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Synthesis and Charecterisation of Methylene Blue Derivatives

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“SYNTHESIS AND CHARECTERISATION OF METHYLENE BLUE DERIVATIVES”

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ABSTRACT

Since its discovery, the fluorescent dye known as methylene blue has been employed in a variety of scientific disciplines. Methylene blue (MB) for intraoperative fluorescence imaging has only lately been employed. 10-N-carbamoyl linkage modification of Methylene blue helps us in developing compounds with off-to-on fluorescence switch in the presence of near infrared (NIR). Due to its naturally induced photodamage to normal tissues and deeper penetration of lesion regions when compared to UV and visible light, near infrared (NIR) light is considered favorable among the broad radiation range. The previously synthesized derivatives are not completely soluble in water, they require other co-solvents, acetone etc. So in this study branched polyethyleneimine which is a water soluble polymer is used to conjugate with methylene blue to effect the 10-N-Carbamoyl linkage . The branched structure of polyethyleneimine has several primary amines , so the amount of methylene blue that can be conjugated can also be varied . The carbamoyl linkage with methylene blue is also sensitive to hydroxyl radical . Using this hypothesis the activity was verified using hemoproteins such as hemoglobin . Subsequently this property can be used in the lysis of the malaria parasite.

MB and PEI conjugate was synthesized and tested in vitro for the cleavage of the 10-N-Carbamoyl linkage .As the result, PEI-conjugated MB successfully demonstrated the cleavage and also sensitivity to ROS-induce cleavage to release intact MB.

ACKNOWLEDGMENTS

I would like to express my deepest regards and gratitude to Dr. Seongbong Jo for taking me under his wing, believing in me, training me and guiding me throughout my master's degree. Dr. Seongbong Jo has extended his constant support, time and valuable advice to me to overcome all kinds of obstacles I faced during my time here at Ole Miss.

I am grateful to Dr. Michael Repka and Dr. Eman Ashour for accepting my request and serving as a part of my thesis defense committee. Their constant support during my time at Ole Miss kept me motivated and aided me in successfully completing my master's thesis and attaining my degree.

Special thanks to my dear friend and my lab mate Jhanvi Desai and Dr.Huy Dao for their kind support during my research project.

DEDICATION

To my brother Nagendra Kumar , Mom and Dad

For your love and support.

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LIST OF ABBREVIATIONS

MB	Methylene Blue
NPC	N-Phenyl Chloroformate
ROS	Reactive Oxygen Species
N-IR	Near-Infra Red
^{1}H NMR	Proton Nuclear Magnetic Resonance
UV	Ultra Violet
ACN	Acetonitrile
THF	Tetrahydrofuran
HCl	Hydrochloric Acid
FT-IR	Fourier Transform Infrared Spectrophotometry
PEI	Polyethyleneimine
ROS	Reactive oxygen species
NP	Nitro phenyl
CDCl3	Deuterated chloroform
EtOAc	Ethyl acetate
D2O	Deuterium oxide
$^{1}O_2$	Singlet oxygen

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CHAPTER I

INTRODUCTION

Methylene blue

One of the synthetic dyes used extensively as a coloring agent for papers, wool, silk, and cotton is methylene blue (3,7-bis(dimethylamine) phenothiazine chloride tetra methylthionine chloride). Additionally, the food, cosmetics, and pharmaceutical industries all use a significant amount of MB dye in their products. It can be used to treat malaria (dose: 36-72 mg/kg over 3 days)[1,2], asplenia during transplant surgery, and heparin neutralization. There are many new properties of MB are being discovered by modifying the phenothiazine ring, the backbone of the MB. In that regard this thesis contains chapters that discuss the synthesis of new MB derivatives and their characterization. Additionally, MB dye is employed in analytical chemistry as a photosensitizer, oxidation-reduction indicator, optical redox indicator, and in the trace measurement of anionic surfactants. When MB is converted to leuco MB, it loses its distinctive deep blue color and turns colorless. The chromophore and auxochrome groups in MB determine its color[1,4]. The core aromatic heterocycle of MB serves as the chromophore group, while the auxochrome group is composed of N-containing groups with lone pair electrons on the benzene ring. When MB is in its oxidized condition, it exhibits a distinctive deep blue color and is methylene blue (MB+), a dye belonging to the thiazine dye family, is frequently employed in many different contexts, such as photodynamic therapy for the treatment of cancer, where it is utilized as a photosensitizer to

produce singlet oxygen[1,2]. Although recent research by this group has proven that the

photobleaching of this dye may be caused by reduction to its leuco form, especially at low dissolved oxygen concentrations, methylene blue is still frequently utilized as a test model pollutant in semiconductor photocatalysis[3,4].

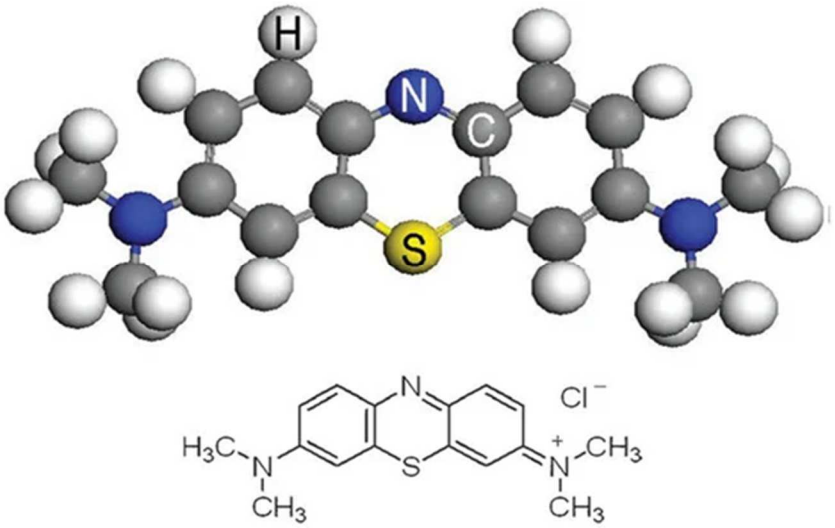

Figure 1 Structure of Methylene blue.

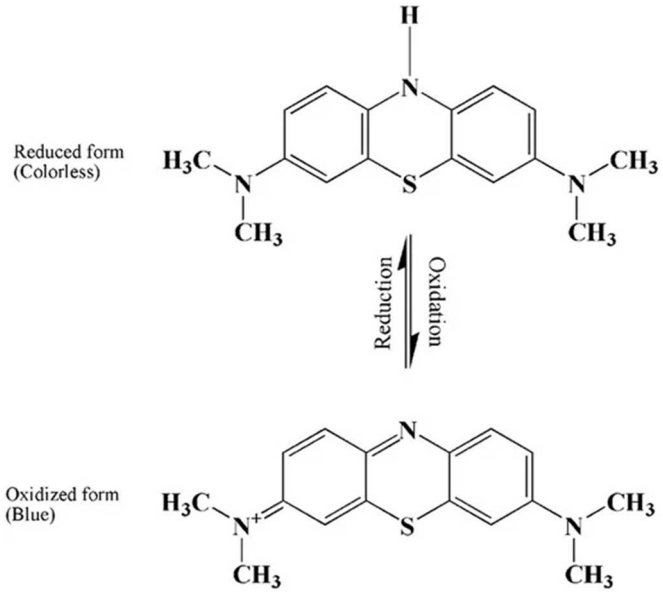

Figure 2 Oxidized and reduced forms of MB

A desirable chemical with numerous features relevant for biomedical applications is MB, a synthetic phenothiazine fluorophore that the body can tolerate. In particular, MB is a potent photosensitizer with substantial photo-cytotoxicity that has been notably demonstrated against pathogenic bacteria and several other organisms, is currently being explored as an antimalarial drug in combination therapy with artesunate and is a strong fluorescent dye.

It's interesting to note that due to the reversal of fluorescence property, MB's fluorescence (FL) becomes latent upon the chemical changes at its 10-N site and is restored with cleavage of the conjugated moiety[3]. It has been reported that a variety of MB-based off-to-on probes take advantage of the induced uncaging at the 10-N position on the phenothiazine ring of caged MB derivatives.

Separate conjugations of ligands capable of stimuli-sensitive activation—such as the nitro benzyl group by nitroreductase and the silyether by fluoride ion—gave the probes the ability to respond to various triggers. Hemoproteins were found to be far more effective than the existing paradigm, which relies on inorganic Fe2+ ion species in excess molar equivalent to H2O2 to start the Fenton reaction at physiologically relevant pH. Malaria-infected erythrocytes (ETCs), known to have increased ROS levels, were selected as the in vitro model and a polyethyleneimine attached MB derivatives were produced to evaluate significantly and accurately the OH sensitivity of caged MB derivatives[4].

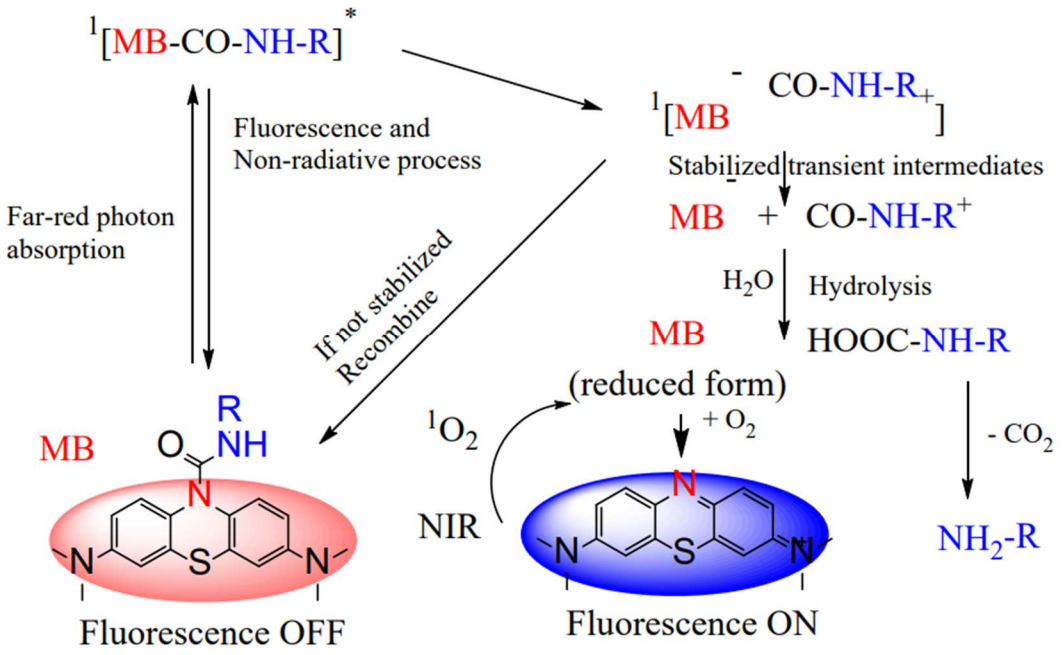

Figure 3 Proposed photolysis mechanism of urea bond containing MB derivatives by far-red light irradiation. Upon photon absorption, the molecule is excited to singlet state and subsequently induce bond cleavage, yielding MB anion and R carbocation.. The transient intermediates, stabilized by electron delocalization within the phenothiazine ring and electron pushing effect of the residual R moiety, will be irreversibly hydrolyzed and oxidized to yield permanent cleavage of the urea bond. Resultant reduced form of MB could be oxidized by dissolved oxygen and later 1O2. ‡[1]

Role of MB in treatment of Malaria:

Regarding the treatment of malaria, studies have shown that MB enhances the effectiveness of chloroquine by lowering resistance and inhibiting Plasmodium falciparum glutathione reductase, an enzyme that prevents the formation of byproducts produced by Plasmodium species that cause the body's immune response[5]. When methemoglobinemia is present, methylene blue works primarily by converting the oxidized form of hemoglobin, Fe3+, to Fe2+. As a result, the ability of hemoglobin to bind oxygen will rise, increasing the amount of oxygen delivered to tissues.

Reactive Oxygen species

Highly reactive molecules called reactive oxygen species (ROS) are created from diatomic oxygen (O2). Peroxides, superoxide, hydroxyl radicals, singlet oxygen, and alpha-oxygen are a few examples of ROS. ROS are byproducts of the typical metabolism of oxygen in a biological setting. ROS play a part in homeostasis and cell signaling. In normal cells, ROS are present at low and stationary levels and are essential for cellular function[5]. This implies that the creation and disposal of ROS at the appropriate time and location must be balanced for them to have a dual role in signaling, protection, or injury[5.6].

Through its contact with water, radiolysis, ionizing radiation can produce harmful intermediates. Since water makes up between 55 and 60 percent of the human body, radiolysis is highly likely in the presence of ionizing radiation[6,7,8]. Water undergoes an electron loss and increases in reactivity as a result. Then, in a three-step chain reaction, water is transformed into oxygen, hydrogen peroxide, superoxide radicals, and hydroxyl radicals in that order (O2). ROS acts both as a bactericide, damaging the bacterial DNA, RNA and proteins. So in the case of malaria, in the erythrocytic cycle due to the effect of schizont there are elevated levels of ROS and with the Fe+2

ion in HB oxidised to Fe+3 ion causes and Fenton reaction[6,7,8]. This in turn helps the cleavage of MB- derivatives and helps in delivery of the drug to specific location.

Fenton's Reaction

A catalytic mechanism turns hydrogen peroxide into a hydroxyl free radical. The process of mitochondrial oxidative respiration often produces the hydrogen peroxide reactant. It's crucial to remember that the hydroxyl free radical produced by the Fenton reaction is extremely poisonous (due to its unstable and reactive nature). Henry John Horstman Fenton, a British chemist, is honored by the name of this reaction[9].

In the presence of hydrogen peroxide, which acts as an oxidizing agent, the ferrous ion Fe^{2+} oxidizes to the ferric ion Fe^{3+} which initiates the Fenton reaction. As a result, a hydroxide ion and a hydroxyl free radical are produced as byproducts.

The ferric ion is now reduced back into the ferrous ion in the presence of an additional hydrogen peroxide molecule in the following stage of Fenton's process. A proton and a hydroperoxyl free radical are produced as a consequence of this reaction. As a result, the catalyst for ferrous ions is renewed.

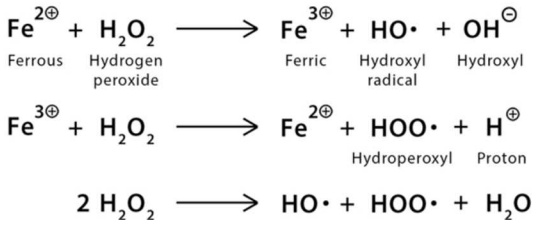

Figure 4 Schematic representation of Fenton reaction.

Stimuli-Responsive Drug Delivery Systems

The two components of drug delivery systems—carriers and therapeutic agents—are typically combined to improve drug stability, desired drug accumulation, and drug release. This offers a potential approach for the treatment of cancer and other infectious diseases[8,9]. Recent advancements in intelligent drug carrier systems have made it possible to administer medications precisely based on the specific tumoral microenvironments or external stimuli, such as pH, enzymes, reactive oxygen species (ROS), photodynamic, and so on[10]. These manufactured stimuli-responsive drug carriers, such as micelles, liposomes, hydrogels, and nanoparticles, cleave chemical bonds or change their shape to release medications in a predetermined sequence. With a thorough understanding of lung cancer research, scientists have discovered the distinct tumor microenvironment that develops during the disease's progression, including an acidic tumoral environment, elevated intracellular glutathione (GSH) conditions, and high levels of ROS[1,10]. This knowledge opens up the possibility of using drug delivery systems for lung cancer therapy. Ultrasound, electric field, light, and magnetic field conditions could be used in addition to stimuli-based nanocarriers that are sensitive to internal (temperature, pH, redox, enzyme reactions) environments to cause rapid drug release at a specific localization.

Table 2 Examples of different types of stimuli responsive drug delivery

STIMULI	SPECIFIC CONDITIONS	NANOCARRIERS	REFERENCE
Light	Near-infrared (NIR) light	Gold nanocage@manganese dioxide ($AuNC@MnO_2$) nanop Articles Titania-coated gold nanobipyramids Poly(L-lysine)-conjugated chlorin e6 (Ce6) derivative nanoparticle	4,11
Ultrasound	Synergistically therapeutic modality	EXO-DVDMS Liposome-based nanobubbles	11,12
pH	pH 5.7–6.9	CHEMS-based liposomes; HA- ERL/BEV-LPH	11

		nanoparticles	

Light has been used extensively for remotely controlled medicine delivery because of its relative safety and noninvasive nature. For on-demand medication release, photolabile groups can be directly destroyed by short-wavelength light, including UV and visible light. Their inability to penetrate deeply, however, limits their potential for use in biomedicine. Near-infrared (NIR) light (780-2500 nm) has the ability to penetrate tissues more deeply than short-wavelength light, making it ideal for remote control of the desired medication release[13].

A moiety with light sensitivity and activated by ROS can be used to load potent, water insoluble chemotherapeutic agents and the release of these agents can be facilitated by exposure of light over the tumor or parasite effected region.

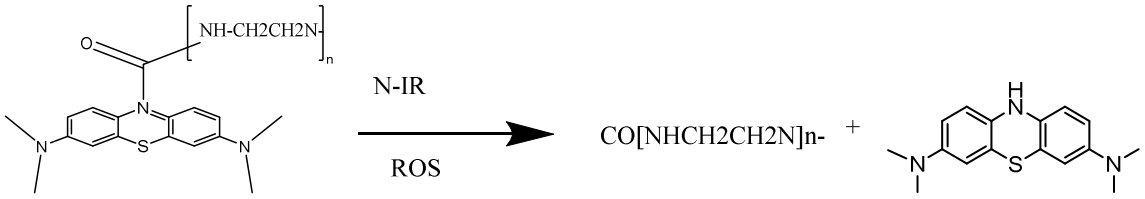

Figure 5 Release of MB from MBPEI on exposure to N-IR and ROS

MB-PEI Methylene blue

Polyethyleneimine

Polyethyleneimine (PEI) or polyaziridine is a polymer with repeating units composed of the amine group and two carbon aliphatic CH2CH2 spacers. In contrast to branching PEIs, which contain primary, secondary, and tertiary amino groups, linear polyethyleneimines only contain secondary amines. Primary amines are the main targets for the conjugation with MB. Aziridine is polymerized with an acid catalyst to form branched PEI (bPEI), whereas 2-ethyl-2-oxazoline is polymerized with a ring opening catalyst to form linear PEI (lPEI), which is then hydrolyzed. Branched PEI are soluble in water and that's one of the reasons for choosing branched PEI. There are several MB conjugates like MB-EA (Methylene blue -Ethanolamine) , MB-ET (Methylene blue-Ethyl chloroformate), and others which are not water soluble , there is always a need of co solvents .

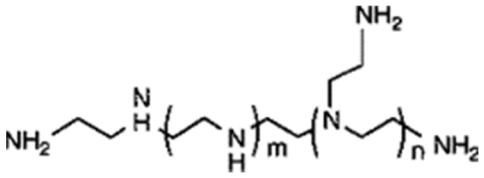

Figure 6 Structure of PEI

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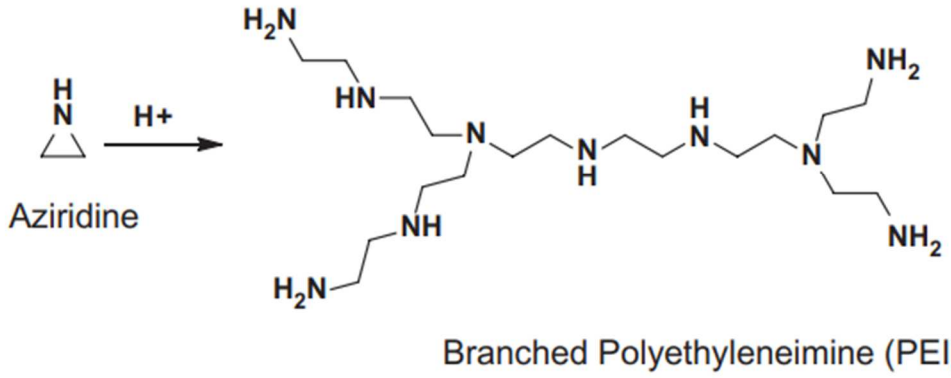


(b)

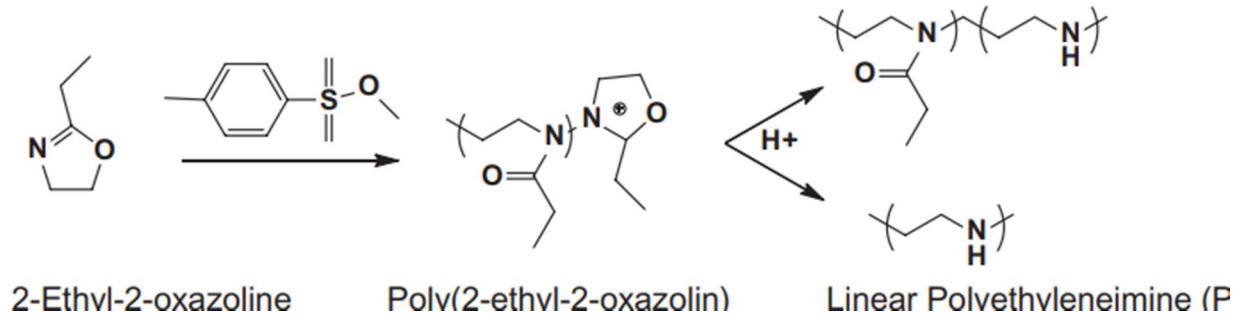


Figure 7 . Synthesis of PEI polymers, (a) b-PEI synthesis by ring opening polymerization of aziridine, and (b) l-PEI synthesis by acid-catalyzed hydrolysis of poly(2-ethyl-2-oxazolin).

CHAPTER II:

METHODOLOGY

Materials

Methylene blue (MB) hydrate,96%, high purity biological stain (Acros Organics), 4-Nitrophenyl chloroformate (NPCs), Polyethyleneimine MW 1800,99%(Polysciences,Inc.)Toluene (Fisher Scientific),Sodium hydrosulfite(Sodium dithionate), Sodium sulphate anhydrous, Triethylamine(TEA),Sodium bicarbonate, Sodium carbonate anhydrous, Hydrochloric acid, Sodium chloride, Toluene, Acetonitrile, Acetone, Tetrahydrofuran(THF), Ethyl acetate, Methanol, Deionized water, Hexane, Activated silica gel.

Methods

Synthesis of Methylene Blue (MB)- Polyethyleneimine (PEI)

This whole synthesis of MB-PEI was performed in three steps i.e., reduction of MB to leuco-methylene blue, conjugation of reduced MB with 4NPC, finally reaction of MB-NP with PEI.

1. **Leuco-methylene blue (L-MB)**

Methylene blue monohydrate (1,200 mg, 3.2 mmol) was dissolved in 100 ml of deionized water. Toluene (150 ml) was added to the solution of MB . Acetone is used to wash the MB on the sides of the wall. The reaction setup is maintained on the oil bath at 60oC, this higher temperature helps evaporate the acetone added and promotes miscibility of toluene and water. It is followed by nitrogen flushing. MB is water soluble so it stays in water phase and toluene will be the top phase. The bottom phase will be dark blue, and the top phase will be pink in color because some amount of MB is soluble in toluene. After a constant nitrogen flow was established, sodium dithionate

(1,116 mg, 6.4 mmol), was added. This helps in reducing the MB. MB turn yellow which is Leuco-methylene blue then it is followed by addition of sodium carbonate anhydrous (680 mg, 6.4 mmol) it is a base, so it helps L-MB to go into toluene phase. After 15 min of vigorous stirring, the reaction mixture turned into yellow, no presence of blue color and both phases became clear indicating that leuco-methylene blue was successfully transferred into toluene phase.

2. MB-NP

Toluene phase containing L-MB was transferred into a nitrogen-discharged flask containing anhydrous sodium sulfate for drying under a nitrogen atmosphere via dropwise addition over 10 min. Make sure all L-MB is in contact with sodium sulfate. Resultant anhydrous L-MB solution in toluene was transferred into a solution of 4-nitrophenyl chloroformate (1.285 mg. 6.4 mmol) in 10 ml of toluene maintained in an ice bath. Because NPC is so reactive, and it will generate lots of heat when L-MB is transferred so it is done on a ice bath. The green undissolved salt is that of HCl formed by the reaction of L-MB and NPC. Upon completion of transferring L-MB into each chloroformate solution, TEA (1,692 ul, 12.8 mmol) was added. TEA helps to absorb the HCl and push the reaction in forward direction. The TEA was added drop by drop, otherwise the position the drop hits the reaction vessel the reaction undergoes rapidly, that will create lot of bi product The reaction was proceeded overnight at room temperature. Then, reaction mixture was collected after rinsing three times with each of the following solutions using a separatory funnel: saturated sodium bicarbonate, 0.01N hydrochloric acid solution, and brine. First it is washed with HCl to get rid of undissolved MB. To maximize the yield, add little amount of toluene and rinse and add it to the separating funnel. After shaking the water-soluble part or undissolved MB will go into the water phase. With every wash the water phase should be more colorless because these is less MB

to dissolve in aqueous phase. The organic phase looks more yellow to orange indicating the right direction of the reaction as MBNP is orange in color. Sodium carbonate wash helps in removal of excess unreacted NPC. The obtained organic phase was dried, evaporated under reduced pressure and recrystallized in acetonitrile 2 times and yielded a bright orange solid crystal of MB-NP. ACN added for recrystallization since MBNP has low solubility in a ACN at low temperature

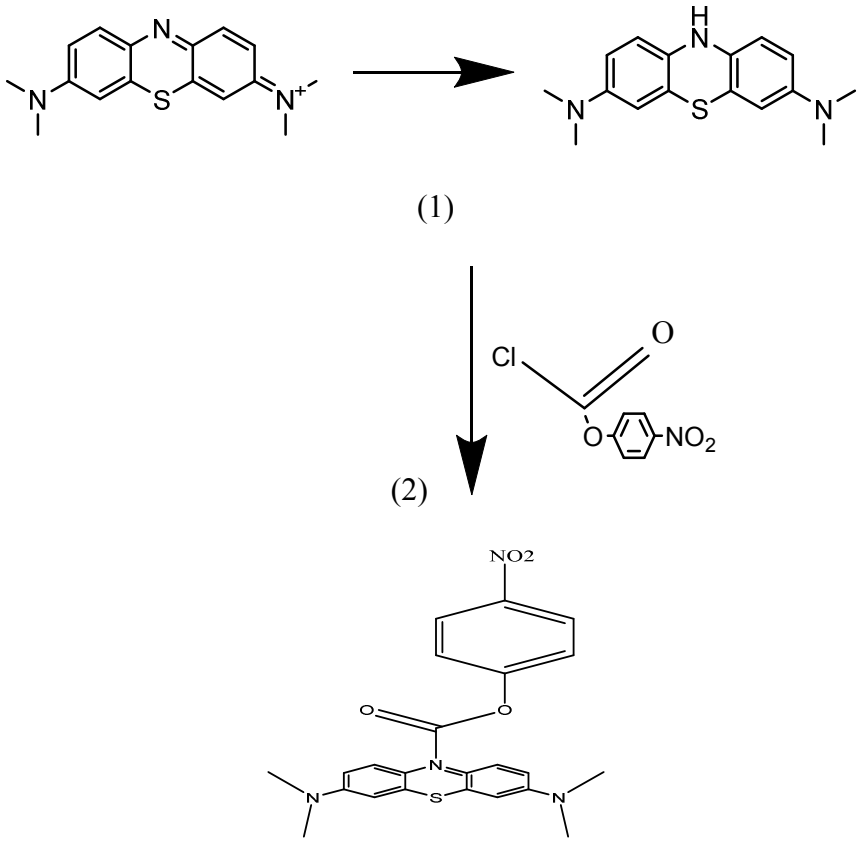


Figure 8 . Synthetic scheme of carbamate bond- containing MB-NP. (1) MB, Sodium dithionate, sodium carbonate, water/Toluene,60oC. (2) 4-nitrophenyl chloroformate, triethylamine (TEA)

3. Conjugation with PEI

MB-NP (100 mg 0.222 mmol) was dissolved in anhydrous THF and added dropwise into Polyethyleneimine (PEI) base (396 mg 0.108 mmol). Since PEI is basic in nature there is no requirement to add TEA. The reaction mixture was protected from light during reaction and refluxed at 60°C for 48 hours. Upon completion of the reaction, the mixture was collected. evaporated under reduce pressure, dissolved in THF and washed three times with hexane to precipitate out the product. The obtained organic phase was dried, evaporated under reduced pressure. The final product is an orange viscous fluid of MB-PEI (47 mg, 47%) which was characterized using ^{1}H NMR. TLC was performed over the course of the reaction to check the yield.

Fluorescence and absorbance spectral measurements

Fluorescence measurements were performed using a LC500 Perkin Elmer fluorescence spectrophotometer at 660 nm excitation wavelength, 600 to 800 nm emission wavelength, 10 nm excitation slit and 10 nm emission slit. MB derivatives were completely dissolved and diluted in a solvent mixture of water :1 μM pH 7.4 phosphate buffer 70:30 unless otherwise stated. The sample concentration was kept below 10 μM to guarantee the linear relationship between fluorescence intensity, MB concentration and minimize MB aggregation. Exactly 1 ml of the tested solution is loaded into fluorescence cell and sealed with a paraffin film to prevent solvent evaporation during the irradiation process. Absorbance scans were performed using a Genesis 8 UV-VIS spectrophotometer. The samples concentration was kept at around 50 μM since the detection sensitivity of absorbance method is lower than that of fluorescence.

Determination of Sensitivity to Reactive Oxygen Species (ROS)

Determination of sensitivity to ROS was conducted by preparing aqueous polymeric solution and exposing it to three conditions i.e. H_2O_2, H_2O_2+polymeric solution + Fe (II) , polymeric solution alone. For this experiment a 10mM solution of H_2O_2, 10 mM solution of HB, 500 µg/ml solution of the synthesized polymer enzyme were prepared. The release was observed using LC500 Perkin Elmer fluorescence spectrophotometer measurement. Absorbance was measured at 660 nm in the fluorescence spectrophotometer measurement.

Yield of the reaction:

The yield of the chemical reaction can be calculated by the formula[14].

$$\text{percent yield} = \frac{\text{actual yield}}{\text{theoretical yield}} \times 100$$

The MB and PEI were taken as per 1:1 molar ratio 100 mg of MB and 389 mg of PEI were taken, and the final yield was 197.2mg so after calculating the percent yield was found to be 39%.

Chapter III: Results and discussion

TLC of the reaction

TLC was performed over the course of the reaction to check the yield. The decrease or disappearance of NPC is an indication for the end of the reaction. TLC was performed using hexane and ethyl acetate in 1: 1 ratio.

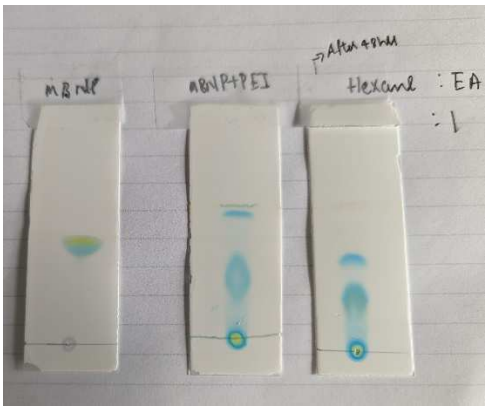
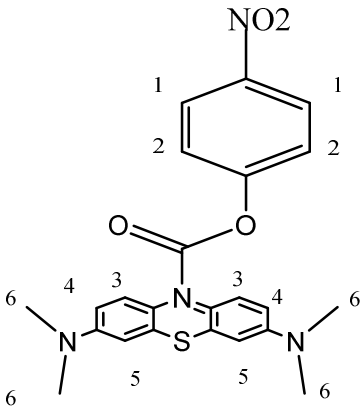


Figure 9 TLC of the reaction showing the disappearance of NP



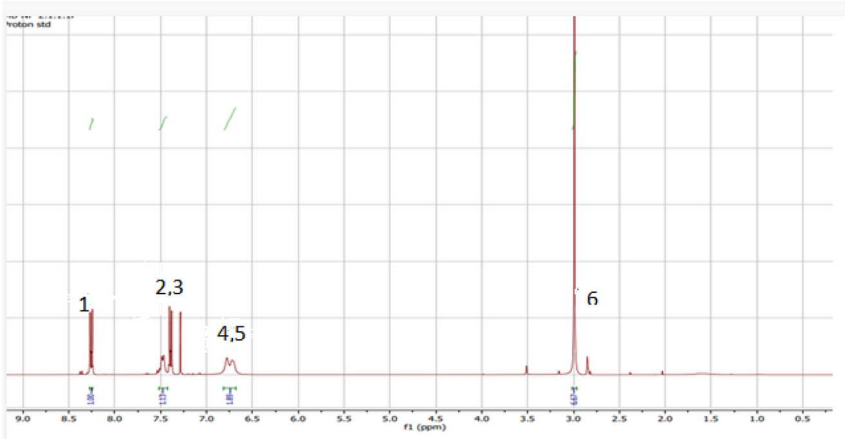


Figure 10 1H NMR spectrum of MB-NP in CDCl3

The 1 indicates the methyl peaks even 2,3 peaks indicate the subsequent methyl groups. The 6thpeak at the 3.0 is the characteristic peak of methylene blue indicating the four methyl grou

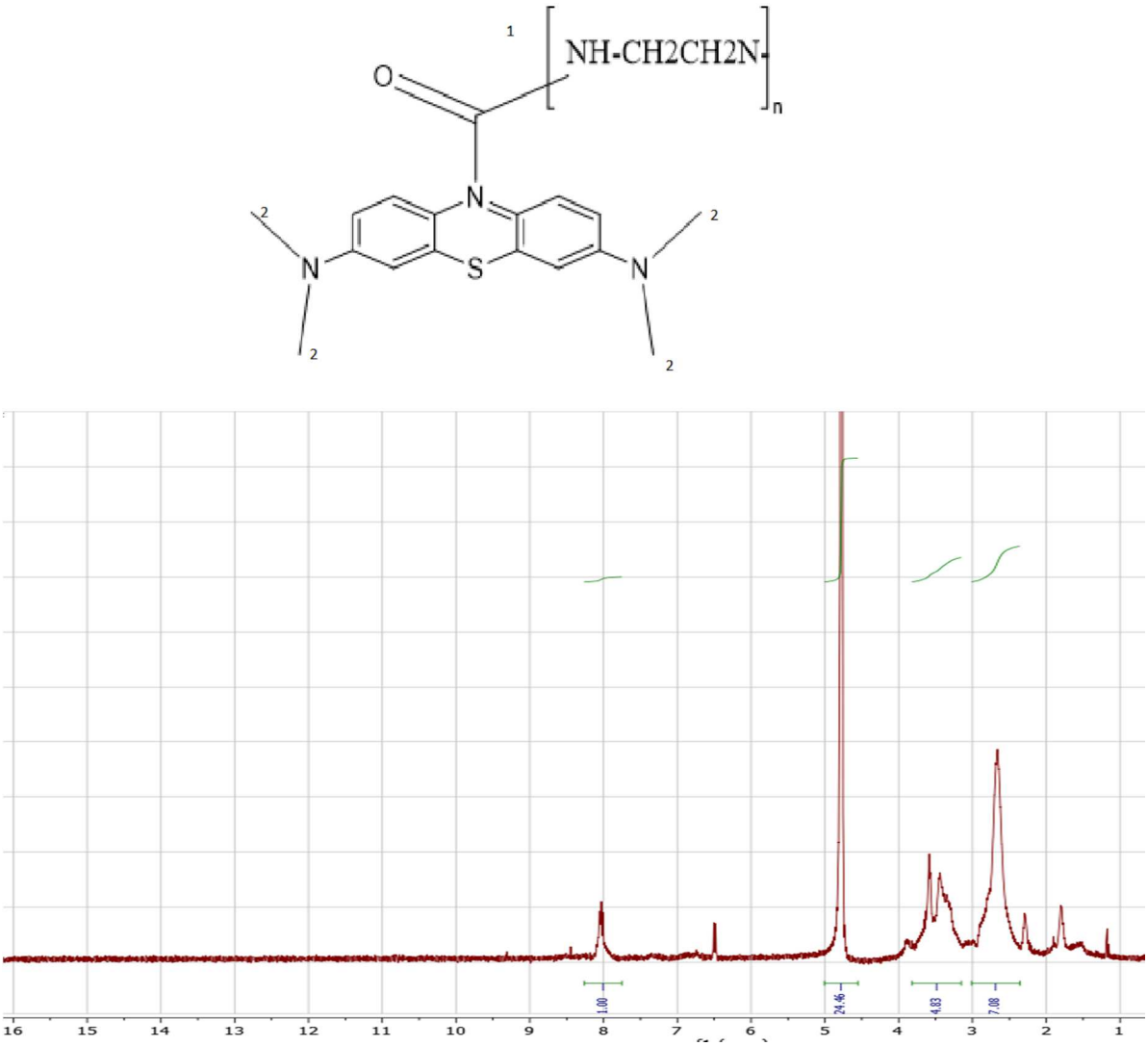


Figure 11 1H NMR of MB-PEI in D2O

The peaks at 3 and 4 indicates the characteristic peaks of MB and PEI respectively.

Determination of light sensitivity by NIR irradiation

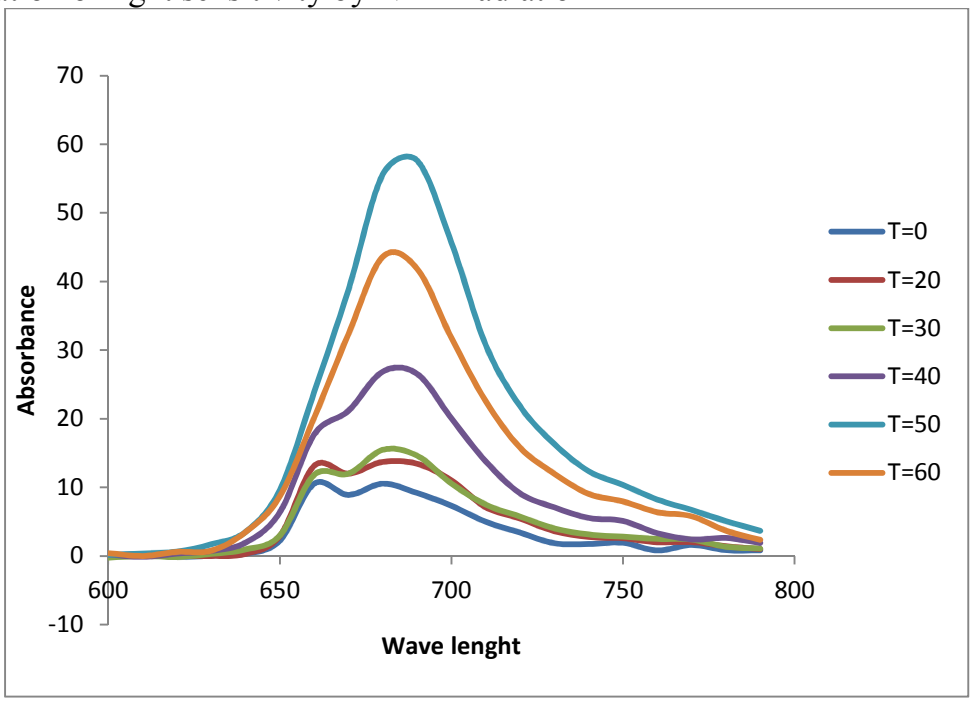


Figure 12 MB release from MB-PEI on exposure to N-IR radiation.

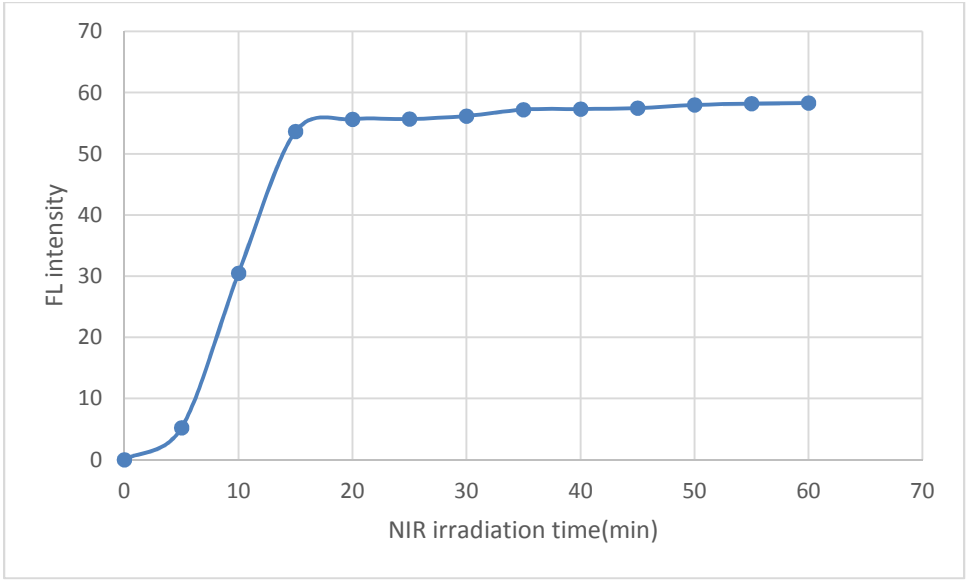


Figure 13 Fluorescence intensity of MB-PEI solution stemming from freed MB after the irradiation of far-red light.

Each timepoint shows an increase in the absorbance of the MB, this suggests that the MB attached to the product is released on exposure to the N-IR radiation. On exposure of the sample concentration to the N-IR radiation over 60 minutes, the increase in absorbance ceases as seen in figure 13, which shows complete release of MB from the synthesized product. This experiment illustrated the N-IR sensitive nature of the synthesized product.

Determination of sensitivity to ROS

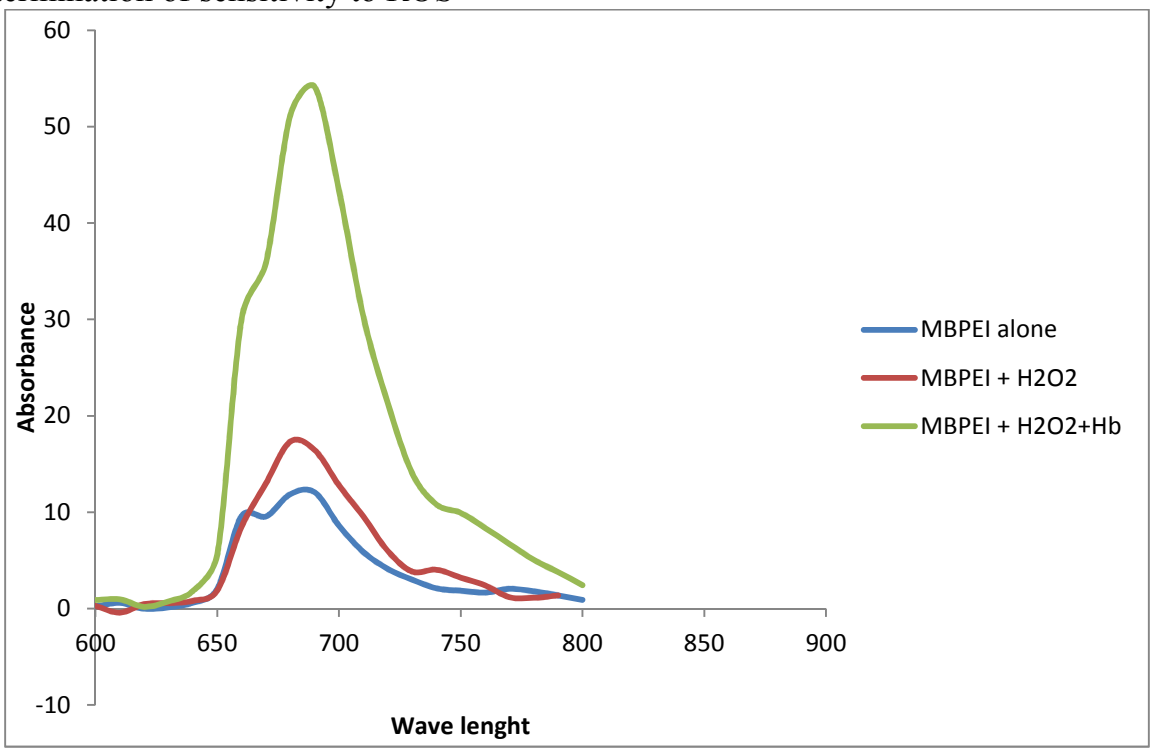


Figure 14 Sensitivity of MB-PEI to hydroxyl free radicals produced by using Hb .

The targeting offered by the synthesized polymer might be further improved by its sensitivity to ROS. The goal of this experiment was to forecast how sensitive the polymer would be to ROS. The conjugated MB was quickly released when the polymer solution was subjected to hydrogen peroxide in the presence of hemoglobin (which produces hydroxyl free radicals), as shown in figure 14. This demonstrates the polymer's sensitivity to the hydroxyl free radical in vitro.

The exposure of the polymeric solution to hydrogen peroxide, which was transformed into hydroxyl free radicals by Fe (II) ions, further supported this theory. This is comparable to the in vivo reaction that the iron in hemoglobin catalyzes. The conjugated MB was instantly released by the polymer, as expected, and this was visible by the polymeric solution's color changing from colorless to dark blue.

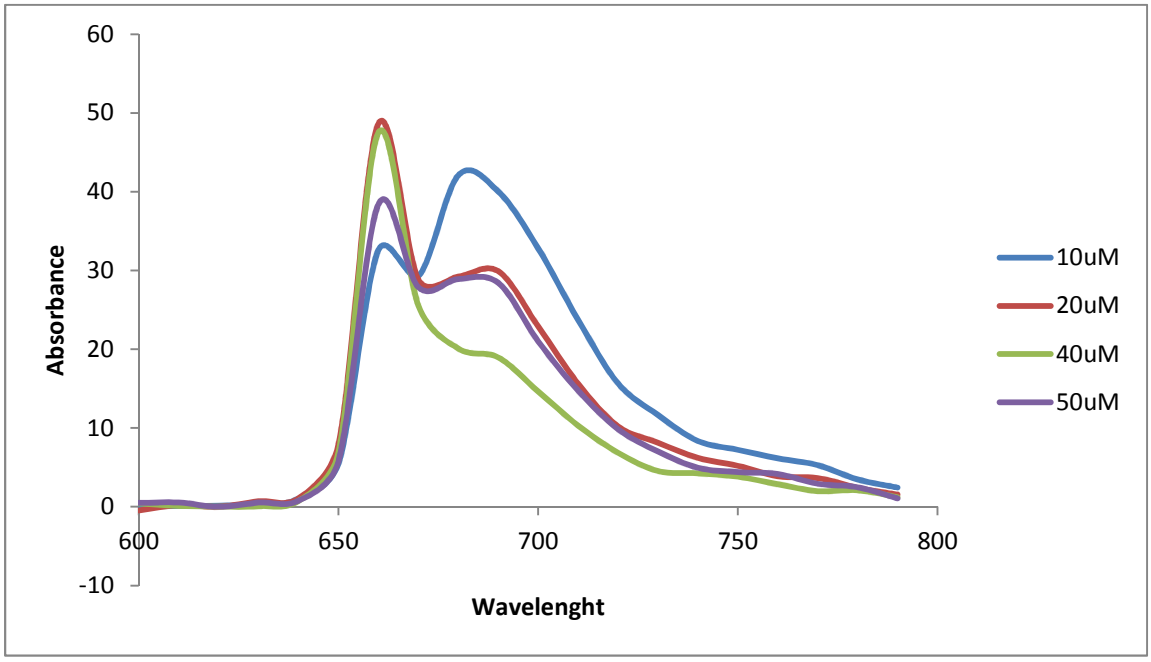
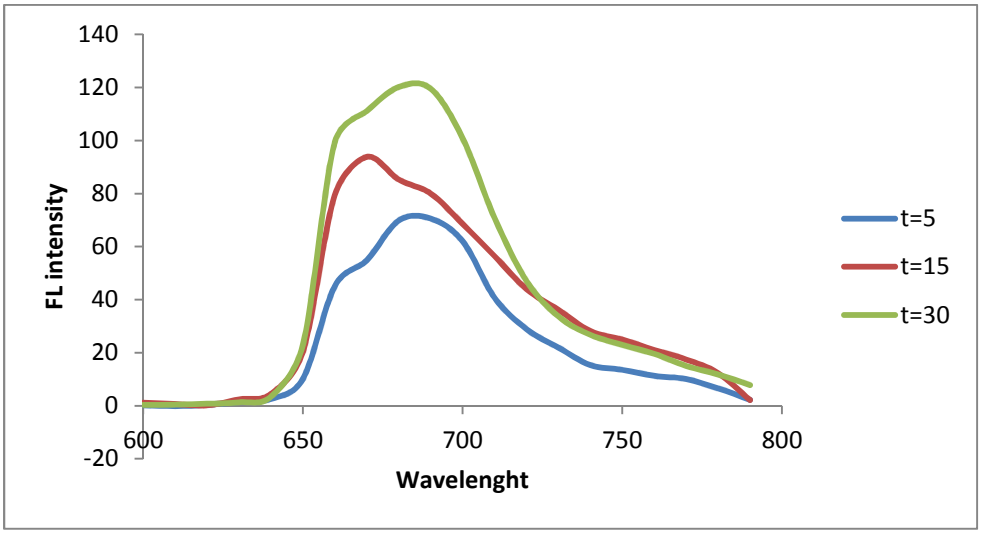


Figure 15 Release of Methylene blue from the polymer with change in concentration of H2O2

The effect of H2O2 of the reaction can be estimated by checking the release in the presence of different concentrations of the H2O2. The concentration of the free radicles will be helpful in estimation of the amount of MB released. From the figure 15 we can estimate that the release of MB was affected by changing the concentration of H202 but the release was increasing till the concentration was increased will 20uM. 40uM showed almost similar results to that of 20uM. This means all the amount of MB that was attached to the polymer was released and reached the

saturation, so the further increase in the concentration showed no effect.

a)



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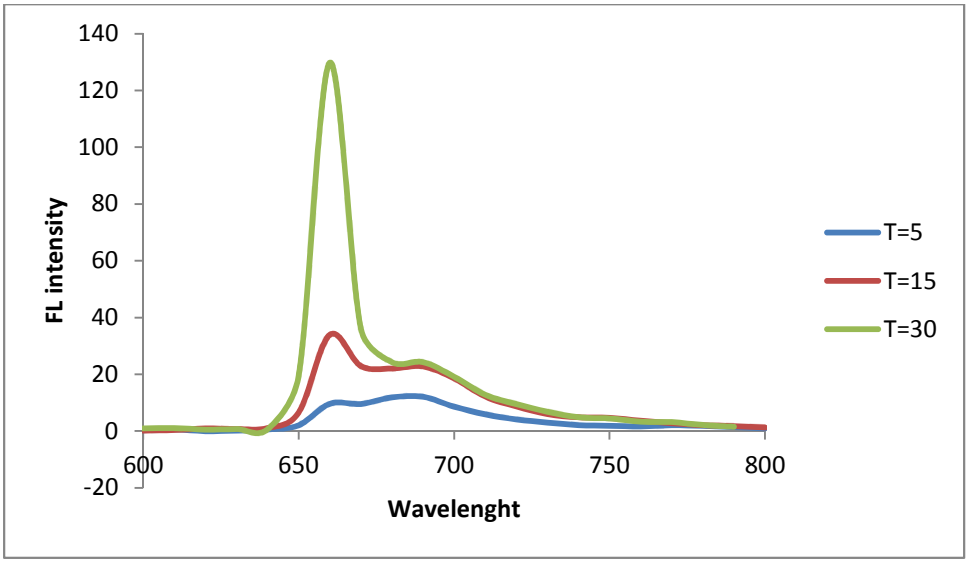

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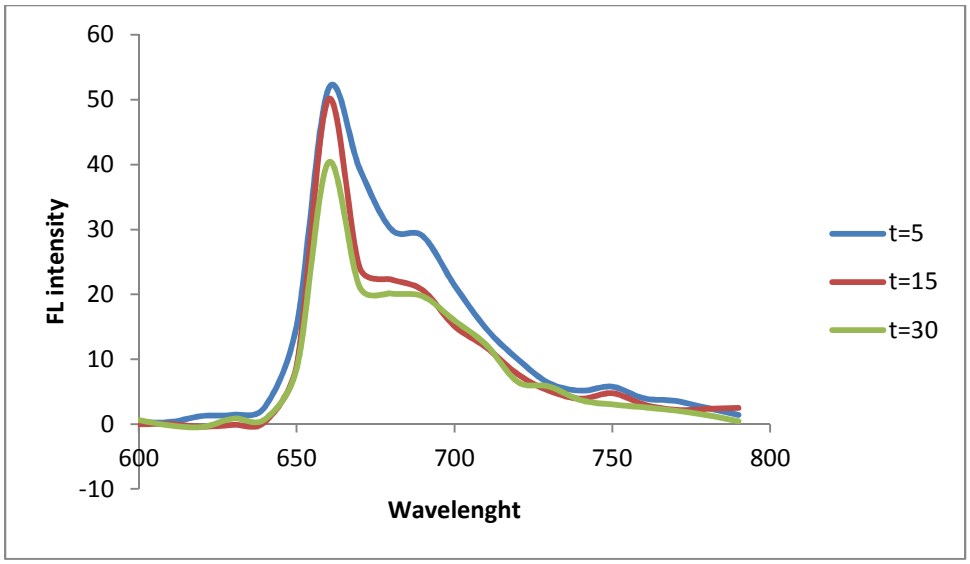
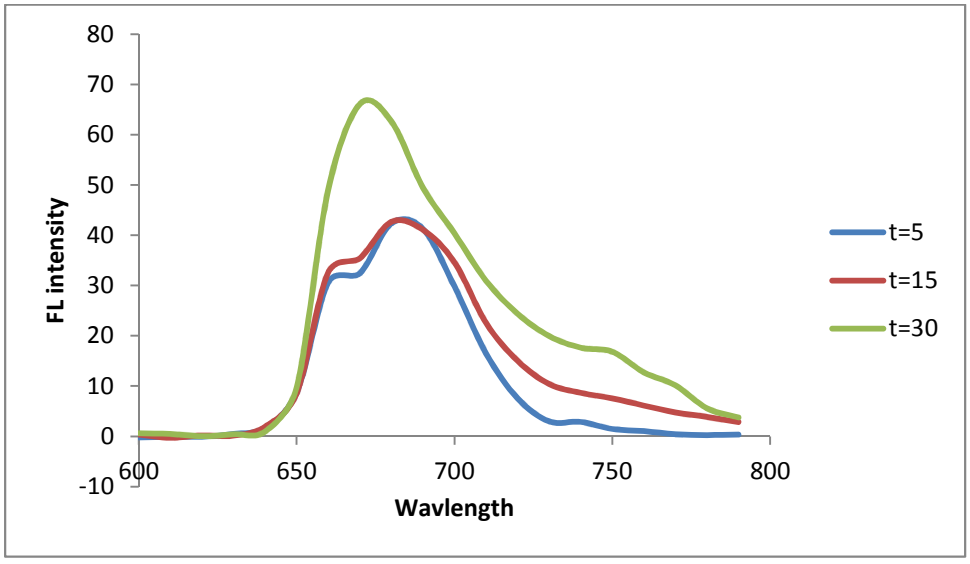

60
50
40
30
20
10
0
-10
FL intensity
600
650
700
750
800
Wavelenght
t=5
t=15
t=30

(d)


80
70
60
50
40
30
20
10
0
-10
FL intensity
600
650
700
750
800
Wavlength
t=5
t=15
t=30

(e)

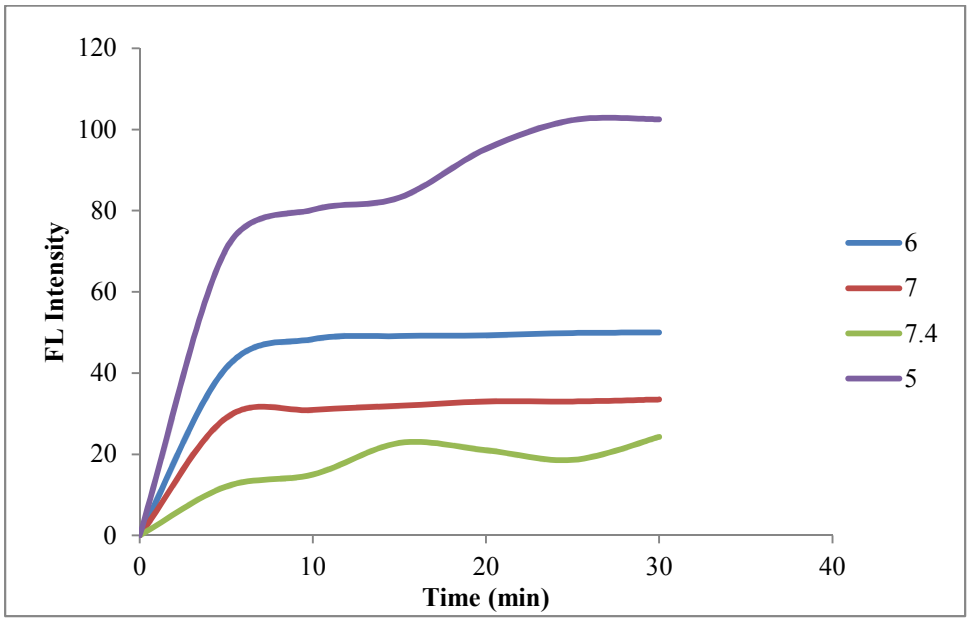


Figure 16 . (a) to (d) Flourescence scan of MB-PEI solutions incubated with HB at different pH values of 5,6,7 and 7.4 respectively, for an increase amount of time. (e) The comparison of FL intensity at maxima of the product in different pH.

CHAPTER IV: CONCLUSION

Targeted drug delivery is slowly becoming important aspect in treatment of various ailments. The objective of synthesizing a novel water soluble, light and ROS sensitive polymer was successfully performed. All the intermediates and the finished product's synthesis and structural characterization were carried out and interpreted effectively. Furthermore, in vitro experimental models were used to illustrate how sensitive the synthesized polymeric entity was to N-IR, and ROS. Furthermore, the effect of hydroxyl ion was performed using different levels of concentration and at different pH. From the synthesis, experimentation, and interpretation of the data Methylene blue -PEI conjugate show the in vitro release of the oxidized form of MB which has fluorescence characteristics and can be used as a bio marker or can be helpful in the treatment of malaria. Further studies such as synthesis using different ratios between the reactants and *ex vivo* cell viability assays, cytotoxicity assays using cell lines and *in vivo* studies on animal models should be performed after optimizing the in number of reactants used for the synthesis to give maximum stimuli to ROS.

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VITA

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