Chapter 4
Vector synthesis & characterization

4 Vector synthesis and characterization

4.1 Introduction

Currently, gene therapy has revolutionized medical science research and thus have become indispensable as a research tool. Gene delivery has widespread applications including diagnostics, target validation, therapeutics etc. Successful delivery of gene can be used to breed knock-in or knock-out animal models which can be utilized for biomarker identification as well as model for therapeutic efficacy purposes [1, 2]. Gene therapeutics include wide variety of substances including plasmid DNA; RNAi therapeutics like siRNA, shRNA and miRNA; antisense oligonucleotides etc. Using these gene therapeutics, impaired gene can either be effectively expressed or silenced regulating optimal gene expression [3-6].

Since inception gene therapeutics have become focal point for research in medical and biological science. But still, there has been limited success which may be attributed to various limitations. Being negatively charged and flexible, nucleic acids and other gene therapeutics pose problems in their successful delivery to the target site as both these characteristics hinder their transport into and across cells [7]. Additionally, degradation by nucleases serve as main limiting factor halting delivery of nucleic acids in naked form. When administered in-vivo, gene therapeutics can interact with plasma components, enzymes, matrix and non-target tissues [8, 9]. This has led to complexation of nucleic acid with vectors to condense the molecule, reduce surface negative charge and protect it from nucleases and other undesirable interactions [10].

Gene therapeutics can be delivered efficiently by employing either viral or non-viral carriers [10]. Currently, recombinant viral vectors are considered as most potent gene delivery vectors [11]. Additionally, viral vectors can effectively modulate packing and unpacking of nucleic acids and thus show higher transfection efficiency. They also have capability to overcome host defense, extracellular and intracellular barriers to gene delivery [12, 13]. Presently, five main classes of viral vectors are applicable clinically - oncoretroviruses, lentiviruses, adenoviruses, adeno-associated viruses and herpes simplex-1 viruses [12]. Furthermore, viruses have capability to affect both dividing as well as non-dividing cells prolonging activity of delivered gene therapeutics. Although, accompanied with such important advantages, it still poses several limitations including immunogenicity, mutagenicity, carcinogenicity, broad tropism, difficulty of vector production, scale-up issues and low packing efficiency [14-16].

In order to overcome above mentioned limitations of viral vectors researchers have diverted their focus for development of non-viral vectors. Such vectors includes cationic cell penetrating peptides, polymeric and dendrimeric carriers, bioconjugates and lipid based carriers. Intensive research has been carried out to make non-viral vectors equivalent to viral vectors in terms of transfection efficiency thereby providing higher transfection with safety.

Amongst all non-viral vectors, PEI is polymeric vector viz. one of the most studied vector. PEI has high charge density at reduced pH which helps in DNA condensation and endosomal escape through proton sponge effect [17]. The positive charge present on PEI can be used for electrostatic binding, aiding effective complexation of nucleic acid with PEI. Still, it has been associated with potential cytotoxicity and blood incompatibility due to electrostatic interactions with cell membrane and intracellular matrix [18, 19]. Additionally, it faces problems of mutagenicity, limited crossing of cell barriers, large molecular size etc [16]. All these issues can be overcome by structural modification of PEI [20-22]. The main aim of structurally/chemically modified PEI is to reduce cationic charge either by converting primary / secondary amines into secondary / tertiary or charge-blocking by conversion of amines to amides [18].

4.2 Synthesis method

4.2.1 bPEI-Hyaluronic acid (HA) copolymer

4.2.1.1 Background

The main objective of modification of PEI by conjugating it with polysaccharide like hyaluronic acid was to impart physico-chemical stability and to reduce non-specific interactions [23, 24]. Presently, hyaluronic acid is employed as a novel carrier for various biopharmaceuticals and its role as gene delivery carrier is explored. HA is natural nonsulfated glycosaminoglycan composed of alternating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine with β(1→4) interglycosidic linkage with a molecular weight ranging from 103 to 107 Da . Additionally, hyaluronic acid can also assist in site specific delivery and cell proliferation [25, 26] as HA receptors such as CD44 [27], RHAMM [28] and LYVE-1 [29] are present on various tissues. It is also reported to act as mucoadhesive agent which enhances brain delivery when used in nasal formulations [30]. Recently, it was concluded that HA has capability to mask cytotoxicity of PEI by conjugation [31] and thus bPEI-HA copolymer was synthesized.

4.2.1.2 Procedure

The bPEI-HA copolymer was synthesized by carbodiimide reaction as shown in Figure 4. 1. Briefly, weighed quantity of HA was dissolved in phosphate buffer (pH 7.0). EDC and NHS were added to HA solution, such that HA:EDC/NHS was 1:5 w/w, and incubated overnight at 40 °C. Later, bPEI (25 kDa) was added such that HA:bPEI was 1:5 w/w and stirred for 24 h. The resultant mixture was dialyzed to purify the copolymer synthesized and later lyophilized. The reaction conditions were optimized for various parameters including reaction temperature, solvent, reactant concentration, incubation time etc. The formation of copolymer was confirmed by FT-IR and NMR. The molecular weight of formed copolymer was characterized by using Malvern Zetasizer by static light scattering technique.

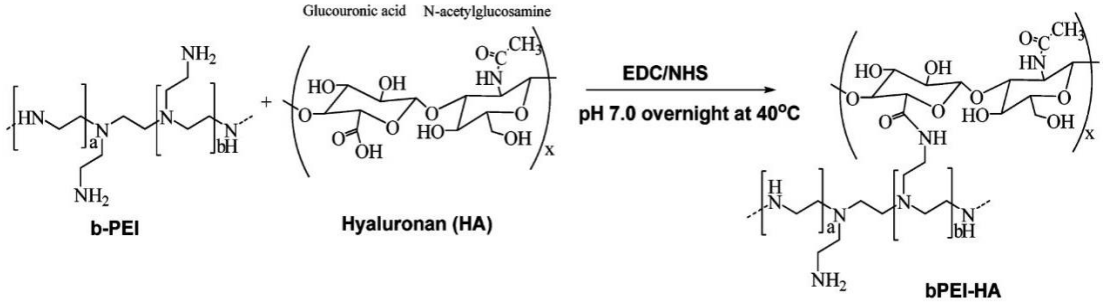

Figure 4. 1: Synthesis of bPEI-HA copolymer by carbodiimide reaction

4.2.2 bPEI-lactoferrin (Lf) conjugate

4.2.2.1 Background

Although, PEI provides carriers for nucleic acid delivery with high transfection efficiency, it still faces disadvantages like inadequate delivery to target tissue or cells, toxicity at higher concentration and limited efficiency for delivery of gene therapeutics [32, 33]. This can be overcome by utilizing receptor mediated gene transfer by complexing PEI with transfection ligands like lactoferrin. This technique, not only improves delivery at targeted tissues or cells, but also aids in internalization of complexed nucleic acid via. receptor mediated endocytosis [34]. Lactoferrin is multifunctional single chain iron-binding glycoprotein belonging to transferrin family [35]. Lactoferrin can modulate several physiological and biological activities which are regulated by lactoferrin (Lf) receptors [36]. Various studies reported presence of Lf receptors in the brain which facilitates receptor mediated transcytosis through blood

brain barrier (BBB) [37-39]. Thus bPEI-Lf conjugate was synthesized to achieve specificity and minimize cytotoxicity of formulated conjugate.

4.2.2.2 Procedure

The bPEI-Lf conjugate was synthesized by periodate oxidation technique and the synthesis scheme is depicted in Figure 4. 2. Briefly, weighed quantity of Lf was incubated with 3 fold molar excess of sodium periodate in 30 mM sodium acetate buffer (pH 5.0) for 90 min on ice. Later, the mixture was incubated for 30 min with bPEI (25 kDa) in weight ratio of 1:5 w/w. Subsequently, sodium cyanoborohydride was added in 4 portions at 1 h interval and incubated for 20 h. Then, the mixture was dialyzed and conjugate was obtained after lyophilization. The reaction conditions were optimized for various parameters including reaction temperature, solvent, reactant concentration, incubation time etc. The formation of conjugate was confirmed by FT-IR and NMR. The molecular weight of formed copolymer was characterized by using Malvern Zetasizer by static light scattering technique.

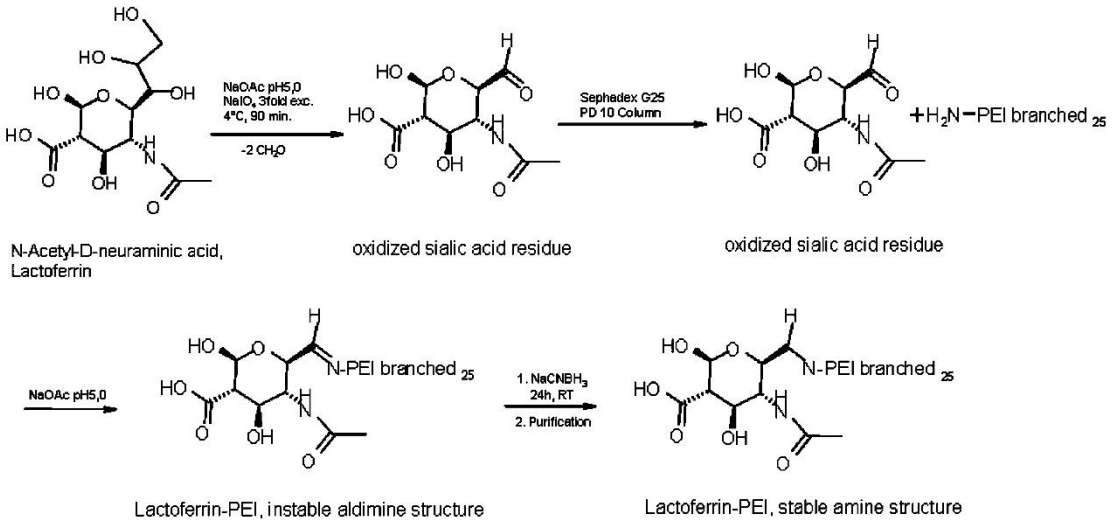

Figure 4. 2: Synthesis of bPEI-Lf conjugate by periodate oxidation method

4.2.3 bPEI-Chitosan (Chi) copolymer

4.2.3.1 Background

As previously discussed in section 4.2.1.1 Background conjugation of PEI with polysaccharides can improve its physicochemical stability and reduce non-specific interactions. Chitosan and PEI are leading cationic polymers to be explored for gene delivery [40]. Chitosan is found in crustaceans and possess some special properties like biocompatibility, biodegradability, low toxicity, ease of modification etc suitable for

gene delivery carrier [41-44]. But, when used alone as a carrier, it has poor solubility, low transfection efficiency and poor proton sponge effect which would impede efficient delivery of gene therapeutics at targeted site [45]. These limitations along with cytotoxicity of PEI can be overcome by synthesis of bPEI-chitosan polymer. Additionally, being mucoadhesive, chitosan can improve bioavailability of hydrophilic molecules in brain across nasal epithelium [46-48]. Various studies concluded that chitosan has ability to enhance paracellular transport through nasal epithelia by increasing permeability through opening of tight junctions and suppression of efflux transporters [49, 50].

4.2.3.2 Procedure

The bPEI-Chi copolymer was synthesized by coupling reaction using carbonyldiimidazole (CDI) as a linker and the reaction mechanism is shown in Figure 4. 3. Briefly, weighed quantity of Chi was dissolved in 1% acetic acid and incubated overnight. After incubation, double quantity of CDI dissolved in water was added and stirred for 1 h. Subsequently, bPEI dissolved in water (Chi:bPEI=1:3 w/w) was added and incubated for 24 h. Then, the mixture was dialyzed and copolymer was obtained after lyophilization. The reaction conditions were optimized for various parameters including reaction temperature, solvent, reactant concentration, incubation time etc. The formation of copolymer was confirmed by FT-IR and NMR. The molecular weight of formed copolymer was characterized by using Malvern Zetasizer by static light scattering technique.

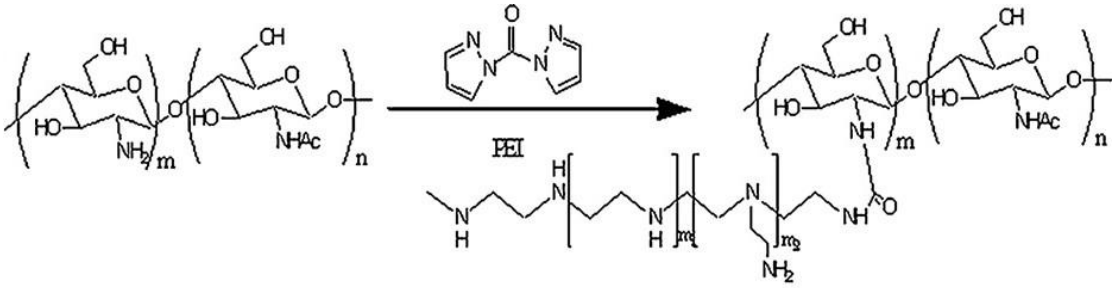

Figure 4. 3: Synthesis of bPEI-Chi copolymer using carbonyldiimidazole as crosslinker

4.2.4 Biodegradable bPEI

4.2.4.1 Background

As described earlier, PEI has evolved as one of the most potent and successful non-viral gene delivery vector due to its property to condense nucleic acid facilitating

endocytosis and proton sponge effect impeding disruption of nucleic acid in endosomes [51, 52]. However, PEI suffers from biodegradability issue which aggravates with increase in molecular weight and branching. This has limited its therapeutic application for gene delivery as it may lead to accumulation toxicity [53]. The low molecular weight PEIs have low cytotoxicity and also linear PEI are more tolerable than branched PEI of same molecular weight. Still, such low molecular weight linear PEI have low transfection efficiency preventing its therapeutic application [54]. To overcome these limitations various degradable PEI prepared by crosslinking have been explored which could provide higher transfection efficiency with low cytotoxicity due to its biodegradation property [55, 56]. Biodegradable PEIs can be classified based on structure and degradable linkages as – linear, branched or grafted PEIs. This work focuses on synthesis of degradable PEI using 1,6-hexanediol diacrylate as a crosslinker which possessed both disulfide and ester linkages improving their bioavailability [57, 58].

4.2.4.2 Procedure

The biodegradable bPEI was synthesized by crosslinking reaction using 1,6-hexanediol diacrylate (HDD) as a crosslinker and the reaction mechanism is shown in Figure 4. 4. Briefly, bPEI (0.8 kDa) was dissolved in sufficient quantity of dichloromethane (DCM) and incubated for 1 h. Later, HDD (HDD:bPEI=1:4 w/w) dissolved in DCM was added dropwise to the PEI solution. The reaction mixture was stirred for 24 h. Then, reaction product was extracted using diethyl ether and dried by subsequently evaporating ether at room temperature. The reaction conditions were optimized for various parameters including reaction temperature, solvent, reactant concentration, incubation time etc. The formation of biodegradable polymer was confirmed by FT-IR and NMR. The molecular weight of formed copolymer was characterized by using Malvern Zetasizer by static light scattering technique.

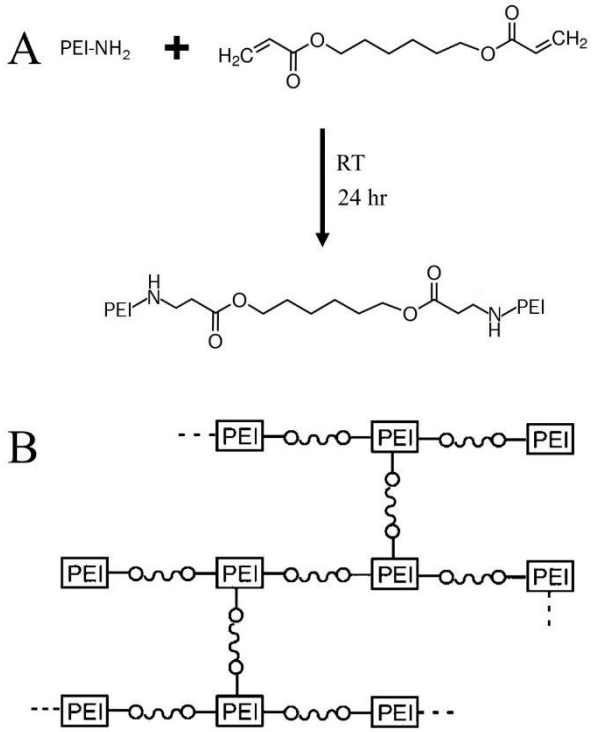

Figure 4. 4: Synthesis of biodegradable bPEI using 1,6-hexanediol diacrylate (A) 0.8 kDa bPEI is reacted with diacrylate crosslinker to generate ester crosslinked polymer (B) Acrylate crosslinker reacts with primary and secondary amines giving crosslinked product

4.2.5 Biodegradable bPEI-Chitosan (Chi) copolymer

4.2.5.1 Procedure

The copolymer was prepared by procedure as described in section 4.2.3. The molecular weight of formed copolymer was characterized by using Malvern Zetasizer by static light scattering technique.

4.2.6 Liposomes

4.2.6.1 Background

Liposomes are vesicular structure that have wide variety of applications in analytical science and drug as well as gene delivery [59] and thus are considered as keystones of biotechnology [60]. Liposomes have several advantages including varied morphologies, compositions, low cost, lack of immunogenic response, protect therapeutic molecules by enveloping it and differential release characteristics [61-63]. These has led to applications of liposomes in various fields including gene delivery [64]. Liposomes for gene delivery can be formulated by cationic, neutral or anionic

lipids. Due to some of the potential limitations of cationic lipids including interaction with serum components, stability issues and cytotoxicity, anionic liposomes are explored as potential carriers for gene delivery [65, 66]. Furthermore, negative surface charge has been associated with reduced clearance and phagocytosis, improving bioavailability of delivered gene therapeutics [67]. Thus this work focuses on development of anionic liposomes using DMPG as negatively charged lipid which also act as permeability enhancer for nasal epithelium [68].

4.2.6.2 Procedure

Liposomes were prepared by ethanol injection method. Briefly, 2 ml of nuclease free water was taken in 10 ml vial. This was kept on magnetic stirrer at around 60 °C temperature with continuous stirring. In another 10 ml beaker HSPC, Cholesterol, DMPG and $mPEG_{2000}$-DSPE were taken at molar ratios of 65:25:8:2 (lipid portion). In lipid portion 1 ml ethanol was added and heated at 60 °C. The lipid portion was added to aqueous phase with continuous stirring and temperature maintained at 60 °C for 15 min till liposomal dispersion was formed. The liposomal dispersion thus obtained was sonicated using probe sonicator with cycle time 0.8 and 80% amplitude.

4.3 Characterization

4.3.1 Nuclear magnetic resonance (NMR)

The prepared copolymers and conjugates were characterized using proton nuclear magnetic resonance (^{1}H NMR). ^{1}H NMR was carried out on 400 MHz JEOL NMR with DELTA processing software. The chemical shifts were referenced to the lock D_2O except bPEI-Chi copolymer and biodegradable bPEI-Chi where CH_3COOD was used as a solvent.

4.3.2 Fourier transform – Infrared spectroscopy (FT-IR)

Purified copolymers and conjugate samples were placed in the sample holder and analyzed by FT-IR spectroscopy using an IRAffinity-1 spectroscope (Shimadzu, Japan)

4.3.3 Molecular weight determination

Molecular weight was determined using Zetasizer NanoZS (Malvern Instruments Ltd., UK) by static light scattering technique. Weighed quantity of the preformed copolymer or conjugate was dissolved in required quantity of solvent to prepare stock solution. Then, the prepared stock solution was diluted with solvent to obtain solution of desired concentration. Before starting the analysis, dark measurement

was performed to eliminate the background effect. This was followed by standard measurement for which methanol was used. Later, the solvent used for dilution was kept to eliminate any solvent effect and finally polymeric solutions of different concentration were analyzed. In this technique, the scattering intensity of a number of concentrations of the sample is measured, and used to construct a Debye plot. From this the average molecular weight and second virial coefficient was calculated.

4.3.4 Muco-adhesion study

Mucoadhesive strength of synthesized copolymers and conjugates was determined by estimating % mucin binding efficiency (%MBE) as proposed by Yin et al [69]. Briefly, synthesized copolymers and conjugates were weighed accurately and dissolved in distilled water (except bPEi-Chi copolymer and biodegradable bPEI-Chi copolymer where copolymers were dissolved in acetic acid solution). Equal volume of porcine mucin suspension (1 mg/ml) was added to polymeric solution and vortexed for uniform mixing. These mixtures were incubated for 24 h at 37 °C. Later, these mixtures were centrifuged at 10000 x g for 20 min and supernatant was collected. Free mucin concentration in supernatant was calculated by UV spectrometry at 258 nm (λmax). % mucin binding efficiency was calculated by employing following equation

$$\%\, MBE \;=\; \frac{Total\ mucin - Free\ mucin}{Total\ mucin} \times 100$$

4.4 Result and discussion

4.4.1 Nuclear magnetic resonance (NMR)

4.4.1.1 bPEI-HA copolymer

The synthesized bPEI-HA copolymer was lyophilized to obtain yellow colored viscous and sticky copolymer. ^{1}H-NMR analysis of aforesaid polymer (Figure 4. 7) gave distinctive peaks than ^{1}H-NMR of bPEI (Figure 4. 5) and HA (Figure 4. 6). The peak at 2.5-2.6 ppm suffered slight shift in δ value due to conjugation of bPEI with HA [70] and indicated presence of NH-CH_2-CH_2-N while peak at ~2.7 ppm and 3.0-3.2 ppm signifies presence of methyl group in bPEI-HA copolymer. These findings suggested successful formation of bPEI-HA copolymer [71, 72].

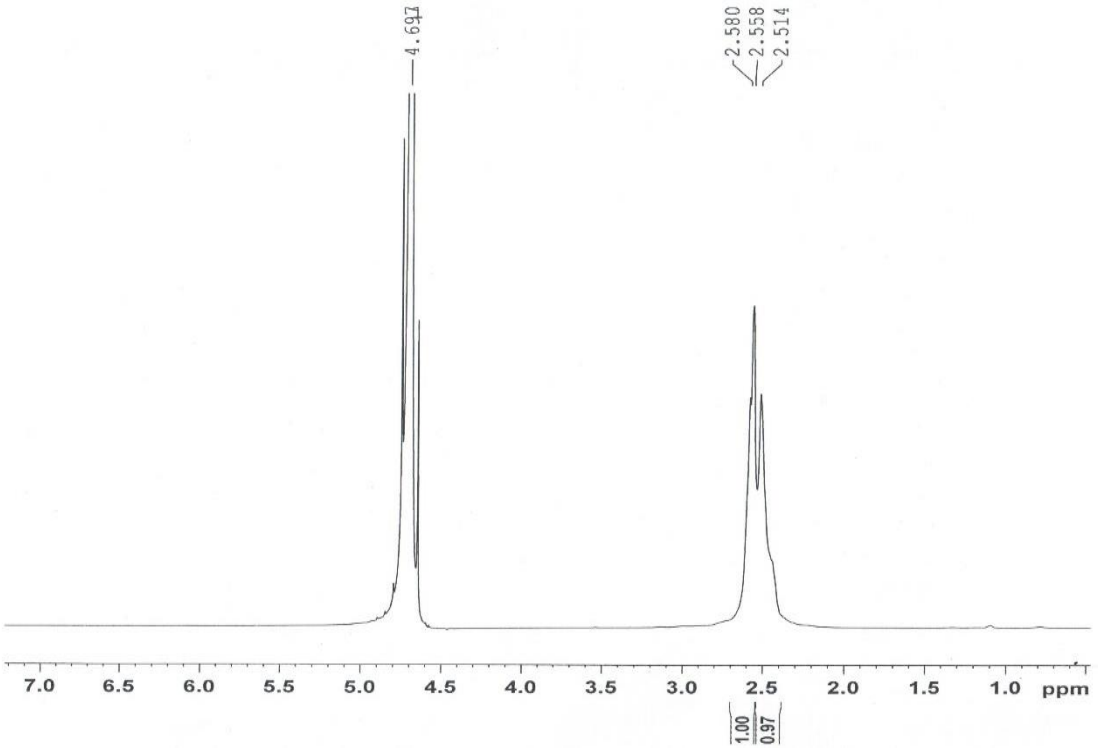

Figure 4. 5: NMR spectra of bPEI (25kDa)

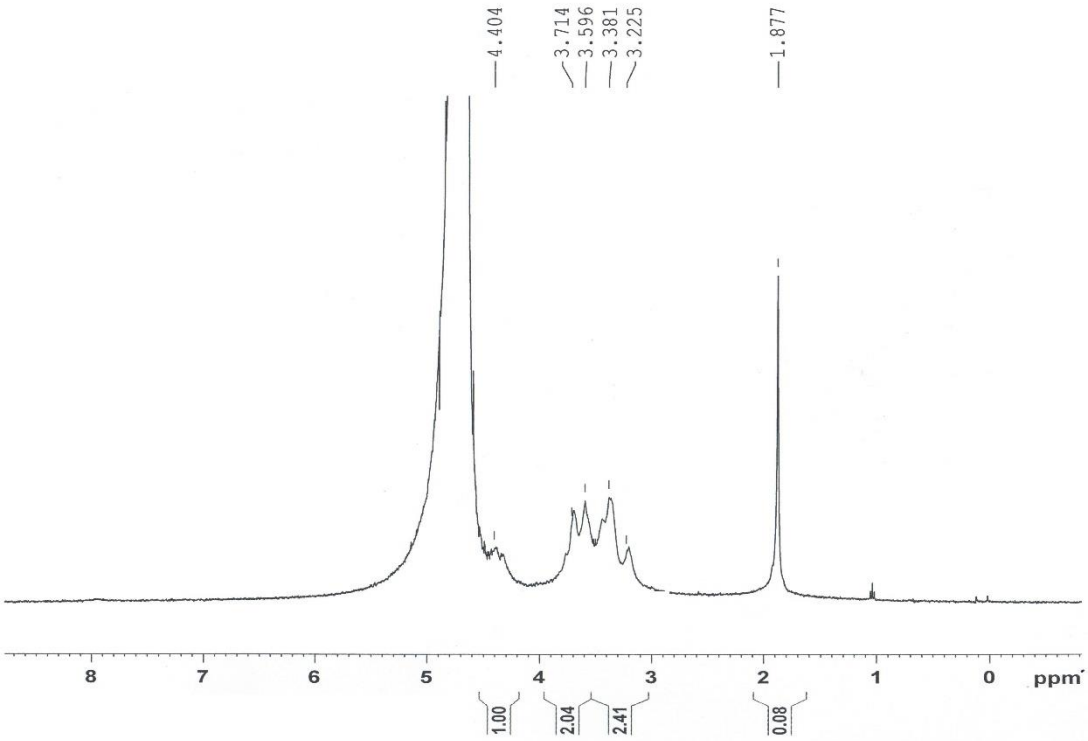

Figure 4. 6: NMR spectra of HA

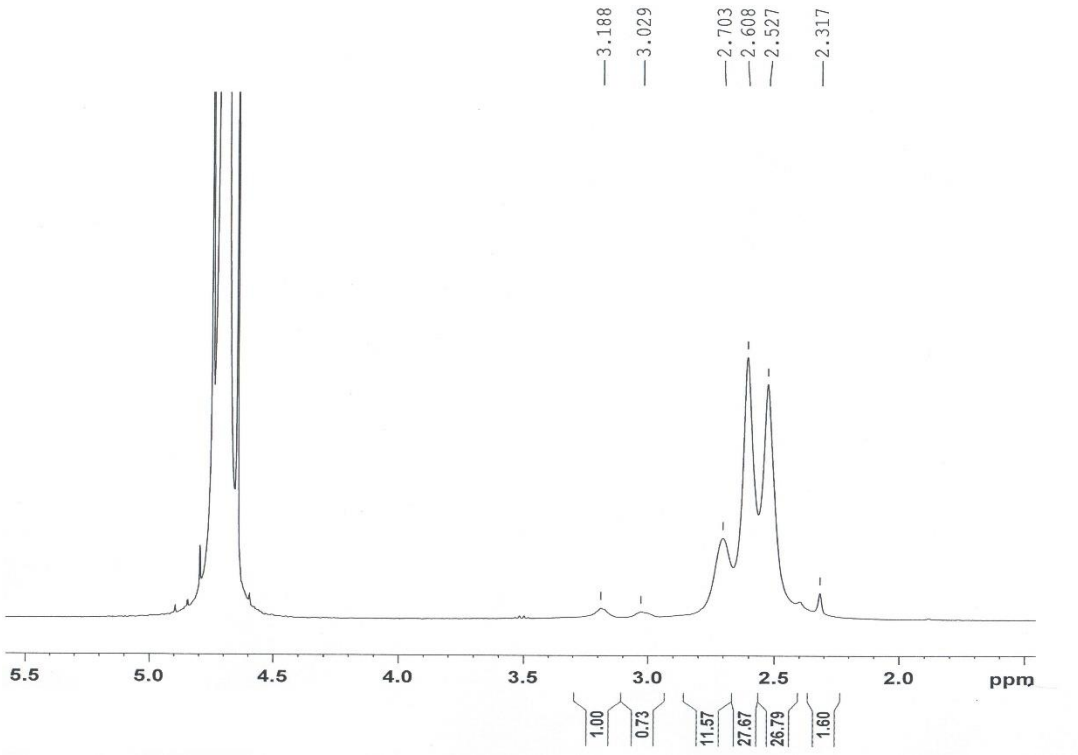

Figure 4. 7: NMR spectra of bPEI-HA copolymer

4.4.1.2 bPEI-Lf conjugate

The ^{1}H-NMR spectra of bPEI-Lf conjugate is represented in Figure 4. 8 which showed peak at ~2.65 ppm which showed slight shift in δ value as compared to peak present in ^{1}H-NMR spectra of bPEI. This showed significant conjugation of bPEI to lactoferrin. Additionally, characteristic peak at 4.5 to 5.0 ppm showed formation of bPEI-Lf conjugate.

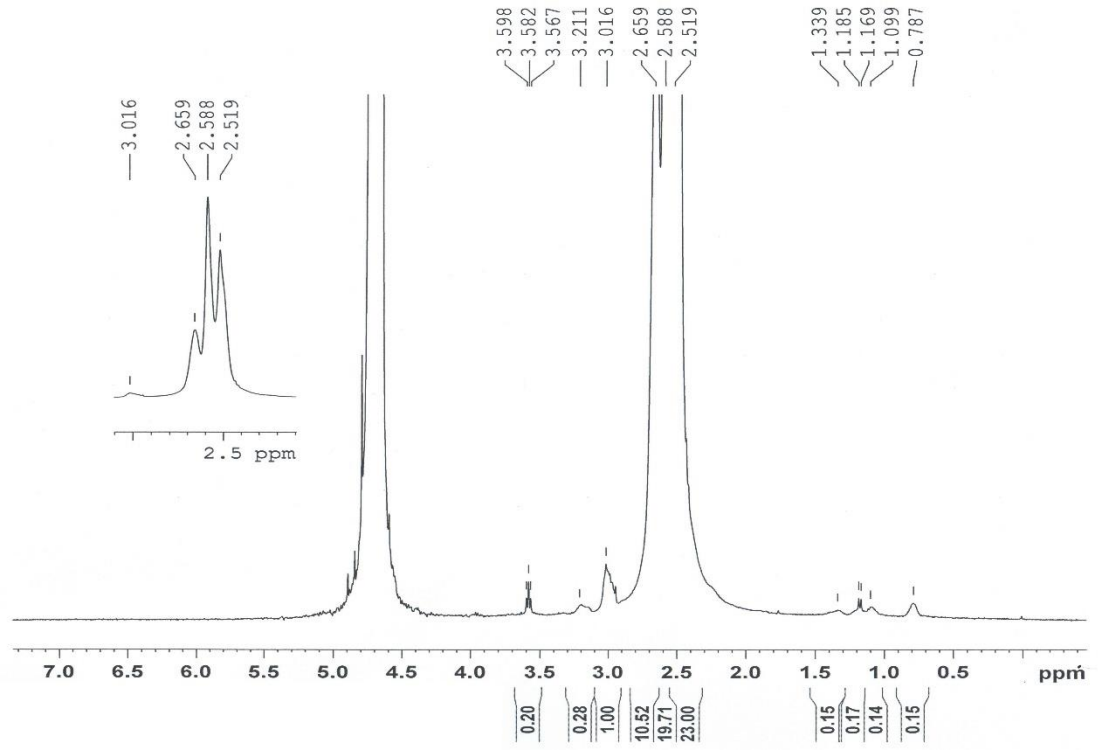

Figure 4. 8: NMR spectra of bPEI-Lf conjugate

4.4.1.3 bPEI-Chi copolymer

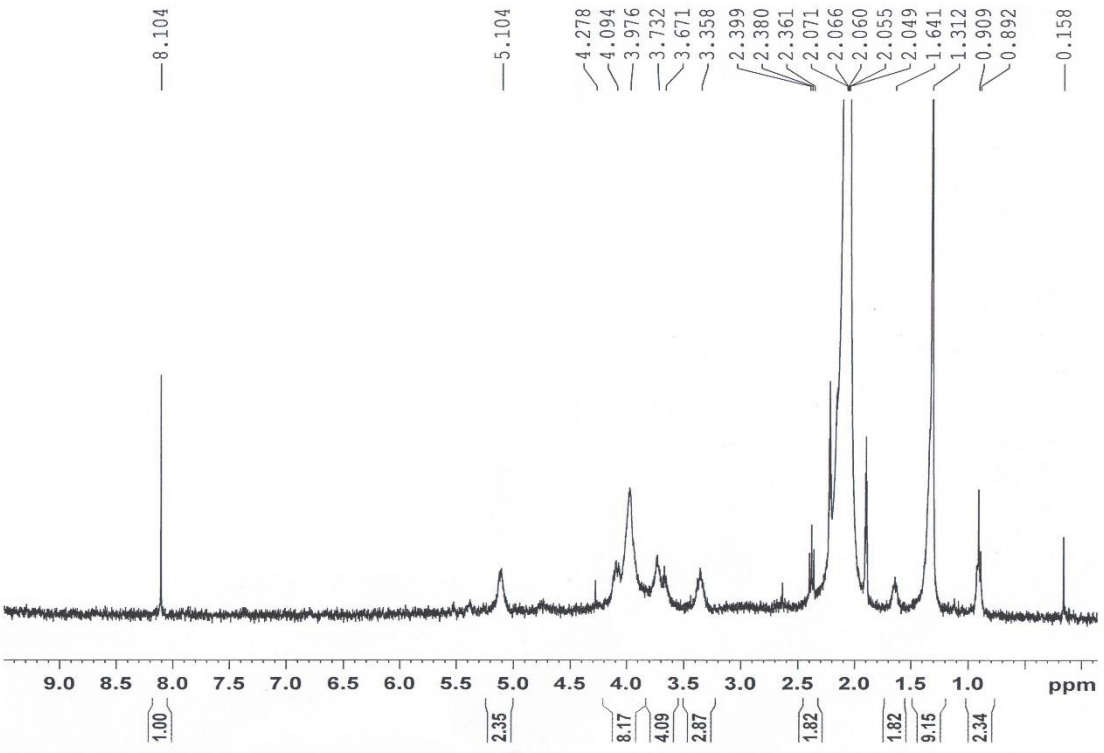

Figure 4. 9: NMR spectra of Chi

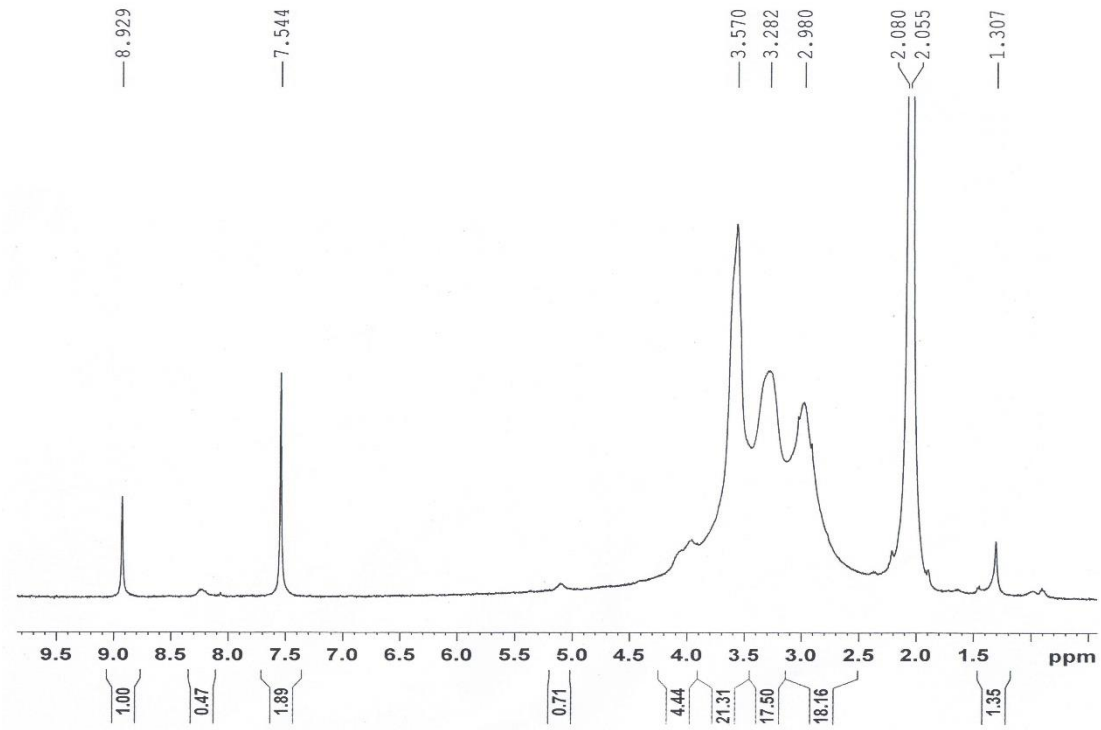

Figure 4. 10: NMR spectra of bPEI-Chi copolymer

The NMR spectra of bPEI-Chi copolymer as depicted in Figure 4. 10 shows proton peaks at 5.1 ppm signifying presence of H1 of glucosamine ring; 3.3 ppm for H2 of glucosamine ring; 3.6 - 4.3 ppm for H3, H4, H5 and H6 of glucosamine ring. Additionally, the characteristic peak of PEI was found around 2.3 ppm which confirmed PEI grafting on chitosan chain [73, 74].

4.4.1.4 Biodegradable PEI

The NMR spectra of bPEI 0.8 kDa and biodegradable crosslinked bPEI are represented in Figure 4. 11 and Figure 4. 12 respectively. The formation of crosslinked biodegradable bPEI from bPEI (0.8 kDa) was confirmed by formation of various ester bonds at δ values of 3.9 – 4.0 ppm ($NHCH_2CH_2COOCH_2$); 3.3 – 3.4 ppm ($OHCH_2CH_2$); 2.5 – 3.3 ppm (PEI ethylenes); 2.5 ppm ($NHCH_2CH_2COOCH_2$); 1.3 ppm ($COOCH_2CH_2$) [75].

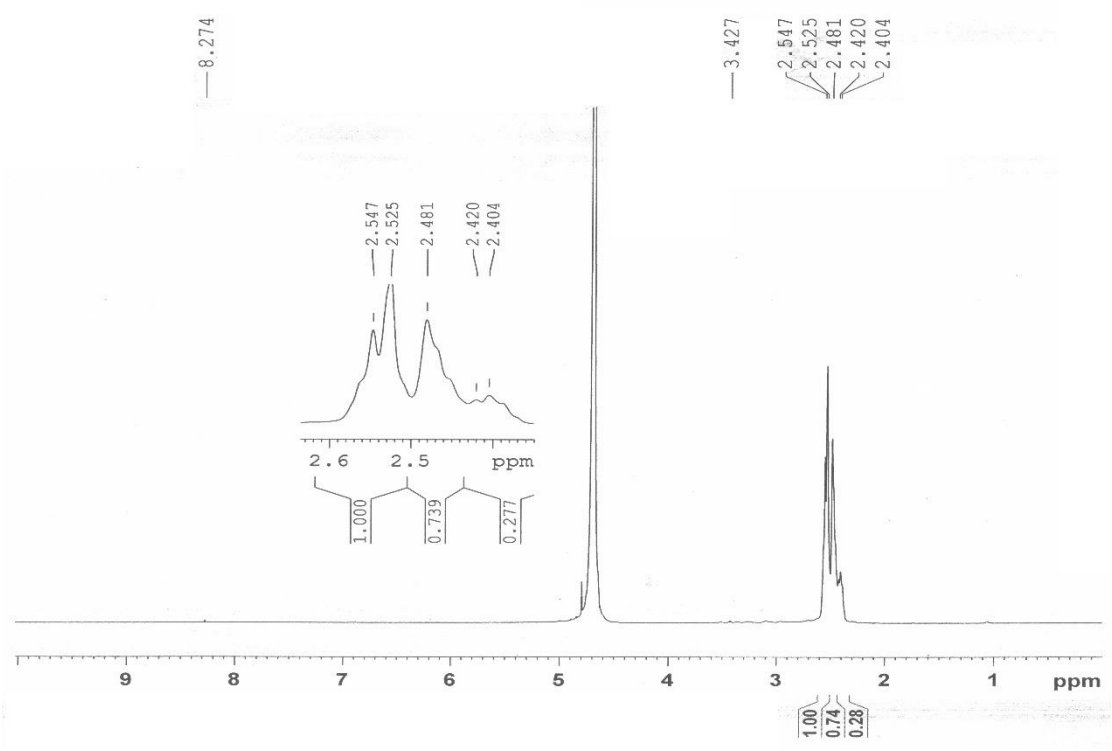

Figure 4. 11: NMR spectra of bPEI (0.8kDa)

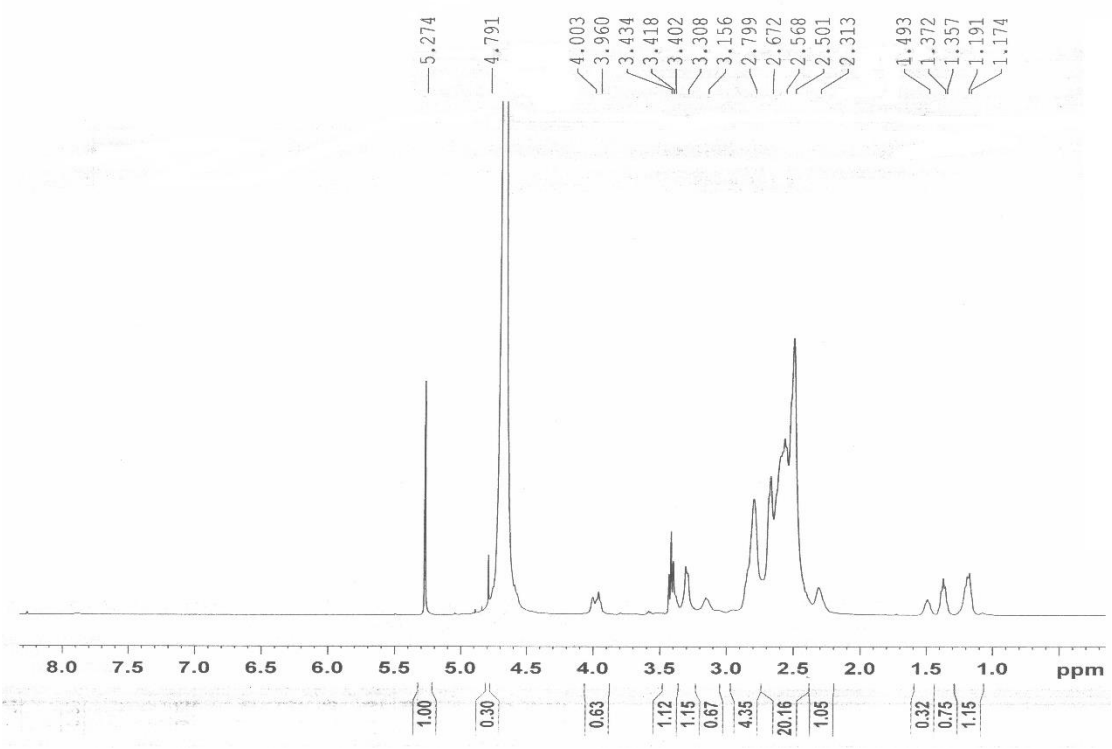

Figure 4. 12: NMR spectra of biodegradable crosslinked bPEI

4.4.2 Fourier transform – Infrared spectroscopy (FT-IR)

4.4.2.1 bPEI-HA copolymer

The FT-IR spectra of bPEI, HA and bPEI-HA copolymer are shown in Figure 4. 13, Figure 4. 14 and Figure 4. 15 respectively. Absorption peaks were observed in the region 3300-3500 cm^{-1} due to N-H streching signifying presence of amine group in bPEI. FT-IR spectra of HA showed characteristic peaks in region 1600-1800 cm^{-1} due to C=O stretching indicating presence of carboxylic acid group. Finally, FT-IR spectra of bPEI-HA showed characteristic peaks in the region of 1500-1650 cm^{-1} and 3200-3300 cm^{-1} due to N-H stretching and C=O stretching indicating presence of amide group. These findings confirm conjugation of HA with bPEI forming bPEI-HA copolymer.

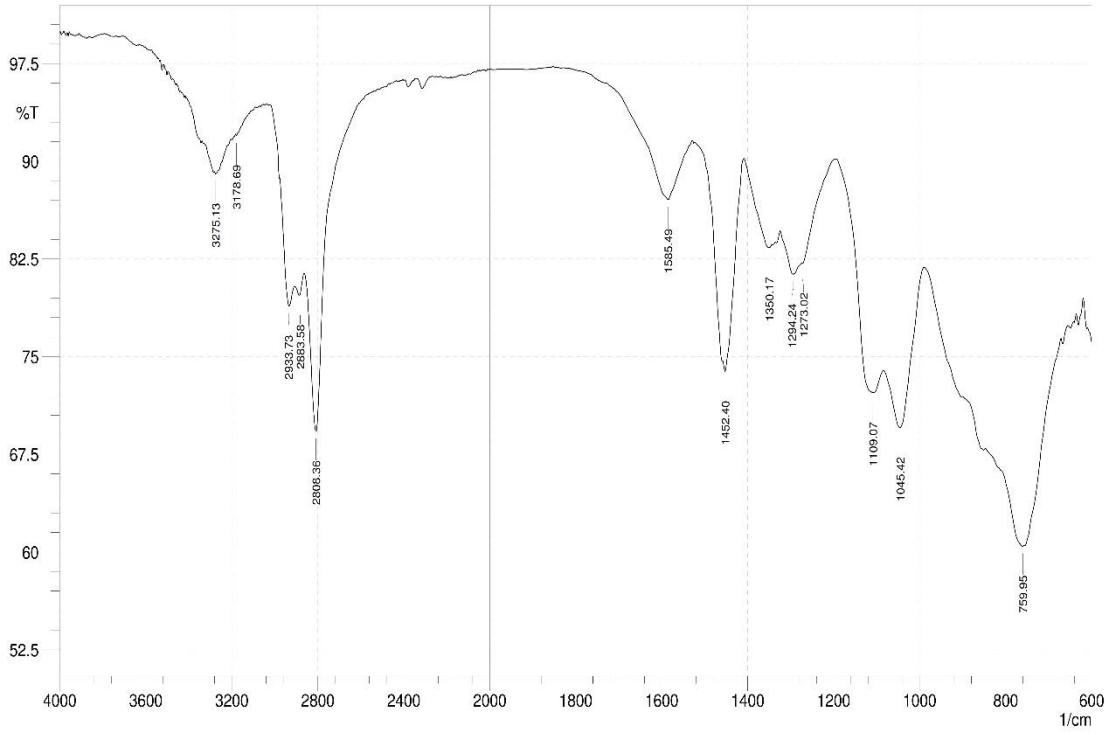

Figure 4. 13: FT-IR spectra of bPEI (25kDa)

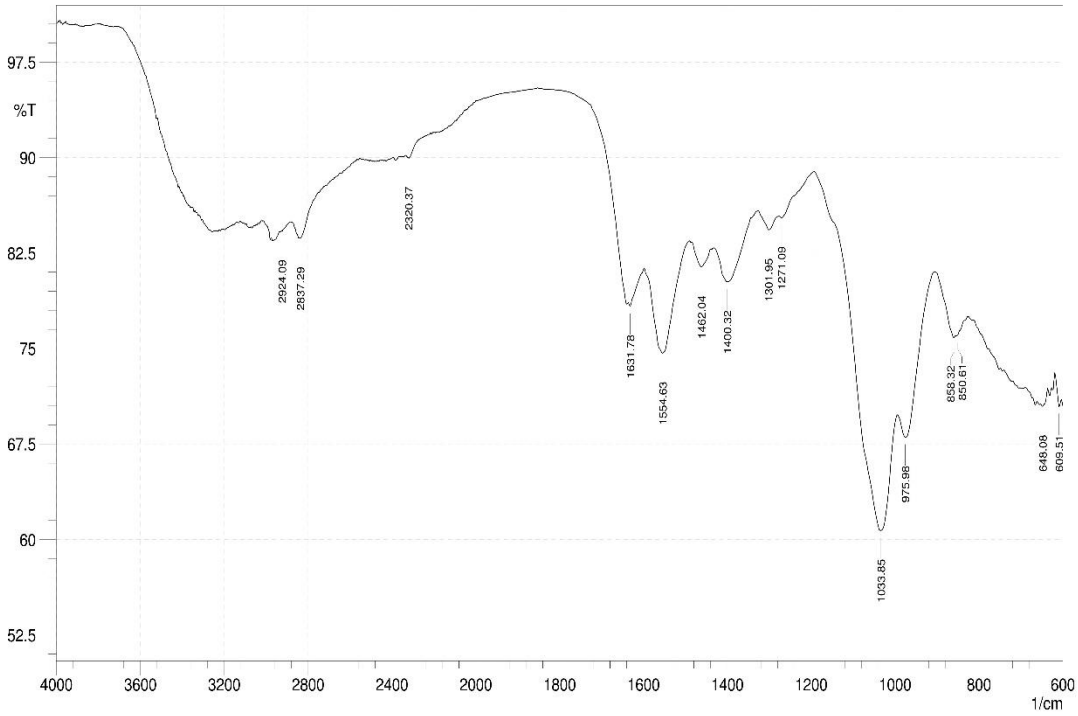

Figure 4. 14: FT-IR spectra of HA

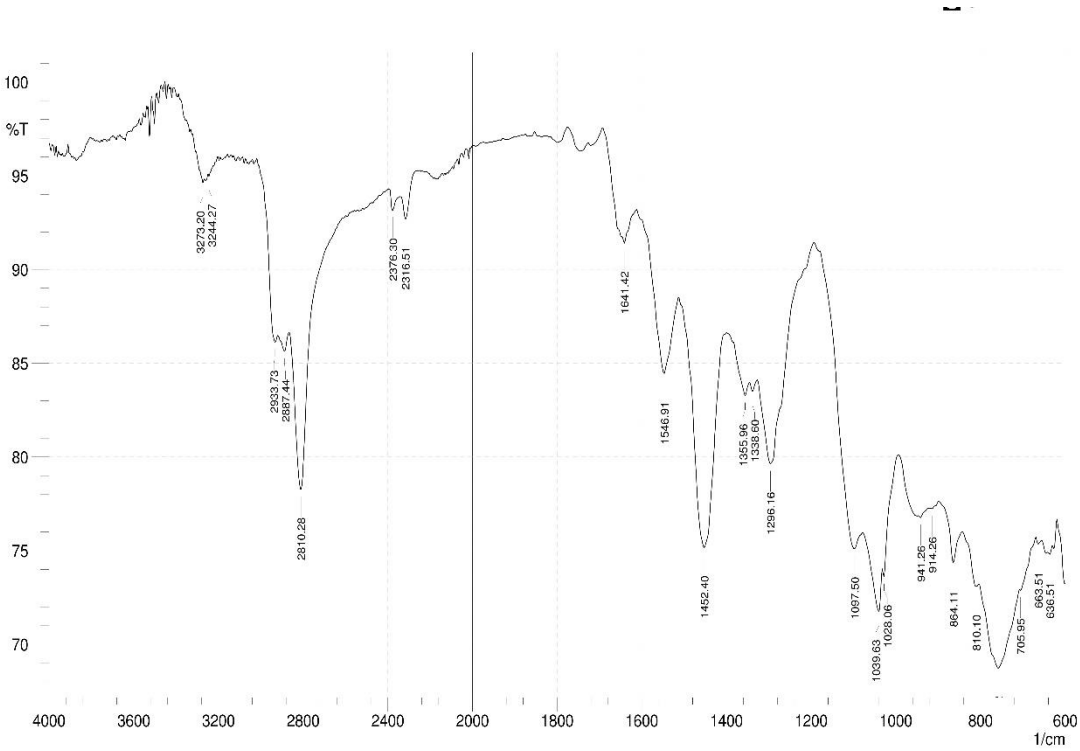

Figure 4. 15: FT-IR spectra of bPEI-HA copolymer

4.4.2.2 bPEI-Lf conjugate

The FT-IR spectra of bPEI, Lf and bPEI-Lf conjugate are shown in Figure 4. 13, Figure 4. 16 and Figure 4. 17 respectively. Absorption peaks were observed in the region 3300-3500 cm^{-1} due to N-H streching signifying presence of amine group in bPEI. FT-IR spectra of Lf showed characteristic peaks in region 1600-1800 cm^{-1} due to C=O stretching indicating presence of carboxylic acid group. Additionally, absorption peaks were observed in the region of 1500-1650 cm^{-1} and 3200-3300 cm^{-1} due to N-H stretching and C=O stretching indicating presence of amide group in lactoferrin. Finally, FT-IR spectra of bPEI-Lf conjugate showed characteristic peaks of both lactoferrin and bPEI with increased intensity of absorption peaks in the region 3200-3300 cm^{-1} due to N-H stretching indicating successful conjugation of bPEI with lactoferrin.

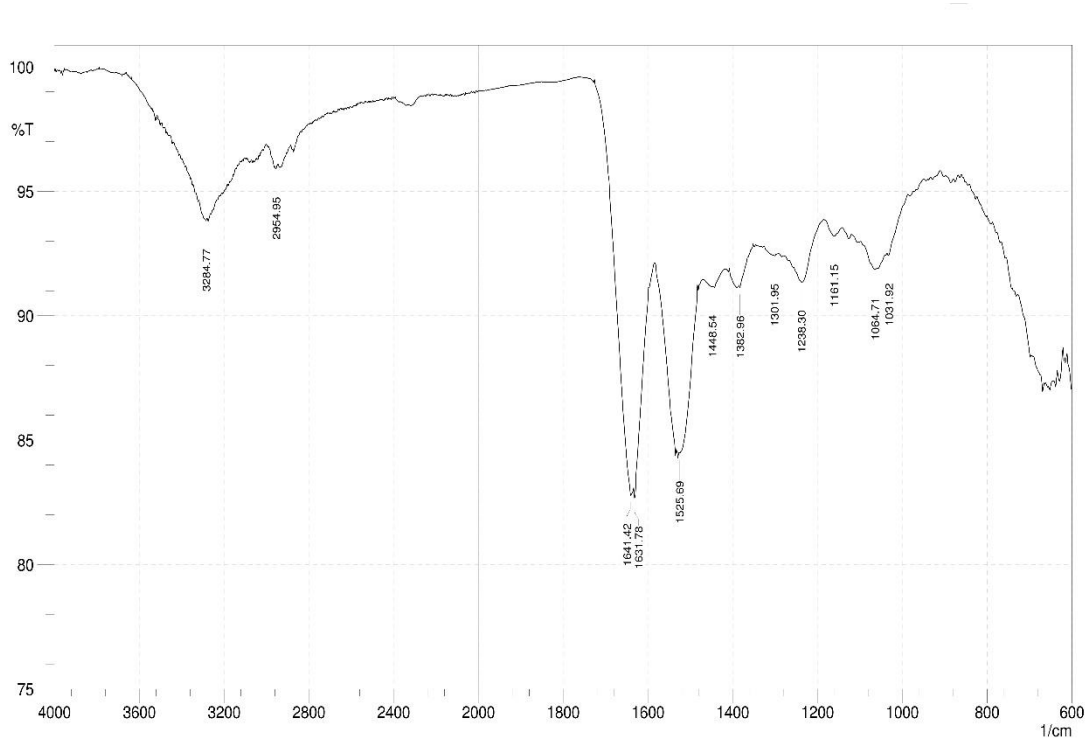

Figure 4. 16: FT-IR spectra of Lf

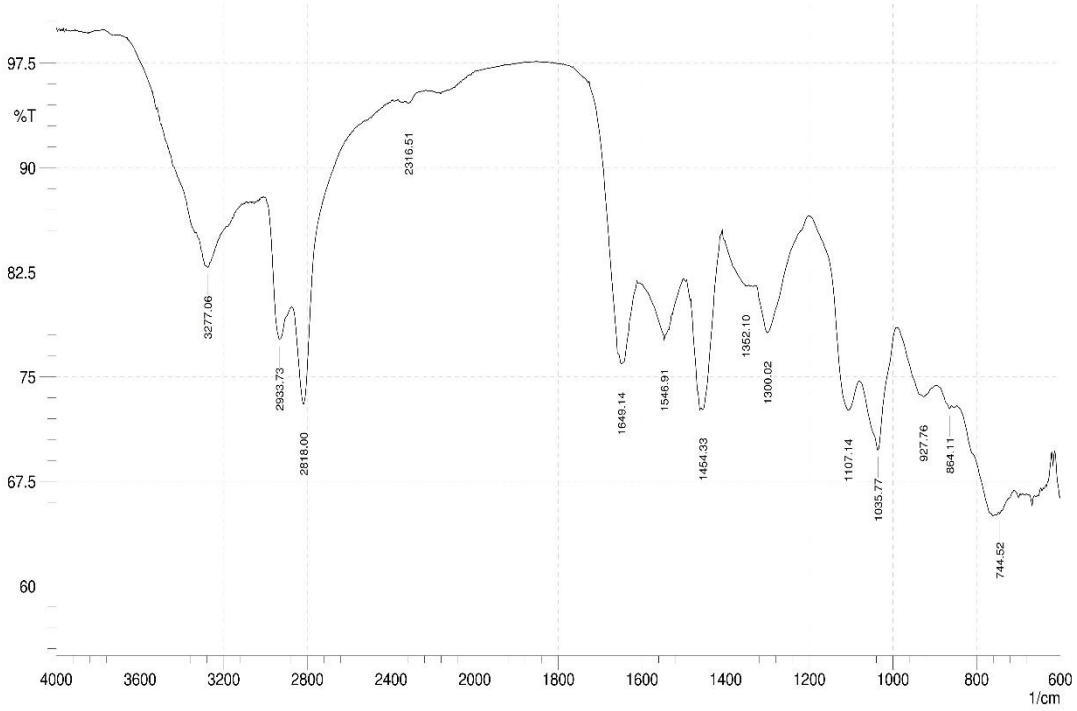

Figure 4. 17: FT-IR spectra of bPEI-Lf conjugate

4.4.2.3 bPEI-Chi copolymer

The FT-IR spectra of bPEI, Chi and bPEI-Chi copolymer are shown in Figure 4. 13, Figure 4. 18 and Figure 4. 19 respectively. Absorption peaks were observed in the region 3300-3500 cm^{-1} due to N-H streching signifying presence of amine group in bPEI. FT-IR spectra of Chi showed characteristic peaks in region 3200-3600 cm^{-1} due to O-H stretching indicating presence of hydroxyl group. Finally, FT-IR spectra of bPEI-Chi copolymer showed characteristic peaks of both N-H stretching and C=O stretching which confirmed formation of amide bond between bPEI and Chi and thus concluded formation of bPEI-Chi copolymer.

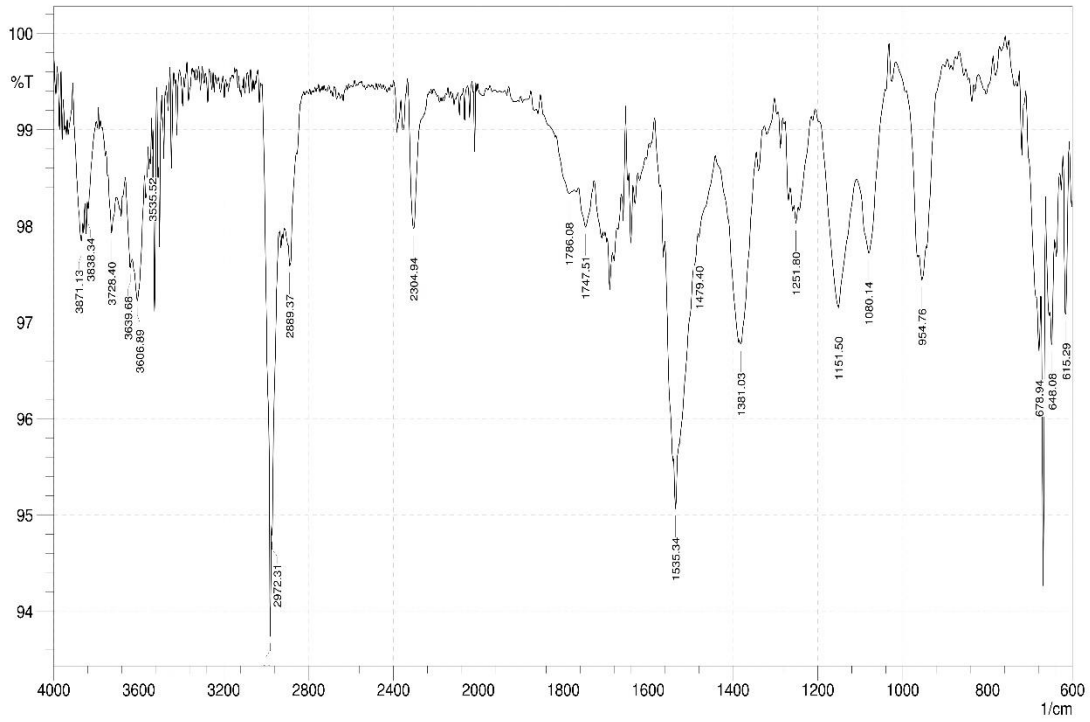

Figure 4. 18: FT-IR spectra of Chi

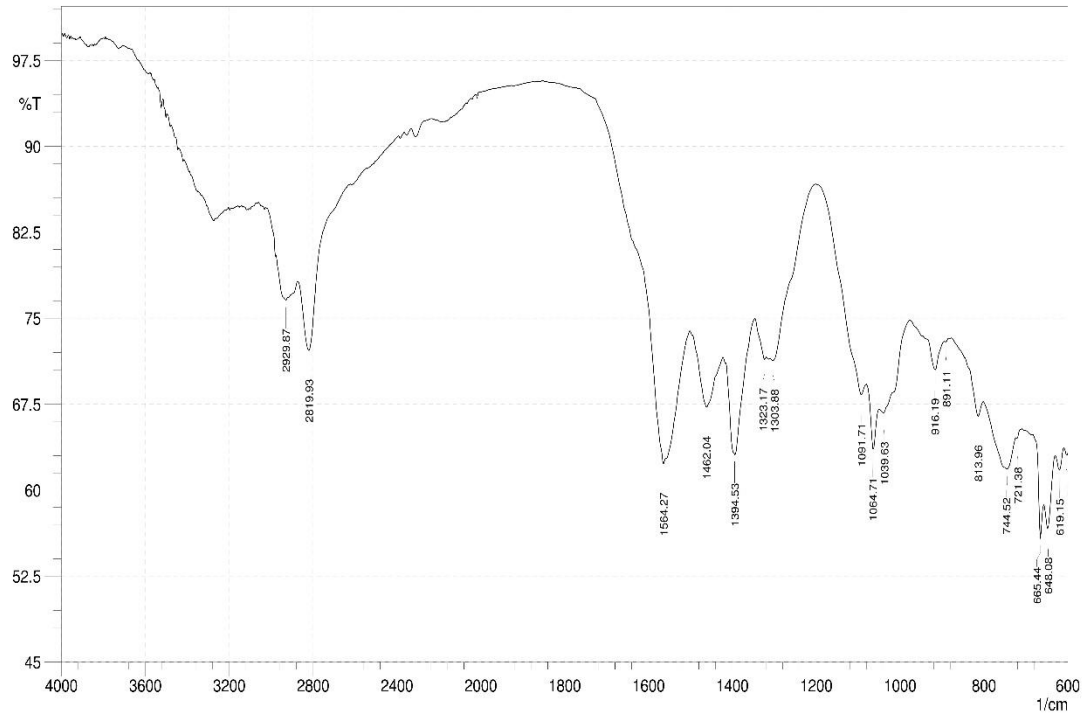

Figure 4. 19: FT-IR spectra of bPEI-Chi copolymer

4.4.2.4 Biodegradable PEI

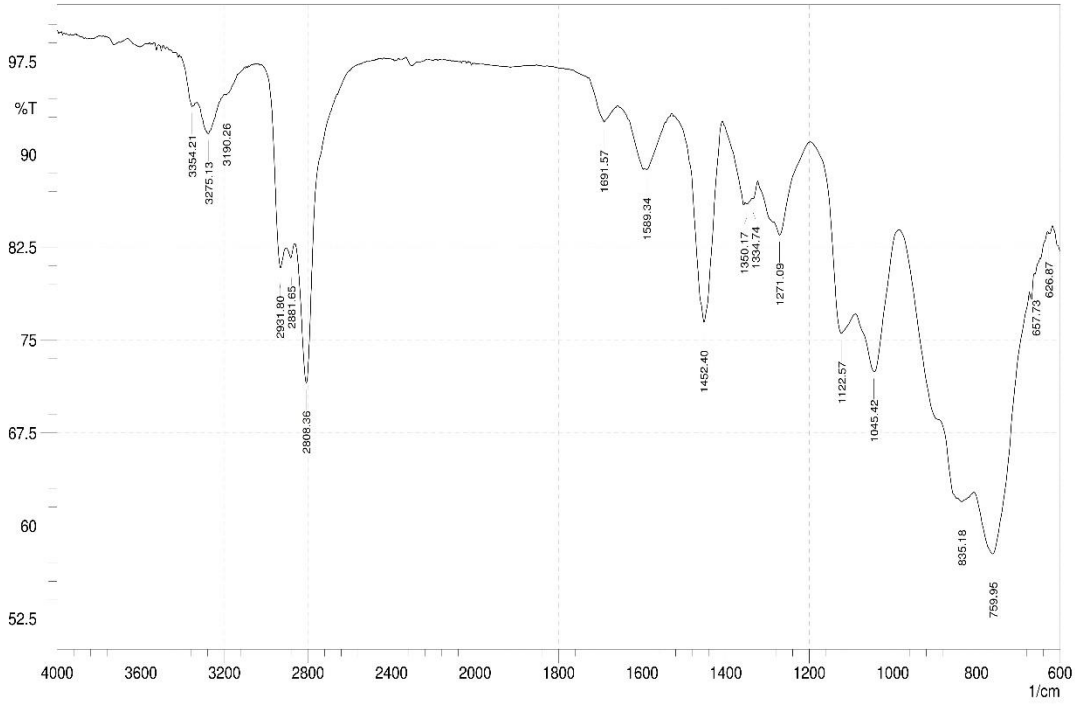

Figure 4. 20: FT-IR spectra of PEI (0.8 kDa)

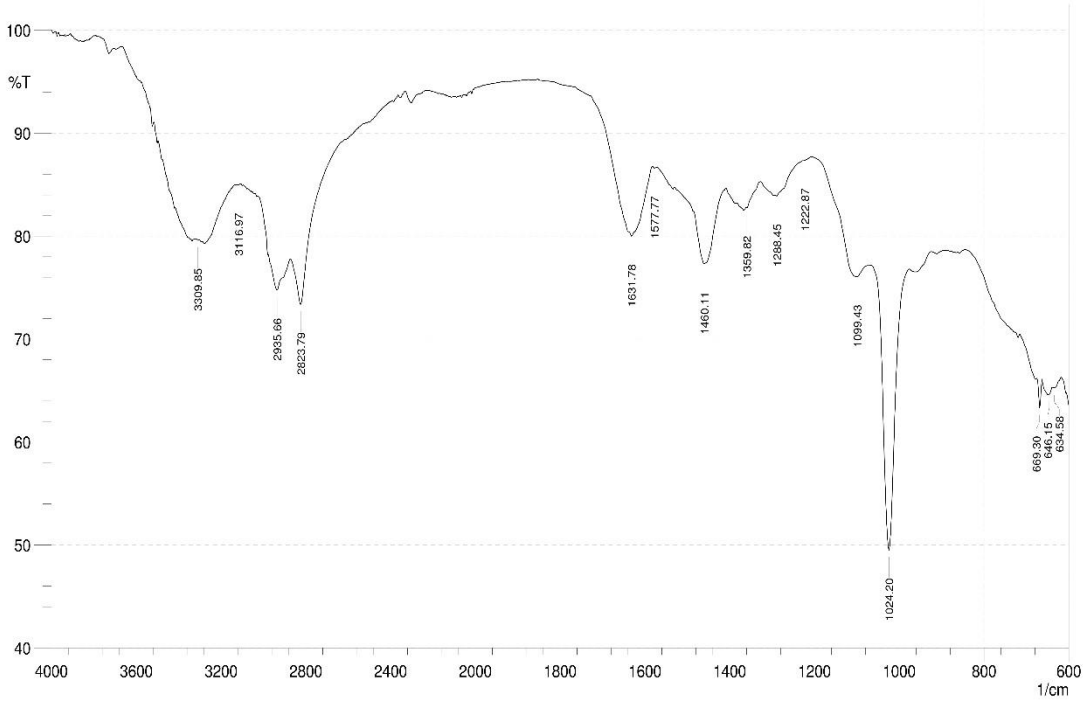

Figure 4. 21: FT-IR spectra of biodegradable bPEI

The FT-IR spectra of bPEI (0.8 kDa) and biodegradable bPEI are shown in Figure 4. 20 and Figure 4. 21 respectively. Absorption peaks were observed in the region 3300-3500 cm^{-1} due to N-H streching signifying presence of amine group in bPEI (0.8 kDa). FT-IR spectra of biodegradable bPEI showed characteristic peak around 1650 cm^{-1} due to C=O stretching indicating presence of ester group. Additionally, it also showed peak for N-H stretching which confirmed formation of crosslinking between bPEI (0.8 kDa).

4.4.3 Molecular weight determination

The results of molecular weight for all polymers, copolymers and conjugates determined using static light scattering are depicted from Figure 4. 22 to Figure 4. 29 and summarized in Table 4. 1.

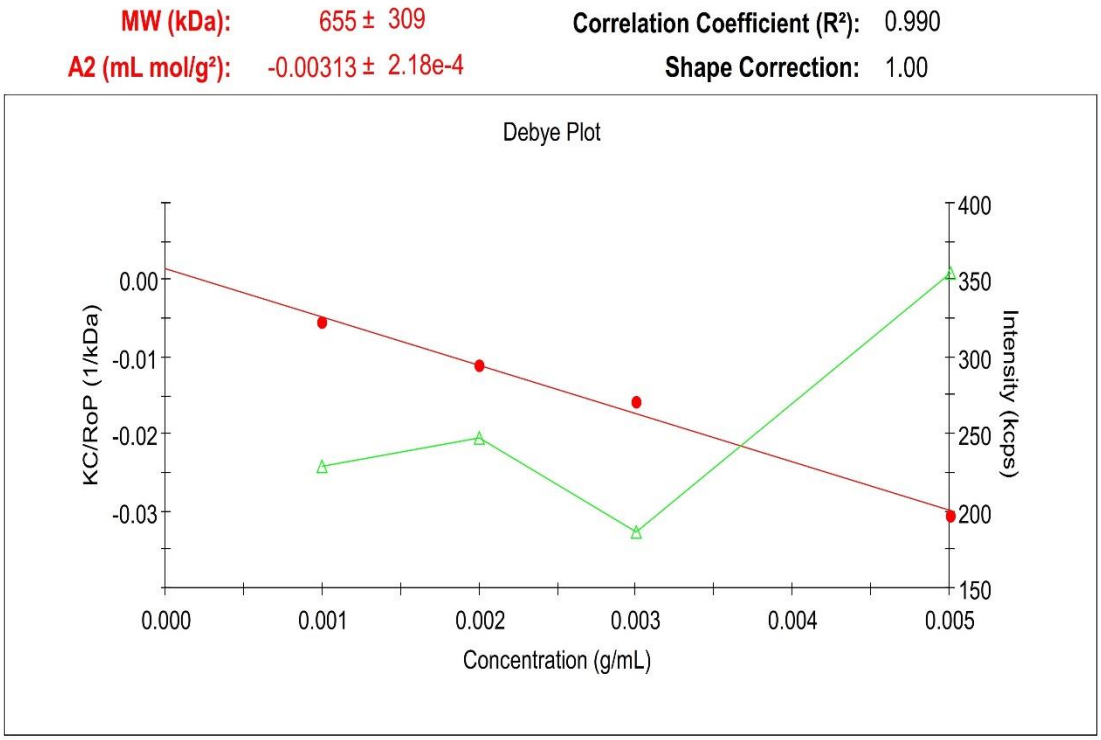

Figure 4. 22: Molecular weight of HA

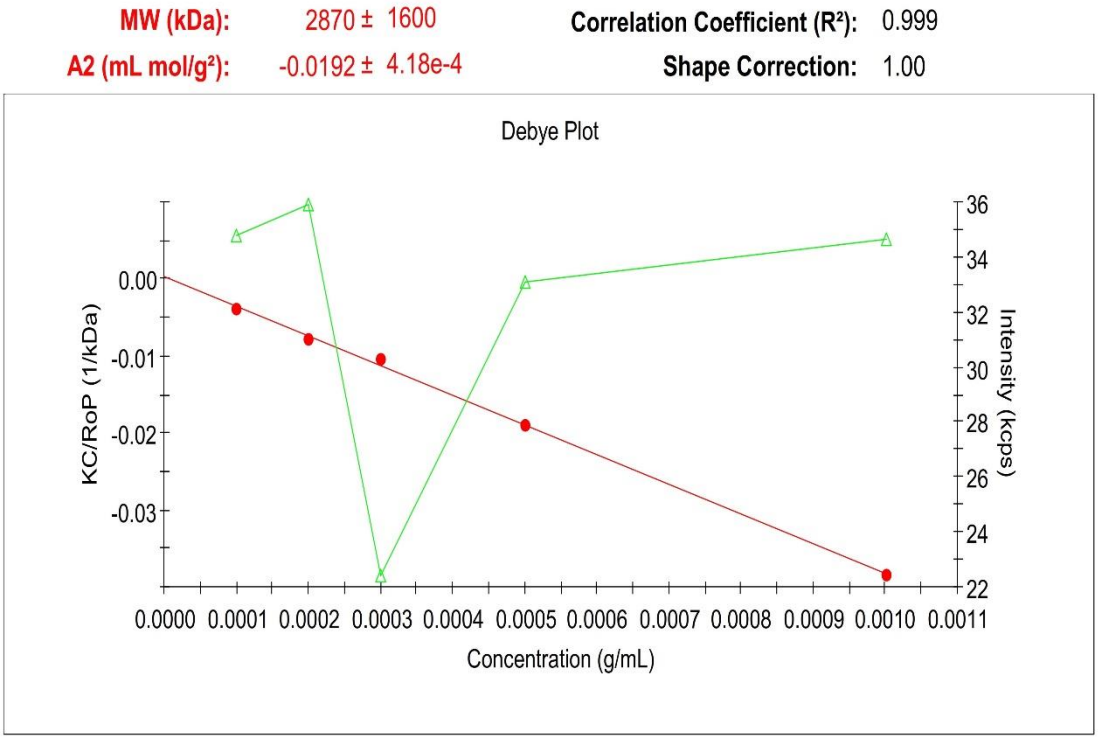

Figure 4. 23: Molecular weight of bPEI-HA copolymer

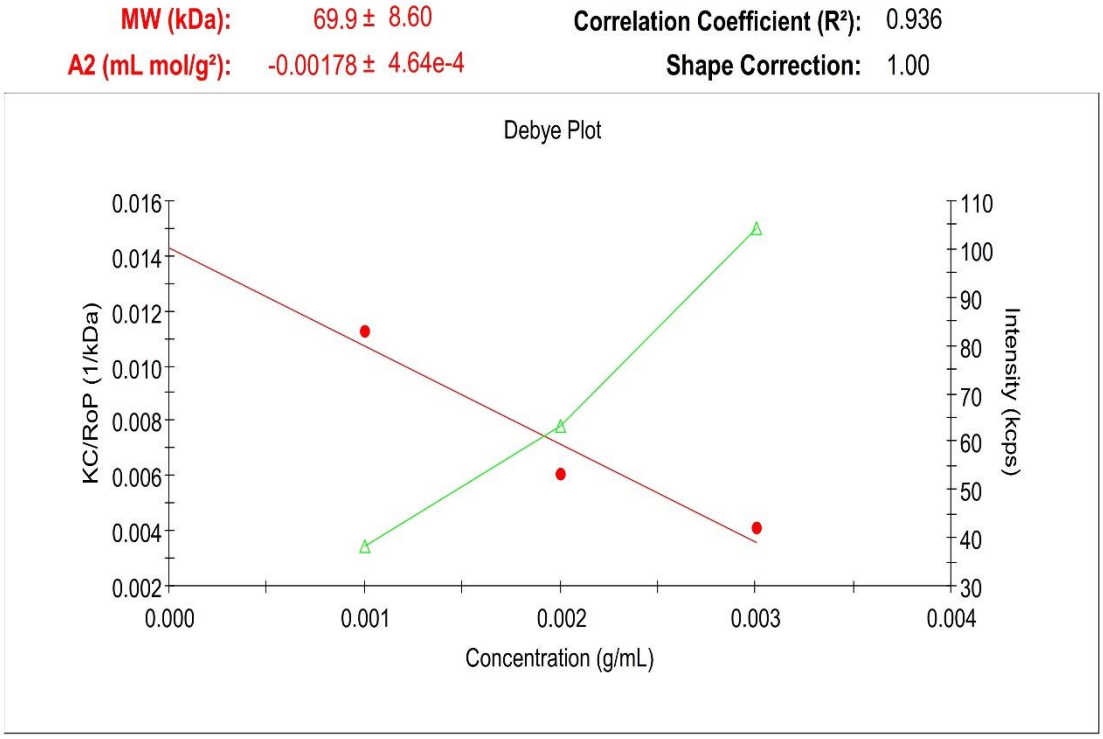

Figure 4. 24: Molecular weight of Lf

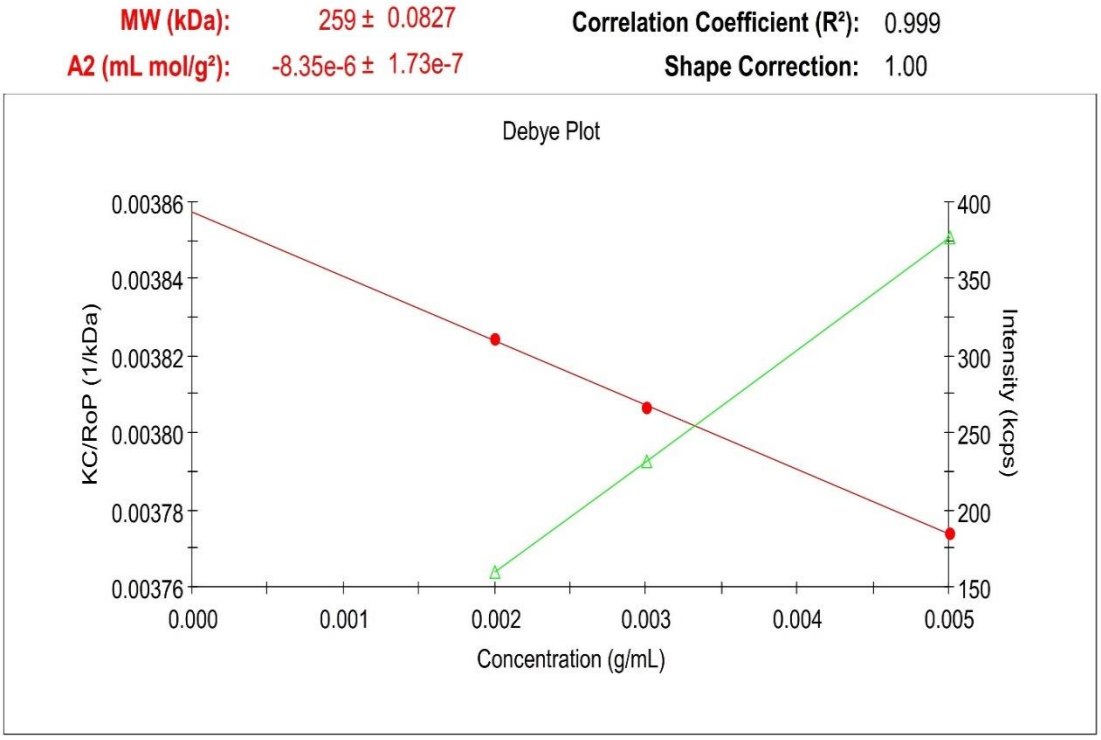

Figure 4. 25: Molecular weight of bPEI-Lf conjugate

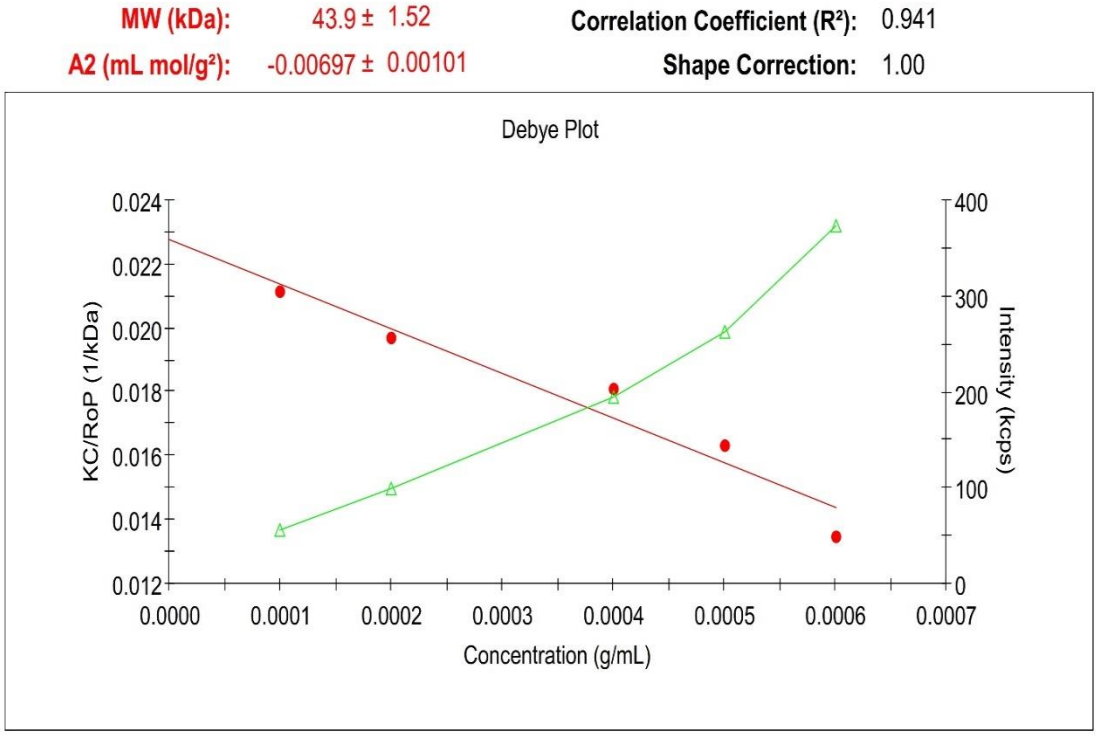

Figure 4. 26: Molecular weight of Chi

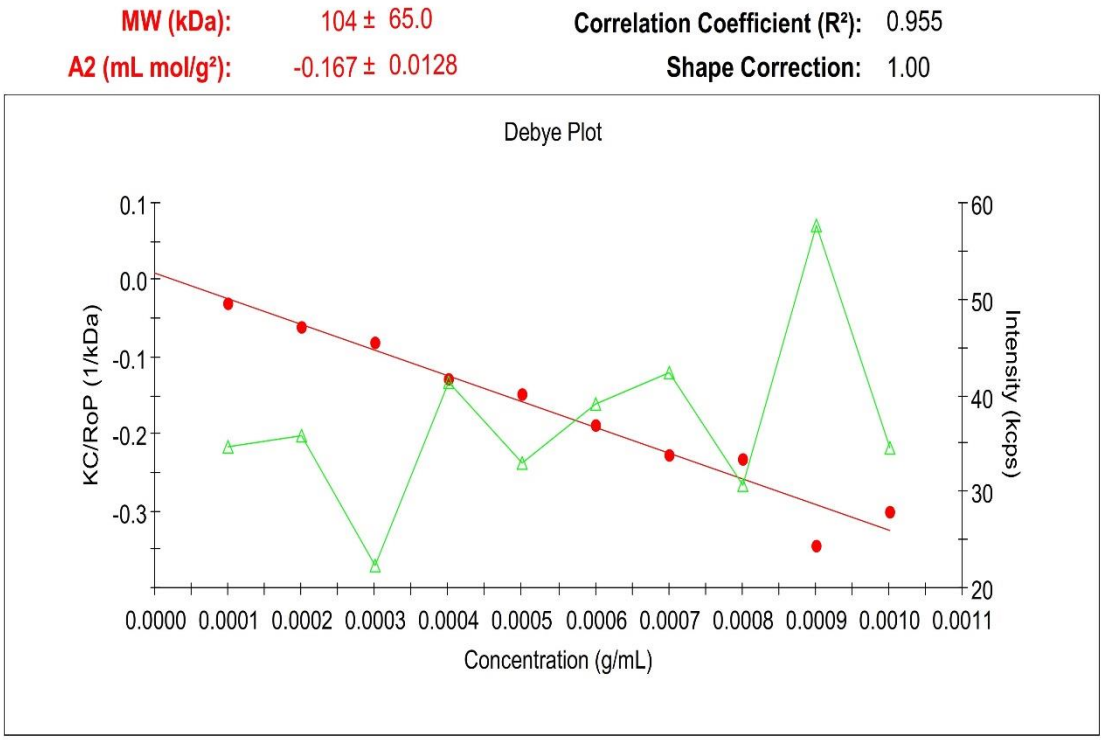

Figure 4. 27: Molecular weight of bPEI-Chi copolymer

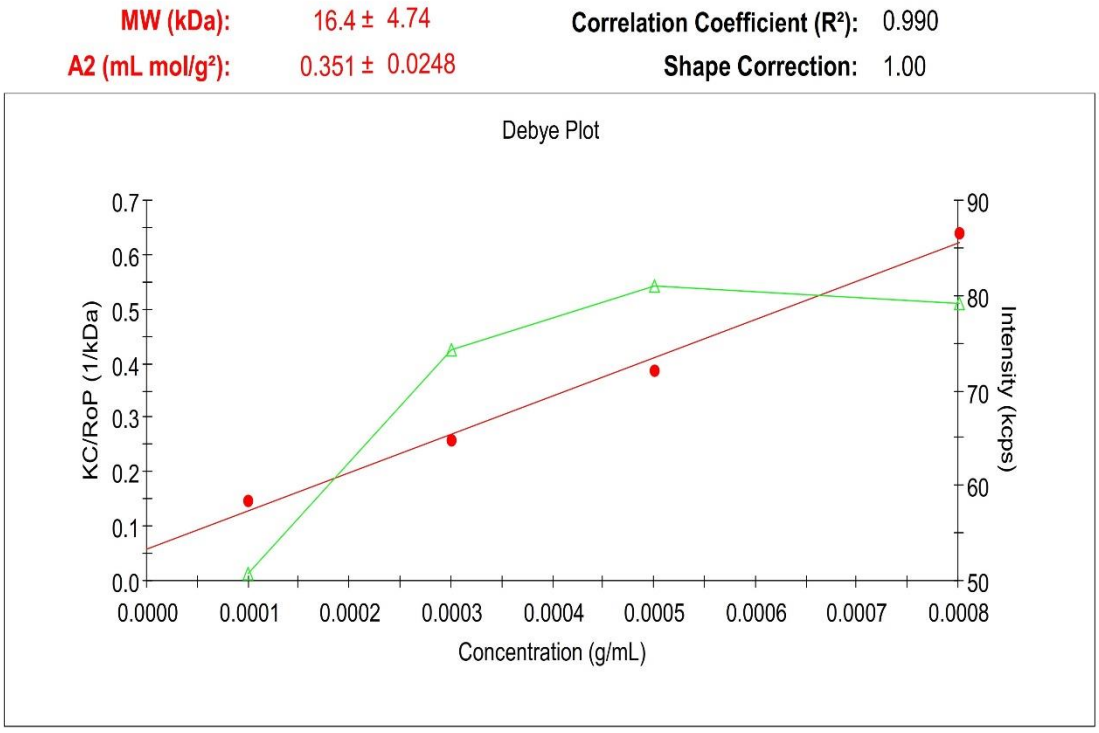

Figure 4. 28: Molecular weight of Biodegradable bPEI

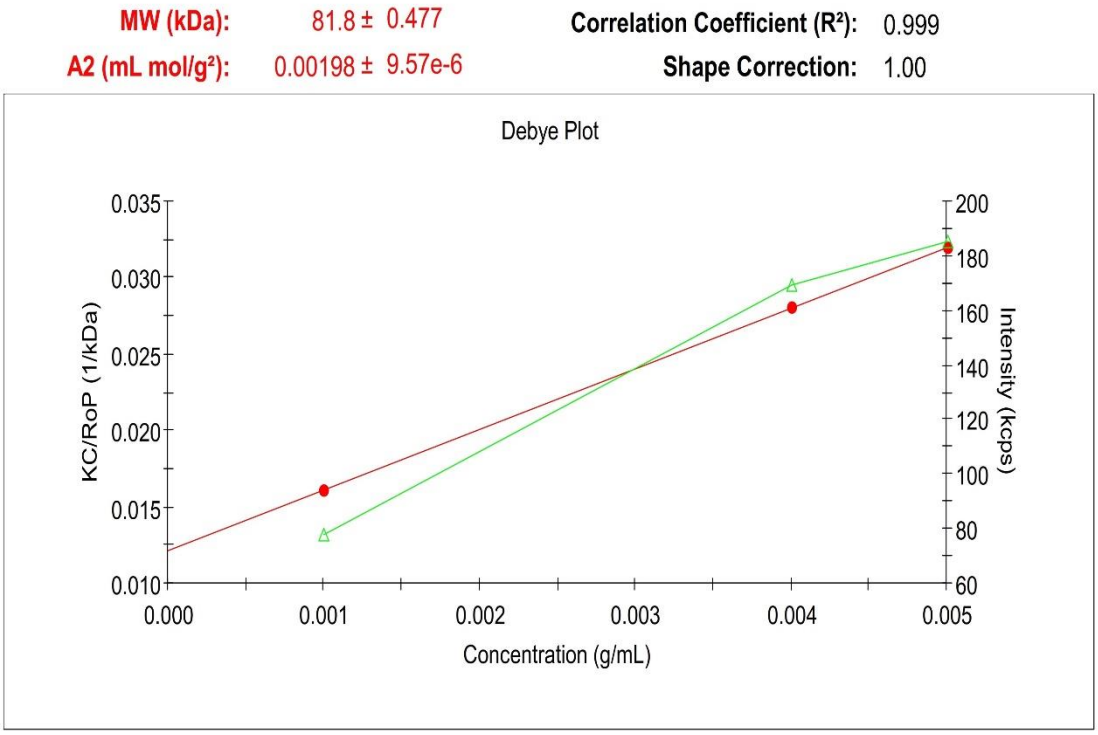

Figure 4. 29: Molecular weight of Biodegradable bPEI-Chi copolymer

Table 4. 1: Molecular weight of polymer/ copolymer/ conjugates

Polymer/ Copolymer/ conjugate	**Molecular weight (kDa) Mean ± SD**
Hyaluronic acid	655 ± 309
bPEI-HA copolymer	2870 ± 1600
Lactoferrin	69.9 ± 8.60
bPEI-Lf conjugate	259 ± 0.0827
Chitosan	43.9 ± 1.52
bPEI-Chi copolymer	104 ± 65
Biodegradable bPEI	16.4 ± 4.74
Biodegradable bPEI-Chi copolymer	81.8 ± 0.477

Additionally, number of molecules attached of each polymer was calculated from average molecular weight of each copolymer and conjugate and the results obtained are depicted in Table 4. 2.

Table 4. 2: Conjugation ratio synthesized copolymers/ conjugates

Polymer/ Copolymer/ conjugate	Theoretical conjugation ratio	Observed conjugation ratio
bPEI-HA copolymer	HA: bPEI = 1:130	~ 89 bPEI (25 kDA) molecules to 1 molecule of HA
bPEI-Lf conjugate	Lf: bPEI = 1:14	~ 8 bPEI (25 kDA) molecules to 1 molecule of Lf
bPEI-Chi copolymer	Chi: bPEI = 1:5	~ 2 bPEI (25 kDA) molecules to 1 molecule of Chi
Biodegradable bPEI	NA	~ 20 bPEI (0.8 kDA) molecules were crosslinked
Biodegradable bPEI-Chi copolymer	Chi: biodegradable bPEI = 1:8	~ 2 bPEI (0.8 kDA) molecules to 1 molecule of Chi

4.4.4 Muco-adhesion study

The results of muco-adhesion study for synthesized mucoadhesive copolymers and conjugates confirmed their mucoadhesive property. The % mucin binding efficiency of bPEI-HA, bPEI-Chi and biodegradable bPEI-Chi copolymers were 28, 16.5 and 14.5 % respectively. This proved mucoadhesive property of synthesized copolymers and conjugates which prolonged nasal residence and thus improved therapeutic efficacy of gene therapeutics. Additionally, on increasing polymer concentration there was increase in % mucin binding efficiency which further significantly improved mucoadhesion.

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