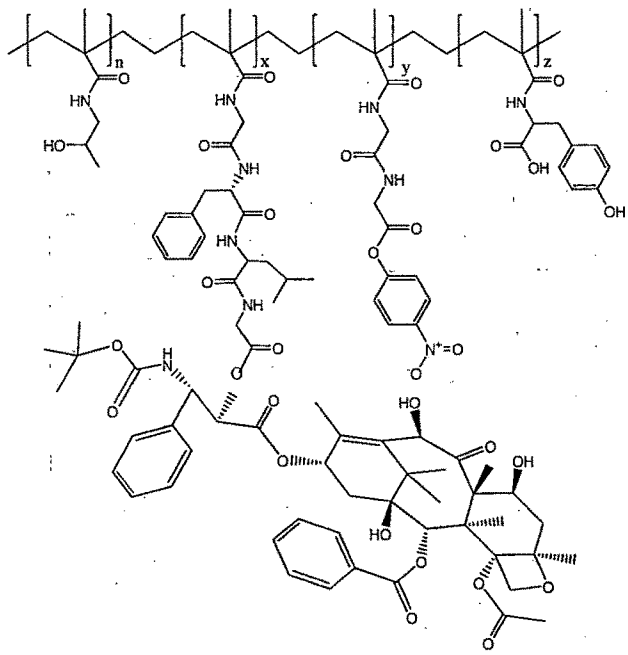
Chapter 8
HPMA -Docetaxel copolymer peptide conjugated system



8.1. Introduction

Polymer conjugates, especially those prepared using N- (2-hydroxypropyl) methacrylate (HPMA) have been extensively studied for intracellular and cytoplasmic drug delivery .HPMA conjugated to oligonucleotides via lysosomally degradable spacers were shown to enter the cytoplasm and nucleus of the cells (Jensen, K.D. et al., 2003). The polymer conjugates offer the flexibility of attaching specific targeting molecules that enable their accumulation in specific intracellular compartments. For example, efficient cytoplasmic and nuclear accumulation of the HPMA conjugates can be obtained by attaching either a NLS (Nuclear localization sequences) or a TAT peptide to the conjugate (Jensen, K.D. et al., 2003).

Polymeric conjugates have been used for targeted delivery of drugs to tumor sites because the attachment of drugs to water soluble polymer increases their solubility, reduces the side effect, and overcomes multi-drug resistance; the large size of conjugates increases blood half life and significantly alters the drug properties and pharmacokinetics; the conjugates can be tailor-made (i.e., side-chain content, molecular weight, charge etc.) for specific targeting and delivery needs; they can be designed to passively (EPR) or actively target tumor sites; and site specific drug release can be achieved by designing biodegradable spacers that can be enzymatically cleaved or that are pH sensitive. The advantages have led to the development of a wide range of polymer-anticancer drug conjugates, some of which are currently in clinical trials.

HPMA copolymer has been employed to modify the in vivo biodistribution of chemotherapeutics agents and enzymes. The advantage of HPMA copolymer over other water soluble polymers is that they can be tailor-made with simple chemical modification to regulate drug and targeting moiety content for biorecognization, internalization, or subcellular trafficking depending on specific therapeutics needs. The over all molecular weight of HPMA copolymers is determined by the polymerization conditions, particularly the concentration of initiator and chain transfer agents. Various side chain moieties (isotope chelator, targeting moieties and drug) may be directly linked to the polymer chain via a biodegradable or non-biodegradable spacer.

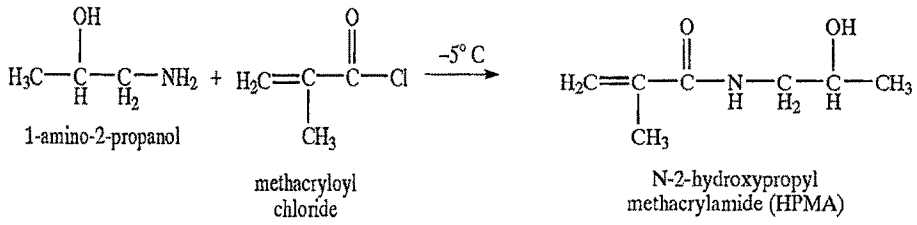
Polymeric based delivery systems have been used as carriers for passive and active targeting of drugs in the treatment of various diseases and as novel imagine agents. Without a specific targeting ligand moderate-size (> 30 kD) polymer can passively (via

EPR) accumulate in tumor tissues. The EPR effect has been used to deliver macromolecular bioactive agents to solid tumors including anti-angiogenic drugs. Peptide-targeted delivery has a basis in nature as many peptides are used as attachment ligands by bacteria and viruses. The use of peptides as ligands for receptor targeting has been investigated by several groups (Shadidi et al., 2003). Peptide ligands have a number of advantages. These include their lower antigenic potential, making them less likely to cause an immune reaction. They are also easier to synthesize and characterize. Their smaller size means multiple peptides could be attached to a single nanoparticle conferring multivalent attachment. The most widely investigated receptor family for targeting with peptide ligands is the integrin family. These receptors are expressed on the neo-vasculature of various tumours and are involved in adhesion and cell signaling (Vander et al., 1994). Their peptide ligands are defined as arginine-glycine-aspartic acid (RGD) peptides, and comprise a range of linear and cyclic peptides containing the RGD motif (Arap et al., 1998). *Arap et al 1998* found that mice treated with RGD-targeted doxorubicin had greater survival than mice treated with doxorubicin alone in a murine tumour model. Many other targets are being investigated with peptide ligands targeting the tumour vasculature, cancer cell surface and surface immunoglobulins (Shadidi et al., 2003).

8.2. Preparation of copolymer

8.2.1. Preparation of HPMA monomer

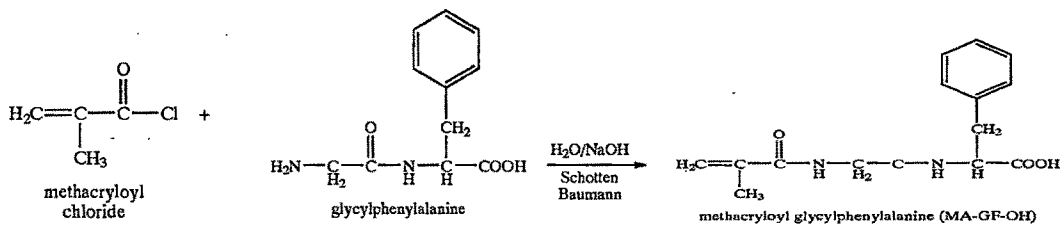
To a solution of 1-amino-2-propanol (23.98ml, 0.3 mol) in 90 ml of acetonitrile, freshly distilled methacryloyl chloride (MA-Cl) (14.8ml, 0.15 mol) was added dropwise under vigorous stirring and cooling to -5^0C. A small amount of inhibitor, tertiary octyl pyrocatechine was added. The reaction mixture was stirred for an additional 30 min at room temperature. 1-amino-2-propanol hydrochloride formed as a byproduct was precipitated and filtered off. The filtrate was cooled to -70^0C and the HPMA precipitated. After equilibrating to room temperature the product was filtered off and washed with pre-cooled acetonitrile. Recrystallization was from acetone and the pure product was isolated.



---- Reaction (1)

8.2.2. Preparation of MA-GF-OH

Glycylphenylalanine (Gly-Phe, 11.85gm, 0.0532mol) was dissolved in 4N NaOH (0.05mol) and cooled to 0^0C. Freshly distilled MACl (7gm, 0.612mol) in 10ml of dichloromethane was added dropwise. A small amount of inhibitor, tertiary octyl pyrocatechine was added to prevent polymerization of monomers. Simultaneously but with slight delay, 0.6 mol of 4N NaOH was added dropwise to the reaction mixture. After addition of MACl and NaOH the reaction mixture was warmed upto room temperature and allowed to react for one hour. The pH was maintained at around 6-7. The dichloromethane layer was separated from water layer, washed with 10ml of water and discarded. The collected water layer was acidified with citric acid to pH 3 and this white emulsion was extracted with ethyl acetate (3x150ml) in presence of inhibitor. The collected organic layer was washed with aq. NaCl and dried over anhydrous sodium sulfate. The organic layer was removed by Rota evaporator to obtain product as a white powder and recrystallized from EtOAc.

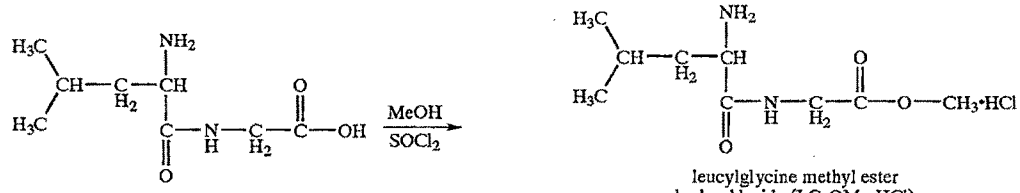


-----Reaction (2)

8.2.3. Synthesis LG-OMe.HCl

Leucylglycine (Leu-Gly, 10gm 53.31mmole) was dissolved in 80ml of methanol and cooled to -5^0C. 4.2ml (55mmole) of $SOCl_2$ was added dropwise under stirring. After equilibrating to room temperature the mixture was refluxed for three hours. The solvent

was evaporated to dryness and the residue was dissolved in methanol and evaporated to remove HCl and $SOCl_2$. The residue was redissolved in toluene and evaporated to obtain white amorphous solid. This was used in subsequent steps without purification.



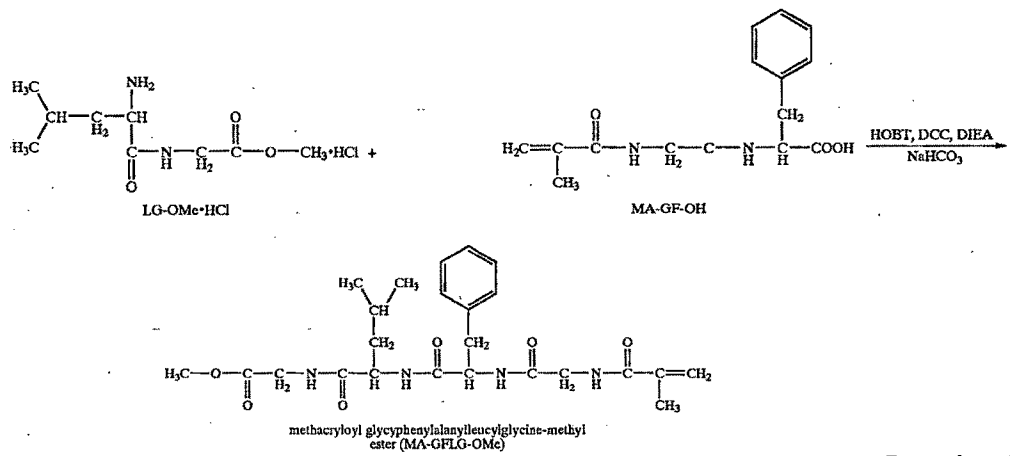
leucylglycine

leucylglycine methyl ester hydrochloride (LG-OMe•HCl)

-----Reaction (3)

8.2.4. Synthesis of MA-GFLG-OMe

To a solution of Leu-Gly-OMe.HCl (9.8gm, 0.0410mole) in 80ml of DMF, was added 6gm(45mmole) of HOBT, 7.83ml of DIEA (45mmole) and the MA-GF-OH (10.74gm, 0.037mol). The reaction mixture was stirred and cooled to -10^{0}C. 9.28gm of DCC in 50ml of DMF was added dropwise within five minutes. The solution was stirred for two hours at 0^{0}C and then for 24hrs at room temperature. After overnight stirring the precipitated byproduct dicyclohexyl urea (DCU) was filtered off. The filtrate was rota-evaporated to remove the DMF completely. The residue was mixed with 100ml of 5% $NaHCO_3$ solution and extracted with EtOAC three times (3x150ml). The extract was washed with (3x100ml) of citric acid 5% solution, 5% $NaHCO_3$ solution and 3x50ml of saturated brine and dried over anhydrous sodium sulfate for two hours. After filtering off the drying agent and addition of a small amount of inhibitor, the filtrated was concentrated under vacuum to obtain the product. Recrystallization was done from EtOAC.



LG-OMe•HCl

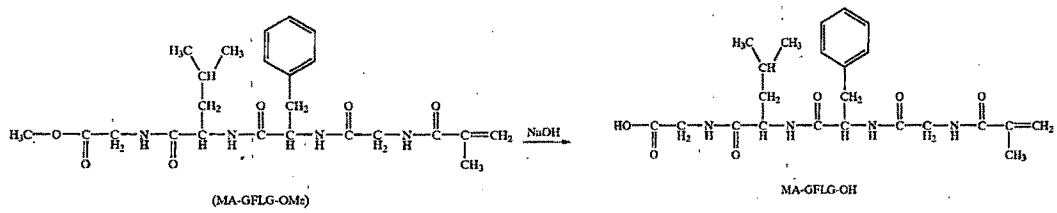
MA-GF-OH

methacryloyl glycyphenylalanylleucylglycine-methyl ester (MA-GFLG-OMe)

-----Reaction (4)

8.2.5. Synthesis of MAGFLG-OH

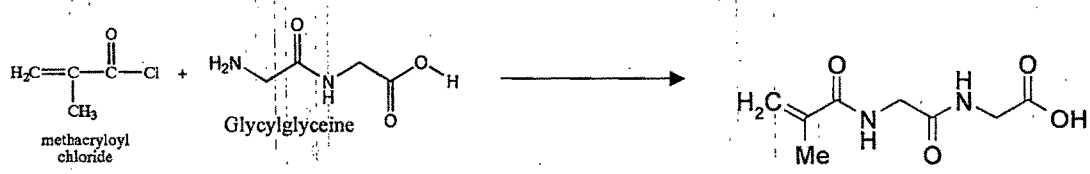
To a solution of MA-GFLG-OMe (16.71gm) in 80ml of methanol and cooled to 0^0C., excess of 2N NaOH was added dropwise under stirring. After addition of a small amount of inhibitor the reaction mixture was stirred for one and half hours at 0^0C and then for two hours at room temperature. The reaction mixture was concentrated under vacuum to remove methanol. 160ml of distilled water was added and the mixture was acidified with concentrated citric acid to pH 2. The free acid was extracted with 4x250ml of EtOAC, washed with saturated brine and dried over anhydrous sodium sulfate overnight. After evaporation of the solvent under vacuum the tetrapeptide product was re-crystallized from EtOAc.



-----Reaction (5)

8.2.6. Synthesis of MA-GG-OH

Glycyleglyceine (Gly-Gly, 26.2gm, 0.2mol) was dissolved in 4N NaOH (0.2mol) and cooled to 0^0C. Freshly distilled MACl (0.2mol) was added dropwise. A small amount of inhibitor was added to prevent polymerization of monomers. Simultaneously but with slight delay, 0.2 mol of 4N NaOH was added dropwise to the reaction mixture. After addition of MACl and NaOH the reaction mixture was warmed up to room temperature and allowed to react for one hour. The pH was maintained at around 6-7. The product was precipitated by acidification with HCl (1:1) at pH 2 in presence of inhibitor. The final product was recrystallized from ethanol: H_2O (50:50) as MA-GG-OH.



MA-GG-OH

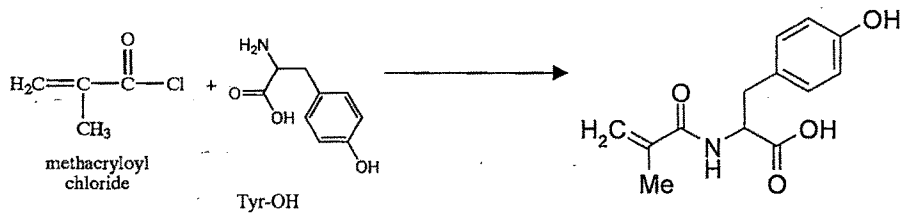
-----Reaction (6)

8.2.7. Synthesis of MA-GG-ONp

To a solution of MA-GG-OH (9gm, 0.45mol) in 150 ml of DMF a solution of 6.15g of p-nitrophenol in 30ml of DMF was added under stirring and cooling to -10^{0}C followed by a solution of 10.31gm of DCC in 10ml of DMF. The reaction mixture was stirred for six hours at -10^{0}C and then overnight at 4^{0}C. The precipitated byproduct DCU was filtered off and the DMF was removed by rotary evaporation. The residue was dissolved in EtOAc and the remaining byproduct was filtered off. EtOAc was evaporated to dryness. The product was soaked in ether to remove excess p-nitrophenol. This procedure was repeated several times and the purity of the final product was determined by calculating extinction coefficient in DMSO.

8.2.8. Synthesis of MA-Tyr-OMe

To a solution of Tyrosine methyl ester (1 gm, 5.16mmol) in 100ml of Acetonitrile a freshly distilled MA-Cl (0.55gm, 5.16mmol) was added dropwise at -5^{0}C. The reaction mixture was brought to room temperature and allowed to react for 2 hours. The solution was concentrated on rota-evaporator to about half of the total volume. The precipitates were extracted with dichloromethane. The organic layer was washed with 5% citric acid solution.



-----Reaction (7)

8.2.9. Synthesis of MA-GFLG-Docetaxel:

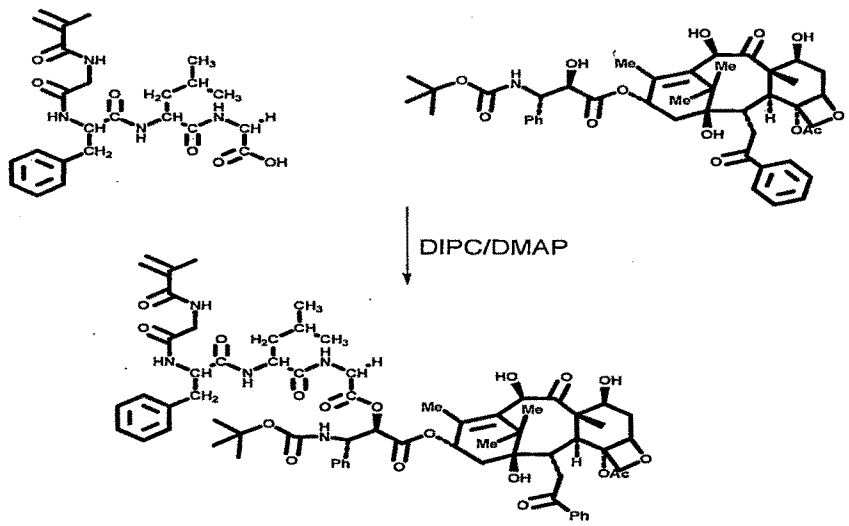
MA-GFLG-OH, Docetaxel and DMAP were weighed and kept in dessicator for 1 hr. This solid mixture was dissolved in anhydrous DMF and to this solution at 0°C DIPC (1.2x) was added. The resulting solution was allowed to warm to room temperature and left for 16 hrs. The completion of reaction was checked by TLC & Mass analysis.

Table: 8.1. Feed ratio of compounds for Docetaxel comonomer reaction

Compound	Mwt	Density	Wt taken(gm)	# of Moles	Molar ratio
MA-GFLG-OH	460.52	-	0.1	6.51438E-05	1
Docetaxel	805.91	-	0.210	7.81725E-05	1.2
DMAP	122.17	-	0.01591722	.000130288	2
DIPC	126.2	0.815	0.00986537	7.81725-05	1.2

MA-GFLG-OH + Docetaxel $\xrightarrow{\text{DMF}}$ MA-GFLG-Docetaxel

-----Reaction (8)



N-methcryloyl-glycyl-phenylanalyl-leucyl-glycyl-docetaxel

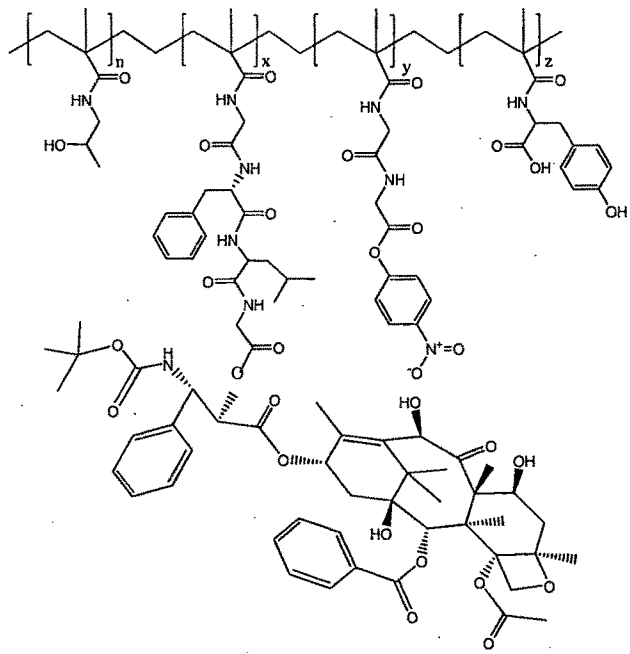
8.2.10. Polymerization of co-monomers

The polymerization was carried out using mixtures of n-HPMA, MA-GG-ONp,MA-GFLG-DC and MA-Tyr.OMe at various molar ratio as shown in Table: 2 using the initiator (2,2-azobisisobutyronitrile, AIBN). The solution containing the monomers in desired molar ratios dissolved in acetone with 1%DMSO and mixed with the initiator was transferred to an ampoule and bubbled with nitrogen for 5 min. The ampoule was sealed and put in an oil bath at 50^0C for 24 hrs under stirring. After 24hrs the copolymer was precipitated out of solution and the ampoule was cooled to room temperature to increase the yield of the precipitated polymer further. The reaction mixture with precipitated

copolymer was added dropwise to ether to improve the yield. It was vacuum dried and further characterized by size exclusion chromatography.

Table: 8.2. Composition of comonomer feed ratio

Compound	Mole percentage	Weight in mg
HPMA	80.5	275
MA-GG-ONp	15	125
MA-GFLG-DC	2.5	57
MA-Tyr-OH	2	11.9
AIBN	-	23
Acetone	-	2.6ml



HPMA-Docetaxel copolymer conjugates

8.2.11. Attachment of RGDfk:

The RGDfk in quantity of 20mg was added to the solution of drug containing polymer (20mg) dissolved in 4ml of anhydrous DMSO. After 20 mins, a drop of Pyridine was added to the reaction mixture. After 48hrs, 100µl of 0.1N NaOH was added dropwise through syringe pump and allowed to stir for 2 hrs to quench the reaction. The peptide conjugated drug containing polymer was obtained after complete removal of solvent via vacuum drying.

8.3. In vitro characterization and biological evaluation of HPMA-docetaxel- RGDfk copolymer conjugates:

8.3.1. Characterization of comonomers by Mass spectrometry

The n-HPMA, MA-GG-OH, MA-GF-OH, LG-OMe, MA-GLFG-OMe, MA-GFLG-OH, MA-GFLG-DC and MA-Tyr-OH were dissolved in methanol to a final concentration of 1mg/ml and analyzed for their respective mass by mass spectrometer.

8.3.2. Characterization of comonomers by NMR

1H NMR spectra were obtained on a Mercury 400 spectrometer (300 MHz for 1H) in CD_3OD solvent for Docetaxel, MA-GFLG-DC, HPMA-DC and blank HPMA. Chemical shifts were reported in ppm with CH_3OH as the internal standard.

8.3.3. Determination of drug content

5 mg of untargeted copolymer conjugates were hydrolyzed by 1 ml of 1 N NaOH. The copolymer was separated from hydrolyzed p-nitrophenyl by Sephadex G-10 (PD 10) column. The collected fractions of copolymer were lyophilized and Docetaxel content was measured by UV spectrophotometer at λmax260nm in DMSO solvent.

8.3.4. Determination of ONp content

2 mg of untargeted copolymer conjugates were hydrolyzed by 1 ml of 1 N NaOH. Serial dilutions were prepared in 1 N NaOH and analyzed by UV spectrophotometer at λmax 400nm for free p-nitrophenol. The ONp content was calculated from the standard plot of pure p-nitrophenol in 1 N NaOH.

8.3.5. Determination of Molecular weight of the copolymer

10 mg of untargeted copolymer conjugates were hydrolyzed by 1 ml of 1 N NaOH. The copolymer was separated from hydrolyzed p-nitrophenyl by Sephadex G-10 (PD 10) column. The collected fractions of copolymer were lyophilized. 5 mg of the dry copolymer was solubilized in 0.5 ml PBS 7.4 pH and injected the Sepharose 12 column for size exclusion chromatography with multi-angle light scattering detector from Wyatt technology. The targeted polymer was directly injected without hydrolysis in 0.5ml of PBS pH 7.4.

8.3.6. Determination of peptide content

1 mg of targeted polymer was dissolved in 1 ml of PBS pH 7.4. The attached peptide was hydrolysed to its individual amino acids and evaluated by amino acid analysis.

8.3.7. Drug release studies

The in vitro release of Docetaxel from HPMA copolymers was evaluated using the model lysosomal enzyme cathepsin B (CPB) according to previously described procedures with minor modifications (Kasuya, Y. et al., 2002, Wang, D. et al., 2000 & Pan, H. et al.,2006) Enzyme incubation mixture consisted of CPB stock solution (0.98 ml, 0.572 mg/ml in 0.1 M ammonium acetate buffer pH=5.5, 1 mM EDTA) and cysteine solution (0.02 ml, 250 mM in acetate buffer pH=5.5, 1 mM EDTA). 5 mg of HPMA copolymer-Docetaxel conjugates P1 and P2 (Table: 8.3) were dissolved in the incubation mixture (1ml, 5 min preincubation at 37°C). At 15, 60 and 180 min, 100 µl samples were removed, drug extracted twice with 1 ml ethyl acetate, organic layer separated and dried under nitrogen. Resulting residue was dissolved in 0.5ml of mobile phase and evaluated for drug content using reverse-phase high performance liquid chromatography (HPLC). Docetaxel was analyzed by HPLC method as previously described method B. *Mallikarjuna Rao et al* with slight modification. The mobile phase consisted of deionized water and HPLC grade acetonitrile, with 34% acetonitrile in deionized water held for 5 min, then linear gradients to 58% acetonitrile in 16 min, then linear gradients to 70% acetonitrile in 2 min, then linear gradients to 34% acetonitrile in 4 min and 34% acetonitrile held for 5 min and was delivered at a flow rate of 1.0 ml/min. UV detection was performed at 227 nm.

8.3.8. In vitro cytotoxicity assay

In vitro cytotoxicity and IC_{50} dose of free drug and copolymers was evaluated on BT-20 and HCT116 cells using a CCK-8 procedure. 6,000 cells per well were seeded in 96-well micro titer plates and plated for a total of 96hrs. On 24hrs cells were treated with drug and copolymer solutions in complete media lasting for 72h. Following treatments, CCk-8 solution was added for 1.5h, media gently aspirated, and absorption measured at 450 nm with blank well correction. Cell growth inhibition was determined by the cell viability at after 72h treatment and expressed as % cell viability compared to untreated cells.

Experiments were routinely conducted in the exponential growth phase. IC_{50} values were determined by nonlinear regression analysis using GraphPad Prism software.

8.3.9. DNA Content Analysis study by Flowcytometer

Cells were plated at the initial density of 0.15 X 10^6 six well plates. After 24hrs cells were exposed to DC and HPMA-DC with the concentration of 10, 30, 60 and 100nM for 24hrs. Cells pellets were collected and washed with PBS pH 7.4 after detaching with Tripsin Like enzyme. Cells were kept in 70% ethanol for overnight. Cells pellet were resuspended in 200µl of staining solution made of Propadium Iodide after washing with PBS and acquired for DNA content by Flow cytometer (FAScan, Becton Dickinson). The data were analyzed by Modfit Software.

8.3.10. Confocal Microscopy

Cells were seeded at the density of 2x 10^4 and 1x10^4 in 35mm glass bottom microwell dishes. After 24hrs both forms of drugs were added in the concentration of 1xIC_{50} and 2x IC_{50}. Control and treated cell were examined under the laser scanning confocal microscope.

8.3.11. Stability studies in mice serum and PBS pH 7.4

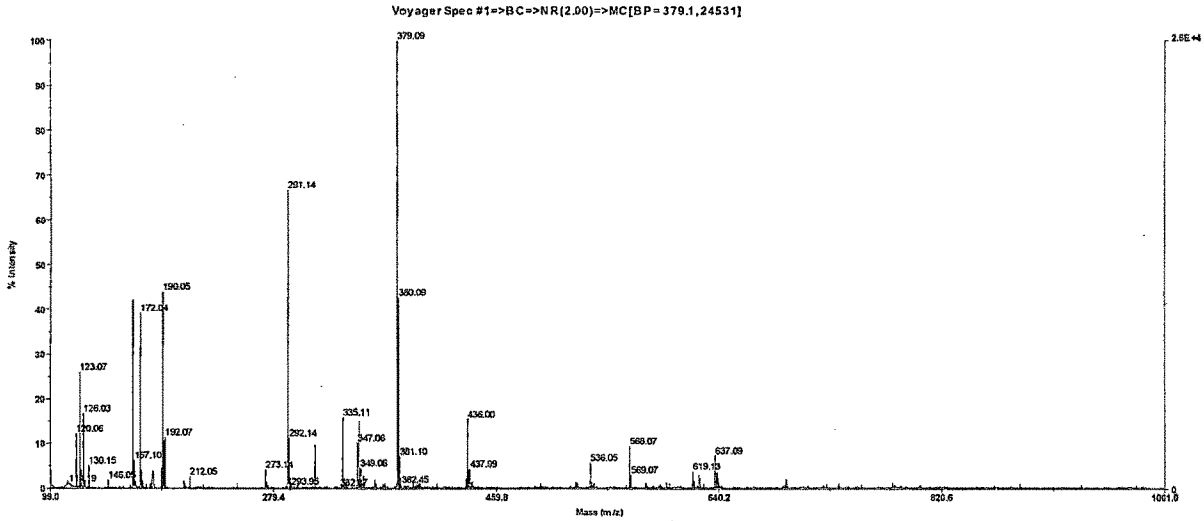
HPMA-DC conjugates were dissolved in 1 ml of 100% mice serum, 10%serum and PBS pH 7.4 respectively in the concentration equivalent to 1 mg of free Docetaxel. The solutions of conjugates were incubated at 37^0C with continuous stirring. At predetermined time intervals, 100µl of sample was withdrawn and free drug was extracted with 300 µl of chloroform. The extraction was repeated three times and the collected choloroform layer was evaporated to dryness under nitrogen. The residue obtained was dissolved in 100 µl of mobile phase and the amount of Docetaxel was analyzed by HPLC.

8.4. Results and Discussion:

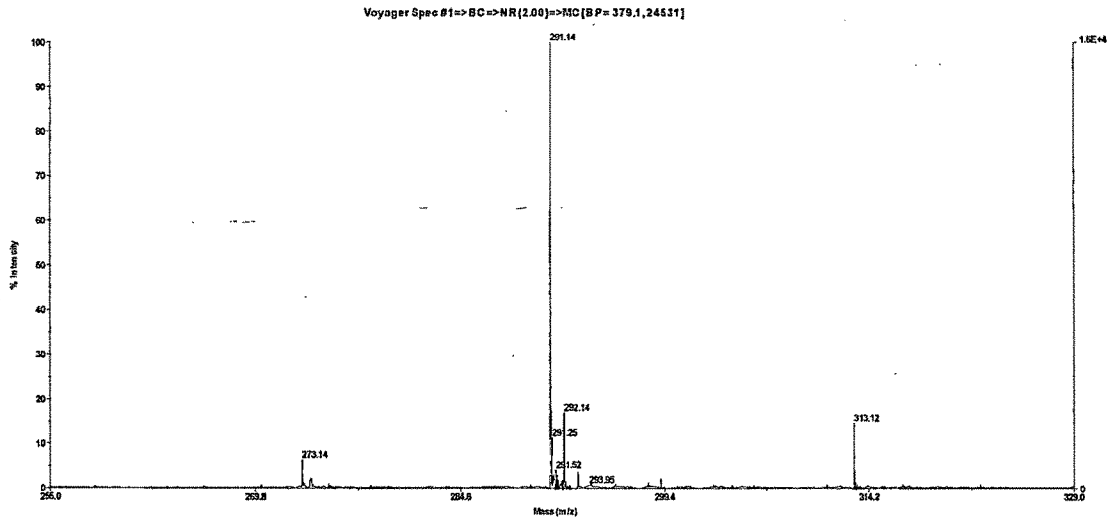
8.4.1. Characterization of monomers by Mass spectrometer

Results for analysis of mass are shown in Figure: 8.1 to Figure: 8.7 for respective monomers. In each figure, (a) and (b) denotes mass spectra of mixture and of individual monomer, respectively.

Figure: 8.1. Mass spectra of matrix (a) and MA-GF-OH (b)

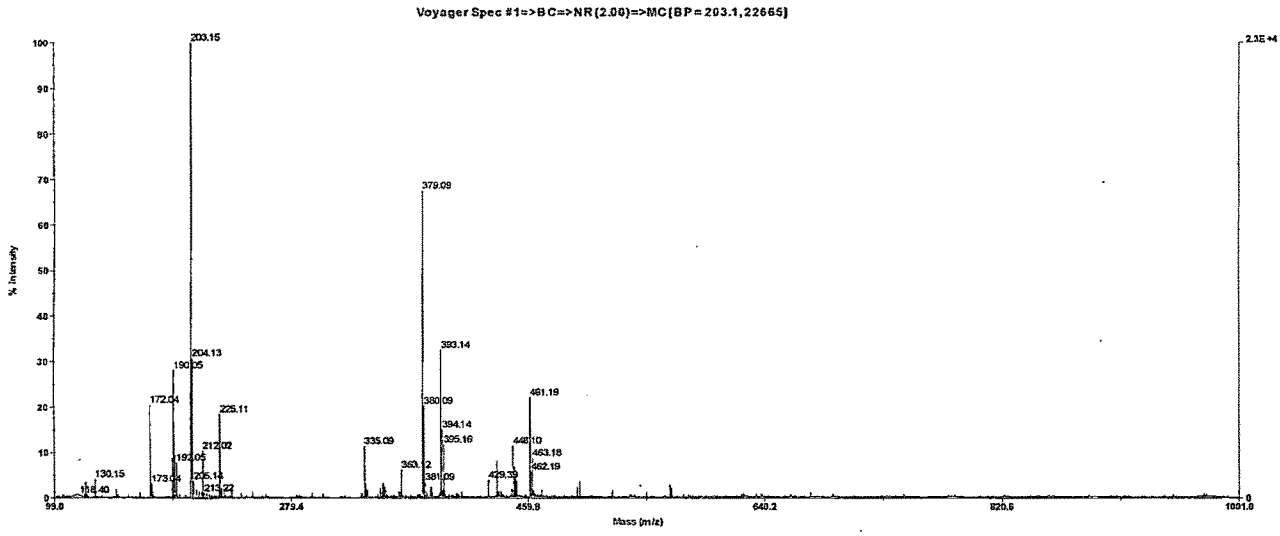


(a)

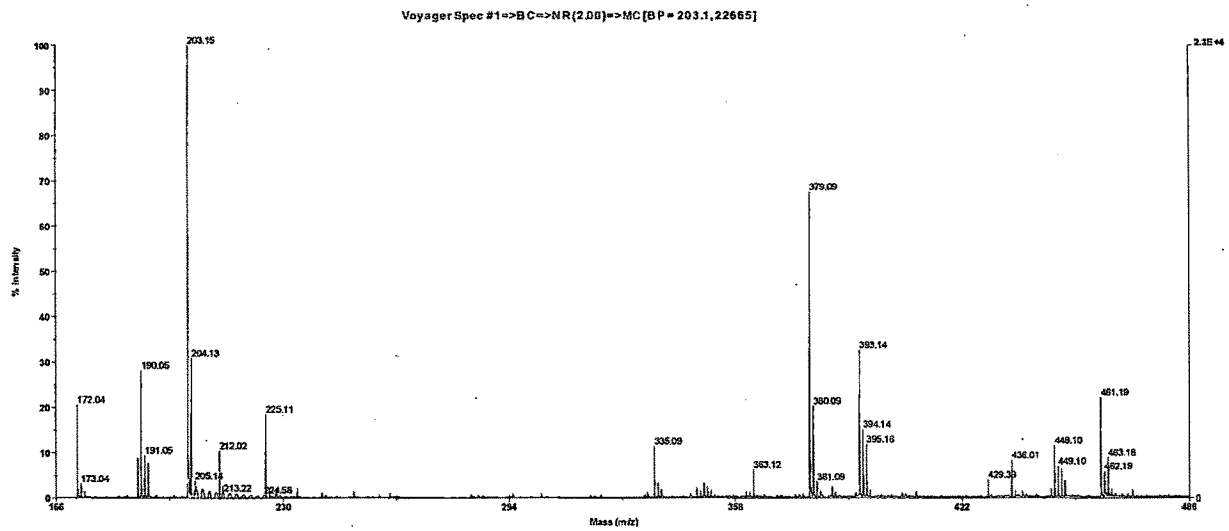


(b)

Figure: 8.2. Mass spectra of matrix (a) and LG-OMe (b)

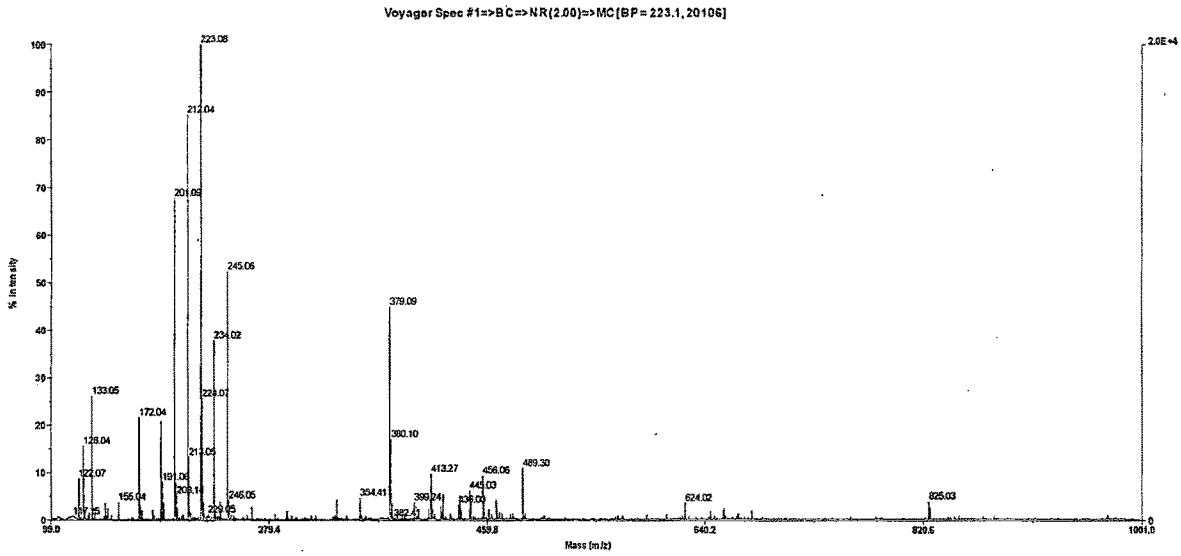


(a)

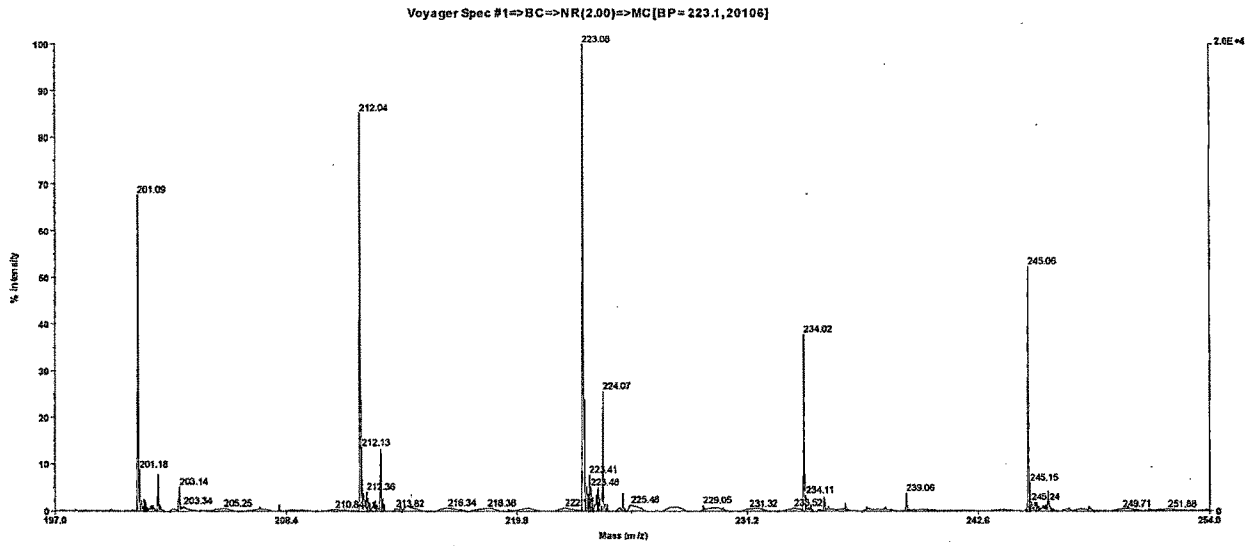


(b)

Figure: 8.3. Mass spectra of matrix (a) and MA-GG-OH (b)

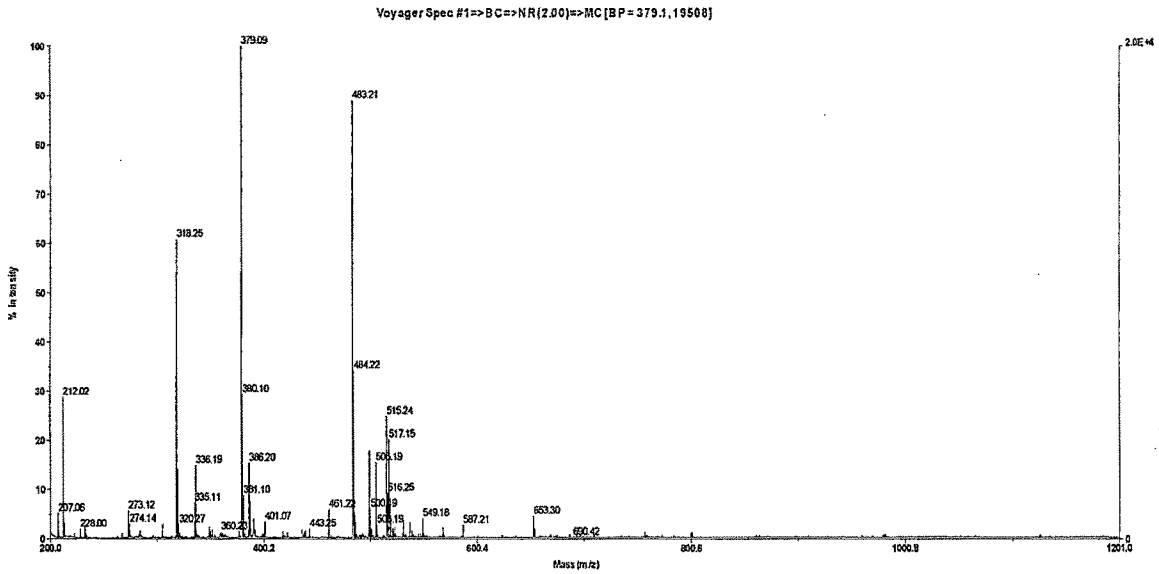


(a)

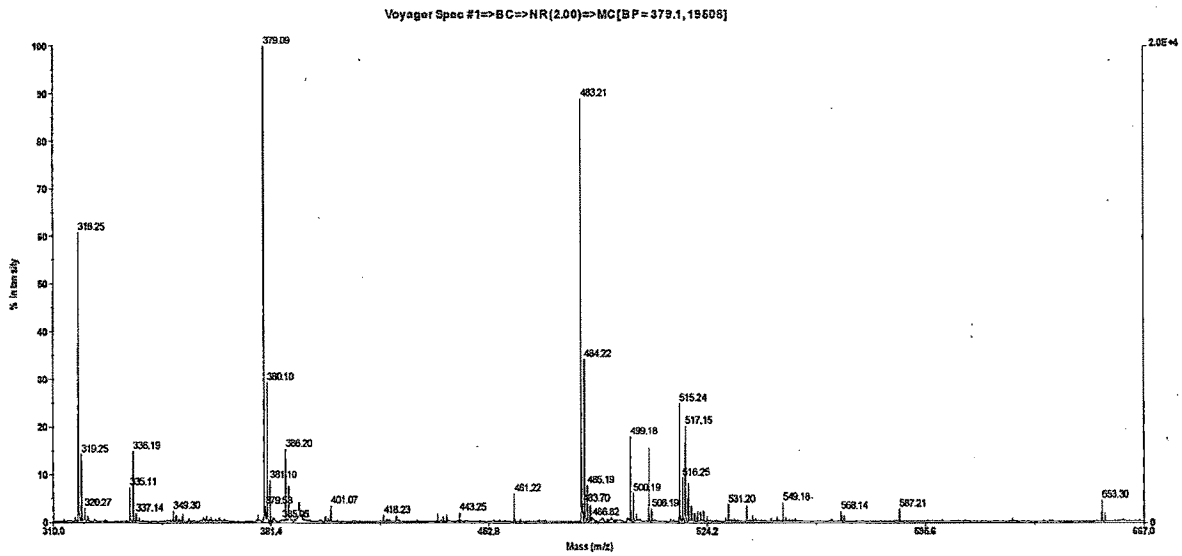


(b)

Figure: 8.4. Mass spectra of matrix (a) and MA-GFLG-OH (b)

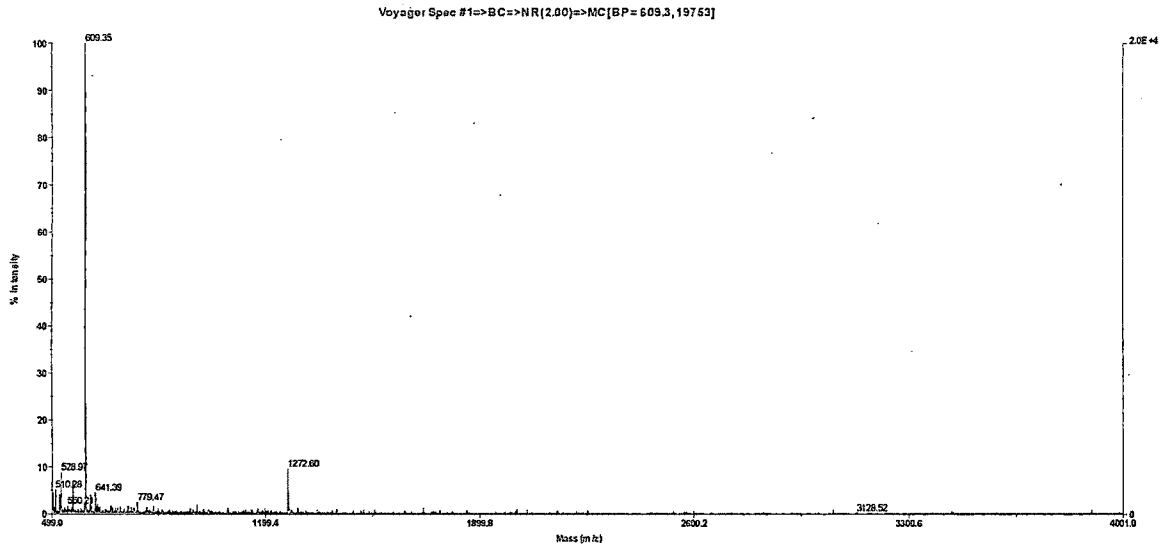


(a)

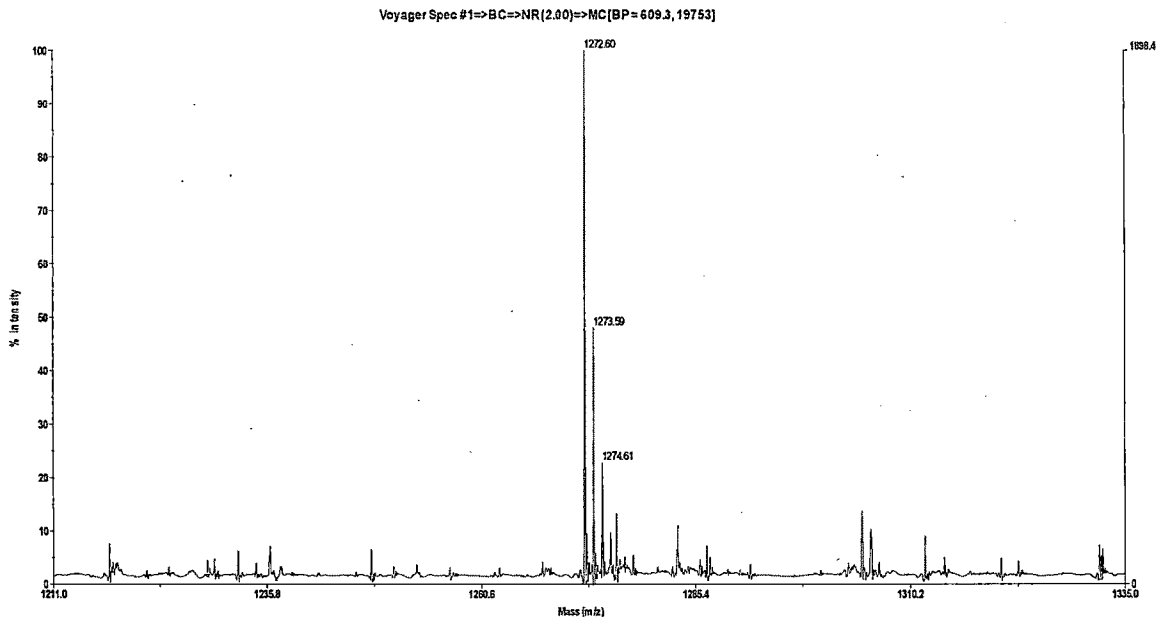


(b)

Figure: 8.5. Mass spectra of matrix (a) and MA-GFLG-DC (b)



(a)

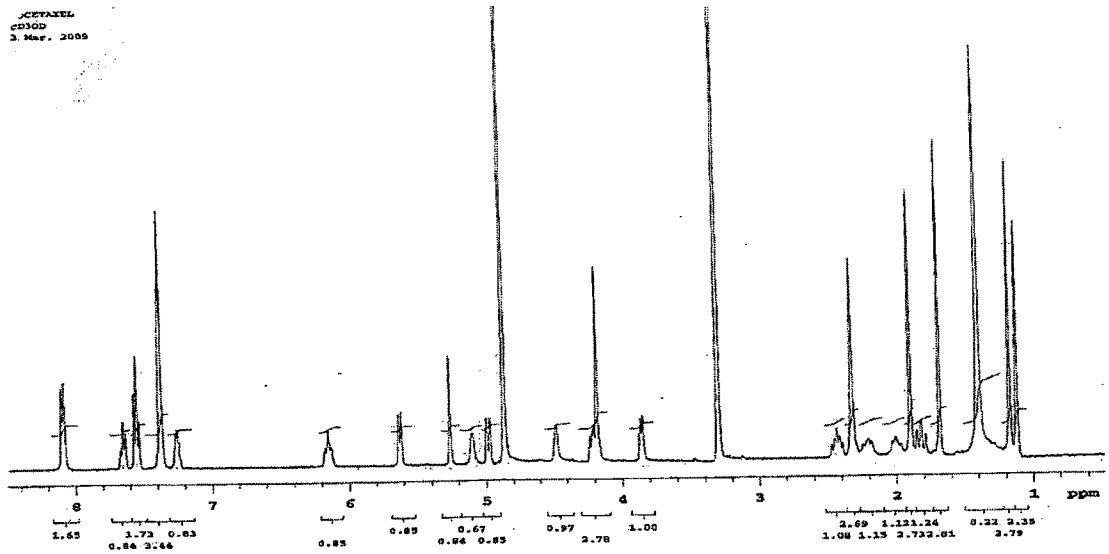


(b)

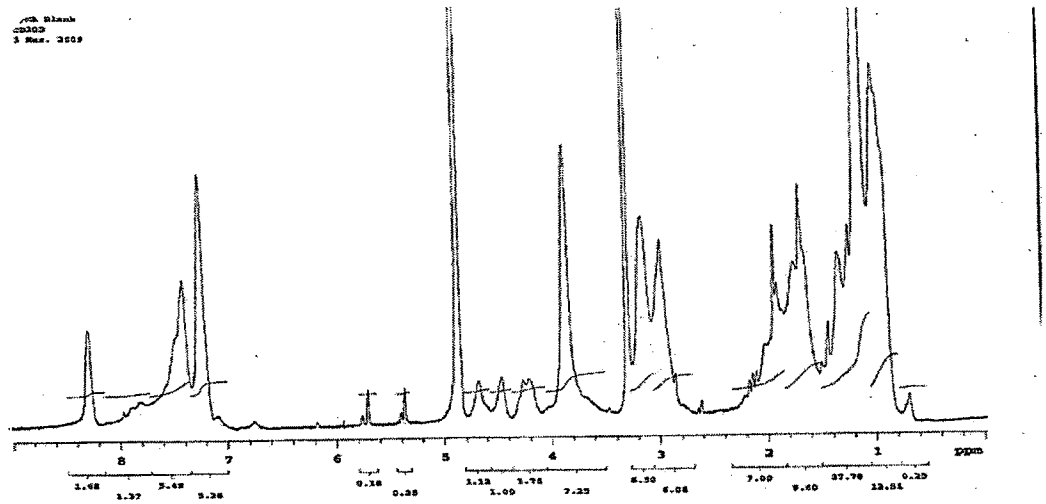
8.4.2. Characterization of drug attachment by NMR

The ^1H NMR analyses as shown in Figure: 8.6 depicts that the presence of drug in monomer as well as in polymer drug conjugates. The characteristic peak of drug at δ 8.1 is present in all drug containing moieties. However, the peak is absent in blank copolymer.

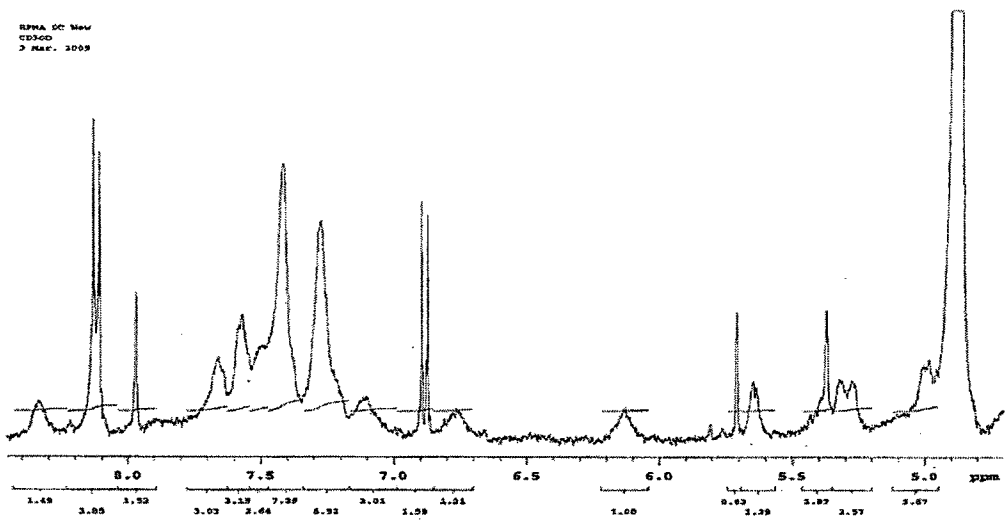
Figure: 8.6. ^1H NMR spectra of a) free Docetaxel b) MA-GFLG-Docetaxel and c) HPMA-Docetaxel d) Blank HPMA



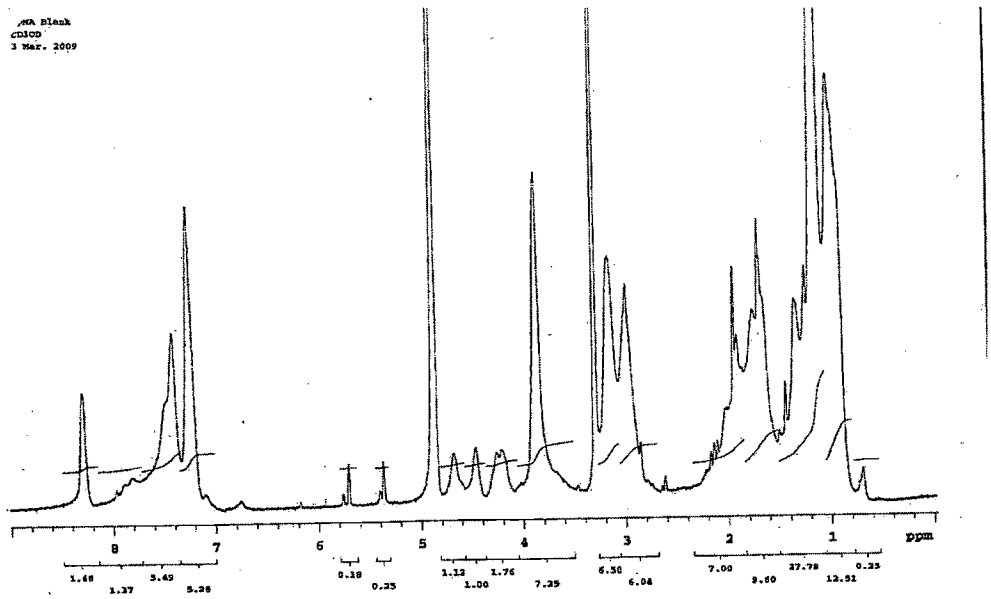
(a)



(b)



(c)



(d)

8.4.3. Determination of drug, peptide content and molecular weight of copolymer

The amount of drug and peptide attached, composition of comonomer feed ratio and molecular weight of copolymer synthesized is enumerated in Table: 8.3. Drug containing copolymers without (P1) and with (P2) RGDfK peptide targeting moiety were synthesized with an estimated molecular weight of *57.0* kDa (for precursor) with 10.3% (wt/wt) drug content. The ^{1}H NMR analyses as shown in Figure: 8.6 depicts that the presence of drug in monomer as well as in polymer drug conjugates.

Table: 8.3. Characterization of copolymer

Polymer no.	Structure	Comonomer Feed Composition (mol %)				Estimated M_w (kD)[a]	M_w/M_n [a,b]	DC content[c]		RGDfK content[d]	
		HPMA	MA-GG-ONp	MA-GFLG-DC	MA-Tyr			mmol /g	% wt/wt	mmol/g	peptides/ backbone
1	P-(GFLG-DC-GGOH) (p1)	80.5	15	2.5	2	28.0	1.4	0.128	10.3	-	-
2	P-(GFLG-DC-GG-RGDfk) (p2)	80.5	15	2.5	2	57.0	0.99	-	-	0.123	7.04

a. Molecular weight, molecular weight distribution (polydispersity) by FPLC
b. Polydispersity
c. Drug content analysis by HPLC method
d. Peptide content determination by amino acid analysis and SEC

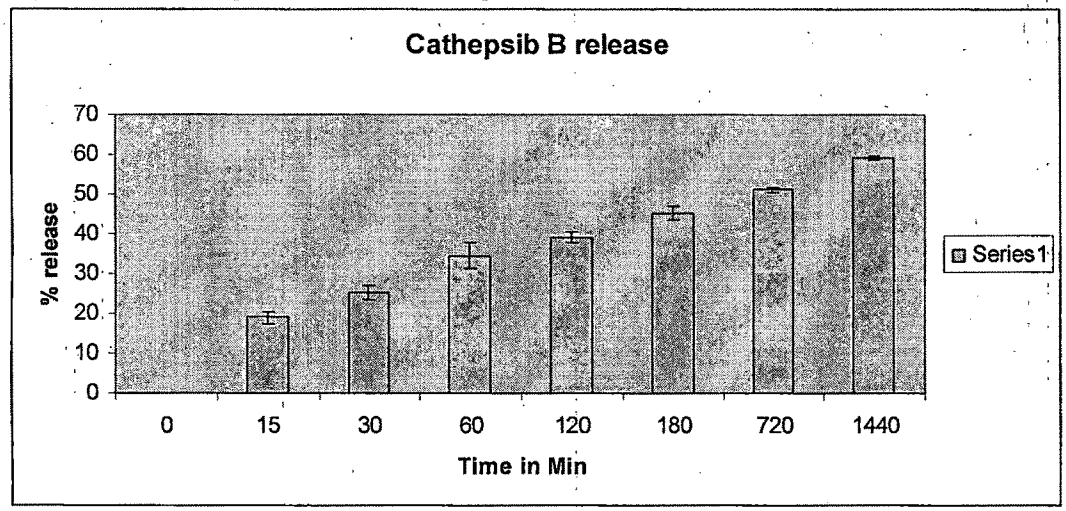
8.4.4. Cathepsin B catalyzed drug release from HPMA copolymer-DC conjugates

Extent of drug release from HPMA copolymer products was evaluated at 15, 60,180,720 and 1440 minutes (Table: 8.4 & Figure: 8.7). The Docetaxel released by CPB catalyzed hydrolysis was quantified by RP-HPLC. Extraction efficiency was calculated to be 97%. The total drug release after 1 and 24hr was found to be 34.5±3.2% and 59.2±0.4% respectively.

Table: 8.4. Cathepsin B release study

Time (hrs)	% release (avg.)	SD
0	0	0
0.25	18.96	1.429452
0.5	25.26	1.817507
1	34.5	3.214032
2	39.03	1.41892
3	45.3	1.873499
12	51.1	0.707107
24	59.2	0.458258

Figure: 8.7. Cathepsin B Release Study



8.4.5. In vitro growth inhibition

Antiproliferative activity of drug and copolymer-drug conjugates in BT-20 and HCT 116 cells are shown in Figure: 8.8a & Figure: 8.8b. IC_{50} values for all treatments were determined by non-linear regression and are given in Table: 8.5 & Table: 8.6 and Figure: 8.8a & Figure: 8.8b. Results for DC drug treatments show that the attachment of DC to HPMA copolymer- conjugates improves its efficacy as compared to blank polymer.

Figure: 8.8. Cytotoxicity curves

a) Cell toxicity study (Free Docetaxel (DC), HPMA-GFLG-DC, HPMA-GG-DC, HPMA-GFLG-DC-RGD,HPMA) for BT-20 cells

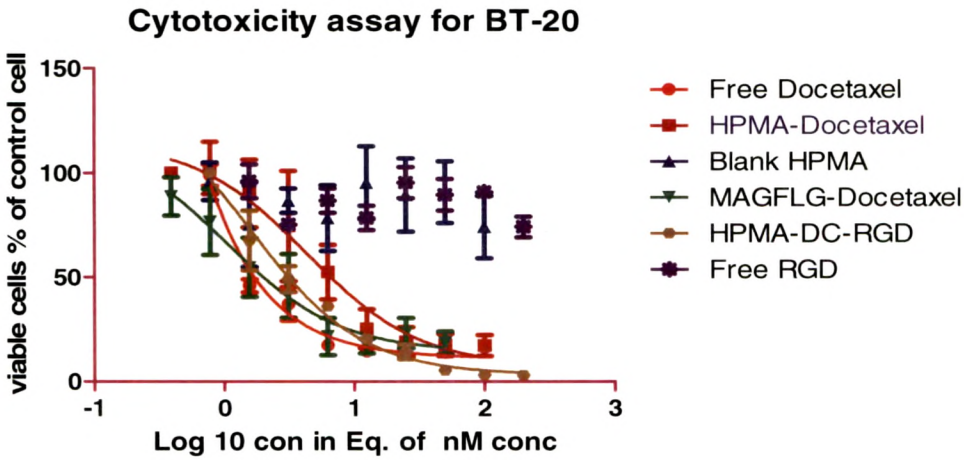


Table: 8.5. IC_{50} for BT-20 cell line

(Mean ± S.D., n = 3)

Components	IC 50 values in nM
Free Docetaxel	2.65±0.3
HPMA-Docetaxel conjugates	7±1.2
MAGFLG-Docetaxel monomer	3.6±0.5
HPMA-DC-RGD conjugate	3.8±0.1

b) For HCT 116 cells (Free Docetaxel (DC), HPMA-GFLG-DC, MAGFLG-DC, blank HPMA without drug)

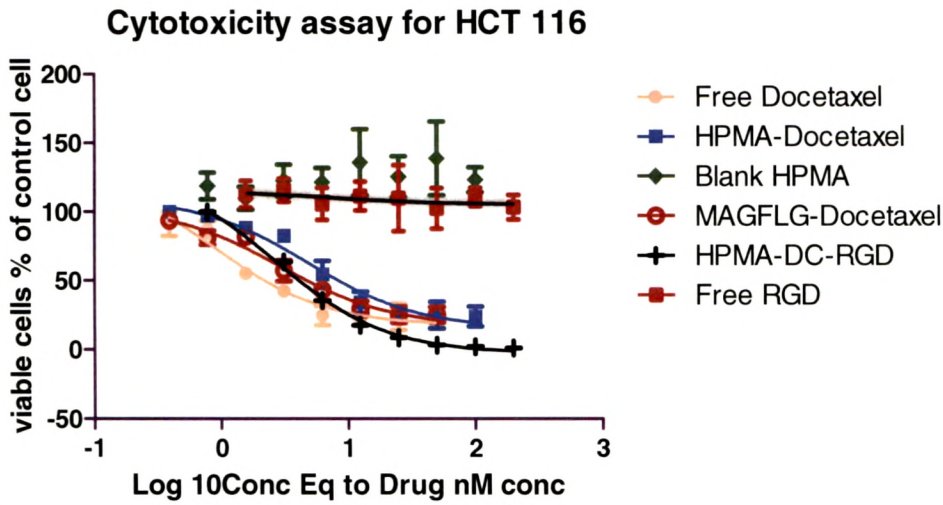


Table: 8.6. IC_{50} values for HCT 116 cell line (Mean ± S.D., *n* = 3)

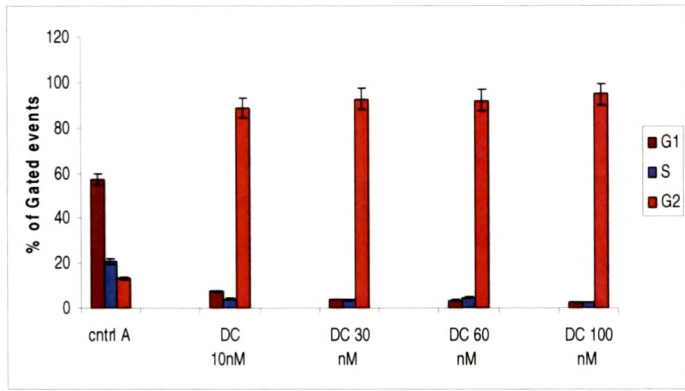
Components	IC 50 values in nM
Free Docetaxel	1.74±0.12
HPMA-Docetaxel conjugates	4.45±0.21
MAGFLG-Docetaxel monomer	4±1.97
HPMA-DC-RGD conjugate	3.7±0.1

8.4.6. DNA Content Analysis study by Flowcytometery

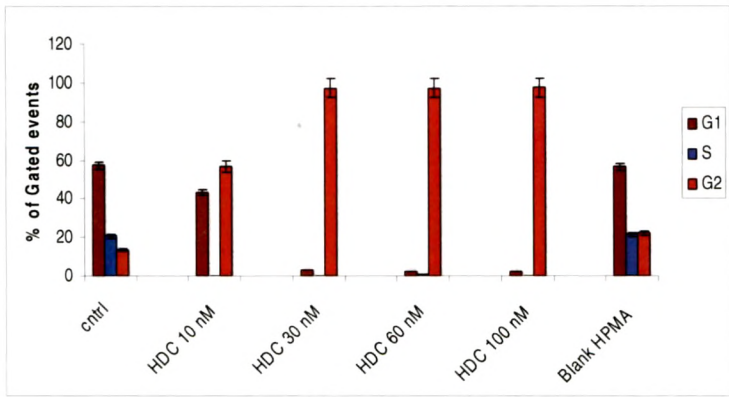
The amounts of DNA content in cells are shown in Figure: 8.9a, Figure: 8.9b & Figure: 8.9c. The data shown hereby are for the higher concentration of both Free DC and HPMA-DC. Results show the both forms for drug arrest cell cycle in G2 phase. However, the percent cell cycle block in G2 phase was insignificant between the 10nM to 100nM concentration for both free and polymer bound drug. The influence of blank HPMA without drug was found to be similar as that of the untreated cells. It was also found that the data at lower concentration was inconclusive because of the difficulties in analysis due to high amount of debris.

Figure: 8.9. DNA content analysis

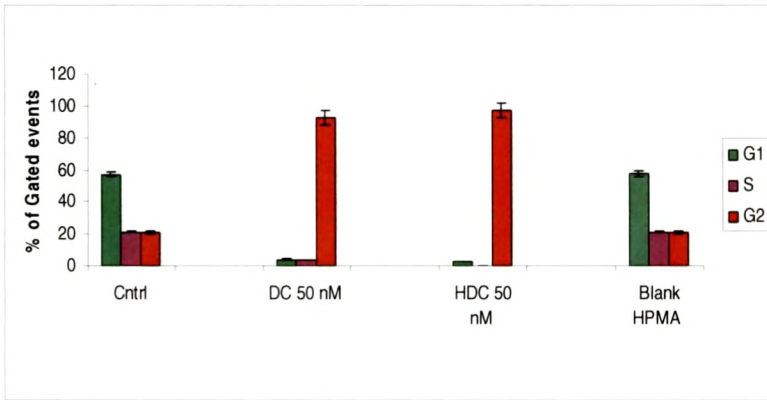
(a) BT- 20 cells with free DC



(b) BT- 20 cells with HPMA-DC and Blank HPMA



(c) HCT 116 cells with free DC and HPMA-DC:

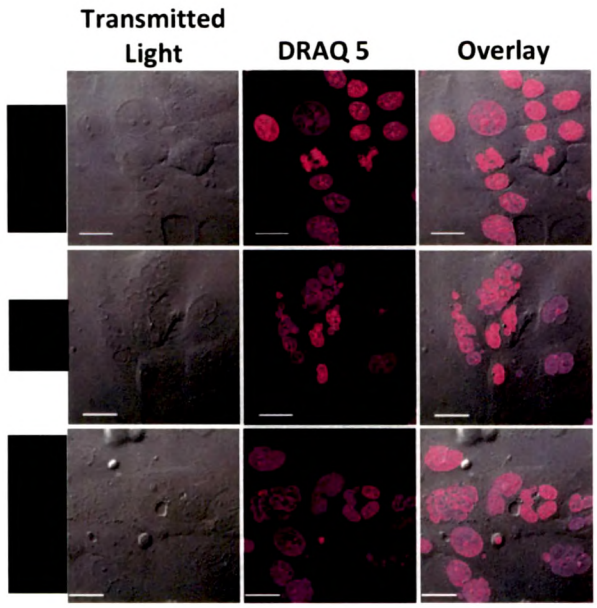


8.4.7. Confocal Microscopy

The results are shown in Figure: 8.10a and Figure: 8.10b. The multinuclear cells were observed for both types of cancer cells with free drug and polymer bound drug.

Figure: 8.10a

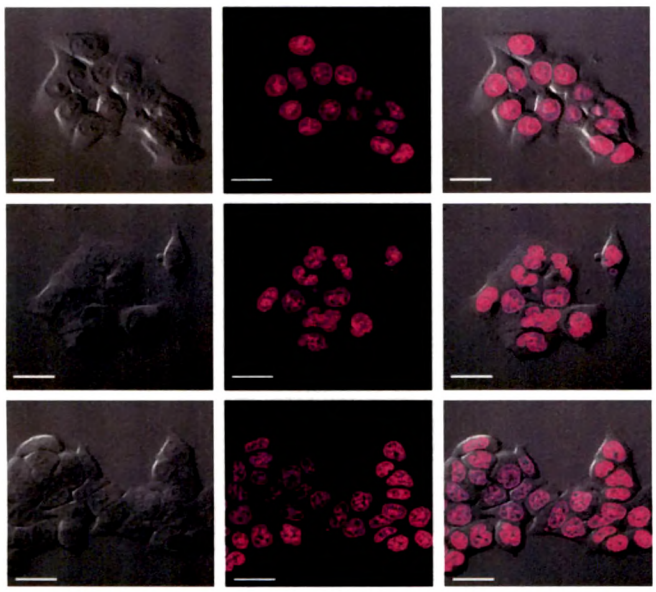
HPMA-GFLG-Docetaxel Induce Mitosis Aberration in Human Breast Cancer BT-20 Cells



Treatment with both forms of docetaxel produces a large multinucleated cells.

Cells are shown after 48 h incubation. Similar cell morphology was observed after 24 h incubation.

Figure: 8.10b. HCT 116 cell line



Cells are shown after 24 h incubation. Similar cell morphology was observed after 48 h incubation.

Nucleus stained with DRAQ 5 (magenta).

Scale bar is 20 μm

8.4.8. Stability study

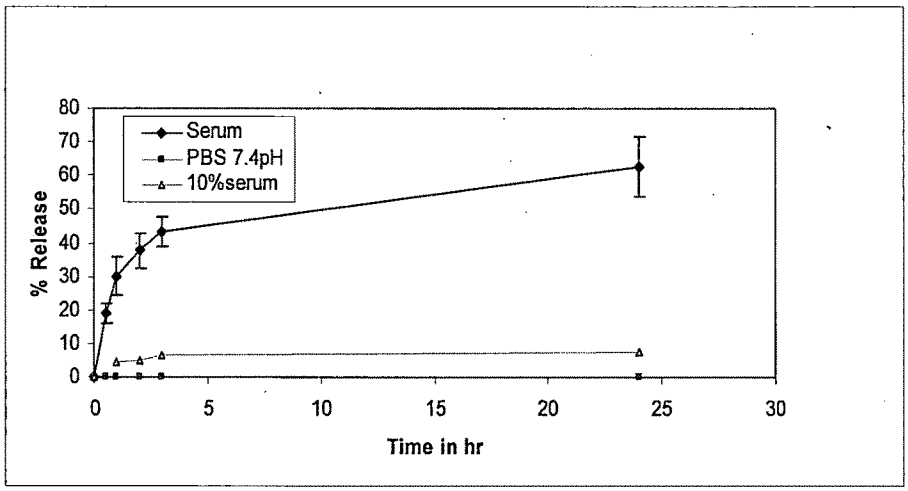
As shown in Table: 8.7 and Figure: 8.11, the Docetaxel conjugated via ester bond with HPMA backbone was found to be stable in PBS pH 7.4 for 24 hrs. While the liberated amount of free drug from the conjugate was 62.6% and 7.29% in serum and 10% serum respectively. The results suggest that the ester bond was instable in the presence of esterase enzymes in serum and the amount of drug cleaved was less in 10% serum than the 100% serum. The rate of drug release was found to be decrease with increase in time with 100% serum.

Table: 8.7. Stability studies in 100% serum, 10% serum and PBS pH 7.4

Time in hr	Serum	PBS 7.4pH	10%serum
0	0	0	0
0.5	18.83±3.06	0	0
1	30±5.54	0	4.39±0.62
2	37.56±5.06	0	5.16±0.58
3	43.46±4.46	0	6.33±0.56
24	62.6±8.88	0	7.29±0.75

(Mean ± S.D., $n = 3$)

Figure: 8.11. Stability studies in 100% serum, 10% serum and PBS pH 7.4



8.4. Discussion:

The synthesis, characterization, in vitro growth inhibition, and cell cycle arrest study of a new HPMA copolymer-RGDfK-Docetaxel conjugate was evaluated in this study. This is the first report of using HPMA copolymer-RGD conjugates for delivery of docetaxel. These copolymer conjugates can be used to actively deliver therapeutic agents to a broad spectrum of cancers with tumor angiogenesis. This work has focused on the synthesis and in vitro cytotoxicity and stability evaluation of HPMA copolymer docetaxel to breast & colon cancer cells.

HPMA copolymers were successfully synthesized and characterized containing RGDfK and docetaxel. The Docetaxel was conjugated via an ester bond with HPMA copolymer backbone. The monocyclic RGDfK was chosen for targeting to $\alpha_v\beta_3$ integrins. This peptide has a higher affinity than linear RGD sequences and greater solution stability than other doubly cyclized or linear peptide versions (Assa-Munt, N. et al., 2001). The RGDfK peptide ligand actively targets the $\alpha_v\beta_3$ integrin and can enhance the cellular entry of HPMA copolymer conjugates through receptor-mediated internalization of conjugates bound to the $\alpha_v\beta_3$ integrin (Schraa. A. J. et al., 2002). This should result in drug release from the copolymers that occurs via enzymatic degradation of GFLG peptide sequences in the lysosomal compartment.

Drug release from the conjugates was evaluated in vitro with the model lysosomal enzyme CPB to understand the release profiles of copolymer. RP-HPLC was employed to accurately quantify the released drug.

Growth inhibition results showed that the IC50 values for free drug is low for both cell lines as compared with the targeted and non targeted polymer drug conjugates. However the HPMA copolymer-DC-RGDfK conjugates affected cell proliferation more that the nontargeted one. This difference may be due to the presence of the RGDfK on the copolymer side chains causing an increased amount of intracellular accumulation of the conjugates due to receptor mediated uptake. The difference in cytotoxicity of free drug and polymer bound drug may be attributed to the lysosomally degradable linker which delays the liberation of free drug from the conjugates. The BT-20 cells were found to be

more sensitive towards all forms of drugs than the HCT-116 cells. The difference in population doubling time might be the responsible for aforesaid difference.

Cell cycle analysis demonstrated that both free DC and HPMA-DC arrested BT-20 and HCT 116 cell in G2 phase of cell cycle. There was no obvious difference in the population appeared the G2 phase in the cells treated with different concentration of both forms of drugs. Hence, cell cycle arrest was not concentration dependent. However, due to the high amount of debris we were unable to determine the cell cycle arrest in the cells treated with 1 to 5nM concentration of free and polymer bound drug.

The stability studies of the conjugates showed that the ester bond between drug and polymer backbone was not stable in serum. The percent drug release was more in 100% serum than the 10% serum. However, this bond was found to be stable in PBS pH 7.4. The study suggests that the amount of drug release was dependent on the available concentration of the esterase enzymes. Hence, the drug release was more with 100% serum. However, hydrolytic cleavage was not found for polymer drug conjugate which reveals the stability of bond in plain buffer.

The mitosis aberration was observed in both types of cells with both forms of conjugates. Docetaxel being a microtubule stablilizing agent, produces mitosis aberration which subsequently leads to formation of multinucei as nuclear envelop forms around chromosomes or group of chromosomes.

8.5. Conclusions:

HPMA copolymer-DC-RGDfK conjugates showed growth inhibition activity in vitro against Breast and Colon cell lines. Our study also demonstrated the cell cycle arrest of cancer cells. The cell cycle analysis study and in vitro stability study reveals the fact that polymer conjugated drug shows toxicity as a result of combined effect of hydrolyzed drug in the medium and enzymatically cleaved drug inside the cancer cells.

8.6. References

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