PYRENE TAGGED FUNCTIONAL POLYCAPROLACTONE FOR BIOIMAGING

Thesis Report submitted towards the partial fulfillment of
B.S. - M.S. dual degree program

BY

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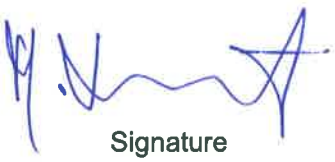
20th March 2017

CERTIFICATE

This is to certify that this dissertation entitled "**Pyrene tagged Polycaprolactone based polymers for bio-imaging**" towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents work carried out by **Abhishek Kumar (20121016)** at **Indian Institute of Science Education and Research(IISER) Pune** under the supervision of **Prof. Manickam Jayakannan,** *Department of Chemistry* during the academic year **2016-2017**.

Date: 29th March' 17

Place: Pune



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DECLARATION

I hereby declare that the matter embodied in the report entitled "**Pyrene tagged Polycaprolactone based polymers for bio-imaging**" are the results of the work carried out by me at the Department of Chemistry, **Indian Institute of Science Education and Research (IISER) Pune** under the supervision of **Prof. Manickam Jayakannan** and the same has not been submitted elsewhere for any other degree.

Date: 29th March'17

Place: Pune

Abhishek Kumar

Signature

ACKNOWLEDGEMENTS

After this long journey of master's thesis, the time has come for honest introspection. I owe my sincere thanks and deepest gratitude to my supervisor **Prof. Manickam Jayakannan**. I would like to thank him for his constant support, motivation, and help throughout the year. His enthusiasm and dedication toward scientific research always amazed me. Motivated by his esteemed guidance, led me to pursue research in my future.

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~ Abhishek

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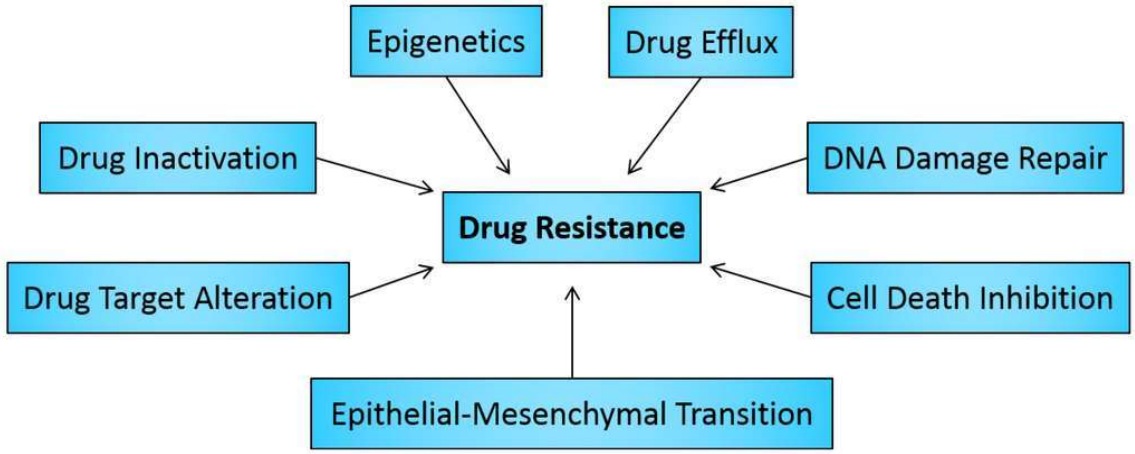
Abstract- Delivering drugs and bio-imaging of cancer cells still remains one of the major challenges for today's chemistry. Here, we report a novel polymeric based scaffolds for bio imaging of cancer cells. These pyrene based polymers were ell characterized by NMR, GPC and MALDI-TOF. Photo physical studies of these polymers were done. Smaller chain length of these polymers shoed more intense peaks of pyrene excimer but the intensity of excimer peak gradually decreased after increase in chain length, showing that the better chain packing efficiency of smaller polymer. Cytotoxicity of these polymer ere checked in Cervical cancer(HeLa) cell lines. These polymers ere nontoxic upto a concentration of 75µg/ml and shoed upto 80 percent of cell viability. Cellular intake studies of polymers ere done using HeLa cell line by CLSM. These polymers accumulated in cytoplasm and a nice blue colored image in cells.

1. Introduction

1.1 -Drug delivery to cancer cells

Cancer remains to be one of the leading causes of deaths in the world. Most of the cancer chemotherapy regimens fail due to the emergence of drug resistance by intrinsic and extrinsic mechanisms.[1], [2].Most of the available anticancer drugs are hydrophobic in nature because of which they face limitations such as poor bio-availability and reduced therapeutic efficacy.[3] The other limitations faced by these drugs also include their interactions with plasma proteins, which in turn provokes RES (reticulo-endothelial system), and these interactions opsonisation by macrophages.[4] Due to these limitations the usage of anticancer drugs are facing major problems.



Fig

Figure 1- Drug resistance in cancer cells (adapted from Sarkar et al. Cancers ***2014***, 6*(3), 1769-1792)*

To overcome these problems associated with conventional drug therapy, several drug carriers such as liposomes, gold nanoparticles, graphene based nanoparticles are being used for delivering the drugs[5]. These delivery vehicles are nanometer in size and hence called Nano carriers. These nano carriers not only help drugs to in improve delivery of poorly water soluble drugs but also possess a lot of advantages, such as targeted delivery of drugs in cell or tissue specific manner, co-delivery of two or more drugs for therapeutic modality for combination therapy, visualization of site of drug delivery by combining imaging modalities, and real time read on *in vivo* efficacy of therapeutic agents[6].Another advantage of using these delivery vehicle is that they can be used for

temporal release of drugs[7]. Controlled release of drugs are important as increasing therapeutic activity compared to the intensity of side effects[8], reducing the number of drug administrations required during treatment, or eliminating the need for specialized drug administration Controlled release over an extended duration is highly beneficial for drugs that are rapidly metabolized and eliminated from the body after administration[7].

1.2 -Polymer based drug delivery and EPR effect: A ideal Nano carrier should have some properties for drug delivery some of them are (i) The carrier should be bio-compatible and biodegradable. (ii) It should be stable enough to not detoxified against the serum but quick enough to release the drug to desired site within given time interval[9]. Using these properties various liposomes, polymers based Nano carriers have been made. Polymer based Nano carriers offer additional privileges like high loading capacity, required stability in bloodstream, selective targeting and long circulating properties. In order to achieve above mentioned properties of the drug, numerous biodegradable polymers have been widely explored due to their unique advantages like lesser side effects, prolonged bioactivity, decreased administration frequency, thereby facilitating patient compliance.

The nannocarriers have a special tendency to accumulate near tumor tissues, this effect is known as Enhanced permeable and Retention(EPR) effect.[10] EPR effect can be explained as, in order for tumor cells to grow quickly, the production of blood vessels must be stimulated. Tumor cells aggregates as small as size 150-200 micrometer start to become dependent on blood supply carried out by neovasculature for their nutritional and oxygen supply. These newly formed vessels are usually abnormal in form and architecture. They are poorly aligned defective endothelial cells with wide fenestrations, lacking a smooth muscle layer, or innervation with a wider lumen and impaired functional receptors for angiotensin. Furthermore, tumor tissues usually lack effective lymphatic drainage. All of these factors lead to abnormal molecular and fluid transport dynamics, especially for macromolecular drug[11]With aim of achieving higher[1]

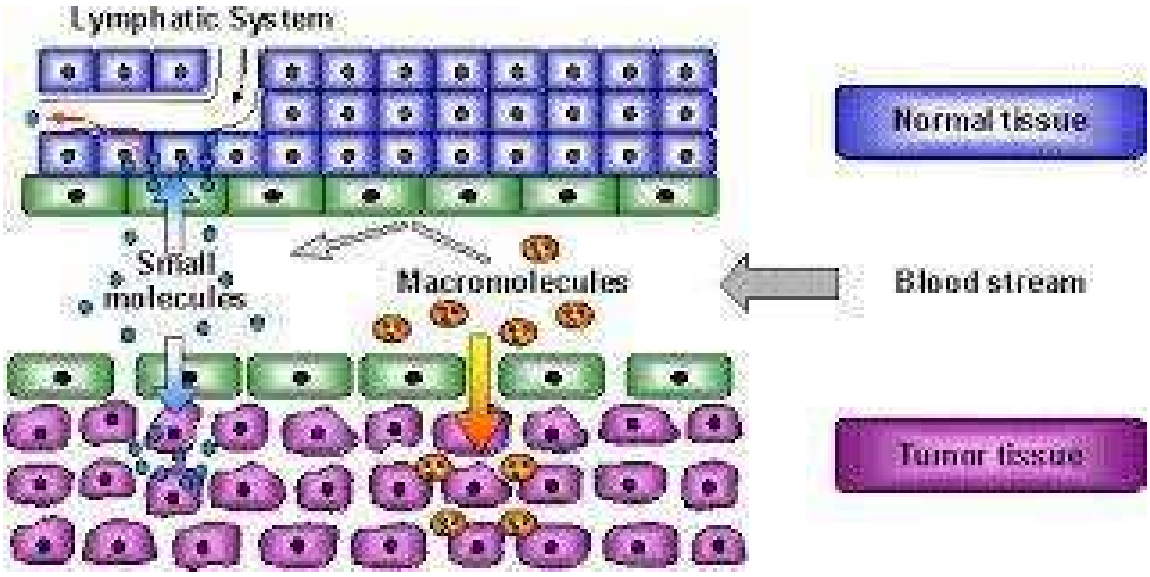


Fig (2) : Schematic representation of EPR effect in Cancer cells(adopted from https://edoc.hu-berlin.de/dissertationen/regehly-martin-2008-07-16/HTML/chapter2.html)

accumulation and release of the chemotherapeutic agent at the desired site variety of synthetic as well as natural occurring polymers based NPs have been tried.

Polycaprolactone for drug delivery and bio-imaging: It is known in cancer cells the concentration of enzymes are very high compared to other part of body, this is due to high metabolism rate in cancer cells compared to other body parts. Esterage is one of the same kind. This enzyme is known to cleave the ester bonds present in the molecules. Making use of this concept several polyesters have been used for drug delivery system. Some of the being Polylactic acid, Polycaprolactone and so on so forth. Polycaprolactone (PCL) is one of the most sought polymers for long term drug delivery systems. Apart from it's drug delivery applications, PCL has been used in various other fields some of them being scaffolds in tissue engineering, in microelectronic, as adhesives and in packaging. Due to it's wide applicability and interesting properties like controlled degradability, miscibility with other polymers, biocompatibility makes PCL a very useful class of polymer.

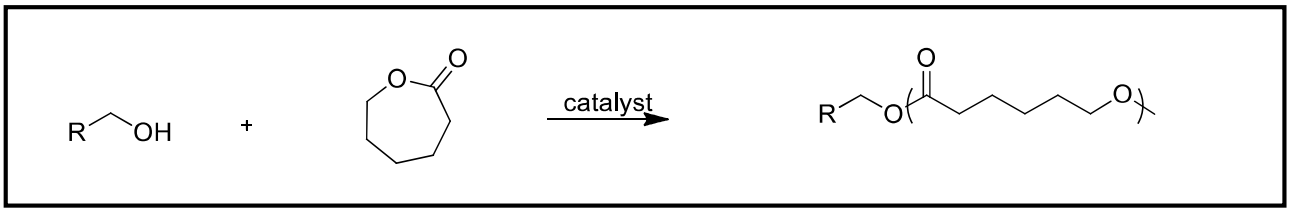


Fig (3) – General scheme of Ring Opening Polymerization

PCL is made from condensation of 6-hydroxycaproic acid and by Ring Opening Polymerization(ROP) of it's monomer called Caprolactone. The second one is generally preferred over first as ROP gives high molecular weight polymers with low polydespersity and this is the reason why ROP have been explored more compared to polycondesation on case of PCL. Depending on the catalysts being used in ROP the mechanism of polymerization may vary. Some of known mechanisms by which it goes are listed below: -

1. Anionic ROP: In this mechanism an anionic initiator attacks at the carbonyl carbon of the monomer. The monomer is the opened at the acyl-oxygen bond and the chain growing is continued. One of the major drawbacks of this process is that it undergoes intramolecular transesterification. Due to this smaller chains of polymer is formed.[12]
2. Cationic ROP: This process involves a formation of cationic species which is attacked by the carbonyl oxygen of monomer through SN2 mechanism.
3. Monomer activated ROP: In this mechanism, the monomer gets activated by catalyst and then this attack polymer chain end.[13],[14]
4. Co-ordination Insertion ROP: It is also called pseudo anionic mechanism, In this, the initiator coordinates to catalysts and then monomer inserts itself into metal-oxygen bond of catalyst. During propagation step, the polymer chain is attached to metal by an alkoxide bond. It is the most commonly used mechanisms of ROP.[12]

There catalysts used in ROP are broadly classified into three systems, (i) metal based, (ii) enzymatic and (iii) Organic. Each have some advantages and disadvantages, for biomedical applications of PCL the commonly used catalyst is Tin Octanoate, due to nontoxic nature of tin over other metals, enzymes and organic catalysts. Tin

Octanoate follows a coordination insertion mechanism of ROP, in this the initiator used is generally some kind of alcohols. Mechanism of this process is given in fig (4)

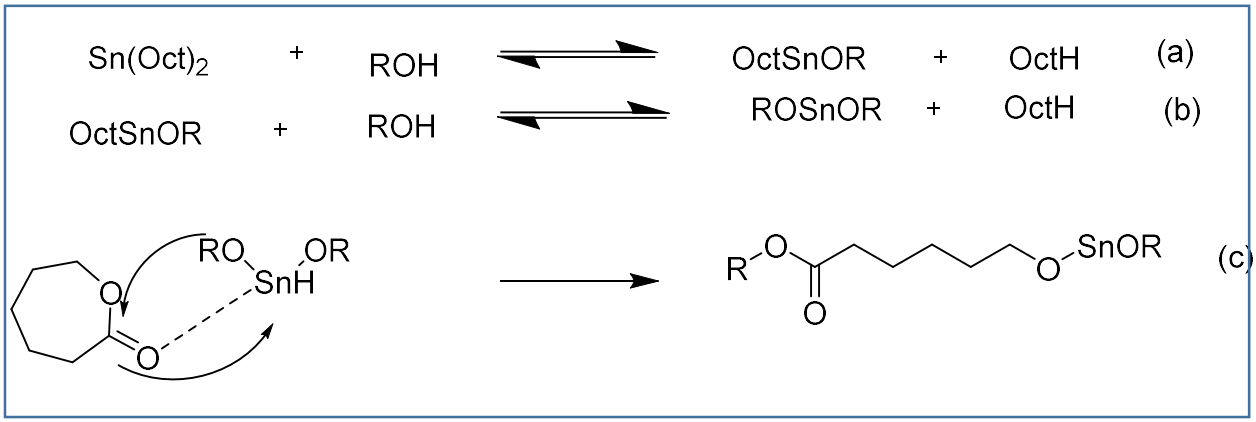


Fig (4)-Coordination insertion mechanism of ROP

PCL based polymers due to biocompatibility have enormously been used in drug delivery approaches. To impart functionality to PCL based polymers several derivatives of PCL have been made. Making different functional group to polymers gives a room of modifications like multiple drug delivery, target specific delivery, improve oral bioavailability, sustain drug/gene effect in target tissue, solubilize drugs for intravascular delivery, and improve the stability of therapeutic agents against enzymatic degradation. Some of the modified Caprolactone based monomers are listed in figure.

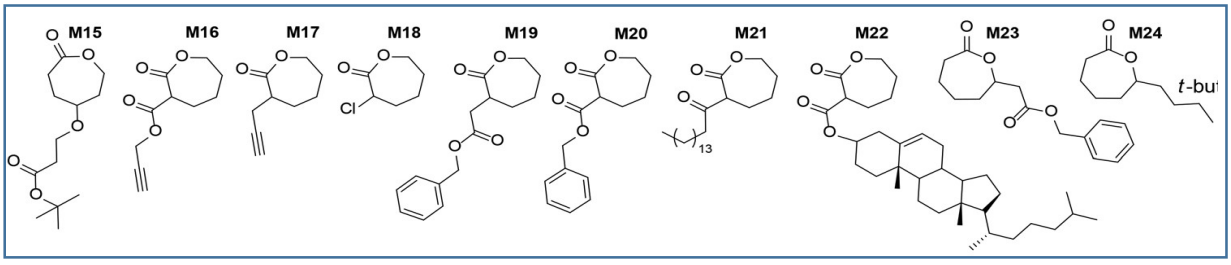


Fig (5)- Different derivatives of Caprolactone reported in the literature

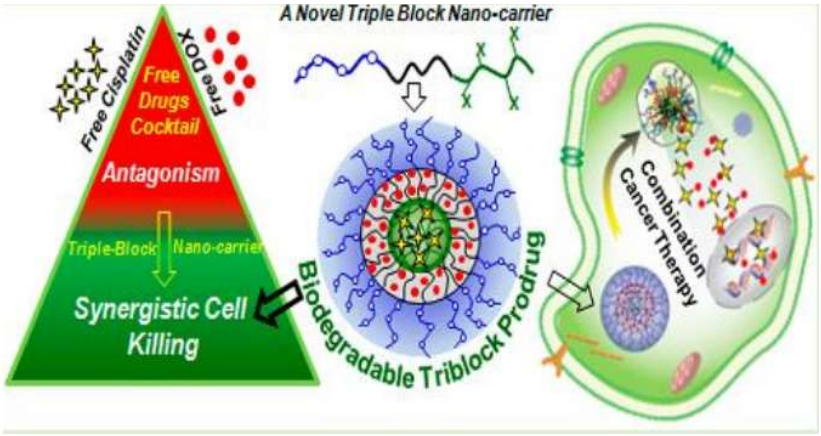
From our research group, Bapu et al. designed a new carboxylic-functionalized caprolactone monomer and further subjected to ring opening polymerization. The carboxylic substituted PCL block copolymers PEG-b-CPCLx resulted in the production of

water soluble 80-250 nm sized pH responsive vesicles capable of loading both hydrophobic and hydrophilic drugs. *In vitro* drug release revealed that the drugs released exclusively under simulated intestinal fluid conditions that is similar to the physiological environment of the small intestine.

They designed a new class of hydrogen bonded and enzyme-responsive (biodegradable) PCL diblock copolymer nanoparticles for loading and delivering anticancer drugs by tuning the biodegradability and hence the release mechanism as "burst" and "controlled" at the intracellular compartments in cervical (HeLa) and breast (MCF-7) cancer cells. New amide and ester substituted ω- caprolactone monomers were tailor-made and their ring opening polymerization was carried out employing PEG-2000 mono methyl ether as an initiator in order to synthesize hydrogen bonded amide and nonhydrogen bonded ester PEG-*b*-PCL diblock copolymers. The nature of the linear or bulky substituent had great impact on the drug loading content of the diblock copolymer nanoparticles. The *in vitro* drug release studies revealed that the aliphatic polyester PCL chain was readily degradable by lysosomal esterase enzyme in PBS at 37 °C to release the loaded drugs. The hydrogen bonded amide copolymer nanoparticles degraded slowly in a much more controlled manner over a prolonged period, whereas the non-hydrogen bonded ester copolymers underwent burst uncontrolled release of drugs. The nascent polymer scaffolds were non-toxic to cells up to 40 μg/mL and the DOX loaded nanoparticles accomplished more than 90 % cell killing in both HeLa and MCF-7 cells. The hydrogen bonding interaction was proven to play an important tool in controlling the drug release profiles of the anticancer drugs at the cancer cells. The cellular uptake of the DOX loaded polymer nanoparticles and their cleavage in the cytoplasm was further supported by the confocal microscope imaging.

Further, Bapu et al. successfully demonstrated the concept of biodegradable diblock copolymer core-shell nanoparticle assemblies for cisplatin delivery against detoxification by cytoplasmic thiol residues in breast cancer cells. They did it by protecting the Cis platin in a core shell NP, made of BPCL by covering a layer of Polyethylene glycol. The nascent polymers were found to be biocompatible and non-toxic to cells. Due to over expression of GSH in MCF-7 cell lines, more than 50% of cells were

viable at higher concentration of free cis platin. The polymer-cisplatin nanoparticles showed enhanced cell killing in MCF-7 and the cell viability was found to be < 10 % at 4 μg/mL drug concentration. This selective and enhanced cell killing in MCF-7 cells by the polymer nanoparticle was attributed to their resistance to drug detoxification by GSH.[15]



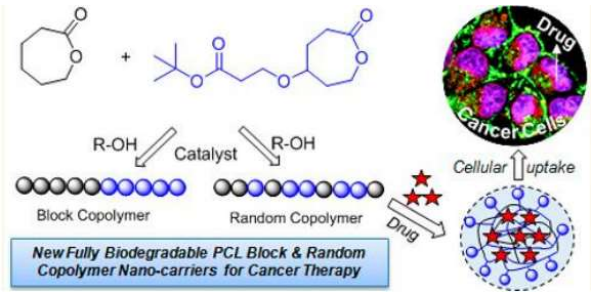
Fig(6) BPCL based polymeric scaffolds for multiple drug delivery(adopted from Bapu et al, acs.biomac.6b01608)

This concept was extended to triblock copolymer nanoparticles in order to study the combination delivery of cisplatin and DOX and achieve synergistic killing in resistant breast cancer cells. In the fabrication of TLNs, cisplatin was chelated to COOH groups of CPCL block and DOX was physically encapsulated into the PCL layer. The TLNs were found to be very stable in water. TLNs stabilized > 90% of cisplatin and < 60 % of DOX in PBS, which is evidently attributed to the protection rendered by the middle PCL layer.

In vitro drug release studies revealed that the PEG shell and PCL layer protected cisplatin drug against detoxification by the cytoplasmic thiol residues, i.e. GSH. Further, the biodegradable aliphatic PCL ester backbone ruptured upon exposure to esterase enzyme at conditions identical to that of intracellular compartments, where cisplatin showed controlled release up to 48 h. Cytotoxicity of the polymer and dual drug loaded nanoparticles was tested in WT-MEF, HeLa, and MCF-7 cell lines. The nascent polymers were found to be biocompatible and non-toxic to cells. In MCF-7 cell lines, free cisplatin drug failed to kill all the cells and more than 50 % of the cells were viable even at very high drug concentration. Over-expression of GSH in MCF-7 is responsible for poor killing by free cisplatin drug. Polymer nanoparticles showed selective and enhanced cell killing in MCF-7 cells, which was due to their resistance against drug detoxification by GSH. The

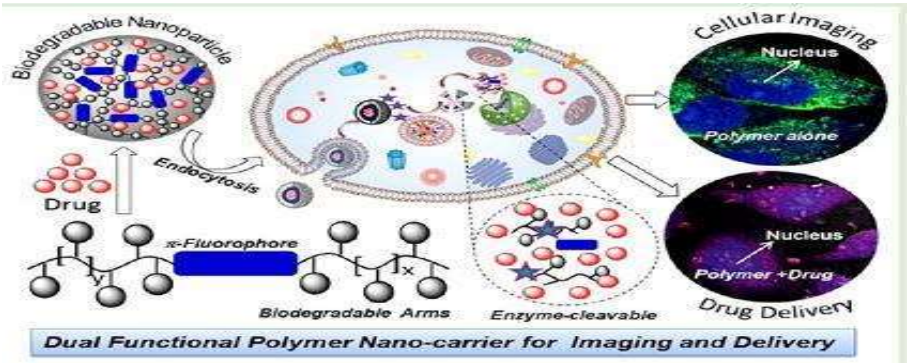
dual drug loaded nanoparticles emanated in 3 fold excess cell killing over polymer cisplatin conjugates, which can be attributed to the increased stability of the DNA-Pt adduct rendered by DOX that retards the DNA repair mechanism efficiency. The dual loaded TLNs containing cisplatin and DOX act synergistically to enhance the killing of breast cancer cells. The CI values showed that the dual loaded nanoparticles exhibited synergistic killing, however when cisplatin and DOX were administered as a cocktail they showed antagonistic effect.[16]

Mehak et al. reported a new class of complete biodegradable amphiphilic random and block copolymer design based on carboxylic functionalized polycaprolactone- copolycaprolactone. Their polymer topology driven enzyme-controlled degradation and delivering capabilities were studied at the intracellular compartments for doxorubicin (DOX) drug in breast (MCF-7) and cervical (HeLa) cancer cells. *In vitro* drug release kinetics revealed the polymer nano-scaffolds' exclusive susceptibility to rupture in the presence of lysosomal esterase enzyme to deliver DOX. Their 'slow' and 'burst' release kinetics was indirectly controlled by the composition of the random copolymers. *In vitro* cytotoxicity studies in MCF-7 and HeLa cell lines revealed that the newly designed polymer scaffolds are non-toxic to cells and their DOX loaded nanoparticles exhibited more than > 95 % cell death. Confocal microscope analysis confirmed the internalization of the polymer-loaded drugs and indicated that the drugs are predominately delivered at the nucleus of the cells for complete cell killing.[17]



Fig(7) completely biodegrable BPCL based polymers for drug delivery to cancer cells(adopted from Mehak et al. **Macromolecules, 2016,** *8098-8112*)

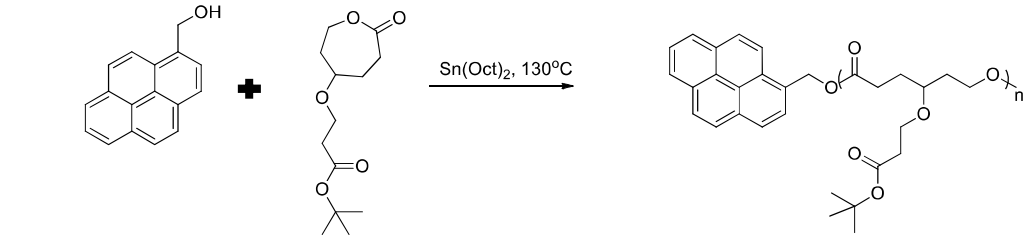
Bhagyashree et. al. reported a new polymer drug delivery concept based on biodegradable Polycaprolactone (PCL) and highly luminescent π-conjugated fluorophore as dual functional Nano-carrier for cellular imaging and delivery vehicles for anticancer drug to cancer cells. The substituted Caprolactone monomer was subjected to ring opening polymerization using a blue bishydroxyl oligo-phenylenevinylene (OPV) fluorophore as an initiator. A series of A-B-A tri-block copolymer building blocks with a fixed OPV π-core and variable chain biodegradable PCL arm length were tailor-made. These tri-blocks self-assembled in organic solvents to produce well defined helical nanofibers whereas in water they produced spherical nanoparticles with blue luminescence. The hydrophobic pocket of the polymer nanoparticle was found to be an efficient host for loading water insoluble anticancer drug such as doxorubicin (DOX). The photophysical studies revealed that there was no cross-talk between the OPV and DOX chromophores and their optical purity was retained in the nanoparticle assembly for cellular imaging. *In vitro* studies revealed that the biodegradable PCL arm was susceptible to enzymatic cleavage at the intracellular lysosomal esterase under physiological conditions to release the loaded drugs. The nascent nanoparticles were found to be non-toxic to cancer cells whereas the DOX loaded nanoparticles accomplished more than 80 % killing in HeLa cells. Confocal microscopic analysis confirmed the cell penetrating ability of the blue luminescent polymer nanoparticles and their accumulation preferably in the cytoplasm. The DOX loaded red luminescent polymer nanoparticles were also taken up by the cells and the drug was found to be accumulated at the peri-nuclear environment.[18]

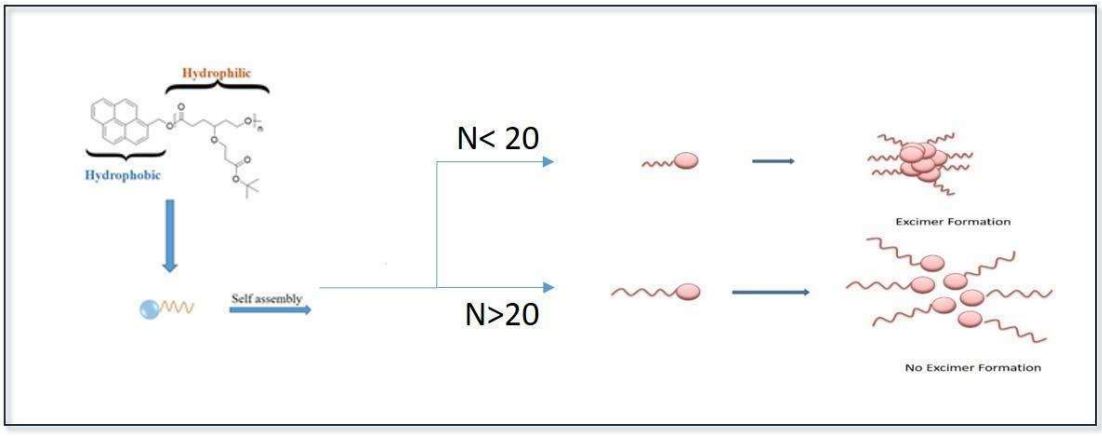


Fig(8)- OPV and BPCL based polymers for drug delivery and bio-imaging(adopted from Bhagyashree et al., **Biomacromolecules, 2016,** *1004-1016)*

Aim of the thesis:

Most of the PCL based block copolymers that are employed as nano-carriers for drug and gene delivery in the literature are non-luminescent in nature. Thus, the probing of the cellular entry of these nano-carriers at the intracellular level is very difficult to study by confocal microscopy, etc. For this purpose, fluorophone dye molecules or drug molecules that were loaded in the PCL nano-carriers was indirectly monitor to assess the cellular internalization of polymer scaffold. The present thesis is aimed to develop new classes of pyrene tagged t-butyl ester substituted PCL(BPCL) polymer as a fluorophore-tagged nano-probe to study the PCL internalization in the cancer cells. Pyrene tagged PCL based polymers have however have been studied, but due to hydrophobic nature of PCL based polymer, It cannot be used for biological applications[19].The present thesis is also aimed to solve this issue. The pyrene probes were found to be highly sensitive to the BPCL arm lengths for exhibiting excimer formation in aqueous medium. The new probe was found to be non-toxic to cells and also blue luminescent for studying their cellular update by confocal microscopy. The schematic representation of the present approach is demonstrated in figure(8)





Fig(9)- Scheme of the excimer formation of smaller and bigger polymers

MATERIALS AND METHODS:

Materials: 1,4-Cyclohexanediol, tert-butyl acrylate, potassium tertiary butoxide, Pyridinium chlorochromate (PCC), molecular sieves (4 Å), m-chloroperbenzoic acid (mCPBA), Pyrene methanol, Nile Red, were bought from sigma Aldrich. Anhydrous sodium sulphate, sodium bicarbonate, sodium thiosulphate pentahydrate and sodium hydroxide were locally purchased.

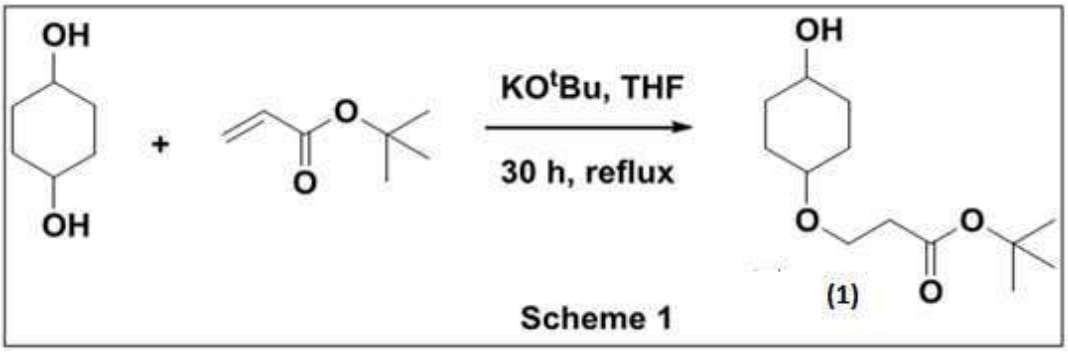
Solvents: tetrahydrofuran (THF), petroleum ether, Ethyl acetate, Methanol, Chloroform, dichloromethane (DCM), dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) were used.

Instrumentation: All the samples for ^{1}H NMR and ^{13}C NMR were prepared in $CDCl_3$ as a solvent and Trimethylsilane was used as an internal standard. The NMR were recorded using JEOLE-400 spectrophotometer. Gel permeation Chromatography(GPC) was done using Viscotek VE 1122 pump, Viscotek VE 3580 RI, 3210 UV-Vis and Light scattering detectors. To do GPC 1mg of polymer was dissolved in 1ml of HPLC grade THF, the solution was heated using water bath to make sure that the polymer is dissolved. Obtained solution was filtered using wattman filter paper to get rid of any bigger impurities. GPC was done using a column which was standardized by using polystyrene. The thermal stability of the polymers were determined by using Perkin-Elmer thermal analyzer STA 6000 model, where the polymers were heated 10°C/min under nitrogen atmosphere. FT-IR study of the monomers was done by using Thermo Scientific Nicolet 6700 spectrometer. The absorption studies and release assays were carried out by using Perkin-Elmer Lambda 45 UV-Visible Spectrophotometer. Steady state emission and excitation spectra were recorded using Fluorolog-3 HORIBA JOBIN VYON fluorescence spectrophotometer. Dynamic Light Scattering (DLS) study of the self-assembled particles was done by using Malvern Instrument -Nano ZS- 90 setup using 633nm laser as the source with the detector collecting the scattered light at 90° angle. The DLS yields information on the correlation function that in turn provides the value of diffusion coefficient (D), the Stock Einstein equation is then used to calculate the diameter of the NPs.

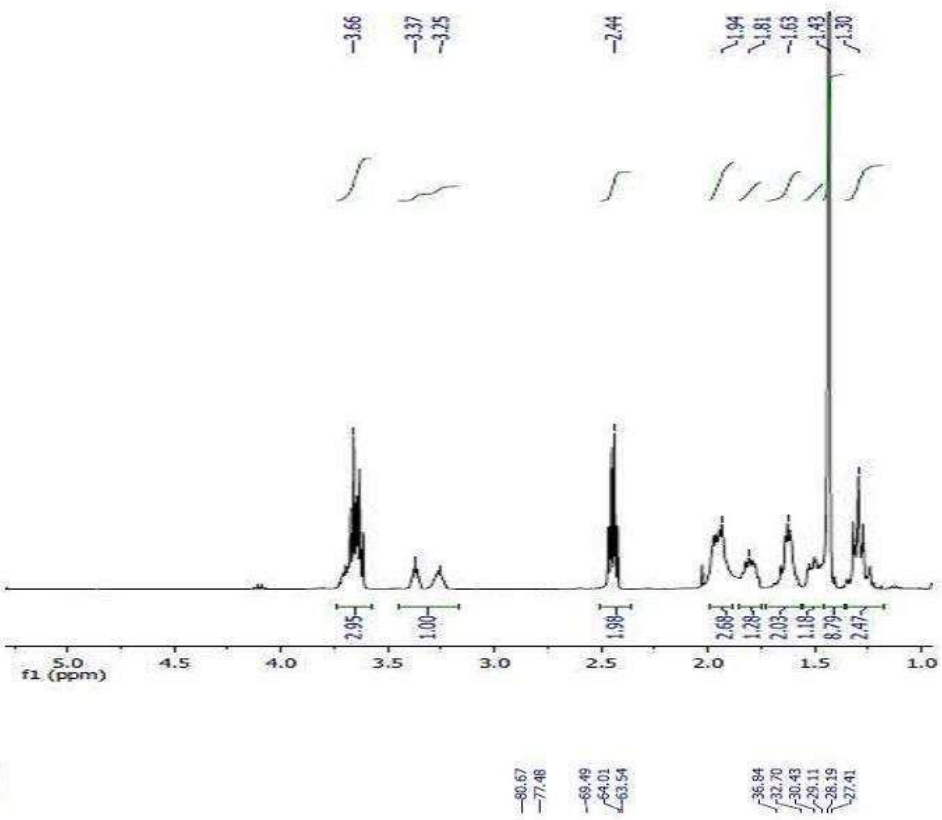
General Procedure: -

2.3.1. Multistep Synthesis of γ-substituted Caprolactone (BPCL):

Synthesis of t-Butyl-3-((4-hydroxycyclohexyl) oxy)-propionate (1) 1,4-Cyclohexane diol (30 g, 258.6mmol) was dissolved in 300 ml of dry tetrahydrofuran in a round bottom flask containing a magnetic bid, to this catalytic amount of tertiary butoxide was added as a base, the reaction mixture was stirred for 15-20minutes on room temperature. Separate mixture of tertiary butyl acrylate (26.52g, 206.9mmol) was prepared in dry THF(50ml), this was added dropwise to the reaction mixture using a dropping funnel over a period of 1 hour. The reaction mixture was heated at 80^{o}C and monitored over a period of 30 hours. After 26 hours no significant change was seen on TLC so reaction was stopped. From the reaction mixture THF was evaporated completely, and Dichloromethane(DCM) was added. Since, 1,4cyclohexane diol is insoluble in DCM, the reaction mixture upon filtration by Buchner funnel separated the unreacted 1,4cyclohexanediol and the products. The product mixture was separated by column chromatography by using 100-200mesh silica as a stationary phase and Ethyl Acetate(EA) and Petroleum Ether(PE) as mobile phase. The desired product was obtained at 5:19(v/v) EA:PE solution. The product was yellow liquid in nature. Yield of this reaction was 40%.**^{1}H-NMR** (400 MHz, CDCl3), δ ppm: 3.64 (m,3H, O−CH_2− and O−CH), 3.29−3.39 (m, 1H, CH−OH), 2.4 (t, 2H, −CH2CO−), 1.96−1.81 (m, 4H, OCH(CH_2)$_2$), 1.64−1.32 (m, 4H, CO(CH_2)$_2$), 1.45 (s, 9H, −C(CH_3)$_3$).**^{13}C-NMR** (100 MHz, $CDCl_3$ δ ppm: 171.01, 80.42, 69.49, 63.84, 63.60, 32.54, 30.32, 29.20 and 27.48. **FT-IR** (cm^{-1}): 3422,2979, 2937, 2863, 1731, 1462, 1393, 1366, 1215, 1158, 1116 and 1134.



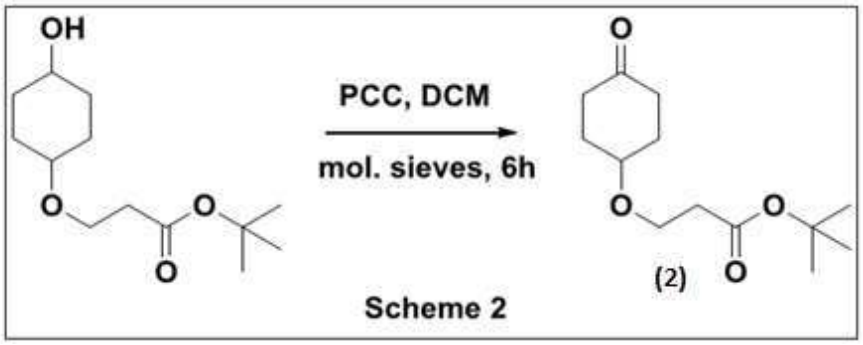
Scheme 1



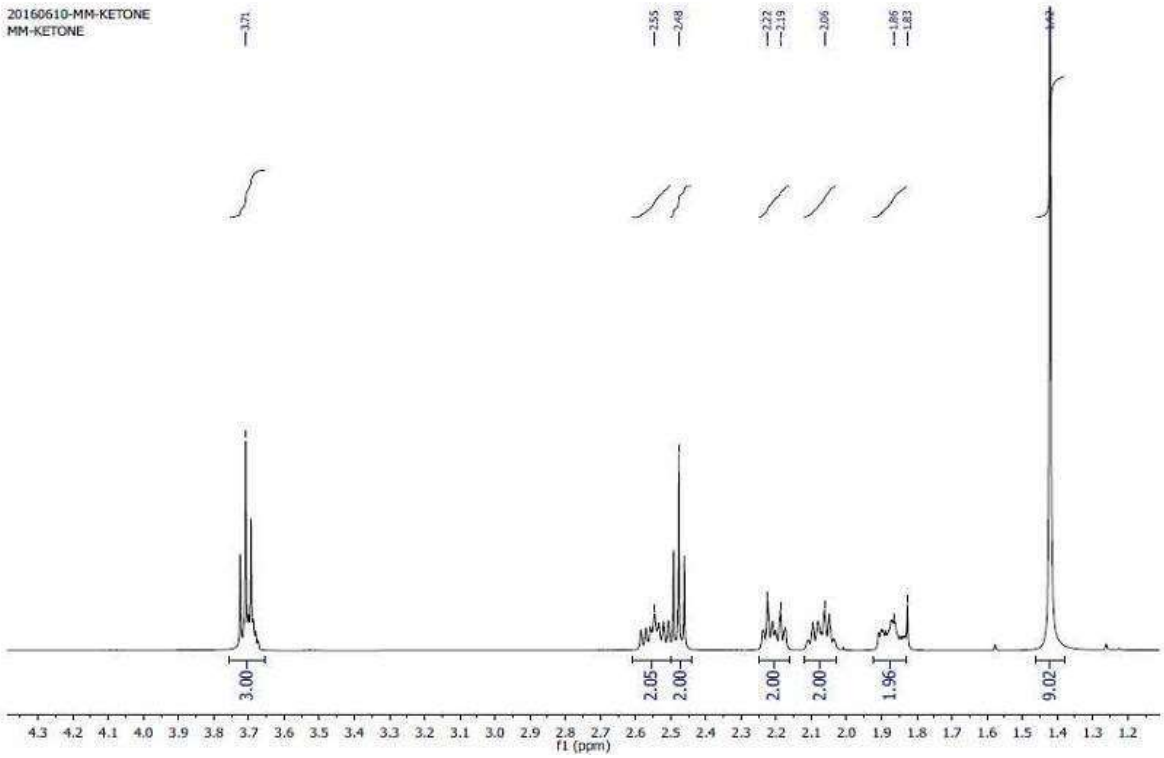
Synthesis of t-Butyl-3-((4-oxocyclohexyl) oxy)-propionate (2):

22 gm of Compound 1 was dissolved in 400ml of dry DCM in inert conditions in round bottomed flask. To this Pyridinium chlorochromate (PCC) was added to the reaction mixture followed by addition of 2-3 spatula of molecular sieves. The stirring was continued

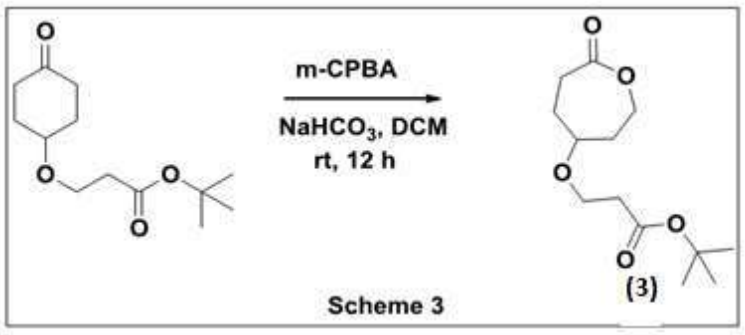
for 6 hours at room temperature. From the reaction mixture the liquid was extracted, and subjected to column chromatography, using Pet Ether Ethyl acetate as eluents. Pure product was obtained at 1:5 v/v mixture of EA:PE. Yield = 71 %. **^{1}H NMR**(400 MHz, $CDCl_3$) δ ppm: 3.56 (m, 3H, O−CH_2 and O−CH), 2.58 (t, 2H, −CH_2−CO), 2.64 (m, 2H, −(C=O)CH_2−), 2.26 (m, 2H, −(C=O)CH_2−), 2.09 (m, 2H, −(CO)CH_2−), 1.90 (m, 2H, −(CO)CH_2−), 1.45 (s, 9H, −$C(CH_3)_3$).**^{13}C-NMR** (100 MHz, $CDCl_3$) δ ppm: 211.40, 170.99, 80.56, 72.74, 64.02, 37.02, 36.56, 30.40 and 20.04. **FT-IR** (cm^{-1}): 2974, 2874, 2360, 1716, 1456, 1419, 1393, 1368, 1316, 1239, 1212 and 1109.



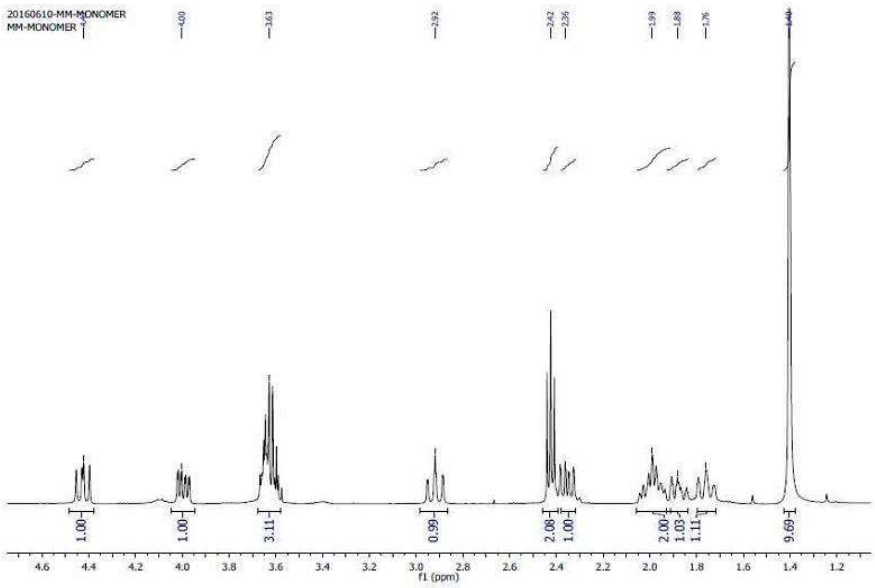
Scheme 2



Synthesis of t-Butyl-3-((7-oxooxepan-4-yl) oxy)-propionate (3): - 10g of compound 2 was taken in Round bottom flask containing magnetic bid. 300ml of dry DCM was added to it and made sure that the compound gets dissolved in it. $NaHCO_3$ was added to the reaction mixture, followed by addition of meta chloroperbenzoic acid (20.7 mg), The reaction mixture was stirred for 12 hours at room temperature. The reaction mixture turned milky after reaction was completed. The DCM from the reaction mixture was evaporated and saturated solution of sodium thiosulfate (in water) and Sodium bicarbonate (in water) was added to it, and made sure that the solid clumps formed during the reaction gets dissolved. To this (50ml*3) of EA was added and the organic layer was extracted and purified using column chromatography using EA and PE as eluent. Product was obtained at 1:5(v/v) EA:PE, and it was colorless liquid. ^{1}**H NMR** (400 MHz, $CDCl_3$) δ ppm: 4.40 (dd, 1H, COOCH), 4.06 (dd, 1H, COOCH), 3.60 (m, 4H, OCH_2, OCH, and COCH), 2.98 (dd, 1H, COCH), 2.48 (t, 2H, $COCH_2$), 2.42−1.81 (m, 4H, $OCH-(CH_2)_2$), 1.46 (s, 9H, $C(CH_3)_3$).^{13}**C-NMR** (100 MHz, $CDCl_3$) δ ppm: 176.35, 171.19, 81.01, 75.24, 64.24, 63.66, 36.80, 34.15, 28.40, 27.84 and 27.61. **FT-IR** (cm^{-1}): 2928, 1727, 1446, 1393, 1367, 1253, 1155, 1110 and 1058.



Scheme 3



Synthesis of Polymers: - BPCL was dried prior to polymerization; this was done by adding 2ml of toluene to BPCL, since toluene forms azeotrope with water, most of water can be evaporated along with toluene. To start synthesis of polymer a clean dry Schleck tube was taken with a small four arm magnetic bid into it. To this pyrene methanol was added, followed by the addition of BPCL and then catalytic amount of tin Octanoate was added. Depending on the number of repeating units in polymer, the value of [M]/[I] was varied for example if the number of repeating unit is 'x', the value of [M]/[I] is kept 'x'.. The reaction mixture was kept on heating at 130^{o}C for 8 hours. After 8 hours, the polymer formed was dissolved in minimum amount of THF, and precipitated in cold pet ether. The polymers were obtained with 80-90 yield. The polymer formed by this process were named as PYBPCLx. Where 'x' is number of repeating units in polymer.

Self-assembly of polymers: In a typical procedure, 2 mg of polymer is solubilized in 2 ml of HPLC grade DMSO. This solution is stirred for 1 hour. After 1 hour stirring; this solution was dropwise added to 3 ml of water with constant stirring. This mixture was additionally stirred for 3-4 hours and then transferred to a Semi Permeable membrane of MWCO 1000 and dialyzed against large volume of distilled water for a period of 48 hours. The water of tank is frequently replaced at an interval of 4 hours to make sure that the DMSO goes out completely for better self-assembly of polymers.

Cell-Viability Assay: - In order to study the cell viability assay of polymers, the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed. A 96-well plate (Corning, USA) was used, For this experiment and each of its wells were seeded with 1000 cells in 100 µL of DMEM with 10% FBS (fetal bovine serum) and these were allowed to adhere for 16 h. The media from each well was aspirated followed by addition of various concentrations of the polymer to each well. These were added in triplicates corresponding to individual experiments. Cells in DMEM with FBS in the absence of polymer were also maintained in triplicates as a blank control. The 96 well plate was incubated for 72 h without changing the media. After 72 h media from each well was aspirated and 100 µL of MTT solution (a freshly prepared stock of MTT in sterile PBS (5 mg/mL) diluted to 50 µg/mL in DMEM) was added. These were allowed to incubate for another 4 h at 37 °C. The formation of purple formazan crystals was observed that developed due to the reduction of MTT by mitochondrial dehydrogenase enzyme, and the media from each well was aspirated and 100 µL of DMSO was added to the resultant crystals giving purple solutions in each well. The absorbance from formazan crystals was immediately recorded using microplate reader at 570 nm (Varioskan Flash), which is representative of the number of viable cells per well. Values corresponding to each triplicate (of control and polymer treated cells) were determined and their mean was used for calculations. In the triplicates, any value that deviated from rest two were discarded. The mean of blank control set as 100% (corresponding to viable cells) and relative percentage values for polymer nanoparticle were calculated with respect to this.

Bio-imaging of polymer loaded cells: - Coverslips were taken in 6 well plate in DMEM medium containing 10% FBS. Coverslips ere Flame-dried prior to use. Cells were seeded at a density of 1×10^5 cells and these were incubated at 37 °C for 16 h, on the surface of these coverslips The cells in each well, thus grown, were treated with required concentrations of PYBPCL5, PYBPCL15 and PYBPCL25 polymer nanoparticles keeping the fluorophore concentration as 2µg throughout. This setup was incubated at 37 °C for 4 h under CO_2 environment and then the media was aspirated from each well, and the cells were washed twice with PBS (2 × 1 mL) and fixed with 4% paraformaldehyde solution in PBS for 10 min at room temperature. The cells were washed twice with PBS

(2 × 1 mL). The coverslips were mounted on slides using Fluoromount mounting medium (Southern Biotech) and were left for drying overnight at room temperature in the dark. The cells were imaged using a confocal microscope using the λ=405 nm laser. Images thus obtained were analyzed using Image J analysis software.

RESULTS AND DISCUSSION:

Synthesis and characterization of monomers: - The monomers were synthesized using multistep synthesis as mentioned in fig (10)

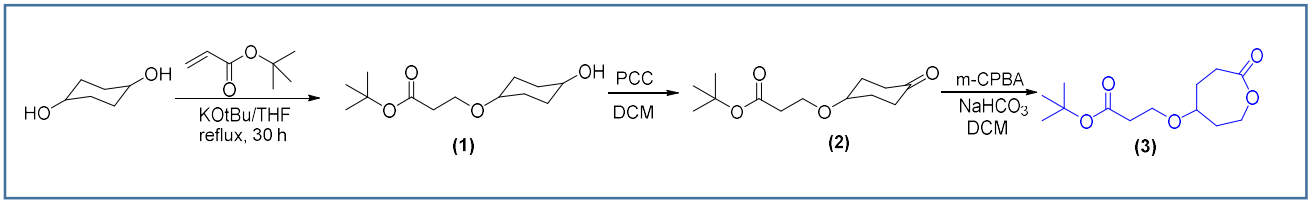


Fig (10)-Scheme of monomer synthesis

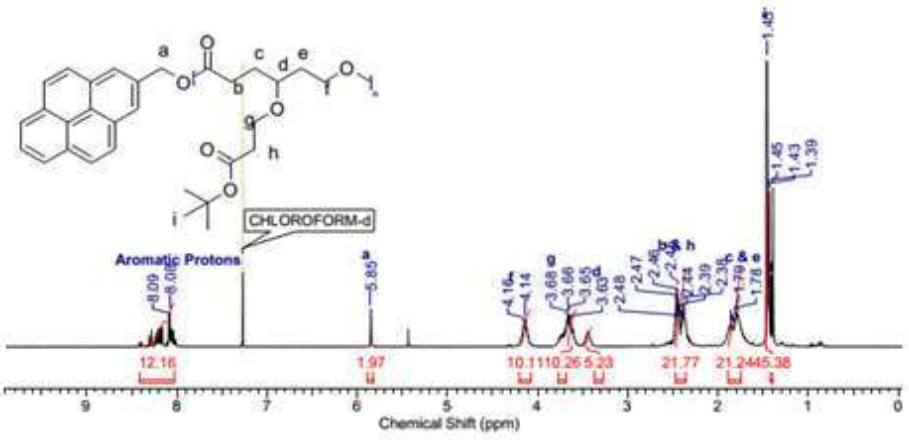
In the first step the 1,4cyclohexane diol undergoes Michael addition with tertibutyl acrylate to give the monosubstituted product. This was confirmed by using ^{1}H NMR, by splitting of signal corresponding to the hydrogen attached next to the oxygen molecule, this is due to formation of two chair confirmations of cyclohexane conformers. Also appearance of additional singlet peak at 1.4ppm, corresponding to tert-butyl group and the monosubstituted product. In the next step the mono coupled product was subjected to oxidation of alcohol group by using Pyridinium chlorochromate(PCC). The formation of this product was confirmed by ^{1}H NMR. In the NMR signal, the signal coming from hydrogen attached to carbon next to alcohol group was vanished, this showed the formation of ketone, also from IR spectra we got a new frequency at around 1716cm^{-1} which is a typical frequency shown by ketone group in a 6 membered ring. In the last step of synthesis, the ketone was subjected to bayers billigars oxidation. The product was confirmed by using ^{1}H NMR, and IR. In NMR there were two new peaks appeared in the NMR spectrum at 4.49 ppm and 4.06 ppm, these appearances in deshielded region confirms the incorporation of oxygen in the ring, also by IR spectra there was a new peak appearing at

1726cm^{-1}, confirms the formation of ester bond in the molecule.

Synthesis and Characterization of polymers**: -** To start synthesis of polymer, a clean and dry schlenk tube is taken, to this monomer, initiator, and catalyst were added. This tube was then subjected to one-hour high vacuum conditions to make it completely free from

moisture. After this is stirred at 130°C for 8 hours to give the polymers. To synthesize polymers of various length the concentration of ratio monomer to initiator was varied. For example, if polymer with 'x' repeating units is required then [M]/[I] will be kept as 'x'. various polymers with chain length 5, 10, 15, 20, 25 were prepared. These polymers were confirmed using ^{1}H NMR. The NMR of polymers were clean, the aromatic peak of pyrene appeared at 8.07 ppm, followed by the 2 protons of –CH2 just outside the pyrene ring. The tertiary butyl group of polymer came as a narrow singlet at 1.45 ppm. In higher analogues of polymers, the 'f' proton (see figure (11)) of last group appeared separately than polymer, this is due to the different environment that the end group proton faces compared to the polymers.

In order to calculate the number of repeating units in polymer the **–CH2** protons of pyrene methanol were taken as standard, and given a standard value of 2 corresponding to 2 protons and any peak from polymer was integrated. For example, the tertiary carbon's proton comes as a pentane at 3.63 ppm, there is one proton in each repeating unit, suppose the number of the polymer is x, then the integration for this proton should be x.



Fig(11)- NMR of PYBPCL5

The stacked NMR of different polymers has been plotted by keeping the intensity of the benzylic proton constant. It can be observed that the intensity of other protons gradually increases with increasing in polymeric chain length.

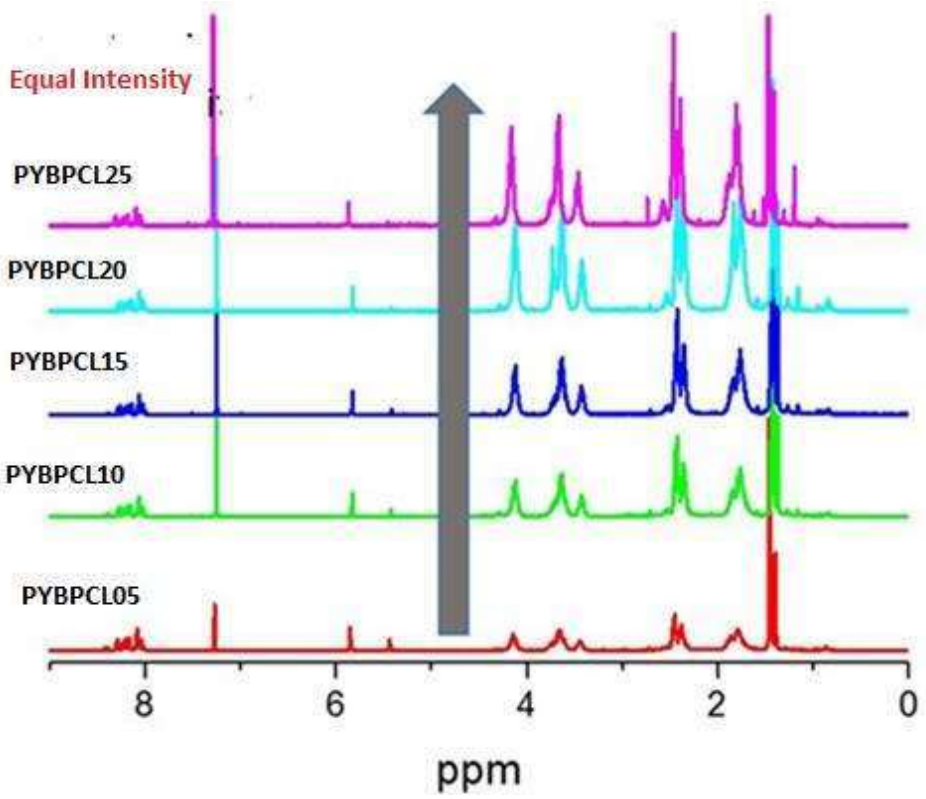
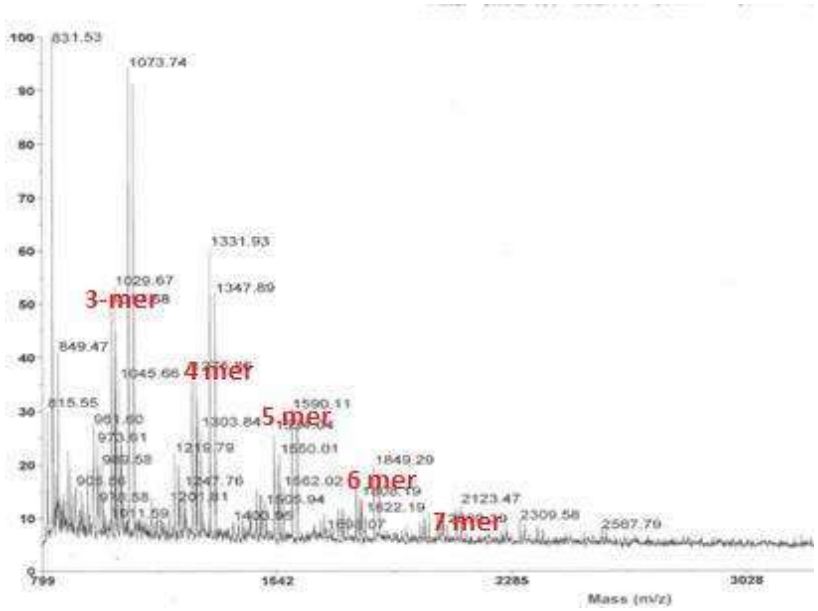


Fig (12) stack NMR plot of different polymers

MALDI-TOF is a very powerful technique used to analyze the end groups in new polymers. For this purpose, the newly made polymers were subjected to MALDI-TOF analysis. MALDI-TOF MS spectra showed a distribution of peaks at an interval of 258 amu, which is the mass of the monomer. Shown below the spectra of PYBPCL-05 polymer, the peak at 1017 is the peak of 3-mer incorporating the pyrene methanol to polymer and potassium peak of the polymers can be seen next to it at 1045 amu.



Fig(13)-MALDI-TOF Spectrum of PYBPCL05

Feed Vs actual incorporation was plotted and It showed almost a linear trend, showing that the polymerization is living in nature.

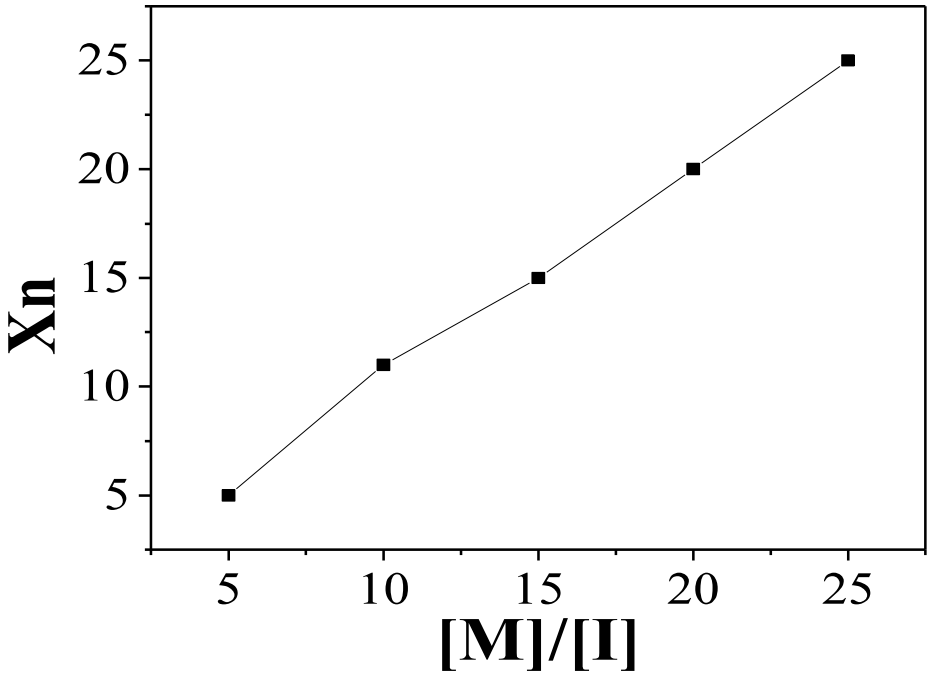


Fig (14)- Feed Vs actual incorporation

The polymers were further confirmed using Gel permeation chromatography, using THF as a solvent and polystyrene as a standard. The GPC chromatogram showed monomodal distribution for polymers.

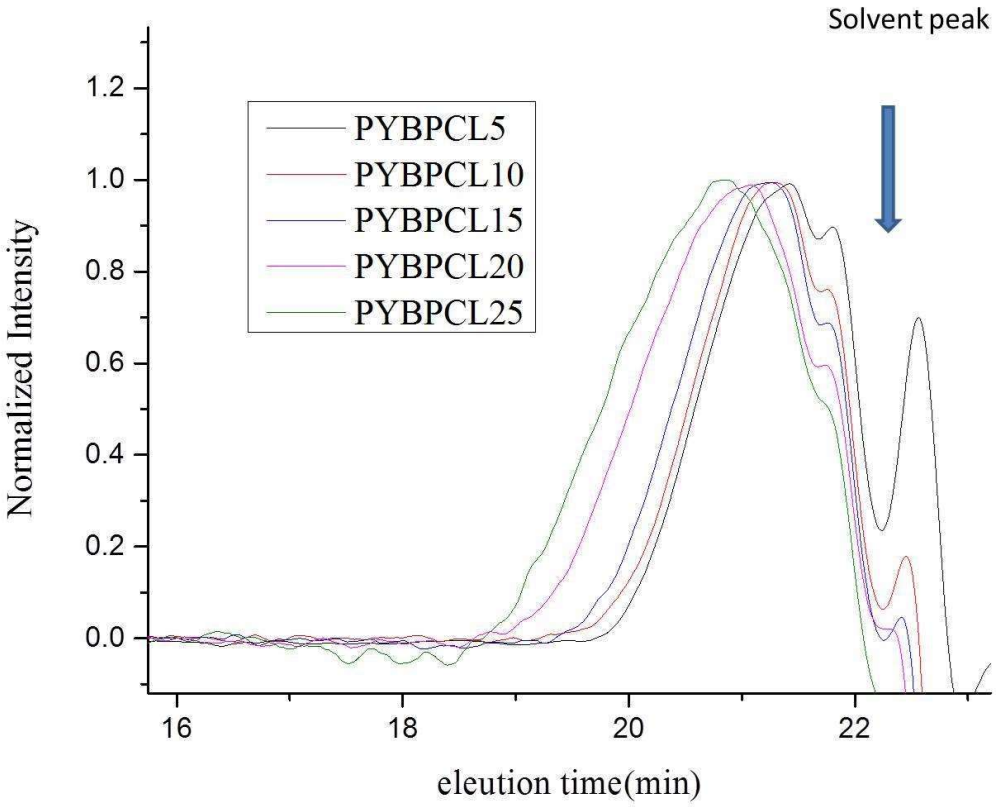


Fig (15)- GPC plots of polymers

. The PDI value of each of polymer was around 2. The GPC chromatogram shows the decrease in retention time due to high hydrodynamic volumes of bigger polymers. The Mn and Mw values of the polymers are tabulated in table (1)

Polymer	No. of Repeating unit(NMR)	M_n(NMR)	M_n(GPC) (g/mol)	M_w(GPC) (g/mol)	PDI
PY5BPCL	5	1500	500	1500	2.9
PY10BPCL	10	2700	1100	2000	1.8
PY15BPCL	16	4200	1200	2200	1.8
PY20BPCL	21	5500	1500	3600	2.4
PY25BPCL	25	6700	1900	4300	2.2

Table (1) – Mn and Mw value of polymer based on GPC and NMR

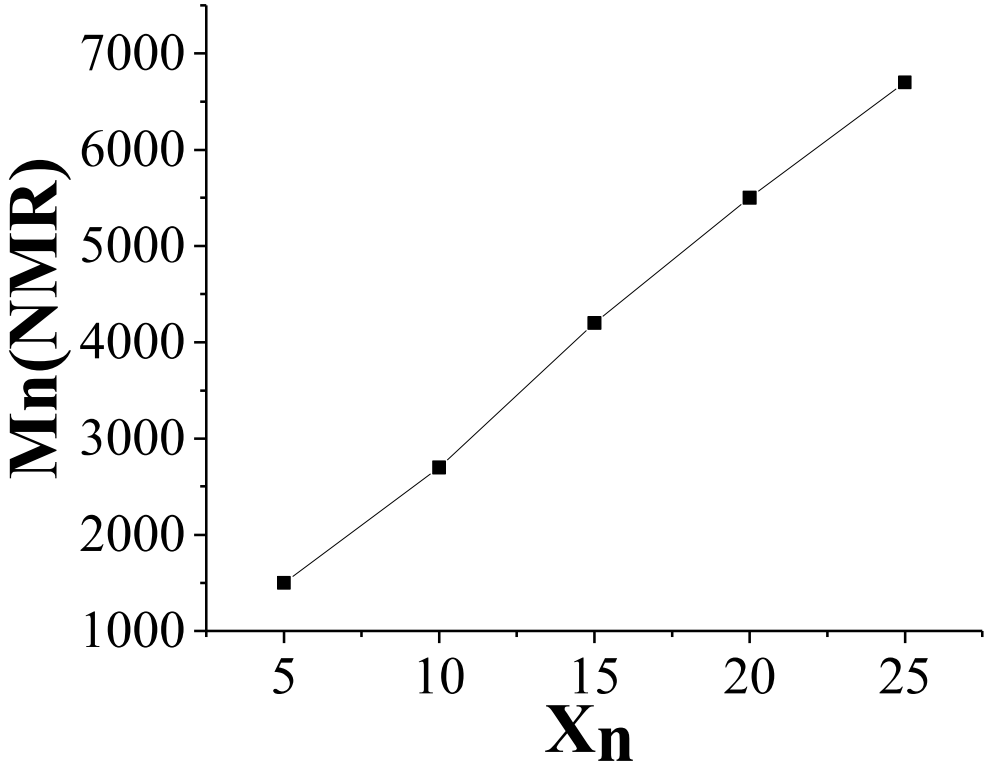


Fig (16)- Mn(NMR) Vs Xn

Ideally, The Number average molecular weight(Mn) estimated by NMR technique and GPC technique should be same and should follow a linear trend, but as from Table, It can be seen that Mn by GPC are not following a linear trend Fig (17). Moreover, the Mn as given by GPC is lesser compared to the Mn value given by NMR. This phenomenon is

called underestimation of molecular weight; this phenomenon is explained by the fact that the GPC works on the principle of calculation of hydrodynamic volume. The GPC is first calibrated by a given polymer of known weight, and depending on the hydrodyanamic volume of polymer which we feed It calculates the molecular weight. The GPC used for this studies uses polystyrene as a standard. The hydrodynamic volume of pyrene based polymer of some Mn (by NMR) may not be same as the hydrodynamic volume of polymers used of same (Mn) so the GPC shows underestimation and overestimation (in some cases) of molecular weights.

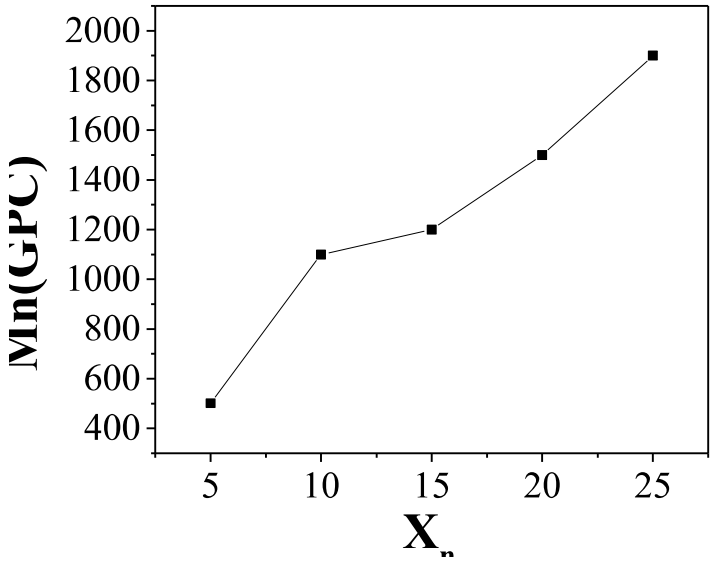


Fig (16) – Mn GPC Vs Xn of different polymers

Thermal stability of polymers: - The thermal stability of polymers were studied by using Thermogravatic analysis (TGA). All the polymers were stable upto 200^{o}C, the mass dissociation peak showed a hump at around 250^{o}C this hump is related to dissociation of tertiary butyl group from the polymer. Polymers were completely degraded by 325^{o}C, all polymers showed similar degradation trend as can be seen in figure.

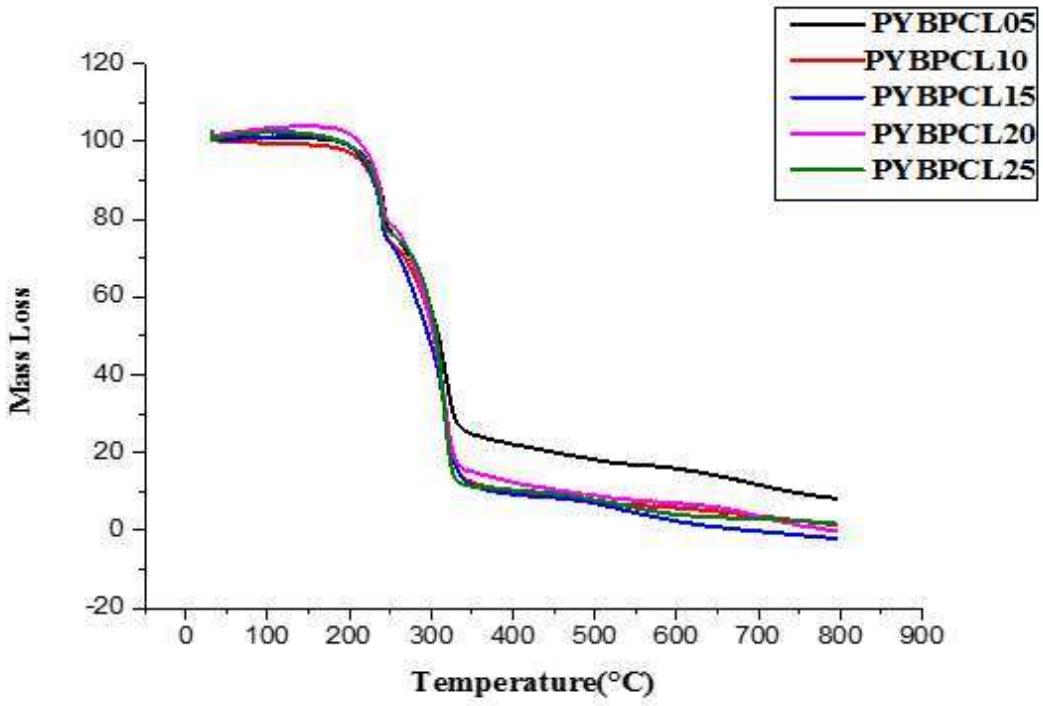


Fig (17) – Thermal stability of polymers

Self-assembly of the polymers: - The self-assembly of polymers were studied in water. For this 2mg of polymer were dissolved into 2ml of DMSO; this solution of DMSO and polymer was then added to 3ml of water in dropwise manner and was additionally stirred for 4 hours. This solution was finally kept for dialysis to get rid of DMSO present in the mixture. The solution was kept in a Semipermeable Membrane(SPM) of Molecular Weight Cutoff (MWCO)= 1000Da (for 5mer and 10mer) and 3500-5000 Da for others. These SPM were kept in beakers filled with distilled water and the water of dialysis was changed over a period of 48 hours at an interval of 2 hours. The size of Nanoparticles(NPs) formed were calculated using Dynamic Light Scattering(DLS). DLS showed an monomodal distribution for each of NPs as can be seen from Fig (19). The average size shown by DLS of 220nm for each of the NPs. The stability of Nanoparticles were checked over a period of 1 month in water as a solvent the DLS data showed a consistent value of ~220 nm for each of the polymers showing that the NPs formed were stable over a month in water.

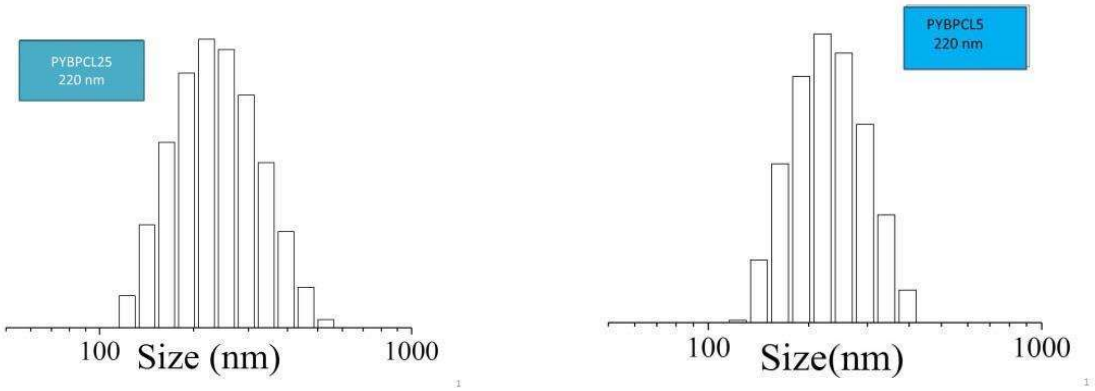


Fig (19)- DLS histograms of polymers

The shape and size of nanoparticles were studied using Field Emission Scanning Electron Microscope(FE-SEM). The samples were prepared in water, and diluted to different concentrations and optimized to get the best image. These samples were drop casted on silicon wafers. Prior to imaging they were dried in a desiccator over a period of 48 hours.

Particles of spherical morphology were seen by FESEM and size of them were around 220 nm for each of them which was consistent with the data obtained from DLS. The distribution of size by DLS technique can be explained by the different aggregates as can be seen by FESEM image. Aggregates of NPs give rise to a distribution in size.

Images of NPs formed by PYBPCL20 is shown in figure (20).

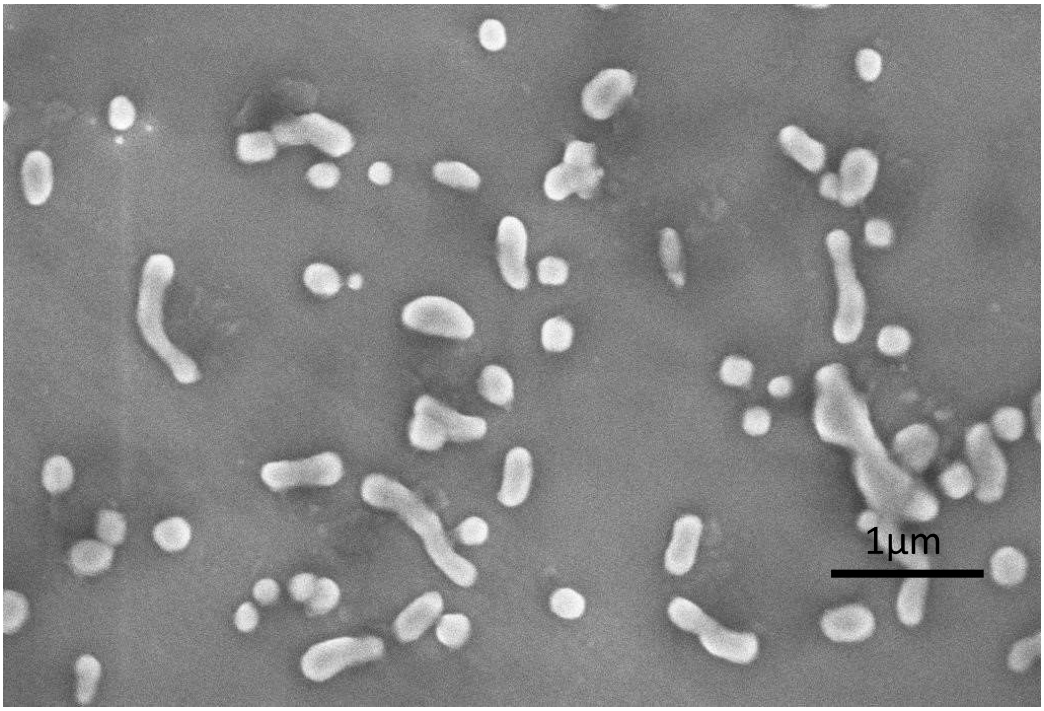


Fig (20)- FESEM images of Nanoparticles

Photophysical Studies of Polymers: Photo physical studies of dialyzed NPs were done in water as a solvent. Absorbance, emission, excitation spectrum of NPs were recorded. Various concentration of solution was prepared and absorbance were studied for seeing if new species were formed or not. The PYBPCL5 showed more absorbance for same concentration compared to its higher analogues this can be explained on the basis that the concentration of fluorophore is higher in case of smaller oligomer compared to the higher one.

Absorbance Spectra: - The typical absorbance spectra of pyrene based NPs in water as a medium looks like fig (21). In this the absorbance at 345 nm corresponds to electronic transition from S_0 to S_1. The three humps in the absorbance spectra comes due to different vibrational transitions, the difference in intensities can be explained by frank-condan principle.

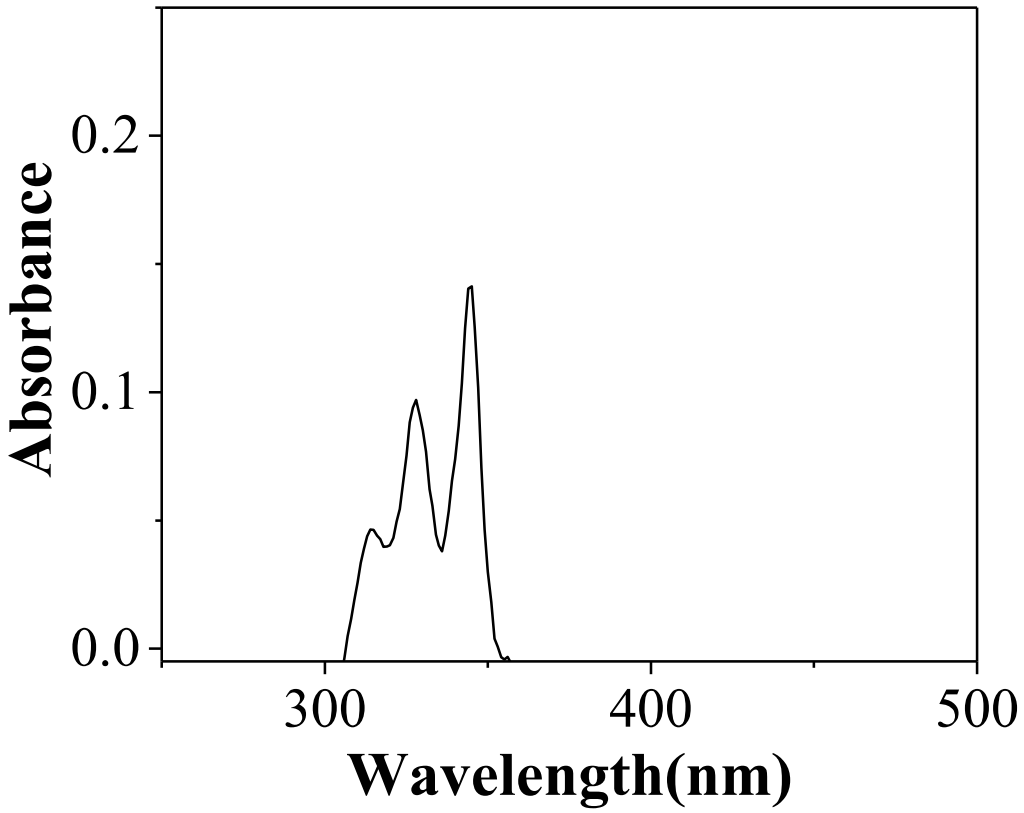


Fig (21)- Absorbance of NPs

Emission of NPs: - The emission of NPs were studied using flurospectrophotometer, the concentration of fluorophore in sample was made equal by setting the absorbance value 0.1 Optical Density(OD) with respect to pyrene. Each of these samples were then excited at the absorbance value i.e. at 345 nm and emission were recorded. The emission of higher oligomers showed a normal emission which was a mirror image of absorbance spectra at 370nm.The smaller oligomers showed a strong emission at,570nm along with the monomeric emission. This process happens when pyrene molecules get closer to each other, the orbital overlaps and thereby the energy of higher electronic state come down and this felicitates the emission at long wavelength. The newly formed species are called excimer.

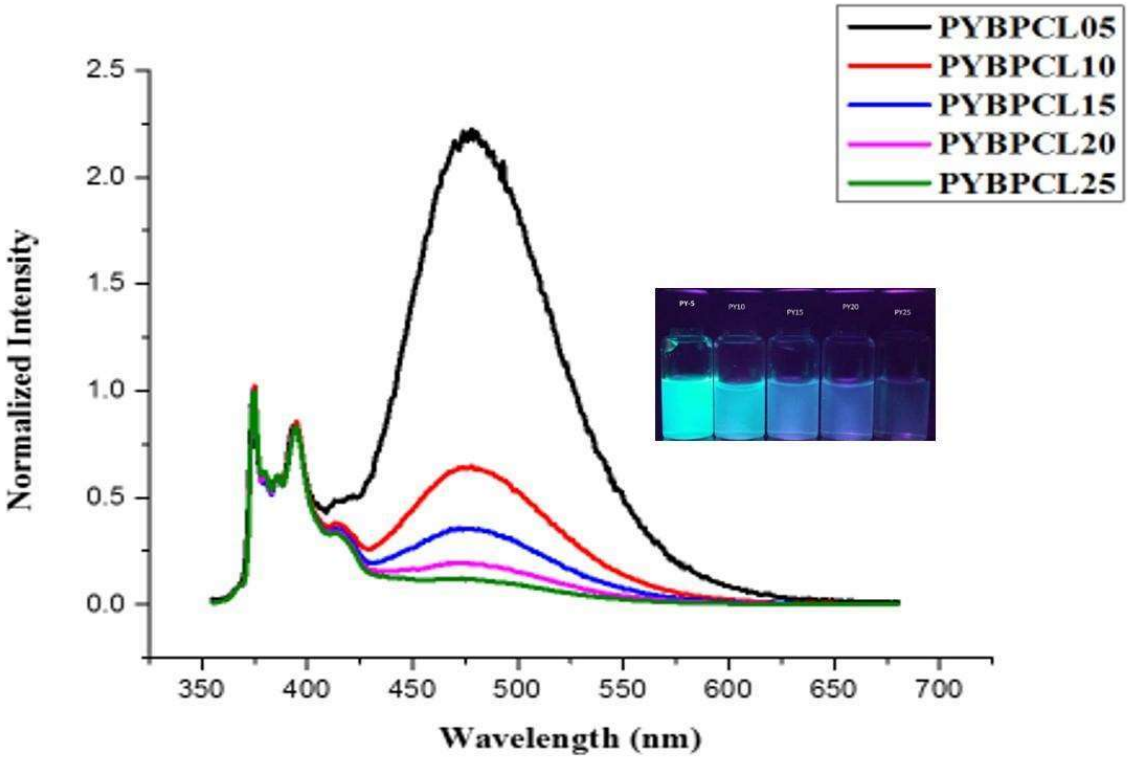
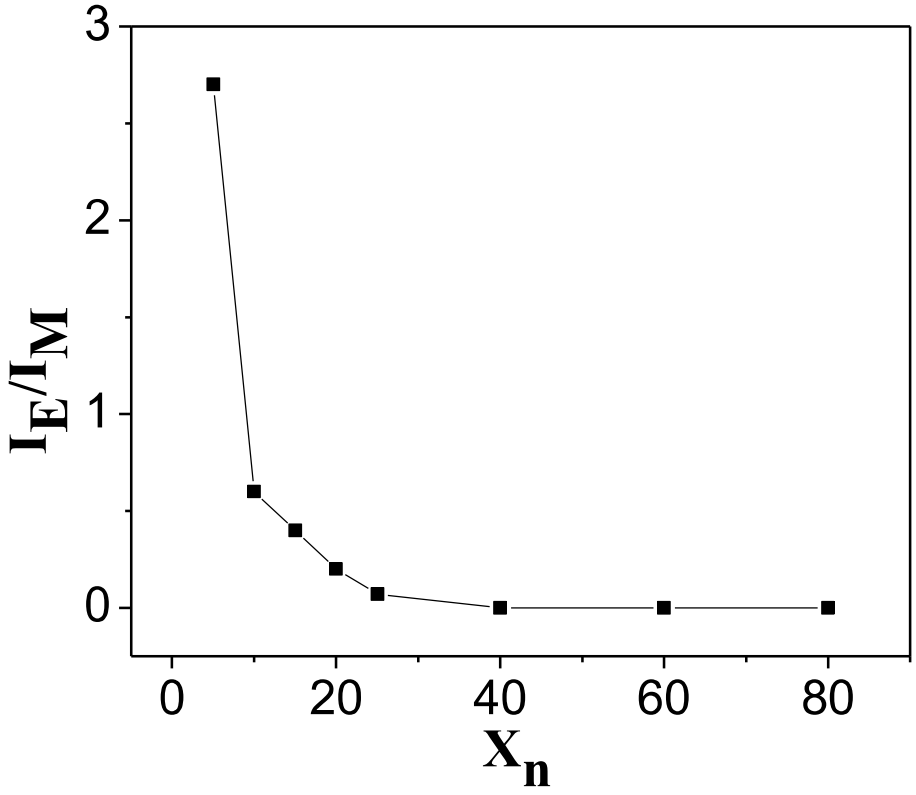


Fig (22) Emission of nanoparticles

As can be seen from the pictures of polymers under UV light that as the length of polymer is increasing the intensity of green emission is decreasing from PYBPCL05 to PYBPCL15 and completely vanishing in PYBPCL25 polymer. This is a direct proof of more excimer emission from PYBPCL05, and PYBPCL10 compared to the longer polymers.

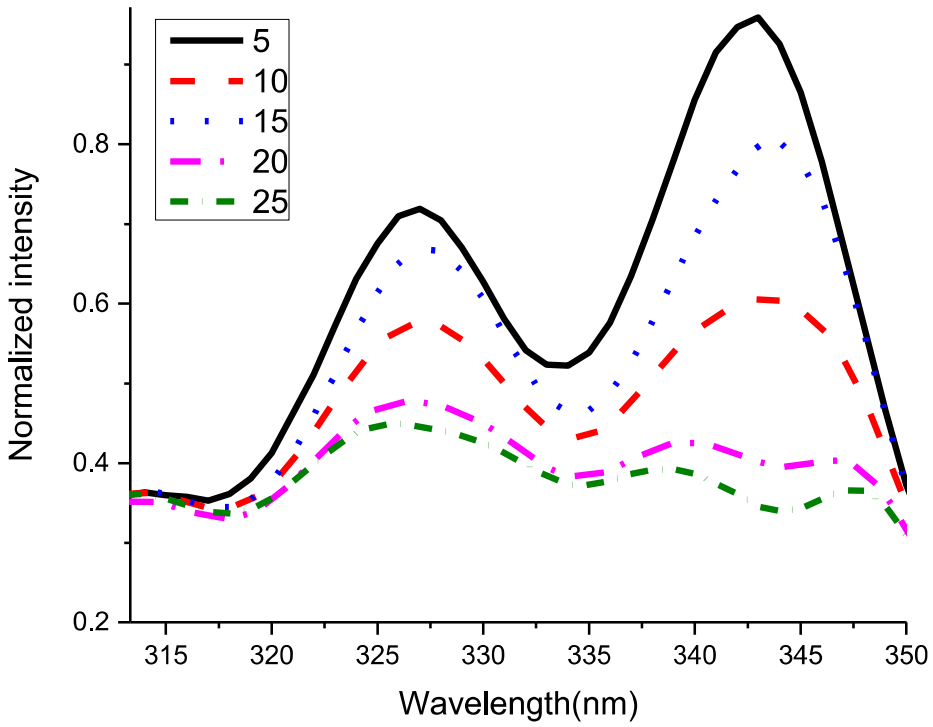
The Ie/Im Vs number of repeating units are plotted in fig (23) from this it is quite evident that the polymers with chain length equal to or lesser than 25 have better packing efficiencies for excimer formation.



Fig(23)- Ie/Im Vs number of repeating unit of polymer

Pyrene forms two types of excimers (i) Static excimer and (ii) Dynamic excimers. Static excimers are formed when a ground state pyrene interacts with other ground state pyrene molecule. Whereas the dynamic excimer is formed when a ground state monomer interacts with an excited state pyrene monomer. There are several ways in which it can be known if the excimer formed is dynamic or static

(i) Excitation Spectra: As stated earlier, Dynamic excimers are formed between excited state monomer and ground state pyrene during the photoexcitation process. The excitation spectra collected at the excimer emission showed the absorption at 345 nm with respect to pyrene monomer in ground state. This is the direct proof for the formation of pyrene dynamic excimer in this process.[20]

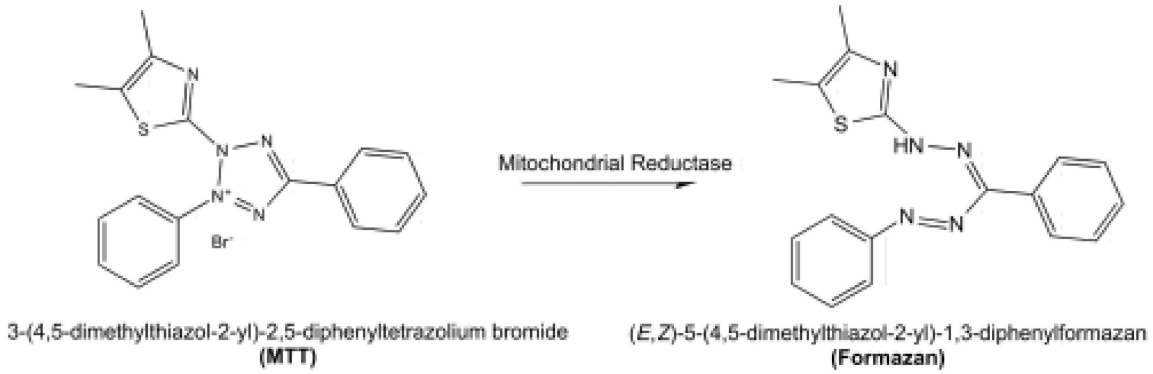


Fig(24)- Excitation of NPs at excimer emission

Cytotoxicity and Bio imaging: - Water dispersed biodegradable polymers are emerging as an important class of luminescent probes for drug delivery and cellular imaging to cancer cells. The pyrene based polymers produced very stable luminescent aqueous NPs, thus these NPs have been employed as a bio imaging probe in various biological experiments such as studying the polymerase chain reaction(PCR) of DNA[21]. Pyrene based polymers have been used to study the photo physics and dynamics of pyrene molecule[19], However to the best of knowledge pyrene based polymers have not been used for biological purposes.

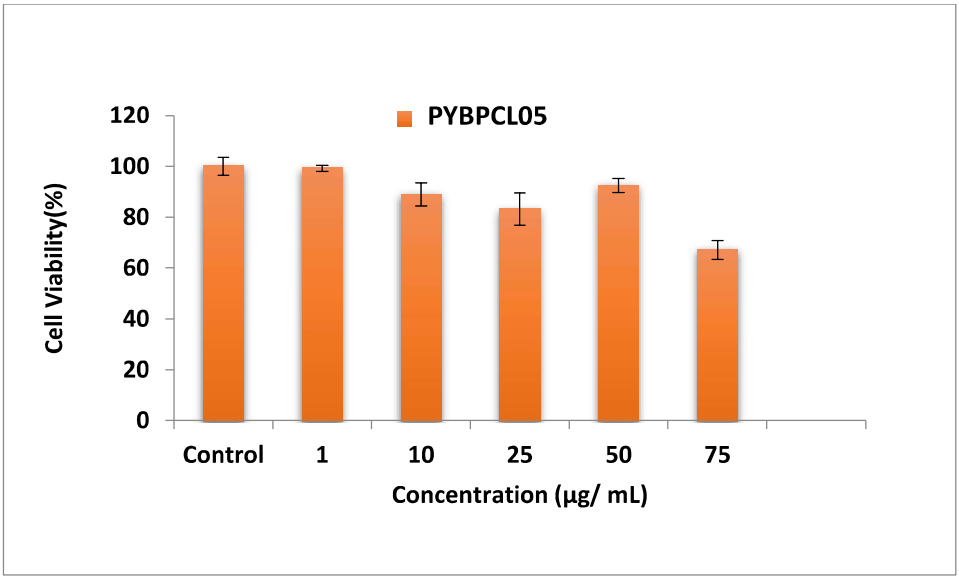
Cytotoxicity of these polymeric NPs were investigated in cervical(HeLa) cell lines. To calculate the cell viability MTT assay have been used, for this assay a dye called 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is used, the principle behind this assay is that the dye is reduced by an enzyme called mitochondrial succinate dehydrogenase, which is only found in living cells. This enzyme reduces the yellow

coloured dye to an insoluble (dark purple) formazan product, the intensity of colour of this insoluble compound gives the percent of cells alive during the experiment.



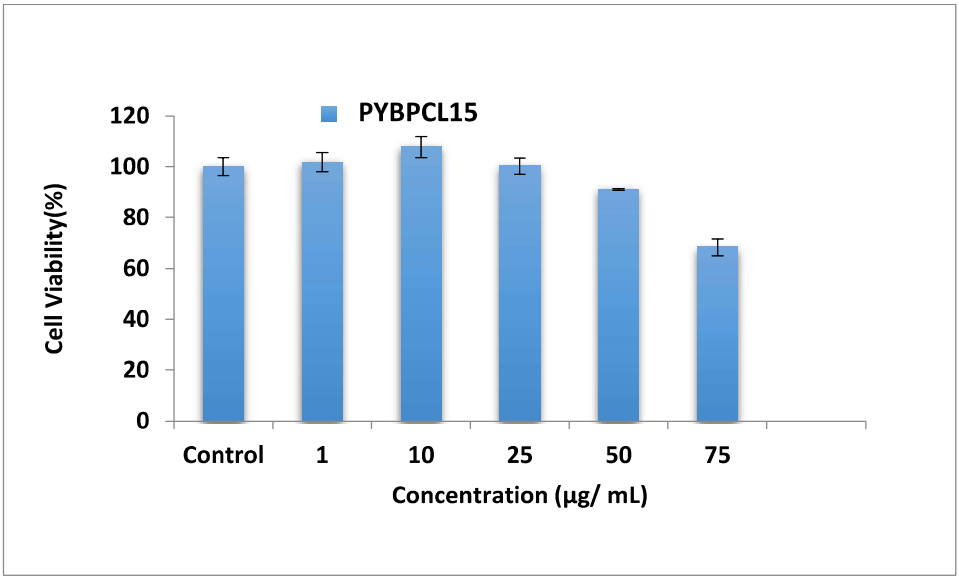
Fig(25)- Mechanism of MTT reduction

The cytotoxicity of these NPs were checked by varying the concentration upto 75 µg/mL of polymers PYBPCL5, PYBPCL15, and PYBPCL25 polymers.



Fig(26)- Cell viability data after treating with PYBPCL05

The PYBPCL-5 NPs upto 50 µg/mL concentration showed 90 percent of cell viability. It can be hence further be used as potential drug carrier. The confocal images of these polymers showed quite intense emission in blue region from cytoplasm of cells Fig(), showing that the polymers have gone inside the cells and can be used as a potential drug carrier as well for bio imaging.



Fig(27)- Cell viability data after treating with PYBPCL15

As can be seen, similar trend can be observed for PYBPCL-15 NPs. Upto a high concentration of 75 µg/mL and till 70% of cell viability can be observed. These polymeric NPs are can be seen as a potential drug carrier as well as bio-imaging probe to cancer cell.

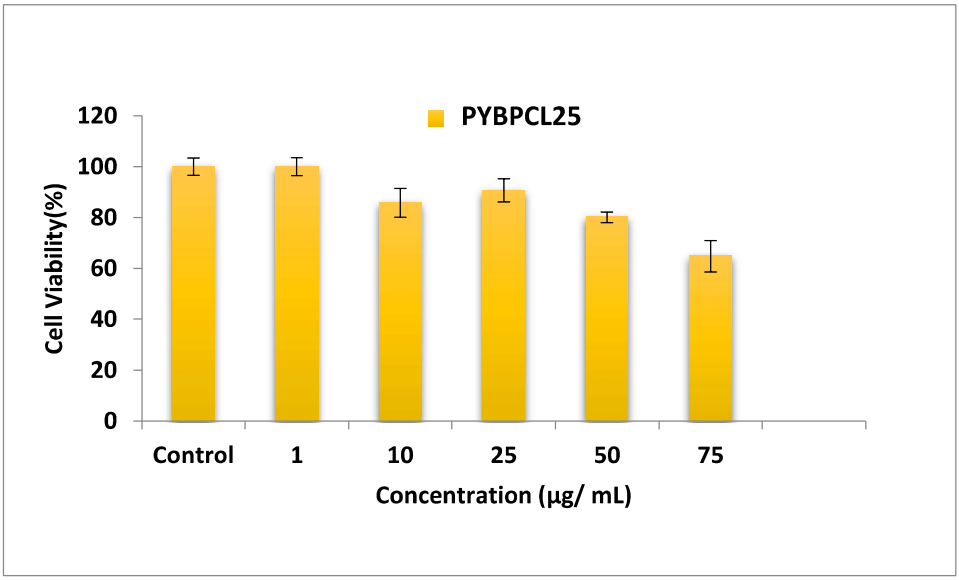
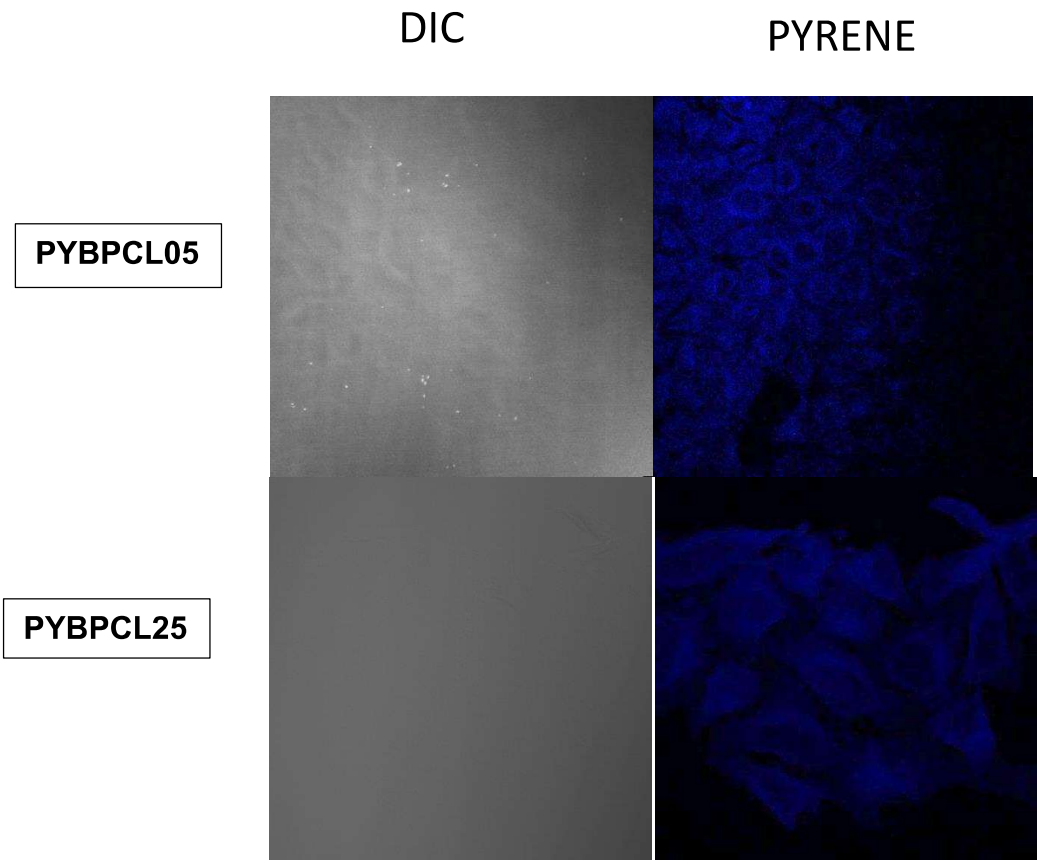


Fig (28)- Cell viability data after treating with PYBPCL25

PYBPCL-25 based NPs also showed 80 percent of cell viability upto 50 µg/mL of concentration. These NPs were also used for bio imaging of cancer cells and showed

nice cellular uptake by cells, these polymers were going to cytoplasm and no fluorescence in nucleus can be seen.



Fig(29) – CLSM images of HeLa cell lines after encapsulation of NPs

Conclusion: - In conclusion, we have synthesized novel pyrene tagged substituted Caprolactone based polymers by melt route. These polymers were thoroughly characterized by NMR, MALDI and GPC. Photo physical studies of polymers have been done. The emission spectra of polymers particularly with smaller chain length showed strong excimer formation, whereas the longer chain length polymers didn't show excimer peaks. The self-assembly of these polymers was done in water as solvent, polymers formed NPs of spherical morphology of an average size of 220 nm. The cytotoxicity of these polymers was checked in HeLa cell lines and polymers were nontoxic to cells upto a concentration of 50 µg/mL, Cellular uptake studies of polymers (PYBPCL5 and PYBPCL25) were carried out in HeLa Cell lines and confocal microscope images exhibited cellular uptake by the cells and they were particularly concentrated in cytoplasm. This new simultaneous imaging concept is not restricted to this particular system; in principle, it can be expanded to other drugs. The present investigation reports the development of new luminescent enzyme-responsive (biodegradable) polymer Nano scaffolds for polymer drug delivery applications and demonstrates the proof of-concept for cellular imaging in cervical cancer cells.

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