other chemicals

- O-dianisidine (D-3252, Sigma)
- Glucose (G5250, Sigma)
• PBS buffer (Cat number: 79382, Fluka)
• Sodium bicarbonate buffer (S-8875, Sigma)
• Acrylic acid (Cat number: 147230)
• Ammonium Persulfate (A9164, Sigma)
• Pyrogallol (P0381, Sigma)
• EDC (03450, Fluka)
• NHS (56485, Fluka)
• DMF - N,N-Dimethylformamide (DMF) (D4551, Sigma)
• Hydrogen Peroxide (349887, Sigma)
APPENDIX D

EQUIPMENT/MATERIALS

1. Scanning fluorescence spectrometer (QMI, Photon Technology International)
2. UV-Vis spectrophotometer (Perkin Elmer, Lambda 45)
3. Scanning Probe Microscope (Atomic Force Microscope Quesant, Model 250)
4. Zeta potential- Zeta Potential Analyzer (Zeta Plus, Brookhaven Instruments Corp.)
5. Gel-filtration columns (Amersham Pharmacia Biotech PD-10 desalting column)
6. pH-meter (Thermo Orion, Model A-210)
7. Sonicator (Branson, 1510)
8. Micro cover glass for imaging with confocal and FRAP experiments (VWR Scientific, Cat number: 48395 252)
APPENDIX E

MATLAB PROGRAM FOR DETERMINATION OF
DIFFUSION COEFFICIENTS USING
FRAP PROCEDURE

removes stored information about variables
clear all
close all

These parameters must be properly identified for file to function
properly. Make sure the current directory contains the files to be
imported

Make sure that the first 2 time/fluorescence data points reflect
the pre-bleach lapse

These are the ONLY parameters that need to be adjusted

molecular_weight='160kDa';
file_name=('matlab input for pss pah 70 diffusion studies 3 19 04');
file_name=('mcdar81ine3');
sheet_name=('mcdar81ine3');
capsule_structure='MF-(PSS/PVS)_2+(PSS/PAH)_2';

Data importing commands
a=xlsread(file_name,sheet_name);

defines the intensity values before and after
bleaching
Iprebleach=a(2,2);
Ipostbleach=a(3,2);

Put fluorescence recovery data in fractional form
f_fractional_form=(a(1:end,2)-a(3,2))/(a(end,2)-a(3,2));

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Since data contains info about prebleach, that data is ignored, by defining time \( t=0 \) as the first recovery point with the corresponding intensity value.

\[
\text{trecovery} = a(3: \text{end}, 1) - a(3, 1);
\]
\[
\text{frecovery} = f_{\text{fractional\_form}}(3: \text{end}, 1);
\]
\[
\text{processed\_time\_and\_recov\_data} = [\text{trecovery}, \text{frecovery}];
\]

Calculation of \( k \)

\[
\text{syms ratio;}
\]
\[
\text{lpre} = \text{num2str}(\text{lprebleach});
\]
\[
\text{lpost} = \text{num2str}(\text{lpostbleach});
\]
\[
\text{ratio} = [\text{lpost} \div ' \text{lpre} ' = \exp(-k)'];
\]
\[
x = \text{solve}(\text{ratio}, 'k');
\]
\[
\text{k\_calc} = \text{eval}(x(1));
\]

FRAP model from Blonk et. al.

Note: model_parm(1) = \( k \)

\[
\text{model\_parm} = [\text{k\_guess}, \text{tau\_guess}];
\]
\[
\text{t\_recovery} = \text{trecovery'};
\]
\[
\text{N} = 50;
\]
\[
\text{n} = [0: \text{N}];
\]

LSQ curve fitting using a calculated \( k \) value, then fitting \( \tau \)

\[
\text{f\_string} = \text{'sum} (( (-\text{num2str(k\_calc)}) \div \text{ones(1, ' num2str(length(frecovery))'))/ (\text{factorialarray}' (\text{num2str(n)})\text{)*ones(1, ' num2str(length(frecovery))') \div 1+(\text{factorialarray}' (\text{num2str(n)})\text{)*ones(1, ' num2str(length(frecovery))')) \text{)*} (1+2*(\text{ones(' num2str(N)' \div 1,1)*t)/\text{tau\_k\_calc})))'));
\]
\[
\text{f} = \text{inline('string', 'tau\_k\_calc', 't');}
\]
\[
[\text{tau\_k\_calc}, \text{resid}] = \text{lsqcurvefit(f, tau\_guess, t\_recovery, frecovery');}
\]

LSQ curve fitting \( \tau \) first, then fitting for \( k \)

\[
\text{f\_string} = \text{'sum} (( (\text{num2str(k\_guess)}) \div \text{ones(1, ' num2str(length(frecovery))'))/ (\text{factorialarray}' (\text{num2str(n)})\text{)*ones(1, ' num2str(length(frecovery))')) \div 1+(\text{factorialarray}' (\text{num2str(n)})\text{)*ones(1, ' num2str(length(frecovery))')) \text{)*} (1+2*(\text{ones(' num2str(N)' \div 1,1)*t)/\text{tau}) ));
\]
\[
\text{f} = \text{inline('string', 'tau', 't');}
\]
\[
[\text{tau}, \text{resid}] = \text{lsqcurvefit(f, tau\_guess, t\_recovery, frecovery');}
\]

\[
\text{f\_string} = \text{'sum} (( (\text{-k}) \div \text{ones(1, ' num2str(length(frecovery))'))/ (\text{factorialarray}' (\text{num2str(n)})\text{)*ones(1, ' num2str(length(frecovery))')) \div 1+(\text{factorialarray}' (\text{num2str(n)})\text{)*ones(1, ' num2str(length(frecovery))')) \text{)*} (1+2*(\text{ones(' num2str(N)' \div 1,1)*t)/\text{tau}) ));
\]
num2str(length(frecovery)) ));*1/(1+(transpose([' num2str(n) '])*ones(1,' num2str(length(frecovery)) ));*1*2*(ones(' num2str(1) '+1,1)*t)/ ' num2str(tau) ' ) ) ));

f=inline(f_string,'kVt');
[k,resid] = lsqcurvefit(f,k_guess,trecovery',frecovery');
model1=eval(f_string);

%LSQ curve fitting using for k anf tau simultaneously
f_string=['sum( ((-model_parm(1)).A (transpose([' num2str(n) '])*ones(1,' num2str(length(frecovery)) ));)/ (transpose(factorialarray([' num2str(n) '])*ones(1,' num2str(length(frecovery)) ));))/(1+2*(ones(' num2str(1) '+1,1)*t)/model_parm(2)) )
);

f=inline(f_string,'model_parm','t');
[model_parm,resid] = lsqcurvefit(f,model_parm0,trecovery',frecovery');
model2=eval(f_string);

%delete first recovery point, since it is 0
mpe_model0=mean(abs(frecovery(2:end)-model_k_calc(2:end))./frecovery(2:end))*100;
km=mean(abs(frecovery(2:end)-model1(2:end))./frecovery(2:end))*100;
mpe_model2=mean(abs(frecovery(2:end)-model2(2:end))./frecovery(2:end))*100;

%solving for diffusion coefficient
%w_squared was wxperimentally determined
w_squared=0.0000557*2;
D0=w_squared/(4*tau_k_calc);
D1=w_squared/(4*tau);
D2=w_squared/(4*model_parm(2));

%spreadsheet information
[file_type, sheet_names]=xlsfinfo(file_name);

%plotting analysis
textplace_x=.1*max(trecovery);
textplace_ymain=.6*max(model1);
textplace_ysub=.45*max(model1);
textplace_ysub2=.3*max(model1);
textplace_ysub3=.15*max(model1);

figure(1)
subplot(2,1,1)
plot(t,model_k_calc,'g',t,model1,'b',t,model2,'--r',trecovery,frecovery, 'k', 'LineWidth',2)
title(['capsule_structure,', FITC-GOx Mw=', molecular_weight, ' (' , sheet_name, ' ) '])
xlabel('Time (sec)')

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ylabel('Fluorescence Intensity (A.U.)')
legend([['Model0 Output (tau)'],['Model1 Output (tau then k)'],['Model2 Output (simul)'],['Exp. Data'],4)
legend('boxoff')
text(textplace_x,textplace_ymain,['M_0: \{\tau\}_D=',num2str(tau_k_calc),'s; D=',num2str(D0),'cm^2/s'; K=',num2str(k_calc)])
text(textplace_x,textplace_ysub,['M_1: \{\tau\}_D=',num2str(tau),'s; D=',num2str(D1),'cm^2/s'; K=',num2str(k)])
text(textplace_x,textplace_ysub2,['M_2: \{\tau\}_D=',num2str(model_parm(2)),'s; D=',num2str(D2),'cm^2/s'; K=',num2str(model_parm(2))])
text(textplace_x,textplace_ysub3,['MPE_0=',num2str(mpe_model0); MPE_1=',num2str(mpe_model1); MPE_2=',num2str(mpe_model2)])

subplot(2,1,2)
plot(a(1:end,1),a(1:end,2), 'k','Linewidth',2)
title(["Unprocessed FRAP Data"])xlabel('Time (sec)')
ylabel('Fluorescence Intensity (A.U.)')
legend('Unprocessed Data',0)
legend('boxoff')
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