Development of L-Aspartic acid Based Polyesters and Their Nano-assemblies in Drug Delivery

A Thesis

Submitted in Partial Fulfilment of the Requirements

Of the Degree of BS-MS

BY

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20131039

UNDER THE SUPERVISION OF

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DEPARTMENT OF CHEMISTRY

INDIAN INSTITUTE OF SCIENCE EDUCATION AND PUNE

CERTIFICATE

This is to certify that this dissertation entitled "**Development of L-Aspartic acid Based Polyesters and Their Nano-assemblies in Drug delivery**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents work carried out by "**ANU PRADEEP** at IISER Pune" under the supervision of "Prof. M. Jayakannan, Professor, Chair, Department of chemistry" during the academic year 2017-2018.

Date: 20/4/2018
Place: Pune

Signature of student

Date: 20/3/2018
Place: Pune

Signature of Supervisor

DECLARATION

I hereby declare that the matter embodied in the report entitled "**Development of L-Aspartic acid Based Polyesters and Their Nano-assemblies in Drug delivery**" are the results of the work carried out by me at the Department of Chemistry, IISER Pune, under the supervision of Prof. M. Jayakannan and the same has not been submitted elsewhere for any other degree.

Date: 20/3/2018
Place: Pune

Signature of student

Date: 20/3/2018
Place: Pune

Signature of Supervisor

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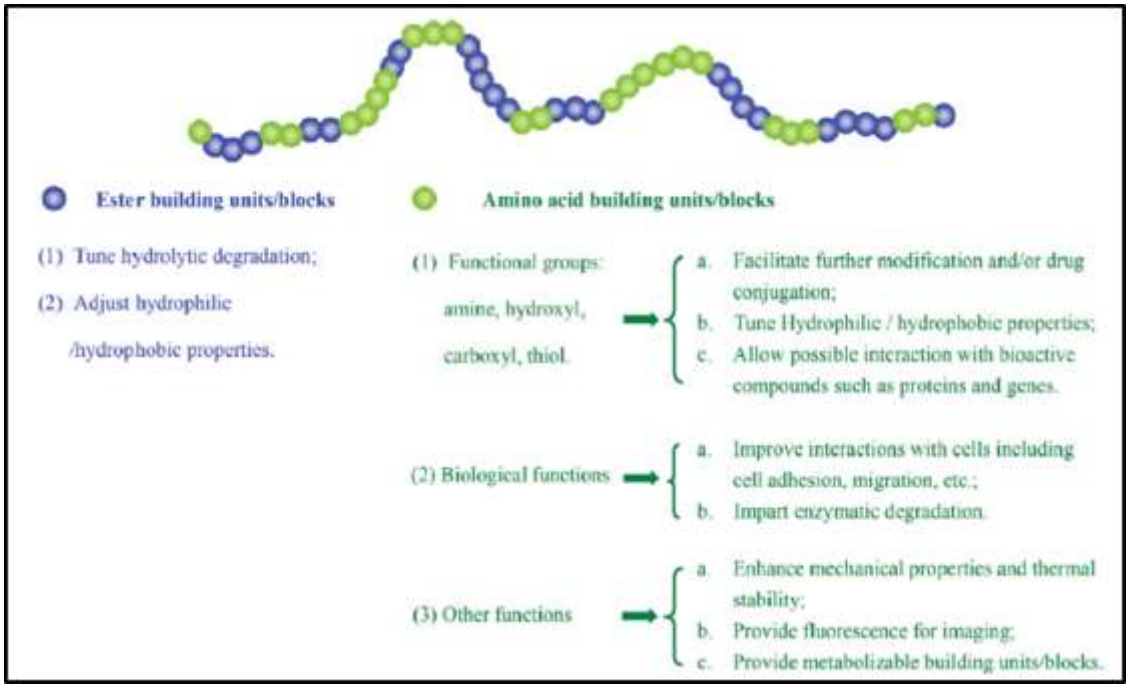
ABSTRACT

New classes of amide-functionalized L-aspartic acid polyesters were designed and developed through solvent-free melt poly-condensation approach and explored self-assembled cationic amphiphilic nanoparticles as drug carrier for cancer cells. For this purpose, natural resource L-aspartic monomers were synthesised through tailor-made approaches with benzoyl, acetyl and naphthoyl amide pendant groups. These aspartic acid monomers were subjected to melt poly-condensation with various aliphatic diols to yield high molecular weight polyesters. The structures of aspartic acid monomers and their corresponding polyesters were confirmed by ^{1}H and ^{13}C NMR spectroscopy. The molecular weight of the polyesters was determined by GPC which showed mono-model distribution. MALDI-TOF MS end group analysis confirmed that the aspartic acid monomers were thermally stable under the melt condensation and there were mainly three types of polymer linear chains. The thermal properties such as glass transition temperature and semi-crystallinity of these new polyesters were significantly varied with the change in the amide functionality in the polymer pendants. Amide and BOC-protected random co-polyesters were made by using L-aspartic acid monomers having acetyl-amide and BOC-pendent units with 1,12-dodecanediol. The selective deprotection of the BOC group in the pendant unit yielded cationic and amphiphilic co-polyesters having positive charge in the backbone. These cationic polymers self-assembled in aqueous medium and showed a size of 210±5nm and they showed excellent capability to encapsulate fluorophore such as Nile red and anticancer drugs such as doxorubicin (DOX). Confocal and flow cytometry analysis proved that these cationic polyester nanoparticles were stable and readily taken up by the cancer cells in significant quantity. Cytotoxic studies of the cationic polyester showed that the nanoparticles are non-toxic to HeLa cells.

INTRODUCTION

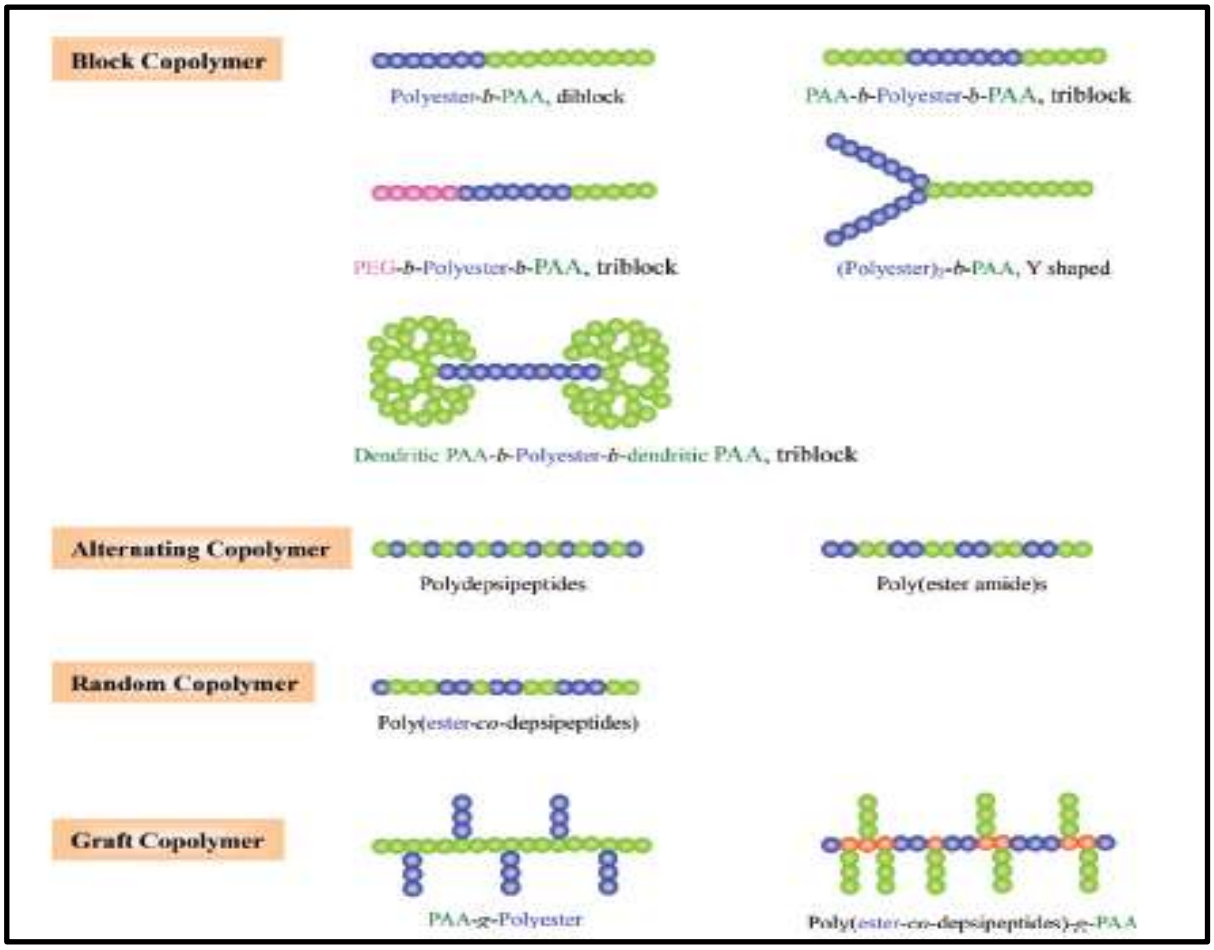
1.1. Introduction to amino acids

L-Amino acids are biological monomers and their peptide sequence and chain length played a crucial role on the size, function and secondary structure of proteins.[20] Synthetic polymers based on amino acids attracted significant interest in chemistry-biology interface due to their possible application in cosmetics, therapeutics, and biocompatible and biodegradable thermoplastics.[20] Until now, all the studies have centred on the development and application of polyesters such as poly(glycolic acid) (PGA)[1], poly (lactic acid) (PLA)[1], and polycarbonates like poly (tri-methylene carbonate (PTMC)[1] and poly (Ɛ-caprolactone) (PCL)[1], also their copolymers. These biodegradable polymers are limited in their application due to several factors like lacking surface recognition sites, hydrophobic nature of the polymers and also acidic products are produced upon degradation. Amino acid as an elementary unit for biodegradable polymers is having several characteristics: (i) impart functional groups like hydroxyl, carboxyl, thiol and amine group which improve hydrophilicity, interactions with protein and genes, also facilitate additional change with bioactive molecules like dyes or drugs; (ii) biological properties are improved including enzymatic degradability and also cell-materials interaction; (iii) mechanical and thermal properties of the materials are improved; (iv) provides metabolizable building (**see scheme1.1).**[1]



Scheme 1.1: *Schematic diagrahm of AA containing polymers and their particular functions (adopted from Zhong. Z. Biomacromolecules, 2011, 12, 1937-1955).*

There has been a remarkable development in the synthesis and biomedical application of these biodegradable polymers. Different types of amino acid containing polymers like block co-polymer[1], alternating co-polymer[1]; random co-polymer[1] and graft co-polymer[1] are shown in the **scheme 1.2**.



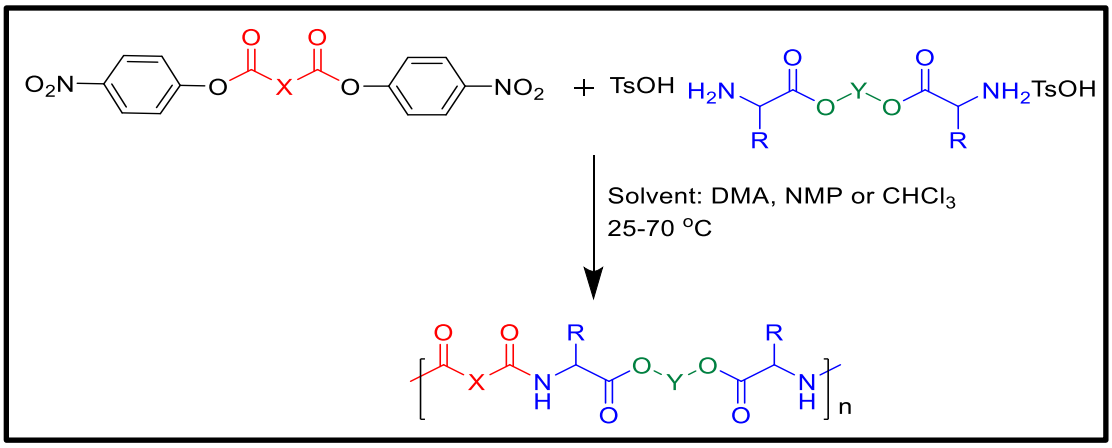
Scheme 1.2: *Schematic depiction of different macromolecular forms of amino acids containing polymers (adopted from Zhong. Z. Biomacromolecules,* ***2011****, 12, 1937-1955).*

1.2. Synthesis of amino acid containing polymers

Different synthetic methods have been investigated for the synthesis of polymers having amino acid including solution poly-condensation, interfacial poly-condensation, solvent-free poly-condensation, ring opening polymerization.

1.2.1. Solution poly-condensation: The solution poly-condensation of polymers containing amino acid was carried out by using di-p-toluenesulfonic acid[1] salts of amino acid di-esters with di-p-nitrophenyl[1] esters of diacids[1](**scheme 1.2.1**). These polyester-amides and polyether-ester-amides can be modified by varying starting di-acids or diols. This polymerization reaction can be completed in mild condition

without using toxic catalytic materials however, this process requires immense purification to avoid the huge quantities of side products produced.

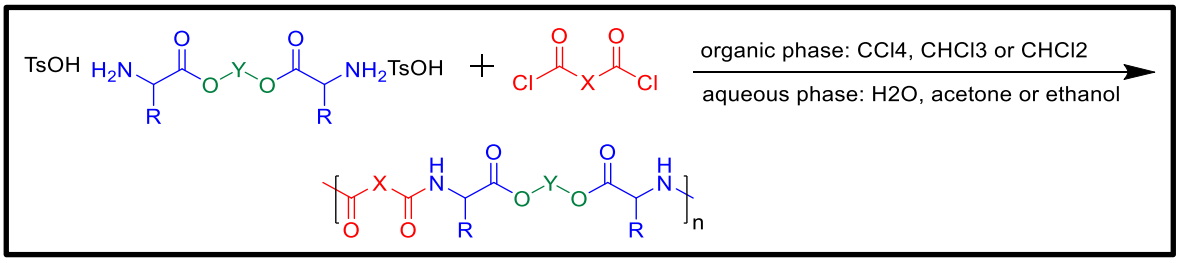


Scheme 1.2.1: *Synthesis of polyester-amides based on Solution Poly-condensation.*

Arabuli and co-workers synthesized a sequence of biodegradable L-Phenyl alanine based polymers which are easy to synthesize and purify. They made polyester-amides by using bis(p-nitrophenyl) adipate[1] with di-p-toluenesulfonic acid[1] salts of 1,2-ethanediol[1] bis-L- Phe diesters at 25 °C for 48 h in NMP or $CHCl_3$.[2,1]

Gillies et al. used amino acids having functional side groups, which provide an opportunity to conjugate drug molecules, targeting and signaling molecules. He and co-workers synthesized a series of amine functionalized polyester-amides by including L-aspartic acid and L-lysine into polymer backbone based on L-phenylalanine and L-alanine[1], diacids like succinic acid[1] and terephthalic acid[1], the diols like 1,4-butanediol and 1,8-octanediol.[3] These protected functional groups can be further de-protected and modified accordingly.

1.2.2. Interfacial polymerization: Interfacial polymerization was conducted at the interface of one monomer in aqueous solution and the other monomer in water immiscible organic solvents.

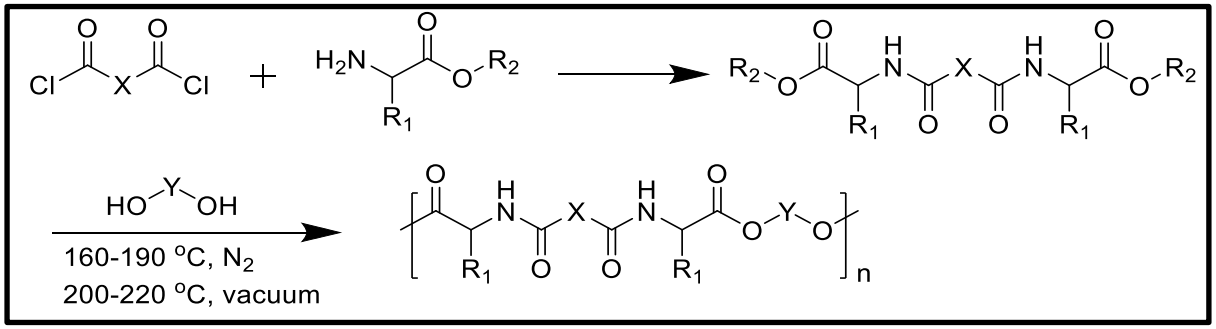


Scheme 1.2.2: *Synthesis of polyester-amides based on Interfacial Polymerisation.*

Polyester-amides are synthesized using di-p-toluenesulfonic acid[1] salts of bis-(amino acid)-alkylene[1] di-esters and di-acid chlorides at the interface of aqueous Na_2CO_3 solution and organic solvents[1] (**scheme 1.2.2**).

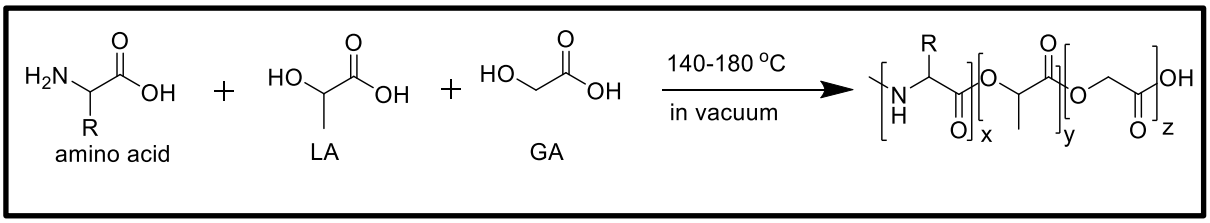
Puiggali et al. synthesised a new polyester-amide from di-p-toluenesulfonic acid[1] salts of bis-L-alanine[1] 1,12-dodecylene diester[1] and sebacoyl chloride[1] at the interface of Na_2CO_3 aqueous solution and a mixture of CCl_4 and acetone as organic solvent.[4]

1.2.3. Solvent-free Poly-condensation: Puiggali and co-workers synthesized polyester-amides with Glycine, di-acid chloride and diols by solvent-free thermal poly-condensation (**Scheme 1.2.3**).[5] The reaction was conducted at 160-190^oC in an N_2 atmosphere followed by vacuum at 200-220^oC. This method yielded polyester-amides of higher molecular weight at higher conversion than interfacial polymerization.



Scheme 1.2.3: *Synthetic pathway polyester-amides from amino acid esters, diols and di-acid chlorides by solvent free poly-condensation.*

Li and co-workers synthesized hyper-branched polyester-amides by self-condensation of monomers based on amino acids and gallic acid (**Scheme 1.2.4**).[6]



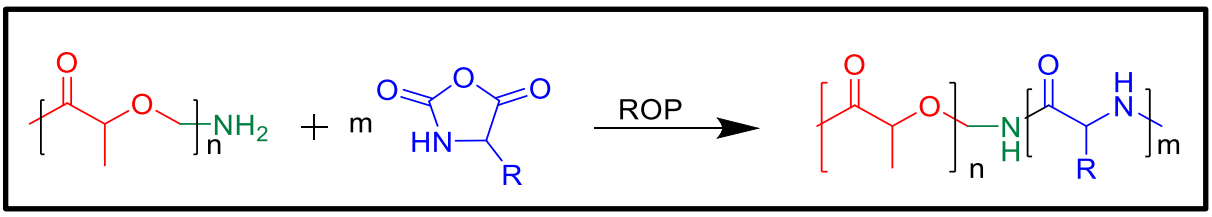
Scheme 1.2.4: *Synthetic pathway polyester-amides from amino acid and hydroxyl acids by solvent free poly-condensation.*

Biodegradable and amine-functionalized poly (LA-co-GA-co-4-hydroxyproline) (poly (LGA-co-Hyp)[1] were synthesized by using lactic acid, gallic acid and N-acetate Hyp and stannous chloride dihydride catalyst by direct melt condensation.[1] The

polymerization reaction was carried out at 180 or 150 ^{o}C for 14 h under vacuum condition.[7] The amphiphilic biodegradable polymers were synthesized using glutamic acid, gallic acid, lactic acid by direct melt condensation with stannous chloride at 180 ^{o}C under vacuum for 10h.[8]

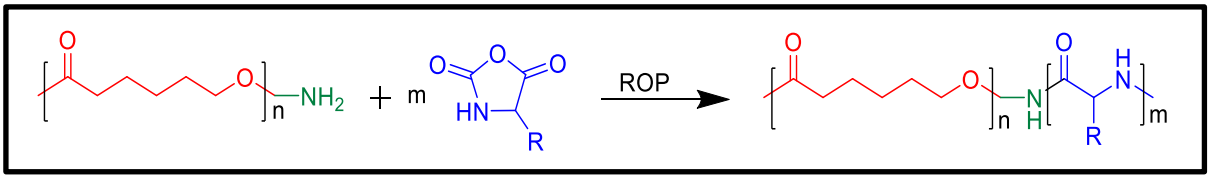
1.2.4. Ring-opening polymerisation of N-Carboxyanhydride (NCA): The ring opening polymerisation of NCA monomer initiated either by alcohol or amine in the presence of a catalyst yielded polyester-polypeptides block copolymers with distinct macromolecular architectures and characteristics.[9] ROP approach has a lot of advantages such as (i) molecular weight of the polymers can be controlled based on the monomer to initiator ratio (ii) narrow dispersity of the polymer (iii) living nature of the ROP can be used to make diverse polymeric structures such as block, graft.[10,11] A sequence of amphiphilic di-block, triblock, and comb-liked graft copolymers has been made using hydrophilic amino acids like glutamic acid, aspartic acid, and lysine.

PLLA-b-PAA (poly(lactic acid)-b-poly(amino acid)) diblock copolymers were synthesized by ring opening polymerization of NCA using amine-terminated PLLA macroinitiator (**scheme 1.2.5**). Caillol and co-workers synthesized PLLA-b-poly (benzyl-L-Glu) (PLLA-b-PBLG)[1] by ring opening polymerization of BLG-NCA[1] using amino-propoxy-terminated PLLA[1] in DCM at rt for 3 h.[12]



Scheme 1.2.5: *ROP of NCA with PLLA-NH$_2$ macro-initiator*

Likewise, PCL-b-PAA di-block copolymers were synthesized by ring opening polymerization of NCA using amine-terminated PCL[1] macroinitiator (**scheme 1.2.6**). Chen and co-workers synthesized PCL-b-PBLG block copolymers in DCM using aminophenyl terminated PCL at rt for 24 hrs. After deprotection, the resulting carboxyl groups were used to antibodies and couple peptides for further applications.[13]



Scheme 1.2.6: *ROP of NCA using PCL-NH$_2$ macro-initiator.*

Tri-block copolymers were synthesized by means of amine terminated PEG-b-polyester[1] or di-amine terminated polyester[1] as a macroinitiator. Kricheldorf et al. synthesized ABA tri-block copolymers of four amino acids like glycine, alanine, phenylalanine and glutamic acid using bis (4-aminobenzoyl) terminated PCL[1] as a macroinitiator. The ring opening polymerization of NCA was conducted in DCM at room temperature for 5 days and they have also done reactions by varying the NCA monomer to macroinitiator feed.[14]

1.2.5. Synthesis of Amino Acids containing polymers via coupling reactions: PLGA –b-PLys block[1] co-polymers was synthesized by coupling of carboxyl terminated PLGA to PZ-Lysine in the presence of DCC and DMAP.[15,1] The biodegradable graft copolymers were synthesized by grafting carboxyl terminated PLGA[1] on to P-Lys in the presence of DCC.[16,1] These polymers were synthesized to demonstrate the micelle formation ability in an aqueous solution which can be used as a gene carrier.

1.2.6. Ring-opening polymerization of Depsipepetides: Poly depsipeptides are made up of alternating copolymers of α- amino acids and α-hydroxy acids.[17] Feijen and co-workers reported a synthetic pathway for poly depsipeptides by ring opening polymerization of morpholine-2,5-dione derivatives.[1,18] Due to controlled and living nature of ROP, depsipeptides provided various polymer structures including alternating, random, di-block, tri-block and graft copolymers. The important features of these polymers are (i) these can be developed without or with pendent functional groups like carboxyl, thiol, hydroxyl and amine groups depending upon the type of amino acids used in the polymerisation (ii) the physicochemical properties and the rate of degradation can be tuned by varying α- amino acids and α-hydroxy acids.[1]

1.3. Biomedical application

Amino acid containing polymers are best materials with various chemical functionalities which improved biological properties, including cell adhesion properties, enzyme degradability, enhanced mechanical and thermal properties. Biomedical application of polymers containing amino acids is in tissue engineering, drug delivery, and bioimaging of which, drug delivery will be discussed in detail.

1.3.1. Drug delivery: Polymer-based controlled drug delivery plays an important role in enhancing drug targeting, guard the bioactive drug [19]from the aggregation of the host, guard the host from the harmful effects of the drug[18], avoid repeated administration, increase the therapeutic index[18], enhance the solubility of hydrophobic drug molecules in water[18] and deliver the drug at pre-determined doses.[19] Many of the pharmaceutical agents are hydrophobic in nature; therefore two approaches can be made to attach drug molecules with the polymer carrier: (i) physical incorporation (ii) chemical conjugation to achieve a better control over drug release and targeting. Various self-assembled amphiphilic polymeric molecules have been used as polymeric drug carriers namely, microspheres, nanoparticles, micelles, vesicles, hydrogels, etc. Polymer-based drug delivery is attractive platforms compared to small molecule drug carrier due to Enhanced Permeability and Retention (EPR) effect.[20] Based on these concept polymeric scaffolds are expected to have an enhanced accumulation of the drug in tumor tissues compared to normal tissues.

From our research group, we published a novel melt poly-condensation chemistry for L-amino acid specialty monomers to produce new series of linear and hyperbranched poly (ester-urethane)s.[20,21,22,23] This solvent-free melt method was further tuned into a thermo-selective poly-condensation approach to enable the syntheses of redox-degradable and biodegradable amphiphilic polymers.[23,24] The amphiphilic polymers were self-assembled in the aqueous solution as 200±10 nm nanoparticles and they showed exceptional encapsulation abilities for anticancer drugs and dyes. These new polymers were also inbuilt with pH, enzyme, and thermo-responsiveness for delivering multiple anticancer drugs like doxorubicin, topotecan, camptothecin, and curcumin, etc. In-vitro drug release studies have shown that the drug-loaded L-tyrosine and L-aspartic acid-based polymer nanoparticles were stable under extracellular conditions and they undertook enzymatic-biodegradation completely at the intracellular level to liberate the drugs.[25,26] The drug administration studies in breast cancer, cervical cancer and normal WT-MEFs cell-lines shown that the L-tyrosine nanoparticles were not toxic whereas the CPT and DOX drug-loaded polymer nanoparticles presented exceptional killing of cells in cancer cells.[27] Fluorescent-tagged biodegradable

polymer nanoparticle probes were also accomplished to track drug dissociation kinetics in the cytoplasm of the cancer cells via FRET mechanism.[27]

1.4. Aim of the thesis work

In this thesis work dicarboxylic acid containing L-aspartic acid has been used and modified into dicarboxylic acid containing monomer. The amine part of the amino acid is deliberately functionalized into amides using molecules like benzoyl chloride, naphthoyl chloride, acetic anhydride and Boc anhydride. The aromatic rings that are present in benzyol chloride and naphthoyl chloride will help in the packing of the polymers due to their intrinsic pi-pi stacking behaviour which may lead to the formation of highly crystalline polymers. Apart from giving high stability to polymers due to aromaticity naphthoyl derivatives would also yield highly luminescent polymers which could be used for bioimaging purposes in cells. All these polymers are hydrophobic in nature and to make these polymers amphiphilic, efforts have been made to make copolymers using acetyl and Boc monomer. Upon chemical de-protection of pendent Boc groups without disturbing the ester backbone yielded cationic functional polyesters. This cationic amine functional group imparts amphiphilicity to the polymers. These amphiphilic cationic polymers formed self-assembled structures in an aqueous medium and were able to load drugs for delivery applications.

EXPERIMENTAL METHODS

2.1. Materials: L-Aspartic acid, 1,12-dodecanediol, 1,10-decanediol, 1,6-hexanediol, Naphthyol chloride, PEG-400,Boc anhydride, Hexaethylene glycol, triethylene glycol, PEG-350 monomethyl ether, Triethylene glycol mono methyl ether, N,N′-Dicyclohexylcarbodiimide and Titanium tetrabutoxide ($Ti(OBu)_4$ were bought from Sigma Aldrich chemicals and utilized without additional purification. Thionyl chloride, Benzoyl chloride, acetic anhydride, 4-Dimethylaminopyridine, 1-Hydroxybenzotriazole, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, sodium carbonate, potassium carbonate and other solvents were bought locally and purified before using.

2.2. General Procedures: ^{1}H and ^{13}C NMR were recorded using 400 MHz JEOL and BRUKER NMR spectrophotometer. NMR spectra for the compounds were recorded in $CDCl_3$ and MeOH-d_4 solvent having TMS as internal standard. The monomer molecular mass were analysed using HRMS-ESI-Q-time of flight LCMS. GPC (Gel Permeation Chromatography) analysis was done using Viscotech RI and Viscotech UV-Vis detector in Tetrahydrofuran (THF) solvent using standards of polystyrene at 25 ^{0}C. Polymer thermal stability was estimated using PerklinElmer thermal analyser at a heating rate of 10 ºC/min in N_2 atmosphere. Thermal analysis for all the polymers was carried out using TA Q20 Differential Scanning Calorimeter. All the polymers were heated to melt prior to recording in order to get rid of previous thermal history. Polymers were heated and cooled at a rate of 10 ºC/min in nitrogen atmosphere. The size of the functionalized polyesters was carried out by dynamic light scattering (DLS) by Nano ZS-90 apparatus from Malvern Instruments. The absorption spectra were noted using Perkin-Elmer Lambda 45 UV-Vis spectrophotometer. The emission studies were completed using SPEX Fluorolog HORIBA JOBIN VYON fluorescence spectrophotometer.

2.2.1 Synthesis of Dimethyl benzoyl Aspartate: L-Aspartic acid (10g, 0.055mol) in dry methanol (80ml) was occupied in a round bottom flask. Thionyl chloride (5.95ml, 0.083mol) was added in a dropwise manner under nitrogen atmosphere at 0 ºC. After complete addition, reaction mixture was let to warm till room temperature and was refluxed for 6 h under nitrogen condition. The additional of thionyl chloride and methanol were eliminated under reduced pressure. A white solid mass of aspartate ester was obtained. This white solid mass was dissolved in triethylamine

and dry DCM. After complete dissolution benzoyl chloride was added in a dropwise manner under nitrogen condition at 0 ºC. After addition, the reaction mixture was permitted to come to room temperature and let the reaction complete for 3 h. The organic solvent was eliminated and the obtained solid was purified by column chromatography using pet ether and ethyl acetate as eluent. Yield = 93% (12g). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 7.83 (d, 2H, C**H**), 7.55 (t, 1H, C**H**), 7.48 (t, 1H, C**H**), 7.26 (d, 1H, N**H**), 5.09 (q, 1H, C**H**), 3.83 (s, 3H, CHCOOC**H**$_3$), 3.73 (s, 3H, CH_2COOC**H**$_3$), 3.20-2.98 (m, 2H, C**H**$_2$). ^{13}C-NMR (100 MHz, $CDCl_3$) δ ppm: 171.88, 171.35, 167.04, 133.64, 132.02, 128.72, 127.25, 53.04, 52.19, 48.96, and 36.14. HRMS (ESI+): m/z [M+H] + calculated for $C_{13}H_{15}NO_5H$ [M+]: 266.0950; found: 266.1028.

2.2.2. Synthesis of Dimethyl (2-Naphthoyl) Aspartate: Aspartic ester (2g, 0.010mol) was dissolved in triethylamine (4.27ml, 0.30mol) and dry DCM. After complete dissolution, naphthoyl chloride (1.73g, 0.009mol) was added under nitrogen condition at 25 ºC. Leave the reaction for 24 h. After completion, the organic solvent was removed and the obtained crude was purified by column chromatography using methanol and dichloromethane as eluent. Yield = 50% (1.57g). ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 8.36 (s, 1H, C**H**), 7.53 (t, 1H, C**H**), 7.98-7.88 (m, 4H, C**H**), 7.59 (m, 2H, C**H**), 7.42 (d, 1H, N**H**), 5.16 (q, 1H, C**H**), 3.85 (t, 3H, CHCOOC**H**$_3$), 3.75 (t, 3H, CH_2COOC**H**$_3$), 3.24-3.04 (m, 2H, C**H**$_2$). ^{13}C-NMR (100 MHz, $CDCl_3$) δ ppm: 171.93, 171.44, 167.06, 135.03, 132.64, 130.84, 129.12, 128.63, 127.94, 127.85, 126.92, 123.66, 53.07, 52.22, 49.07 and 36.21. HRMS (ESI+): m/z [M+H] + calculated for $C_{17}H_{17}NO_5H$ [M+] 316.1107; found: 316.1185.

2.2.3. Synthesis of Dimethyl Acetyl Aspartate: Aspartic ester (2g, 0.01mol) was dissolved in triethylamine (1.69ml, 0.012mol) and dry DCM. After complete dissolution, acetic anhydride (1.15ml, 0.012mol) was added in a dropwise manner under nitrogen condition at 25 ºC. Leave the reaction for 12 h. After completion, the organic solvent was eliminated and the reaction mixture was extracted into ethyl acetate. The obtained product was further purified by column chromatography using ethyl acetate and pet ether as eluent. Yield = 50% (1g). ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 6.63 (d, 1H, N**H**), 4.81 (q, 1H, C**H**), 2.76 (s, 3H, CHCOOC**H**$_3$), 3.64 (s, 3H, CH_2COOC**H**$_3$), 3.01-2.78(m, 2H, C**H**$_2$), 1.99 (s, 3H, C**H**$_3$). ^{13}C-NMR (100 MHz,

$CDCl_3$) δ ppm: 171.75, 171.29, 169.97, 52.94, 52.15, 48.50, 36.15 and 23.24. HRMS (ESI+): m/z [M+H] + calculated for $C_8H_{13}NO_5H$ [M+H]: 204.1940; found: 204.0872.

2.2.4. Synthesis of Dimethyl (tert-butoxy carbonyl)-L-aspartate: To the aspartate ester (5g, 0.025mol), sodium carbonate (8.03g, 0.07mol) in distilled water (80ml) and THF (80ml) were added with stirring. BOC anhydride (5.82ml, 0.025mol) in THF was added in a dropwise manner in an ice-cold condition. The reaction mixture was warmed to room temperature (rt) and the reaction was allowed to continue for 12 h. THF was removed under reduced pressure and the compound was extracted into ethyl acetate. The organic layer was secluded and dried over anhydrous sodium sulfate. The solvent was evaporated and the obtained product was further purified by column chromatography using ethyl acetate and pet ether as eluent. Yield = 83.3% (5.5g). ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 5.51 (d, 1H, N**H**), 4.60 (q, 1H, C**H**), 3.79 (s, 3H, CHCOOC**H**$_3$), 3.72 (s, 3H, CH_2COOC**H**$_3$), 3.06-2.82(m, 2H, C**H**$_2$), 1.48 (s, 9H, NHCOOC (C**H**$_{3)3}$). ^{13}C-NMR (100 MHz, $CDCl_3$) δ ppm: 171.55, 171.43, 80.19, 52.75, 52.64, 49.93, 36.69 and 28.31. HRMS (ESI+): m/z [M+H] + calculated for $C_{11}H_{19}NO_6H$ [M+H]: 262.1212; found: 262.1290.

2.3.1. Synthesis of Homo-polyesters: Benzoyl aspartate monomer (0.5g, 0.002mol) and 1,12-dodecanediol (0.4g, 0.002mol) were taken in a test-tube shaped polymerisation tube and allowed to melt by keeping the tube in the oil bath at 100ºC. The polymer tube was made moisture and oxygen free by nitrogen purging and consequent evacuation by vacuum (10^0 m bar) thrice. Titanium tetrabutoxide (1 mol%) as catalyst was added and purged with nitrogen for 4 h at 140 ºC with constant stirring. The viscous melt was then exposed to high vacuum (10^{-2} m bar) for 2 h at 140 ºC. Methanol was removed during this stage. At the end of the polymerisation, a transparent high viscous polymer was obtained. Yield = 97% (0.69g). ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 7.83 (d, 2H, C**H**), 7.53 (t, 1H, C**H**), 7.46 (t, 1H, C**H**), 7.27 (d, 1H, N**H**), 5.05 (q, 1H, -C**H**), 4.20 (t, 2H, CHCOOC**H**$_2$), 4.10 (t, 2H, C**H**$_2$COOCH_2) 3.81 (s, 3H, CHCOOC**H**$_3$), 3.72 (s, 3H, CH_2COOC**H**$_3$), 3.65 (s, 1H, O**H**), 3.17-2.96 (m, 2H, C**H**$_2$), 1.67-1.25 (m, 2H, CH_2C**H**$_2$OH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ ppm: 171.41, 170.95, 167.00, 133.81, 131.93, 128.68, 127.20, 66.18, 65.38, 49.11, 36.37, 29.57, 29.29, 28.54, 25.96 and 25.88.

2.3.2. Melt Poly-condensation Procedure for the synthesis of Naphthoyl Aspartate Polymer: Similarly, Naphthoyl Aspartate monomer (0.5g, 0.001mol) was polymerised with 1, 12 –dodecanediol (0.33g, 0.002mol) to produce highly viscous polymer. Yield = 97% (0.7g) ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 8.34 (s, 1H, C**H**), 7.53 (t, 1H, C**H**), 7.89 (m, 4H, C**H**), 7.56 (m, 2H, C**H**), 7.43 (d, 1H, N**H**), 5.11 (q, 1H, C**H**), 4.21 (t, 2H, CHCOOC**H**$_2$), 4.11 (t, 2H, CH_2COOC**H**$_2$), 3.20-3.02 (m, 2H, C**H**$_2$), 1.68-1.20 (m, 20H, C**H**$_2$). ^{13}C-NMR (100 MHz, $CDCl_3$) δ ppm: 171.37, 170.94, 132.58, 130.93, 129.01, 128.51, 127.75, 126.80, 123.59, 66.14, 65.32, 49.19, 36.37, 29.51, 29.21, 28.56, 28.49 and 25.89.

2.3.3. Melt Poly-condensation Procedure for the synthesis of Acetyl Aspartate Polymer: Similarly, Acetyl Aspartate monomer (0.5g, 0.001mol) was polymerised with 1, 12 –dodecanediol (0.52g, 0.002mol) to produce highly viscous polymer. Yield = 97% (0.79g).^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 6.5 (d, 1H, N**H**), 4.85 (q, 1H, C**H**), 4.16 (t, 2H, CHCOOC**H**$_2$), 4.09 (t, 2H, C**H**$_2$COOCH_2), 3.06-2.85 (m, 2H, C**H**$_2$), 2.05 (s, 3H, C**H**$_3$), 1.66-1.29 (m, 20H, C**H**$_2$). ^{13}C-NMR (100 MHz, $CDCl_3$) δ ppm: 171.41, 170.96, 167.10, 133.80, 131.93, 128.68, 127.20, 65.23, 48.65, 36.35, 29.57, 29.27, 28.55, 28.47, 25.89 and 25.80.

2.3.4. Melt Poly-condensation Procedure for the synthesis of Boc Aspartate Polymer: Similarly, Boc Aspartate monomer (0.5g, 0.001mol) was polymerised with 1, 12 –dodecanediol (0.4g, 0.002mol) to produce highly viscous polymer. Yield = 97% (0.74g).^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 5.52 (d, 1H, N**H**), 4.57 (q, 1H, C**H**), 4.17 (t, 2H, CHCOOC**H**$_2$), 4.08 (t, 2H, C**H**$_2$COOCH_2), 3.03-2.80 (m, 2H, C**H**$_2$), 1.47 (s, 9H, NHCOOC(C**H**$_{3)3}$), 1.66-1.28 (m, 20H, C**H**$_2$). ^{13}C-NMR (100 MHz, $CDCl_3$) δ ppm: 171.25, 171.14, 155.51, 80.09, 68.06, 65.96, 65.27, 50.10, 42.10, 36.88, 32.87, 29.66, 29.60, 28.38, 25.95, 25.88 and 25.69.

2.3.5. Synthesis of Co-polyesters: The procedure described here in detail for the copolymer with 50:50 mol ratio of Acetyl Aspartate, BOC monomer and 1,12-dodecanediol. Acetyl aspartate (0.25 g, 0.0012 mol) and BOC monomer (0.32 g, 0.0012 mol) and 1,12-dodecanediol (0.50 g, 0.0024 mol), titanium tetrabutoxide (0.01 g, 0.96 mol %) were taken and melt polymerisation was conducted as described for homopolymers. Yield = 98% (0.87g). ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 6.54 (d, 1H, N**H**), 5.52 (d, 1H, N**H**), 4.84 (q, 1H, C**H**), 4.56 (q, 1H, C**H**), 4.15 (t,

2H, CHCOOC**H**$_2$), 4.08 (t, 2H, CH_2COOC**H**$_2$), 3.05-2.79 (m, 2H, C**H**$_2$), 2.05 (s, 3H, **C**H_3), 1.48 (s, 9H, NHCOOC (C**H**$_3$)$_3$), 1.68-1.28 (m, 20H, C**H**$_2$). ^{13}C-NMR (100 MHz, $CDCl_3$) δ ppm: 171.31, 170.90, 169.91, 155.52, 80.08, 66.09, 65.96, 65.31, 50.09, 48.65, 36.88, 36.37, 32.87, 29.51, 29.60, 28.38, 25.95, 25.87 and 253.23.

Copolymers were made by changing the composition of acetyl aspartate and Boc monomer from 10 to 90% feed from procedure.

2.3.6. De-protection of functional polyesters: Copolymer (0.500g, 0.001mol) was dissolved in dichloromethane (5 ml). Trifluoroacetic acid (1 ml, 0.013mol) was added dropwise into the polymer solution in an ice-cold condition. The reaction mixture was permitted to stir at room temperature for 2 hr. After the completion of the reaction DCM and TFA were removed under reduced vacuum. Yield = 80% (0.288g). ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 4.85 (q, 1H, C**H**), 4.37 (q, 1H, C**H**), 4.18 (t, 2H, CHCOOC**H**$_2$), 4.09 (t, 2H, CH_2COOC**H**$_2$), 3.16-2.86 (m, 2H, C**H**$_2$), 2.15 (s, 3H, **C**H_3), 1.65-1.29 (m, 20H, C**H**$_2$). ^{13}C-NMR (100 MHz, $CDCl_3$) δ ppm: 187.81, 165.75, 165.53, 159.74, 88.42, 80.91, 66.50, 65.65, 49.08, 40.06, 36.02, 31.04, 29.56, 28.42, 28.15 and 25.81.

2.4. Encapsulation of drug/dye in polymer nanoparticle: In this drug / dye loading, DOX was used as a drug and was loaded into the de-protected polymer nanoparticle via nanoprecipitation technique. De-protected polymer (3 mg) and DOX (0.3 mg) were dissolved in 500 µL of acetone, and this polymeric solution was added in a dropwise manner in 5 mL of deionized water (DI) in magnetic stirring at 500 rpm.[29]The drug-loaded nanoparticles were formed instantly and the solvent was eliminated during slow evaporation at room temperature.[28]The subsequent dispersions were centrifuged for 30 min at 4000 rpm to remove the aggregated particles and non-entrapped drug.[28]After that, decant the dispersion in order to remove the precipitate. By following the above procedure, Nile red (NR) was also loaded in the de-protected co-polymer. Drug loading content (DLC) and drug loading efficiency (DLE) were calculated by the help of UV-vis spectra of each drug and dye, using the following equation:

DLE (%) = [weight of drug/dye in NP / weight of drug / dye in feed] X 100

DLC (%) = [weight of drug/dye in NP / weight of polymer taken] X 100

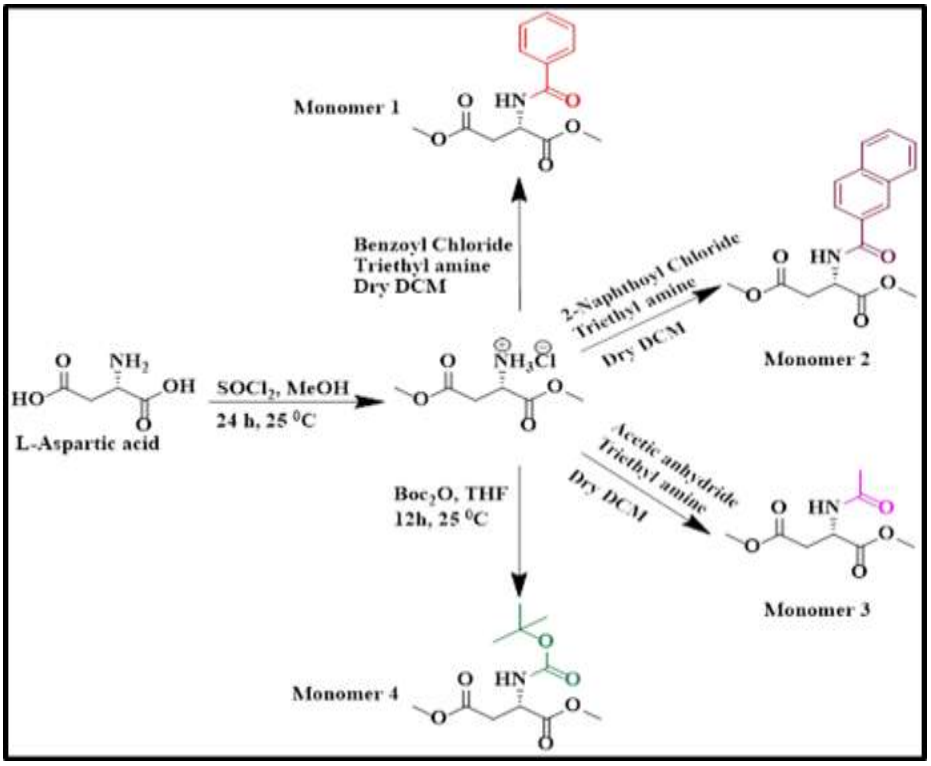
2.5. Cell Viability Assay: A-50:B-50 polymer nano scaffold was exposed to HeLa cell line in order to calculate the cell viability in normal cells using MTT assay, which is centred on the activity of tetrazolium bromide, 3-4,5-dimethylthiazol-2,5-phenyletrazolium bromide in living cells. A 96-well plate having 1000 cells per well in 100 μL of DMEM with 10% FBS was taken and cells were cultured for a period of 16 h. The media was aspirated and the resultant cells were administrated with polymer nanoparticle. As a control, cells without polymers were also retained in triplicates. The cells were permitted to cultivate in the presence polymers for 72 h, and after that the media was changed by 100 μL freshly prepared stock solution of MTT. Cells were later incubated for 4 h at 37 ^{o}C. 100 μL of DMSO was added to the media having purple formazan crystals which is formed due to treatment of living cells to MTT. The absorbance from the purple formazan crystals were noted using microplate reader at 570 nm (Varioskan Flash). This gave the characteristic number of viable cells per well.

2.6. Confocal Imaging: A 6-well cell culture plate was laden with glass coverslips and MCF-7 cells were cultivated in them in the presence of DMEM and 10 % FBS having a cell density of 1 x 10^5. These cells were incubated for 16 h prior to experiments and then treated with DOX-loaded polymer scaffolds. After incubation for 4 h, DOX containing medium was aspirated and fixed with 4 % paraformaldehyde solution in PBS for 10 min at room temperature. For the cells treated with polymer loaded nanoparticle, the cells were stained with DAPI. The cells fixed on the glass slide were then imaged by means of confocal microscopy.

2.7. FACS Analysis: The quantification of drugs taken by the cells can be estimated by using flow cytometry cell analyser. HeLa cells were cultured at a density of 1x10^5 in a six-well plate and were incubated and permitted to grow for 16 h in the presence of DMEM with 10 % FBS at 37 ^{o}C. **A-50:B-50** polymer loaded with DOX and NR also free DOX and NR were treated with cells. Further cells were trypsinated and were centrifuged. The suspension of cells was analysed using BD LSRFortessa SORP cell analyser.

RESULT AND DISCUSSION

3.1. Synthesis of L-aspartic acid-based monomers: The synthesis of L-amino acid-based monomers was carried out from naturally available L-aspartic acid. These multifunctional amino acids are having two carboxylic groups and one amine group. These carboxylic groups were transformed into representative esters and amine into amide group using benzoyl chloride, naphthoyl chloride, acetic anhydride and Boc anhydride (**see scheme 3.1.1**).



Scheme 3.1.1: *Synthesis of L-aspartic acid based monomers.*

3.2. NMR characterization of L-aspartic based monomers: The formation of monomers was confirmed by proton NMR spectroscopy (**see figure 3.1**). The peaks in the monomers **M1**, **M2**, **M3** and **M4** equivalent to the COOC**H**$_3$ protons appeared at 3.81 to 3.72 ppm confirms the formation of corresponding functional aspartate ester. ^{13}C-NMR spectroscopy further confirms the formation of monomers (**see figure 3.2**). The carbon atoms of O**C**H$_3$ ester groups appeared at 48-54 ppm values further confirms the formation of the required product.

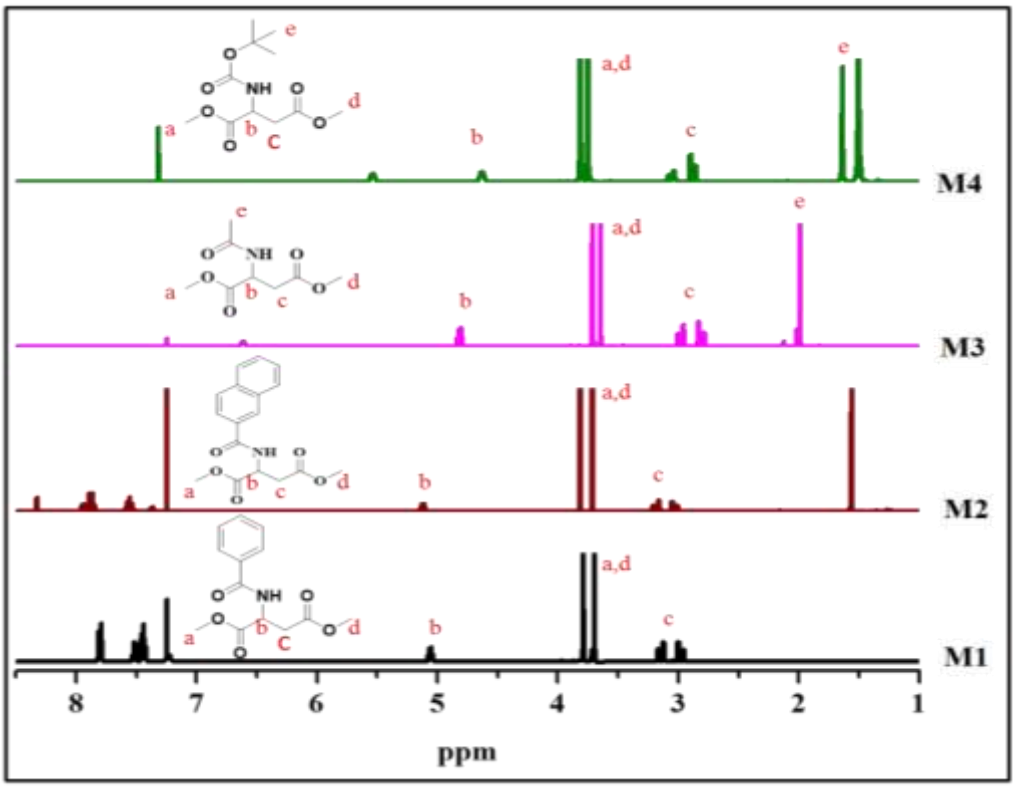


Figure 3.1: *^{1}H NMR spectra of L-Aspartic acid monomers (i) Benzoyl aspartate (M1) (ii) Naphthyol aspartate (M2) (iii) Acetyl aspartate (M3) and (iv) Boc aspartate (M4).*

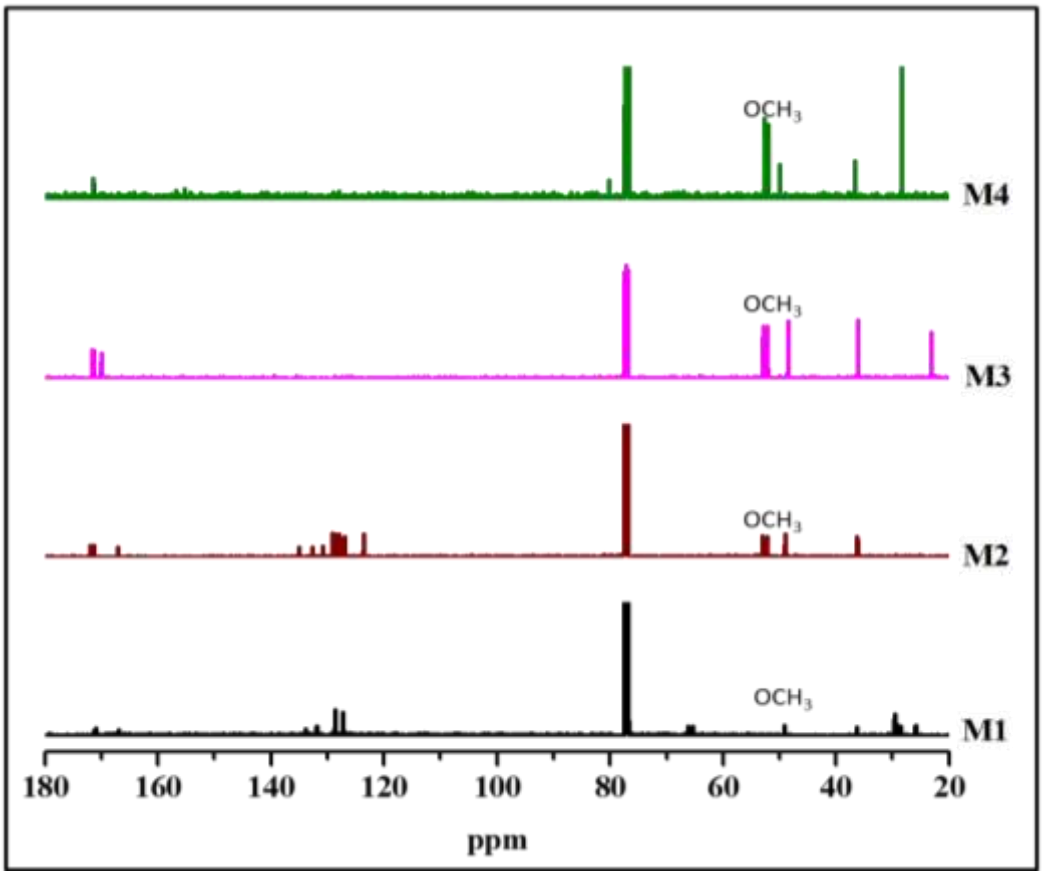
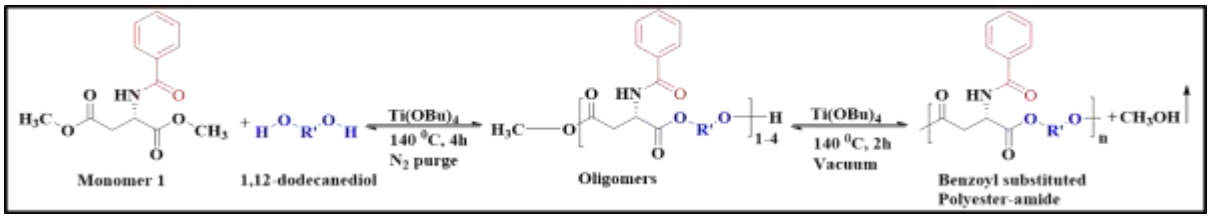


Figure 3.2: *^{13}C NMR spectra of L-Aspartic acid monomers (i) Benzoyl aspartate (M1) (ii) Naphthyol aspartate (M2) (iii) Acetyl aspartate (M3) and (iv) Boc aspartate (M4).*

Melt condensation of ester and urethane is a thermo-selective process in which the ester functionalized part of the amino acid monomer can undergo trans-esterification in the presence of $Ti(OBu)_4$ (1 mol%) at 120 ºC and urethane starts to react only at 150 ºC and above.[23] In this polymerization, the multi-functional monomers underwent poly-condensation with the diols to form linear polyesters (**see scheme 3.1.2**).



Scheme 3.1.2: *Synthesis of Benzoyl aspartate based polyester-amide.*

Thermal stability of these monomers and various diols were tested by thermogravimetric analysis (**fig 3.3**). The TGA studies confirmed that these monomers **M1** and **M2** are stable up to 200 ºC and **M3**, **M4** are stable up to 150 ºC. So in order to optimize the polymerization reaction, various reactions were carried out by varying the temperature and diols. Since trans-esterification will undergo from 120 ºC onwards and the monomers (**M1**, **M2**) were stable up to 200 ºC, model reactions were performed at 140 ºC, 150 ºC, 160 ºC and limited up 160 ºC because most the diols were not stable up to 170 ºC. The details of the reaction that were carried out are shown in **Table 1**.

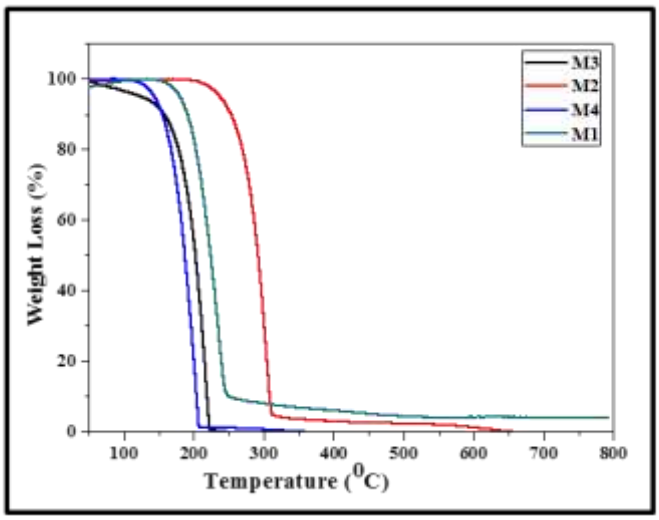


Figure 3.3: *TGA profiles of L-aspartic acid based monomers.*

Polymer	Monomer	Diol	Temperature (^{0}C)	Mn (g/mol)	Mw (g/mol)	Mw/Mn
P1	Benzoyl aspartate	1,12-dodecane diol	140^{0}C	13,500	31,000	2.3
P2	Benzoyl aspartate	1,12-dodecanediol	150^{0}C	9,500	23,000	2.4
P3	Benzoyl aspartate	1,12-dodecane diol	160^{0}C	13,000	40,000	3
P4	Benzoyl aspartate	1,10-decanediol	140^{0}C	8,000	16,500	2
P5	Benzoyl aspartate	Tri-ethylene glycol	140^{0}C	2,000	3,000	1.1
P6	Benzoyl aspartate	PEG-400	140^{0}C	7,500	14,500	1.9

Table 1: *Polymerisation of Benzoyl monomer with various diols at various temperatures.*

3.3. Molecular weight of polymers: Synthesis of benzoyl aspartic polymers were carried out at 140 ^{0}C with continuous nitrogen purging for 4 h and followed by 2 h vacuum and it yielded decently good molecular weight polymers (Mn = 13,500 g/mol) with a polydispersity (PDI) ~ 2.3. Since monomers (**M1**, **M2**) were stable up to 200 ^{0}C efforts have been made to synthesize polymers at reaction temperatures 150 ^{0}C and 160 ^{0}C. The reactions carried out at 150 ^{0}C and 160 ^{0}C yielded polymers and molecular weights were found to be in the range of Mn = 9,500-13,000 g/mol with PDI~ 3. The effect of increasing temperature did not reflect on the building up of the polymer molecular weight therefore further polymers were synthesized at 140 ºC. Also, the reaction carried out with 1, 10-decanediol was also yielded high molecular weight as the previous one and has a PDI ~ 2. Tri-ethylene glycol and PEG-400 diols were further used in polymerization in order to make the hydrophilic polymers. While molecular weight build-up could not be achieved using tri-ethylene glycol, however, PEG-400 graciously gave good molecular weight of 7,500 g/mol with PDI ~ 1.9. The

molecular weights and the PDI are tabulated in **table 1**. It was observed that these polymers were stable up to 300 ºC and these were found to be amorphous in nature.

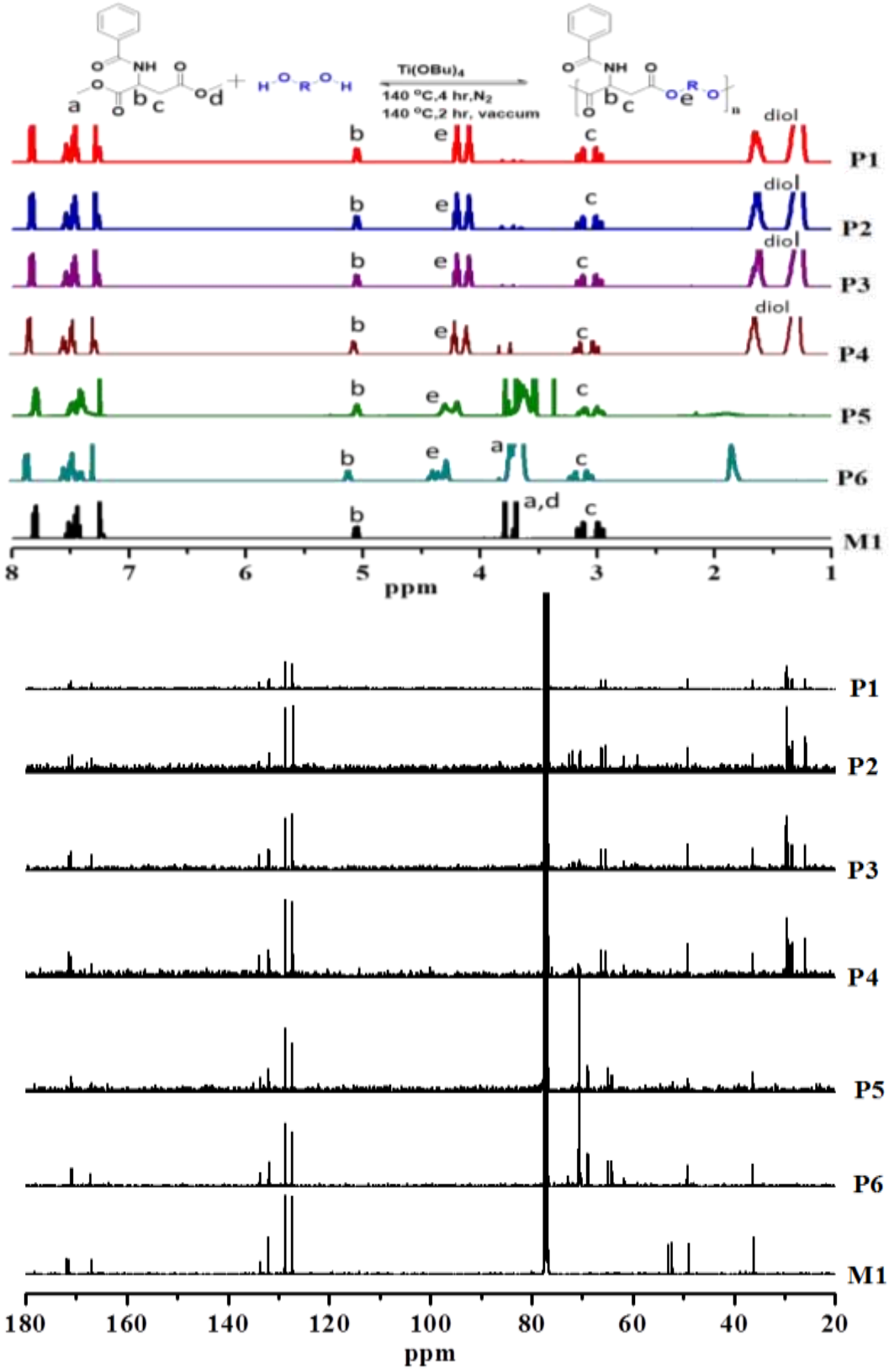


Figure 3.4: ^{1}H and ^{13}C NMR spectra of various L-aspartic acid based functional polyester-amides.

The polymer structures were confirmed by proton NMR spectroscopy (see **figure 3.4)**. The vanishing of benzoyl aspartic monomer ester protons –CHCOOC**H**$_3$, –CH_2COOC**H**$_3$ at 3.81, 3.72 ppm and the emergence of new ester peaks at 4.20 and 4.10 ppm confirmed the formation of polyesters. All other protons of the 1,12-dodecane diol appeared around 1-2 ppm for polymers **P1** to **P3**. 1,10 decanediol peaks also appeared in the similar range. For polymers, **P5** and **P6** TEG and PEG proton peaks were observed at 3.5 ppm. Since the chiral protons (3.17-2.96), -C**H** protons (5.05), amide protons (7.46) and all other aromatic protons (7.83-7.46) in the polymer NMR spectrum is perfectly matching with the monomer spectrum reveals that no other additional reaction has gone except trans-esterification. ^{13}C-NMR spectroscopy further confirmed the formation of the polymer. (**see figure 3.4**). The carbon atoms in the ester group -OCH_3 at 52 ppm vanished and the new peak corresponds to ester –COO**C**H_2CH_2 carbon atoms were observed at 72 ppm. Thus, the formation of a new class of poly-ester having amide pendants from the trans-esterification of functionalized amino acids monomers were both confirmed by proton and ^{13}C NMR spectroscopy.

Gel permeation chromatography (GPC) technique was used to estimate the molecular weights of the newly synthesized polymers using THF solvent as eluent. The GPC chromatogram of polyester amides is shown in **fig 3.5** and their molecular weights are brief in **table 1**. All the polyester-amides exhibited mono-modal distribution designating that polymer chains are of uniform lengths. The molecular weights of the synthesized polymers were gained in the range of Mn = $2x10^3$ – $14x10^3$ and Mw = 3.0 $X10^3$ – 4.0$X10^4$ g/mol with polydispersity ~2.2.

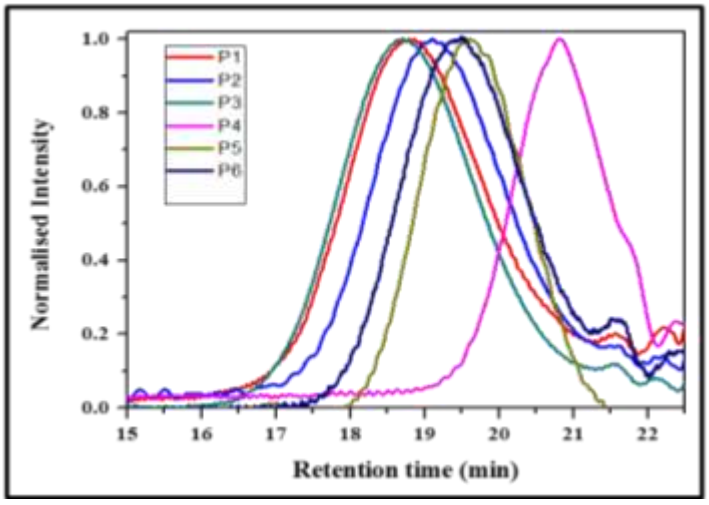


Figure 3.5: *Gel permeation chromatograms of benzoyl polymers formed at various diols and reaction temperatures.*

The number average degree of polymerization (Xn) of the newly synthesized polymers was calculated from the given equation, Mn = Mo x Xn, where Mn is the number average molecular weight and Mo is the molecular weight of the repeating unit mass. The Xn for step growth polymerization is given by Carothers equation, Xn=1/(1-p), where p is the percentage conversion. [20] Xn and p for melt process were gained as 25 units and 96 %.

3.4. Kinetics of polymerization: To study the kinetics of the polymerization, Benzoyl monomer (**M1**) with 1,12-dodecanediol polymerization was conducted and aliquots were taken at regular intervals to check the polymer thermal stability (**see table 2**). The TGA plots (**see fig3.6 (a)**) of the aliquots showed a continuous increase in thermal stability of the polymers due to the formation of high molecular weight polymer chains from 200 to 350 ^{o}C.

Aliquot	Time (hr)	Mn (g/mol)	Mw (g/mol)	Mw/Mn
1	0	200	280	1.4
2	1	510	720	1.4
3	2	1,000	1,800	1.8
4	3	1,650	2,900	1.8
5	4	2,350	4,700	2.1
6	5	5,700	14,700	2.5
7	6	7,400	17,000	2.4

Table 2: *Molecular weights of aliquots of the polymerisation reaction of Benzoyl aspartate and 1,12-dodecanediol.*

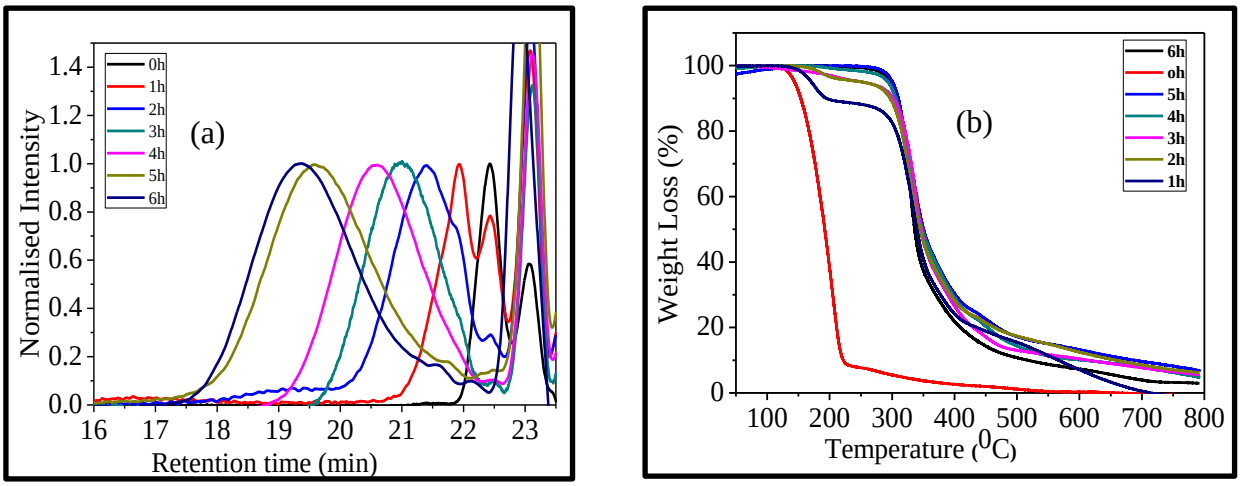


Figure 3.6: (a) *Gel permeation chromatograms and (b) TGA plots of benzoyl polyester-amide aliquots that were taken at regular intervals of time.*

The GPC of the of the aliquots were done (**see fig 3.6 (b)**) and it showed an increase in the molecular weight and the chromatogram of polymeric samples moved from multi-model distribution to mono-model distribution due to the formation of higher molecular weight polymer chains.

The unreacted monomers and products can be quantified using proton NMR spectra of the aliquots. Proton NMR spectra of the aliquots for the polymerization is shown in **fig 3.7**. In the early stage, peaks matching to the protons HO-(C**H**$_2$)$_{10}$-OH, $CH_2COOC\mathbf{H}_3$, $CHCOOC\mathbf{H}_3$ appeared at 2.87, 3.01, 3.05 ppm respectively. At 140 ^oC, esters of the monomer underwent reaction with 1,12-dodecanediol, as a result, new ester peaks were formed at 4.16 and 4.09 ppm and the old ester peaks almost got vanished. The appearance of new ester peaks at 1 hr suggests that the trans-esterification started at the initial stage itself. The gradual disappearance of the old ester peaks confirmed the consumption of monomer molecules and diols to form polyesteramides.

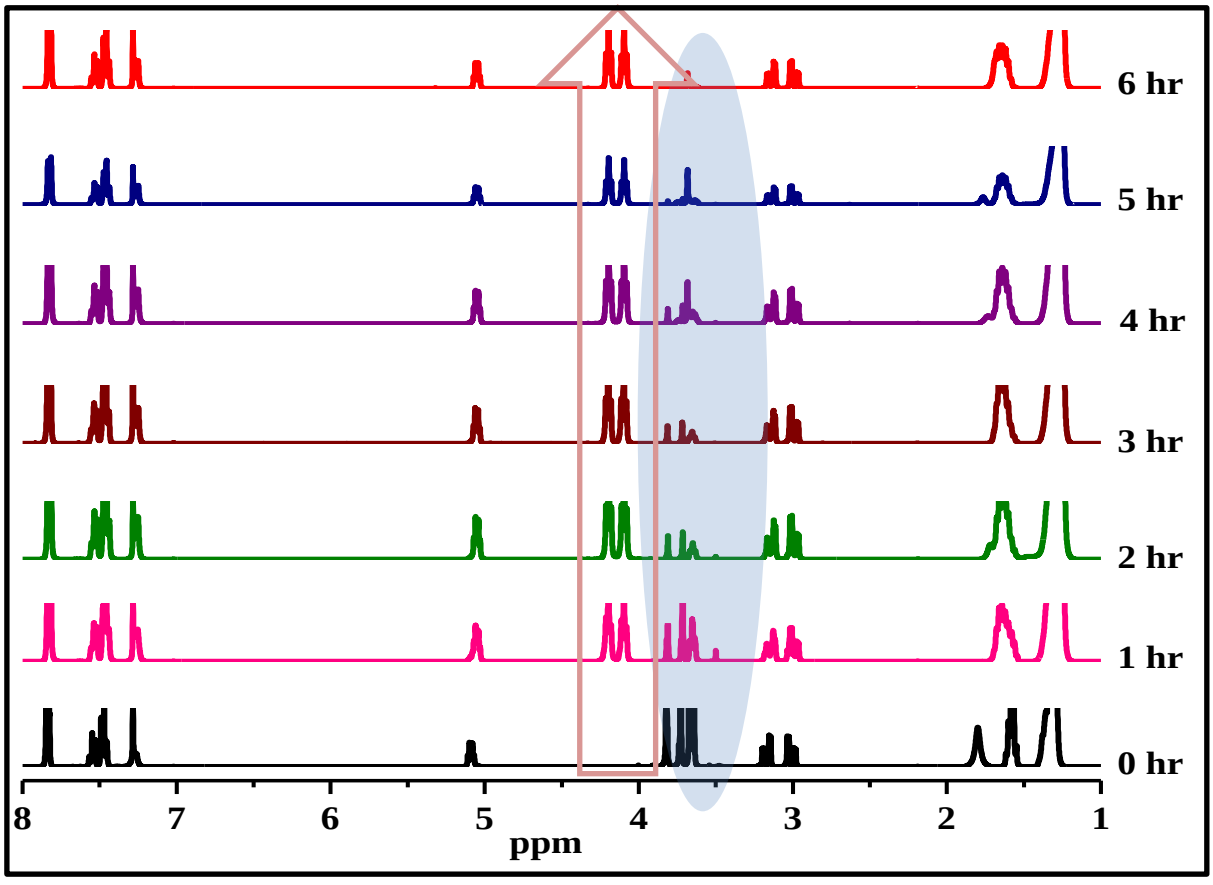


Figure 3.7: *^{1}H NMR spectra of benzoyl polyester-amide aliquots* that were taken at regular intervals of time.

By comparing the intensities of the new ester peaks formed with the end group disappearing ester groups from ^{1}H NMR, the Mn (number average molecular weight) of the aliquots was determined. Mn can also be deducted from the equation, Mn = Xn Mo, where Mo is molecular weight of the repeating unit mass, the number average molecular weight (Mn) can be deduced.[20] The molecular weight gained from NMR and GPC techniques was plotted against the polymerization time (**see figure 3.8**). The molecular weights of the polymers increased linearly with the increase in the reaction time. The molecular weight enhancement at greater conversion was observed after 5 hrs which indicates that application of vacuum is extremely crucial for the higher molecular weight build-up.

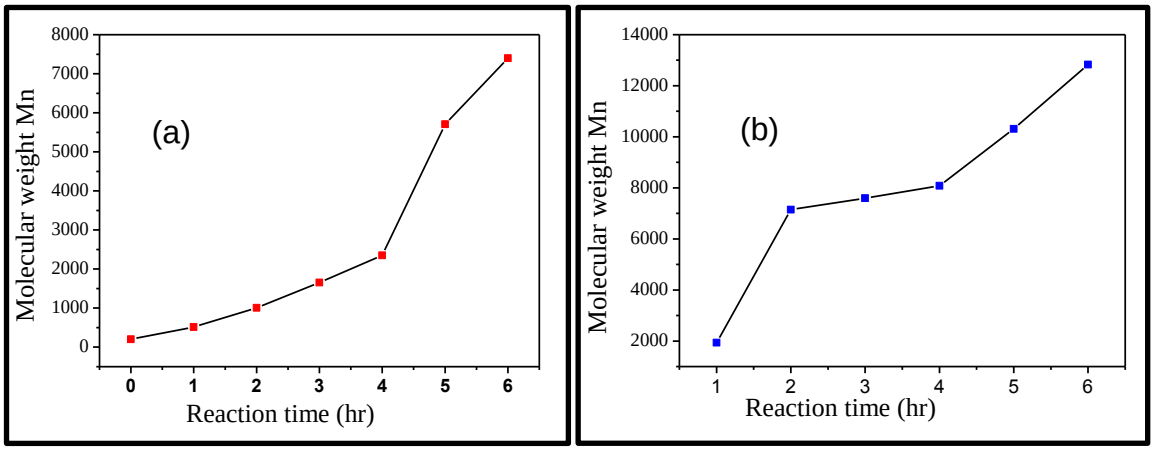


Figure 3.8: *The Mn determined by GPC (a) and NMR (b) technique was plotted for aliquots at various polymerisation reaction times.*

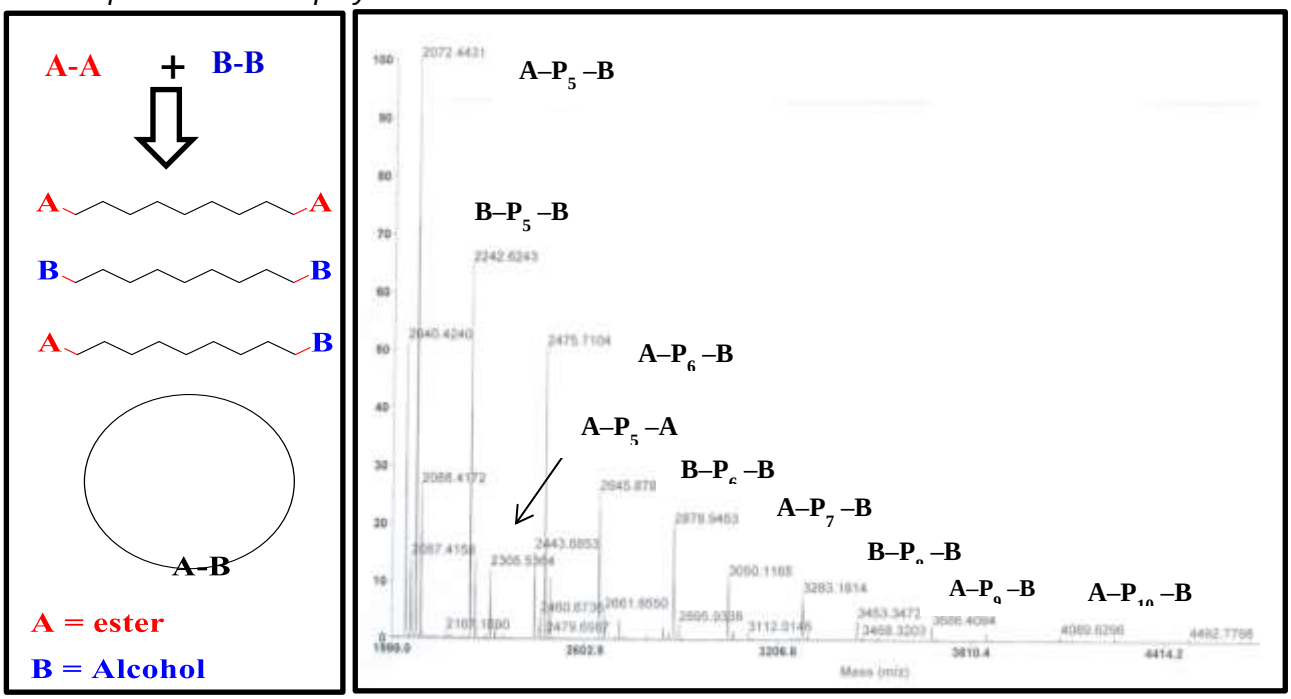


Figure 3.9: *Schematic representation of possible end group formation in A-A + B-B type condensation and MALDI-TOF spectra of benzoyl monomer and 1,12-dodecanediol polymer at 6 h.*

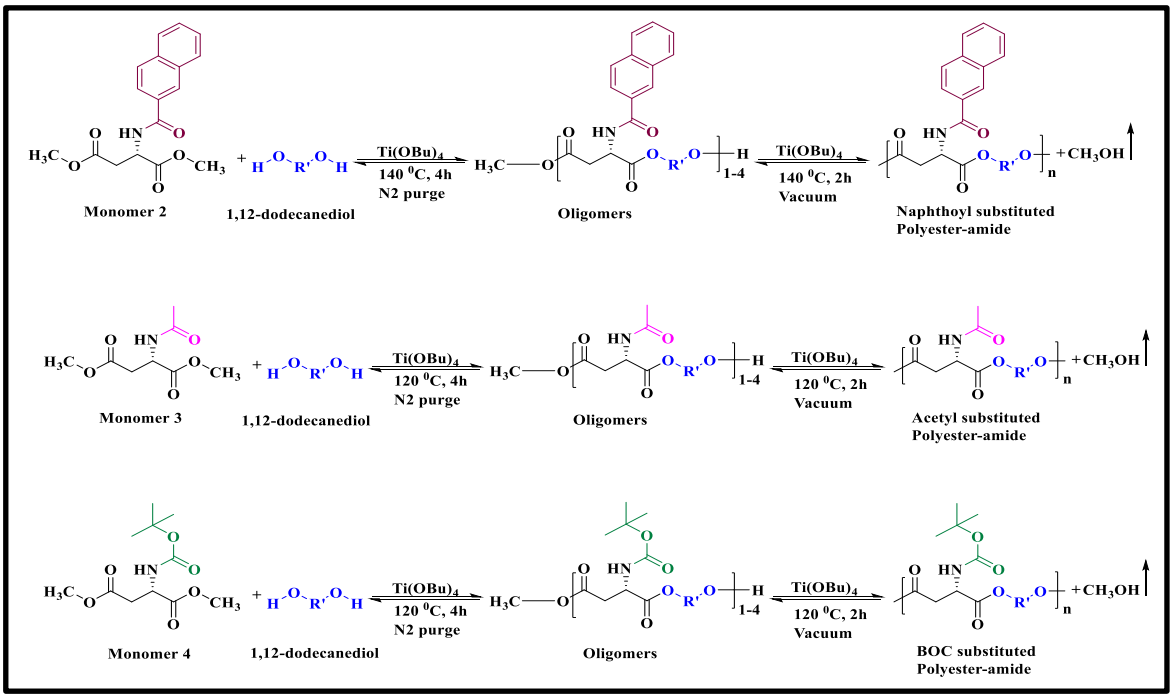
3.5. End group analysis by MALDI-TOF-MS: The number of types of end groups available in the polymer chains plus the formation of macrocycles can be directly inferred by MALDI-TOF-MS.[22] Polymerisation involving **A-A** and **B-B** type monomer can form 4 different types of polymer chains. (i) Both end groups having A functional moiety (**A-P-A**), (ii) Both end groups having B functional moiety (**B-P-B**), (iii) One of the end group having A functionality and other end having B functionality (**A-P-B**), (iv) cyclised one having no end group (**see fig 3.9**). The formation of the cyclised product can be distinguished only by MALDI mass spectrometry.

MALDI-TOF MS analysis was done to find the end group, functional groups. For this aliquots were taken at time intervals for the polymerization reaction of benzoyl monomer and 1,12-dodecanediol. The polymer showed four sets of peaks having linear and cyclic units. Every repeating unit mass was solved for the repeating unit formula: (i) (403)n + 265 + 23 for sodium ion peaks [(403)n + 265 + 39 for potassium ion peaks] for end groups having A functionality, (ii) (403)n + 202 + 23 for sodium ion peaks, for end groups having B functionality, (iii) (403)n + 32 +23 for sodium ion peaks and this is for polymer having both A and B functionality, (iv) (403)n + 23 for sodium ion peaks and this corresponds to cyclised one. In **Fig. 3.9**, the mass peaks are observed up to 8 repeating units.

From MALDI-TOF MS analysis few conclusions can be made: (i) under high-temperature melt poly-condensation process the ester functionalities of the amino acid monomers were stable (ii) increased polymerisation reaction time enhanced the molecular weight of the polymer chains (iii) all possible three different types of polymer chains like A-A, B-B, and A-B was formed in the polymerisation reaction (iv) macrocycles were not formed in the polymerisation process. The absence of macrocycles in the polymerization process indicates that amino acid monomers undergone linear chain formation instead of cyclisation which is necessary for high molecular weight polymers.

3.6. Synthesis of other functional polyesters: The polymerisation reaction was carried out with functionalised amino acid monomers and 1,12-dodecanediol at a mole ratio of 1:1 in the presence of $Ti(OBu)_4$ (1 mol%) catalyst at 120 ºC (except for **M2** which was at 140 ºC to form polyester-amides (**see scheme 3.1.3**). Melt

condensation underwent under a continuous N_2 purge followed by vacuum (10^{-2} bar) for 2 h which yielded a polymer of high molecular weight.



Scheme 3.1.3: *Synthesis of Naphthoyl (M2), Acetyl (M3) and Boc (M4) based polyester-amide*.

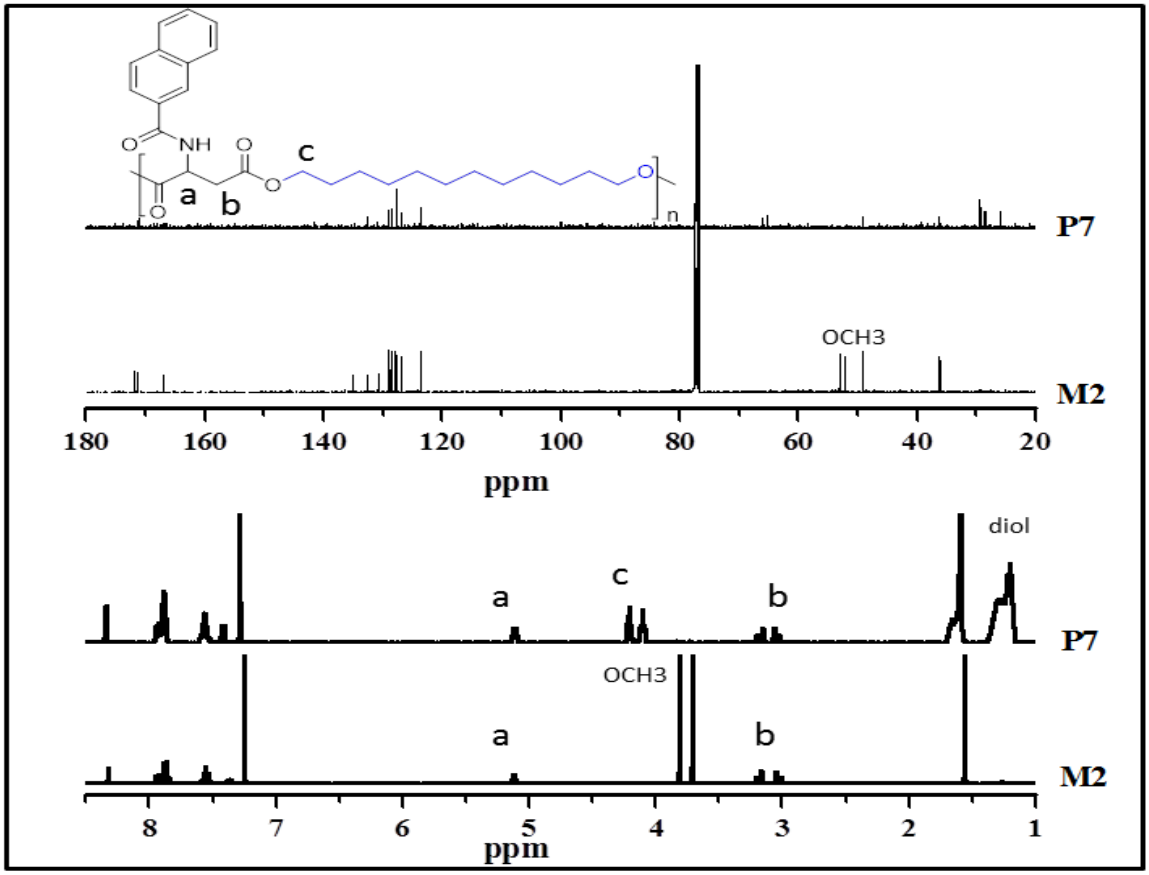


Figure 3.10: *^{1}H and ^{13}C NMR spectra of Naphthoyl monomer and polymer.*

3.7. NMR characterization of other functional polyester-amides: The melt polymerization process was confirmed by proton NMR spectroscopy (**see fig 3.10**). The peaks in the **M2** matching to the ester -COOC**H**$_3$ protons emerged at 3.85 and 3.75 ppm. After polymerization, these protons disappeared and new ester peaks at 4.20, 4.09 ppm confirmed the formation of naphthyol polyester. As the other peaks like –C**H**$_2$ (3.20-3.02), -C**H** (5.11), -N**H** (7.43) and all aromatic peaks corresponding to 12 protons (8.34-7.56) are retained in both the monomer and polymer NMR spectrum. ^{13}C NMR spectroscopy further confirms the formation of polyester-amide (**see fig 3.10**). The O**C**H$_3$ ester group carbon atoms at 48 and 54 ppm vanished and new ester carbon atoms –COO**C**H$_2$CH$_2$ appeared at 68 ppm.

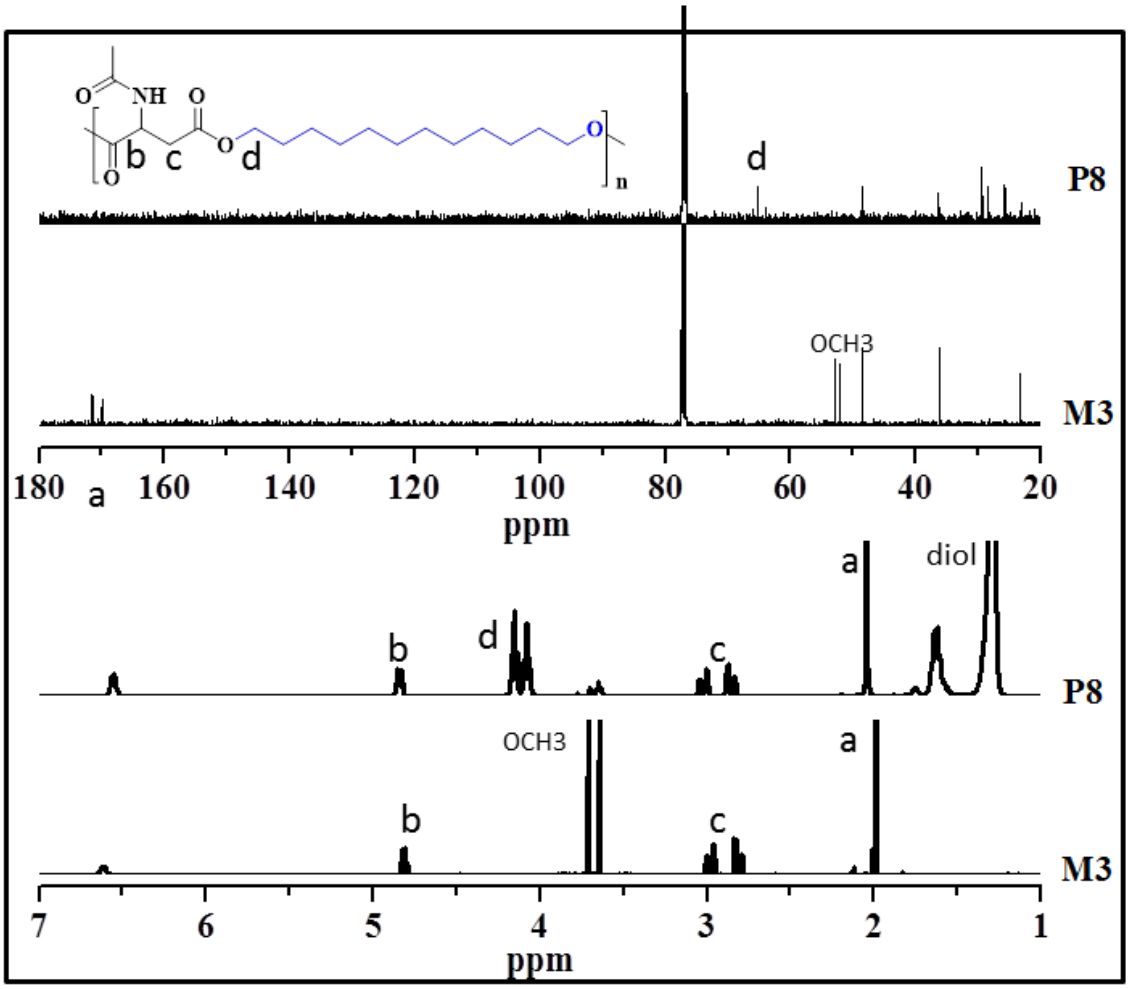


Figure 3.11: *^{1}H and ^{13}C NMR spectra of acetyl monomer and polymer.*

As explained above the disappearance of ester peaks at 3.71, 3.64 ppm and the appearance of new ester linkages at 4.16, 4.09 ppm confirms the formation of acetyl polyester (**see fig 3.11**). All the other diol peaks were same from 2-1 ppm. As the other peaks like –C**H**$_2$ (3.01-2.78), -C**H** (4.81), -N**H** (6.63) and the significant –C**H**$_3$ (1.99) peaks are retained in both the monomer and polymer NMR spectrum, which confirms the formation of polyester. ^{13}C NMR spectroscopy further confirms the formation of acetyl polyester (**see fig 3.11**). The O**C**H$_3$ ester group carbon atoms at 54 ppm vanished and new ester carbon atoms –COO**C**H$_2$CH$_2$ was appeared at 68 ppm.

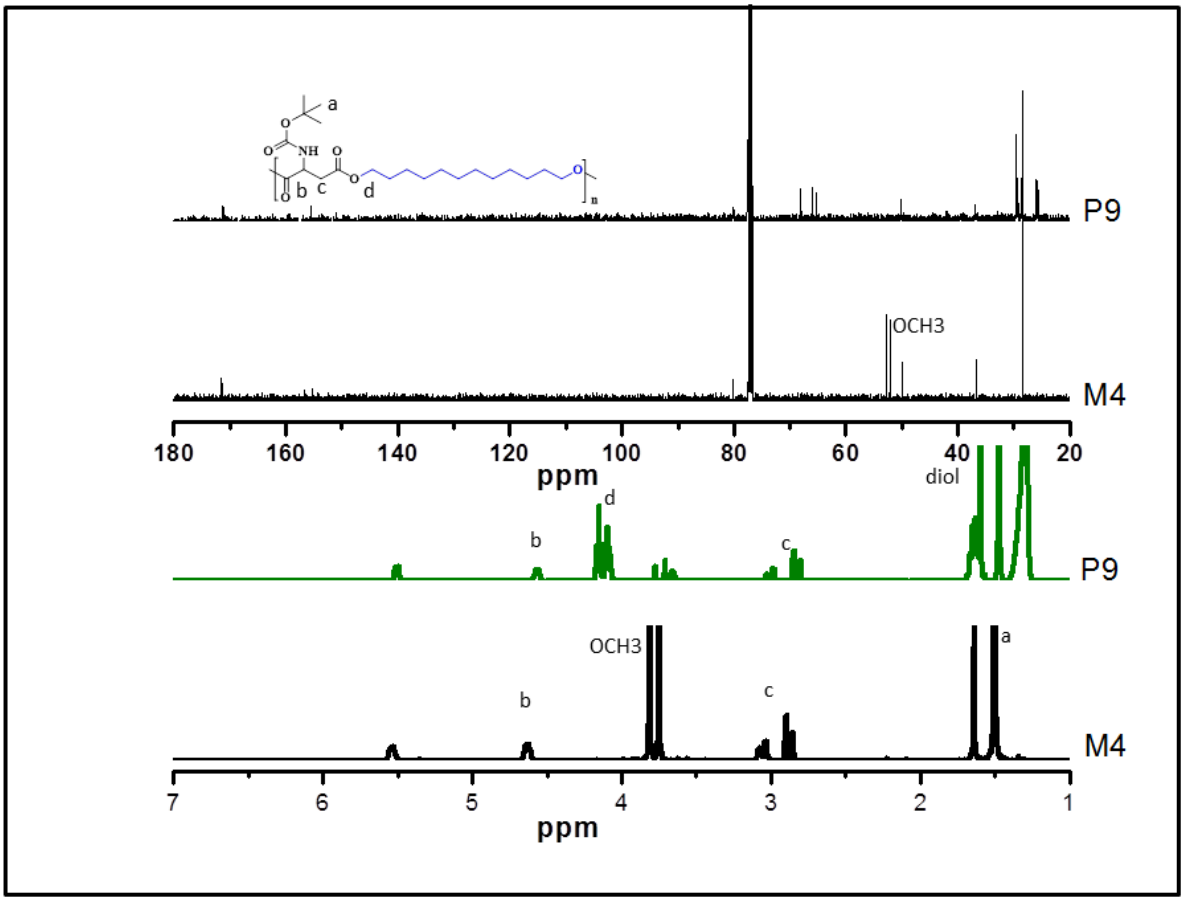


Figure 3.12: *^{1}H and ^{13}C NMR spectra of Boc monomer and polymer.*

The disappearance of ester peaks at 3.79, 3.65 ppm in the polymer and the appearance of new ester linkages at 4.15, 4.09 ppm confirm the formation of Boc substituted polyester (**see fig 3.12**). All other diol peaks are also present from 2-1 ppm. As the other peaks like –C**H**$_2$ (3.01-2.78), -C**H** (4.81), -N**H** (6.63) and the significant –C**H**$_3$ (1.99) peaks are retained in both the monomer and polymer NMR

spectrum, which confirms the formation of polyester. By comparing the intensities of the Boc group –NHCOOC(C**H**$_3$)$_3$ protons with the other protons in the polyester structure indicates that Boc group was not affected by the melt polycondensation reaction and it was found to be totally inert during polymerization reaction at 120^{o}C. ^{13}C NMR spectroscopy further proves the formation of the product (**see fig 3.12**). The -O**C**H$_3$ ester group carbon atoms at 52.65, 51.95 ppm vanished and new –COO**C**H$_2$CH$_2$ ester carbon atoms appeared at 64.71 and 68.89 ppm.

3.8. GPC molecular weight of other functional polyester-amides: Gel permeation chromatography (GPC) technique was used to estimate the molecular weights of the newly synthesized polymers using THF solvent as eluent (**see table 3**). The GPC chromatograms of polymers are shown in the **figure 3.13**. All the newly synthesized polymers showed mono-modal distribution designating that polymer chains are of uniform lengths. The molecular weights of the polymers were gained in the range of Mn = 4x10^3 – 18x10^3 and Mw = 9.0 X10^3 – 3.0X10^4 g/mol with polydispersity ~2.0. The Xn for step growth polymerization is given by Carothers equation, Xn=1/(1-p), where p is the percentage conversion. [20] Xn and p for melt process were gained as 25 units and 95 %.

Polymer	Monomer	Diol	Temp (^{0}C)	Mn (g/mol)	Mw (g/mol)	Mw/Mn
P7	Naphthoyl aspartate	1,12-dodecanediol	140^{0}C	18,000	34,000	1.8
P8	Acetyl aspartate	1,12-dodecanediol	120^{0}C	5,000	10,000	2
P9	Boc aspartate	1,12-dodecanediol	120^{0}C	4,000	9,400	2.4

Table 3: *Molecular weight of other L-aspartic acid based functional polyester-amides*

3.9. Thermal analysis of functional polyester-amides: The thermal analysis of these functional polyester-amides was done by means of thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). TGA measures the amount of weight loss as a function of increasing temperature and here these polyester-amides were thermally stable up to 200 ºC (**see fig 3.13**). DSC plots showed that all polyesters are amorphous in nature (**see fig 3.13**).

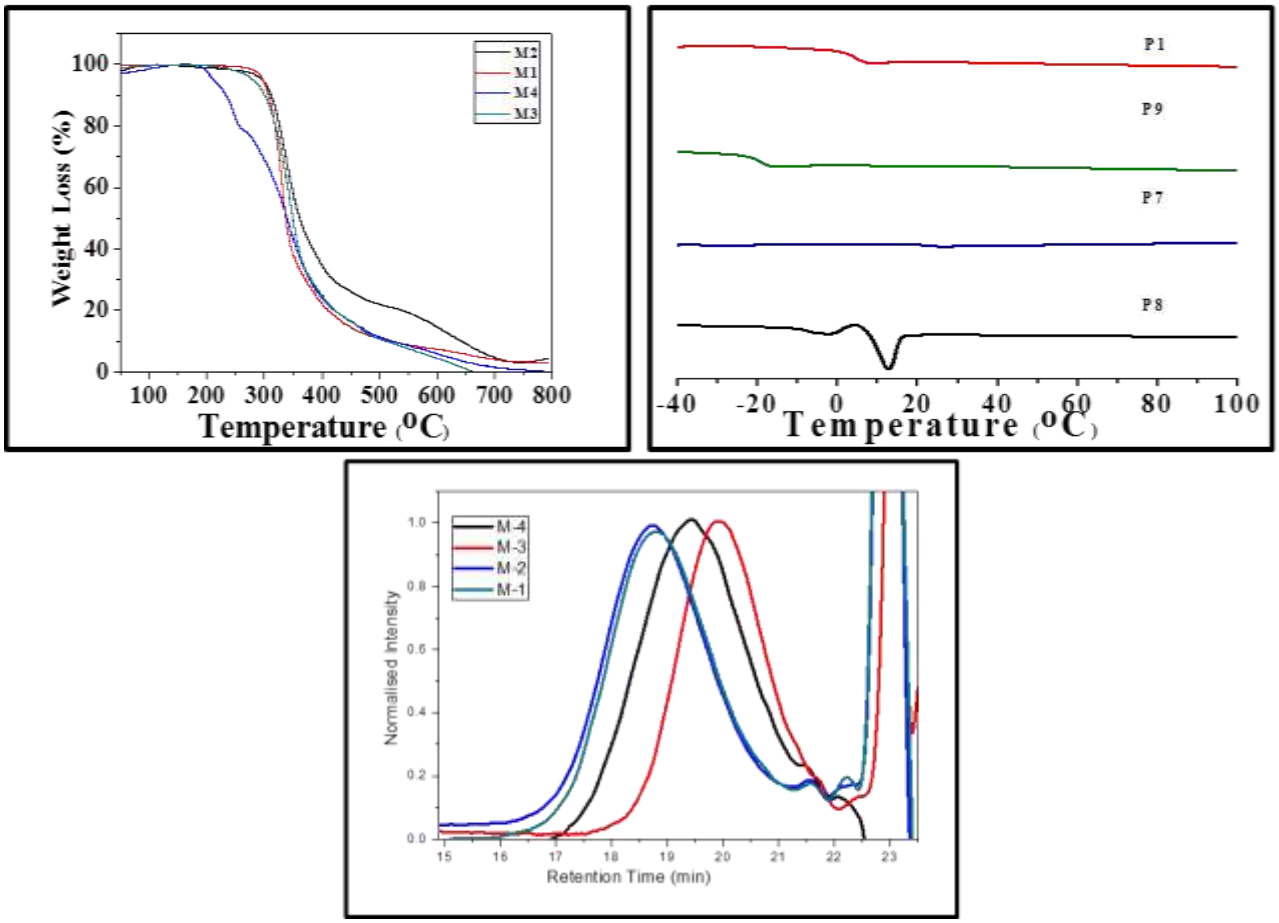
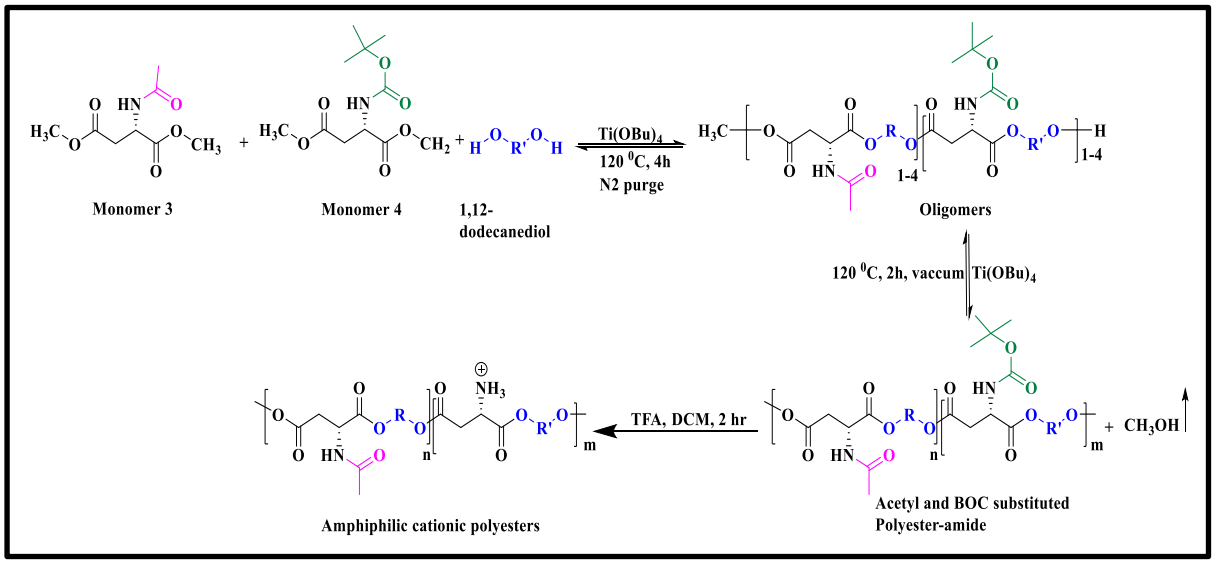


Figure 3.13: *TGA, DSC and GPC profiles for L-aspartic acid based polyester-amides*.

3.10. Synthesis of Amphiphilic cationic polyesters: In this random co-polymerisation process, the monomers **M3** and **M4** underwent reaction with 1,12-dodecanediol to form linear polyester-amides (**see scheme 3.1.4**). Random copolymers were made by changing the composition of Acetyl and Boc monomer from 10 to 90 % in the feed. Polymerization reaction was carried out with these two monomers and 1, 12-dodecanediol at a mole ratio of 1:1 in the presence of $Ti(OBu)_4$ catalyst at 120 ºC. Melt condensation underwent in a continuous N_2 purge for 4h followed by 2h vacuum (10^{-2} bar) which yielded a polymer of high molecular weight. The synthesized co-polyesters were having Boc moiety as the pendant unit in the main chain. The careful de-protection of the -(-NHCOO-$C(CH_3)_3$) Boc group in the pendant unit without interrupting the polyester main chain yielded amphiphilic cationic polyesters. These synthesized copolymers were de-protected with trifluoroacetic acid (TFA) in DCM to form amine functionalized polyester (**see scheme 3.1.4**).



Scheme 3.1.4: *Synthesis of amphiphilic cationic polyester.*

3.11. NMR characterization of amphiphilic cationic polyesters: The melt co-polymerisation process was confirmed by proton NMR spectroscopy (**see fig 3.14**). The peaks in the monomers matching to the ester -COOC**H**$_3$ protons emerged at 3.79-3.71 and 3.72-3.64 ppm. After polymerization, these protons vanished and new ester peaks appeared at 4.15, 4.07 ppm confirmed the formation of polyester. The protons corresponding to –C**H**$_3$ (from acetyl monomer) and the –NHCOO-C(C**H**$_3$)$_3$ (from Boc monomer) segments were well separated as singlet peaks at 2.04 and 1.45 ppm. This facilitated to determine the exact compositions incorporated in the copolymer by ^{1}H NMR. Based on this, the real incorporation of acetyl units in the copolymers was determined to be 11, 23.5, 36, 50.58, and 73.62 % for their feed molar ratios of 10, 25, 35, 50 and 75 % respectively.

3.12. Molecular weight for functional random co-polymers: GPC technique was used to determine the molecular weight of the polymers. The GPC chromatograms of polymers are shown in the **figure 3.15**. All the copolymers showed mono-modal distribution designating that polymer chains are of uniform lengths. The molecular weights of the polymers were gained in the range of Mn = 5x10^3 – 15x10^3 and Mw = 7.0 X10^3 – 7.0X10^4 g/mol with polydispersity ~2.5.

The thermal analysis of these functional polyester-amides was done by means of thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). TGA

measures the amount of weight loss as a function of increasing temperature and here these polyester-amides were thermally stable up to 250 ºC (**see fig 3.15**). DSC plots showed that all polyesters are amorphous in nature (**see fig 3.15**).

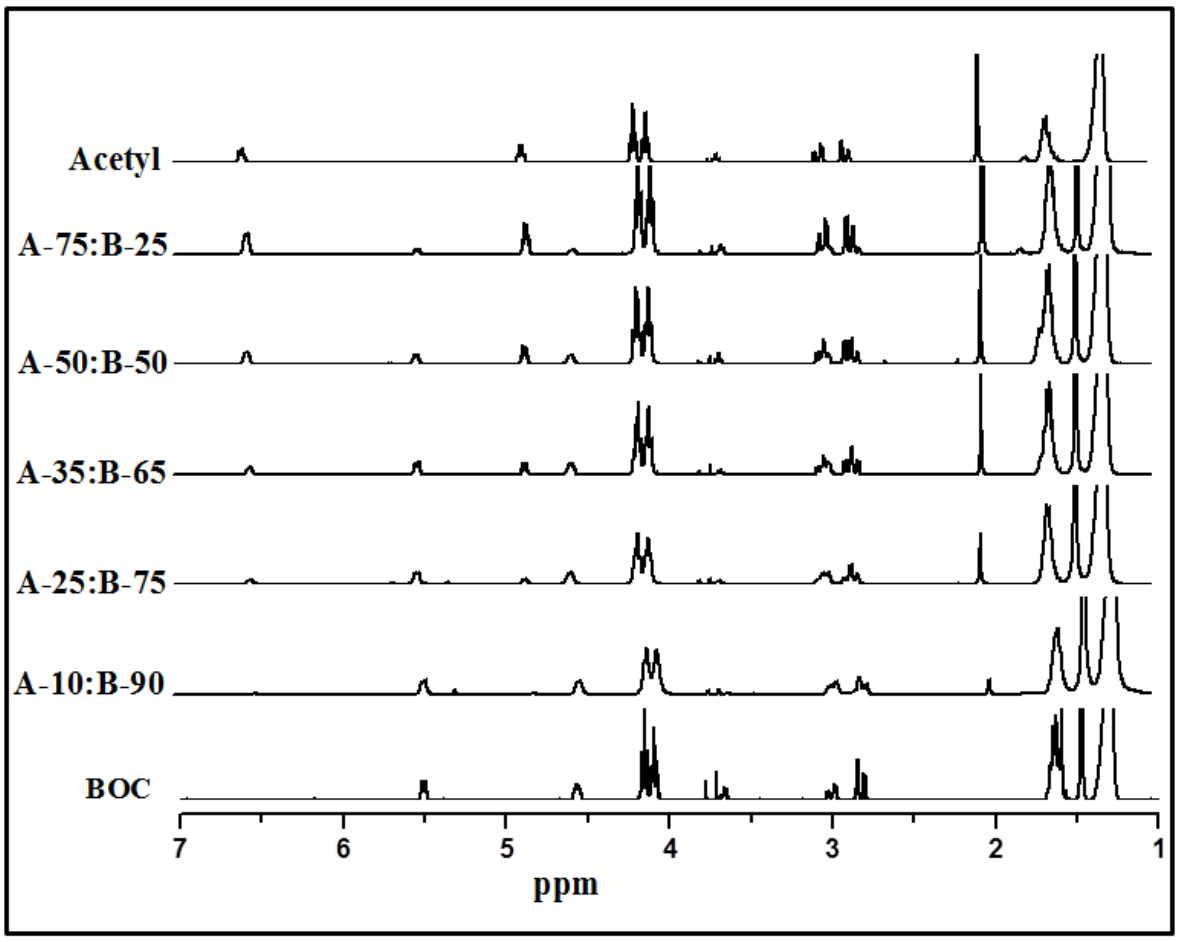


Figure 3.14: *^{1}H NMR spectra various functional random co-polymers made by varying the composition of monomers*.

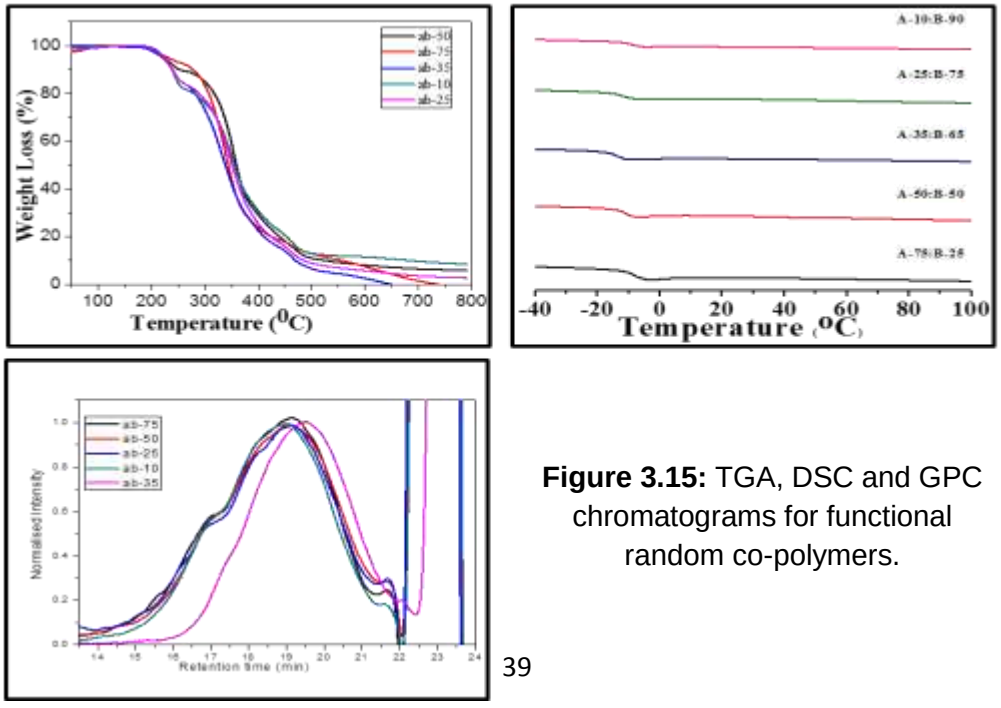


Figure 3.15: TGA, DSC and GPC chromatograms for functional random co-polymers.

The de-protection of polymers was confirmed by proton NMR spectroscopy (**see figure 3.16**). The protons corresponding to the -(NHCOO-C(C**H**$_3$)$_3$) Boc moiety in the copolymer completely vanished after de-protection. This clearly indicates that Boc group was particularly de-protected to produce amphiphilic cationic polyesters.

3.13. Self-assembly and loading capabilities: The purpose of these amphiphilic cationic polyester amines for drug delivery can be verified by two means: (i) the capability to form self-assembled and stable nanostructures in the aqueous medium[26] (ii) the encapsulation ability of the nano-assemblies for anti-cancer drugs.[26] For this objective, the polymer was dissolved in water + DMSO mixture and exposed to nanoprecipitation method. The loading capacities of **A-50:B-50** polymer was tested for hydrophobic drug doxorubicin and hydrophobic dye Nile red. The drug loading efficiency (DLE) for **A-50:B-50** were determined by UV-vis spectroscopy as 31.13% and 5.45% for DOX and NR correspondingly. The drug loading content (DLC) for DOX and NR were found to be 3.1% and 0.54% respectively. Dynamic light scattering (DLS) studies of **A-50:B-50** polymer sample in the aqueous solution showed mono-model distribution due to the formation of homogenous nanoparticles of 210 ± 5 nm (**figure 3.17**). The amphiphilic cationic polymers were exposed to zeta potential analysis since the de-protected polymers are having cationic $NH_3^+X^-$ units. The zeta potential values were detected in the range of 47 to 50 mV.

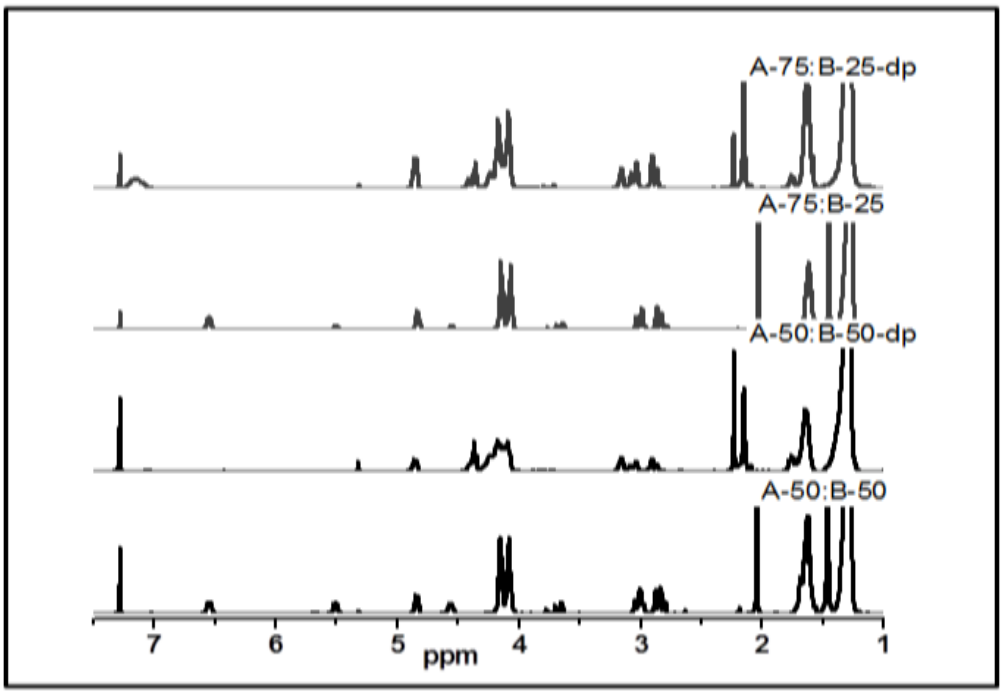


Figure 3.16: *^{1}H NMR spectra of amphiphilic cationic polyesters and random co-polyesters.*

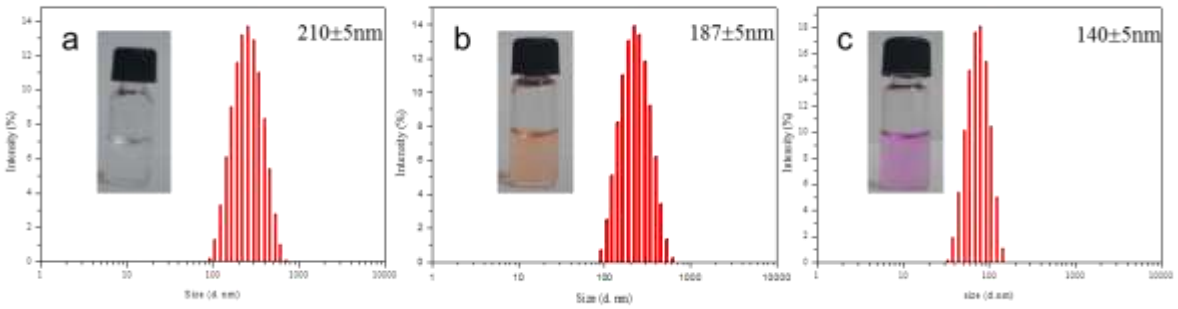


Figure 3.17: *DLS histograms and photographs of vials containing nano-precipitated polymer samples of (a) A-B:B-50 (b) DOX loaded A-50:B-50 and (c) NR loaded A-50:B-50.*

3.14. Cytotoxicity and Cellular uptake: The cytotoxicity of the amphiphilic cationic polymeric nanoparticle was studied in breast cancer (MCF-7) cell line by MTT assay method. The cells were exposed to different polymeric concentrations and their respective histogram is shown in the **figure 3.18**. The newly synthesized amphiphilic cationic polymer **A-50:B-50** was found to be non-toxic at these concentrations. The polymer is displaying more than 80 % cell viability even at a concentration of 60µg/ml.

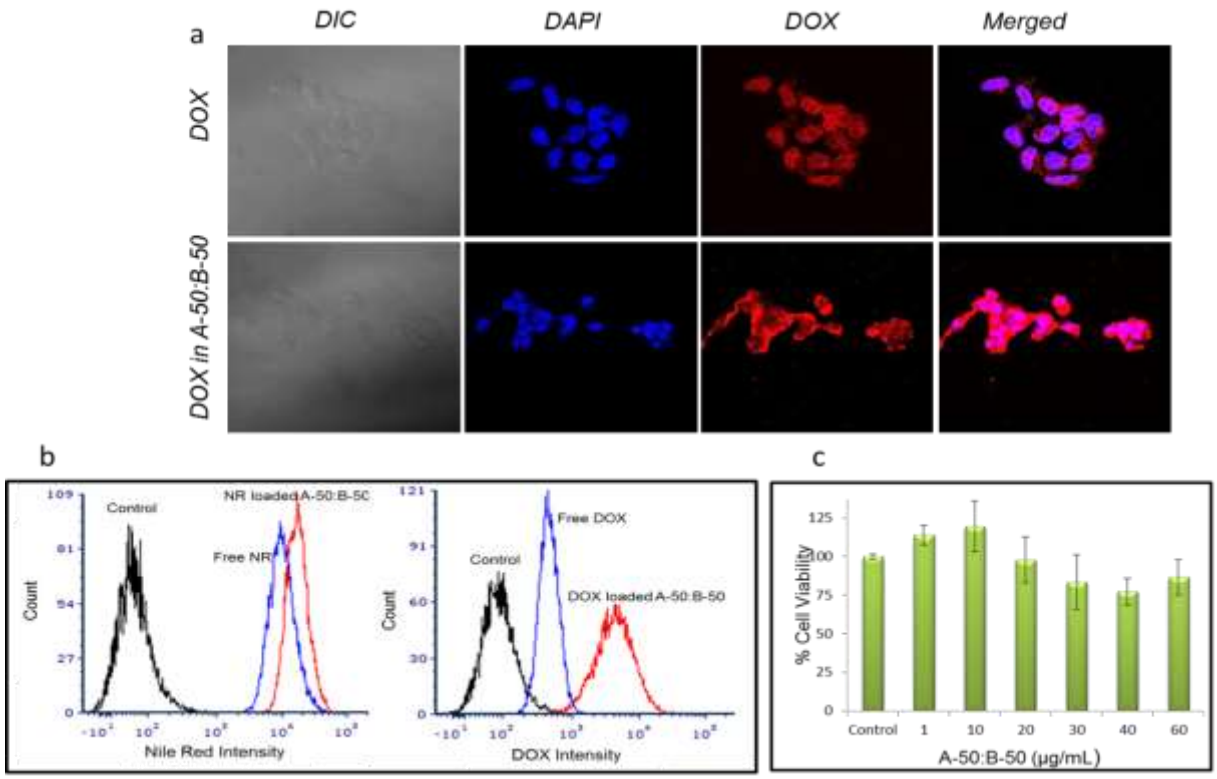


Figure 3.18*: (a) CLSM images of DOX-loaded and free DOX **A-50:B-50** incubated in MCF7 cells (b) Flow cytometry plots for (i) control, free NR, NR loaded **A-50:B-50** and (ii) control, free DOX, DOX loaded **A-50:B-50** in HeLa cell lines(10,000 cells were counted) (c) Cytotoxicity in MCF7 cell line for **A-50:B-50** nanoparticles.*

To picturise cellular internalization of these polymeric nanoparticles, MCF-7 cells were treated with **A-50:B-50** polymer loaded with DOX exposed to confocal laser scanning microscopy (CLSM). For DOX-loaded polymeric samples, the images were pictured at the red channel (λ=561nm) and the cell nucleus was stained with DAPI which was visualized at the blue channel (λ=405nm). The confocal microscopy images for DOX-loaded nanoparticles and free DOX were shown in the **figure 3.18**. The image equivalent to free DOX shows that drug occupied both nucleus and cytoplasm of the cell. This was obvious from the merged image which is displaying magenta color. In the instance of DOX-loaded polymer nanoparticle, the DOX gathered in both nucleus and cytoplasm but predominantly in the nucleus. The uptake of DOX-loaded nanoparticle and NR-loaded nanoparticle was later confirmed by flow cytometry study (**see figure 3.18**). The cytometry plots show that DOX taken up by the cells are almost 10 fold better while delivering the DOX from the polymer **A-50:B-50** as compared to free DOX. The plots for NR showed that NR-loaded nanoparticle showed 2 fold better delivery of NR from the polymer.

CONCLUSION

L-Aspartic acid was altered into their equivalent esters and amides and these multi-functional monomers were condensed with different diols in solvent-free melt condensation process. In this method, oligomers of 1-4 repeating units were formed initially under nitrogen purging condition which upon further condensation under reduced vacuum condition yields polyester amides of higher molecular weight. The formation of polyester amides was confirmed by ^{1}H and ^{13}C NMR spectroscopies. The molecular weights of the newly synthesized polymers were gained in the range of $2x10^3$-$15x10^3$ with a polydispersity of ~2.2. The end group analysis by MALDI-TOF MS showed that there were 3 types of polymer linear chains as well as the cyclised product. Monomers and aliphatic diols that were taken at a mole ratio of 1:1 in the presence of $Ti(OBu)_4$ (1 mol%) catalyst at 140 ºC. Melt condensation underwent in a continuous N_2 purge for 4h followed by vacuum yielded a polymer of high molecular weight. Amide and BOC-protected random co-polyesters were synthesized by using L-aspartic acid monomers having acetyl-amide and BOC-pendent units with 1,12-dodecanediol. The selective deprotection of the BOC group (-NHCOO-$C(CH_3)_3$) in the pendant unit yielded cationic and amphiphilic co-polyesters having a positive charge in the backbone. These amphiphilic cationic polymers self-assembled in an aqueous medium and showed a size of 200 $\pm$ 5 nm. Zeta potential analysis of the de-protected polymer was observed in the range of 47 to 50 mV. The loading capacities of **A-50:B-50** polymer was tested for hydrophobic drug doxorubicin and hydrophobic dye Nile red. The drug loading efficiency (DLE) for **A-50:B-50** were observed as 31.13% and 5.45% for DOX and NR and the drug loading content (DLC) for DOX and NR were found to be 3.1% and 0.54% respectively. Cytotoxic data revealed that newly synthesized amphiphilic cationic polymers are not toxic to HeLa cells and showed cell viability more than 80 % at 60 μg/ml polymers. The uptake of the nanoparticles loaded with DOX and NR was confirmed by CLSM and flow cytometry technique.

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