TOWARDS DEVELOPMENT OF A NONINVASIVE & COLORIMETRIC

GLUCOSE SENSOR

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ABSTRACT

This study was focused on development of a novel glucosensor for continuous and noninvasive monitoring of tear glucose. This was an effort to relieve diabetic patients of pain while assaying their blood glucose by conventional invasive methods like finger pricking. The project consisted of two parts: synthesis of a catechol based dye and improvement of its properties through further modification; secondly, synthesis of a novel pyridinylboronic acid framework. The target dye was synthesized through a series of reactions. Subsequently, the dye was tested for its utility as an indicator by using dye-glucose-pyridinylboronic acid system; but it failed to respond positively at the physiologic pH of 7.4.

In an interesting reaction, furfurylamine was ring expanded to a bromopyridinol, which was successfully converted to 3-allyloxy-5-bromopyridinol. Allyl functionality was introduced to serve two purposes, one being to utilize its C=C double bond as an anchoring site for a siloxane oligomer. The other purpose of protecting the hydroxyl group during sensitive reactions was badly defeated when both the alkyl lithium and palladium catalyzed cross-coupling route to pyridinylboronic acid synthesis failed. This interference by allyl group could be avoided by using acryloyl moiety. Anyway, in terms of future prospect, the idea of glucosensor seemed to be pretty much realizable. Proper fine-tuning of the dye system and pyridine moiety could lead to successful completion of the project.
DEDICATION

To my parents, sisters and late grandfather
ACKNOWLEDGEMENTS

I am grateful to my advisor, Dr. Jun Hu for guiding and supporting me throughout this study with great patience and care. I am also thankful to him for his enlightening discussions on various stages during this study. I also extend my sincere thanks to Dr. Weiping Zheng for his helpful suggestions regarding writing this thesis. I would also like to thank my group mates for helping me on numerous occasions.

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CHAPTER I
GLUCOSE SENSORS

1.1 Introduction

Carbohydrates, the most abundant biomacromolecules, are diverse both in terms of their structure and function. Carbohydrate-based bioconjugates, such as glycoproteins and glycolipids, govern many biological phenomena. These natural glycoconjugates are the key elements for cell to cell signal transduction and intra- and extra-cellular communications. Their functions can be varied and modified depending on the location, structure, physical and chemical properties of the related carbohydrates.1

1.1.1 Functions of Glucose

Complex carbohydrates contained in foods are broken down into corresponding monosaccharides. Glucose is one of those monosaccharides and is an essential element in many physiological processes. Glucose present in blood provides nourishment from cell to cell to sustain the life processes. The carbohydrate itself gets burnt and provides vital energy during respiration. There are many other constructive roles of glucose in human body.

1.1.2 When Glucose plays foul

Unfortunately, not all the consequences of glucose action on human body are beneficial. The physiological systems of the human body is so adjusted that it allows only a certain range of glucose concentration in blood stream; anything beyond that range is
strongly disfavored. If the maximum tolerance limit is exceeded, then a physiological disharmony occurs; this diseased state is then called hyperglycemia or diabetes mellitus. If the blood glucose level is not kept below this threshold limit, many other dangerous complications in heart, liver, kidney may arise. Thus, foul play of excess blood glucose can lead to heart attack and multi-organ failure due to chronic malfunctioning of glucose.

1.1.3 Tackling Diabetes

Diabetes is spreading across the world at an alarming rate. It is recorded as one of the prime killers during the past few years. Each successive year, the rate of patients increases in a fearsome way. Proper treatment of a diabetic patient can help him lead a normal life without much trouble. To achieve success in fighting diabetes mostly depends on continuous monitoring of blood glucose. Strict control of blood glucose level is the key factor in determining the diet, dosage and timing of medication. In fact, success in diabetes treatment runs parallel with effective monitoring of blood glucose level.

1.2 Available Glucose Monitoring Techniques

Numerous glucose monitoring techniques have been developed since sixties. These may be categorized broadly as invasive and noninvasive techniques.\(^2\)-\(^7\)

1.2.1 Invasive Glucosensing Techniques

Invasive methods of glucose monitoring are the most prevalent and these sensors mainly operate on principles of electrochemistry where a direct contact between a body fluid (mainly blood and urine) and a suitable electrode is essential.\(^8\) Urine based detection is disadvantageous and not also reliable as a real-time glucose sensing method. Collecting blood samples from random pricking is the most prevalent of all invasive techniques;

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however this is not only costly and painful but also gives rise to other complications as well. Blood sample collection by laser based technique is preached to be less painful than finger pricking by a needle or lancet; but overall, this method is also painful. Implantable probes containing insulin operate through a sensor which releases insulin whenever blood glucose level is high. However, this technique is also invasive in nature and works well for a single day only.

1.2.2 Noninvasive Glucosensing Techniques

Almost all the diabetic patients hate invasive methods of glucose estimation because these are not only costly but also painful. Thus noninvasive methods of glucose monitoring are needed badly, since it will greatly improve the patient compliance.

1.2.2.1 Glucosensing Watch and Tattoos

GlucoWatch® is the first noninvasive glucosensor approved by FDA in 2001. It works on the principle of microdialysis and reverse iontophoresism. This system is not only very costly, but also is highly recommended to be used in conjunction with another standard glucose monitoring device. Thus GlucoWatch® can, by no means, stand as a standard and unbiased method of continuous glucose monitoring. Glucose sensing skin patches and tattoos are among other noninvasive techniques of glucose monitoring; but these did not enjoy much success as well due to other complications.

1.2.2.2 Arylboronic Acid based Glucosensors

Carbohydrates are well known for their strong interaction with a variety of arylboronic acids. Pronounced Lewis acidity of arylboronic acids makes them prone to attack by Lewis bases like water, alcohols, diols and carbohydrates. This interaction is actually a result of an intrinsic affinity of arylboronic acids to single or multiple cis-diol
groups present in carbohydrates. This interaction between the afore-mentioned units leads to reversible covalent bonding.\textsuperscript{10} Naturally arylboronic acid based molecules have been proven to be excellent glucosensors. Fortunately, this interaction, being covalent, is strong enough to survive in aqueous solution, but again, reversible, so that sensors could be designed utilizing and manipulating this equilibrium.

1.2.2.2.1 Stability of Arylboronic Acid-Carbohydrate Conjugates

Favorable orientation of two close-lying hydroxyl groups is very important factor in determining the stability of the tetrahedral anionic intermediate formed between the arylboronic acid and the diol. This explains why various diol and triol arrangements in carbohydrates exhibit differential affinities towards arylboronic acids. Thus depending on the O-C-C-O dihedral angle\textsuperscript{11}, ease of ester formation varies with different sugars. Cis-1,2- & 1,3-diols of various carbohydrates form relatively less-strained 5- and 6-membered cyclic arylboronate esters which are thermodynamically stable. The lesser the strain in the 5- or 6-membered ring (arylboronate ester) is, the greater is the strength of complexation which is reflected in the binding constant values of various sugars. Nature of the aryl group (such as phenyl, pyridyl etc) is another important decider in complexation equilibria. Introducing electron withdrawing moieties (such as –F, -Cl, -NO\textsubscript{2} ) in the aromatic system does lower the pk\textsubscript{a} values of the arylboronic acids resulting in stronger interaction.

1.2.2.2.2 Thermodynamics Favors Complexation

Lewis acidity of boronic acids is prominent in aqueous neutral or alkaline solution. When suitable diols or triols are added to aqueous solution of aryl boronic acids then two water molecules are expelled at the expense of each diol or triol is complexed. This raises
the entropy of the system and thus chelating effect makes the change in Gibbs free energy value go to negative. So arylboronic acid-sugar complexation in aqueous solution is an entropy-driven phenomenon, and as a consequence, thermodynamically favorable process.\textsuperscript{12}

1.2.2.2.3 Various Arylboronic Acid-based Carbohydrate Sensors

In his pioneering research on phenylboronic acid-diol complexation, Lorand et al\textsuperscript{13} calculated binding constants of various diols successfully by pH depression method. Since then many carbohydrate-sensing devices based on arylboronic acids have been formulated with various degrees of success and promise. Czarnik\textsuperscript{14} group and Shinkai\textsuperscript{15} group reported two fluorescence-based glucosensors. After that sugar-sensing ability of arylboronic acids was explored comprehensively. There have been many other realized examples of arylboronic acid based biosensors.

1.2.2.3.1 Fluorescent Probe Containing Contact Lens

Contact lens approach regarding tear glucose monitoring has been first introduced by Badugu et al. Their method of glucose sensing involves utilization of a series of fluorescent arylboronic acids as a detection element/probe of glucose. According to their claim, this fluorescent probe is capable of tracking down glucose present even in milimolar or submilimolar quantities.\textsuperscript{8,9}

1.2.2.3.2 Photonic Crystal based Glucosensor

Asher and coworkers developed a photonic crystal based noninvasive glucosensor which, according to their claim, could monitor ocular glucose concentration with significant accuracy. The group developed a type of acrylamide polymerized crystalline colloidal array hydrogel films which contained different types of substituted
phenylboronic acids as glucosensing elements. When these compounds form complexes with glucose a change in charge distribution occurs resulting in a blue shift in the diffracted light. They claimed that this blue shift corresponds well with tear-glucose concentration. Applying this technology in ocular devices or contact lenses they expected to monitor tear glucose continuously.

1.3 Author’s Approach: Colorimetric Contact Lens based Glucosensor

This project involves development of a colorimetric contact lens which will operate noninvasively and will be able to monitor tear glucose continuously indicating automatic threshold reminder. This method will enable diabetic patients to monitor their extent of hyperglycemia ceaselessly and painlessly in a cost-effective way. Instead of a phenylboronic acid, a pyridinylboronic acid had been used as glucosensing element due to its greater affinity towards glucose. The patient, after looking at the mirror, will compare the color in contact lens spot with a previously calibrated colorimetric paper. The comparison will help him evaluate the hyperglycemia level and take necessary action. Thus patient can be his own doctor.
CHAPTER II
DESIGN OF THE GLUCOSE SENSOR PROJECT

2.1 Introduction

Continuous noninvasive techniques for glucose estimation are the needs of the hour in diabetes treatment. Our project aims at realizing a convenient glucosensing device (based on pyridinylboronic acid) that can function uninterruptedly at or around the physiologic pH 7.4.

2.2 Basis of the Method

It is a colorimetric estimation method of tear glucose in millimolar and micromolar quantities. This novel approach is based on the direct and linear correlation between blood glucose and ocular glucose. Increase in glucose concentration in blood results in an equivalent increase in glucose concentration in tears. Ocular glucose concentration is about ten times or even lower than that in blood and it takes almost 30 minutes for establishment of a correspondence between blood and tear glucose level.

2.3 Sensing Element

Pyridinylboronic acids (PBAs) are known, so far, as the best glucose binding arylboronic acids. The unusually high binding affinity of this acid to glucose has been reflected in the association constant of the complex formation which is 272±25 M⁻¹. This
information is promising in terms of development of a highly sensitive glucosensor. Robust yet reversible complexing ability of 3-PBA with glucose at physiological pH (7.4) has given an opportunity to develop a glucosensor based on noninvasive estimation of ocular glucose.

2.4 The Idea

The project requires syntheses of few dyes which can complex with PBA. Main requirement is that the interaction be strongest at the physiological pH of tears. Colors of PBA-dye assemblies should be distinctly different as compared to free dyes. Thus these dyes can also act as molecular switches. PBA can bind tightly to various monosachharides and polysachharides as well. The working principle of this molecular switch is based on indicator displacement assay. This technique allows an indicator to form complex with a receptor. Complexation leads to a modified or shifted emission wave length of the indicator. Upon addition of an external analyte, complexation equilibrium is switched and free indicator, being replaced by the analyte, emits its characteristic wave length. In this project a catechol dye is an indicator; pyridinylboronic acid is the receptor and glucose is the analyte. So if PBA, dye and sugar all three are present in a system, there will be two competitive interactions: PBA-dye interaction and PBA-sugar interaction. From previous studies done by the author’s group, it has been noticed that addition of definite concentration of sugar solution to PBA-dye conjugate results in a visible color change due to dissociation of the conjugate forming free dye anion. The addition leads to the formation of a new conjugate PBA-sugar. This encouraging result clearly gives an indication that the project can be realized.
2.5 Synthetic Strategies

Earlier studies on contact lens method have not been successful because the pyridinylboronic acid (PBA), being small and polar, leaked out of the lens. To prevent leaching out of the sensor system during testing in artificial tears, a unique pyridinylboronic acid skeleton has been chosen. This pyridinylboronic acid can be synthesized in several steps from a versatile reagent furfurylamine. A hydroxyl group has been incorporated to introduce hydrophilicity in the pyridine system. Again the same group can act as a link to other functional groups like allyl or acryloyl group. In case of introduction of an allyl group it will be further linked to a siloxane oligomer which, through its bulkiness, can make the boronic acid immovable. Then siloxane-bound-PBA will be imprinted on a polymeric contact lens. PBA, being linked to the oligomer, cannot diffuse out of the contact lens. Alternatively an acryloyl or analogous group can also be connected to the hydroxyl group of the pyridine which can be oligomerized to form a macromolecular pyridinylboronic acid; and thus this PBA will also be stationed at a particular point of the contact lens, eliminating the tendency of its escape during testing. Eventually, several dye systems will be anchored on the contact lens and the lens system will then be tested with artificial tears containing various glucose concentrations.
CHAPTER III
EXPERIMENTAL SECTION

3.1 Synthesis of a Catechol Dye

Arylsulfonic acids were less suitable in lithiation reactions because of their greater hygroscopicity. Therefore crude 90% benzenesulfonic acid was converted to lithium salt of benzenesulfonic acid. In a round bottom flask 2 g of crude PhSO₃H was dissolved in ethanol. 2N solution of lithium hydroxide is added drop wise until the pH of the solution becomes approximately 7. Now the solvent system is removed by rotary evaporation. The salt was dissolved in minimum amount of hot ethanol. Then cold toluene was added drop-wise to recrystallize the lithium salt of benzenesulfonic acid crystals. That powder was heated at 60 °C under vacuum overnight. This way residual water and ethanol were removed and the salt is now ready for water-sensitive reactions.

Protection of the catechol group is a necessary step during the synthesis of the dye. In a round bottom flask, 4 mmol catechol was taken. 10 ml anhydrous DMF was added and it was stirred to dissolve the catechol. Then 12 mmol potassium carbonate was added and the resulting slurry was stirred for 15 minutes. 5 mmol of bromochloromethane was added drop-wise.¹⁸ A reflux condenser was connected to the flask. Temperature of the solution was raised to 85 °C gradually and the system was kept at that temperature for 3 hours. The reaction was monitored by thin layer chromatography. After completion of the
reaction, water and toluene were added to the DMF solution. DMF mixed up with water and the product, being more hydrophobic dissolved preferentially in the toluene layer. Separatory funnel was used to collect toluene layer. Toluene layer was washed two more times with water. In column chromatographic separation, 10% ethyl acetate in hexane was used as eluent to afford pure product. Protected catechol being a very high boiling liquid, was vacuum dried for 24 hours at room temperature. Then it was ready to do the lithiation reaction. A round bottom flask and clean magnetic stirrer was flame dried under vacuum and then the flask was cooled down under argon. This process was repeated two more times. 3 mmol of lithium salt was taken in the flask and the flask was stoppered with a two-way adapter. Flask was again kept under vacuum. 15 ml of freshly distilled & anhydrous THF was added to the flask by oven dried syringe under argon flow. The system was again evacuated and purged with argon. 3.3 mmol of n-BuLi was added similarly via a syringe. The system was kept at 0 °C by using ice-water bath. Within 10-15 minutes, the reaction was complete. The product was exclusively lithiated on the ortho position of the benzenesulfonic acid moiety. Generation of the dilithiated species resulted in a distinctive color change from colorless to orange-yellow. In another 250 ml flame-dried round bottom flask, 3 mmol protected catechol (PC) and 10 ml freshly distilled THF were taken. The flask was stirred to dissolve the PC. Then the solution in the second flask was then transferred to the reaction flask containing dilithiated benzene sulfonate (already under argon) via a double-headed needle (cannula). The reaction proceeded for three hours at 0 °C under argon atmosphere and then the reaction was allowed to proceed overnight at room temperature (20 °C).
Scheme 3.1 Synthetic diagram of catechol dye
Initial color of the product mixture was dirty yellow. A sample of the solution was taken in a test tube and 4N HCl was added and the test tube was shaken for a couple of minutes. The color turned to orange red. Here acid deprotected the ether linkage and unfolded the catechol group. On raising the pH of the solution by gradual addition of base (aqueous sodium hydroxide solution), no color change was observed until pH 9.4. Above this pH, the color of the dye turned deep red.

The solution was filtered and most of the undissolved lithium hydroxide and salts were removed. Then 4N HCl was added dropwise to help the solution reach pH 1. The mixture was shaken for few minutes to deprotect the catechol group. Under acidic
condition the dye was more soluble in 1-butanol and under alkaline condition it dissolved more in water in comparison to 1-butanol. Grabbing this opportunity, the acidic form of the dye was preferentially extracted in 1-butanol layer. Remaining salts were left in aqueous layer. This procedure was repeated thrice and almost pure dye is obtained. An accurate melting point could not be obtained since the product decomposed above 300°C. 

\[^1\text{H NMR (CDCl}_3\text{):} \delta 6.1 \text{ (s, 2H), 6.88 (d, 1H, } J = 12\text{Hz), 7.2 (m, 4H), 7.37 (m, 2H), 7.48 (m, 2H), 7.59 \text{ (m,1H), 7.76 (m, 2H) ppm.}; FTIR: 767, 819, 859, 933, 1020, 1040, 1126, 1189, 1248, 1344, 1448, 1488, 1503, 1588, 1725, 2960, 3418 \text{ cm}^{-1}.\]

Figure 3.2 \[^1\text{H NMR spectrum of the catechol dye}\]
Scheme 3.2 Interconversion of various dye forms

3.2 Synthesis of 5-Bromo-3-hydroxypyridine

7 ml of 48 % HBr (0.062mol) was taken in a round bottom flask and 28 ml water was added to it to make ~ 11.5 % HBr. Acid solution was cooled down to 10 °C. 0.1 mol (8.9 ml, d =1.09) 2-furfurylamine was added drop-wise at 10 °C under stirring condition. Temperature was lowered to -10 °C by using ethanol – dry ice mixture. 11 ml (.22mmol) of bromine was added drop-wise at -10 °C. Addition should be extremely slow and the entire volume of bromine should be added in 7 hour, under stirring condition. Resultant deep red solution was then stirred under inert argon atmosphere for 16 hours at -15 °C to -20 °C. To expel excess and unreacted bromine from the reaction mixture, argon gas was passed through the solution for one hour at -10 °C. At that point the pH of the solution
was negative. So concentrated aqueous sodium hydroxide was added drop-wise at -10 °C to raise the pH of the system to 1. At this point the color of the solution was deep red. The system was then allowed to equilibrate with the ambient temperature gradually and the reaction mixture was slowly heated to reach the reflux temperature in 55 minutes (bath temperature 135 °C). The solution was then kept at that temperature for five minutes and resultant dark brown solution was cooled down to 80 °C and the mixture was held at that temperature for few minutes. During that time, aqueous NaOH solution was added drop-wise and the pH of the solution was frequently checked with pH paper.\textsuperscript{20}

Scheme 3.3 Reaction sequence for the synthesis of 5-bromo-3-pyridinol
Addition of the alkali was continued till the pH became 5-6. Ideally pH of 5.3 was the best but it was hard to get to reach that much accuracy with pH paper. During the second phase of addition of concentrated alkali, color became blackish brown. Precipitation commenced at pH higher than 4 and it completes at about pH 5-6.

Black precipitate was filtered and dried under vacuum at 50 °C overnight and then 50 ml hot methanol was added to the precipitate and shaken well for few minutes. Then the mixture was filtered hot and the filtrate was collected. Upon cooling, some yellow

Figure 3.3 FT-IR spectrum of 5-bromo-3-hydroxypyridine
crystals separated from the of toluene and excess of hexane was added drop-wise under stirring condition to the dark brown solution. This led to preferential precipitation of impurities. Repeating this procedure thrice [after dissolving the crude product in minimum amount of ethanol and adding excess cyclohexane (ethanol : cyclohexane = 1:3)] yielded 75 mol% of pure light yellow crystals of 3-hydroxy-5-bromopyridine. Then pure product was recrystallized from hot toluene. Melting point of the product turned out to be 161-164 °C which supported literature. $^1$H NMR (DMSO): $\delta$ 4.0 (s, 1 H), 7.43 (m, 1H), 8.15 (m, 2H) ppm.; $^{13}$C NMR (DMSO): $\delta$ 120, 125, 137, 140, 154 ppm.; FTIR: 767, 880, 992, 1152, 1225, 1369, 1424, 1502, 1546, 1614, 1636, 1755, 2931, 3410 cm$^{-1}$.

Figure 3.4 $^1$H NMR spectrum of 5-bromo-3-hydroxypyridine
Scheme 3.4 Plausible mechanism for the conversion of 2-furfurylamine to 5-bromo-3-hydroxypyridine
Figure 3.5 $^{13}$C NMR spectrum of 5-bromo-3-hydroxypyridine

3.3 Synthesis of 3-allyloxy-5-bromopyridine

2 g pure 3-hydroxy-5-bromobromopyridinol was taken in a clean and oven-dried 250 ml round bottom flask. 125 ml acetone was added to it and the system was stirred to dissolve the HBP. Previously, 7 g potassium carbonate was dried at 100 °C (in oil bath) under vacuum for 6 hours. This affords anhydrous potassium carbonate. Then the potassium carbonate was cooled under argon atmosphere. 3 equivalent of vacuum dry K$_2$CO$_3$ (4.8 g) was added to the reaction vessel. 1.5 equivalent of allyl bromide (1.56g) was added drop-wise to the reaction flask and stirred for 15 hours at room temperature under argon atmosphere. The reaction progress was monitored by thin layer chromatography. After 4 hours, the reaction was complete.
Scheme 3.5 Synthesis of 3-allyloxy-5-bromopyridinol

Figure 3.6 FT-IR spectrum of 3-allyloxy-5-bromopyridine

The product 3-allyloxy-5-bromopyridinyl ether was separated by column chromatography. The product was relatively nonpolar as compared to most of the impurities present in the mixture. Thus 1:9 ethyl acetate and hexane mixture was used to elute the product. First column operation was good enough to remove 95% of the impurities; but a second column is essential for complete removal of the impurity and get
almost colorless and liquid product. $^1$H NMR (CDCl$_3$): $\delta$ 4.55 (d, 2H, $J = 5$Hz), 5.37 (m, 2H), 5.98 (m, 1H), 7.35 (m, 1H), 8.24 (m, 2H) ppm.; $^{13}$C NMR (CDCl$_3$): 69, 119, 120, 124, 132, 137, 143, 155 ppm.; FTIR: 862, 910, 1033, 1122, 1197, 1365, 1416, 1573, 1635, 2832, 2935 cm$^{-1}$.

Figure 3.7 $^1$H NMR spectrum of 3-allyloxy-5-bromopyridine
3.4. Synthetic Trials of Pyridinylboronic Acid

Pyridinylboronic acid synthesis from the 3-allyloxy-5-bromopyridine seemed to be a formidable task. The target synthesis was tried in several different ways; but all the routes ended in failure.

3.4.1 Synthetic Trial via Reverse Addition Method of n-Butyl lithium

A 250 ml reaction flask equipped with a two way septum and a magnetic stir bar was flame-dried under vacuum and then the flask was flushed with dry and pure argon. This process was repeated thrice and then the flask was cooled down under argon flow. 7 ml anhydrous toluene was introduced into the reaction vessel and the system was cooled
down to -78 °C. Then 1.3 mmol. n-butyl lithium (2.5 M in hexane) was added to the solvent with an oven-dried syringe. After 5 minute, solution of 1 mmol 3-allyloxy-5-bromopyridine in 5 ml toluene was added drop-wise to the reaction flask with a double-headed needle. An orange colored slurry resulted and it was stirred for 15 minutes at bath temperature -78 °C. Subsequently, 4 ml. THF was introduced inside the flask and the system was aged for 15 additional minutes. After the aging was complete, addition of 1.1 mmol of triisopropyl borate was continued for 3 minutes via an oven-dried syringe. The resultant red solution was allowed to get warmer and reach -15 °C temperature. That time the product mixture was quenched with -2.5 N HCl and transferred to a separatory funnel and stirred. The aqueous layer was collected in a conical flask and the organic layer was washed twice with water.21 All the aqueous portions were united and neutralized with ml. 10N sodium hydroxide solution to pH 7. Then the aqueous fraction was again extracted thrice with 10 ml THF each time. THF fractions were collected and concentrated to dryness via rotary evaporation. Resultant residue was again dissolved in anhydrous THF and anhydrous toluene mixture and evaporated to dryness. A sample product mixture was ground well and vacuum dried overnight. Crude 1H NMR study in d-DMSO indicated that the desired pyridinylboronic acid was not obtained.

3.4.2 Synthetic Trial via Pd-catalyzed cross-coupling reaction

Two oven-dried 50 ml round bottom flasks were flame-dried under vacuum. While cooling the flasks were purged with argon. 3 mmol of potassium acetate and 5 mmol% of catalyst were weighed and kept in the first flask along with a magnetic stirrer. Potassium acetate was found to be the most suitable base for this palladium catalyzed coupling
reaction. The second flask was charged with 1.2 mmol bis(pinacolato)diboron and 1 mmol 3-allyloxy-5-bromopyridinyl ether. Both the flasks were evacuated and flushed with pure argon. 4 ml of pure DMF (flushed under argon) was added to both the flasks by syringe. Argon pressure was used to transfer solvent from sealed bottle to the reaction flasks. Both the flasks are stoppered with two-way adapters. Mixtures under both the flasks were stirred under argon for ten minutes. Solutions of both the flasks were frozen with the help of liquid nitrogen. After solidification of both the mixtures, the flasks were degassed to remove oxygen. An external liquid nitrogen trap was connected to the vacuum line. Then the solutions were allowed to thaw at room temperature under vacuum (while the vacuum line closed). This way dissolved gas (especially oxygen) was collected over the solution. The solutions were then again frozen by applying liquid nitrogen bath and degassed. This degassing & thawing procedure was repeated two more times. Then both the flasks were allowed to attain the ambient temperature (water bath accelerates the attainment of room temperature). Solution in the second flask was transferred to the first flask by cannula after applying vacuum in the first flask. That reaction mixture was degassed and thawed three times to ensure complete elimination of molecular oxygen (which may harm the catalyst and thus upsetting the desired reaction). Completely degassed system would render no further bubbling. The reaction mixture looked dark brown in color. Finally, the system was purged with pure argon and the reaction continues overnight at room temperature. The reaction progress was monitored by TLC.

Rotary evaporation of the solvent DMF at moderate temperature (50 – 60 °C) was a fearsome job and thus solvent extraction procedure was adopted; and it successfully
worked because of the differential affinity of the product and other chemicals towards water and methylene chloride The product, as expected to be more organophilic, should be extracted in the methylene chloride layer. DMF, potassium acetate and some fractions of the catalyst were water soluble and hence could be removed without much trouble. The organic layer containing the product was dark brown in color. After column chromatographic separation the fractions were characterized by the proton NMR, but the product was not the pyridinylboronic acid; rather it was 5-bromo-3-pyridinol.

3.4.3 Transmetallation Route for the Synthetic Trial of Pyridinylboronic Acid

5-bromo-3allyloxypyidine was unable to be converted into the corresponding boronic acid whereas 3-bromopyridine could be easily converted into the boronic acid analog. Thus reluctance of the former compound to undergo the palladium-catalyzed cross-coupling reaction might be attributed to the presence of a sensitive allyl group. Subsequently another familiar route was given a try.23

A 250 ml reaction flask equipped with a 2-way adaptor and a magnetic stir bar was flame-dried under vacuum and then the system was charged with dry argon. This procedure was repeated two more times. The flask was then charged with 6 ml of anhydrous (freshly distilled) THF, 12 ml dry toluene, 1.130 g (5.28 mmol, 1 equivalent) of 5-bromo-3allyloxypyidine and 1.215 g (1.2 equivalent) triisopropyl borate. Normally the lithiation reactions of the halopyridines were carried out in -40 to -60 °C and the temperature was normally attained by dry ice/acetone bath. Our compound contained a highly sensitive allyl group and accordingly a lower temperature of -80 to -100 °C was used to carry out the reaction. This temperature was arrived at by using liquid nitrogen/acetone. The reaction mixture was allowed to reach -100 °C temperature and
then 4 ml (1.2 equivalent) cold n-butyl lithium (1.6 M in hexane) was added drop by drop with an oven-dried 10 ml syringe. The addition was completed in 20 minutes. During the addition color of the solution changed from pale yellow to orange. The reaction mixture was then stirred at -80 °C in presence of argon for an additional 15 minutes. Temperature was maintained at that temperature by gradual addition of liquid nitrogen. The cooling bath was removed after half an hour and the mixture was slowly allowed to reach -20 °C. At this temperature 2N HCl solution was added drop wise to the mixture. The addition of the hydrochloric acid solution was continued until the pH of solution reached ~ 1. The worked-up reaction mixture was gradually allowed to attain the ambient temperature whereupon the mixture is transferred to a 250 ml separatory funnel. Aqueous layer was collected in a conical flask and 5N NaOH solution was added drop wise to the aqueous layer under stirring condition till the pH of the solution rose to ~ 7. As the pH approaches 7 a prominent white precipitation became visible. On saturation of the aqueous layer with solid sodium chloride more of the white solid crystallized out. The aqueous layer was transferred to a 250 ml separatory funnel and extracted thrice with 25 ml THF each time. THF extracts were then mixed in a flask and the solvent was removed by rotary evaporation. The solid was ground well and a portion of the product was vacuum dried. On ¹H NMR, this sample did not give optimistic result. It seemed from the ¹H NMR that the suspected product is actually 5-bromo-3-hydroxypyridine.
CHAPTER IV
RESULTS & DISCUSSION

4.1 Rationale behind the Synthesis of the Dye

A catechol dye bearing two successive phenolic group in its moiety, was synthesized. The dye bearing a 1,2-cisdiol group could be expected to make complex with pyridinylboronic acid (PBA). The synthesis of the dye was inspired by the behavior of another dye pyrocatechol violet in the PBA-dye-glucose interaction system; but this latter dye responded in a very complicated way with PBA-glucose system. Foul play of the dye, pyrocatechol violet, was thought to be due to the presence of two pairs of hydroxyl groups in two phenyl rings of the dye. Accordingly, the plan was to construct a dye, similar to pyrocatechol violet, containing a single pair of hydroxyl groups in one of its three phenyl rings.

4.2 Behavior of the dye across the pH scale

The main characteristic of these types of dyes was that they exhibited a pronounced color change on varying their pH around certain distinct value. It was noticed that the dye had two color regions: from pH negative to below pH 9.3 the color was pale red but after pH 9.3 the color drastically darkened. This behavior was also supported by the UV-Vis spectroscopy which recorded a drastic shift of absorption wave length on going beyond
pH 9.3. This was an indication that at biological pH the dye might not change its color when it would bind PBA or be present in the medium as a free dye molecule.

4.3 Incapability of the Dye as a Molecular Switch

It was expected that the dye and PBA would form a reversible complex in physiological pH; and when glucose concentration of the test solution increased, glucose

Scheme 4.1 Proposed mechanism of glucosensing
should displace the dye from PBA-dye complex. This would, in turn, generate a new PBA-glucose complex, releasing free dye anion which was expected to be in another distinct form. Thus there should be distinct and visible color changes. But it did not happen to be the case. No color change was noticed on addition of glucose solution to PBA-dye complex. This was a direct consequence of inability of the dye to ionize and change its form at physiological pH 7.4.

4.4 Problems Associated with Pyridinylboronic Acid Synthesis

The precursor of the pyridinylboronic acid, 6-membered 5-bromo-3-pyridinol, was synthesized from a 5-membered furan-based compound successfully. The hydroxyl group could be converted into a good handle which should be conveniently linked to other siloxane based oligomers. The entire bulky system would show much lesser tendency to diffuse out of the contact lens system. Thus allyl handle was attached to this hydroxyl group to serve dual function: firstly, it was expected to protect highly active hydroxyl group so that alkyl lithium and palladium catalyzed cross-coupling reactions could be carried out safely on the other part of the bromohydroxypyridine; secondly, it carried a double bond and so should serve as a very exciting handle for siloxane based oligomers containing a silicon-hydrogen bond. After the synthesis of 3-allyloxy-5-bromopyridine, the next step of the project was to synthesize the pyridinylboronic acid. Several different routes, including two n-butyllithium routes and one palladium-catalyzed cross-coupling route, were tried, only to give negative results. Indeed in all the cases mentioned above, one common product was obtained and this product was none but a precursor of the 3-allyloxy-5-bromopyridine, 5-bromo-3-pyridinol. That unexpected observation found out
the main culprit that disallowed the reaction to proceed successfully. It was believed to be the allyl group on all the occasions. So, probably, replacing the allyl segment with an alternative group like acryloyl might solve the problem. In summary it could be stated that the results and findings of the research looked promising for the further advancement of the glucosensor project.
CHAPTER V
CONCLUSION

The dye could not respond positively at the physiological pH and thus is unsuitable for its proposed application as a molecular switch. Though the initial picture is not so bright, there are rays of hopes as well. The dye contains three benzene rings and so modification of the dye keeping the principal functional part intact seems to be a sensible and feasible idea. Especially, introducing one or more electron withdrawing groups in one or more benzene rings, without tampering with the functional unit of the dye, can be an interesting idea. Some promising functional groups may be chloro, fluoro and nitro groups which should drastically reduce the \( pK_a \) of the phenolic –OH groups. Thus, fine-tuning the original dye can lead to the development of a modified dye which would precisely function at the desired physiological pH. So synthesis of the catechol dye is a promising part of the project which, on further extension, can be realized. Similarly, the pyridylboronic acid part can be synthesized by anchoring a suitable protection group on the phenolic –OH linkage. The protecting group should be such that it directly or indirectly helps the PBA to be joined to a macromolecular segment.

In spite of presence of a lot of organic and inorganic compounds in human tears, chemicals other than carbohydrates are not capable of influencing the dye-PBA equilibrium. Incidentally, other carbohydrates (like fructose, mannose, galactose etc.)
that can be suspected to unsettle the equilibrium are present in so small quantities in comparison to glucose that these will not at all affect the equilibrium significantly.

There are some serious disadvantages regarding the contact lens based glucosensor. Tears track blood glucose with a lag time of ~ 20/30 minutes. This delay can be disadvantageous on many occasions. Again for drastic and sudden physiological changes blood glucose concentration may rise to very high values. Thus equilibration between blood and other interstitial body fluids may not be possible. In this special case blood glucose test knows no other option.

The range of glucose concentration that can be monitored through this colorimetric contact lens procedure should also be determined precisely. Such a glucosensor should be able to sense milimolar and if possible micromolar amount of glucose in tears within very short time. While designing a contact lens based sensor, it is highly desirable that the lens material remains as much unaltered as possible, violation of which may lead to tampering of the desired biological and optical properties of the lens. Deterioration of the biocompatibility can be another serious concern.

So far, the outcome of this glucosensor project is promising and encouraging. Some modified synthetic strategies can help realize this novel colorimetric contact lens based glucosensor project. In near future, the contact lens probe can also prove to be a highly exciting analytical technique for multiple purposes. For example, if a correlation is established between the chemicals in tears and those in other biological fluids, then this contact lens based estimation of tear glucose can open up new noninvasive estimation methods of other physiologic analytes as well.


