Synthesis and characterization of self-assembled polymeric nanoparticles for dual drug delivery in cancer.

A thesis submitted towards partial fulfilment of

BS-MS dual degree program

by

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under the guidance of

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Certificate

This is to certify that this dissertation entitled "Synthesis and characterization of self-assembled polymeric nanoparticles for dual drug delivery in cancer" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research,Pune represents original research carried out by Deepali Madhusudan Koturkar, Indian Institute of Science and Research under the supervision of Dr. Sudipta Basu, Assistant Professor, Department of Chemistry during the academic year 2013-2014.

Supervisor

Science

Date:

Place:

Head Bio/Chem/Phy/Math

Date:

Place:

Declaration

I hereby declare that the matter embodied in the report entitled "Synthesis and characterization of self-assembled polymeric nanoparticles for dual drug delivery in cancer" are the results of the investigations carried out by me at the Department of Chemistry, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Sudipta Basu and the same has not been submitted elsewhere for anyother degree.

Student:

Deepali M Koturkar

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Any accomplishment requires converted efforts of many people and this project work is no different. I am highly obliged and grateful to all those who have helped me and provided all encouragement that has helped me immensely in the development of my project work. It is my pleasure to present the project entitled **"Synthesis and characterisation of self-assembled polymeric nanoparticles for dual drug delivery in cancer"**.

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Abstract

Chemotherapy is the important primary treatment for cancer. However traditional chemotherapeutic drugs fail to selectively accumulate at the tumor site, resulting in undesired side effects and inadequate drug concentrations reaching the tumor. Harnessing nanotechnology based platforms could reduce these side effects and improve the efficacy of therapeutics through enhanced permeation and retention (EPR) effect. Moreover, using single chemotherapeutic drug eventually leads to drug resistance. Hence drug combinations are used in cancer chemotherapy, although free drugs lead to unwanted side effects. To overcome toxic side effects and drug resistance, in current study, we have developed self-assembled polymeric nanoparticles from poly*(isobutylene-alt-maleic anhydride)* (PMA) to deliver dual cytotoxic drugs (proflavine, and cisplatin), and PI3K signalling inhibitor (PI103) simultaneously in cancer. The size, shape and morphology of nanoparticles were characterized by FESEM, AFM, DLS and confocal microscopy. The nanoparticles released dual drugs in slow and sustained manner over a long period of time at pH 5.5 mimicking lysosomal compartment in cells. Present study put forward the potential of PMA as a promising and versatile nano-vector for dual drug delivery in cancer.

Chapter 1: Introduction

1.1 Nanotechnology as a tool for chemotherapy in cancer

Cancer is one of the leading causes of death in the world.[3]The current view of cancer is that the malignancy arises from a transformation of the genetic material of a normal cell, followed by successive mutations, ultimately leading to the uncontrolled proliferation of progeny cells. All types of human cancer have six hallmarks like self-sufficiency in growth signals, insensitivity to antigrowth signal, sustained angiogenesis, evasion of apoptosis , limitless replicative potential, tissue invasion and metastasis which collectively dictate malignant growth.[2] Most malignant diseases, collectively referred to as cancer, are treated with some combinations of surgery, radiation therapy and drug treatment. Surgery and radiation are used to treat cancer that is confined locally and drug therapy is essential to kill cancer cells that have spread (metastasized) to distant sites in the body. Until now, drug treatment mainly involved cytotoxic chemotherapy that kills all rapidly dividing cells, both tumor and normal. Traditional chemotherapeutic drugs are difficult to selectively accumulate at tumor site, resulting undesired side effects and inadequate drug concentrations reaching the tumor. Nanotechnology-based delivery systems, which carrychemotherapeutic drug by chemical bonding or physicalentrapment, can improve pharmacokinetics and biodistribution profile of drugs. Such nanovectors accumulate preferentially in the tumors due to the unique leaky tumor vasculature coupled with impaired intratumoral lymphatic drainage, which leads to an enhanced permeation and retention (EPR) effect[1](Fig. 1) and thereby increase anticancer efficacy of drugs. To acquire satisfactory effect, an ideal nanotechnology-based drug delivery system following systemicadministration should

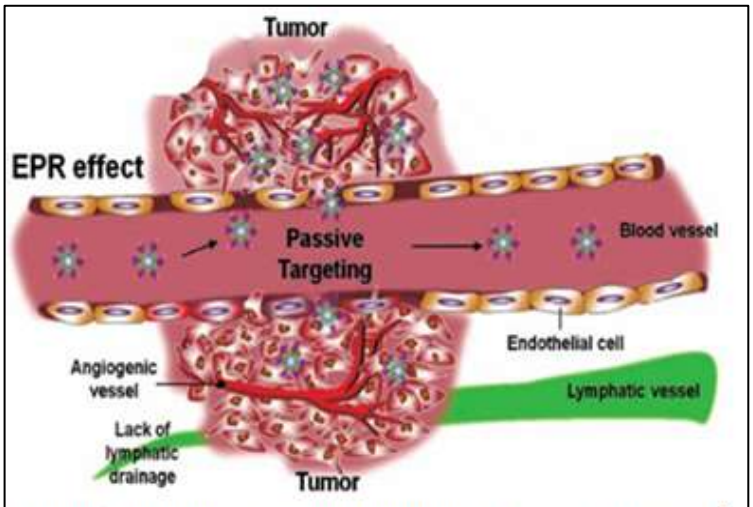

Figure 1. Enhanced permeation and retention (EPR) effect.[8]

possess a number of attributes, mainly including (i) prolonged circulation, (ii) enhanced accumulation in the tumor, (iii) facilitated cellular internalization, (iv) rapidly or sufficiently intracellular drug release, (v) biocompatible and biodegradable nanovectors.

1.2 Need of combination therapy

The design of cancer chemotherapy has become increasingly sophisticated, however there is no cancer treatment that is 100% effective against disseminated cancer because of emergence of drug resistance. Resistance to treatment with anticancer drugs results from a variety of factors including individual variations in patients and somatic cell genetic differences in tumors, including those from the same tissue of origin. Most of the times, resistance is intrinsic to the cancer, however as therapy becomes more and more effective, acquired resistance has also become very frequent. The most important reason for acquisition of resistance to a broad range of anticancer drugs is expression of one or more energy-dependent transporters that detect and efflux anticancer drugs from cells, also other mechanisms of resistance including insensitivity to drug-induced apoptosis and induction of drug-detoxifying mechanisms probably play an important role in acquired anticancer drug resistance. Explicit studies on mechanisms of cancer drug resistance have yielded important information about how to circumvent this resistance to improve cancer chemotherapy and have implications to improve pharmacokinetics of many commonly used drugs. It is been observed that chemotherapy drugs are most effective when given in combination as combination chemotherapy. The rationale of combination chemotherapy is to use drugs that inhibit different targets, thereby decreasing the likelihood that resistant cancer cells will develop. When drugs with different cellular targets are combined, each drug could be used at its optimal dose, without inducing intolerable side effects.

Individual targeted agents that simultaneously affect multiple oncogenic signal transduction pathways could be used either as single agents or in combination with other signalling inhibitors to yield even more powerful antitumor effects and block the induction of resistance mechanisms. Most cancer chemotherapy discovery and development is focused on targeted agents that induce apoptosis or cell cycle arrest

in malignant cells. Powered by genomic technologies, there is been astonishing progress in our understanding of the genetics and biology of human cancers and in the discovery of molecularly targeted therapies for personalized medicine. At the same time, the major challenges of extensive tumor heterogeneity, adaptive feedback loops and clonal selection are now been understood, providing a new biochemical and genetic explanation for the enduring and shape-shifting nature of acquired drug resistance. So far it is clear that, drug combinations currently provide a route, and possibly the only route to overcome the complexity of human cancers.[2] Combining drugs based on increasingly well-understood molecular interactions and attacking complementary cancer hallmarks or distinct cell populations in heterogeneous tumors is now imperative.

In this way, using nanotechnology based combination chemotherapy. We can achieve truly personalized and precision medicine for individual cancer patients suffering from different types of cancer. We face major challenges, but we have powerful tools to tackle it.[2]

1.3 Mechanism of action of drugs (used in the project)

1.3.1 *Proflavine* (Fig. 2)

DNA intercalation[12,13](insertion between base pairs) is themechanism by which a certain class of anticancer drugs function. Intercalation subsequently leads to inhibition oftopoisomerase action followed by cell death.[14] It is been observed that proflavine serves as cytotoxic agent by direct intercalation from minor groove-bound state.proflavine inhibit total RNA synthesis, while it does not induce discrete elongation stops of RNA polymerase.[4] Moreover, due to its fluorescence properties, proflavine can serve as a tool to visualise its localization and mechanism of action. However, the full potential of proflavine is yet to be determined in cancer chemotherapy.

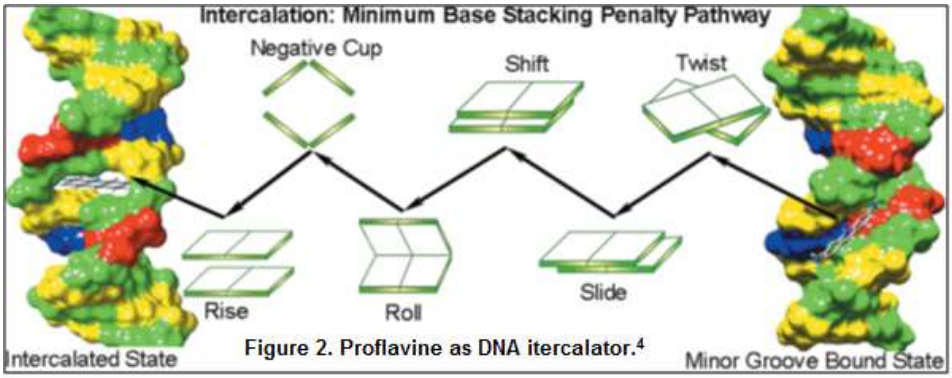

Figure 2. Proflavine as DNA itercalator.[4]

1.3.2 *Cisplatin* (Fig. 3)

Cisplatin is a first line chemotherapy for most types of cancer. Cisplatin functions as bifunctional and non-cell cycle specific alkylating agent. Cisplatin has activity in lymphoma and in many different solid tumors. It has revolutionized the chemo treatment for testicular carcinoma and made previously deadly disease potentially curable. As opposite to classic alkylating agents, cisplatin binds with DNA at guanine residues to form intrastrand crosslinks and adducts that cause changes in the conformation of the DNA and affect DNA replication. Other mechanisms of cisplatin cytotoxicity include decreased ATPase activity, mitochondrial damage, altered cellular transport mechanisms. As cisplatin is considered to be cell cycle non-specific, cytotoxicity is increased with exposure during the S-phase. Cisplatin arrests cell cycle in the G2-phase and then induces programmed cell death or apoptosis. However, free cisplatin use is dose-limited due to severe nephrotoxicity. As a result, delivering cisplatin specifically to the tumor would reduce nephrotoxicity.[7]

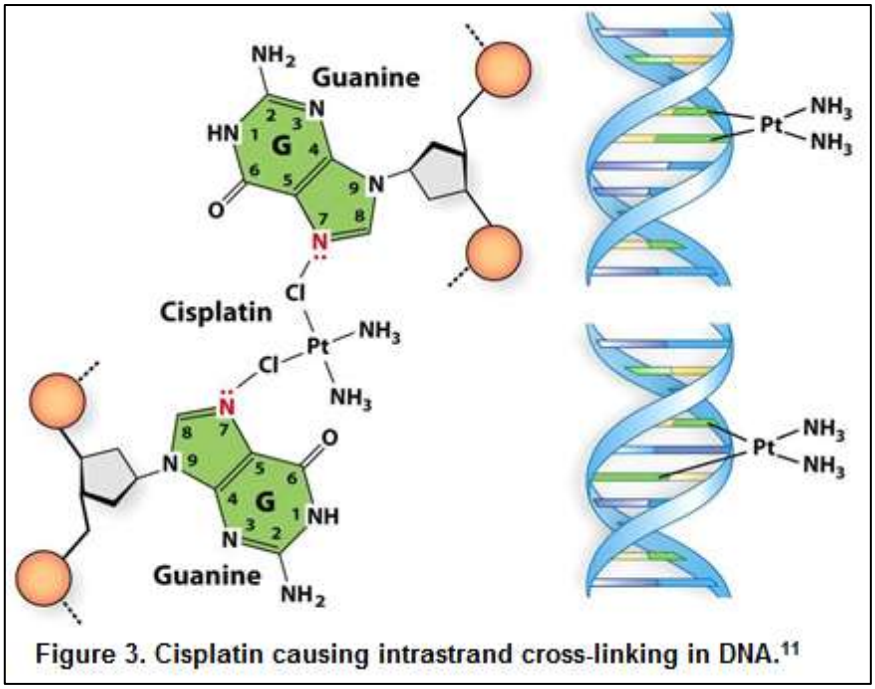

Figure 3. Cisplatin causing intrastrand cross-linking in DNA.[11]

1.3.3 *PI103* (Fig. 4)

PI3-kinase (PI3K)-Akt signalling pathway plays critical roles in cell growth and survival and is commonly deregulated in most types of cancer. PI3K-AKT signalling is implicated in ovarian, breast, colon and pancreatic cancer. Estimates indicate that mutations in one or more PI3K pathway components account for up to 30% of all human cancers. As a result PI3K-AKT signaling pathway has emerged as important target for cancer tharapeutics.[5]As a multi-target inhibitor, PI-103 inhibits p110 and both mTORC2 and the mTORC1 in PI3K/AKT/mTOR signalling pathway which leads to inhibition of cell proliferation, tumor growth and induces apoptosis.[9]However poor water solubility is the major challenge in administration and limits the use of free PI103 in cancer.

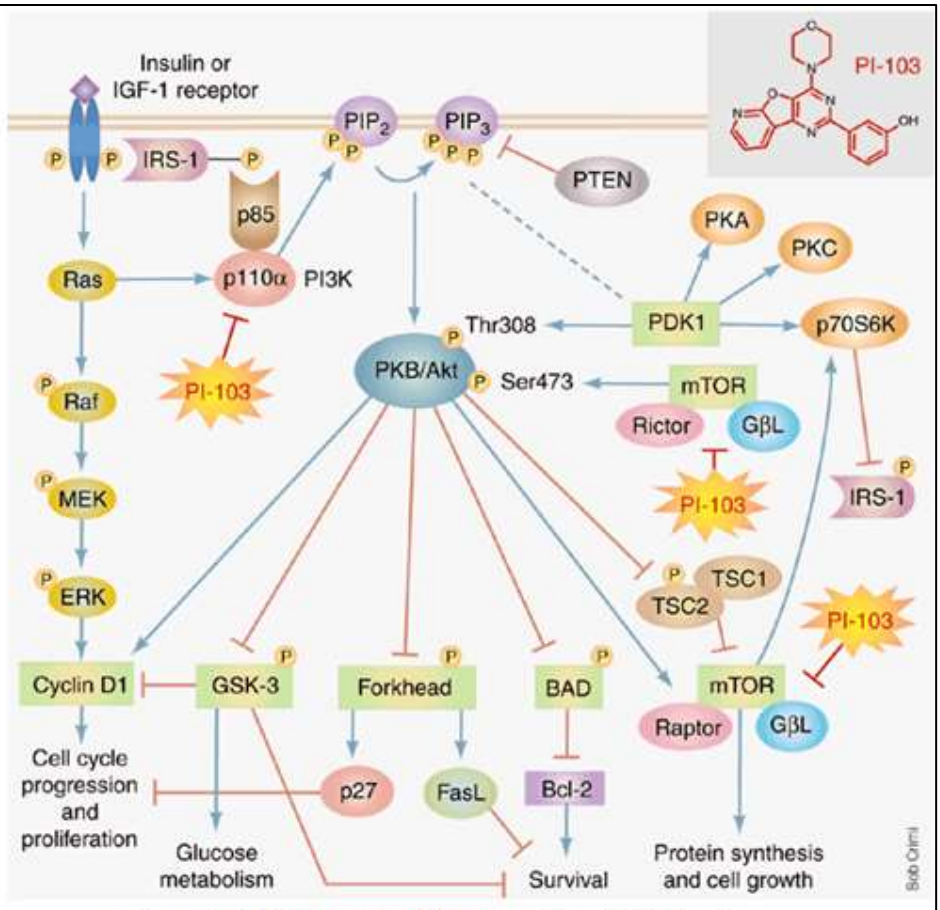

Figure 4. PI103 causing PI3K signalling inhibition.[9]

1.4 Hypothesis

Considering the major challenges of unwanted toxic effects of free drug treatments and emergence of drug resistance over a period in chemotherapy of cancer, we thought of developing a nanotechnology based platform which can be used todeliver dual drugs in cancer to have optimum efficacy. This idea would help to overcome both the major challenges in this field. As it is been observed, if drugs inhibiting different targets in a cell are given in combination, they show synergistic effects.Herein we have attempted to build a polymeric nanoplatform which could be used to deliver dual drugs. These polymeric dual drug nanoparticles are expected to improve pharmacokinetic and biodistribution profiles of the loaded drugs and deliverdrugs after internalization in cell into lysosomal compartment due to lower pH 5.5.

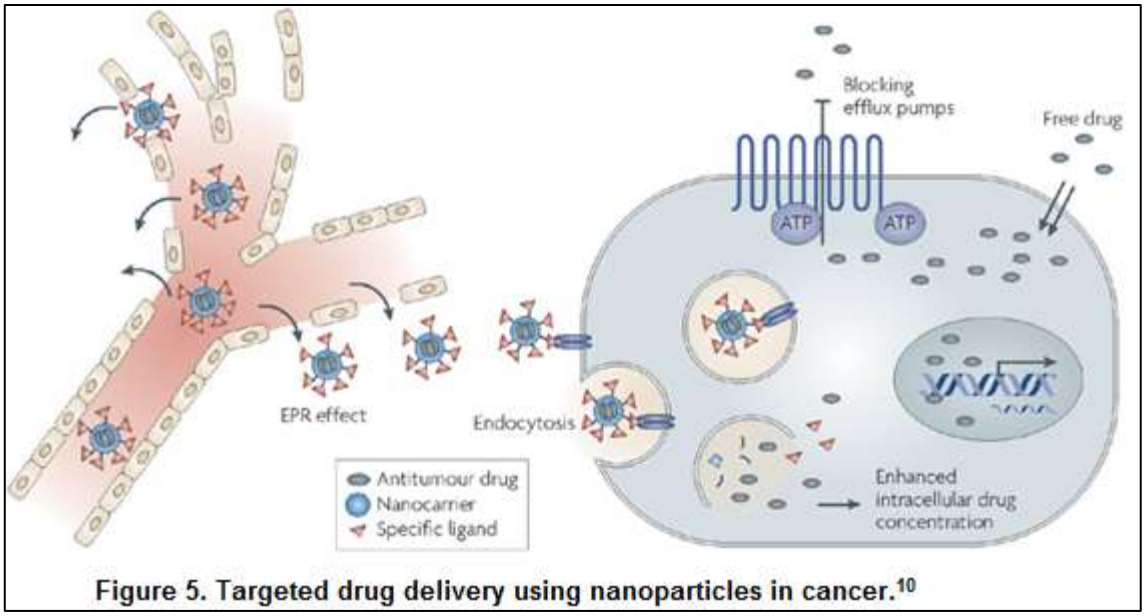

Figure 5. Targeted drug delivery using nanoparticles in cancer.[10]

Chapter 2: Methods

2.1 Materials and Instruments

All reactions were performed under inert conditions unless otherwise indicated. All commercially obtained compounds were used without further purification.Chemicals were of the best grade available and were supplied by Sigma-Aldrich chemicals and Selleck chemicals. Poly(isobutylene-*alt*-maleic anhydride) (PMA) , dry dimethylformamide(DMF) , 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) , 4-Dimethylaminopyridine (DMAP) , Proflavine, dialysis bags, release membranes and silicon wafers for FE-SEM are obtained from Sigma-Aldrich. PI-103 was bought from Selleck Chemicals.^{1}H-NMR spectra were recorded using a 400 MHz Jeol NMR spectrometer in DMSO D_6ahd D_2O containing a small amount of TMS as an internal standard. The absorption studies were done by a Perkin-Elmer Lambda 45 UV–visible spectrophotometer. The size determination of the NPs is carried out by dynamic light scattering (DLS), using a Nano ZS-90 apparatus utilizing a 633 nm red laser from Malvern instruments. Atomic force microscope images were recorded for drop cast samples using JPK instruments attached with Nanowizard-II setup. AFM is also attached with a Zeiss

inverted optical microscope. Field Emission Scanning Electron Microscope (FE-SEM) images were recorded using utra plus ziess microscope.

2.2 Procedures and Protocols

2.2.1 Synthesis of PMA-proflavine-cisplatinnanoparticle

Synthesis of PMA-proflavine-cisplatin nanoparticle was carried out in following steps

2.2.1.1 Synthesis of PMA-proflavine conjugates.

Optimization of proflavine equivalents

Different equivalents (10, 20, 40) of proflavine were conjugated to PMA and different proflavine-PMA conjugates(compound **1,2,3**respectively) were obtained. To optimise the final equivalent which is to be used further reactions, loading values were compared for the above compounds (Table.1) and as a result, compound 2 (20 equivalents) was taken forward to proceed with further use because of maximum loading and water solubility.

Compound	Amount of drug per mg
1	256 µg
2	806 µg
3	476 µg

Table 1. Loading of Proflavin

Synthesis of compound 2 (Fig. 6)

10 mg of *Poly(isobutylene-alt-maleic anhydride)* (PMA) **(a)** (MW=6000 Da, 0.0017mmol, 1 equiv) was dissolved in 1 ml of dry DMF under nitrogen atmosphere in round bottom flask. Proflavine**(b)** (8.19 mg, 0.033mmol, 20 equiv) was the added to the flask. The reaction mixture was stirred at room temperature for 48 hr in dark conditions as proflavine is a light sensitive compound. After 48 h, solvent was removed under high vacuum at 50^{o} C. 4 mL of water was then added to the dry product and mixture was dialysed (MWCO=3.5 kDa) against MQ water for48 h. After dialysis, solution was kept for lyophilization for 24 h to remove water and 11.5 mg of dry compound**2** was obtained.

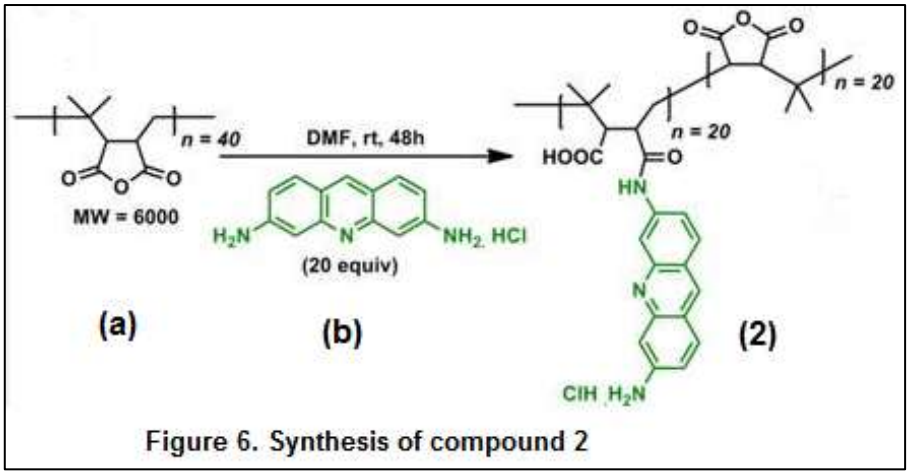

Figure 6. Synthesis of compound 2

^{1}H NMR (400 MHz,D_2O) δ = 8.65 (s, 9H), 7.78 (d, *J* = 9.1 Hz, 18H), 6.99 (dd, *J* = 9.0, 2.0 Hz, 18H), 6.71 (d, *J* = 1.9 Hz, 17H), 3.68 – 3.61 (m, 195H), 3.58 (dd, *J* = 11.2, 4.8 Hz, 383H), 3.50 (dd, *J* = 11.1, 6.0 Hz, 386H), 3.32 – 3.28 (m, 193H), 2.69 (s, 3H), 1.18 (d, *J* = 75.4 Hz, 54H).

2.2.1.2 Synthesis of aquated cisplatin (compound 4) (Fig. 7)

50 mg of cisplatin**(c)** was dissolved in 10 ml of water and 28 mg of silver nitrate was added to the solution. Reaction mixture was then stirred at room temperature under dark conditions for 24 h. Reaction mixture turned milky white, and was centrifuged for 15 to 20 minutes to precipitate silver chloride. The supernatant is then filtered through 0.2 μm filter and compound **4** was obtained for further reaction.

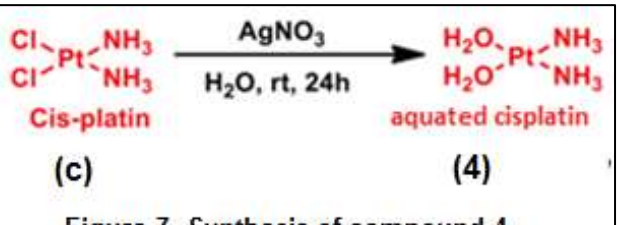

Figure 7. Synthesis of compound 4

2.2.1.3 Synthesis of compound 5 (Fig. 8)

5 mg of dry compound 2 (estimated MW=10180, 0.00049 mmol, 1 equiv) was dissolved in 519.4 μL of 5 mg per ml aquated cisplatin (compound 4) solution (MW=265 Da, 0.0098mmol, 2.6mg, 20equiv). Reaction mixture was stirred for 24 h in dark. Reaction mixture was then and dialysed (MWCO=3.5 kDa) for 8 h to remove excess unconjugated drug molecules. Water was removed by overnight lyophilization and 4.76 mg of dry compound **5** was obtained.

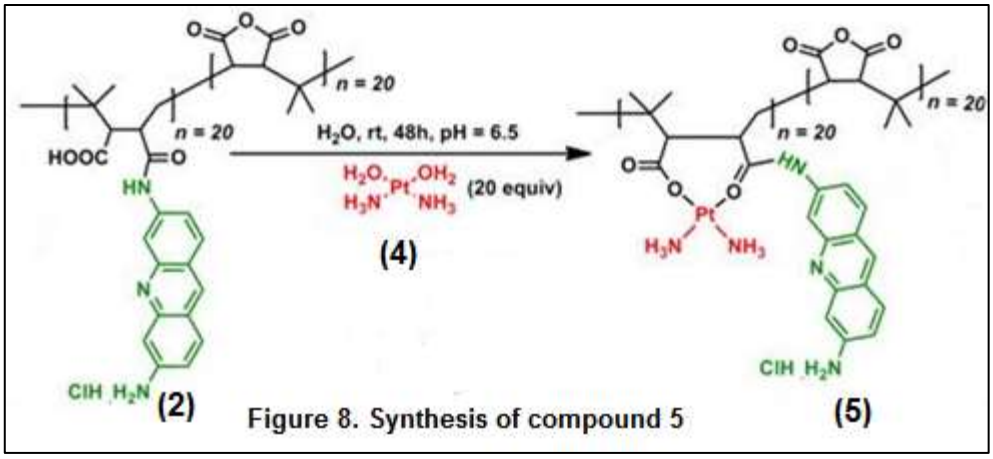

Figure 8. Synthesis of compound 5

2.2.2 Synthesis of PIMA-PI103-cisplatin nanoparticle

Synthesis of PIMA-PI103-cisplatin nanoparticle was carried out in following steps.

2.2.2.1 Synthesis of poly(isobutylene-*alt*-maleic acid) from poly(isobutylene-*alt*-maleic anhydride) (PIMA) (Compound 6) (Fig. 9)

20 mg of PMA **(1)** was dissolved in mixture of 3 mL DMF and 3 mL of water. Reaction mixture was then heated at 60^{o}C with constant stirring for 48 h. Solvent was removed under high vacuum at 50^{o}C. Dry compound was then again suspended in 4 mL of water and dialysed (MWCO=3.5 kDa) against water for 24 h. Dry PIMA was then obtained after removing water by lipholization.

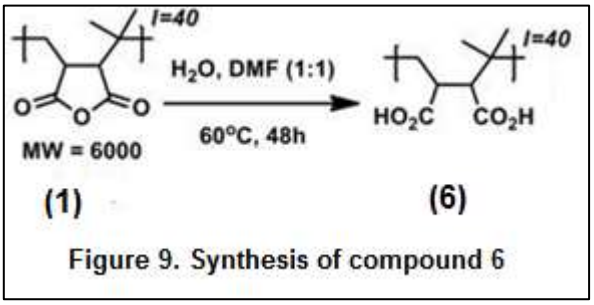

Figure 9. Synthesis of compound 6

2.2.2.2 Synthesis of PIMA-PI103 conjugates (compound 7) (Fig. 10)

5 mg of PIMA (compound **6**) (MW=6700 Da, 0.000746 mmol, 1 equiv) was dissolved in 1 mL of dry DMF in round bottom flask. 2.6 mg of PI103**(d)** (MW=348.4 Da, 0.00746 mmol, 10equiv)to the flask followed by 2.15 mg of EDC (0.0112 mmol, 15 equiv) and 0.5 mg of DMAP (MW=122 Da, 0.0037 mmol, 5 equiv). Reaction was carried out under inert condions for 48 h under inert conditions. Then solvent was removed using and crude compound was resuspended in 0.5 ml of DMF into a

falcon tube. 10mL of diethyl ether was then added to precipitate the PIMA-PI103 conjugates. Mixture was centrifuged for 15 minutes and ether layer was removed. Same procedure is repeated 3 times to obtain pure polymer-drug conjugate by precipitation the product was insoluble in diethyl ether. Precipitated conjugate was suspended in 4 mL of water and dialysed (MWCO=3.5 kDa) for 24 h against water. Water is then removed by over lyophilisation and 3.85 mg of dry compound **(7)** is obtained.

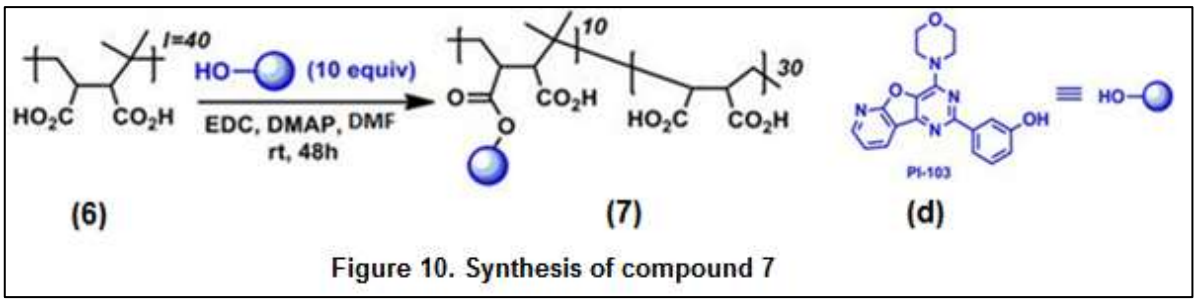

Figure 10. Synthesis of compound 7

2.2.2.3 Synthesis of PIMA-PI103-cisplatin nanoparticles (compound 8)(Fig. 11)

7.6 mg of compound **7** (estimated MW=10,004 Da, 0.00076, 1 equiv) was dissolved in 806 μL (4.03mg, 0.0152 mmol, 20 equiv) of 5mg per mL solution of aquated cisplatin (compound **4**). Reaction mixture was then stirred for 24 h at room temperature under dark conditions. After 24 h, reaction mixture was diluted to 3 mL and dialysed (MWCO = 3.5 kDa) against water for 8 h. 8.2 mg of dry compound **8**was then obtained by removing excess water by lyophilization.

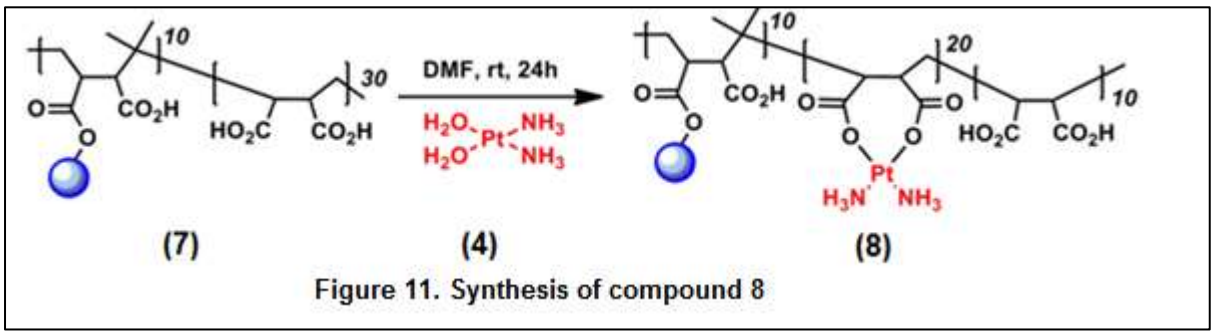

Figure 11. Synthesis of compound 8

^{1}H NMR (400 MHz, D_2O) δ = 7.98 (d, *J* = 7.7 Hz, 2H), 7.91 (s, 9H), 7.39 – 7.23 (m, 2H), 6.86 (d, *J* = 10.3 Hz, 2H), 5.43 (s, 8H), 3.12 (dd, *J* = 30.0, 14.5 Hz, 42H), 2.99 (s, 29H), 2.85 (d, *J* = 10.9 Hz, 62H), 1.95 – 1.80 (m, 18H), 1.05 (t, *J* = 7.3 Hz, 59H).

2.2.3 Detemination ofstandard calibration curves of drugs

2.2.3.1 Standard calibration curve of proflavine by UV-Vis spectrophotometry (Fig. 12)

1 mM aqueous stock of proflavine was prepared. Further dilutions ranging from 15 µM to 40 µM were obtained from the stock. The absorbance was measured at 445 nm (which is characteristic λmax for proflavine) against water as blank. Linear relationship was obtained by plotting the absorbance values (A) against corresponding concentrations (C) with R^2 value 0.9635 and the slope equation y = 0.0153x.

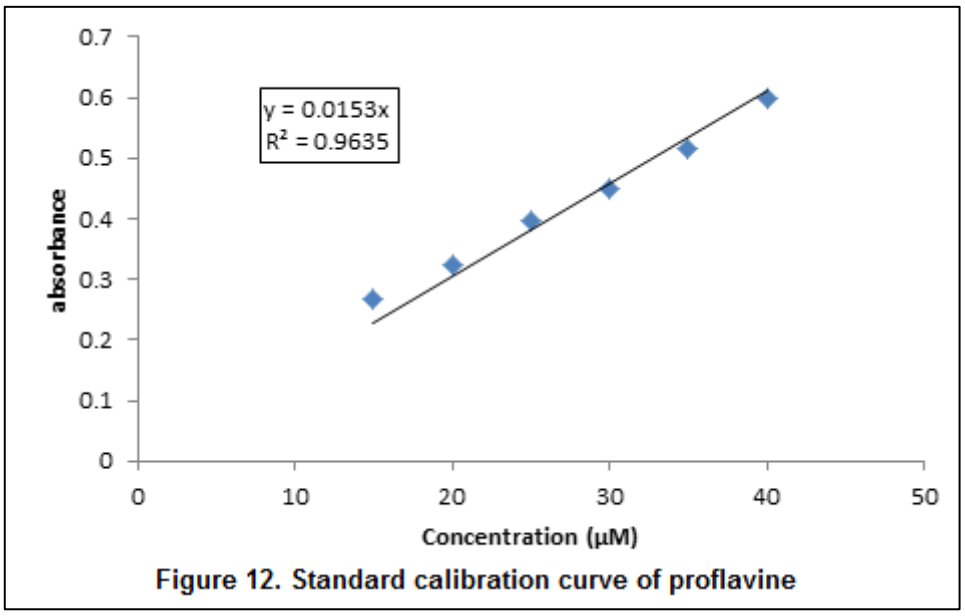

Figure 12. Standard calibration curve of proflavine

2.2.3.2 Standard calibration curve of cisplatin by UV-Vis spectrophotometry (Fig. 13)

1 mM stock was prepared by dissolving cisplatin into 1.2 mg per mL solution of o-phenylenediamine in DMF and heated at 100^{o}C for 4 h, till the solution turned deep green in colour. Range of further dilutions from 2.5 µM to 15 µM was obtained by diluting stock solution with DMF.The absorbance was measured at 706 nm (which is characteristic λmax for cisplatin) against DMF as blank. Linear relationship was obtained by plotting the absorbance values (A) against corresponding concentrations (C) with R^2 value 0.9537and the slope equation y = 0.0355x.

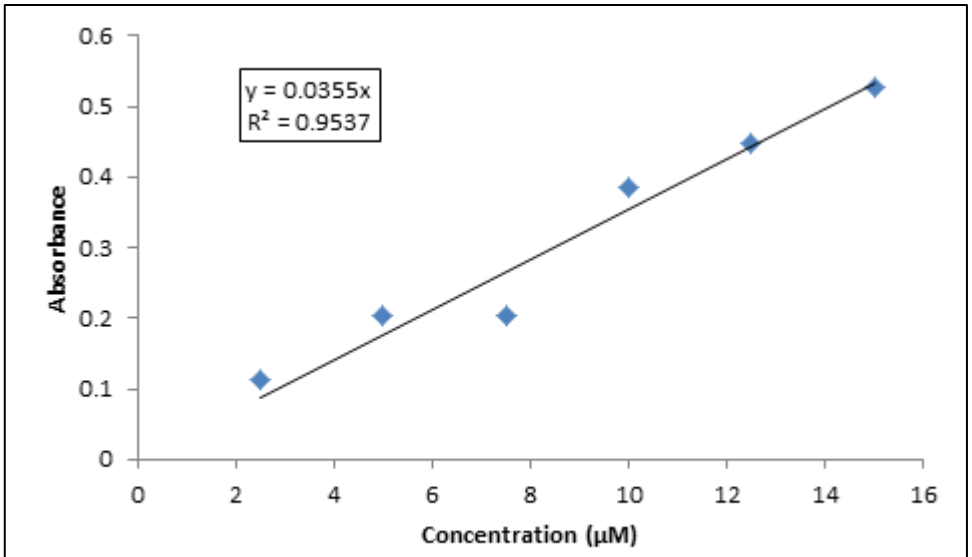

Figure 13. Standard calibration curve of cisplatin

2.2.3.3 Standard calibration curve of PI103 by UV-Vis spectrophotometry (Fig. 14)

A calibration curve was plotted in the concentration range of 2.5 to 25 µM by diluting the 1 mM standard stock solution with dimethyl sulfoxide (DMSO). The absorbance was measured at 293 nm (which is characteristic λmax value for PI103) against the corresponding solvent blank. The linearity was plotted for absorbance (A) against concentration (C) with R^2 value 0.9945 and with the slope equation y = 0.036x.

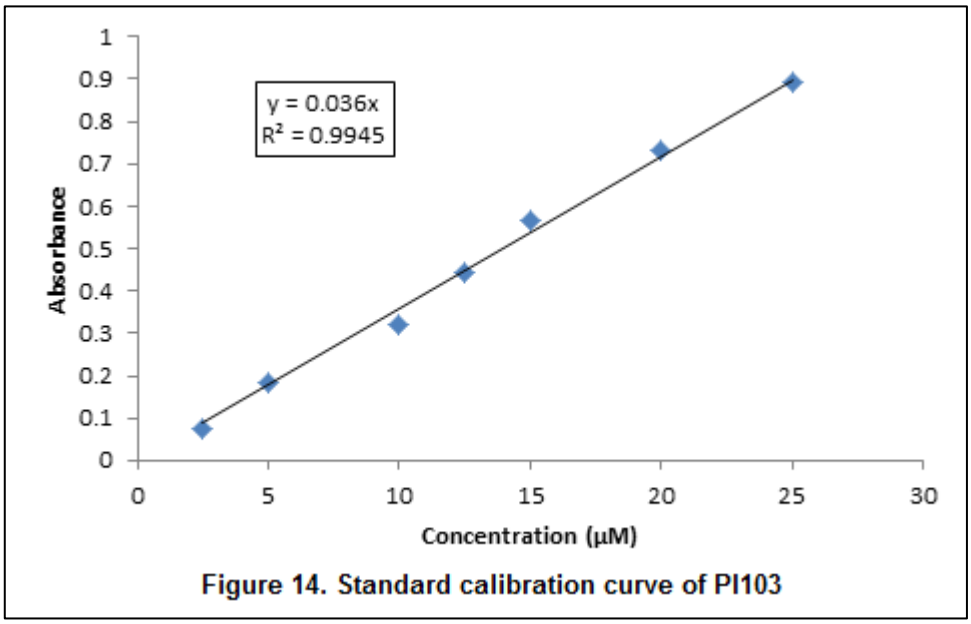

Figure 14. Standard calibration curve of PI103

2.2.4 Release kinetics of drug loaded NPs at pH 5.5

Drug-loaded NPs were suspended in aqueous HCl solution (pH = 5.5) and sealed in a dialysis membrane (MWCO = 500 Da). The dialysis bags were incubated in 10 mL of aqueous HCl solution (pH = 5.5) at room temperature with gentle shaking. A 200-µL portion of the aliquot was collected from the incubation medium at predetermined time intervals. Aliquots were concentrated on speed vaccum. The released drugs were quantified using UV-VIS spectrophotometer following the similar procedureused for drug loading.

2.3 Instrumentation

2.3.1 Atomic Force Microscopy

One droplet of dilute aqueous solution of NPs was drop-casted with micropipette (5 µL) on to freshly cleaved mica surface. The solution was allowed to dry (evaporation) in air overnight before AFM measurement.

2.3.2 Field-Emission Scanning Electron Microscope (FESEM)

Five micro litter of freshly prepared dilute solution of NPs were drop casted onto silicon wefer and sample were allowed to dry in air, followed by gold coating. Images were obtained using Ultra-55, Zeiss NTS GmbH, at an operating voltage of 4.0 KV. Multiple images taken at different locations were used for image analysis.

Chapter 3: Results and discussion

3.1 Rationale of using PMA as a nanovector.

Nanocarriers are nanosized materials (diameter 1-200 nm) that can carry a single drug or multiple drugs or a combination of drug and imaging agent simultaneously. Nanocarriers help to increase local drug concentration by carrying the drug within and control-releasing when reach the target. Currently, many natural and synthetic polymers and lipids are typically in use as nanovectors. The family of nanocarriers include polymer-drug conjugates, polymeric nanoparticles, lipid-based carriers like liposomes and micelles, dendrimers, carbon nanotubes, metallic nanoparticles etc. in present study, we have used a polymer as a nanovector as

they provide precise morphological control and surface modification,their sizes, structures and functions could be readily tailored through careful selection of polymer structures. Polymeric NPs exhibit higher stability, sharper size distribution,more tunable physicochemical properties, sustained and more controllable drug-release profiles and higher loading capacity also for poorly water soluble drugs like PI-103.

Herein we have used PMA as polymeric nanovector because of its unique structural properties.Each monomeric unit of 40 mer PMA could serve as a site for conjugation of drug like proflavine by amide linkage. Also conjugation of proflvine, leads to opening of anhydride groups and provides acid groups for further conjugation of aquated cisplatin andaids to the hydrophilicity of polymer.

Moreover a simple hydrolysis of polymer results in a 40 units of maleic acid linked linearly through isobutylene linker. This *Poly-isobutylene-maleic acid* (PIMA) could be used to conjugate a hydrophobic drug like PI-103 through ester linkage and open acids could form of complex with aquated cisplatin through dicarboxylato linkage.

In this way PMA provides 40 sites of drug conjugation for a single molecule which would help to load maximum amount of drug in a nanoparticle. Hence,present study demonstrates the potential of PMA as a nanocarrier for conjugation and targeted delivery of various hydrophilic (proflavine and cisplatin) as well as hydrophobic drug (PI103).

3.2 Self assembled polymeric nanoparticles (Fig.15, 16)

According to the above mentioned strategy, 20 equivalentsof a cytotoxic agent, proflavine was first conjugated to PMA and 10 equivalents of a PI3K signalling inhibitor, PI103 was conjugated to PIMA to obtain polymer-mono drug conjugates. These conjugates did not self assemble to form any nanoparticles. Interestingly, conjugation of 20 equivalents of aquated cisplatin to each of these polymer-drug conjugates resulted in self-assembled nanoparticles in both the cases, PMA-proflavine-cisplatin (fig. 15) and PIMA-PI103-cisplatin (fig. 16). Both the polymer dual drug nanoparticles exhibited spherical shape and sub 200 nmsize to explicit EPR effect.

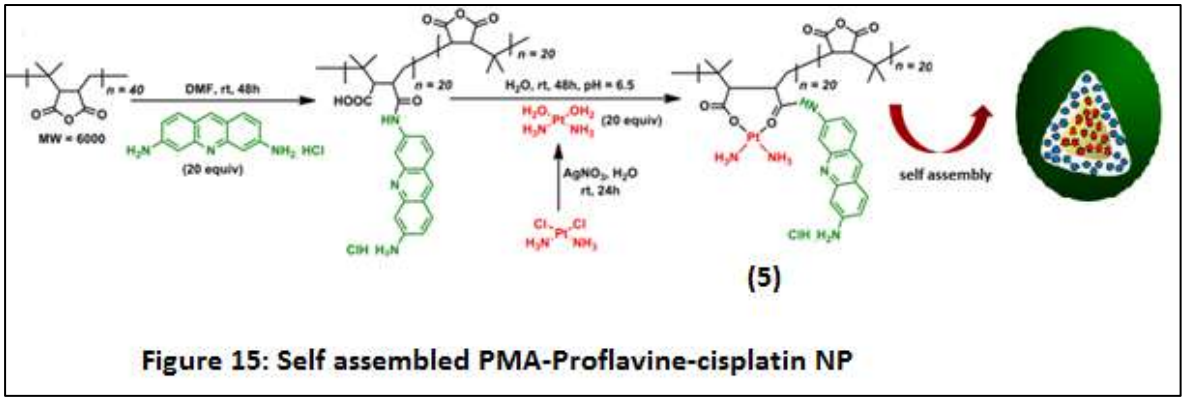

Figure 15: Self assembled PMA-Proflavine-cisplatin NP

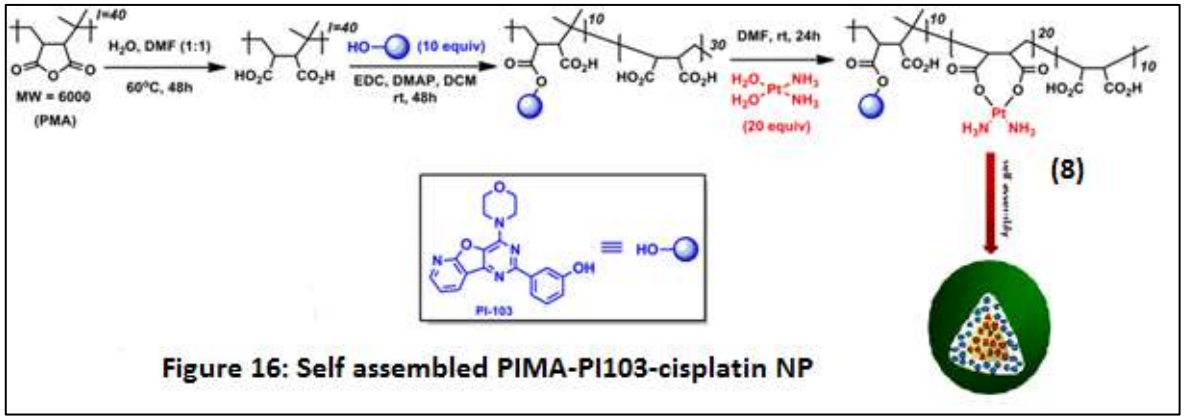

Figure 16: Self assembled PIMA-PI103-cisplatin NP

3.3 Characterization of size and shape of NPs

3.3.1 PMA-proflavine-cisplatin NPs (compound 5) (Fig. 17)

The morphology of self assembled NPs was examined by Field emission scanning electron microscopy (FE-SEM) (fig. 18 a), Atomic force microscopy (AFM) (fig. 18 b,18 c) and confocal microscopy (fig. 18 d)using proflavine as a fluorescent molecule. FE-SEM and AFM images clearly show the spherical shaped nanostructure of NPs with diameter 100 to 120 nm. Laser scanning confocal microscopy also supported the similar results.

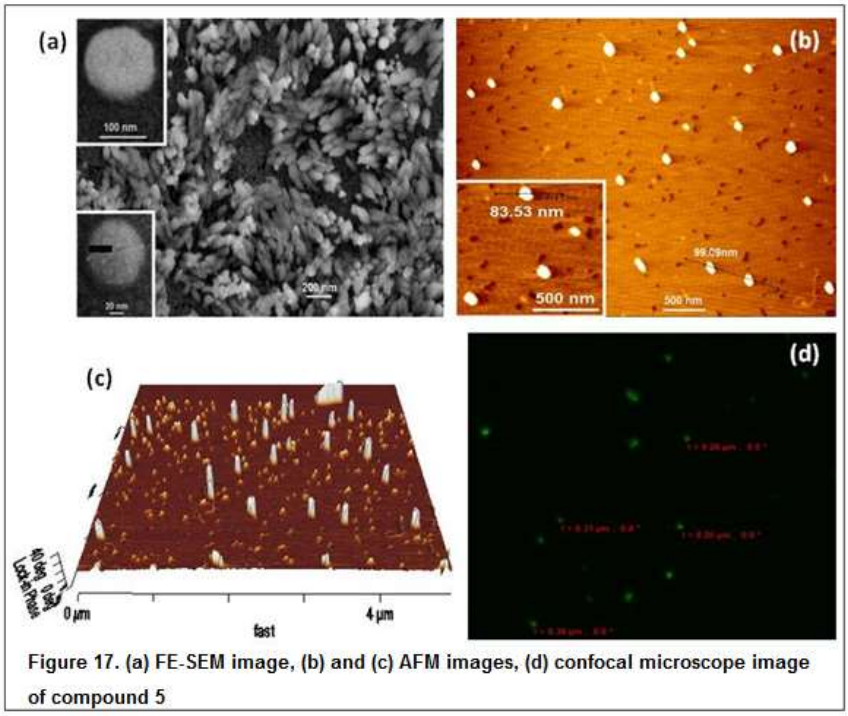

Figure 17. (a) FE-SEM image, (b) and (c) AFM images, (d) confocal microscope image of compound 5

3.3.2 PIMA-PI103-cisplatin NPs (compound 8) (Fig. 18)

PIMA-PI103 conjugate self assembled into nanoparticles after conjugation of aquated cisplatin. FE-SEM (fig. 19a) and AFM (19 b, 19 c) images and DLS (19 d) clearly showed the sub 200 nm size, spherical shape and morphology of NPs which is ideal for EPR effect

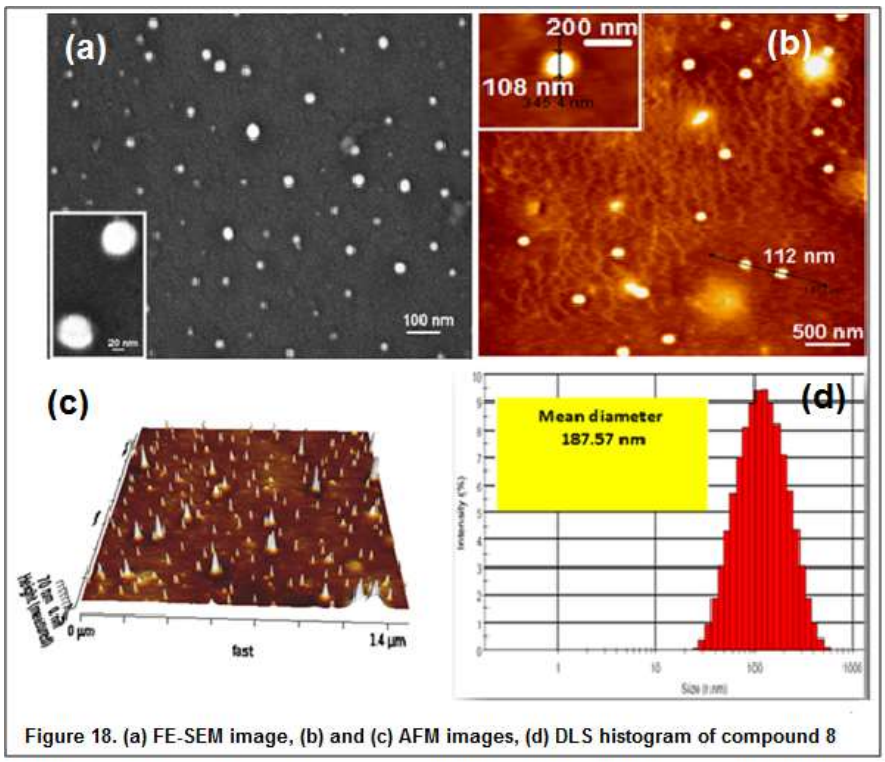

Figure 18. (a) FE-SEM image, (b) and (c) AFM images, (d) DLS histogram of compound 8

3.4 Evaluation of drug loading in NPs

3.4.1 PMA-proflavine-cisplatin NPs (compound 5)

3.4.1.1 Proflavine loading (Table. 2)

1 mg of freshly synthesized NPs was dissolved in 1 ml of water and stock solution was prepared. Further dilutions were obtained from stock and absorbance was measured at 445 nm against the corresponding solvent (water) as blank in 200 µL quartz cuvette. Loading was calculatedwith the standard absorbance curve for proflavine(Fig. 12) and the observed loading was 103 µg of proflavine per mg of NPs.

Dilution	Amount of drug
1%	97.9 µg
3%	111.8 µg
5%	100.8 µg
mean	**103.5 µg**

Table 2. Proflavin loading in compound 5

3.4.1.2 Cisplatin loading (Table. 3)

For stock solution, 1mg of NPs was dissolved in 1 mL of 1.2mg of o-phenylene diamine per mL of DMF solution. The stock solution was then heated upto 100^{o} C for 3 to 4 h till the solution becomes deep green (due to platinum conjugation). Further dilutions were obtained from stock and absorbance was measured at 706 nm against the corresponding solvent (DMF) as blank in 200 µL quartz cuvette. Loading was estimated using the standard absorbance curve for cisplatin (Fig. 13) and calculated loading was 433.13 µg of cisplatin per mg of NPs.

Dilution	Amount of drug
0.67%	434.2 µg
1%	432.3 µg
1.33%	432.9 µg
mean	**433.13 µg**

Table 3. Cisplatin loading in compound 5

3.4.2 PIMA-PI103-cisplatin NPs (compound 8)

3.4.2.1 PI103 loading (Table. 4)

For PI103 loading, 1mg of prepared NPs was dissolved in 1ml of spectroscopic grade DMSO. Further dilutions were prepared from the above stock solution. Absorbance was measured at 293 nm against the corresponding solvent (DMSO) as blank in 200 µL quartz cuvette. Loading was estimated using standard curve of PI103(Fig. 14). Loading was observed as 74 µg of PI103 per mg of NPs.

Dilution	Amount of drug
2%	69.1 µg
5%	76.8 µg
10%	76.35 µg
mean	**74.1 µg**

Table 4. PI103 loading in compound 8

3.4.2.2 Cisplatin loading (Table. 5)

CIsplatin loading was calculated using above mentioned procedure (3.4.1.2). The calculated loading was (178.26) µg of isplatin per mg of NPs.

Dilution	Amount of drug
1%	182.6 µg
2%	185.5 µg
4%	166.7 µg
mean	**178.26 µg**

Table 5. Cisplatin loading in compound 8

3.5 Water solubility of NPs.

3.5.1 PMA-proflavine-cisplatin NPs (Fig. 19)

PMA is a hydrophobic polymer which becomes partially water soluble when proflavine is conjugated due to opening of anhydride rings. After conjugation of aquated cisplatin, self assembled NPs were completely hydrophilic (fig 20), which makes the NPs compatible to use further for biological studies.

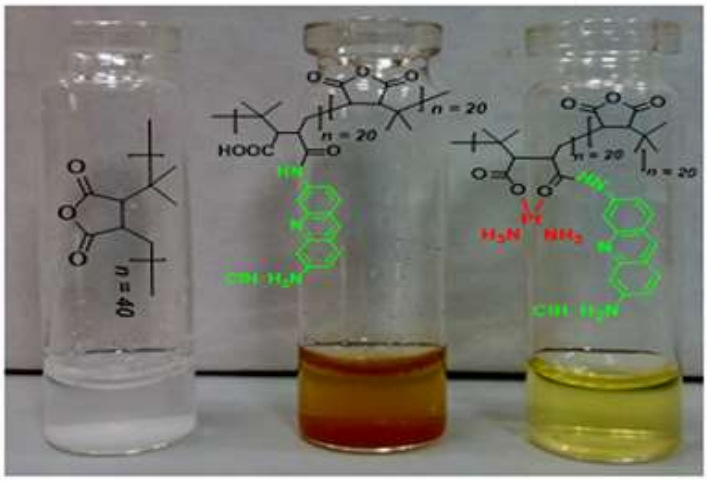

Figure 19. Water solubility image of compound 5

3.5.1 PIMA-PI103-cisplatin NPs (Fig. 20)

PIMA is a hydrophilic polymer which is a hydrolysed product of PMA. NPs formulated from conjugation of PI103 and cisplatin were completely water soluble (fig 21). These NPs serve as delivery system for hydrophobic drugs like PI103. The water solubility of the NPs carrying dual drugs makes them compatible for further biological investigation.

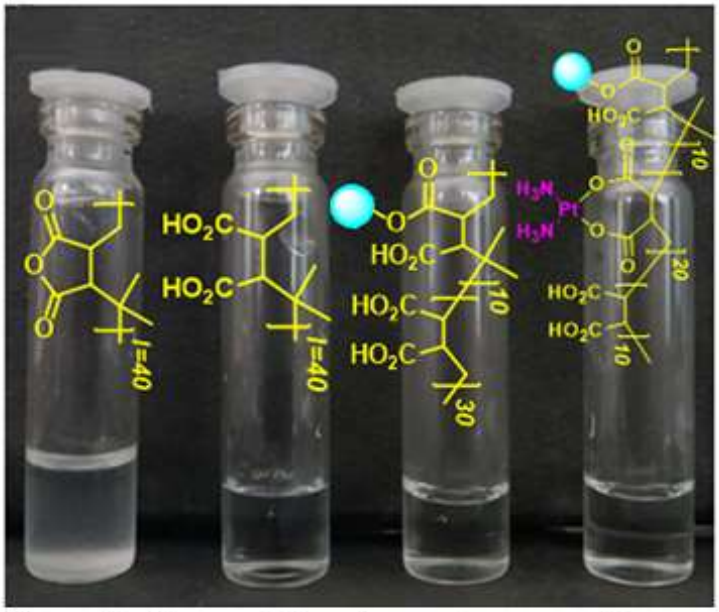

Figure 20. Water solubility image of compound 8

3.6 Release kinetic studies of drugs from NPs

3.6.1 PMA-proflavine-cisplatin NPs (Fig. 21)

The *in vitro* release profile of PI103 was obtained at 37°C in pH 5.5 (mimicking lysosomal compartment) by representing the percentage released drugs with respect to the loaded amount in NPs. It is observed that both the drugs released almost equally with time also the release is gradual and less, around 10% and 15% in 72 h for proflavine and cisplatin respectively (fig 22). As proflavine is a green fluorescent molecule, it could be seen as a marker to observe the internalization of NPs and hence the nanoparticles could have great application as 'Theranostic agent' in cancer therapy.

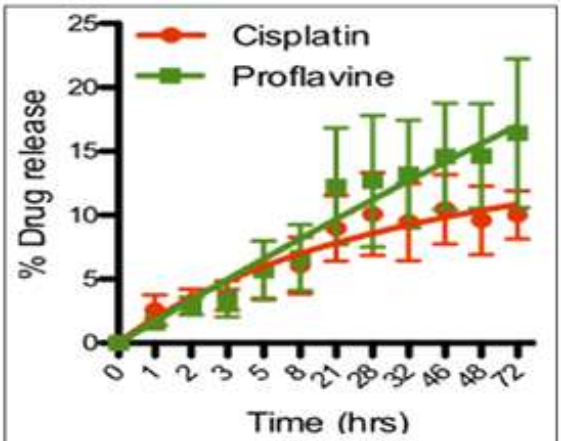

Figure 21. Release kinetic profiles of proflavine and cisplatin in compound 5

3.6.2 PIMA-PI103-cisplatin NPs (Fig. 22)

At pH 5.5, it is clearly observed that the release profilesof both the drugs, PI103 and Cisplatin are completely different. PI103 released gradually almost 80 % over a period of 72 h whereas cisplatin released even more slowly and release is less that 20% (fig 23). It was already observed that, if cells are treated with different drugs attacking different targets at different time points, shows synergistic effects(). As the NPs also deliver two differently targeting drugs (PI3K signalling pathway and DNA) with varying release profiles, are expected to work synergistically.

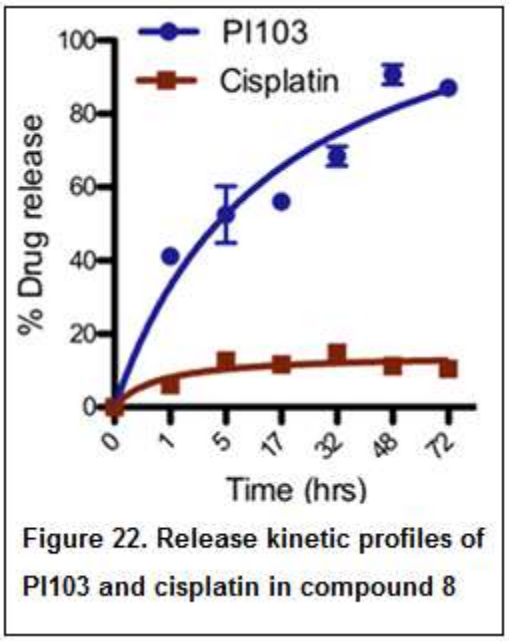

Figure 22. Release kinetic profiles of PI103 and cisplatin in compound 8

However in both the cases the dual drugs released from NPs in slow and sustained manner over 72 h which would lead to controlled drug delivery in vitro.

Conclusion

Herein we have successfully demonstrated the synthesis of efficient self-assembled polymeric dual drug NPs using biocompatible, biodegradable and non-toxic nano vector PMA. The NPs carry and deliver dual anticancer drugs, which leads to overcome both the major challenges of toxic side effects and drug resistance simultaneously in cancer. Using the present idea of combining nanotechnology and combination therapy, all the three drugs proflavine, PI-103 and cisplatin can be used to their optimum dosages. The size, shape and morphology of synthesized NPs are less than 200 nm and hence compatible with EPR effect. Moreover, the release kinetic profiles of NPs at pH 5.5(mimicking lysosomal compartments) demonstrate sustained release of active drugs over a period. This strategy would serve as a novel platform technology and various personalized therapeutics could be formulated.

Outlook

- Understanding the mechanism of self-assembly of NPs by molecular dynamic simulation.
- In vitro biological evaluation of NPs.

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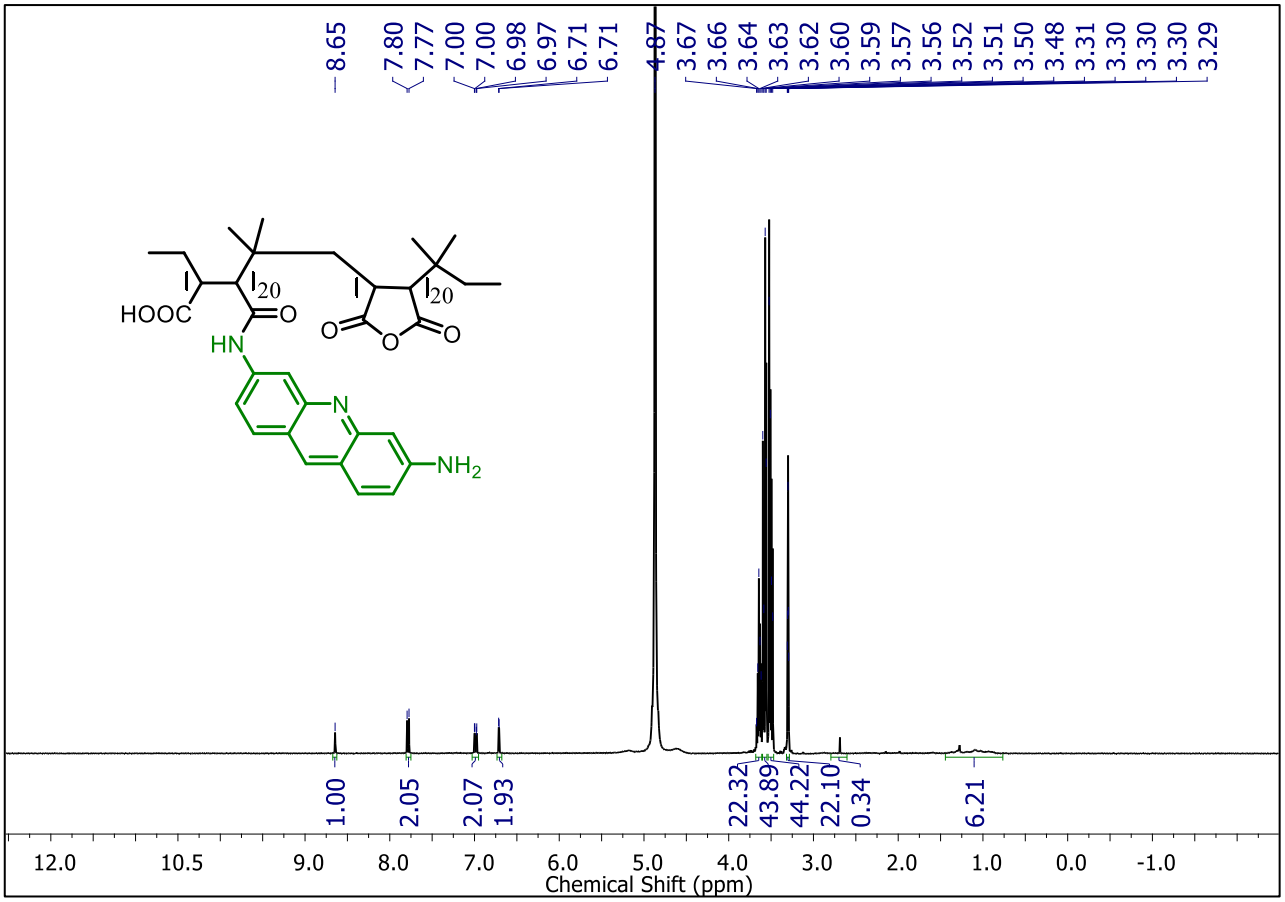
^{1}HNMR of compound 2



^{1}HNMR of compound 8